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Protamines and DNA Integrity as A Biomarkers of Sperm Quality of Smokers and Non-Smokers Patients Undergoing Assisted Reproduction Therapy

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"For anything worth having

one must pay the price;

and price is always

work, patience, love,

and self-sacrifice"

Dedication

This work is dedicated to the persons whom supported me all through my life and never saved an effort to lead me to what I am now, my father and mother.

My wife and our children as a sign of love and appreciation for enduring my absence during the long stay abroad, their patience and moral support to complish this piece of work

Contents

1- Summary 7
1-1- Summary 7
1-2- Zusammenfassung 10

2- Introduction 13
2-1- Human sperm chromatin structure 13
2-2- Sperm Nuclear Proteins 16
2-2-1- Histones 16
2-2-2- Transition nuclear proteins (TPs) 17
2-2-3- Replacement of Histones by Protamine 18
2-2-4- Protamines 20
2-2-4-1- Genes of protamines 21
2-2-4-2- Synthesis of protamines 22
2-2-4-3- Characteristic structural features 25
2-2-4-3-1- Protamine P1 25
2-2-4-3-2- Protamine P2 25
2-2-4-4- Protamines Functions 26
2-2-4-5- Structural – functional relationships 26
2-2-4-6-Sperm DNA integrity and Protamines 27
2-2-4-7-Protamines and infertility 27
2-2-4-8- Determination of protamines 28
2-2-4-8-1- Direct determination 28
2-2-4-8-2- Indirect assessment of sperm chromatin structure by histochemical procedures 30
2-3- Reactive Oxygen Species (ROS) 30
2-3-1- Physiologic role of ROS 31
2-3-2- Consequences of excessive generation of ROS 31
2-3-3- (8-hydroxy-2'-deoxyguanosine (8-OHdG)) level 32
2-3-4- Lipid peroxidation (Malondialdehyde, MDA) 32
2-4- Smoking and sperm parameters 33
2-4-1- Introduction 33
2-4-2-Smoking and semen quality 33
2-4-3- Effects of Smoking on Semen Volume 34
2-4-4- Effect of Smoking on Sperm Concentration 34
2-4-5- Effect of Smoking on Sperm Motility 34
2-4-6- Effect of smoking on sperm Morphology 35
2-4-7- Effect of smoking on sperm chromatin integrity (DNA) 35
2-4-8- Cotinine level in seminal plasma 36
2-5- Factors that influence sperm chromatin structure 36
2-5-1- Temperature 36
2-5-2- Chemicals and toxins 37
2-5-3- Disease 37
2-5-4- Molecular disturbances 37
2-5-5- Age and spermatozoal DNA damage 37
2-6- Origin of sperm DNA damage 37
2-6-1- Defects in recombination 39
2-6-2- Spermatid maturation abnormalities 39
2-6-3- Oxidative stress 39
2-6-4- Abortive apoptosis 41
2-7- Clinical significance of sperm DNA damage 42
2-7-1- DNA damage and semen parameters 42
2-7-2- The relationship between sperm DNA integrity and fertilization 43

2-7-3- Sperm DNA damage and preimplantation development 43
2-7-4- Intrauterine insemination (IUI) 43
2-7-5- In vitro fertilization (IVF) and Intracytoplasmic sperm injection (ICSI) 43
2-7-6- Embryonal loss 44
2-7-7- Effect of sperm DNA quality on offspring 44
2-8- Assessment of sperm DNA damage 44
2-8-1- Factors that influence the predictive value of DNA damage tests 45
2-8-2- Assessment Methods 45
2-8-2-1- Chromatin structural probes using nuclear dyes 45
2-8-2-1-1- Acridine Orange Assay (AOA) 46
2-8-2-1-2- Chromomycin–A_3 Staining (CMA_3) 46
2-8-2-1-3- Sperm chromatin structure assay (SCSA) 47
2-8-2-1-4- DNA breakage detection–fluorescence in situ hybridization test (DBD-FISH) 47
2-8-2-1- 5- Acidic Aniline Blue Assay (AB) 47
2-8-2-1- 6- Toluidine blue Assay (TB) 48
2-8-2-2- Tests for direct assessment of sperm DNA fragmentation 48
2-8-2-2-1- In situ nick translation assay (ISNT) 48
2-8-2-2-2- Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick-end abelling assay (TUNEL) 48
2-8-2-2-3- COMET assay (Single Cell Gel Electophoresis) Assay 48
2-8-2-3- Sperm nuclear matrix assays 49
2-8-2-3-1- Sperm chromatin dispersion assay (SCDA) 49
2-9- Aims of the study 50

3- Materials and Methods 51
3-1- Materials 51
3-1-1- Semen samples 51
3-1-2- Chemicals and laboratory materials 51
3-1-3- Buffers and Solutions 52
3-1-4- Apparatus and Instruments 53
3-1-5- Antibodies 54
3-1-5-1- Primary antibodies 54
3-1-5-2- Secondary antibody 54
3-1-6- ELISA Kits 54
3-2- Methods 54
3-2-1- Ejaculates and Sperm Preparation 54
3-2-2- Assessment of Sperm Morphology 55
3-2-3- Assessment of Sperm Vitality (Eosin Test) 55
3-2-4- Assessment of the Sperm Membrane Integrity (Hypo-Osmotic Test; HOS) 55
3-2-5- Assessment of Chromatin Condensation (Chromomycin, CMA_3) of Spermatozoa 55
3-2-6- DNA Fragmentation Analysis (Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling, TUNEL Test) 56
3-2-6- Protamine Quantification 56
3-2-7-1- Determination of protein concentration according to Bradford 56
3-2-7-2- Total Nuclear Protein Extraction: 57
3-2-7-2-1- Tail Dissociation 57
3-2-7-2-2- Protein Extraction 57
3-2-7-3- Production of a Protamine Standard 59
3-2-7-4- Protein Extraction Control Sample 59
3-2-7-5- Acid-Urea Polyacrylamide Gel Electrophoresis (AU-PAGE) 59
3-2-7-5-1- AU-PAG preparation 59
3-2-7-5-2-Electrophoresis 60
3-2-7-5-3- Staining 60
3-2-7-6- Western Blot 60

3-2-8- Reactive oxygen species measurement 61
3-2-9- 8-hydroxy-2-deoxyguanosine (8-OH-dG) measurement 62
3-2-10- Lipid peroxidation assay
(Measurment of Malondialdehyde, MDA) In Seminal Plasma 63
3-2-11- Cotinine measurement 64
3-2-12- Statistical analysis 65

4- Results 66
4-1- Semen and Sperm Parameters for all Participants 66
4-2- Normal volunteers versus Patients 76
4-2-1- Normal Volunteers 77
4-2-2- Patients 81
4-3- Non-smokers versus Smokers 83
4-3-1- All participants 83
4-3-2- Normal volunteers 85
4-3-3- Patients 87
4-3-4- Normal smokers versus patient smokers 89
4-4- Protamines Quantification 90
4-4-1- Volunteers versus Patients 92
4-4-2- Non-smokers versus smokers 95
4-4-2-1-Volunteers non-smokers versus smokers 96
4-4-2-2-Patients non-smokers versus smokers 97
4-4-2-3- Patients smokers versus volunteers smokers 98
4-4-3- P1/P2 ratio grouping 98
4-4-3-1- Relationship between P1/P2 ratios and seminal parameters 100
4-4-3-1-1- All participants 100
4-4-3-1-2- Smokers 101
4-5- Clinical Data 108

5- Discussion 111
5-1- Sperm DNA Integrity and Semen Parameters (CMA_3 and TUNEL) 111
5-2- Oxidative stress 114
5-2-1- Oxidative Stress and Semen Parameters 115
5-3- Smoking and infertility 117
5-3-1- Smoking and oxidative damage 117
5-3-1-1- Smoking and ROS 117
5-3-1-2- Cotinine and sperm parameters 119
5-3-1-3- Smoking cigarette and MDA levels 119
5-3-1-4 Smoking cigarette and 8-OHdG levels 120
5-3-2- Smoking and Semen Parameters 121
5-3-3- Smoking and Semen volume 122
5-3-4- Smoking and Sperm motility 123
5-3-5- Smoking and Sperm Morphology 123
5-3-6- Smoking and DNA damage 124
5-4- Protamines Quantifications 125
5-4-1- P1 and P2 Concentrations 125
5-4-2- Protamines and DNA integrity 127
5-4-3- P1/P2 Content and Sperm Quality 129
5-4-4- P1/P2 Content and Semen Parameters 130
5-4-5- P1/P2 Content and Fertilization and Pregnancy Rates 130
5-4-6- P1/P2 ratio Grouping 131
5-4-7- P1/P2 ratios and seminal characteristics 132
5-4-8- Relationship between Protamines and Smoking 134
Conclusion 135

References 136
Acknowledgment 168
Curriculum Vitae (CV) 169

1- Summary

1-1- Summary

Unlike the chromatin structure of somatic cells, sperm chromatin is very tightly compacted by virtue of the unique associations among the DNA, the nuclear matrix, and sperm nuclear proteins. Protamines (P1 and P2 family) are the most abundant proteins in human sperm nucleus and are important for condensation of the paternal genome of the spermatozoa that produce a more compact and hydrodynamic nucleus. This compact structure protects the genetic materials during transport of the paternal genome through the male and female reproductive tracts, also, allow proper fusion of male and female gametes and correct expression of the genetic information by the developing embryo. Human sperm protamine deficiency has been correlated significantly with reduced semen quality parameters (counts, motility and morphology), sperm penetration ability, sperm DNA integrity, and male infertility.

Sperm DNA damage may occur at testicular, epididymal or post-ejaculatory levels. DNA damage in the male germ line has been associated with poor semen quality, low fertilization rates, impaired embryonal preimplantation development, increased abortion and an elevated incidence of disease in the offspring, including childhood cancer.

Oxidative stress resulting from an imbalance in the productions of reactive oxygen species (ROS) and the antioxidant capacity appears to be the major cause of DNA damage in the male germ line. Spermatozoa are particularly susceptible to damage induced by excessive ROS; their plasma membrane contains large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes. Smoking was associated with an increase in ROS levels and a decrease in ROS-Total antioxidant capacity (TAC) scores. Many studies revealed that semen samples from smokers have a significantly higher ratio of single-strand-to double-strand breaks of DNA spermatozoa.

The current study evaluates the relationship between the oxidative stress, smoking and human sperm DNA integrity. Other evaluations include the effect of smoking and sperm parameters, cotinine and oxidative stress biomarkers (ROS, MDA, and 8-OHdG) levels in seminal plasma, the effect of smoking on protamines, and the ratios of protamine 1 to protamine 2 in human spermatozoa and their effect on intracytoplasmic sperm injection (ICSI) and standard semen parameters.

Semen samples of patients who underwent ICSI therapy (n=116) and healthy volunteers (n=50) were included in this study. The patients group and volunteers group were divided into two groups: smokers (where n=53 patients and n=19 volunteers) and non-smokers (n=63 patients and n= 31 volunteers). Each semen sample was evaluated according to the WHO guideline (1999) except the morphology, which was evaluated undergo strict criteria. Chromatin condensation, DNA integrity, and protamines concentrations were investigated. In addition, the concentrations of ROS, MDA, 8-OHdG and cotinine in seminal plasma were measured. Smears were prepared for evaluation of: sperm vitality (Eosin test) and morphology by normal staining; chromatin condensation (CMA_3 assay) and DNA integrity (TUNEL assay) by fluorescence technique. Aliquots of each sperms sample were used for protamine extraction and evaluation using acid-urea polyacrylamide gel electrophoresis. ROS, cotinine and 8-OHdG were evaluated by ELISA, while MDA were evaluated by chemical reactions.

The results from this study showed significant differences ($p<0.010$) between volunteers and patients in sperm's concentrations (80.2 ± 27.5 vs. 61.4 ± 31.1), motility (37.1 ± 14.7 vs. 27.4 ± 12.0), vitality (45.2 ± 16.9 vs. 34.7 ± 17.3), sperm membrane integrity (53.4 ± 15.8 vs. 45.1 ± 20.8), morphologically normal spermatozoa (45.9 ± 13.9 vs. 29.4 ± 12.7), chromatin condensation (positive CMA_3) (23.3 ± 7.0 vs. 32.9 ± 8.2), and DNA fragmentation (Tunel) (9.6 ± 5.4 vs. 14.2 ± 5.6). In addition, an analysis of all samples showed that chromatin condensation evaluated by

CMA_3 assay (bad sperms) showed significant negative ($p<0.010$) correlation with sperm concentration ($r=-0.289$), motility ($r=-0.384$), morphologically normal sperm ($r=-0.514$), viability ($r=-0.458$), and membrane activity ($r=-0.343$). Similarly, DNA integrity evaluated by TUNEL assay showed significant negative correlation ($p<0.010$) with motility, morphologically normal sperm, viability, and membrane activity ($r=-0.330$, $r=-0.509$, $r=-0.406$, $r=-0.263$ respectively). A significant positive ($p<0.010$) correlation between non-condensed chromatin and DNA integrity was also shown ($r=0.479$).

All smokers samples (volunteers and patients) (n=72) showed significantly higher ($p<0.010$) levels than all non-smokers samples (volunteers and patients) (n=94) in sperm's motility (35.8 ± 14.6 vs. 23.4 ± 8.0), vitality (41.2 ± 17.3 vs. 33.5 ± 17.6), membrane integrity (54.1 ± 18.4 vs. 39.2 ± 18.4), morphologically normal spermatozoa (41.4 ± 13.7 vs. 26.6 ± 12.0), non-condensed chromatin (34.6 ± 8.1 vs. 26.4 ± 8.0), and DNA fragmentation (16.8 ± 5.1 vs. 9.6 ± 4.3).

The oxidative stress biomarkers ROS, MDA, and 8-OHdG were significantly higher ($p<0.010$) in patients than in volunteers; ROS (103.85 ± 5.68 vs. 67.6 ± 42.2, $p<0.010$), MDA (7.82 ± 1.79 vs. 6.45 ± 1.43, $p<0.010$), and 8-OHdG (1.95 ± 1.76 vs. 1.15 ± 0.82, $p<0.050$). In addition, these oxidative stress biomarkers (ROS, MDA, and 8-OHdG) of all samples showed significantly negative ($p<0.010$) correlations with sperm's motility ($r=-0.537$, $r=-0.513$, $r=-0.532$), vitality ($r=-0.298$, $r=-0.309$, $r=-0.373$), membrane integrity ($r=-0.343$, $r=-0.362$, $r=-0.335$) and morphologically normal sperm ($r=-0.547$, $r=-0.514$, $r=-0.513$). In contrast, significant positive ($p<0.010$) correlations between these biomarkers and non-condensed chromatin ($r=0.629$, $r=0.555$, $r=0.545$) and DNA fragmentation ($r=0.630$, $r=0.624$, $r=0.600$) were demonstrated. Fertilization and pregnancy rates were negatively affected by these biomarkers.

Evaluation of smoking effects of both volunteers and patients smokers (n=72) compared to non-smokers (n=94) on seminal plasma oxidative stress biomarkers showed significantly higher ($p<0.010$) concentrations in smokers compared to non-smokers; MDA (8.51 ± 1.43 vs. 6.10 ± 1.18), ROS (128.75 ± 39.46 vs. 52.89 ± 28.48), and 8-OHdG (2.63 ± 1.54 vs. 0.71 ± 0.53).

Seminal plasma smoking marker cotinine levels were found to be significantly higher in smokers than non-smokers (88.22 ± 58.05 vs. 2.34 ± 2.20, $p<0.010$). In general, negative significant ($p<0.010$) correlations were found between smoking marker cotinine levels in seminal plasma with sperm's motility, vitality, membrane integrity and morphologically normal sperm ($r=-0.489$, $r=-0.322$, $r=-0.356$, $r=-0.555$ respectively). Whereas, positive correlations ($p<0.010$) with sperm non-condensed chromatin ($r=0.549$) and DNA damage ($r=0.627$) were demonstrated.

Moreover, concentrations of P1 (416.30 ± 101.70) and P2 (363.60 ± 114.9) in patients were higher than that of volunteers (378.20 ± 100.00, 347.30 ± 77.30) and the P1/P2 ratio of patients was significantly higher than that of volunteers (1.22 ± 0.36 vs. 1.10 ± 0.20, $p<0.050$). The percentage of P2 in patients (46.7%) was lower compared to that of volunteers (47.9%). In addition, these findings showed that the concentrations of P1 were higher in samples of smokers than non-smokers (407.60 ± 128.80 vs. 402.63 ± 93.96, $p>0.050$) while the concentrations of P2 were significantly lower in smokers compared to non-smokers (335.60 ± 110.60 vs. 376.81 ± 97.36, $p<0.050$). The P1/P2 ratios were significantly higher ($p<0.010$) in smokers group compared to non-smokers (1.30 ± 0.42 vs. 1.09 ± 0.18).

In the present study, direct quantitative measurements of sperm protamine concentrations revealed that P1 and P2 inversely correlated with DNA fragmentation. However, the concentrations of P1 showed negative ($p>0.050$) correlations with non-condensed chromatin ($r=-0.011$, $r=-0.091$, $r=-0.167$) and DNA fragmentation ($r=-0.004$, $r=-0.053$, $r=-0.136$) in all participant samples — volunteers and patients. Similar results were detected with P2 concentration ($r=-0.126$, $r=-0.097$, $r=-0.208$); ($r=-0.060$, $r=-0.161$, $r=-0.036$). Also, abnormalities in P1/P2 ratios inversely affected the sperm quality and function. The results indicated that in all participant samples P1/P2 ratios have

significant positive correlations ($p<0.010$) with the concentrations of the oxidative biomarkers; MDA ($r=0.384$), ROS ($r=0.410$), 8-OhdG ($r=0.393$) and cotinine ($r=0.411$).

Smoking effect on protamines 1 showed that total amounts of protamines (1+2) in all non-smokers were higher than smokers (779.44 vs. 743.20). P1 concentrations in non-smokers and smokers were almost equal (402.63 ± 93.96; 407.60 ± 128.80, $p>0.050$), whereas, P2 concentrations in non-smokers were significantly higher (376.81 ± 97.36 vs. 335.60 ± 110.60, $p<0.050$) than those of smokers. The P1/P2 ratios of all smokers, volunteers and patients, were significantly higher in comparison to those of non-smokers —(1.30 ± 0.42, 1.19 ± 0.27, 1.34 ± 0.46) vs. (1.09 ± 0.18, 1.04 ± 0.11, 1.11 ± 0.20), respectively. These findings indicated that the causative agent for higher P1/P2 ratios in smokers was the under-expression of P2.

In conclusion, the results of this study indicated that high levels of oxidative stress biomarkers (ROS, MDA, and 8-OHdG) induce lipid peroxidation of the sperm membrane and induce DNA damage which markedly influences sperm quality. It is quite probable that such a deleterious effect may account for some cases of male infertility, and the evaluation of oxidative stress biomarkers may be a part of infertile male workup in the near future. Also, oxidative stress inversely affects fertilization and pregnancy rates. Reactive oxygen species status may be used as an important indicator for clinical evaluation and treatment of malefactor infertility.

In addition, in this study smokers showed high levels of oxidative stress biomarkers and decreased sperm quality as compared to non-smokers. This study also demonstrated that cigarette smoking impairs sperm function by reducing the quality and fertilizing capacity. This is probably because of high levels to toxic components of smoking such as cotinine that cause an increase of seminal plasma free radicals and oxidative stress.

Quantification of the protamines revealed that most of the cases had high P1/P2 ratios and the majority was due to under-expression of P2. Results also showed that aberrant P1/P2 ratios are associated with reduced sperm quality and percentages of fertilization and pregnancy rates. Protamine concentrations (P1 and P2) were inversely correlated with the DNA fragmentation and non-condensed chromatin. Evaluation of the effects of smoking on protamines demonstrated a reduction in protamines concentrations and elevation of P1/P2 ratio in smokers as compared to non-smokers.

This is the first study conducted to evaluate the effect of smoking on the protamination process. Given the potential adverse effects of smoking on fertility, physicians should advise infertile patients who smoke cigarettes to quit smoking. Additional studies with large number of subjects are needed to confirm the effect of smoking on protamines and to clarify the potential mechanism behind this effect.

1-2- Zusammenfassung

Im Gegensatz zu Körperzellen, hat das Chromatin der Spermien eine eigene Kondensation zwischen den DNA Strängen, der nukleären Matrix und den nukleären Proteinen. Die Protamine (P1 und P2 Familie) sind die am häufigsten vorkommenden Proteine des Spermnukleus und von entscheidender Bedeutung bei der Chromatinkondensation des paternalen Genoms. Diese Kondensation ist notwendig um das genetische Material während des Transportes des männlichen Genomes im weiblichen als auch männlichen Reproduktionstraktes, sowie des Gametenfusionierung und der Geschlechtsdifferenzierung, zu schützen. Ein Mangel an Protaminen im menschlichen Sperma korreliert signifikant mit einer Verminderung der Spermienqualität (Motilität und Morphologie), ebenso wie schlechte Penetrationsfähigkeit der Eizelle, DNA Integrität und männliche Infertilität.

Ein DNA Schaden in den Spermien kann im Hoden, Nebenhoden oder auch erst nach der Ejakulation stattfinden. Solch ein Schaden führt nicht nur zu einer eingeschränkten Spermienqualität sondern auch zu einer verminderten Fertilisationsrate, verminderte Implantation der Embryonen, höhere Abortrate und zunehmende Erkrankungen beim Neugeborenen, wie z.B. Krebserkrankungen.

Oxidativer Streß, der durch ein Ungleichgewicht zwischen höheren Produktionen an ROS (reactive oxygen species) und eine verminderte antioxidantien Kapazität, scheint der Hauptverursacher von DNA Schäden in männlichen Gameten zu sein. Das Sperma ist besonders empfindlich für ROS, da die membran aus vielen ungesättigten Fettsäuren besteht und keine Reparaturenzyme, aufgrund mangelndem Zytoplasma, besitzt. Andererseits erhöht das Rauchen den Anteil der ROS und vermindert die antioxidative Kapazität. Viele Studien konnten zeigen, das die Spermien von Rauchern einen höheren Anteil an Einzel- und Dopplestrangbrüchen aufzeigen.

Das Ziel dieser vorliegenden Arbeit war es den Einfluß des Rauchens auf die Entstehung des oxidativen Streß und daraus resultierende DNA- Schäden in Spermatozoen zu untersuchen. Darüberhinaus sollte der Einfluß des Rauchens auf andere Spermienparameter, die Cotininekonzentration im Seminalplasma und anderen Biomarkern des oxidativen Streß (ROS, MDA, 8-OHdG) analysiert werden. Weiterhin sollte der Einfluß des Rauchens auf das Protamin 1-Protamin 2 Verhältnisses in der Spermien-DNA sowie deren Zusammenhang mit anderen Spermienparametern und deren Einfluß auf die ICSI Ergebnisse untersucht werden.

Ejakulate von ICSI Patienten (n=116) und gesunde Probanden (n=50) wurden im Rahmen der vorliegneden Doktorarbeit untersucht. Die Patienten-, als auch die Probandengruppe wurden in eine Raucher (Patienten n=53; Probanden n=19) und eine Nichtrauchergruppe (Patienten n=63; Probanden n= 31) unterteilt. Jede Spermaprobe wurde anhand der WHO Kriterien (1999) untersucht, die Morphologie nach Kruger- Kriterien. Darüberhinaus wurden die Chromatinkondenation, DNA Integrität und Protaminkonzentration beurteilt. Die Konzentrationen an ROS, MDA, 8-OHdG und Cotinine wurden im Seminalplasma gemessen. Es wurden Abstriche angefertigt zur Bestimmung der Spermienvitalität (Eosintest) und Morphologie, Chromatinkondensation (CMA_3 Test) und DNA Integrität (TUNNEL Test) mittels Fluoreszenstechniken. Ein Teil jeder Spermienprbe wurde zur Bestimmung der Protamine genutzt durch Urea-Acid Polyacrylamid Gel Elektrophorese. ROS, Cotinine und 8-OhdG wurden durch Anwendung des ELISA bestimmt, während MDA durch chemische Reaktionen nachgewiesen wurde.

Die Ergebnisse dieser Arbeit zeigten einen signifikanten Unterschied ($p<0.010$) zwischen den Probanden und den Spermienkonzentrationen der Patienten (80.2 ±27.5 mill./ml vs.61.4 ± 31.1 mill./ml), Motilität (37.1 ± 14.7% vs. 27.4 ± 12.0 %), Vitalität (45.2 ± 16.9% vs. 34.7 ± 17.3%), Spermienmembranintegrität (53.4 ± 15.8% vs. 45.1 ± 20.8%), morphologisch normale Spermien (45.9 ± 13.9% vs. 29.4 ± 12.7%), Chromatinkondensation (positive CMA_3) (23.3 ± 7.0% vs.32.9 ± 8.2%), und DNA Bruchstücke (Tunel) (9.6 ± 5.4% vs. 14.2 ± 5.6%). Darüberhinaus zeigte eine

Analyse aller Spermienproben dass die Chromatinkondensation, dargestellt mittels CMA_3 assay (schlechte Spermien) eine negative Korrelation ($p<0.010$) mit der Spermienkonzentration (r=-0.289), Motilität (r=-0.384), morphologisch normale Spermien (r=-0.514), viability (r=-0.458), und Membran und DNA Integrität (r=-0.343).

Gleichermaßen zeigte die DNA-Integrität, gemessen mittels TUNNEL Assay, eine signifikante negative Korrelation ($p<0.010$) mit der Motilität, morphologisch normale Spermien, Vitalität und Membranaktivität (r=-0.330, r=-0.509, r=-0.406, r=-0.263). Darüberhinaus konnte eine positive Korrelation ($p<0.010$) zwischen nicht-kondensiertem Chromatin und DNA-Integrität gezeigt warden (r=0.479).

Weiterhin, alle Proben von Rauchern (Probanten und Patienten) (n=72) zeigten einen signifikant höheren Anteil motiler Spermien ($p<0.010$) als die Proben der Nicht-Raucher (Probanden und Patienten) (n=94), (35.8 ± 14.6% vs. 23.4 ± 8.0%), Vitalität (41.2 ± 17.3% vs. 33.5 ± 17.6%), Membranintegrität (54.1 ± 18.4% vs. 39.2 ± 18.4%), morphologisch normale Spermien (41.4 ± 13.7% vs. 26.6 ± 12.0%), nicht kondensiertes Chromatin (34.6 ± 8.1% vs. 26.4 ± 8.0%), und DNA Fragmentierung (16.8 ± 5.1% vs. 9.6 ± 4.3%).

Biomarker des oxidativen Stresses ROS, MDA, und 8-OHdG waren signifikant höher ($p<0.010$) bei der Gruppe der Patienten als der der Probanden; ROS (µmol/ml) (103.9 ± 5.7 vs. 67.6 ± 42.2, $p<0.010$), MDA (µM) (7.8 ± 1.8 vs. 6.5 ± 1.4, $p<0.010$), und 8-OHdG (ng/ml) (2.0 ± 1.8 vs. 1.2 ± 0.8, $p<0.050$). Diese Marker des oxidativen Stresses (ROS, MDA, and 8-OHdG) aller Proben zeigten eine significant negative Korrelation ($p<0.010$) mit der Spermienmotilität (r=-0.537, r=-0.513, r=-0.532), Vitalität (r=-0.298, r=-0.309, r=-0.373), Membranintegrität (r=-0.343, r=-0.362, r=-0.335) und morphologisch normale Spermien (r=-0.547, r=-0.514, r=-0.513). Im Gegensatz dazu wurde eine signifikant positive Korrelation ($p<0.010$) zwischen diesen Biomarkern und nicht-kondensiertem Chromatin (r=0.629, r=0.555, r=0.545) und DNA Fragmentation (r=0.630, r=0.624, r=0.600) gezeigt. Die Fertilisation als auch die Schwangerschaftsrate wurden von diesen Biomarkern negativ beeinflusst.

Effekte des Rauchens in beiden Gruppen Probanden als auch Patienten (n=72) verglichen mit Nicht-Rauchern (n=94) wurden die Marker des oxidativen Stresses im Seminalplasma untersucht und zeigten significant höhere Konzentrationen ($p<0.010$) bei Rauchern, verglichen mit Nicht-Rauchern; MDA (µM) (8.5 ± 1.4 vs. 6.1 ± 1.2), ROS (µ mol/ml) (128.8 ± 39.5 vs. 52.99 ± 28.5), und 8-OHdG (ng/ml) (2.6 ± 1.5 vs. 0.7 ± 0.5).

Darüberhinaus war der Cotininegehalt (ng/ml) im Seminalplasma von Rauchern signifikant höher als bei Nicht-Rauchern (88.22 ± 58.05 vs. 2.34 ± 2.20, $p<0.010$). Zusammenfassend konnte eine signifikant negative ($p<0.010$) Korrelation zwischen dem Rauchermarker Cotinine im Seminalplasma und den Spermienmarkern Motilität, Vitalität, Membranintegrität und morphologisch normale Spermien (r=-0.489, r=-0.322, r=-0.356, r=-0.555). Weiterhin konnte eine positive Korrelation ($p<0.010$) zwischen Spermien mit nicht-kondensiertem Chromatin (r=0.549) und DNA Schäden (r=0.627) gezeigt werden.

Die Konzentrationen von P1(ng/10^6 sperm) (416.3 ± 101.7) und P2 (ng/10^6 sperm) (363.6 ± 114.9) waren bei den Patienten höher als bei den Probanden (378.2 ± 100.0, 347.3 ± 77.3), ebenso war das P1/P2 Verhältnis bei den Patientensignifikant höher als in der Probandengruppe (1.2 ± 0.4 vs. 1.1 ± 0.2, $p<0.050$), jedoch der prozentuale Anteil an P2 war bei der Patientengruppe niedriger (46.7%) verglichen mit der Probandengruppe (47.9%). Diese Ergebnisse zeigen, dass die Konzentrationen an P1 (ng/10^6 sperm) bei den Raucherproben höher waren als bei den Nicht-Rauchern (407.6 ± 128.8 vs. 402.6 ± 94.0, $p>0.050$) die Konzentrationen an P2 (ng/10^6 sperm) waren signifikant niedriger bei der Rauchergruppe als bei den Nicht-Rauchern (335.6 ± 110.6 vs. 376.8 ± 97.4, $p<0.050$). Das P1/P2 Verhältnis war significant höher ($p<0.010$) in der Raucher-Gruppe verglichen mit der Nicht-Rauchergruppe (1.3 ± 0.4 vs. 1.1 ± 0.2).

In der vorliegenden Untersuchung wurde außerdem ein direkter quantitativer inverse Korrelatione zwischen der Konzentration an Spermienprotaminen P1 und P2 (ng/10^6 sperm) und der DNA Fragmentation (%) gemessen. Darüberhinaus zeigte die Konzentration an P1 (ng/10^6 sperm) eine negative Korrelation (p>0.050) mit nicht-kondensierten Chromatin % (r=-0.011, r=-0.091, r=-0.167) und DNA Fragmentation % (r=-0.004, r=-0.053, r=-0.136) in allen Teilnehmenden, Patienten und Probanden. Ähnliche Ergebnisse zeigten sich für die P2 (ng/10^6 sperm) Konzentrationen (r=-0.126, r=-0.097, r=-0.208); (r=-0.060, r=-0.161, r=-0.036). Ebenso konnten Abnormalitäten bei der P1/P2 Ratio gezeigt werden mit inversem Effekt auf die Spermien Qualität und Funktion. Diese Ergebnesse zeigen, dass in allen Proben (Probanden als auch Patienten) die P1/P2 Ratios eine signifikant positive Korrelations (p<0.010) mit den Konzentrationen an oxidativen Biomarkern zeigt MDA (µM) (r=0.384), ROS (µmol/ml) (r=0.410), 8-OhdG (ng/ml) (r=0.393) und Cotinine (ng/ml) (r=0.411).

Der Einfluß des Rauchens auf die Protamine 1 und den gesamt Anteil aller Protamine (1+2) (ng/10^6 sperm) bei allen Nicht-Rauchern war höher als bei den Rauchern (779.4 vs. 743.2). Die P1 (ng/10^6 sperm) Konzentrationen waren bei den Nicht-Rauchern und Rauchern fast identisch (402.6 ± 94.0; 407.6 ± 128.8, p>0.050), wohingegen die P2 (ng/10^6 sperm) Konzentration bei Nicht-Rauchern signifikant höher war (376.8 ± 97.4 vs. 335.6 ± 110.6, p<0.050) als bei den Rauchern. Die P1/P2 Ratio aller Raucher, Probanden und Patienten, war signifikant höher im Vergleich zu der Nicht-Raucher (1.3 ± 0.4, 1.2 ± 0.3, 1.3 ± 0.5) vs. (1.1 ± 0.2, 1.0 ± 0.1, 1.1 ± 0.2). Diese Ergebnisse zeigen daß der ursächliche Indikator für eine erhöhte P1/P2 Ratio ein verminderter Anteil an P2 ist.

Zusammenfassend belegen die Ergebnisse dieser Arbeit, dass ein hoher Anteil an oxidativen Stressmarkern (ROS, MDA und 8-OHdG) eine Lipidperoxidation in der Spermienmembran verursachen, als auch einen Schaden in der DNA und somit die Spermienfähigkeit beeinflußt. Es ist möglich, dass diese Schäden durch Peroxidation eine männlichen Subfertiliträt verursachen und daher kann die Evaluierung des oxidativen Stresses als entscheidender klinischer Parameter in der Beurteilung als auch Behandlung der männlichen Subfertilität dienen.

Darüberhinaus zeigte diese Arbeit, dass Oxidativer Stress durch Rauchen ensteht und dadurch die Spermienqualität bei Rauchern herabgesetzt ist im Vergleich zu Nicht-Rauchern.

Sowohl die Parameter als auch die Funktionsfähigkeit der Spermien wird beeinsträchtigt. Die Ursache hierin liegt in der toxischen Substanz des Rauchens einerseits und dem Cotininanstieg andererseits im Seminalplasma.

Die qualitative Bestimmung der Protamine zeigt, dass in den meisten Fällen die P1/P2 Ratio höher waren durch eine P2 Minderexpression. Dieses Verhältnis führt zu einer Verminderung der Spermienqualität und damit Verminderung der Fertilisationsfähigkeit und Schwangerschaftsrate. Die Bestimmung des Protamines bei Rauchern zeigt , dass Raucher weniger Protamin besitzen und die P1/P2 Ratio erhöht ist im Vergleich zu Nicht-Rauchern.

Nach unseren besten Kenntnissen und Literaturrecherche ist dies die erste Untersuchung über einen Zusammenhang zwischen Rauchen und Protaminkonzentrationen und P1/P2 Ratio in Spermien. Unter Berücksichtigung des negativen Effektes des Rauchens auf die Fertiltät sollte der Arzt bei einem subfertilen Patienten immer ein Rauchverbot empfehlen. Weitere Studien sind notwendig um durch eine größere Patientenzahl diese Ergebnisse zu untermauern.

2- Introduction

Infertility affects approximately 15% of couples trying to conceive and a male cause is believed to be a sole or contributing factor in approximately half of these cases (Oehninger, 2001), up to 40% of men in the study had sperm abnormalities, without known specific etiological factors (de Kretser, 1997). Disorders of the male reproductive system have become an important public health issue as they can cause infertility, miscarriages and abnormal outcomes in the offspring. Basic semen analysis consists of measuring seminal volume, pH, sperm concentration, motility, morphology and vitality (Centola and Ginsburg, 1996). These parameters may describe important aspects of the functions of the testes and sperm, but they do not address the integrity of the genetic material of the male gamete.

Mammalian spermatogenesis is a complex process through which physiological, biochemical and morphological changes occurs, resulting in a transformation of round diploid spermatogonia into fully differentiated haploid spermatozoa with a condensed nucleus, acrosome and flagellum. These reactions include a variety of mechanisms including DNA methylation, phosphorylation, acetylation and ubiquitination of the histone, the main proteins of chromatin, component (Berger, 2002; Govin *et al.,* 2004; Li *et al.,* 2008). Spermatogenesis is characterized by two distinct stages, spermatocytogenesis and spermiogenesis (D'Occhio, 2007).

Spermatocytogenesis involves mitotic divisions including proliferation and maintenance of spermatogonia (Senger, 1999), which will undergo meiosis to form primary spermatocytes. These spermatocytes will develop into secondary spermatocytes, which then differentiate into spermatids. Spermatids can be divided into three categories: early spermatids with round nuclei, intermediate spermatids with elongating nuclei, and mature spermatids with condensed nuclei (Dadoune, 2003). In the mid-spermiogenesis histone nucleoproteins of the meiotic germ cells are replaced by protamine nucleoproteins leading to changes in the shape and size of the nucleus and the compaction state of the chromatin (Dadoune, 2003).

The final stage of spermatogenesis includes spermiogenesis in which a fully differentiated, mature sperm produced from an early round spermatid. The remodeling that occurs during spermiogenesis including replacement of somatic histones with protamines produces a cell in which the nucleus is transcriptionally inactive, and the majority of the cytoplasm is shed in the form of a residual body (Sakkas *et al.,* 2002; Aoki and Carrell, 2003).

Sperm DNA is organized in a specific manner that keeps the nuclear chromatin compact and stable (Agarwal & Said, 2003). Packaging of the sperm DNA with specific nucleoproteins; protamines into tight and crystalline status at least six times more condensed than in mitotic chromosomes, sperm chromatin structure protects the integrity of the genetic materials during transport of the paternal genome through the male and female reproductive tracts or injection into oocyte cytoplasm (Fuentes-Mascorro *et al.,* 2000; Zini *et al.,* 2001; Evenson *et al.,* 2002). Spermatozoa retain some of their original histone content that forms less-compact nucleosome structures, which is important in the sperm nucleus to respond to oocyte signals for pronucleus formation after sperm penetration (Bench *et al.,* 1996; Gineitis *et al.,* 2000).

2-1- Human sperm chromatin structure

Sperm chromatin differs from somatic cells in structure and composition. Sperm chromatin has a somatic cell-like structure during the last meiotic division of spermatocytes leading to round spermatids and consists of histone-DNA complex formed classical nucleosome core particles (Evenson, 1999). The nucleosome comprises DNA enveloped around an octamer of core histones (Kornberg, 1974; Noll, 1974). Each nucleosome is connected by linker DNA to give somatic chromatin the appearance of beads on a string (Wolffe, 2001). Concomitant with visible changes in sperm chromatin organization, histones are removed from the DNA of spermatocytes in early spermatids and replaced by transition proteins. Transition proteins are then replaced by protamines

that are responsible for the final condensation and stabilization of sperm chromatin (Steger, 1999; Kierszenbaum, 2001; Zhao *et al.,* 2001).

Therefore, sperm chromatin is a highly organized, compact structure consisting of DNA and nucleoproteins. Protamines are the most abundant nucleoproteins in mature sperm. They are positively charged and replace histones during spermiogenesis (Carrell *et al.,* 2007). This condensed, insoluble and highly organized sperm chromatin structure protects the genetic materials during transport of the paternal genome through the male and female reproductive tracts, also, allow proper fusion of male and female gametes and correct expression of the genetic information by the developing embryo (De Jonge 2000; Solov'eva *et al.,* 2004). Humans express two protamines, protamine 1 (P1) and protamine 2 (P2), both of which are expressed in roughly equal quantities with a mean P1/P2 ratio of approximately 1.0 (Balhorn *et al.,* 1999; Carrell and Liu, 2001; Oliva, 2006).

Human sperm nuclei contain approximately 85% protamines in their nucleoprotein component, considerably fewer than those of a bull, stallion, hamster and mouse (Gatewood *et al.,* 1987; Bench *et al.,* 1996). Human sperm chromatin, therefore, is less regularly compacted and frequently contains DNA strand breaks (Sakkas *et al.,* 1999b; Irvine *et al.,* 2000).

Sperm DNA interacts with protamines in a unique fashion that involves the coiling of sperm DNA into toroidal subunits, also known as doughnut loops. Toroid containing 50–60 kb of DNA is the packaging unit of the sperm chromatin. Each toroid represents a DNA loop-domains highly condensed by protamines and fixed at the nuclear matrix. They are cross linked by disulfide bridges of cysteine residues of the protamines (Fuentes-Mascorro *et al.,* 2000). Thus, chromosome contains many toroids; at the completion of spermiogenesis there can be up to 50,000 toroidal structures packed within the sperm nucleus (Balhorn *et al.,* 1999). Toroids and all 23 chromosomes are clustered by centromeres into a compact chromocenter positioned well inside the nucleus with telomere ends united into diamers exposed to the nuclear periphery (Zalensky *et al.,* 1995; Solov'eva *et al.,* 2004) The retention of 15 % histones, which are less basic than protamines, leads to the formation of less compact chromatin structure. This structure facilitates the decondensation process after fertilization. Reduction of the protamine disulphide bonds that allows protamine removal is one of the first steps to occurr (Gatewood *et al.,* 1987). Protamines replacement by histones may also be necessary for silencing of the paternal genome and reprogramming of the imprinting pattern of the gamete (Aoki and Carrell, 2003). Figure 1 represents the major chromatin changes occurring during the nucleohistone–nucleoprotamine transition.

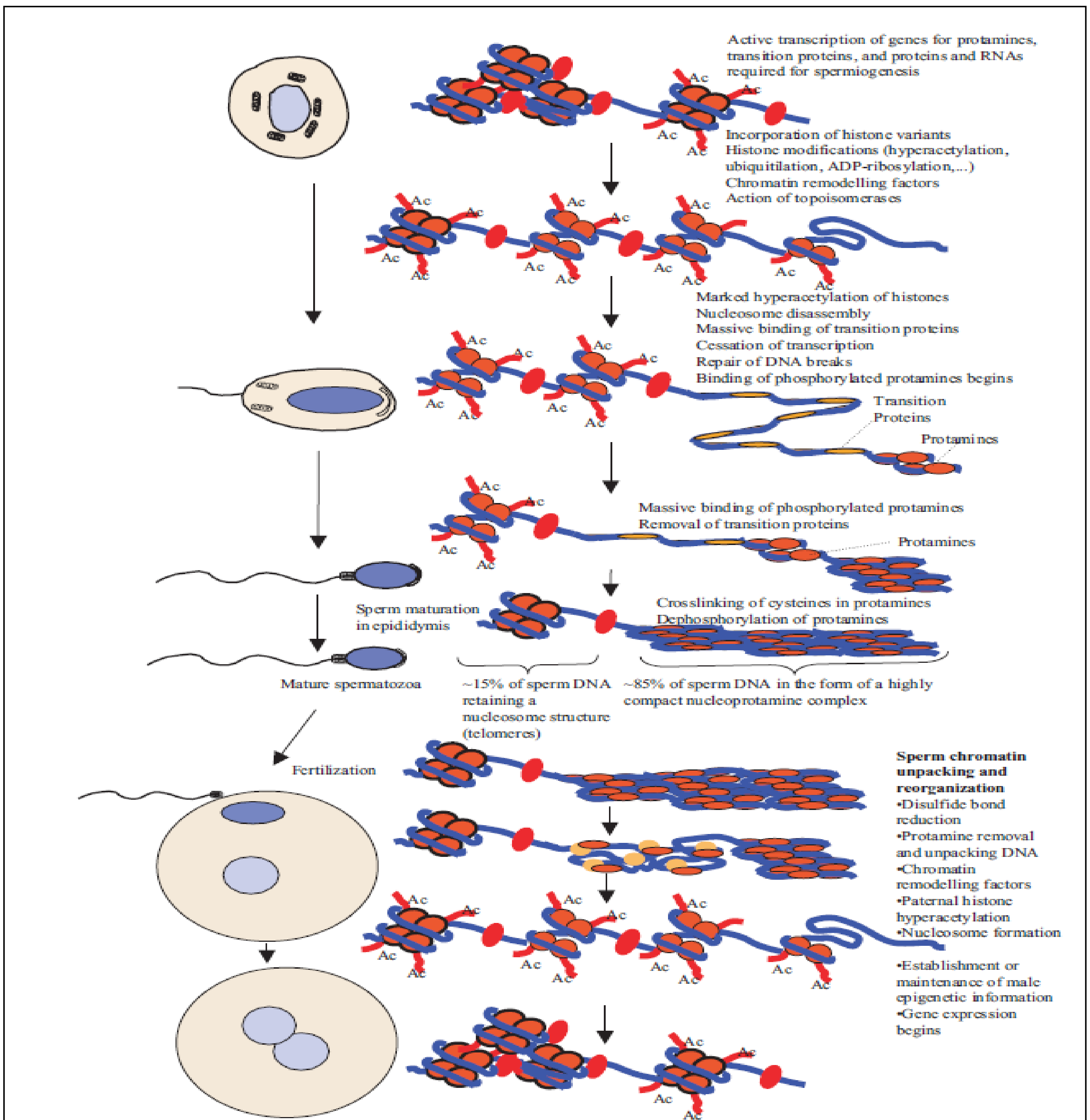


Figure 1. Schematic representation of the major chromatin changes occurring during the nucleohistone–nucleoprotamine transition in spermiogenesis and the subsequent nucleoprotamine unpacking and nucleohistone structure reconstitution at fertilization. The round spermatid (top left) has a chromatin structure similar to that present in all somatic cells, with the DNA organized in nucleosomes and many genes being actively transcribed. During the initial stages of spermiogenesis, histones are hyperacetylated and undergo other modifications, nucleosomes are disassembled, topoisomerase II unwinds superhelicity of the DNA, transcription ceases and transition proteins (TNPs) bind the DNA. At the final stage of spermiogenesis, TNPs are removed and protamines progressively bind the DNA. During sperm maturation in the epididymis, the formation of disulphide bonds in protamines further stabilizes the nucleoprotamine complex. At fertilization, the highly compact nucleoprotamine structure must be unpacked and reorganized into a nucleosomal structure. Histones are represented in red and DNA is represented by blue lines. The presence of hyperacetylation in the N-terminal histone tails is indicated by ‘Ac’. Transition proteins are represented as orange elongated ovals. Protamines are represented as red elongated ovals (Adapted from Oliva, 2006).

2-2- Sperm Nuclear Proteins

2-2-1- Histones

Histones, the main proteins of chromatin, are essential for packaging of the DNA in chromosome (Redon *et al.,* 2002). Conventional histones are mostly synthesized and assembled into nucleosomes during S phase progression; whereas, replacement histones can be produced and incorporated throughout the cell cycle (Govin *et al.,* 2004). The highly basic nature of histones facilitates DNA-histone interactions, and contributes to the water solubility of histones. They are characterized by relatively high levels of lysine and arginine.

The mutagen family encoding the five classes of replication-dependent histones has been identified from the human genome sequence. The human replication-dependent histone genes are found in clusters: the largest cluster is on chromosome 6 (6p21–p22) and contains 55 histone genes (Albig and Doenecke, 1997; Albig *et al.,* 1997) and is termed *HIST1*. Two smaller clusters of histone genes are present in *HIST2* the first on chromosome 1 (located at 1q21), which contains six genes, and the second, termed *HIST3* on human chromosome 1(located at 1q42), which contains three histone genes (Marzluff *et al.,* 2002).

Histones are part of the building structural unit of chromatin,the nucleosome, which consists of 146 base pairs of DNA wrapped around an octamer of core histones, including two molecules of H2A, H2B, H3 and H4 (Luger *et al.,* 1997). There is also a fifth histone H1 that protects additional DNA fragments linking neighboring nucleosomes (Khochbin, 2001). More specifically, the histones H3 and H4 form a dimer, two H3–H4 dimers associate into a (H3–H4) 2 tetramer. DNA wraps around this tetramer, forming a tetrameric particle. Histones H2A and H2B heterodimerize and heterodimers associate on each side of the tetrameric particle to form a nucleosome (Luger *et al.,* 1997). Variants of histones H2A, H2B, H3 and H1 also have been identified (Table 1).

Table1. Variants of histones in mammalian spermatogenic cells (adapted from Govin *et al.,* 2004; D'Occhio *et al.,* 2007)

Histone	Somatic Variants	Gametogenic variant	Testis specific variant
H1	H1b, H1c, H1d, H1e, H1°	H1a	H1t, HILS1
H2A	H2A.1, H2A.2, H2A.Z	H2A.X	TH2A
H2B	H2B.1	--	TH2B
H3	H3.1, H3.2, H3.3	--	TH3/H3t
H4	No variants	No variants	No variants

In addition to the somatic-type histone variants, spermatogenic cells express testis-specific histones that replace somatic histones (Dadoune, 2003). A structural difference had been observed between testis-specific histones and somatic histones in the N-terminal region, the core region and the C-terminal region. The testis specific histone variants did not undergo polyadenylation and was translated early during spermatogenesis (Zalensky *et al.,* 2002). H2B variant had been localized in telomeres and contain a phosphorylatable amino acids instead of four prolines found in the N-terminal region of the somatic H2B (Churikov *et al.,* 2004a).

Moreover, histones are subject to post translational modification by enzymes primarily on their N-terminal tails, but also in their globular domains. Such modifications include methylation, citrullination, acetylation, phosphorylation, ubiquitination, and ADP-ribosylation. This affects their function in gene regulation.

Recently, Li *et al.,* (2008) found that the core histones and chromosome 16 telomeric regions were localized at the base of the sperm nuclei. Also, previous studies reported that the nucleohistone components localized in the posterior region of the nuclei and extend to the region occupied by the sperm nuclear annulus, likely provide an anchor (Ward and Coffey, 1989; Barone *et al.,* 1994).

Other reports showed that histones associated with specific regions that include the telomeres, promoter and repeating regions and certain small gene-rich regions (Wykes and Krawetz, 2003; Namekawa *et al.,* 2006).

The highly packaging of the sperm nucleus might be necessary for proper relaxing of the male genome during fertilization (Zalensky and Zalenskaya, 2007). van der Heijden *et al.,* (2006) suggested that nucleohistone component remains in the nucleus could serve as a template for the replacement of protamines by histones after fertilization. While others have suggested that this histones could influence transcription of some genes after fertilization (Gardiner-Garden *et al.,* 1998). Perhaps this unique packaging is essential for appropriate decondensation and reorganization of the paternal genome following fertilization. In contrast, both protamines were distributed more evenly throughout the nucleus (Li *et al.,* 2008).

2-2-2- Transition nuclear proteins (TPs)

Replacement of histones with protamines pass first through another type of nuclear proteins called transitional proteins (TPs). Despite the presence of at least four transition proteins, in man (Luerssen *et al.*, 1988) transitional proteins 1 (TP1) and 2 (TP2) are the major ones. Transition proteins, TP1 and TP2 are unique to mammalian spermatogenesis and play an important role in the spermiogenesis process. The transition nuclear proteins (TPs) constitute 90% of the chromatin basic proteins during the steps of spermiogenesis between histone removal and the deposition of the protamines (Yu *et al.*, 2000).

They are arginine- and lysine-rich proteins. They are of intermediate basicity. TP1 is a 6.2 kDa protein of about 54 amino acid rich in arginine (20%), lysine (20%), histidine and serine. They are distributed uniformly throughout the molecule, but contains no cysteine (Kistler *et al.,* 1975; Dadoune, 1995; 2003). It is important for destabilization of the spermatozoa DNA probably due to the presence of two tyrosine residues flanked by basic amino acids (Schumacher *et al.,* 1998). In vitro studies have shown that the possible functions of TP1 includes relaxation of the DNA in nucleosomal core particles (Singh and Rao 1988), decreasing the melting temperature of DNA (Akama *et al.,* 1998), and stimulating the DNA- relaxing activity of topoisomerase I (Akama *et al.,* 1999). Also TP1 can stimulate single-strand break repair in vitro and in transfected somatic cells (Caron *et al.,* 2001).

TP2 is a 13 kDa protein (117–138 amino acids) containing proline (13%), serine (22%), arginine (14%), lysine (9%) and cysteine (5%) basic residues (Grimes *et al.,* 1975; Dadoune, 2003). Also it has a highly basic C terminal domain and a zinc finger N-terminal domain (Meetei *et al.,* 2000), which is essential for the recognition of CpG islands in the genome (Pradeepa and Rao, 2007). Contrary to TP1, TP2 can increase the compaction of the DNA in nucleosomal cores, increasing the melting temperature of DNA. It has been proposed to be a DNA-condensing protein (Baskaran and Rao 1990). Pradeepa and Rao, (2007) showed that TP1 is a DNA melting protein while TP2 is a DNA condensing protein and TP2 has zinc, which is essential to the recognition of CpG islands in the genome.

The sequence of TP1 is highly conserved in various mammals, while the TP2 sequence is poorly conserved (Kremling *et al.,* 1989; Alfonso and Kistler 1993). The mRNA of these proteins (TP1 and TP2) first appears in the post-meiotic, round spermatid stage in mice and are degraded around stages, 13–14 of spermiogenesis, while the proteins are observed in stages 12 and 13 and are removed by stage 14 when replaced with protamines (Kistler *et al.,* 1996). In addition, in transgenic mice, failure of complete chromatin compaction and increase in DNA denaturation was indicated due to the reduced amount of protamine 2 that resulted in animals without TP2 (Zhao *et al.,* 2001; Cho *et al.,* 2003; Meistrich *et al.,* 2003).

The work of Zhao *et al.,* (2004) on mice showed that TP1 and TP2 had partially complemented each other and both are required for normal chromatin condensation, for reducing the number of

DNA breaks, and for preventing the formation of secondary defects in spermatozoa, eventual loss of genomic integrity, and sterility.

TP1 and TP2 are encoded by single copy genes, Tnp1 and Tnp2. Tnp2 is found in the same chromosome with the two protamine genes (Engel *et al.,* 1992), which suggests that they arose by gene duplication and might have retained common functions (Meistrich *et al.,* 2003). On the other hand, Tnp1 was found on a separate chromosome (Heidaran *et al.,* 1989).

Transition protein 3 (76–103 amino acids) has been isolated and characterized in the rat, ram and boar (Dadoune, 2003). Transition protein 4 also has been characterized in rat and boar late spermatid nuclei; it is a basic protein of 138 amino acids comprising a highly basic amino-terminal region and a less basic carboxy-terminal region (Wouters-Tyrou et al., 1998). In the rat, transition protein 4 is a unique protein, is immunologically distinct from transition proteins 1–3, protamine 1 and histones, and shares no sequence homology with other known proteins (Unni and Meistrich, 1992).

2-2-3- Replacement of histones by protamines

Replacement of histones by protamines involved many steps, including the expression and incorporation of testis-specific histone variants, histone hyperacetylation, replacement of histones with transition proteins and protamine incorporation and phosphorylation (Aoki and Carrell, 2003; Churikov *et al.,* 2004b). At the end of the spermatogenesis, the nucleosomal structure is disassembled and replaced by transition proteins, then finally by protamines. This transition is continuing with many changes in the chromatin activities (Kierszenbaum and Tres, 2004; Li *et al.,* 2008). Although, protamines replaced most of the histones —approximately 85%. The human male gamete retains approximately 15% of its genome in a histone-bound state (Gatewood *et al.,* 1987, Li *et al.,* 2008) in camparison to other species such as the mouse where replacement reached more than 98% (de Yebra *et al.,* 1993; Zalensky *et al.,* 2002. This structure facilitates the decondensation process after fertilization. Reduction of the protamine disulphide bonds to allow protamine removal is one of the first steps occurring. Figure 2 highlights the key events in the transition of somatic histones to replacement by protamines. Most of these spermatozoal histones are variants of their somatic histone counterparts, whichpackage the DNA into nucleosomes that are more closely packed than in somatic chromatin. The DNA that is packaged by histones in mature sperm includes the genes for ε-globin and γ-globin (Gardiner-Garden *et al.,* 1998) and telomeric DNA (Banerjee *et al.,*1995; Zalenskaya *et al.,* 2000).

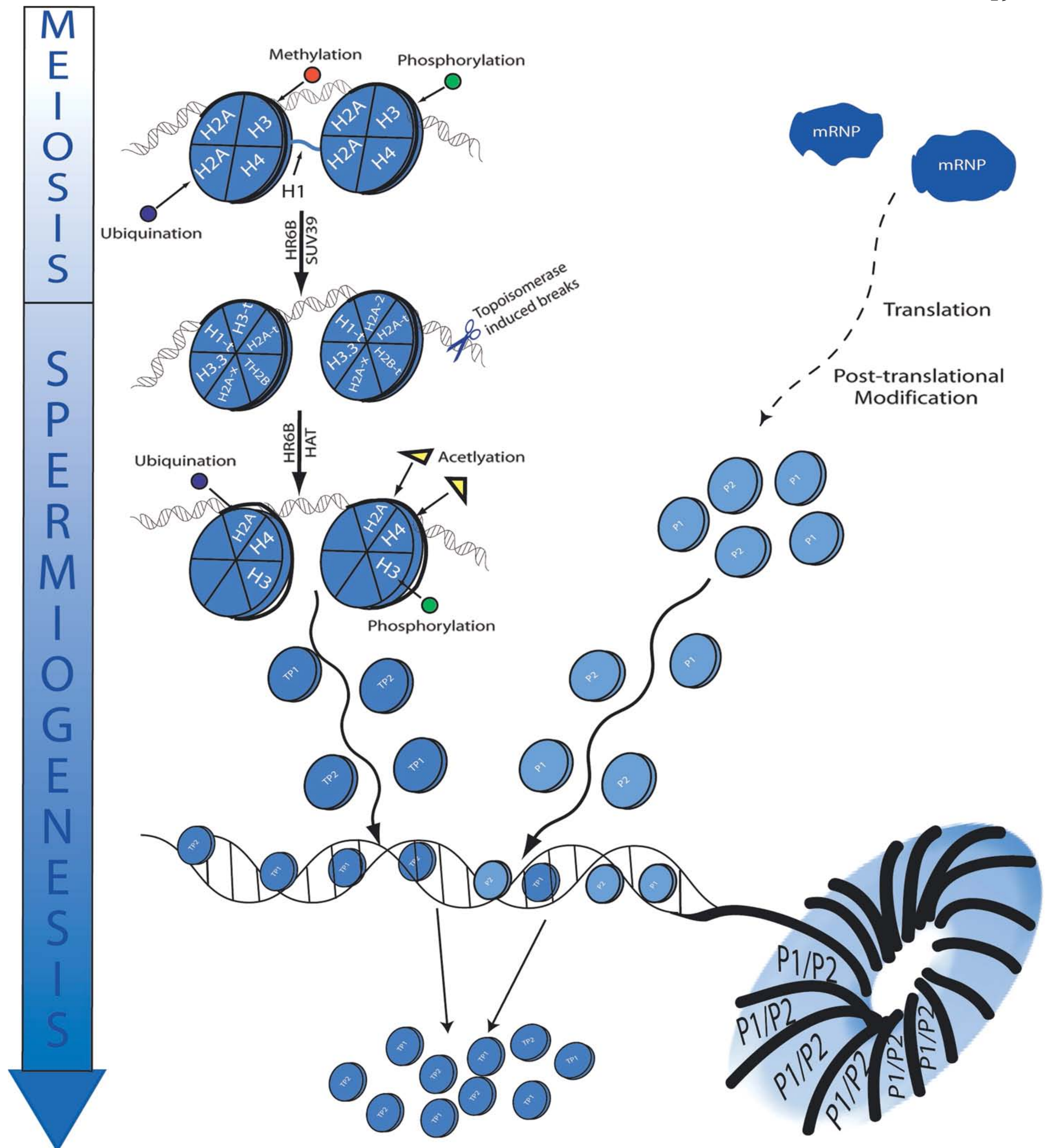


Figure 2. Diagram highlighting the key events in the transition of somatic histones to replacement by protamines. Somatic histones undergo site-specific methylation, phosphorylation and ubiquitination which facilitates their replacement by testis-specific histones (t) during meiosis. Hyperacetylation of H4-t is a key factor in relaxation of the DNA coil to facilitate replacement of the testis-specific histones by the transition proteins, whereas topoisomerase 1 relieves torsional stress by causing double-strand breaks which are subsequently re-ligated. Protamines 1 and 2, processed from a pool of RNP particles, undergo maturation before and during binding to the DNA and replacemnt of the transition proteins. HR6B, ubiquitin-conjugating enzyme E2B (UBE2B) (RAD6 homolog); HAT, histone acetyltransferase; Suv39, H3 Lys 9 histone methyltransferase (adapted from Carrell *et al.,* 2007).

Hyperacetylation of the histones regulation by histone acetyl transferases and histone deacetylases reduces the binding between nucleosomes and DNA, leading to chromatin relaxation and is also associated with the activation of topoisomerases that inducing strand breaks (Hazzouri *et al.,* 2000; Marcon and Boissonneault, 2004). This histone hyperacetylation and rapid turnover of acetyl groups could rapidly and reversibly expose binding sites in chromatin for subsequent binding of chromosomal proteins (Oliva and Mezquita, 1982). It was proposed that hyperacetylation of core histones may facilitate the replacement of histones by protamines (Oliva and Mezquita, 1986; Oliva *et al.,* 1987; Sonnack *et al.,* 2002).

However, DNA relaxation under the effect of histones hyperacetylation and topoisomerases activity allows the replacement of histones with transition proteins (Kierszenbaum, 2001; Meistrich *et al.,* 2003). The transition nuclear proteins (TPs) replaced about 90% of the chromatin basic proteins during the steps of spermiogenesis (Yu *et al.*, 2000). Finally, protamines replace the transition proteins to form a highly compact nucleoprotamine complex. After binding to the DNA, the formation of disulphide bonds between protamines further stabilizes the nucleoprotamine complex (Balhorn *et al.,* 1992). A significant overlap in the expression of histones, transition proteins and protamines was proposed (Meistrich *et al.,* 2003). Therefore, expression of the proteins has been shown to have some overlap in human-elongating spermatids (Aoki *et al.,* 2006b).

Immediately after the translation of P1 and P2 intermediate, they were phosphorylated (Green *et al.,* 1994; Papoutsopoulou *et al.,* 1999). This phosphorylation is necessary for proper binding of the proteins to DNA. However, once protamines were bound to DNA, dephosphorylation occurred and this process appears to be essential for proper condensation of the chromatin (Gusse *et al.,* 1986; Aoki and Carrell, 2003).

A conformational change in the packaging of the chromatin was induced once the transition proteins were replaced by protamines. A loop of less than half the size of somatic cell histone loops formed followed by formation of toroidal structures, which have a 6–20-fold increase in packaging compaction (Ward and Coffey, 1991; Balhorn *et al.,* 2000). P1 and P2 may bind to the major and minor groove of DNA or to the DNA surface by interacting electrostatically with phosphate residues (Balhorn *et al.,* 1999; Fuentes-Mascorro *et al.,* 2000).

2-2-4- Protamines

Protamines are a diverse family of small and highly basic proteins (5–8 kDa) which are about half the size of a typical histone. They are short of about 50-110 amino acids, and they are positively charged due to the high content of positive charged amino acid, the arginine residues (the major of the amino acid residues) that form about 55 to 79% of the amino acid residues, permitting a strong binding with DNA. But, the total number of amino acids and the positions of the arginine residues have changed considerably (Rooney *et al.,* 2000). It was proposed that the driving forces for this arginine-rich selection could be (i) the DNA-binding function of the protamine P1 resulting in a more compact sperm nucleus and (ii) the interaction and strong activation of oocyte creatine kinase II by protamine (Ohtsuki *et al.,* 1996; Rooney and Zhang, 1999).

A significant amount of cystein residues that are very important in final sperm nuclear maturation by forming multiple inter- and intra protamine disulfides cross bonds (Loir and Lanneau, 1984). Formation of these inter and intra molecular disulphide bonds between cysteine residues will strongly stabilize the nucleoprotamine structure in the sperm nucleus and stabilize the folding of different protamine domains (Vilfan *et al.,* 2004).

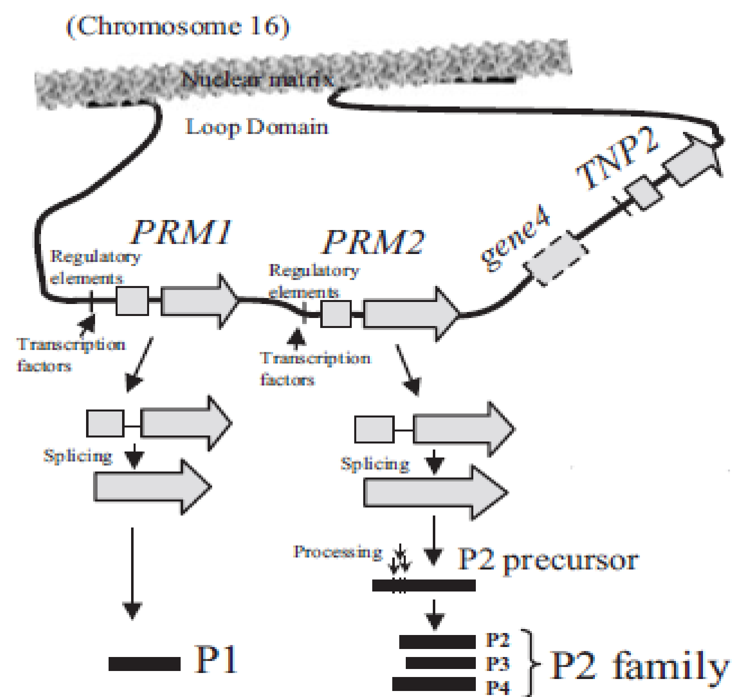
2-2-4-1 - Genes of protamines

Humans have one copy of the protamine 1 gene (*PRM1*) and one copy of the protamine 2 gene (*PRM2*) per haploid genome, located on chromosome 16 at 16p13.3 (Figure 3; Oliva and Dixon, 1991; Wykes and Krawetz, 2003). The mammalian P1 and P2 genes contain a single intron. The sequences of these genes (*PRM1* and *PRM2*) are organized in the form of a loop domain together with the transition protein 2 gene (*TNP2*) and a sequence called *gene4* or protamine 3 (Engel *et al.,* 1992; Martins *et al.,* 2004). The P1-P2-TP2 locus spans a 28.5-kb region and is organized in a linear array, a structural feature affording concurrent expression of the P1, P2 and TP2 genes (Choudhary *et al.,* 1995). The P1 gene is present in all mammalian species, whereas P2 is present in the mouse, hamster, rat, stallion and man (Calvin, 1976; McKay *et al.,* 1986; Bower *et al.,* 1987; de Yebra *et al.,* 1993). However, in some species, the protamine 2 gene is present but the protein is absent (Maier *et al.,* 1990).

The protamine 3 (*gene 4*) contains a repeating glutamic and aspartic acid residues similar in number and distribution to the clusters of arginine and lysine residues found in the DNA-binding domains of protamines. The amino-acid sequence for this protein approximately has the same size as protamine P2. This composition with a high content of negatively charged amino acids suggests that it is not binding or condensing DNA and may have other functions (Balhorn, 2007).

The structure of the protamine genes plays a major role in their transcriptional regulation (Aoki and Carrell, 2003): First, the highly conserved sequence of cAMP-response element (CRE) that is present in all protamine genes and is found residing from position -57 to -48 (Johnson *et al.,* 1988), regulates transcription by binding various CRE proteins to this regulatory region (Tamai *et al.,* 1997). Second, upstream regulatory sequences in the individual P1 and P2 promoters bind other trans-acting proteins, thereby directing transcriptional activation or suppression (Peschon *et al.,* 1989; Zambrowicz *et al.,* 1990). Third, in round spermatids, the P1 and P2 genes are located in a large methylated domain which facilitates nuclear matrix attachment and potentiation of the P1-P2-TP2 gene locus (Choi *et al.,* 1997). The repetitive alanine (Ala) elements that is found in the matrix attachment regions (MAR) serve as sites of methylation that flanked this P1-P2-TP2 multigenic locus (Schmid *et al.,* 2001). Fourth, the presence of a TATA-box in all protamine genes facilitates binding of transcription factors to their promoters, thereby playing a major role in transcription initiation.

A



B

Protamine 1 (P1) ARYRCCRSQSRSRYYRQRQRSRRRRRRSCQTRRRAMRCCRPRYRPRCRRH

Protamine 2 family

P2 RTHGQSHYRRRHCSRRRLHRIHRRQHRSCRRRKRRSCRHRRRHRRGCRTRKRTCRRH

P3 GQSHYRRRHCSRRRLHRIHRRQHRSCRRRKRRSCRHRRRHRRGCRTRKRTCRRH

P4 ERTHGQSHYRRRHCSRRRLHRIHRRQHRSCRRRKRRSCRHRRRHRRGCRTRKRTCRRH

Figure 3: Transcription of the protamine genes and translation and processing of human protamines. (**A**) Schematic representation of the genomic structure of protamine genes (*PRM1* and *PRM2*) and the transcription, translation and processing involved in the synthesis of mature protamine. *TNP2,* gene-encoding transition protein 2. (**B**) Amino acid sequences for human P1 and for the main components (P2, P3 and P4) of the protamine 2 family. It should be noted that P2 is the most abundant component, while P3 and P4 are minor components of the P2 family. The arginine, histidine and lysine residues are shown in bold. Cysteines are underlined (adapted from Oliva, 2006).

2-2-4-2 - Synthesis of protamines

Protamines are synthesized in the late-stage spermatids of many animals and plants and bind to DNA, condensing the spermatid genome into a genetically inactive state (Balhorn, 2007). They are synthesized in soluble polyribosomes in the cytoplasm of elongating spermatids (Kleene, 1996). Protamine P1 is synthesized directly as a mature protein, whereas the protamine P2 family generated by proteolysis from a precursor encoded by a single gene (Wouters-Tyrou *et al.,* 1998). The P2 family members differ from each other only by the N-terminal extension of 1–4 residues, although the P2 component is the most abundant (Figure 4) (Yoshii *et al.,* 2005). The ratio of the contents of protamine P1 and protamine P2 (P1/P2 ratio) in the human sperm nucleus is roughly similar, P1/P2 ratio is about 1 (Balhorn *et al.,* 1988; Corzett *et al.,* 2002; Mengual *et al.,* 2003; Aoki *et al.,* 2005a). Transcription and translation of protamine mRNAs has been shown to occur in specific spermatid stages (Millar *et al.,* 2000), and protamine mRNA has not been detected in sertoli or interstitial cells or in other tissues (Wykes *et al.,* 1995).

Post-translational modifications of protamines included the phosphorylation-dephosphorylation process and formation of the disulfide bonds that form during the final stages of sperm maturation and epididymal transit in eutherian mammals. However, during chromatin remodeling, two distinct stages have been described involving protamines (Love and Kenney, 1999; Dadoune, 2003). Stage 1 involves the phosphorylation and dephosphorylation of serine and threonine residues of

protamines (Dadoune, 2003) and Stage 2 involves the stabilization of DNA by the formation of inter- and intra-molecular disulfide bonds between cysteine residues of protamine molecules (Love and Kenney, 1999). Direct phosphorylation of the protamines after synthesis occurred. This step may be required for the proper binding to DNA (reviewed in Oliva and Dixon, 1991); but, after binding to DNA, most of the phosphate groups are removed and cysteine residues are oxidized, forming disulfide bridges that link the protamines together (Balhorn, 2007). In humans, it has been shown that phosphorylated protamines are still present in mature spermatozoa and the corresponding phosphorylation sites of P1 and P2 have been determined (Papoutsopoulou *et al.,* 1999). Given the importance of phosphorylation in regulating protein function, the possibility that altered protamine phosphorylation could also be associated with infertility or assisted reproduction outcomes deserves to be evaluated (Oliva, 2006).

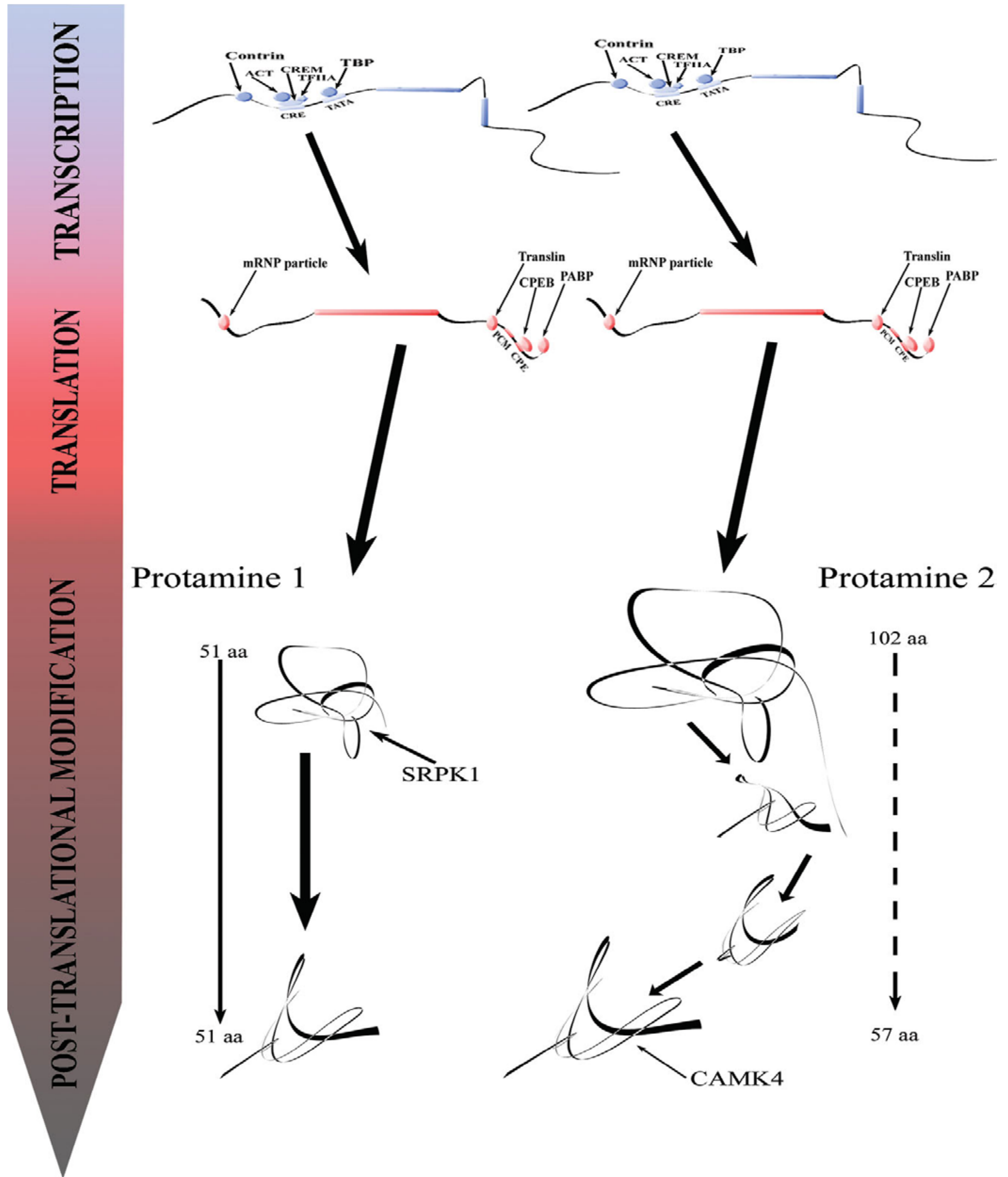


Figure 4. A schematic drawing of key regulators of expression of protamines 1 and 2 at the levels of genomic transcription, translation and post-translational modifications to yield the mature protamine products. The key regulating protein complexes are shown for each stage of protein maturation. CREM, cAMP response element modulator; ACT, activator of CREM in the testis; TFIIA, transcription factor II alpha; CRE, CREM response element; TBP, TATA box binding protein; PABP, poly-a binding protein; CPE, cytoplasmic polyadenylation element; CPEB, cytoplasmic polyadenylation element binding protein; PCM, polyadenlyation consensus motif; SRPK1, serine/arginine protein-specific kinase-1; CAMK4, calcium/calmodulin-dependent protein kinase IV (adapted from Carrell et al., 2007).

2-2-4-3- Characteristic structural features

Mammals including humans express two protamines (protamines P1 and P2). They are the most abundant nuclear proteins present in the human sperm nucleus (Aoki and Carrell, 2003; Carell *et al.,* 2007). Most of the information about the structure of protamines and DNA-protamine complexes were obtained from studying the protamines P1 and P2 of placental mammals, and from the fish protamines salmine and clupine (Balhorn, 2007). The human P1 and P2 genes encode a 50 amino-acid protein and a final processed protein of 57 amino-acids, respectively. Overall, there is approximately 50 % identity between human P1 and P2.

2-2-4-3-1- Protamine P1

The P1of the mammalian placenta is exactly 49 or 50 amino acids long and contains three domains. A central arginine-rich DNA-binding domain flanked on both sides by short peptide segments containing cysteine residues. In most species, the central DNA binding domain typically consists of a series of anchoring sequences containing 3-11 consecutive arginine residues, which bind the protein to DNA (Balhorn, 2007). P1 synthesized as a mature protein coded by a single gene. Also, P1 was found in all studied vertebrates (Yoshii *et al.,* 2005; Torregrosa *et al.,* 2006).

2-2-4-3-2- Protamine P2

P2 has several features that distinguish it from P1: the gene *PRM2* codes for a precursor protein that has been shown to bind to DNA first, then undergo proteolytic processing (de Yebra *et al.,* 1993; Queralt *et al.,* 1995). P2 is synthesized as a precursor protein of 103 amino acids and undergoes proteolytic cleavage of its amino-terminus (Balhorn *et al.,* 1999). In humans, there are two differently processed forms of protamine P2 (P2 and P3) bounded to DNA. The two forms of the P2 protein differ only in their three amino-terminal amino acids; P3 is three amino acids shorter (at 54 amino-acid residues) than P2 (57 amino acids) and they seem to be products of the same PRM2 gene. A third protamine P2 (named P4) sequence variant has also been detected in humans (Balhorn *et al.,* 1977; Gusse *et al.,* 1986). In addition, protamine P2 differs from P1 in that P2 binds to zinc. Roughly equal amounts of P1and P2 are considered normal in human spermatozoa (Bianchi *et al.,* 1992; de Yebra *et al.,* 1993; Oliva, 2006).

However, many hypotheses discuss the differences between P1 and P2 protamines:

(i) Unlike P1 protamine, P2 protamines are zinc-finger proteins with one Cys2–His2 motif (Bianchi *et al.,* 1992).

(ii) Although the arginine content of both proteins is similar, P2 is slightly more basic due to its higher histidine and lysine content.

(iii) P2 proteins are expressed only in some mammals; whereas, P1 is invariably present in all mammals, indicating a more basic and conserved function for P1 and an accessory function for P2 protamines in some species.

(iv) Alterations of P1 or P2 protamines in infertile patients impact differently on the integrity of the DNA and in the assisted reproduction outcome (Aoki *et al.,* 2005b).

Both protamines will undergo post-transcriptional modifications before binding to the DNA and generating the highly compact nucleoprotamine complex (Oliva, 2006).

Protamines phosphorylation is required to facilitate their binding with DNA. The physiological significance of protamine phosphorylation had been proposed: first, Oliva and Dixon, 1991 suggested that P1/P2 phosphorylation is required for correct binding to DNA and their subsequent dephosphorylation induces chromatin condensation in the sperm nucleus (Oliva and Dixon, 1991). Second, phosphorylation (not dephos-phorylation) may also promote correct chromatin

condensation (Papoutsopoulou *et al.,* 1999). In humans, it has been shown that phosphorylated protamines are still present in mature spermatozoa and the corresponding phosphorylation sites of P1 and P2 have been determined (Papoutsopoulou *et al*., 1999).

2-2-4-4- Protamines Functions

Several proposed functions of the protamines had been reviewed by Oliva and Dixon, (1991):

(i) Condensation of the paternal genome of the spermatozoa that produce a more compact and hydrodynamic nucleus. Those allow the sperm to move faster and more efficient to fertilize the oocyte.

(ii) Protecting the paternal genome from the effect of nucleases or mutagens potentially present in the internal or in the external media.

(iii) Competition and removal of transcription factors and other proteins from the spermatid resulting in a blank paternal genetic message, devoid of epigenetic information, therefore allowing its reprogramming by the oocyte.

(iv) Involvement in the imprinting of the paternal genome during spermatogenesis.

(v) Protamines could be part of a checkpoint during spermiogenesis.

(vi) They may play a role in the fertilized oocytes.

Balhorn, (2007) demonstrated that of the above proposed functions for protamines, only one has been unequivocally demonstrated. The synthesis and deposition of protamine in spermatid chromatin has been shown to correlate temporally with the condensation of the genome of the elongating spermatid and the concomitant termination of transcription. Each protamine P1 molecule binds to 10-11 bp of DNA; protamine P2 binds to a slightly larger segment of DNA (around 15 bp). This binding neutralizes the negative charge along the phosphodiester backbone of DNA and enables adjacent DNA molecules to pack close together. Protamine binding to DNA does result in the production of an uncharged chromatin complex that enables the DNA molecules to be condensed into a volume some 1/20th that of a somatic nucleus. This condensation enables the production of a smaller, more hydrodynamic head, and contributes, albeit indirectly, to head shape (Balhorn, 2007).

Formation of intermolecular and intramolecular disulphide bonds between cysteine residues in the protamines will strongly stabilize the nucleoprotamine structure, which in turn stabilizes the folding of different protamine domains in the sperm nucleus (Vilfan *et al*., 2004). Glutathione peroxidase activity could be involved in disulphide cross-linking in protamines (Conrad *et al*., 2005).

2-2-4-5- Structural – functional relationships

Oliva, (2006) had reviewed many structural and functional roles of protamines in the cell:

1- By providing positive charge residues, they bind the strong negatively charged paternal genomic DNA allowing the formation of highly condensed complex (Lewis *et al.,* 2003).

2- By containing cysteines, they allow the formation of the disulphide bridges between the adjacent protamine molecules, therefore stabilizing the nucleoprotamine complex (Balhorn *et al.,* 1992).

3- They are of the highest rates of evolutionary variation among proteins (Oliva, 1995; Lewis *et al.,* 2003). A positive Darwinian selection has been proposed as a cause of this rapid evolution rate (Rooney and Zhang, 1999; Wyckoff *et al.,* 2000).

2-2-4-6- Sperm DNA integrity and Protamines

Spermatozoa DNA fragmentation may occur due to the attack of different endo- or exogenous factors such as nucleases, free radicals, mutagens and other agents if protamination of the DNA is not completed (Szczygiel and Ward, 2002; Sotolongo *et al.,* 2003). One of the hypotheses about the function of protamines is that they could be involved in the protection of the genetic message delivered by the spermatozoa (Mengual *et al.,* 2003).

It is important to note that many groups showed that DNA fragmentation is more frequent in protamine-deficient spermatozoa and a significant negative correlation with fertilization rate was found (Nasr-Esfahani *et al.,* 2004b; 2005; Aoki *et al.,* 2005b; Torregrosa *et al.,* 2006). Greco *et al*., (2005) showed that the use of ICSI with testicular sperm had improved the pregnancy rates in patients with poor pregnancy rates and decreased DNA integrity of ejaculated spermatozoa. Thus, a reasonable explanation could be that incomplete or abnormal protamination, as observed in many studies (Table 2), could lead to incomplete disulphide bond formation and incomplete DNA protection during epididymal passage in these patients.

Sperm DNA fragmentation is correlated significantly with low P1/P2 ratio compared with normal and high P1/P2 ratios (Aoki *et al.,* 2005b). Moreover, patients who under-expressed P1, P2 or both P1 and P2 had significantly elevated levels of DNA fragmentation compared with patients normally expressing P1and P2.

Heterogeneity in protamine expression and a clear correlation between under-expression of protamines, DNA damage and lack of viability was detected using many techniques (TUNEL assay, CMA_3 and Aniline Blue staining assays) (Manicardi *et al.,* 1995; Hammadeh *et al.,* 2001; Aoki *et al.,* 2006b).

2-2-4-7-Protamines and infertility

Human sperm protamine deficiency had been correlated significantly with diminished semen quality parameters (counts, motility and morphology), sperm functional ability, and sperm DNA integrity (de Yebra *et al.,* 1993; 1998; Balhorn *et al.,* 1999; Carrell and Liu, 2001; Mengual *et al.,* 2003; Aoki *et al.,* 2005a). Many studies correlated the male infertility with aberrations in protamine expression (Chevaillier *et al.,* 1987; Balhorn *et al.,* 1988; Chevaillier *et al.,* 1990; Belokopytova *et al.,* 1993; de Yebra *et al.,* 1993; Carrell and Liu, 2001; Aoki *et al.,* 2005a).

The abnormality in protamines (P1 or P2) may be explained by reduced protamine transcription, altered translation of the transcript or failed post-translational modifications, but none of these explanations would directly explain the associated decline in sperm counts and function unless the regulation of protamine exchange is linked to a broader control of spermatogenesis (Carrell *et al.,* 2007). In addition, a number of these studies have described infertile male populations with abnormally elevated ratios of P1 to P2 (P1/P2) (Balhorn *et al.,* 1988; Chevaillier *et al.,* 1990; de Yebra *et al.,* 1993; 1998; Carrell and Liu, 2001; Aoki *et al.,* 2005a). The main reason for high P1/P2 ratio is the under expression of P2, but subsequent studies have demonstrated that P1 dysregulation also accounts for some abnormalities (Aoki *et al.,* 2005a). However, P2 dysregulation is more common and this may be explained by the fact that the P2 gene is derived more recently than the P1 gene, which may suggest that the regulatory mechanisms governing P2 gene expression are not as stringent and are more susceptible to variation than the P1 gene (Lewis *et al.,* 2003).

Moreover, complete selective absence of P2 had been documented in a small population of infertile men (de Yebra *et al.,* 1993; Carrell and Liu, 2001). In another report, a population of infertile males with deregulated P1 expression and abnormally reduced P1/P2 ratios was identified (Aoki *et al.,* 2005a).

Protamines are critical for proper sperm chromatin packaging (Balhorn *et al.,* 2000). In another work by Aoki *et al.,* (2005b) they indicated that protamine concentrations (P1, P2, and total protamine) inversely correlate with the DNA fragmentation index and patients with low P1/P2 ratios have markedly increased DNA damage. Besides, for the first time the same group in 2006 demonstrated existence of significant variations in the protamine levels of individual human sperm cells. These protamine variations related significantly to sperm cell viability and DNA damage and may be of clinical significance (Aoki *et al.,* 2006a).

Torregrosa *et al.,* (2006) also, demonstrated that sperm cells of infertile patients and controls contain different levels of pre-P2. The presence of pre-P2 in infertile patients correlates negatively with sperm count, normal morphology and motility of the sperms and correlates positively with the P1/P2 ratio, with the presence of other proteins and, at low pre-P2 levels, with decreased DNA integrity. The correlation detected between pre-P2 and the P1/P2 ratio in the patient samples is consistent with the hypothesis that, incomplete processing of pre-P2 may result in lower levels of mature P2 and therefore an increased P1/P2 ratio (Bench *et al.,* 1998; de Yebra *et al.,* 1998). Also, the correlation between pre-P2 and the presence of other proteins could indicate that a general failure in the histone to protamine replacement occurs in the samples with higher pre-P2. This hypothesis would be consistent with the inverse correlation of pre-P2 levels and sperm count, normal morphology and motility.

2-2-4-8- Determination of protamines

2-2-4-8-1- Direct determination

Direct protamine extraction and applying Acid Urea Polyacrylamide Gel Electrophoresis (AU-PAGE) is the standard method to measure and quantify the protamines (Mengual *et al.,* 2003; Aoki *et al*. 2005a). Moreover, due to the use of protamines as drugs, there are many sensitive pharmaceutical methods developed for detection of protamines (Shvarev and Bakker, 2005) and new proteomic approaches based on liquid fractionation mass spectrometry or new fluidic devices that have the potential to make protamine quantification even easier and faster in the near future.

Table 2 summaries the studies in which protamines were detected directly after extraction from sperm samples and separated by polyacrylamide gel electrophoresis.

Table 2. Studies in infertile patients where protamines were detected directly after extraction from sperm samples and separated by polyacrylamide gel electrophoresis (modified from Oliva, 2006)

Reference	Main findings
Silvestroni *et al.,* 1976	Protamines not detected in the spermatozoon of infertile patients
Chevaillier *et al.,* 1987	Proteins additional to the normal ones were found in infertile patients
Balhorn *et al.,* 1988	-P1/P2 ratio = 0.98 ± 0.12 in normal samples (*n* = 17) -P1/P2 ratio = 1.58 ± 0.24 in infertile patients (*n* = 7) -Increased P1/P2 ratio in six of the seven patients studied
Lescoat *et al.,* 1988	Heterogeneous protamine fraction observed in patients with altered seminal parameters (*n* = 11) compared with samples with normal parameters (*n* = 11)
Bach *et al.,* 1990	Percentage of protamines is different in the patients with abnormal seminal parameters compared to patients with normal parameters
Blanchard *et al.,* 1990	-Round-headed spermatozoa from patients (*n* = 2) contain less protamines and more histones and intermediate proteins than the normal spermatozoa (*n* = 2) -Expression of P2 proteins is lower in round-headed sperm
Belokopytova *et al.,* 1993	P1/P2 ratio = 0.99 ± 0.06 in normal samples (*n* = 20)
de Yebra and Oliva, 1993	Description of an optimized method to extract and analysis protamines by gel electrophoresis to allow easier and faster clinical application
de Yebra *et al.,* 1993	-P1/P2 ratio = 1.10 ± 0.08 (normal) in 22.4% of infertile patients (*n* = 26) -P1/P2 ratio = 3.00 ± 2.84 (abnormal) in 74.1% of infertile patients (*n* = 86) -Absence of detectable P2 in 3.4% of the patients (*n* = 4)
Colleu *et al.,* 1996	The densest Percoll gradient fractions were enriched in less-intermediate proteins and more P2 in patient samples with normal count and motility (*n* = 12)
Khara *et al.,* 1997	-P1/P2 ratio = between 0.55 and 1.29 in patients with FI ≥50% (*n* = 18) -P1/P2 ratio = outside the 0.55–1.29 range in patients with FI <50% (*n* = 3)
de Yebra *et al.,* 1998	Detection of increased protamine P2 precursors by western analysis in patients with an increased P1/P2 ratio
Bench *et al.,* 1998	P1/P2 ratio varied in patients' samples obtained at different times
Carrell *et al.,* 1999	Differences in protamine content and sperm ultrastructure found in two siblings associated with different ICSI outcomes
Evenson *et al.,* 2000	Appearance of protamine P2 precursors detected by electrophoresis between 33 and 39 days post-hyperthermia in one patient
Carrell and Liu, 2001	-12 of 13 patients without detectable P2 had a reduction in the sperm penetration assay in comparison with the patients with P2 -P2 precursor bands associated with reduction in the penetration capacity
Mengual *et al.,* 2003a	-P1/P2 ratio = 1.01 ± 0.15 in control fertile men (*n* = 10) -P1/P2 ratio = 1.51 ± 0.48 in oligozoospermic patients (*n* = 12) -P1/P2 ratio = 1.23 ± 0.65 in asthenozoospermic patients (*n* = 13) -Little heterogeneity between Percoll fractions from individual samples and marked differences between patients and controls
Nasr-Esfahani *et al.,* 2004b	Negative significant correlation of fertilization rate with protamine deficiency and P1/P2 ratio
Chen *et al.,* 2005	Altered levels of protamines present in infertile patients are shown to improve upon patient treatment
Aoki *et al.,* 2005a	-P1/P2 ratio = 1.06 ± 0.01 in fertile donors (*n* = 87) -P1/P2 ratio < 0.8 in 13.6% of the patients (*n* = 37) -P1/P2 ratio = between 0.8 and 1.2 in 46.7% of the patients (*n* = 127) -P1/P2 ratio > 1.2 in 39.7% of the patients (*n* = 108) -P1/P2 ratio correlates with sperm penetration score and fertilization rate
Aoki *et al.,* 2005b	DNA fragmentation raised in low P1/P2 samples versus normal/high P1/P2 ratio
Aoki *et al.,* 2006a	Correlations between P1 and P2 proteins and mRNA detected by real-time PCR
Aoki *et al.,* 2006b	Protamine-deficient sperm appear to be more susceptible to DNA strand breaks, evidenced by the significantly increased DNA damage
Zhang *et al.,* 2006	Increased proportion of H2B to protamine in infertile men
Torregrosa *et al.,* 2006	P2 precursors related to protamine content and DNA integrity
Zini *et al.,* 2007	Semen samples from asthenospermic infertile men possess a significantly higher ratio of sperm nuclear histone (H2B) to protamine (PRM1+PRM2) than do fertile men.
De Matio *et al.,* 2009	P2 precursors related to poor pregnancy outcome
Hammoud et al., 2009	Inverse correlation between P1/P2 ratio and the level of histone expression in the different layers of the density gradient.

FI, fertilization index; P1, protamine 1; P2, protamine 2; H2B, histone 2B.

2-2-4-8-2- Indirect assessment of sperm chromatin structure by histochemical Procedures

Indirect detection methods based on different staining procedures or fluorochromes techniques. Of these methods:

1- Chromomycin A_3 (CMA_3) assay

In situ competition between protamine and CMA_3 indicated that CMA_3 staining inversely correlated with the protamination state of spermatozoa (Bizzaro *et al.,* 1998). CMA_3 staining has been shown to be increased in the sperm cells of infertile patients (Nasr-Esfahani *et al.,* 2004a, 2005). However, CMA_3 staining cannot distinguish whether the potential protamine deficiency is due to a lack of P1, P2 or a combination of both.

2- Sperm chromatin structure assay (SCSA)

SCSA based on the Acridine Orange (AO) stain red–green shift to differentiate double- versus single-stranded DNA (Evenson and Wixon, 2005).

3- Aniline Blue Staining

Aniline blue staining procedures were used to detect the presence of histones and therefore indirectly infer the presence of lower amounts of protamines in the sperm nucleus (Chevaillier *et al.,* 1987).

4- Other new sperm chromatin structure tests based on sperm chromatin dispersion are also being proposed (Jager, 1990).

Depending on the results of these indirect methods on studying the sperm chromatin composition, structure, accessibility and integrity of the DNA it is difficult to interpret these results (Erenpreiss *et al.,* 2006).

2-3- Reactive Oxygen Species (ROS) and Male Infertility

Reactive oxygen species (ROS) are free radicals that carry unpaired electrons which tend to bind other molecules and alter them. The ROS of primary interest are the superoxide anion ($^{\cdot}O_2^{-}$), hydroxyl radical ($^{\cdot}$OH), and hypochlorite radical ($^{\cdot}$OHCl). ROS are highly disruptive to cellular function, and play a role in male-factor infertility. They may affect and damage all types of cells and may play a role in as much as 40% of male-factor infertility (Sharma and Agarwal, 1996). Agarwal *et al.,* (2006) reported that high ROS may serve as a marker of male-factor infertility. They also suggested that ROS measurement be included in clinical diagnosis of idiopathic infertility.

The unique structure of sperm makes it highly susceptible to damage by these molecules. The head of the sperm is covered with unique lipid membrane rich in polyunsaturated fatty acids that make up approximately 40% of the lipids in the sperm head, which are important for membrane fluidity, sperm motility, capacitation, and sperm binding to the egg zona pellucida. These polyunsaturated fatty acids are extremely susceptible to oxidative damage. 'Oxidative Stress' is an imbalance between the ROS generating factors and ROS scavenging systems (Sikka, 2001). Furthermore, it has been shown that ROS can damage DNA by causing deletions, mutations, and other lethal genetic effects (Tominga *et al.,* 2004). Damage of the sperm DNA by free radicals induces activation of the poly (ADP ribose) synthetase enzyme. This splits NAD+ to aid the repair of DNA. Depletion of the NAD^{+} concentration will disrupt the function of the cells and cause cells death. The relative proportion of ROS–producing immature sperm was directly correlated with nuclear DNA damage value in mature sperm and inversely correlated with recovery of motile mature sperm (Aitken *et al.,* 1998b)

Agarwal, *et al.,* (2003) proposed that oxidative damage of mature sperm by ROS-producing immature sperm during their co-migration from seminiferous tubules to the epididymis may be an important cause of male infertility. Also, sperm may be damaged by oxidative stress in epididymis where it is stored after its production (Vernet *et al.,* 2004). Mustafa *et al.,* (2004) demonstrated that infertile patients with high ROS levels in their seminal plasma had a higher percentage of apoptosis than normal healthy donors.

Furthermore, sperm head contains the acrosomal enzymes as well as the chromosomes that will eventually fuse with the maternal chromosomes. The mid-piece of the sperm contains mitochondria that generate the energy to propel the tail of the sperm. These mitochondria is considered the internal source of reactive oxygen molecules. Mitochondria generate hydrogen peroxide (H_2O_2) and the sperm plasma membrane NADPH oxidase system (Agarwal *et al.,* 2003). Another important external source of ROS is the leukocytes that is found in semen. They produce H_2O_2 and Oxygen (O_2). The phagocytes and -lymphocytes produce $-O_2$ which appear to use NADPH oxidase like enzymes. Furthermore, ROS may be increased with certain medications, radiation, pollutants and in patients with spinal cord injuries (Potts and Pasqualotto, 2003). The quality of the sperm may increase ROS generation as a consequence of the presence of excess residual cytoplasma that indicate immaturity of the sperm and functionally defective (Aziz *et al.,* 2004) and are capable of producing increased amounts of ROS (Sikka, 2001). A strong positive correlation exists between immature spermatozoa and ROS production, which in turn negatively affects the sperm quality (Said *et al.,* 2004). The excessive production of ROS that exceeds critical levels can overwhelm all the antioxidant defense systems of spermatozoa and seminal plasma causing oxidative stress (de Lamirande *et al.,* 1997).

2-3-1- Physiologic role of ROS

Spermatozoa may require ROS for their functions depending on the nature and the concentration of the ROS as well as the location and length of exposure to ROS (Agarwal and Saleh, 2002). Under certain physiological conditions, spermatozoa produce small amounts of ROS, to facilitate their ability for fertilization the oocytes “capacitation” and acrosomal reaction. Also, ROS is required for the progressive movement during epididymial transit (Visconti and Kopf, 1998).

2-3-2- Consequences of excessive generation of ROS

Excessive generation of ROS in semen could be a cause of infertility (Sharma and Agarwal, 1996). The main ROS produced in spermatozoa is hydrogen peroxide. Moderately elevated concentrations of hydrogen peroxide did not affect sperm viability but did affect the motility of the sperm through depletion of intracellular ATP and the subsequent decrease in the phosphorylation of axonemal proteins (Kemal Duru *et al.,* 2000; Misro *et al.,* 2004). Also, high concentrations of hydrogen peroxide induce cell death as a result of lipid peroxidation and were negatively correlated with the quality of sperm in the original semen (Gomez *et al.,* 1998). However, pathological levels of ROS detected in semen of infertile men are more likely a result of increased ROS production rather than reduced antioxidant capacity of the seminal plasma (Zini *et al.,* 1993).

A meta-study conducted by Agarwal and Prabakaran, (2005) showed that ROS levels correlated significantly with in vitro fertilization (IVF) rate. Recently Hammadeh *et al.,* (2008a) showed that ROS and TAS concentration in seminal plasma did not differ significantly between the patients undergoing IVF or ICSI therapy, but negative correlation was shown between ROS concentration in seminal plasma and sperm vitality, membrane integrity, sperm density, chromatin condensation, and DNA single stand breaks in both IVF and ICSI groups.

2-3-3- (8-hydroxy-2'-deoxyguanosine (8-OHdG)) level

8-hydroxy-2′-deoxyguanosine (8-OHdG) resulted from oxidative DNA damage following specific enzymatic cleavage after 8-hydroxylation of the guanine base. 8-OHdG induced the G:C to T:A conversion during DNA replication. Hydroxylation of guanosine occurs in response to both normal and metabolic processes and variety of environmental factors. 8-OHdG used as a biomarker of oxidative stress in many cases including renal carcinogenesis (Toyokuni *et al.,* 1997), diabetes mellitus (Leinonen *et al.,* 1997) and ageing (Kaneko *et al.,* 1996). It was reported that sperm DNA damage is closely related to male infertility, and 8-OHdG is a sensitive biomarker of oxidative DNA damage caused by ROS in human sperm (Shen and Ong 2000).

Furthermore, the significance effect of oxidative stress on sperm DNA integrity was supported by Loft *et al.,*(2003) who reported that pregnancy occurring in a single menstrual cycle was inversely associated with the level of 8-hydroxy-2′-deoxyguanosine, an indicator of oxidative DNA damage in spermatozoa (Loft *et al.,* 2003). The oxidized deoxynucleoside, 8-OHdG if not repaired 8-OHdG modifications in DNA are mutagenic and may cause embryonic loss, malformations, or childhood cancers (Agarwal *et al.,* 2006).

A recent study conducted by Ishikawa and colleages demonstrated that increased 8-OHdG expression in the testis was associated with deficient spermatogenesis in infertile men with varicocele (Ishikawa *et al.,* 2007). In addition, the work of Kao *et al.,* (2008) showed significant negative correlations between sperm motility and 8-OHdG and between motility and lipid peroxides. They concluded that oxidative stress and oxidative damage were increased significantly in spermatozoa with declined motility, and the antioxidant capacities in the spermatozoa and seminal plasma were lower in males who had infertility or subfertility.

2-3-4- Lipid peroxidation (Malondialdehyde, MDA)

Lipid peroxidation is a well established-mechanism of cellular injure and is used as an indicator of oxidative stress (Yagi, 1998). Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decomposed to form a complex series of compounds that include reactive carbonyl compounds, such as Malondialdehyde (MDA). MDA is a naturally occurring product of lipid peroxidation. Malondialdehyde (MDA) is hydrophilic, and released from low density lipoproteins (LDL) into aqueous surroundings.

The measurement of Thiobarbituric Acid Reactive Substances (TBARS) is a well-established method for screening and monitoring lipid peroxidation (Armstrong and Browne, 1994). The MDA-TBA compound formed by the reaction of MDA and TBA under high temperatures (90-100 ^{o}C) and acidic conditions is measured colorimetrically at 530-540 nm or fluorometrically at an excitation wavelength of 530 nm and emission wavelength of 550 nm.

Human sperm plasma membranes are enriched with polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid with six double bonds (Khosrowbeygi and Zarghami, 2007). Physiologically, the high concentrations of PUFA in sperm are important for maintaining membrane fluidity and flexibility during fertilization process. Lipid peroxidation is known to cause impairments such as membrane damage, damage to sperm chromatin (Oyawoye *et al.,* 2003), reduced sperm-oocyte fusion (Pasqualotto *et al.,* 2004), and decrease in a chromosomal function (Aziz *et al.,* 2004). The mechanism by which oxidative stress induced motility loss in mammalian spermatozoa involved the induction of peroxidative damage to the sperm plasma membrane (Tavilani *et al.,* 2005). Seminal plasma malondialdehyde, which is the stable lipid peroxidation product, is a simple method to evaluate the effect of lipid peroxidation on sperm (Geva *et al.,* 1998).

Tavilani *et al.,* (2008) observed a higher content of lipid peroxidation product malondialdehyde (MDA) in spermatozoa of asthenozoospermic compared with normozoospermic samples ($p<0.05$). Although, the difference between MDA of seminal plasma was not significant between two groups.

On the contrary, measurement of the seminal levels of malondialdehyde and antioxidants in men with both normal and abnormal seminogram by Nabil *et al.,* (2008) showed that malondialdehyde level was significantly elevated in oligozoospermic and azoospermic men. They concluded that Lipid peroxidation plays a significant role in disrupting sperm functions and semen quality especially sperm motility and morphology and may account for some cases of male infertility.

2-4- Smoking and sperm parameters

2-4-1- Introduction

Cigarette smoking is a serious health problem for most societies. Consumption of tobacco exerts widely adverse effects on different aspects of health (O'Dowd, 2006). Studies about the relation between smoking and semen quality reveal conflicting results. Also, cigarette smoking has mutagenic properties, and having been associated with an overall reduction in semen quality, and specifically a reduction in sperm count and motility and increase in number of abnormal cells (Potts *et al.,* 1999). In addition, Taszarek *et al.,* (2006) and Agarwal *et al.,* (2005) demonstrated that cigarette smoking alters semen quality, which could worsen the fertilizing capability in infertile men, while others found that the average values of conventional sperm parameters (sperm density, motility, normal morphology, viability) were normal (Sepaniak *et al.,* 2006).

A strong body of evidence indicates that the negative effects of cigarette smoking on fertility comprises fairly every system involved in the reproductive process. Therefore, couples in their reproductive age should be strongly discouraged to smoke (Soares and Melo, 2008). In addition, cigarette smoking has hazardous effects on spermiogenesis either by direct action or by its associated compounds such as cotinine on sperm kinetic variables. Besides, smoking effects on sperm parameters could be mediated by decreases in seminal insulin-like growth factor-I (IGF-I) (Hassan *et al.,* 2009).

2-4-2-Smoking and semen quality

Several published studies have conquered and supported the fact that tobacco smoking significantly affects semen quality (Chen *et al.,* 2004, Marinelli *et al.,* 2004, Martini *et al.,* 2004, Stutz *et al.,* 2004, Pasqualotto *et al.,* 2005). Mostafa *et al.,* (2006) demonstrated that ascorbic acid in seminal plasma decreased significantly in smokers and infertile men in comparison to non-smokers and fertile men, and is significantly correlated with the main sperm parameters (count, motility and normal morphology). Gaur *et al.* conducted a study on semen samples of 100 cigarette smokers and 100 strictly non-smoking (primary infertility patients) and showed a decrease in sperm motility in smokers in comparison to non-smokers. Therefore, they pointed out that asthenozoospermia (decreased in motility) may be an early indicator of reduced semen quality in light smokers. In addition, they reported significantly high teratozoospermia (poor morphology) in heavy smokers compared to non-smokers. It has been shown that maternal smoking affects reproductive parameters of their offspring (male) during adolescence (Gaur *et al.,* 2007).

In addition, studying semen samples provided from fertile smokers (n=25), fertile non-smokers (n=21), infertile smokers (n=23) and infertile men non-smokers (n=32), showed that sperm quality parameters in men smokers was approximately lower than those in men non-smokers (Colagar *et al.,* 2007). Also they showed that cigarette smoking has dosedependent effect on sperm parameters and is associated with reduced sperm quality and the risk of idiopathic male infertility.

Pasqualotto *et al.,* (2008) evaluated the effect of cigarette smoking on antioxidant levels and the presence of leukocytospermia in infertile men. They found a lower level of superoxide dismutase

and catalase in infertile patients and men who smoke cigarettes compared to fertile donors. Therefore, cigarette smoking decreases sperm motility and antioxidant levels and may be a possible reason for infertility in men who smoke cigarettes. They recommended that infertile patients who smoke cigarettes should quit smoking.

On the contrary, other previous studies are not in agreement with these results (Trummer *et al,.* 2002; Belcheva *et al.,* 2004; and Pasqualotto *et al.,* 2006).

2-4-3- Effects of Smoking on Semen Volume

Pasqualotto *et al.,* (2005) carried out a study to assess the effect of smoking on the semen parameters and concluded that male smoking does not significantly impair the usual sperm parameters, namely, concentration, motility and morphology. However, they found that sperm volume is affected by smoking in a dose-depended manner. More precisely, sperm volume declined significantly between non-, mild, moderate and heavy smokers, with a mean 2.8ml, 2,4ml, 2,3ml and 2.1ml, respectively. Ramlau-Hansen *et al.,* (2007) presented an overall study two years later, which supported that there is an inverse dose-response relation between smoking and semen volume. This study incorporated seven separate occupational or environmental semen studies performed from 1987 to 2004, resulting in a total of 2562 men evaluated.

On the contrary, Sobreiro *et al.,* in (2005) evaluated the effect of smoking, among other parameters, on semen characteristics of 500 fertile men, who attended a clinic for vasectomy for sterilization purposes. These men were asked to provide samples before the vasectomy and the group's results showed that smoking has no influence on semen parameters whatsoever. Others showed that testicular and ejaculate volume were similar in smokers and non-smokers (Richthoff *et al.,* 2008).

2-4-4- Effect of Smoking on Sperm Concentration

An association between cigarette smoking and sperm density (10^6/ml) was found to exist according to the investigation carried out by Kunzle *et al.,* (2003). The men they included in their study had attended the infertility clinic with their spouses, seeking causative factors concerning their infertility issues and the overall result was a significant decrease in sperm density of smoking males, compared to non-smoking controls (Kunzle *et al.,* 2003).

Ramlau-Hansen *et al.,* (2007) in a study carried out in 2007, found a 19% lower sperm concentration in smoking men, compared to non-smokers. Moreover, Bouvet *et al.,* (2007) showed that tobacco consumption has an adverse effect on spermatogenesis process resulting in a decrease sperm concentration and increase in the mean percentage of morphologically abnormal spermatozoa. Also, in a recent study researchers found a significant reduction in total sperm counts in smokers when compared to non-smokers (Richthoff *et al.,* 2008*)*.

On the other hand, Pasqualotto *et al.,* (2005) evaluated a group of men who attended the clinic for vasectomy for sterilization purposes, by dividing them into groups: non-smokers, mild smokers, moderate smokers and heavy smokers. All conventional semen parameters were evaluated and they found no significant differences in sperm concentration among the four groups. Additionally, no significant negative correlation between tobacco smoking and sperm concentration was detected by Hassa *et al.,* (2006).

2-4-5- Effect of Smoking on Sperm Motility

Heavy smokers seem to have an enhancing effect on progressively rapid motile sperm compared to light-smoking infertile men (Ozgur *et al.,* 2003). The possible explanations for this enhancement of rapidly progressive motility due to heavy smoking in comparison to light smoking is definitely related to number of cigarettes per day, which may constitute a threshold for the enhancement of rapid progressive motility in smokers.

Many studies had found a negative significant correlation between smoking and sperm motility or morphology and lower sperm density (Vine *et al.,* 1994; Zenzes, 2000). Zavos *et al. (*1998) confirmed this effect; by exposing isolated spermatozoa of non-smokers *in vitro* to separated seminal plasma from smokers, they observed a decreased in sperm motility. In a study conducted by Gaur *et al.* on semen samples from cigarette smokers and strictly non-smoking (primary infertility patients) showed a decrease in sperm motility in smokers in comparison to non-smokers (Gaur *et al.,* 2007).

In another study, Kumosani *et al.,* (2008) showed that cadmium (Cd) concentration was found to be significantly higher in smokers than in non-smokers either in fertile or infertile groups. Together with this increase in seminal Cd a significant decrease in $Ca^{(2+)}$-ATPase activity, decrease in seminal zinc and decrease in sperm motility were found. Also, recently Hassan and team found that the percentage of motile spermatozoa was significantly lower in smokers than in the controls. This sheds light on the harmful effect of cigarette smoking on sperm motility (Hassan *et al.,* 2009).

2-4-6- Effect of smoking on sperm Morphology

Abnormal sperm morphology is related to sperm head abnormalities, and may involve the acrosome, nucleus and post acrosomal region (Zamboni, 1987). Guo *et al.,* (2006) investigated the impact effect of cigarette, alcohol consumption and sauna on spermatozoa and demonstrated that normal morphologic sperm rates in cigarette, alcohol consumption and sauna groups were lower than those in the corresponding control groups. In addition, heavy smoking produces teratozoospermia, which further reduces semen quality (Gaur *et al.,* 2007).

Moreover, the relationship between cigarette smoking and the blood lead levels (BLL) (lead as a component of cigarette smoke) and their effect on sperm morphology, sperm chromatin and DNA integrity of factory workers revealed that BLL level was considerably higher among smokers as compared to non-smokers and workers with higher BLL were found to be at a higher risk of sperm morphological abnormality and chromatin DNA integrity (Hsu *et al.,* 2008).

2-4-7- Effect of smoking on sperm chromatin integrity (DNA)

The etiology of sperm DNA fragmentation is still poorly understood, but relationship between cigarette smoking and increased DNA damage has been shown in infertile smokers compared of non-smokers (Zenzes, 2000; Saleh *et al.,* 2003a).

Sepaniak *et al.,* (2006) showed that smokers' spermatozoa have a significantly higher DNA fragmentation than that of non-smokers (32% versus 25.9%, $p<0.01$). Besides, there was no significant difference in conventional parameters between smokers and non-smokers. These findings suggested that cigarette smoking may have deleterious effects on sperm nuclear DNA. Recently, Elshal *et al.,* (2008) found that cigarette smoking of idiopathic infertile men was significantly associated with DNA fragmentation index (DFI %), high DNA stainability (HDS %) as indicator of immature spermatozoa and lipid peroxidation (TBARS) of sperm plasma membrane and decreased superoxide dismutase (SOD) levels. There are currently various techniques for the evaluation of sperm DNA fragmentation. These techniques have been recently reviewed (Evenson and Wixon, 2006 a; b).

In a meta-analysis study conducted by Collins *et al.,* (2008) to evaluate the predictive value of sperm DNA integrity tests for pregnancy from *in vitro* fertilization treatment, a small but statistically significant association between sperm DNA integrity test results and pregnancy in IVF and ICSI cycles was found. But it was also concluded that this is not strong enough to provide a clinical indication for routine use of these tests in infertility evaluation of men. It is possible that yet to be determined subgroups of infertile couples may benefit from sperm DNA integrity testing.

2-4-8- Cotinine level in seminal plasma

Exposure to tobacco smoke can be detected by nicotine and its metabolites. Nicotine [3 - (1- methyl – 2 - pyrrolidinyl) pyridine] is the principal alkaloid in tobacco and is present as a major component of tobacco smoke. It is absorbed in measurable quantities by both active and passive smokers, the latter having been shown to inhale an amount of nicotine proportional to the product of concentration, duration of exposure and respiration rate (Bently *et al.,* 1999). It has been shown in human liver microsomes that nicotine and cotinine [1 – methyl – 5 - (3 - pyridinyl)- 2 - pyrrolidinone] are oxidized primarily *via* CYP 2A6 (Messina *et al.,* 1997). Nicotine is primarily metabolized to cotinine, and cotinine is further metabolized to trans-3'-hydroxycotinine in the human liver, which is a major metabolite of nicotine in humans (Nakajima *et al.,* 1996).

Nicotine has a short half life and is not used as a marker for tobacco smoke exposure. Cotinine and hydroxycotinine are the major metabolites of nicotine, and cotinine is the major psychoactive substance found in cigarette smoke (Zenzes et al 1996). Cotinine, which is easily detectable in human body fluids, such as urine, saliva (Zenzes et al 1996), and seminal plasma (Pacifici *et al.,* 1993), had been used as a specific biomarker of cigarette smoking (Hulka, 1991). Also, cotinine with a half-life in sperm of 5–7 days is a better indicator of long-term exposure to cigarette smoke than urinary metabolites index (Jarvis *et al.,* 1988). However, the concentration of cotinine and hydroxycotinine in the seminal plasma is significantly correlated with total sperm motility of spermatozoa (Pacifici *et al.,* 1993). Zavos *et al.,* (1998) found that smoking affected sperm viability and had a strong detrimental effect on motility of spermatozoa. There is a small but significant correlation between cotinine concentration in seminal plasma and abnormal sperm morphology, but not for other semen variables (Wong *et al.,* 2000). Cotinine concentration of 400-800 ng/ml impairs sperm motility, membrane function and their ability to undergo capacitation (Sofikitis *et al.,* 2000).

In a study conducted on 35 smoking and 30 non-smoking students, it was shown that cotinine levels, sperm density and total number of motile spermatozoa would differ significantly between the groups (Chen and Kuo, 2007). In addition, some previous work from our group showed an inverse correlation between cotinine concentration and ejaculate volume, sperm concentration, motility, vitality, morphology membrane integrity, DNA fragmentation (TUNEL), TAS concentration and fertilization (Hammadeh *et al.,* 2008b).

2-5- Factors that influence sperm chromatin structure

Sperm chromatin structures can be altered by many factors including temperature, chemicals and toxins, disease and molecular disturbances (Karabinus *et al.,* 1997; Sailer *et al.,* 1996; Ahmadi and Ng, 1999; Bochenek *et al.,* 2001; Love *et al.,* 2002).

2-5-1- Temperature

Sperm production requires a temperature, which is three to five degrees below body temperature. The scrotum has a built-in thermostat, which keeps the sperm at the correct temperature while they are being stored. This is why the testes hang away from the body, so sperm can develop at the temperature they need (35° - 36° C). A study conducted by Karabinus *et al.,* (1997) demonstrated that bulls showed a reduction in sperm chromatin stability around 12 days after exposed to heat stress. The exposure of mice testes to 40°C for 60 minutes induced moderate changes in sperm chromatin, whereas, exposure to 42°C for 60 minutes induced marked chromatin abnormalities in virtually all stages of spermatogenesis (Sailer *et al.,* 1995). In fact, it had been shown that testicular hyperthermia, either direct or indirect, can cause DNA damage via an increase in the histone/protamine ratio (Evenson & Libman, 2000; Zini and Libman, 2006).

2-5-2- Chemicals and toxins

A causal relationship between cigarette smoking and impaired reproductive function is highly suspected due to the fact that smokers inhale a range of toxins such as nicotine, carbon monoxide, cadmium and other mutagenic compounds (Zavos *et al.,* 1998). Smoking has been shown to increase sperm DNA damage (Sun *et al.,* 1997; Potts *et al.,* 1999) which may be a result of seminal oxidative stress (Saleh *et al.,* 2002a). The latter was supported by reports that oxidative stress not only damages the sperm plasma membrane but also induces high frequencies of single- and double-strand DNA breaks (ssDNA, dsDNA) (Twigg *et al.,* 1998a; Aitken, 1999).

2-5-3- Disease

Genital tract infections may cause spermatozoal DNA damage and have been associated with increased levels of ROS (Aitken & De luliis, 2007). Ureaplasmal (*Ureaplasma urealyticum*) infection in men did not alter sperm motility but was associated with a high incidence of chromatin instability (SCSA, 60%) that was reversed by treatment with antibiotic (Reichart *et al.,* 2000). Similar sperm chromatin instability was reported in men suffering from prostatitis, which also returned to normal after antibiotic treatment (Evenson *et al.,* 1991). Varicocele constitutes a serious cause of male infertility although it is, also, present in 15% of patients who tried to have children (Marmar, 2001). Significant higher levels of spermatozoal DNA damage were noticed in infertile men with varicocele compared to normal controls, due to high levels of ROS and elevated intratesticular temperature (Saleh *et al.,* 2003b).

2-5-4- Molecular disturbances

A proportion of infertile men have diminished levels of protamine 2 in ejaculated sperm, which has been attributed to the persistence of histones or incomplete processing of protamine 2 precursors (Foresta *et al.,* 1992; de Yebra *et al.,* 1998).

2-5-5- Age and spermatozoa DNA damage

According to Barroso *et al.,* (2000) and Aitken *et al.,* (2003b) the age-related effect in older men is due to a higher exposure to oxidative stress in their reproductive tracts that may damage the sperm DNA and mitochondrial and nuclear membranes. Singh *et al*., (2003) demonstrated an increase in sperm double stranded DNA breaks and a decrease in apoptosis with age. They found DNA that damages in men aged 36–57 years were highly significant compared to those aged 20–35 years. However, the percentage of apoptosis was significantly lower in the older group. Moreover, Kuhnert and Nieschlag, (2004) showed that paternal age is associated with a variety of anomalies, such as diminished semen quality, numerical and structural chromosomal abnormalities, that can decrease reproductive capacity and fertility and increase the frequency of spontaneous abortions.

2-6- Origin of sperm DNA damage

A successful fertilization, and normal embryo and fetal development resulting in a healthy offspring require normal sperm genetic material. For this reason any damage in sperm chromatin or DNA structure may cause an alteration in the health of the offspring. The source(s) of sperm DNA damage in the male germ line have not yet been elucidated. Damage may occur at testicular, epididymal or post-ejaculatory levels. DNA damage in the male germ line has been associated with poor semen quality, low fertilization rates, impaired pre-implantation development, increased abortion and an elevated incidence of disease in the offspring, including childhood cancer (Lewis & Aitken, 2005). Thoroughly, this damage is thought to arise due to environmental stresses, gene mutations or chromosomal abnormalities (Agarwal and Said, 2004). Sperm chromatin abnormalities may occur during, or as a result of, DNA packaging at spermiogenesis (Sailer *et al.,* 1995), free-radical (reactive oxygen species; ROS) (Aitken *et al.,* 1998a) or apoptosis (Gorczyza *et al.,* 1993). The precise source or mechanism of mammalian sperm damage has not been completely understood (Lewis & Aitken 2005). Therefore, abnormalities in proteolytic cleavage of the

processing protamine precursors may act as an additional source of human sperm chromatin heterogeneity and potential infertility (de Yebra *et al.,* 1998; Evenson *et al.,* 2000).

Several studies have shown that sperm from infertile men are also more susceptible to DNA damage under the effect of several factors; both H_2O_2 and X–irradiation (McKelvey-Martin *et al.*, 1997; Hughes *et al.,* 1998), cryopreservation (Donnelly *et al.,* 2001) and the routine *in vitro* incubation of the testicular spermatozoa prior to ICSI injection from men with obstructive azoospermia (Dalzell *et al*., 2003).

DNA fragmentation is characterized by both single and double DNA strand breaks, and is particularly frequent in the ejaculates of subfertile men (Irvine *et al.,* 2000). Torregrosa *et al.,* (2006) listed several hypotheses proposed to explain the origin of decreased DNA integrity in the sperm cells of infertile patients, which include:

(1) Incomplete repair during meiosis (Baarends *et al.,* 2001)

(2) Incomplete repair of DNA breaks arising from the action of topoisomerase II during spermiogenesis (Marcon and Boissonneault, 2004)

(3) Incomplete removal of apoptotic cells (Sakkas *et al.,* 2002; Weng *et al.,* 2002)

(4) Incomplete protamination resulting in increased susceptibility of DNA (Cho *et al.,* 2003; Aoki *et al.,* 2005a)

(5) Defects in the expression of transition proteins (Adham *et al.,* 2001; Meistrich *et al.,* 2003; Shirley *et al.,* 2004; Zhao *et al.,* 2004; Suganuma *et al.,* 2005)

(6) Damage mediated by heavy metals or toxins interacting with protamines (Bal *et al.,* 1997; Quintanilla-Vega *et al.,* 2000)

(7) Increased ageing and oxidation of the spermatozoa during passage and storage in the male tract (Ollero *et al.,* 2001; Suganuma *et al.,* 2005)

(8) Increased action of exogenous factors such as infection and increased oxidant action of leukocytes (Alvarez *et al.,* 2002)

In two separate studies on mice sperm by (Shaman *et al.,* 2007 and Yamauchi *et al.,* 2007a) researchers proposed that sperm DNA degradation occurs due to sperm chromatin fragmentation (SCF)—induced pathway. They reported a mechanism by which the DNA is degraded to facilitate decondensation of the tight toroid. This process was initiated by topoisomerase IIB and ended by sperm nuclease action. During the passing of the sperm through epididymis, this process may be executed (Yamauchi *et al.,* 2007a). Physiologically, this mechanism is associated with DNA replication in oocytes and is thought to be part of the process by which paternal DNA is prevented from influencing initial cleavage of the embryo and may help in preventing the passing of the damaged DNA to the fertilized oocytes (Yamauchi *et al.,* 2007b). Pathologically, infertile sperm may possess an aberrant SCF induced DNA degradation pathway explaining the increased load of DNA damage (reviewed by Aitken and De Luliis 2007).

Even though, the causes of DNA damage are still uncertain, up-to-date abnormal sperm chromatin/DNA structure is thought to arise from four potential sources (listed below) (Sakkas *et al.,* 1999b; Agarwal and Said, 2004; Erenpreiss *et al.,* 2006). Because markers of sperm DNA damage or chromatin integrity are still relatively new, factors contributing to these sources of damage remain largely unclear. However, their discovery is vital for the development of preventative or treatment measures (Aitken and De Luliis, 2007).

2-6-1- Defects in recombination

Deficiencies in recombination during spermatogenesis due to defect in checkpoint system during ligation of DNA double strand breaks may lead to persistent sperm DNA fragmentation in ejaculated spermatozoa, which usually lead to cell abortion (Bannister and Schimenti, 2004; Erenpreiss *et al.,* 2006).

2-6-2- Spermatid maturation abnormalities

DNA strand breaks have been found in round and elongating spermatids. DNA breaks are necessary for transient relief of torsional stress, favoring removing of the nucleosome histone cores, and aiding their replacement with transitional proteins and protamines during maturation in elongating spermatids (Marcon and Boissonneault, 2004; Laberge and Boissonneault, 2005). Ligation of these breaks is important for restoring the DNA integrity and for reassembly of the important unit of genome expression, the DNA loop-domain.

Chromatin re-packaging requires decondensation of the chromatin by hyperacylation of the histone and introduction of breaks by topoisomerase II (Laberge and Boissonneault, 2005) during epididymal transit replacement of histones by protamines completed and chromatin condensation restored. However, if these temporary breaks are not repaired, DNA fragmentation in ejaculated spermatozoa may occur.

Animal models have proven the relationship between aberrant chromatin condensation and presence of DNA breaks (Cho *et al.,* 2003; Zhao *et al.,* 2004).

Human sperm DNA contains a segment of partially denatured DNA that represent a potential DNA break if induced by any factors (Muriel *et al.,* 2004)

The relative spermatid DNA/chromatin conformational fragility may be responsible for the presence of higher levels of spontaneous DNA damage in sperm than in somatic cells (Fernandez *et al.,* 1998). In addition, elongating chromatids have a lower repair capacity for strand breaks (van Loon *et al.,* 1993).

2-6-3- Oxidative stress

The first indication that oxidative stress might be involved in the disruption of normal sperm function came from John MacLeod (1943), who observed that human spermatozoa rapidly lose their motility when incubated under high oxygen tensions.

High levels of reactive oxygen species (ROS) are toxic to sperm quality and function (Saleh and Agarwal, 2002a; Hammadeh *et al.,* 2006). About 25–40% of the infertile patients had elevated levels of ROS (Padron *et al.,* 1997). Oxidative stress resulted by an imbalance between the productions of ROS and the antioxidant capacity (Sharma and Agarwal, 1996). Many studies showed that oxidative stress appears to be the major cause of DNA damage in the male germ line (Figure 5) (Saleh *et al.,* 2003a; Aitken and Sawyer 2003; Aitken *et al.,* 2003a, b).

Hydrogen peroxide, superoxide and free radicals are part of the ROS that are highly reactive oxidizing agents (Warren *et al.,* 1987). Low levels of ROS are necessary for modulating gene and protein activities that is vital for sperm proliferation, differentiation and function. The major sources of ROS are the defective spermatozoa (specifically those with retained cytoplasm), semen leukocytes and mitochondria (Vernet *et al.,* 2001; Zini and Libman, 2006). Other ROS-generating systems are the enzymes nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX5) that is potentially capable of generating ROS in the presence of calcium and NADPH (Aitken *et al.,* 1997; Banfi *et al.,* 2001), lipoxygenase (Oliw and Sprecher, 1989), tumor-associated cell surface NADH oxidase (tNOX) (Morré and Morré, 2003) and cytochrome P450 reductase (Baker *et al.,* 2004).

One of the mechanisms by which oxidative stress impacts upon sperm function, particularly motility and sperm–oocyte fusion, is through the stimulation of a lipid peroxidation in the plasma membrane (Aitken and Clarkson 1987; Aitken *et al.,* 1998a; b). Spermatozoa plasma membrane has high unsaturated fatty acid content that makes it particularly susceptible to ROS–mediated damage, as well as, spermatozoa lack of appropriate repair mechanisms. Protection of the sperm DNA from oxidative stress can be achieved by tight DNA packaging and high levels of antioxidants present in seminal plasma (Twigg *et al.,* 1998b).

A defect in spermatid protamination and disulphide bridge formation due to inadequate oxidation of thiol-groups will negatively affect the sperm chromatin packaging, making sperm cells more vulnerable to ROS induced DNA fragmentation (Erenpreiss *et al.,* 2006).

Many studies have indicated a significant correlation between DNA damage and high levels of ROS in infertile patients (Fraga *et al.,* 1996; Kodama *et al.,* 1997; Sun *et al.,* 1997; Twigg *et al.,* 1998a, b; Kemal Duru *et al.,* 2000; Barroso *et al.,* 2000; Aitken and Baker, 2004). Furthermore, the significant effect of oxidative stress on sperm DNA integrity was supported by Loft *et al.* (2003) who reported that pregnancy occurring in a single menstrual cycle was inversely associated with the level of 8-hydroxy-2′-deoxyguanosine, an indicator of oxidative DNA damage in spermatozoa.

Tremellen, (2008) had summarized the routine laboratory tests signs that suggest the possible existing of oxidative stress in seminal plasma as follow:

1. Poor sperm motility (Asthenozoospermia)

2. Poor sperm morphology (Teratozoospermia)

3. High number of round cells in semen ((Leukocytes?)

4. Increased semen viscosity

5. Poor sperm membrane integrity on hypo-osmolar swelling test (HOS-test)

6. Poor fertilization on routine IVF

7. Poor sperm motility after overnight incubation with the oocyte

8. Poor blastocyst development in the absence of a clear female factor (advanced maternal age/poor ovarian reserve)

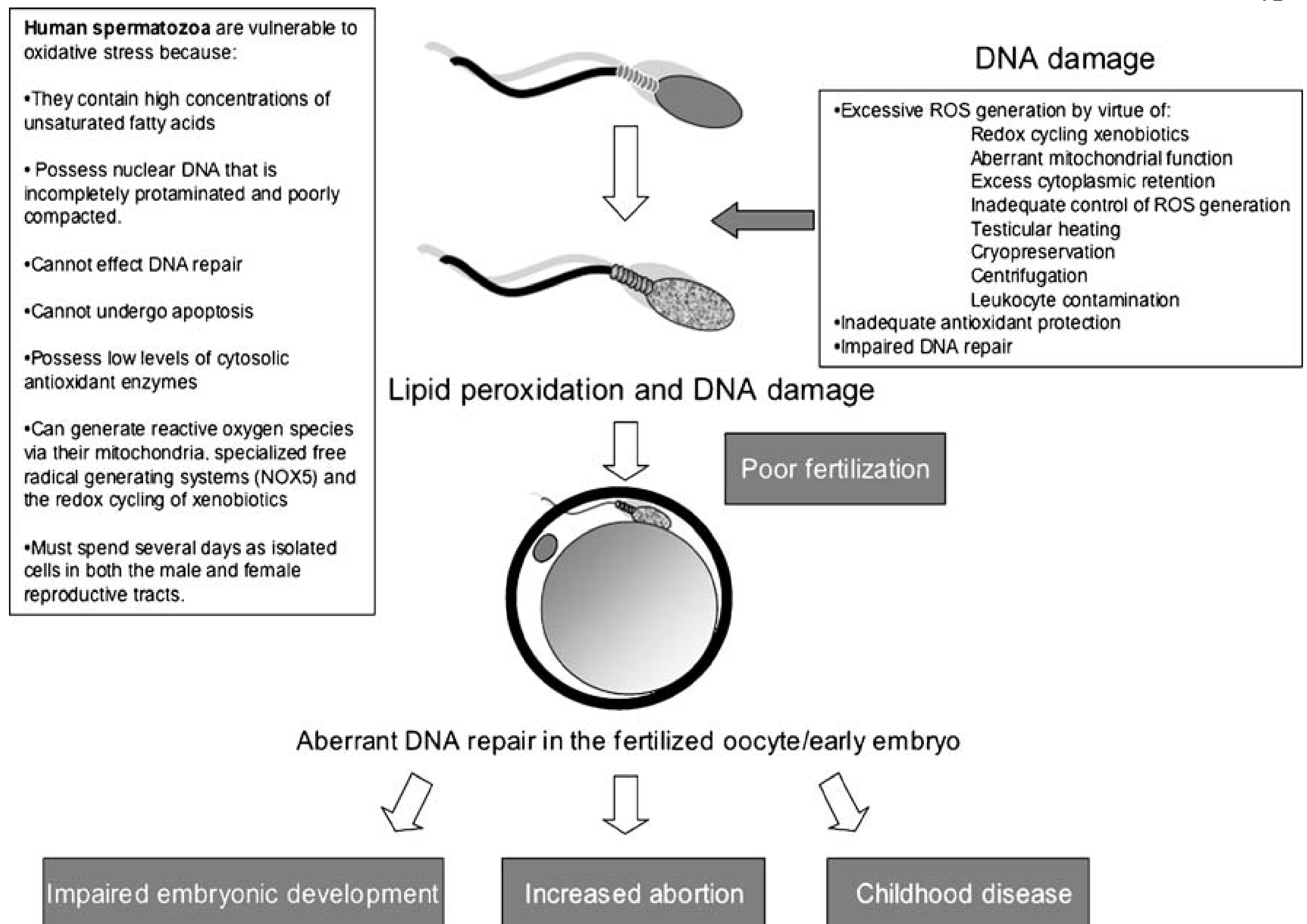


Figure 5. Oxidative stress as a major cause of defective sperm function and DNA damage. Summary of some of the factors that render human spermatozoa vulnerable to oxidative stress and the way in which such stress can generate pathology (adapted from Lewis & Aitken 2005).

2-6-4- Abortive apoptosis

Another cause for the DNA double-strand breaks (DSBs) in the spermatozoa of infertile patients can arise through an abortive apoptotic pathway (Cisternas and Moreno, 2006). Spermatozoa that are marked for apoptotic degradation may have normal mitochondrial activity, high or low motility (Barroso *et al.,* 2000) as well as normal morphology (Host *et al.,* 2000a; b).

It has been suggested that an early apoptotic pathway, initiated in spermatogonia and spermatocytes, is mediated by Fas protein. Fas is a type I membrane protein that belongs to the tumor necrosis factor–nerve growth factor receptor family (Suda *et al.,* 1993; Krammer *et al.,* 1994). It has been shown that sertoli cells express Fas ligand, which by binding to Fas leads to cell death through apoptosis (Suda *et al.,* 1993), limiting the size of the germ cell population to numbers sertoli cells can support (Rodriguez *et al.,* 1997). Some studies have not found correlations between DNA damage and Fas expression (Muratori *et al.,* 2000), or, in contrast, have not revealed ultra-structural evidence for the association of apoptosis with DNA damage in sperm (Barroso *et al.,* 2000).

Furthermore, 20% of ejaculated spermatozoa that showed DNA strand breaks and the apoptotic marker annexin V, was reported (Oosterhuis *et al.,* 2000). Whereas, according to an investigation by Sakkas *et al.,* (1999a), it was reported that DNA strand breaks and apoptotic markers did not co-exist together in the same mature spermatozoa. The percentage of spermatozoa with DNA fragmentation in normal donors and different groups of patients is illustrated in figure 6.

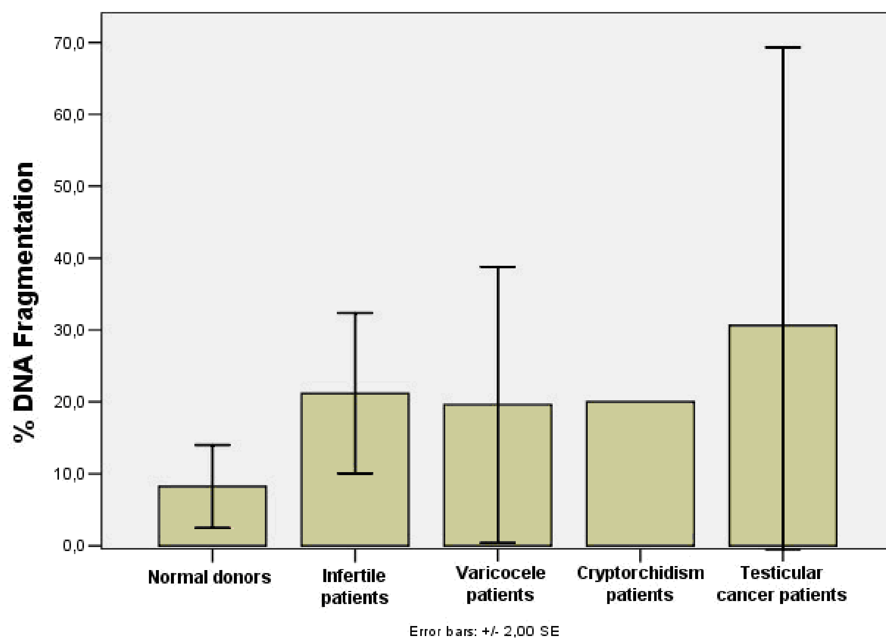


Figure 6. Frequency of DNA fragmentation in human ejaculated spermatozoa. The percentage of DNA fragmentation in the above groups was determined using TUNEL assay. Five studies evaluated normal donors. Three of the studies also assessed a group of infertile patients, two evaluated varicocele patients, one evaluated cryptorchidism patients and two assessed testicular cancer patients (adapted from Angelopoulou *et al.,* 2007).

Alternatively, if the apoptotic cascade is initiated at the round spermatid phase, when transcription (and mitochondria) is still active, abortive apoptosis might be an origin of the DNA breaks. Although many apoptotic biomarkers have been found in the mature male gamete, particularly in infertile men, their definitive association with DNA fragmentation remains elusive (Lachaud *et al.,* 2004; Henke *et al.,* 2004; Paasch *et al.,* 2004). It was demonstrated in elongated spermatozoa stage that high levels of DNA breaks were associated with high level of topoisomerase II (Roca and Mezquita, 1989; McPherson and Longo, 1993a). Topoisomerase II possibly needed to relieve torsional stress caused by the negative supercoiling associated with histone to protamine transition (Balhorn, 1982). It was indicated that topoisomerase II may act by causing double-strand breaks, which are re-ligated again (termed sperm chromatin fragmentation) or acting by conjuncting with an extracellular nuclease to cause regulated double-strand breaks in protamine-bound DNA (termed sperm DNA degradation) (Sotolongo *et al.,* 2005; Shaman *et al.,* 2006). This topoisomerase/ nuclease-induced DNA degradation may be a specialized apoptotic pathway in sperm, different from the normal function of topoisomerase in relieving torsional stress, followed by re-ligation of the DNA break (Shaman *et al.,* 2006).

In ejaculated human sperm both apoptosis and necrosis are seen. However, it is uncertain whether ejaculated sperm retains the ability to activate the apoptotic cascade or whether the detected apoptotic markers in spermatozoa are, simply, the expression of an apoptotic process that has began before the event of ejaculation (Sakkas *et al.,* 2002). Moreover, during *in vitro* processing, spermatozoa cannot enter in the apoptotic pathway and they are eliminated by necrosis (Greco *et al.,* 2005).

2-7- Clinical significance of sperm DNA damage

2-7-1- DNA damage and semen parameters

Different studies reported either weak or no correlation between conventional semen parameters and sperm DNA damage, most of them indicated that spermatozoa from patients with abnormal sperm count, morphology and motility had increased levels of DNA damage (reviewed in Erenpreiss *et al.,* 2006).

If sperm DNA damage resulted as adverse effect of ROS, then a relationship to sperm motility could be expected. This is due to the effect of ROS on the lipid peroxidation of sperm membranes rich with unsaturated fatty acids (Erenpreiss *et al.,* 2006). Besides, some studies report a correlation between sperm DNA damage and motility (Gandini *et al.,* 2000; Giwercman *et al.,* 2003). However, it should be remembered that these processes are inter-related. Unrepaired DNA DSB's can lead to defective sperm packaging which, in turn, as a consequence of persistent DNA fragmentation or due to the other reasons, can cause increased access to ROS attack.

2-7-2- The relationship between sperm DNA integrity and fertilization (Natural conception)

In vivo fertilization probability almost close to zero in patients with sperm DNA damage exceeds 30% as detected by SCSA (Evenson *et al.,* 1999; Spano *et al.,* 2000). The DNA in human spermatozoa is extensively damaged at high levels of oxidative stress, resulting in severely disrupted in fertilizing potential of the spermatozoa as a consequence of collateral peroxidative damage to the sperm plasma membrane (Aitken *et al.,* 1998a). A recent meta-analysis by Evenson and Wixon, (2008) indicated a strong correlation between sperm DNA damage and failure to achieve a natural pregnancy.

2-7-3- Sperm DNA damage and pre-implantation development

Post-fertilization development of the embryo can be seriously disrupted by DNA damage. Such disruption is associated with the abortive transcription of damaged genes originating from the paternal genome (Tesarik *et al.,* 2002; 2004). Embryonal pre-implantation development is negatively correlated with DNA damage as detected by nick translation (Sakkas *et al.,* 1998), TUNEL (Ahmadi and Ng 1999), Comet (Morris *et al.,* 2002), and SCSA (Virro *et al.,* 2004) assays.

Furthermore, chromatin structure abnormalities are associated with reduced rates of embryo cleavage, again emphasizing the link between chromatin integrity and developmental potential. To avoid the inheritance of an abnormal paternal genome after ICSI, Spano *et al.,* (2000) suggested the use of blastocyst for implantation.

2-7-4- Intrauterine insemination (IUI)

High levels of sperm DNA damage have generally been associated with lower IUI pregnancy rates (Duran *et al.,* 2002; Muriel *et al.,* 2006; Bungum *et al.,* 2007). In patient samples with >12% sperm DNA damage detected by TUNEL used for insemination, no pregnancy was achieved (Duran *et al.,* 2002). Also, if the proportion of sperm cells with DNA damage exceeded 30% as detected by SCSA, then the probability of fertilization by IUI seemed to be close to zero (Saleh *et al.,* 2003a; Bungum *et al.,* 2004). Thus, the artificial insemination cycles in which DNA fragmentation is high had been found to correlate negatively with pregnancy (Duran *et al.,* 2002). Therefore, sperm DNA damage assessment has a high predictive value for the outcome of both natural conception and IUI.

2-7-5- In vitro fertilization (IVF) and Intracytoplasmic sperm injection (ICSI)

Many of systemic review and meta-analysis of IVF and IVF/ICSI studies showed that sperm DNA damage is associated with a significant increase in the rate of pregnancy loss after IVF and ICSI (reviewed by Zini and Sigman, 2009). Tomlinson *et al.,* (2001) reported a significant negative correlation between sperm DNA damage and embryo quality in IVF cycles. Many studies reported that a sperm DNA fragmentation index (DFI) less than 27%, detected by SCSA, is necessary to obtain a successful pregnancy by IVF and ICSI (Larson *et al.,* 2000; Larson-Cook *et al.,* 2003). However, other studies did not have similar results (Bungum *et al.,* 2004; Gandini *et al.,* 2004; Check *et al.,* 2005); these results indicated that successful pregnancies in IVF/ICSI cycles can even be obtained using semen samples with a high proportion of DNA damage. These data are in accordance with and confirm previous investigations by Hammadeh *et al.* (1998), Host *et al.* (2000b), and Larson-Cook *et al.* (2003), who showed that sperm DNA damage is more predictive in IVF and much less in ICSI. Successful fertilization was achieved by ICSI technique, despite the high levels of DNA damage in the injected spermatozoa, regardless of whether ejaculated or testicular spermatozoa are used during therapy (Hammadeh *et al.,* 1998; Esterhuizen *et al.,* 2000;

Lewis *et al.,* 2004; Aitken, 2004). A recent meta-study by Collins *et al.,* (2008), suggested that sperm DNA damage has no measurable impact on pregnancy rates in ICSI. Whereas, other studies showed an inverse relationship between pregnancy rates with ICSI and the level of DNA fragmentation in testicular and ejaculated sperm (Lewis *et al.,* 2004; Bungum *et al.,* 2004; Virro *et al.,* 2004).

2-7-6- Embryonal loss

DNA abnormalities have been associated with unexplained recurrent pregnancy loss including sperm DNA fragmentation (Carrell *et al.,* 2003a; Saleh *et al.,* 2003a). Depending on the severity of the genetic damage and the ability of the oocyte to repair it, the embryo may fail at any stages of pregnancy or might develop to term with abnormalities. Low pregnancy rates were obtained in patients with sperm DNA damage (DFI≥30%) (Check *et al.,* 2005). Also, it had been shown by Carrell *et al.,* (2003b) that the proportion of sperm with DNA damage was significantly higher in men from couples with recurrent pregnancy loss, compared with the general population or fertile donors.

2-7-7- Effect of sperm DNA quality on offspring

Many studies indicated that sperm DNA damage can affect the health of the embryo, fetus, and offspring (Bonde *et al.,* 2003; Perreault, 2003; Savitz, 2003). A possible consequence of sperm DNA damage is infertility in the offspring (Aitken and Krausz, 2001; Silber and Repping, 2002). Association between paternal occupations involving exposure to metals, solvents and pesticides and an increase in birth defects and childhood diseases had been indicated (Olshan and Mattison, 1994). Moreover, one concern raised from studies of smokers is the increased risk of childhood cancer of the offspring of men with a high proportion of sperm DNA fragmentation in their sperm. It was shown that the offspring of these men, whose ejaculate are under oxidative stress (Manicardi et al., 1995) and whose semen is characterized by high chromatin fragmentation, are four to five times more likely to develop childhood cancer than the children of non–smoking fathers (Ji *et al.,* 1997; Virro *et al.,* 2004). Another study has demonstrated that 15% of all childhood cancers are directly attributed to paternal smoking (Sorahan *et al.,* 1997). A powerful association exist between childhood disease and paternal occupation (Sawyer *et al.,* 2003).

However, the linkage between sperm DNA damage and abnormalities in offspring is not confirmed to smokers.

Aitken and Krausz, in 2001 proposed that sperm DNA damage is promutagenic and can give rise to mutations after fertilization, as the oocyte attempts to repair DNA damage prior to the initiation of the first cleavage. Mutations occurring at this point will be fixed in the germ line and may be responsible for the induction of not only such pathologies as described above (infertility and childhood cancer in the offspring), but also for a higher risk of imprinting diseases (Cox *et al.,* 2002; DeBaun *et al.,* 2003).

2-8- Assessment of sperm DNA damage

Spermatozoa DNA damage detection may be considered a good indicator to identify males with reduced fertilization capacity or to initiate a healthy pregnancy. A number of direct and indirect assay methods have been used for the assessment of sperm DNA damage; of the common methods used for direct detection the Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick-end labeling assay (TUNEL) and Comet assay. The most common indirect method for assessing DNA damage include the sperm chromatin integrity assays like sperm chromatin structure assay (SCSA) (Andrabi, 2007).

2-8-1- Factors that influence the predictive value of DNA damage assays

The predictive value of DNA fragmentation tests depends on several factors associated with the DNA damage itself (Lewis *et al.,* 2008). These include:

1. Single- vs. double-stranded DNA fragmentation. Single strand breaks are easier to repair than double strands breaks.

2. Percentage of spermatozoa with DNA damage. The probability for fertilization decreases as the number of affected sperm increases.

3. Degree of DNA fragmentation per spermatozoon. The higher the degree of DNA fragmentation, the lower the probability the oocyte or the embryo of repairing the damage.

4. Whether there is primary and/or secondary DNA damage. Apoptosis during spermatogenesis and nicks produced during the process of spermiogenesis are considered primary, while damages due to hydroxyl radical or damage produced due to exposure to ionizing radiation are considered secondary damage. Primary damage in this case consists of, for the most part, double-stranded DNA fragmentation.

5. Type of DNA fragmentation test used. Tests that measure DNA damage under physiological conditions like COMET at neutral pH or TUNEL have higher predictive values than tests that measure DNA damage or susceptibility to DNA denaturation under non-physiological conditions.

6. Whether DNA damage affects coding sequences. The probability that DNA damage affects a protein coding sequence is relatively low because more than 90% of DNA consist of non-coding sequences.

7. Ability of the oocyte to repair sperm DNA damage in ART. Repairing ability depends on the extent of sperm DNA damage and on the quality of oocytes

8. Ability of the embryo to repair DNA damage. It depends on quality of the embryo and the extent of DNA damage introduced in the embryo's genome by the fertilizing spermatozoon.

9. Number of metaphase II oocytes.

10. Sample processing. Processing of the sperm-like centrifugation and incubation, for example, may increase the percentage of DNA fragmentation.

2-8-2- Assessment Methods

In general, all assays can be divided into three groups: the assays' principles, as well as the advantages and disadvantages of assays from all three groups, are described:

2-8-2-1- Chromatin structural probes using nuclear dyes

Nuclear dyes are both sensitive and simple to use and therefore attractive for clinical use. Many factors affect the staining of the chromatin by planar ionic dyes:

1. DNA secondary structure and conformation. Fragmented DNA is easily denatured (Darzynkiewicz, 1994). However, even a single DNA strand break causes conformational transition of the DNA loop-domain from a supercoiled state to a relaxed state. Supercoiled DNA takes up intercalating dyes like acridine orange (AO) because this reduces the free energy of torsion stress.

2. Chromatin packaging density. If the chromatin is regularly arranged and sufficiently densely packed, dye co-planar polymerization providing metachromatic shift (change of color) is favored (Erenpreisa and Zaleskaya, 1983). However, if the chromatin is packaged even more densely (as in normal sperm), the polymerization of the dye is hindered (Erenpreisa *et al.,* 1992) and may even

prevent dye binding, especially by large, bulky dyes at an unfavorable pH. The latter case is seen with aniline blue (AB) at low pH where it stains basic proteins loosely associated with DNA and is unable to bind to the chromatin of normal sperm, which is very densely packaged.

3. Chromatin proteins. Chromatin proteins affect the binding of DNA dyes in the way that they themselves bind differently to relaxed/fragmented or supercoiled DNA. DNA supercoiling requires covalent binding of some nuclear matrix proteins and tighter ionic interactions between DNA and chromatin proteins to support negative supercoils (Benyajati and Worcel, 1976). Chromatin proteins in sperm nuclei with impaired DNA appear to be more accessible to binding with the acidic dye, as found by the AB test (Auger *et al.,* 1990; Erenpreisa *et al.,* 2001). An increase in the ability to stain sperm by acid AB indicates a looser chromatin packaging and increased accessibility of the basic groups of the nucleoprotein. This is due to the presence of residual histones (Terquem *et al.,* 1983) and correlates well with the AOT (Liu and Baker, 1992). Chromomycin–A3 (CMA_3) is another staining technique, which has been used as a measure of sperm chromatin condensation anomalies. CMA_3 is a fluorochrome specific for GC-rich sequences and is believed to compete with protamines for association with DNA. The extent of staining is therefore related to the degree of protamination of mature spermatozoa (Bianchi *et al.,* 1993; Manicardi *et al.,* 1995).

In turn, it can be inferred that the phosphate residues of sperm DNA in nuclei with loosely packed chromatin and/or impaired DNA will be more liable to binding with basic dyes. Such conclusions were also deduced from the results of staining with basic dyes, such as toluidine blue (TB), methyl green and Giemsa stain (Erenpreisa *et al.,* 1992; Andreetta *et al.,* 1995). The most widely used techniques for sperm chromatin structure assessment are the SCSA (Larson-Cook *et al.,* 2003; Bungum *et al.,* 2004), AO (Tejada *et al.,* 1984; Hoshi *et al.,* 1996) and TB tests (Erenpreiss *et al.,* 2004; Beletti and Mello, 2004).

2-8-2-1- 1- Acridine Orange Test (AOT)

The acridine orange test (AOT) was introduced by Tejada *et al.,* (1984). The AOT measures the susceptibility of sperm nuclear DNA to acid-induced denaturation *in situ* by quantifying the metachromatic shift of AO fluorescence from green (native or dsDNA) to red (denatured or ssDNA). AOT is inexpensive and simple but fading of the color occurs in a short time which requires fast evaluation. In recent studies by Chohan *et al.,* (2006) and Martin *et al.,* (2007a; b) it is found that AOT is not that much sensitive as TUNEL and SCSA for evaluating sperm DNA fragmentation. The percentage of DNA fragmentation, also referred to as DNA Fragmentation Index (DFI) is yielded by the ratio of red/red+green.

In 1996, Hoshi *et al.,* demonstrated that when > or = 50% of spermatozoa in semen samples exhibited green AO nuclear fluorescence, IVF was always successful and when green AO nuclear fluorescence was < 50%, only 39% of IVF treatment cycles were successful. When the incidence of green AO fluorescent spermatozoa was < 50%, no pregnancy resulted even though an average of 26% of the oocytes could be fertilized by ICSI (Hoshi *et al.* 1996). Also, Virant-Klun *et al.,* 2002; Shibahara *et al.,* 2003; Cebesoy *et al.,* 2006, indicated that the percentage of sperm showing > 56% red fluorescence is considered as the "cut-off" value to characterize an abnormal chromatin status.

2-8-2-1- 2- Chromomycin–A_3 Staining (CMA_3)

CMA_3, a polymerase inhibitor that is used to evaluate the normal protaminosis by indirect approach, this assay is inversely correlated with the protamination state of spermatozoa based on the *in situ* competition with protamine (Bizzaro *et al.,* 1998). A Chromomycin A_3 is a guanine–cytosine-specific fluorochrome that binds to the DNA as a Mg^{2+} -coordinated dimer at the minor groove of GC-rich DNA and prevent the accessibility of DNA polymerase I to the DNA and, almost none of the CMA_3- negative spermatozoa present nicked DNA, as CMA_3 is unable to access DNA in the presence of protamines and normally formed disulphide bonds (Manicardi *et al.,* 1995; Lolis *et al.,* 1996).

Evaluation of CMA_3 is done by differentiation between spermatozoa nuclei with bright (CMA_3 positive) or dull yellow staining (CMA_3 negative) or by comparing bright and dull yellow (CMA_3 positive) with spermatozoa nuclei counterstained blue with DAPI (CMA_3 negative) or with bright or dull green if counterstain is not used. However, the disadvantage of this staining technique is the disability to discriminate whether the potential protamine deficiency is due to a lack of P1, P2 or a combination of both, and this can be solved by gel electrophoresis (Oliva, 2006).

Sakkas *et al.,* reported that semen samples with high CMA_3 positivity (> 30%) may have significantly lower fertilization rates if used for ICSI (Sakkas *et al.,* 1998). Also, the work of Tarozzi and her team demonstrated the good predictive value of CMA_3 analysis in IVF outcome and the possibility of using this test as a prognostic tool for IVF patients. They propose a calculated threshold value for CMA_3 staining (cutt-off value) equal 29.25% by using Raceiver Operating Characteristic (ROC) curve (Tarozzi *et al.,* 2009).

2-8-2-1- 3- Sperm chromatin structure assay (SCSA)

The sperm chromatin structure assay (SCSA) depends on the fact that abnormal sperm chromatin has a greater susceptibility to the physical induction of partial DNA denaturation *in situ* (Agarwal and Said, 2004). The SCSA exploits the metachromatic properties of acridine orange (AO) to monitor the susceptibility of sperm chromatin to heat or acid-induced denaturation (Agarwal and Said, 2004; Fraser, 2004). Similar to AOT the percentage of DNA fragmentation, also referred to as DNA Fragmentation Index (DFI), is yielded by the ratio of red/red+green. The percentage of sperm showing > 56% red fluorescence is considered as the "cut-off" value to characterize an abnormal chromatin status (Virant-Klun *et al.,* 2002; Shibahara *et al.,* 2003 ; Cebesoy *et al.,* 2006).

Evenson *et al.,* (1999) reported that if the proportion of sperm cells with DNA damage exceeds 30% as detected by SCSA, then the probability of fertilization *in vivo* is almost zero for natural conception. Also, Larson *et al.,* (2003) reported that no patients achieved pregnancy following IVF or ICSI if >27% of sperm in a sample of semen showed DNA fractionation in the SCSA (i.e. DNA fragmentation index (DFI) >27%). The same authors also observed that patients with morphologically abnormal sperm could achieve pregnancy following ICSI if the same sample of sperm had a DFI of 0–15%.

2-8-2-1-4- DNA breakage detection–fluorescence *in situ* hybridization test (DBD-FISH)

DNA breakage detection–fluorescence *in situ* hybridization (DBD-FISH) is a procedure that allows *in situ* detection and quantification of DNA breaks in the whole genome or within specific DNA sequence areas, cell by cell (Fernandez *et al.,* 1998).

Sperm cells embedded within an agarose matrix on a slide are exposed to an alkaline solution, which transforms DNA-strand breaks into single-stranded DNA (ssDNA) motifs. After neutralizing and protein removal, ssDNA is accessible to hybridization with whole genome or specific DNA probes. The probe highlights the chromatin area to be analyzed. Fluorescence intensity and surface area of the FISH signal increase as DNA breaks increase, which means the more ssDNA produced by the alkaline solution, the more the probe hybridizes (Fernandez *et al.,* 2000).

2-8-2-1- 5- Acidic Aniline Blue Assay (AB)

The AB is an acidic stain, which distinguishes between lysine-rich histones and arginine/cysteine-rich protamines (Hofmann and Hilschler, 1991). This assay gives a specific positive reaction for lysine and reveals differences in the basic nuclear protein composition of ejaculated human spermatozoa. Histones are rich in lysine while protamines are rich in arginine and cystine. Immature spermatozoa nuclei are rich in histones that are rich in lysine and will be stained blue. On the other hand, nuclei of mature spermatozoa are rich protamines that are rich in arginine and cysteine and contain relatively low levels of lysine, which means they will not be stained by aniline blue (Hammadeh *et al.,* 2001).

The results of the work of Hammadeh *et al.,* (1998) indicated that chromatin condensation as visualized by aniline blue staining is a good predictor for IVF outcome, although it cannot determine the fertilization potential, cleavage, and pregnancy rate following ICSI. AB method is simple and inexpensive but has limited repeatability.

2-8-2-1- 6- Toluidine blue Assay (TB)

Toluidine blue is a basic nuclear dye used for metachromatic and orthochromatic staining of chromatin. It stains phosphate residues of the DNA of sperm nuclei with both loosely packed chromatin and fragmented (Erenpreisa *et al.,* 2003). Sperm heads with good chromatin integrity stain light blue, and those of diminished integrity stain violet (purple). Nevertheless, TB staining may be considered a fairly reliable method for assessing the sperm chromatin. Like AB, TB method is simple and inexpensive but has limited repeatability.

2-8-2-2- Tests for direct assessment of sperm DNA fragmentation

2-8-2-2-1- *In situ* nick translation assay (ISNT)

This *in situ* nick translation (ISNT) assay quantifies the incorporation of biotinylated-dUTP at ssDNA breaks in a reaction catalyzed by the template-dependant enzyme DNA polymerase I. The ISNT may detect spermatozoa that contain appreciable and variable levels of endogenous DNA damage. Currently, however, ISNT assay thresholds for post-fertilization embryo viability have not been established, which severely limits the clinical usefulness of this assay (Andrabi, 2007).

ISNT test has the ability to indicate if there is damage arising from factors such as the generation of ROS following exposure to leukocytes within the male reproductive tract or heat exposure (Aitken *et al.,* 1991; Setchell *et al.,* 1998). Furthermore, it has a very clear relationship with sperm motility and morphology and, to a lesser extent, sperm concentration (Irvine *et al*. 2000; Tomlinson *et al*. 2001).

2-8-2-2-2- Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick-end labeling assay (TUNEL)

The TUNEL assay quantifies the incorporation of deoxyuridine triphosphate (dUTP) at single strand (ss)- and ds-DNA breaks in an enzymatic reaction by labeling the free 3′-OH terminal and modified nucleotides (5-bromo-2′-deoxyuridine-5′-triphosphate nucleotide) with terminal deoxynucleotidyl transferase. Sperm with normal DNA therefore have only background staining/fluorescence, while those with fragmented DNA (multiple 3′-OH ends) stain/fluoresce brightly (Gorczyza *et al.,* 1993), which can be quantified by flow cytometry, or by fluorescent microscopy or light microscopy (Agarwal and Said, 2003). The microscopic TUNEL assay has been modified by some researchers to include a peroxidase enzyme labeling system that catalytically generates an intense signal from chromogenic substrates. This labeling technique eliminates the problems associated with fluorescence fading in the microscopic method, thereby giving operators more time to analyze a greater number of cells for more accuracy (Host *et al.,* 2000a; b).

In 2000, Sergerie *et al.,* demonstrated that the calculated threshold value “cut-off value“ for TUNEL assay to distinguish between fertile controls and infertile men was 20%. They demonstrated that sperm DNA fragmentation, as measured by TUNEL assay, is a highly valuable indicator of male fertility. In another study the proposed “cut-off value“ calculated from receiver operating characteristics (ROC) curve analysis for IVF patients was 36.5% and for ICSI patient 24.3% (Henkel *et al.,* 2003; 2004).

2-8-2-2-3- COMET assay (Single Cell Gel Electrophoresis) Assay

This assay was first introduced by Ostling and Johanson (1984), for the analysis of DNA damage in an individual cell. In this assay, spermatozoa are stained with a fluorescent DNA-binding dye. One of the principles of the comet assay is that nicked double-stranded DNA tends to remain in the

comet head, whereas short fragments of nicked double and single stranded DNA migrate into the tail area (Klaude *et al.,* 1996). Therefore, spermatozoa with high levels of DNA strand breaks would show increased comet tail fluorescent intensity (Hughes *et al.,* 1998) and comet tail length (Singh and Stephens, 1998). The results are interpreted by comparing the number of cells with comet tails with total number of spermatozoa (Anderson *et al.,* 1997). Also the assay is classified as alkaline and neutral COMET assay depending on the type of damage being investigated (Klaude *et al.,* 1996; Hughes *et al.,* 1998).

Regarding the alkaline COMET assay (pH>10), it denatures sperm DNA and therefore, identifies both ss and dsDNA breaks. Whereas, the neutral COMET assay may be more sensitive to dsDNA breaks and therefore, better able to identify DNA damage related to infertility as the conditions of the assay (pH 9) do not denature DNA. Neutral COMET assay is better able to identify DNA damage related to infertility (Fraser, 2004). The major problem with COMET assay is that it is a labor-intensive test.

The assay has been successfully used in the evaluation of DNA damage after cryopreservation (Duty *et al.,* 2002). It may also predict embryo development after IVF and ICSI, especially in couples with unexplained infertility (Morris *et al.,* 2002).

2-8-2-3- Sperm nuclear matrix assays

2-8-2-3-1- Sperm chromatin dispersion assay (SCDA)

More recently, sperm chromatin dispersion assay (SCDA) has been described as a simple and inexpensive method for analysis of mammalian sperm DNA fragmentation (Fernandez *et al.,* 2003). The SCDA is based on the principal that spermatozoa with non-fragmented DNA are immersed in an agarose matrix and exposed to lysing solution; the resulting deproteinized nuclei show extended halos of DNA dispersion as monitored by fluorescent or light microscopy. On the other hand, the SCDA does not give much information about the extent of spermatozoal DNA damage, since its endpoints consist of subjectively quantifying the percentage of spermatozoa with dispersed or non-dispersed nucleoids. SCD test does not require the determination of color or fluorescence intensity; the test is simple, fast, and reproducible, and its results are comparable to those of the SCSA. Because the SCD test was recently introduced, little is known about its limitations and its clinical significance (Fernandez *et al.,* 2003; Andrabi, 2007).

2-9- Aims of the study

The aims of the present study were to:

1- Investigate the relationship between the human sperm DNA integrity under oxidative stress and smoking using; TUNEL assay, Chromomycin A_3 and protamine concentration by electrophoresis.

2- Evaluate the level of oxidative DNA damage by measuring the reactive oxygen species (ROS), 8-hydroxy-2'- deoxyguanosine, (8-OHdG) and of lipid peroxides (TBA, Malondialdehyde) in seminal plasma.

3- Investigate the effect of smoking on sperm parameters including protamines and measuring the level of cotinine concentration in seminal plasma.

4- Evaluate the consequence of oxidative stress on the fertilization and pregnancy rates of patients undergoing ICSI treatment.

5- Determine the ratio of protamine 1 to protamine 2 in human spermatozoa and relate it to in intracytoplasmic sperm injection (ICSI) and standard semen parameters.

3- Materials and Methods

3-1- Materials

3-1-1- Semen samples

166 Semen samples were collected. Ejaculates from volunteers with normozoospermia (n =50) served as controls and ejaculates from unselected male partners (n=116 patients) of couples consulting for infertility at department of obstetrics and gynaecology, university of Saarland/Homburg/Saar. Ejaculates were obtained in the early morning at the clinic (7.00–9.30 AM) after three to five days of sexual abstinence.

3-1-2- Chemicals and laboratory materials

- Acetic Acid	Fluka, Neu-Ulm, Germany
- Acetone	Merck, Darmstadt, Germany
- Acrylamide-Stock solution: Rotiphorese® Gel 30 Rotiphorese® Gel 40	Carl Roth GmbH, Karlsruhe, Germany
- Ammonium Persulphate (APS)	Sigma, Munchen, Germany
- Bovin-IgG protein	Bio-Rad, Munchen, Germany
- Bradford Protein Assay Kit	Bio-Rad, Munchen, Germany
- Chromomycin	Sigma, Munchen, Germany
- Coomassie Blue Stain Brilliant R 250 Brilliant G 250	Serva, Heidlberg, Germany
- Disodium hydrogenphosphate	Merck, Darmstadt, Germany
- Dithiothreitol (DTT)	Sigma, Munchen, Germany
- Dry milk powder (non-fat_	J.M. Galber Saliter GmbH & Co.KG, Obergunzburg, Germany
- Eosin Y Stain	Sigma, Munchen, Germany
- Ethanol Absolute	Merck, Darmstadt, Germany
- Ethylene diamine tetraacetic acid Disodium salt (EDETA)	Sigma, Munchen, Germany
- Glycerin	Fluka, Neu-Ulm, Germany
- Guanidine Hydrochloride	Sigma, Munchen, Germany
- HAM's F10 medium	PAN Biotech, Germany
- Hexadecyltrimethylammonium bromide (CTAB)	Sigma, Munchen, Germany
- Hydrochloric acid	Merck, Darmstadt, Germany
- Lumi-Light Western Blotting Substratee	Roche, Mannheim, Germany
- ß-Mercaptoethanol	Merck, Darmstadt, Germany
- Methanol	Merck, Darmstadt, Germany
- Methyl Green	Sigma, Munchen, Germany
- Paraformaldehyde	Merck, Darmstadt, Germany
- Penylmethylsulfonyl fluoride (PMSF)	Sigma, Munchen, Germany
- Potassium Acetate	Sigma, Munchen, Germany
- Potassium Chloride	Merck, Darmstadt, Germany
- Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
- Pure sperm gradient	Nidacon International AB, Sweden
- PVDF membrane	Roche, Mannheim, Germany
- Sodium Chloride	Merck, Darmstadt, Germany
- *tri*-Sodium citrate. $2H_2O$	Merck, Darmstadt, Germany
- Sodium Iodoacetate	Sigma, Munchen, Germany
- Sucrose	Sigma, Munchen, Germany
- N,N,N$^{/}$,N$^{/}$- Tetramethylendiamine	GA Healthcare, Freiburg

(TEMED)

- 1,1,3,3-Tetramethoxypropan (Malonaldehyde bis,dimethyl acetal, MDA)	Sigma-Aldrich, Germany
- Thiobarbituric acid (TBA)	Merck, Darmstadt, Germany
- Trichloroacetic acid (TCA)	Sigma, Munchen, Germany
- Tris(hydroxymethyl)aminomethane (Tris)	Sigma, Munchen, Germany
- Triton X-100	Sigma, Munchen, Germany
- Tween 20	Serva, Heidlberg, Germany
- Urea	Sigma, Munchen, Germany

3-1-3- Buffers and Solutions

1. Ammonium Persulphate (APS) Solution
   - 1.6 % (w/v) Ammonium Persulphate
2. Blocking Buffer
   - 1 x PBS, pH 7.4
   - 0.1% (v/v) Tween 20
   - 5% (w/v) Non fat-Dry milk
3. CMA3 Solution
   - 0.25 gm/ml
   - 1 x PBS, pH 7.4
   - 10 mmol $MgCl_3$
4. Coomassei Blue Stain
   - 0.2% Brilliant R250
   - 0.01% Brilliant G250
   - 50% methanol
   - 10% acetic acid
   - 40% Disttild Water
5. Decondensation Buffer 1
   - 6 M Guanidine Hydrochloride (Gn-HCl)
   - 575 mM Ditheothreitol (DTT)
6. Decondensation Buffer 2
   - 522 mM Sodium Iodoacetate
7. Denaturing Solution
   - 0.5 N HCl (Hydrochloric acid)

8- Destaining buffer
   - 20 % methanol
   - 10 % acetic acid

9. Electrophoresis Buffer
   - 5% (v/v) Acetic acid
10. Fixation Solution for CMA_3
    - 3 X Methanol
    - 1 X Glacial acetic acid
11.Fixation Solution for TUNEL
    - 4% Paraformaldehyde
    - PBS, pH 7.4
12. Hypo Osmotic Solution
    - 150 mmol/L Fructose (13.5 g/L)
    - 150 m/mol/L Sodium Citrate.$2H_2O$ (7.35 g/L)
13. Loading Buffer
    - 0.375 M Potassium Acetate, pH 4.0
    - 15% Succrose
    - 0.05% Methyl Green (MG)
14. Phosphate Buffer Saline (PBS), pH 7.4
    - 137 mM Sodium Chloride (NaCl)

- 2.7 mM Potassium Chloride (KCl)
- 8 mM Disodium hydrogenphosphate (Na_2HPO_4)
- 1.5 mM Potassium dihydrogen phosphate (KH_2PO_4)

15. Permeability Solution
    - 0.10 % Triton X-100
    - 0.10 M Sodium citrate, pH 6.0
16. Precipitating solution
    - 100% Trichloroacetic acid (TCA)
17. Thiobarbituric acid - Trichloroacetic acid (TBA–TCA) Extraction reagent
    - 15% (w/v) TCA (Trichloroacetic acid)
    - 0.375% (w/v) TBA (Thiobarbituric acid)
    - 0.25N HCl (Hydrochloric acid)
18. Transfer buffer
    - 0.0009N Acetic acid
19. Washing Buffer 1
    - mmol/L (1mM) phenylmethylsulfonyl fluoride (PMSF)
20. Washing Buffer 2
    - 100 mM Tris, pH(8.0) (Tris(hydroxymethyl)aminomethane,
    - 1 mM phenylmethylsulfonyl fluoride
    - 20 mM EDTA (Ethylenediaminetetraacetic acid, EDETA).
21. Washing Buffer 3
    - 1 % β- Mercaptoethanol (β-MSH)
    - 100 % Acetone
22. Washing buffer 4
    - 1X PBS, pH 7.4
    - 0.1 % (v/v) Tween 20
    - 1 % (w/v) Non-Fat Dry milk
23. Washing buffer 5
    - 1X PBS, pH 7.4
    - 0.1 % (v/v) Tween 20

3-1-4- Apparatus and Instruments

Automatic Pipettes	Eppendorf, Hamburg, Germany
Bio-Rad Microplate Reader 3550-UV	Bio-Rad, Munchen, Germany
Electrophoresis Chamber	Home Made
Enhance Chemilumenscece (ECL) system	Bio-Rad, Germany
Eppendorf 5414C table centrifuge	Eppendorf, Hamburg, Germany
pH-Meter: pH537	Carl Roth GmbH & Co, Karlsuhe, Germany
Incubator with CO_2	ZAPF Instrument, Germany
Light Microscope	Olympus,
Fluorescence Microscope	Zeiss, Oberkochen, Germany
Macklar Counting Chamber	Macklar Co.
Magnetic stirrer	Eppendorf, Hamburg, Germany
Magnetic Bars	Eppendorf, Hamburg, Germany
Power supply	Amersham Bioscience, Freiburg, Germany
Simple beam photometer	Amersham Bioscience, Freiburg, Germany
Ultrasonic bath Transonic 460	Elma GmbH, Singen, Germany
Vortex	Elma GmbH, Singen, Germany

3-1-5- Antibodies

3-1-5-1- Primary antibodies

1- Protamine 1 (Hup 1N) — Mouse polyclonal antibody (Gift from Prof Dr Rod Balhorn, Lawrenece Livemore National Laboratory, USA)

2- Protamine 2 (Hup 2B) — Mouse polyclonal antibody (Gift from Prof Dr Rod Balhorn, Lawrenece Livemore National Laboratory, USA)

3-1-5-2- Secondary antibody

- Western Blot — Horseradish peroxidase conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany)

3-1-6- ELISA Kits

Table 3. ELISA Kits

Kit	Company
OxyStat ELISA, Reactive Oxygen Species (Peroxidase)	Biomedica Medicine product GmbH & Co KG, Wien, Austria
Cotinine ELISA	Calbiotech, CA, USA
8-Hydroxy-2-deoxy Guanosine (8-OH- dG) ELISA Kit	Cayman Chemical Company, USA
In Situ Cell Death Detection Kit, Fluorescein (TUNEL)	Roche Diagnostics GmbH, Mannheim, Germany

3-2- Methods

3-2-1- Ejaculates and Sperm Preparation

The semen samples were prepared with modification as in previously thoroughly described by Hammadeh *et al.*, (1996). Briefly:

1- The semen ejaculates obtained by masturbation collected in separate polypropylene containers and assayed within 2 hours after collection.

2- Semen ejaculates were kept at 37°C for 30 minutes until complete liquefaction had occurred. (If liquefaction is not completed, syringes were used to liquefy the samples).

3- In all samples a standard semen analysis (semen volume, sperm concentration, total sperm count and motility) was performed within one hour of obtaining the sample according to the WHO criteria (WHO, 1999). Morphology evaluated according to strict criteria.

4- The following volumes of seminal plasma were used to prepare smears:

- 10 µl to prepare smears (6 smears prepared)
- 100 µl for Hypo-osmotic (HOS) test
- 5 µl for Eosin test
- 5 µl for MAR test

5- Samples were prepared by discontinuous pure sperm gradient (Nidacon International AB, Sweden) by layering 1ml of each different concentration of pure sperm solution 80%, and 40% in a sterilized tube, beginning at the bottom of the tube with that of 80% density.

6- Two ml of liquefied semen was placed on the surface of the pure sperm fraction of lowest density (40%).

7- Centrifugation of the samples was done at room temperature (RT) for 25 minutes at 3000 rpm.

8- After centrifugation, the supernatant (seminal plasma) was immediately separated, and examined before storage to rule out the presence of spermatozoa in the supernatant. The seminal plasma was aliquot into storage ampoules and stored at -80°C until used.

9- The 40% layer aspirated and discarded with Pasteur pipette.

10- The final fraction that contains motile spermatozoa transferred into a new tube and washed by adding 4-5 ml HAM's F10 medium using 10 min of centrifugation at 3000 rpm at room temperature.

11- The pellet then was overlaid with 2-3 ml HAM's F10 medium and counting the sperm numbers.

12- Aliquot contains 20 - 40 X 10^6 sperm of each sample transferred into eppendorf and centrifugated for 10 min at 3000 rpm at room temperature, then the pellet stored at – 80 ^{o}C until used for sperm nuclear protein extraction.

13- The rest of the sperms also treated the same and kept at – 80 ^{o}C.

3-2-2- Assessment of Sperm Morphology

Sperm morphology was evaluated according to Kruger's strict criteria (Kruger *et al.*, 1986).

1- Smears were prepared by spreading 10 µl of seminal plasma on a glass slide, dry at room temperature.

2- The slides then were stained using Papnucleou method (WHO, 1999).

3- A total of 100 spermatozoa from each slide were evaluated under oil immersion at a magnification of 1000 X using bright field illumination. At least 10 high power fields from different areas of the slide were evaluated.

3-2-3- Assessment of Sperm Vitality (Eosin Test):

Test was carried out according to the method described by Eliasson and Treich, (1971), briefly:

1- On a glass slide, 5 µl of seminal fluid was mixed with 5 µl of 0.5% aqueous yellowish eosin Y solution.

2- The mixture covered with a cover slide, then evaluated after 1-2 min by distinguishing between the dead spermatozoa (Red stained) and the live spermatozoa (not stained). 100 spermatozoa from each slide were evaluated.

3-2-4- Assessment of the Sperm Membrane Integrity (Hypo-Osmotic Test; HOS)

HOS test performed according to the method described by Jeyendran *et al.,* (1984) in brief:

1- 0.1 ml of fresh ejaculate was mixed with 1.0 ml of the hypo osmotic solution

2- The mixture then incubated at 37°C for 30-60 minutes.

3- A sample was evaluated by taking 10 µl of the mixture on a slide and examined under phase contrast microscope. A minimum of 200 spermatozoa were examined per slide.

4- Calculate the percentage of spermatozoa that showed typical tail abnormalities (good sperms).

Percentage of spermatozoa with tail swelling = [(number of spermatozoa with tail swelling) / (total number of spermatozoa counted)]* 100%

3-2-5- Assessment of Chromatin Condensation (Chromomycin A_3, CMA_3) of Spermatozoa

Chromomycin A_3 (CMA_3) performed as previously described by Bianchi *et al*, (1993).

1- A semen aliquots were washed using Ca-Mg free phosphate buffered saline (PBS).

2- Then samples were centrifuged at 3000 rpm at RT for 10 minutes.

3- The spermatozoa then washed once again as in steps 1 and 2.

4- Resuspended the spermatozoa in few drops of the PBS.

5- Then 10 µl of each sample was smeared on microscopic slide and air dried.

6. Smears were then fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) at room temperature for 2 hours, then air dried.

7- Each slide was treated for 20 minutes with 100 µl of CMA_3 solution at 25°C in the dark.

8- Slides then were washed in PBS.

9- Slides were covered by cover slips and mounted with buffered-glycerol.

10- For evaluation, a total of 200 spermatozoa were analyzed on each slides, by distinguishing spermatozoa stained bright green (CMA_3 positive, with bad chromatin condensation) from those stained dull green (CMA_3 negative, with good chromatin condensation). A Zeiss Photomicroscope III, used for the fluorochrome evaluation via a combination of exciter dichromic barrier filter of BP 436/10: FT 580: LP 470.

3-2-6- DNA Fragmentation Analysis

(Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling, TUNEL Test)

DNA fragmentation was assessed using the Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay as previously described (Borini *et al.,* 2006). The TUNEL assay was performed using the In-Situ Cell Death Detection Kit: Fluorescein following the manufacturer's guidelines (Roche Diagnostics GmbH, Mannheim, Germany).

1- Ejaculated sperm samples were washed from seminal plasma by slow speed centrifugation (250 g for 10 minutes)

2- Smears were prepared using 10 µL of sperm suspension on microscope slides, and allowed to air dried

3- Smears were fixed with 4% paraformaldehyde phosphate–buffered saline, pH 7.4 at room temperature for tow hours then rinsed with PBS.

4- Smears then permeabilized with 0.1% triton X-100 in 0.1% sodium citrate, pH 6.0 for 10 minutes at RT.

5- 50 µl of the TdT-labeled nucleotide mixture (50 µl of enzyme solution and 450 µl of label solution) was added to each slide and incubated in a humidified chamber at 37°C overnight in the dark. Negative controls without TdT enzyme were run in each replicate.

6- Slides were rinsed twice in PBS.

7- Slides were covered by cover slips and mounted with buffered-glycerol.

8- For evaluation, a total of 500 spermatozoa were analyzed on each slides, by distinguishing spermatozoa stained bright green (TUNEL positive, with bad chromatin condensation) from those stained dull green (TUNEL negative, with good chromatin condensation). A Zeiss Photomicroscope III, used for the fluorochrome evaluation via a combination of exciter dichromic barrier filter of BP 436/10: FT 580: LP 470. A negative control was performed for each sample by using fluorescein-isothiocyanate-labelled dUTP without enzyme.

3-2-7- Protamine Quantification

3-2-7-1- Determination of protein concentration according to Bradford

A- Preparation of protein standard curve

Bovin-IgG protein was used to prepare the standard curve as following:

50 mg of IgG-bovine was dissolved in 5 ml of distilled water (DW) as a stock solution (A, 10mg/ml =10 µg/µl)), then the standard concentrations were prepared by taking:

- 700 µl of stock A in 1 ml DW, this will give a concentration of 7 µg/ml (solution B)
- 500 µl of stock A in 1 ml DW, this will give a concentration of 5 µg/ml (solution C)
- 300 µl of stock A in 1 ml DW, this will give a concentration of 3 µg/ml (solution D)
- 200 µl of stock A in 1 ml DW, this will give a concentration of 2 µg/ml (solution E)
- 100 µl of stock A in 1 ml DW, this will give a concentration of 1 µg/ml (solution F)

B- Determination of protein concentration

Measuring the concentration of proteins in the extractions was performed using the Bio-Rad method which based on Bradford reagent as following:

1- Reaction mixtures were prepared as following (table 4): each labelled tube contains:

Table 4. Reaction mixtures used for preparing the protein standard curve.

T.T. No.	Volume of concentration (µl)	D. H_2O (µl)	Protein assay reagent (µl)	Final Volume (µl)
1	1µl stock F (1 µg/ml)	799	200	1000
2	1µl stock E (2 µg/ml)	799	200	1000
3	1µl stock D (3 µg/ml)	799	200	1000
4	1µl stock C (5 µg/ml)	799	200	1000
5	1µl stock B (7 µg/ml)	799	200	1000
6	1µl stock A (10 µg/ml)	799	200	1000
7	1.5 µl stock A (15 µg/ml)	798.5	200	1000
8	2 µl stock A (20 µg/ml)	798	200	1000
9	2.51µl stock A (25 µg/ml)	797.5	200	1000

Note: Concentrations prepared in triple

2- Mixed by vortex and then incubated 5 minutes at room temperature.

3- The absorbance was measured against blank of distilled water at wavelength 595 nm.

(For measuring the proteins in the extraction, absorbance measured against blank with extraction buffer)

3-2-7-2- Total Nuclear Protein Extraction:

3-2-7-2-1- Tail Dissociation

Sperm's tail dissociation was done as previously described by Balhorn *et al.,* (1977), with modifications as follows:

1- Sperms Aliquot contains 20 - 40 X 10^6 sperm prepared in section (3-2-1) was suspended in 200 µl of 0,05 M Tris-HCl, pH 8.0 and 10 mM dithiothreitol (DTT) and sonicated for 10 seconds then incubated at 4 ^oC for 15 minutes.

2- 50 µl of 5% Hexadecyltrimethylammonium bromide (CTAB) was added to obtain a final concentration of 1% CTAB and the sample was sonicated for 30 seconds, mixed and incubated for additional 30 minutes at 4 ^oC.

3- The heads then pelleted by centrifugation at 3000 g and the supernatant discarded.

4- The heads were washed twice in 0.5 ml of 1% CTAB in 0.01 M Tris-HCL, pH 8.0 and then twice in 0.5 ml of Tris-Saline.

5- The supernatant discarded and the pellets then stored at – 80 ^oC until used for protein extraction.

3-2-7-2-2- Protein Extraction

Sperm nuclear proteins extraction of each sample (n= 166) was performed by using an established protein extraction protocol described by Carrell and Liu, (2001) with modifications as following:

1- The heads pellets were taken out from – 80 ^oC and used.

2- The pellets were washed in 1 ml of the washing buffer I containing 1 mM (mmol/L) of phenylmethylsulfonyl fluoride in distilled water (PMSF) and centrifuge at 3000 rpm for 5 minutes at room temperature.

3- Then, each pellet was resuspended in 100 µl of the washing buffer 2 containing 20 mM EDTA, 1 mM PMSF in 0.1 M Tris (pH 8.0) and mixed by vortex for 15 seconds at a high speed.

4- 100 µl of the decondensation buffer 1 composed of 6 M guanidine and 575 mM dithiothreitol (DTT) was added and mixed by vortex for 15 seconds at a high speed.

5- Then, 200 µl of decondensation buffer 2 composed of 522 mM sodium iodoacetate was added to the mixture then mixed by vortex at high speed for 30 seconds.

6- The mixture then incubated in dark place (protected from light) at room temperature for 30 min.

7- 1 ml of ice cold absolute ethanol was added and after mixing incubated at – 20°C for one minute.

8- The preparation then centrifuged at 13,000rpm for 10 min at 4°C and the supernatant discarded. (Repeat this step once).

9- The pellet was resuspended in 0.8 ml of denaturing solution 0.5 M HCl, then mixed and incubated for 15 min at 37 ^{o}C.

10- The preparation then centrifuged at 13,000rpm for 10 minutes at 4°C.

11- Then the supernatant was transferred to another tube each contained 200 µl of 100% trichloroacetic acid (TCA). (The final concentration TCA should be 20%, adjust the volume as needed).

12- The mixture was incubated in ice for 3 minutes and centrifuged at 13,000rpm for 10 min at 4°C.

13- Carefully, the supernatant was removed. (The precipitate may be invisible, do not disturb the precipitate).

14- The precipitate then washed in 1 ml of washing buffer 3 containing 1% ß-mercaptoethanol in 100% acetone then mixed by vortex for 15 seconds at a high speed. (Generally the precipitate can be seen).

15- The preparation was centrifuged at 13,000rpm for 8 minutes at 4°C and the supernatant removed as much as possible. (Repeat this step once).

16- The final pellet was air-dried at 4°C overnight and stored at -20 ^{o}C.

3-2-7-3- Production of a Protamine Standard

A human protamines standard was prepared as described by Mengual *et al.,* (2003). Sperm samples of 20 known fertile donors were pooled to give a total of $1x10^9$ sperm.

1- The semen samples were centrifuged at 3000 rpm for 10 minutes at RT to remove the seminal plasma.

2- The sperms then washed with PBS and then divided into eppendorfs each contained $1x10^8$ sperms then centrifuged at 3000 rpm for 10 minutes at RT and discarded the supernatant.

3- The pellet of each sample was resuspended in 0.5 ml of the denaturing solution (0.5 M HCl) and incubated at 37 ^{o}C for 15 min.

4- The samples then centrifuged at 3000 rpm for 5 min at RT then the supernatant removed.

*NOTE: This incubation will extract the histone fraction from the sperm nuclei.

5- The pellets washed with 0.25 ml of washing buffer 2 (20 mM EDTA, 1 mM PMSF in 0.1 M Tris (pH 8.0)) then centrifuged at 3000 rpm for 5 min at RT then discarded the supernatant.

*NOTE: This step neutralizes the pH of the suspension for subsequent protamine extraction.

6- Go back to step 2 in section "3-2-7-2, Total Nuclear Protein Extraction" and complete the extraction protocol.

7- Each sample of the extracted protamines was resuspended in 50 µl sterile DW.

8- All samples were collected in one tube.

*NOTE: If precipitate was present, additional 100µL of DW added until completely soluble.

9- The final protein concentration was determined by using the RC DC protein assay kit (BioRad Laboratories, USA).

10- The protamine extract was run using acid-urea polyacrylamide gel electrophoresis to determine the ratio of P1 and P2 (Refer to section 3-2-7-5 below). Bands of P1 and P2 can be seen in figure 24, lanes 6-9.

11- The intensity of the P1 and P2 bands were used to draw the standard curve and to calculate the linear equation and the r^2 value of the regression curve (Figure 26).

3-2-7-4- Protein Extraction Control Sample

Semen samples were pooled from 20 individuals treated as in standard preparation (3-2-7-3).

1- A 40 x10^6 sperm aliquots prepared as in sample preparation and stored at at – 80 ^{o}C.

2- One aliquot was extracted in tandem with test samples for every run.

3- Follow total sperm extraction protocol as above (section 3-2-7-2).

* Refere to figure 24, lane 10

3-2-7-5- Acid-Urea Polyacrylamide Gel Electrophoresis (AU-PAGE)

Protamine extracts were tested by using acetic acid-urea polyacrylamide gel electrophoresis (AU-PAGE) method as described by Carrell and Liu, (2001) with modifications.

3-2-7-5-1- AU-PAG preparation:

1- Acid-urea polyacrylamide gel was prepared in a vertical stand. Tow glass plates and spacers were placed vertically and fixed between clamps in a sandwich module, making by spacers to fill the gel in.

2- The resolving gel (20%) was prepared and filled in about 10 cm of the space (the glass plates).

3- 400 µl of N-Butanol were added on the top of the resolving gel to avoid air bubbles.

4- After polymerization, N-Butanol discarded and stacking gel (7.5%) was prepared and filled in the space.

5- Then comb was placed to make columns to fill the protein extracts.

6- After gel was polymerized it was kept in moist atmosphere in refrigerator until used.

For resolving gel preparation we used an acrylamide solution with 40% (w/v) acrylamide and 0.8% (w/v) N,N′-Methylenebisacrylamide, while for stacking gel we used an acrylamide solution with 30% (w/v) acrylamide and 0.8% (w/v) ′N,N -Methylenebisacrylamide and 0.375M potassium acetate (pH 4.0). For both gels we used 2.5 M urea and 43% acetic acid. In addition, for polymerization we used 1.6% (w/v) ammonium persulphate for both and 0.5% and 2% (w/v) TEMED for resolving and stacking gels respectively. Table (5) summarizes the volumes of solutions used for preparing both gels.

Table 5. Solution's volumes used for preparing polyacrylamide gels.

Resolving gel	20 %
40 % polyacrylamide gel (19:1) (ml)	9
10 M Urea (ml)	4 (final concentration 2.5M)
43 % Acetic Acid (ml)	2
1.6 % Ammonium Persulphate (ml)	2 (freshly prepared)
TEMED (µl)	80

Stacking gel	7.5 %
30 % polyacrylamide gel (19:1) (ml)	1
10 M Urea (ml)	1 (final concentration 2.5M)
43 % Acetic Acid (ml)	1
1.6 % Ammonium Persulphate (ml)	0.5 (freshly prepared)
3 M Potassium Acetate (pH 4.0) (ml)	0.5
TEMED (µl)	80

3-2-7-5-2-Electrophoresis

Acid-Urea gel electrophoresis was performed using the vertical electrophoresis system (home made). Three steps (A, B, and C) separating process was applied. A reverse polarity and 5% acetic acid as running buffer were used in all steps.

A- Pre-electrophoresis

1- The AU-polyacrylamide gel was electrephorised before loading with samples.

2- The electrophoresis conditions were 200 volts, 40 mA for 1.5 hours (reverse polarity was used).

3- The running buffer discarded and a new one used for the next step.

B- Slow run electrophoresis

1- The extracted nuclear proteins were solublized in 80 µL sample loading buffer (0.375 M potassium acetate (pH 4.0), 15% sucrose, 0.05% Methyl Green).

2- Lanes of each gel loaded with 10 µL of each sample, control sample and human protamine standards (0.25, 0.50, 1.00, 1.50, and 2.00 µg).

3- The electrophoresis conditions were 100 volts, 40 mA for 45 minutes (reverse polarity was used).

C- Fast run electrophoresis

The same gels with the same running buffer used but the electrophoresis conditions were changed to be 200 volts, 80 mA for 5-6 hours (reverse polarity was used).

3-2-7-5-3- Staining

Gels were stained with Coomassie Blue stain.

1- The gel was taking out of the glass plates.

2- The stacking gel was cut away

3- The gel was transferred to a container contains Coomassie Blue stain for 20 minutes at RT with shaking.

4- The stain removed from the container and the gel was washed with water to remove the excess of the stain.

5- The gels then destained using destaining buffer (20% methanol and 10% acetic acid) over night with shaking at RT.

6- The staining buffer was removed and gel was washed in DW for 1-2 hours with shaking at RT.

7- The gels then were scanned using a Bio-Rad scanner system.

8- The intensity of the bands corresponding to P1 and P2 were quantified. Figure 24, lanes 1-5 and 11-12 represents the bands of different sperm samples.

9- P1 and P2 quantities were calculated against the standard curve generated from the human protamine standard as described above in section (3-2-7-3) (figure 26).

3-2-7-6- Western Blot

Proteins were transferred from gel to a PVDF- membrane (Roche, Germany).

1- Gels were transferred to the transfer set contained a wetted PVDF- membrane (2 gels and 2 membranes prepared).

2- The transfer set then transferred to the blotting tank contained the transfer buffer (0.0009 N acetic acid).

3- The transfer conditions were 150 mA, 75 V for 2 hours (reverse polarity was used)

4- Then Membranes were blocked in blocking buffer (1X PBS, pH 7.4, 0.1% (v/v) Tween 20, and 5% (w/v) non-fat dry milk) for 1 hour at room temperature (RT) with shaking.

5- The membranes then were washed with washing buffer 4 (1x PBS, pH 7.4, 0.1% (v/v) Tween 20, 1% (w/v) non-fat dry milk) three times, each for 5 minutes with shaking at RT.

6- The membranes were incubated with the primary antibody diluted in washing buffer 4 (1X PBS, pH 7.4, 0.1% (v/v) Tween 20, 1% (w/v) non-fat dry milk) overnight at 4 ^{o}C with shaking (two membranes used, each in a separated jar):

- One membrane incubated with Hup 1N (anti-protamine 1) primary antibody diluted into 1:100000

- Second membrane incubated with Hup 2B (anti-protamine 2) primary antibody diluted into 1:500000

7- The membranes were washed with washing buffer 4 three times, each for 5 minutes with shaking at RT.

8- Then membranes were incubated with the horseradish peroxidase-conjugated secondary antibody diluted in washing buffer 5 for 1 hour at RT with shaking. (Horseradish peroxidase-conjugated Goat anti-mouse secondary antibody was diluted into 1:10000).

9- Then membranes were washed with washing buffer 5(1X PBS, pH 7.4, 0.1% (v/v) Tween 20) three times, each for 5 minutes with shaking at RT.

10- Signals were developed by using the Lumi-light western blotting substrate 1 and 2 (1 ml of each for one minute) (Roche, Mannheim, Germany).

11- Then the signals were visualized by using the Enhance Chemilumenscece (ECL) system (Bio-Rad, Germany).

* Figure 25 represents the replica of the gel of P1 (A) and replica of the gel of P2 (B).

3-2-8- Reactive oxygen species measurement

The concentration of ROS was measured by a colorimetric assay for the quantitative determination of peroxides in EDTA–plasma, serum and other biological fluids using ELISA kit (Oxy Stat; Cat. No. BI-5007 Biomedica Medicine product GmbH & Co KG, Wien, Austria).

Principal of the assay: The peroxide concentration is determined by the reaction of the biological peroxides with peroxidase and a subsequent color–reaction using TMB (3, 3, 5, 5-Tetramethylbenzidine) as a substrate. After the addition of the stop solution, the colored liquid was measured photometrically at 450nm. A calibrator was used to calculate the concentration of circulating biological peroxides in the sample (one point calibrator).

Assay characteristics: The Biomedica Oxy Stat assay measures the total concentration of peroxides, which is formed in the propagation-phase of the low density lipoprotein oxidation process using peroxidase/TMB. 12x8 well microtiter plate format (96 tests per kit). Reference values EDTA plasma <400µmol/L, serum <350µmol/L. Measuring range 7-6000µmol/L; Detection limited 7µmol/L. Sample volume 10µL /test. Assay time 30 minutes.

Assay protocol

Following the manufacturer's guidelines:

1- 10 µl of each control, calibrator and sample was added to respective well

2- 100 µl of solution A (Sample Buffer) was added to each well and mixed mix.

3- The absorbance was determined with ELISA reader at 450 nm (OD1)

4- Then 100 µl of the freshly prepared ABC-reaction mixture (5 ml of solution A + 100 µl of solution B (reaction Buffer) + 5 µl of solution C (enzyme solution)) was added to each well and mixed.

5- Plate then was incubated for 15 minutes at 37 ^{o}C.

6- 100 µl of solution D (stop solution) was added to each well and mixed.

7- The absorbance was determined with ELISA reader at 450 nm (OD2)

8- Calculate the concentration of peroxide (µ mol/L) according to the equation

Peroxide (µ mol/L) = ([ΔOD sample X (µ mol/L) calibrator]/ ΔOD calibrator)

ΔOD = OD2 – OD1

3-2-10- [8-hydroxy-2-deoxyguanosine (8-OH-dG)] measurement

8-OH-dG Enzyme immunoassay (EIA) kit (Cayman Chemical Company, USA) is a competitive assay that can be used for the quantification of 8-OHdG in urine, cell culture, plasma, and other sample matrices. The EIA utilizes the anti-mouse IgG-coated plate and tracer consisting of an 8-OHdG-enzyme conjugate. This format has the advantage of providing low variability and increase sensitivity compared to assays that utilize an antigen-coated plate. This EIA typically display IC_{50} (50% B/B_o) and IC_{80} (80% B/B_o) values of approximately 100 and 30 pg/ml, respectively.

It is important to note that 8-OH-dG-antibody used in this assay recognizes both free 8-OHdG and DNA-incorporated 8-OHdG. Since complex samples such as plasma, cell lysates, and tissue are comprise mixtures of DNA fragments and free 8-OH-dG, concentrations of 8-OHdG reported by EIA methodology will not coincide with those reported by LC-MS where the single nucleoside is typically measured.

Precision

The intra- and inter-assay CV's have been determined at multiple points on the standard curve.

Table 7. Intra- and inter- assay variation.

Dose (pg/ml)	%CV* Intra-assay variation	%CV* Inter-assay variation
3000	6.2	8.4
1333	6.1	4.6
592.6	9.6	4.8
263.4	4.7	5.5
117.1	9.3	4.5
52.0	11.6	10.7
23.1	+	+
10.3	+	+

* %CV represents the variation in concentration as determined a reference standard curve.
+ Outside of recommended usable range assay.

Specificity

Table 8. Specificity of 8-hydroxy-2-deoxy Guanosine monoclonal antibody.

Compound	Cross-reactivity
8-hydroxy-2-deoxy Guanosine	100%
8-hydroxy Guanosine	23%
8-hydroxy Guanine	23%
Guanosine	<0.01%

Assay Procedures

Procedure applied below was followed in sequence following the manufacturer's guidelines:

1- A 100 µl of the EIA buffer was added to Non-Specific Binding (NSB) wells.

2- A 100 µl of the EIA buffer was added to Maximum Binding (B_o) wells.

3- A 50 µl of each standard concentration (refer to standard preparation down) was added into the wells in duplicate.

4- A 50 µl of seminal plasma was added into the wells in duplicate.

5- Then 50 µl of 8-hydroxy-2-deoxy Guanosine AChE tracer was added to each well except the wells of Total Activity (TA), and the Blank (Blk) wells.

6- Then 50 µl of 8-hydroxy-2-deoxy Guanosine Monoclonal antibody was added to each well except the wells of Total Activity (TA), Non-Specific Binding (NSB), and the Blank (Blk) wells.

7- The plate then was covered with plastic film and incubated for 18 hours at 4 °C.

8- The wells then were empty and rinsed 5 times each with 300 µl with wash buffer.

9- A 200 µl of Ellman's Reagent was added to each well.

10- A 5 µl of 8-hydroxy-2-deoxy Guanosine AChE tracer was added to the total activity wells.

11- Then the plate was covered with plastic film and incubated for 1.5-2 hours at room temperature with shaking in dark.

12- Absorbance was read on ELISA reader at 405- 420 nm. The plate should be read when the absorbance of B_o wells is in the range of 0.3-1.0 A.U. (blank subtracted).

13- The concentrations of the samples were calculated using the equation obtained from the standard curve plot (Plot $\%B/B_o$ for standard (vertical, linear) versus 8-OH-dG concentrations (horizontal, Log).

Standard preparation

A 30 ng/ml bulk solution standard was prepared by transferring 100 µl from the tube that contains 8-OH-dG EIA standard (300 ng/ml) into a clean test tube, then dilute it with 900 µl ultra pure water.

To prepare the standard for use: eight clean test tubes were obtained and numbered them # 1 through # 8. Then aliquot 900 µl of µl EIA buffer to tube # 1 and 500 µl EIA buffer to tube # 2 – 8. Then:

- 100 µl from the bulk standard (30ng/ml) was transferred to tube # 1 and mix thoroughly (3000 pg/ml). Then serial dilutions were prepared by transferring:

- 400 µl from tube # 1 to tube # 2 and mix thoroughly, this will give 1333.0 pg/ml.
- 400 µl from tube # 2 to tube # 3 and mix thoroughly, this will give 592.6 pg/ml.
- 400 µl from tube # 3 to tube # 4 and mix thoroughly, this will give 263.4 pg/ml.
- 400 µl from tube # 4 to tube # 5 and mix thoroughly, this will give 117.1 pg/ml.
- 400 µl from tube # 5 to tube # 6 and mix thoroughly, this will give 52.0 pg/ml.
- 400 µl from tube # 6 to tube # 7 and mix thoroughly, this will give 23.1 pg/ml.
- 400 µl from tube # 7 to tube # 8 and mix thoroughly, this will give 10.3 pg/ml.

3-2-11- Lipid Peroxidation Assay in Seminal Plasma

(Measurement of Malondialdehyde, MDA)

Lipid peroxidation level of biological samples (serum, sperm, seminal plasma, and follicular fluid) was measured by determining the Malondialdehyde (MDA) production, using a modified thiobarbituric acid (TBA) from the method of Buege and Aust (1978) and of Suleiman *et al.,* (1996).

Briefly,

1- Take 250 µl of seminal plasma in a clean glass tube.

2- Add 2.0 ml of the extraction reagent (section 3-1-3) and mix by vortex 15 sec.

3- Boil samples at 95 ^{o}C for 30 min in water bath.

4- Coal the samples at room temperature.

5- Centrifuge the samples at 3000 rpm for 10 min at RT.

6- Take the supernatant and read the absorbance at wave length 535 nm against blank.

7- Calculate the concentration of MDA from the standard curve equation.

For Standard Curve preparation, the same procedures applied using a 250 µl sample of each standard concentration in duplicate.

Standard Solutions

Malondialdehyde standard was prepared by modifying the method described by Sangalli et al., (2003). Briefly,

10 mM stock solution (A) was prepared by mixing 25.97 µl of 1,1,3,3-tetramethoxypropane (6 M) with 1M hydrochloric acid at 37 ^{o}C 30 minutes. This solution was then further diluted with distilled water to prepare various working standard solutions as following:

- 50 µl of stock A in 5 ml DW, this will give a conc. of 100 µM (solution B)
- 500 µl of stock B in 5 ml DW, this will give a conc. of 10 µM (solution C)
- 375 µl of stock B in 1 ml DW, this will give a conc. of 7.5 µM (solution D)
- 250 µl of stock B in 1 ml DW, this will give a conc. of 5 µM (solution E)
- 125 µl of stock B in 1 ml DW, this will give a conc. of 2.5 µM (solution F)
- 50 µl of stock B in 1 ml DW, this will give a conc. of 1.0 µM (solution G)
- 25 µl of stock B in 1 ml DW, this will give a conc. of 0.5 µM (solution H)

* Standard solutions stored at 4 ◦C.

3-2-9- Cotinine measurement

Summary and Explanation

The Calbiotec Cotinine Direct Elisa Kit (Calbiotech, CA, USA) is design to detect the presence of Cotinine in serum and urine. It can also be adapted for other fluids.

Principles of the test

The calbiotech cotinine kit is a solid phase competitive ELISA. The samples and cotinine enzyme conjugate are added to the wells coated with anti-cotinine antibody. Cotinine in the samples competes with a cotinine enzyme (Horse Radish Peroxidase, HRP) conjugate for binding sites. Unbound cotinine and cotinine enzyme conjugate is washed off by washing step. Upon the addition of the substrate, the intensity of colour is inversely proportional to the concentration of Cotinine in the samples. A standard curve is prepared relating colour intensity to the concentration of the Cotinine.

Performance Characteristics

Accuracy

20 urine samples from non smokers were screened with this cotinine ELISA method. All 20 samples screened negative with the ELISA method. 15 samples from smokers which contained various amounts of cotinine were screened with this cotinine direct ELISA Kit. All 15 samples showed a presence of Cotinine at a level greater than 500ng/ml. Three urine samples submitted by individuals exposed to passive inhalation for over 30 days all showed levels of 5 to 10 ng/ml of Cotinine when extrapolated of a dose response curve.

Sensitivity

Assay sensitivity based on the minimum cotinine concentration required to produce a three standard deviation from assay Ao is 1 ng/ml.

Specificity

The specificity of this Cotinine ELISA was determined by generating inhibition curves for each of the compounds listed below and the antisera cross-reactivity below:

Table 6. Specificity of cotinine antibody

Compound	Approximal ng/ml equivalent to 100 ng cotinine/ml	Cross-reactivity
Cotinine	100	100
Nicotine	>10000	<1
Nicotinamide	>10000	<1
Nicotinic acid	>10000	<1

Assay Procedures

All reagents must be brought to room temperature (18-26 °C) before use. The procedure described below was followed in sequence using manual pipettes. Following the manufacturer's guidelines:

1. 10 µl of standards, controls and specimens were pipette into selected well in duplicate.

2. Then 100 µl of the Enzyme Conjugate was added to each well. Then the plate was shacked for 10-30 seconds to ensure proper mixing.

3. The plate then incubated for 60 minutes at room temperature (18-26° C) in the dark.

4. The wells were washed 6 times with 300 µl distilled water (take care not to cross contaminate wells).

5. The plate was inverted and vigorously slapped dry on absorbent paper to ensure all residual moisture was removed. This step was critical to ensure that residual enzyme conjugate, did not skew results.

6. Then 100 µl of Substrate reagent was added to each well.

7. Plate was incubated for 30 minutes at room temperature in the dark.

8. 100 µl of Stop Solution was added to each well and the plate was shacked gently to mix the solution.

9. Absorbance was read on ELISA reader at 450nm with in 15 minutes after adding the stopping solution.

10- The concentration of cotinine was calculated against the standard curve generated from the standards (0.0, 5.0, 10, 25, 50, 100 ng/ml) applied in the kit in the same plate (Absorbance for cotinine standards (vertical axis) versus cotinine standards concentrations (horizontal axis)). Samples with concentrations higher than 100 ng/ml were diluted and measured once more.

3.2.12. Statistical analysis

Data analysis was performed using the personal computer of the local area computer network of the Institute of Medical Biometrics and Medical Information, University of Saarland, Homburg/Saar, Germany, using the SPSS 17 for Windows Software Package (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD and range. The relationship between protamines 1 and 2, P1/P2, ROS, cotinine, 8-hydroxy-2-deoxy-Guanosine, MDA, DNA integrity, chromatin condensation, sperm viability, membrane integrity, concentration, motility and morphology and seminal volume were analyzed using nonparametric correlation (Spearman's test). Furthermore, semen characteristics, ROS, MDA, 8-OHdG and cotinine concentrations were analyzed in seminal plasma in smokers and non-smokers and their effect on ART results were analyzed using parametric and nonparametric methods. The Mann-Whitney (U-test) was used for non–paired data. One way-ANOVA was used to compare different parameters within groups of protamines ratios; Post HOC Test- Bonferroni test was used. The results were presented by the mean ± SD values. Correlations were analyzed by means of chi-square test or exact Fisher-test and by Spearman's test. They were considered statistically significant when $p<0.050$ and highly significant when $p<0.010$.

4- Results

Semen samples were collected from an unselected group of patients consulting for infertility and from a cohort of volunteer donors. In all samples, data on semen quality, ROS production, seminal plasma TBAR substances (MDA), Cotinine, and 8-OHdG, and sperm DNA fragmentation were collected of 50 normal volunteer donors and of 116 male patients (Fig. 7). In addition, protamine 1, protamine 2, and P1/P2 ratios data were collected for all samples. All members of the donor group had normal values for sperm concentration (more than 20 X 10^6/mL), overall motility (40% motile), and normal morphology (30%) (WHO, 1999).

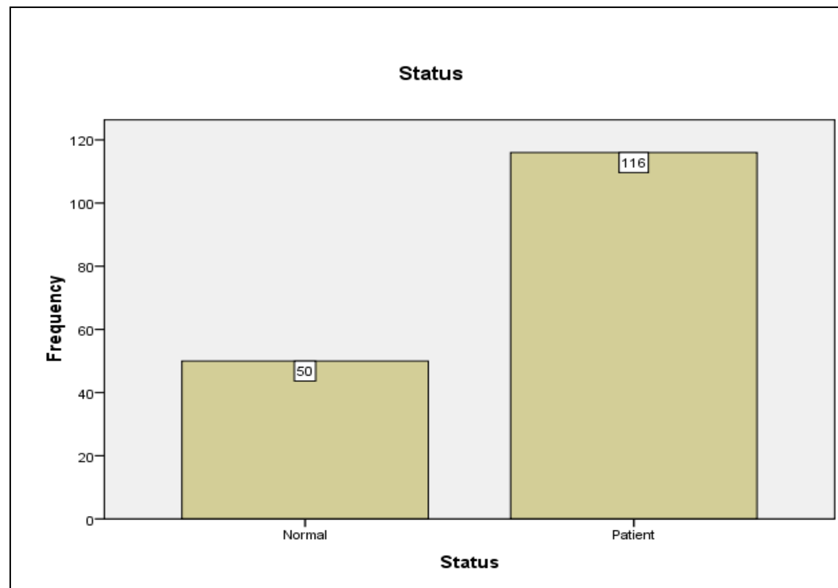


Figure 7. The distribution of samples (n=166) into normal volunteers (n=050) and patients (n=116).

4-1- Semen and Sperm Parameters for all Participants

Table 9. Statistical data for all participants

	Mean ± STD	Minimum	Maximum	25 %	50 %	75 %
Age (year)	34.14 ± 7.81	18.0	51.0	26.8	35.5	40.0
Volume (ml)	3.42 ± 1.63	0.0	10.0	2.0	3.5	4.1
pH	8.66 ± 0.38	8.0	10.0	8.5	8.5	9.0
Count (mill/ml)	67.08 ± 31.23	10.0	100.0	36.0	70.0	100.0
Motility (% motile)	30.33 ± 13.61	5.0	75.0	20.0	30.0	40.0
Sperm vitality (Eosin) (%)	37.83 ± 17.81	5.0	80.0	20.0	37.5	50.0
Membrane integrity (HOS) (%)%	47.59 ± 19.79	5.0	90.0	30.0	50.0	60.0
Leukocytes (%)	2.53 ± 1.48	1.0	3.0	2.0	2.0	3.0
Chromatin condensation (positive CMA_3) (%)	30.00 ± 9.01	11.9	75.5	24.6	30.0	35.2
DNA Fragmentation (positive TUNEL) (%)	12.77 ± 5.89	2.2	33.7	8.2	12.4	16.8
Morphologically Normal spermatozoa (%)	34.77 ± 14.92	5.0	76.0	25.0	34.0	44.3
Malondialdehyde (MDA) (µM)	7.21 ± 1.77	3.18	13.84	6.1	7.2	8.1
Reactive oxygen species (ROS) (µmol/l)	87.80 ± 50.85	7.0	255.6	47.1	87.8	118.3
Cotinine (ng/ml)	41.86 ± 58.18	1.0	233.3	1.2	5.9	73.4
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml)	1.59 ± 1.47	0.02	8.02	0.5	1.2	2.2
Protamine 1 (P1) (ng/10^6 sperm)	404.81 ± 102.37	171.8	656.8	324.3	396.8	476.1
Protamine 2 (P2) (ng/10^6 sperm)	358.69 ± 105.08	84.8	615.0	282.6	344.6	435.9
P1/P2 ratio	1.18 ± 0.33	0.63	3.63	0.99	1.17	1.32

Table 9 demonstrated the means ± standard deviations data of all sperm and seminal plasma parameters for all participants (n=166). A normal semen volume (ml) and sperm concentration (mill/ml) were indicated (3.42 ± 1.63; 67.08 ± 31.23 respectively). Sperm motility (%) was ranged from 5.0 % to 75.0 % while sperm vitality (Eosin, %) (5.0-80.0 %) and sperm membrane integrity (HOS, %) (5.0-90.0 %). Besides, the percentages of chromatin condensation (CMA_3 %, bad sperm) was (30.00 ± 9.01%), and DNA fragmentation (Tunel %, bad sperm) (12.77 ± 5.89 %%). in addition, the percentage of morphologically normal sperm was 34.77 ± 14.92%. Besides, the seminal plasma parameters MDA (µM), ROS (µmol/l), Cotinine (ng/ml), and 8-OHdG (ng/ml) concentrations were (7.21 ± 1.77, 87.80 ± 50.85, 41.86 ± 58.18, and 1.59 ± 1.47 respectively). Protamine 1 concentration (ng/10^6 sperm) was ranged (171.8-656.8), while minimum concentration of Protamine 2 (ng/10^6 sperm) was 84.8 and the maximum was 615.0. The P1/P2 ratio was ranged from 0.63 to 3.63.

Table 10a: Correlation coefficient of sperm parameters of all participant samples (n=166)

		Count (mill/ml)	Motility (%motile)	Sperm vitality (Eosin) (%)	Membrane integrity (HOS) (%)%	Chromatin condensation (positive CMA_3) (%)	DNA Fragmentation (positive TUNEL) (%)	Morphologically Normal spermatozoa (%)	Protamine 1 (ng/ 10^6 sperm)	Protamine 2 (ng/ 10^6 sperm)	P1/P2 Ratios
Count (mill/ml)	R =	1.000	0.194*	0.160*	0.113	-0.289**	-0.098	0.250**	0.122	0.115	-0.050
	P =	.	0.012	0.039	0.146	0.000	0.211	0.001	0.118	0.142	0.524
Motility (%motile)	R =	0.194*	1.000	0.494**	0.446**	-0.458**	-0.406**	0.479**	-0.023	0.094	-0.235**
	P =	0.012	.	0.000	0.000	0.000	0.000	0.000	0.766	0.230	0.002
Sperm vitality (Eosin) (%)	R =	0.160*	0.494**	1.000	0.598**	-0.343**	-0.263**	0.229**	-0.063	-0.005	-0.139
	P =	0.039	0.000	.	0.000	0.000	0.001	0.003	0.421	0.949	0.074
Membrane integrity (HOS) (%)%	R =	0.113	0.446**	0.598**	1.000	-0.384**	-0.330**	0.378**	0.090	0.146	-0.168*
	P =	0.146	0.000	0.000	.	0.000	0.000	0.000	0.251	0.060	0.031
Chromatin condensation (positive CMA_3) (%)	R =	-0.289**	-0.458**	-0.343**	-0.384**	1.000	0.479**	-0.514**	-0.011	-0.126	0.264**
	P =	0.000	0.000	0.000	0.000	.	0.000	0.000	0.889	0.107	0.001
DNA Fragmentation (positive TUNEL) (%)	R =	-0.098	-0.406**	-0.263**	-0.330**	0.479**	1.000	-0.509**	-0.004	-0.060	0.227**
	P =	0.211	0.000	0.001	0.000	0.000	.	0.000	0.962	0.439	0.003
Morphologically Normal spermatozoa (%)	R =	0.250**	0.479**	0.229**	0.378**	-0.514**	-0.509**	1.000	-0.189*	-0.037	-0.253**
	P =	0.001	0.000	0.003	0.000	0.000	0.000	.	0.015	0.633	0.001
Protamine 1 (ng/ 10^6 sperm)	R =	0.122	-0.023	-0.063	0.090	-0.011	-0.004	-0.189*	1.000	0.650**	0.237**
	P =	0.118	0.766	0.421	0.251	0.889	0.962	0.015	.	0.000	0.002
Protamine 2 (ng/ 10^6 sperm)	R =	0.115	0.094	-0.005	0.146	-0.126	-0.060	-0.037	0.650**	1.000	-0.525**
	P =	0.142	0.230	0.949	0.060	0.107	0.439	0.633	0.000	.	0.000
P1/P2 Ratios	R =	-0.050	-0.235**	-0.139	-0.168*	0.264**	0.227**	-0.253**	0.237**	-0.525**	1.000
	P =	0.524	0.002	0.074	0.031	0.001	0.003	0.001	0.002	0.000	.

Table 10a, 10b, and 10c illustrated the correlation coefficients between all parameters of semen and sperm quality of all participant samples (n=166). In table 10a, a significantly positive correlation was found between sperm concentrations (mill. /ml) with sperm motility (r=0.194' p<0.050), sperm vitality (r=0.16, p<0.050), and morphologically normal sperm (r=0.250' p<0.010), whereas, a significant negative correlation was demonstrated with chromatin condensation (CMA_3 positivity, r=-0.289, p<0.010). The mean percentage of the sperm motility showed significantly positive (p<0.100) correlations with sperm vitality (Eosin-test, r=0.494), sperm membrane integrity (HOS-test, r=0.446), and morphologically normal sperm (r=0.479). In contrast, sperm motility showed highly negative significant correlations (p<0.010) with chromatin condensation, (CMA_3-test), DNA integrity as assessed by Tunel-test, and P1/P2 ratios, (r=-0.458; r=-0.406; r=-0.235; respectively).

In addition, a significant increased (p<0.010) correlations observed between sperm vitality with membrane integrity (r=0.598), and morphologically normal sperm (r=0.229), and significantly decreased correlations were found with non-condensed chromatin (r=-0.343, p<0.010), and DNA fragmentation (r=-0.263, p<0.010).

The mean percentage of the sperm membrane integrity was correlated significantly positive with sperm morphologically normal sperm (r=0.378, p<0.010), with non-condensed chromatin (%) (r=-0.384, p<0.010), and DNA fragmentation (%) (r=-0.330, p<0.010). P1/P2 ratios showed significant negative correlations (r=-0.168, p<0.050).

Figure 8 represents the significant positive correlations between the sperm non-condensed chromatin (CMA_3) and DNA fragmentation (Tunel), while figure 9 represents the significantly negative correlation with morphologically normal sperm. Also, sperm chromatin condensation showed positive significant correlation with P1/P2 ratio. It was introduced that sperm DNA integrity was correlated significantly positive with P1/P2 ratios (r=227, p<0.010), and correlated negatively with morphologically normal sperm (r=-0.509, p<0.010).

The mean percentage of morphologically normal sperm showed a high negative significant correlation with P1/P2 ratios (r=-0.384, p<0.010) and negative significant correlation with P1 concentration also observed (r=-0.189, p<0.050). In addition, the correlations between protamines 1 and 2 (P1, P2) and P1/P2 ratios were found to be highly significant positive (p<0.010) between P1 with P2, and P1/P2 ratios (r=0.650; r=0.237 respectively). Whereas, P2 showed a decreased significant correlation with P1/P2 ratios (r=-0.525, p<0.010).

Table 10b. Correlation coefficient of semen parameters of all participant samples (n=166)

		Volume (ml)	pH	Leukocytes (%)	Malondialdehyde (MDA) (µM) (N=113)	Reactive oxygen species (ROS) (µmol/l) (N=113)	Cotinine (ng/ml) (N=113)	8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)
Volume (ml)	r =	1.000	-0.025	-0.012	0.170	-0.176	-0.151	-0.193*
	p =	.	0.751	0.882	0.072	0.062	0.110	0.040
pH	r =	-0.025	1.000	-0.049	0.015	0.011	0.044	-0.049
	p =	0.751	.	0.534	0.876	0.911	0.641	0.604
Leukocytes (%)	r =	-0.012	-0.049	1.000	0.170	0.170	0.042	0.027
	p =	0.882	0.534	.	0.072	0.072	0.656	0.775
Malondialdehyde (MDA) (µM) (N=113)	r =	-0.205*	0.015	0.170	1.000	0.645**	0.632**	0.580**
	p =	0.030	0.876	0.072	.	0.000	0.000	0.000
Reactive oxygen species (ROS) (µmol/l) (N=113)	r =	-0.176	0.011	0.077	0.645**	1.000	0.737**	0.580**
	p =	0.062	0.911	0.417	0.000	.	0.000	0.000
Cotinine (ng/ml) (N=113)	r =	-0.151	0.044	0.042	0.632**	0.737**	1.000	0.786**
	p =	0.110	0.641	0.656	0.000	0.000	.	0.000
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)	r =	-0.193*	-0.049	0.027	0.580**	0.580**	0.786**	1.000
	p =	0.040	0.604	0.775	0.000	0.000	0.000	.

Table 10b showed that the pH and concentration of leukocytes in semen had no significant effect on other parameters. On the contrary, volume (ml) of semen showed a significant negative correlations (p<0.050 with MDA (µM) and 8-OHdG (ng/ml) (r=-0.205; r=-0.193), and ROS (µmol/l) (r=-0176, p=0.062).

Moreover, the concentration of MDA (µM) showed a significant positive correlations (p<0.010) with ROS (µmol/l), cotinine (ng/ml), 8-OHdG (ng/ml) (Figures 10, 112, and 12 respectively), and P1/P2 ratios of spermatozoa. ROS (µmol/l) was correlated significantly (p<0.010) with concentrations of cotinine (ng/ml) (r=0.737), and 8-OHdG (ng/ml) (r=0.580). Cotinine (ng/ml) and the 8-OHdG (ng/ml) of the spermatozoa showed similar correlation (r=0.786, p<0.010).

Table 10c. Correlation coefficient of sperm and semen parameters of all participant samples (n=166)

		Count (mill/ml)	Motility (%motile)	Sperm vitality (Eosin) (%)	Membrane integrity (HOS) (%)%	Chromatin condensation (positive CMA_3) (%)	DNA Fragmentation (positive TUNEL) (%)	Morphologically Normal spermatozoa (%)	Protamine 1 (ng/ 10^6 sperm)	Protamine 2 (ng/ 10^6 sperm)	P1/P2 Ratios
Volume (ml)	R =	0.073	0.118	0.084	0.075	-0.166*	-0.159*	0.224**	0.088	0.115	-0.061
	P =	0.348	0.129	0.281	0.338	0.033	0.041	0.004	0.258	0.141	0.437
pH	R =	-0.219**	-0.134	-0.085	-0.039	0.106	0.092	-0.095	-0.054	-0.050	-0.008
	P =	0.005	0.085	0.278	0.617	0.174	0.239	0.222	0.487	0.520	0.917
Leukocytes (%)	R =	0.001	-0.010	0.026	-0.106	0.071	0.009	0.013	-0.045	-0.115	0.110
	P =	0.994	0.902	0.741	0.172	0.362	0.903	0.872	0.562	0.141	0.160
Malondialdehyde (MDA) (μM) (N=113)	R =	-0.069	-0.513**	-0.309**	-0.362**	0.555**	0.624**	-0.514**	0.140	-0.052	0.384**
	P =	0.465	0.000	0.001	0.000	0.000	0.000	0.000	0.140	0.581	0.000
Reactive oxygen species (ROS) (μmol/l) (N=113)	R =	-0.133	-0.537**	-0.298**	-0.343**	0.629**	0.630**	-0.547**	0.065	-0.141	0.410**
	P =	0.160	0.000	0.001	0.000	0.000	0.000	0.000	0.496	0.137	0.000
Cotinine (ng/ml) (N=113)	R =	-0.137	-0.489**	-0.322**	-0.356**	0.549**	0.627**	-0.555**	0.074	-0.127	0.411**
	P =	0.148	0.000	0.001	0.000	0.000	0.000	0.000	0.438	0.180	0.000
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)	R =	-0.169	-0.532**	-0.373**	-0.335**	0.545**	0.600**	-0.513**	-0.028	-0.221*	0.393**
	P =	0.073	0.000	0.000	0.000	0.000	0.000	0.000	0.771	0.019	0.000

Table 10c demonstrated that there are an adverse correlations (p<0.010) between sperm motility and MDA (μM), ROS (μmol/l) (Fig. 13), cotinine (ng/ml), and 8-OHdG (ng/ml) concentrations in seminal plasma (r=-0.513, r=-0.537, r=-0.489, and r=-0.532 respectively). Moreover, significant negative correlations (p<0.010) were found between sperm vitality and MDA (r=-0.309), ROS (r=-0.298), cotinine (r=-0.322), and 8-OHdG (r=-0.373). Sperm membrane integrity also was negatively correlated (p<0.010) with MDA, ROS, cotinine, and 8-OHdG (r= -0.362, r=-0.343, r=-0.356, and r=-0.335 respectively). Likewise, a significant negative correlation (p<0.010) found between morphologically normal spermatozoa with MDA (Fig. 15), ROS, cotinine, and 8-OHdG (Fig. 14; Table 10c). However, the percentage of sperms with reduced chromatin condensation showed significant positive correlations (p<0.010) with MDA (Fig. 15), ROS, cotinine, and 8-OHdG (Fig. 16) (r=0.555, r=0.629, r=0.549, r=0.545 respectively). Besides, Sperm DNA fragmentation was correlated positively (p<0.010) with MDA (r=0.624), ROS (r=0.630, cotinine (r=0.627), and 8-OHdG (r=0.600) (Fig. 17). However, "but not significant" positive correlation observed between Protamine 1 concentrations with MDA, ROS, and cotinine, and negative correlation with 8-OHdG. whereas, protamine 2 concentration showed negative correlations with MDA, ROS, and cotinine, and significant negative correlation with 8-OHdG (r=-0.221, p<0.050). On the opposite side P1/P2 ratios showed significant positive correlations (p<0.010) with MDA, ROS (Fig. 18), cotinine, and 8-OHdG (r=0.384, r=0.410, r=0.411, r=0.393 respectively).

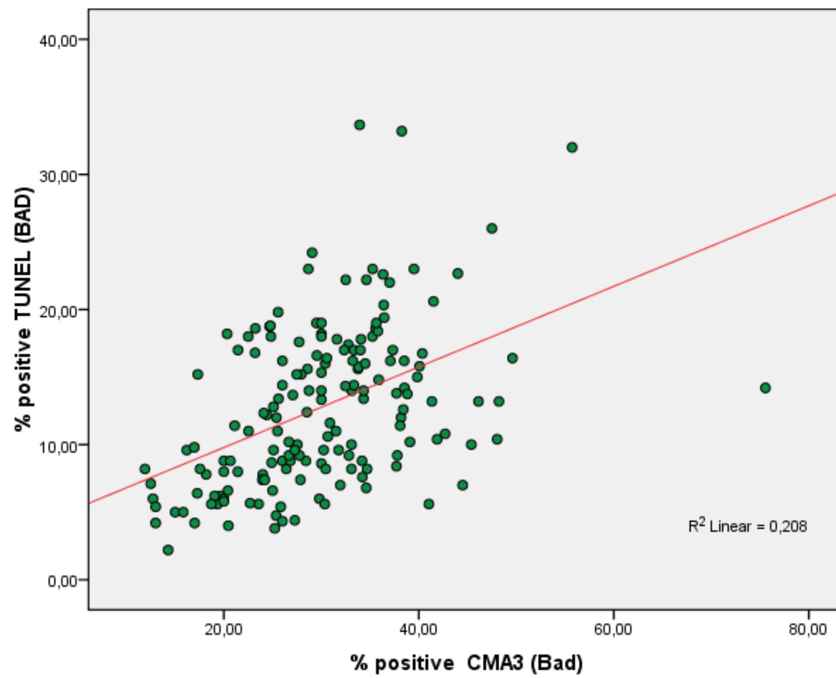


Figure 8: Scatter plot of correlation between sperm chromatin condensation (CMA_3, %) and sperm DNA fragmentation (Tunel, %) of all participants. A significant positive correlation was found ($r=0.479$, $p<0.010$).

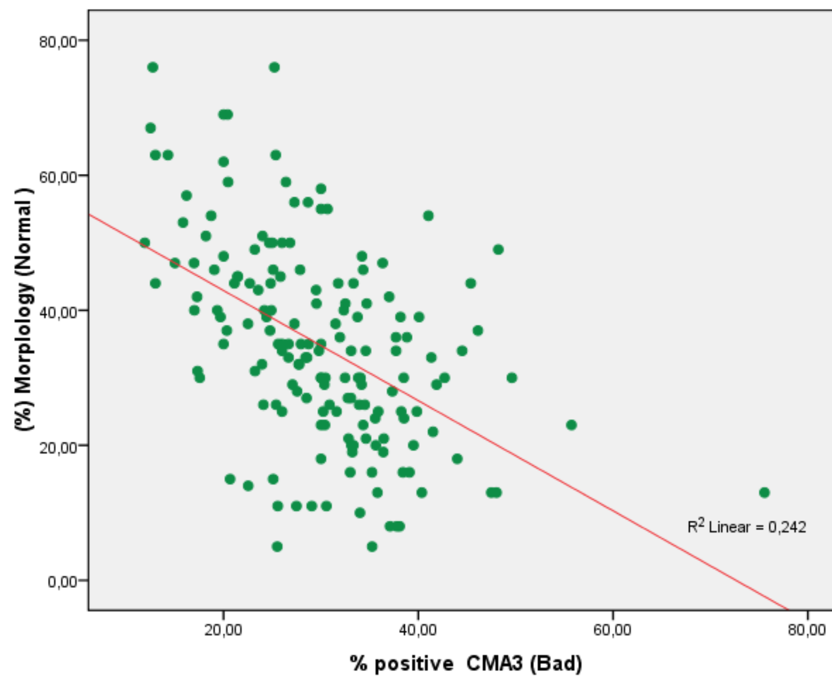


Figure 9: Scatter plot of correlation between sperm chromatin condensation (%) and morphologically normal spermatozoa of all participants. A significant negative correlation was detected ($r=-0.514$, $p<0.010$).

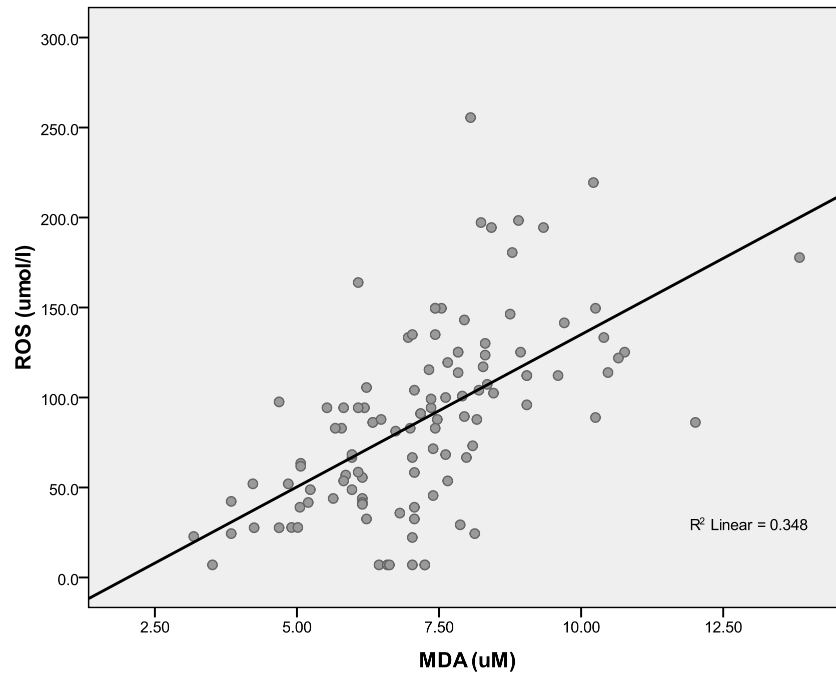


Figure 10: Scatter plot of correlation between MDA (µM) and ROS (µmol/l) concentrations in seminal plasma of all participants. A significant positive correlation was found (r=0.645, p<0.010).

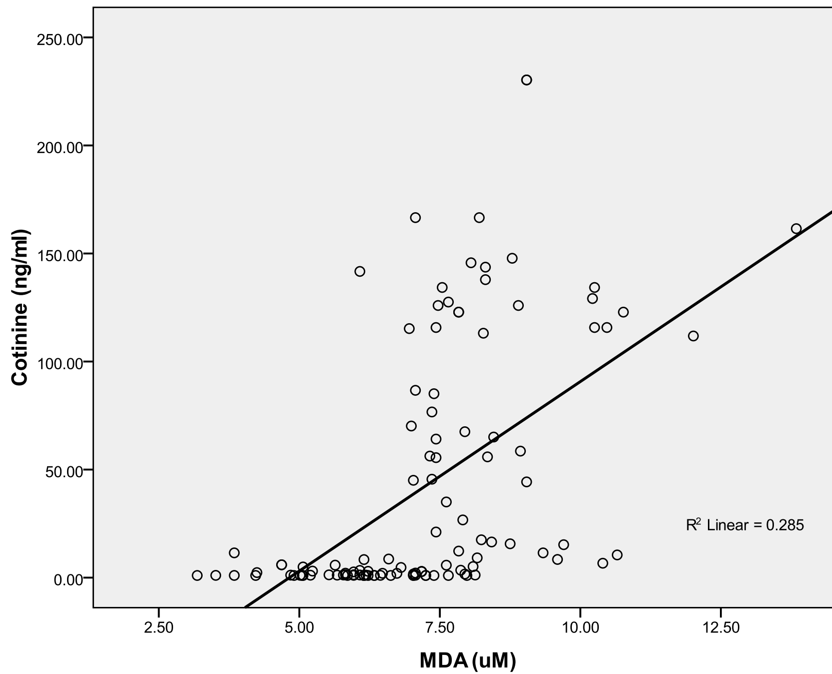


Figure 11: Scatter plot of correlation between MDA (µM) and cotinine (ng/ml) concentrations in seminal plasma of all participants. A significant positive correlation was illustrated (r=0.632, p<0.010).

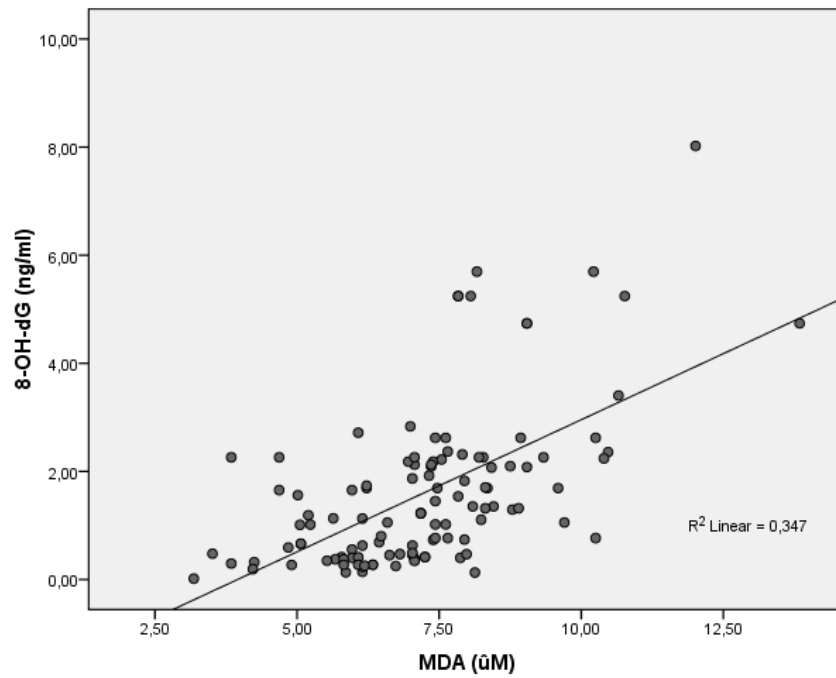


Figure 12: Scatter plot of correlation between MDA (μM) and 8-OHdG (ng/ml) concentrations in seminal plasma of all participants. A significant positive correlation, was found (r=0.580, p<0.010).

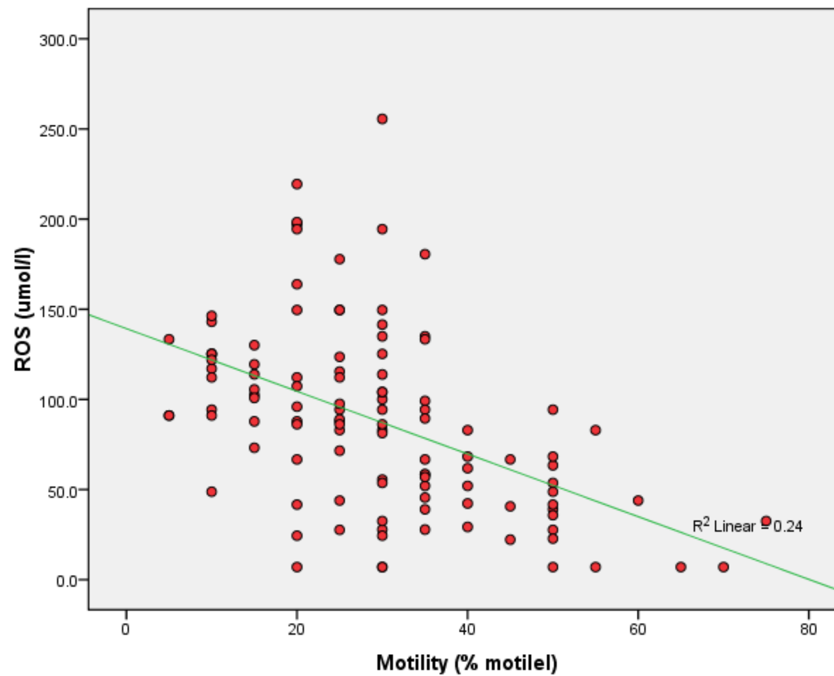


Figure 13: Scatter plot of correlation between sperm motility (%) and ROS (μmol/ml) concentration in seminal plasma of all participants. A significant negative correlation was found (r=-0.537, p<0.010).

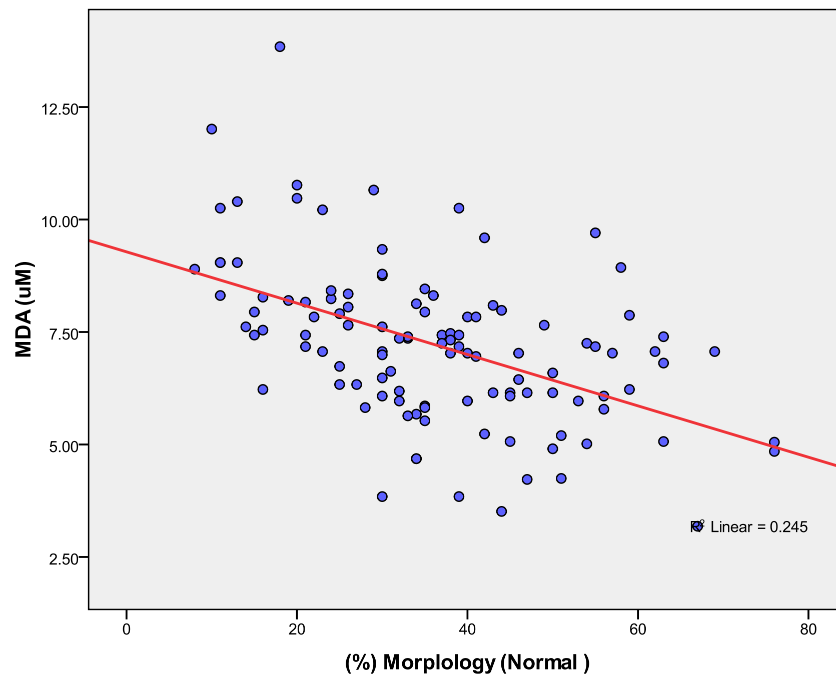


Figure 14: Scatter plot of correlation between the mean percentage of morphologically normal spermatozoa (%) and MDA (μM) concentration in seminal plasma of all participants. A significant negative correlation ($r=-0.514$, $p<0.010$) was found.

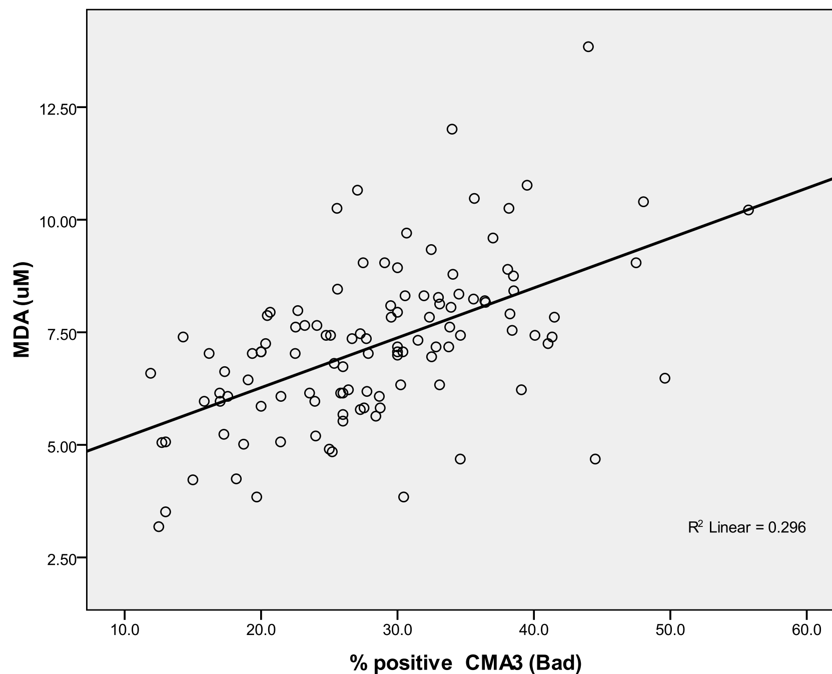


Figure 15: Scatter plot of correlation between the mean percentage of chromatin condensation spermatozoa (CMA_3, %) and MDA (μM) concentration in seminal plasma concentration of all participants. A significant positive correlation was demonstrated ($r=0.555$, $p<0.010$).

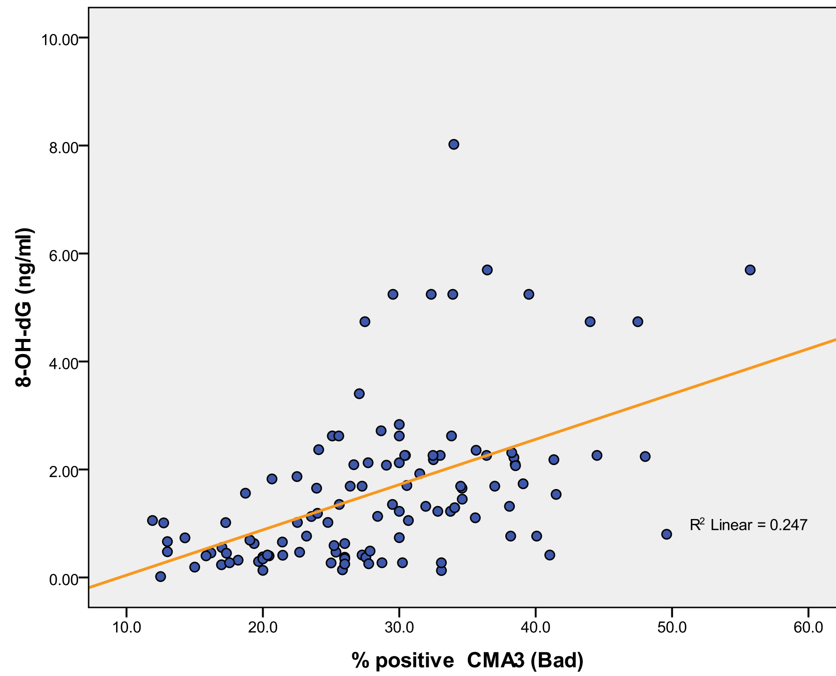


Figure 16: Scatter plot of correlation between the mean percentage of chromatin condensation spermatozoa (CMA_3, %) and 8-OHdG (ng/ml) concentration in seminal plasma of all participants. A significant positive correlation (r=0.545, p<0.010) was found.

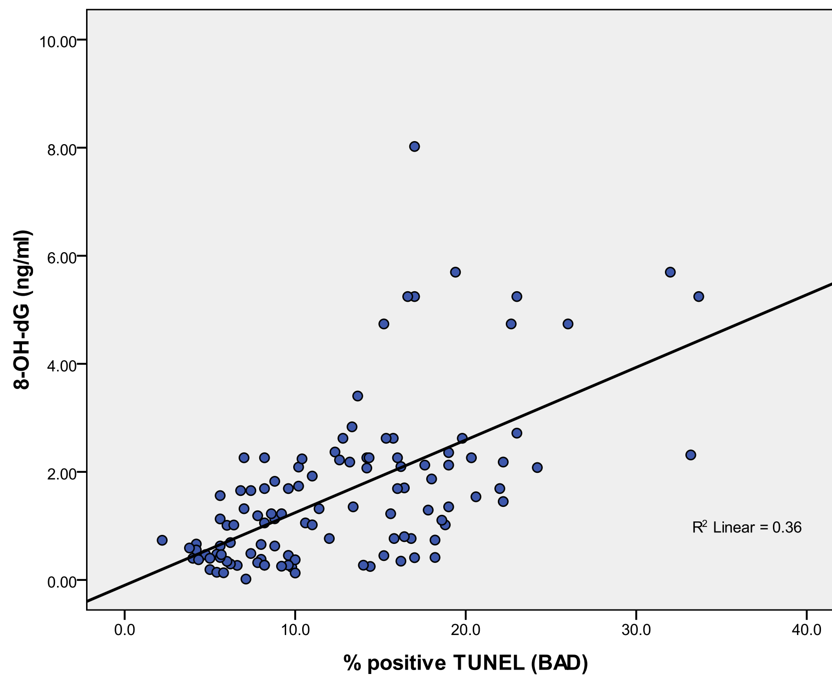


Figure 17: Scatter plot of correlation between the mean percentage of sperm DNA fragmentation (%) as assessed by Tunel and 8-OHdG (ng/ml) concentration in seminal plasma of all participants. A significant positive correlation (r=0.600, p<0.010) was showed.

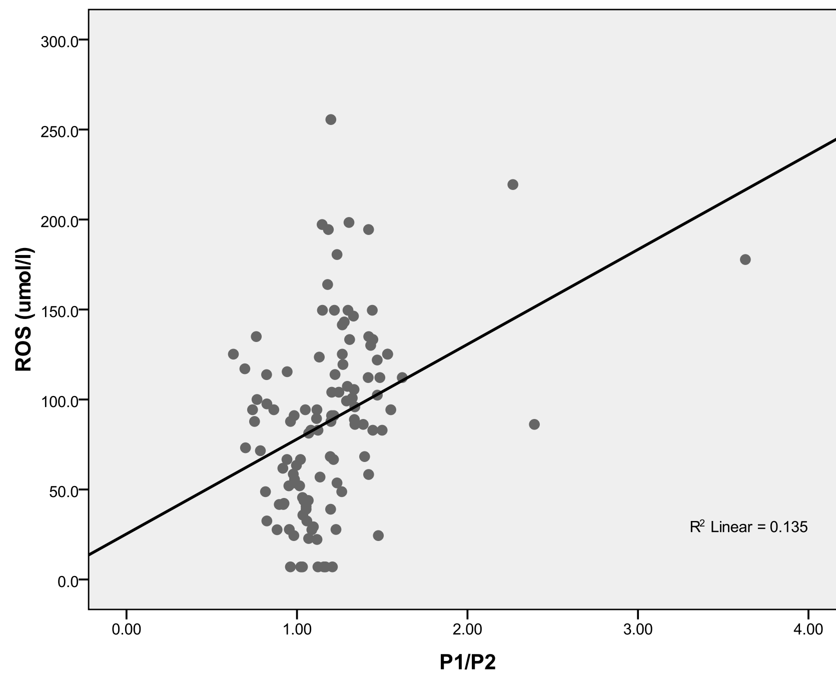


Figure 18: Scatter plot of correlation between P1/P2 ratios of the spermatozoa and ROS (µmol/ml) concentration in seminal plasma of all participants. A significant positive correlation (r=0.410, p<0.010) was found.

4-2- Normal volunteers versus Patients

Table 11 illustrated the mean ± standard deviation and the *p*-values of the semen and sperm characteristics of 166 semen samples; normal volunteers (n=50) and patients (n=116) (refer to Figure 7), who underwent therapy in the IVF laboratory. Samples were analyzed according to the WHO criteria with the exception of morphology which evaluated by strict criteria. The results include the sperm concentration, motility, morphology, DNA integrity, DNA fragmentation, protamines concentrations, and ROS, MDA, cotinine and 8-OHdG concentrations in seminal plasma.

Table 11. Comparison of semen and sperm parameters between all normal volunteers (n=50) and all patients (n=116) participants.

Parameters	Normal	Patients	p-value
Samples	50	116	
Age (year)	25.5 ± 4.5	37.9 ± 5.7	0.000
Volume (ml)	3.9 ± 1.4	3.2 ± 1.7	0.004
pH	8.6 ± 0.3	8.7 ± 0.4	0.060
Count (mill/ml)	80.2 ±27.5	61.4 ± 31.1	0.000
Motility (% motile)	37.1 ± 14.7	27.4 ± 12.0	0.000
Sperm vitality (Eosin) (%)	45.2 ± 16.9	34.7 ± 17.3	0.001
Membrane integrity (HOS) (%)%	53.4 ± 15.8	45.1 ± 20.8	0.010
Leukocytes (%)	2.4 ± 0.5	2.6 ± 1.7	0.959
Chromatin condensation (positive CMA_3) (%)	23.3 ± 7.0	32.9 ± 8.2	0.000
DNA Fragmentation (positive TUNEL) (%)	9.6 ± 5.4	14.2 ± 5.6	0.000
Morphologically Normal spermatozoa (%)	45.9 ± 13.9	29.4 ± 12.7	0.000
Malondialdehyde (MDA) (µM)	6.45 ± 1.43	7.82 ± 1.79 (n=63)	0.000
Reactive oxygen species (ROS) (µmol/l)	67.6 ± 42.2	103.85 ± 5.68 (n=63)	0.000
Cotinine (ng/ml)	27.30 ± 40.17	53.42 ± 67.33 (n=63)	0.014
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml)	1.15 ± 0.82	1.95 ± 1.76 (n=63)	0.027
Protamine 1 (P1) (ng/10^6 sperm)	378.20 ± 100.00	416.30 ± 101.70	0.007
Protamine 2 (P2) (ng/10^6 sperm)	347.30 ± 77.30	363.60 ± 114.9	0.424
P1/P2 ratio	1.10 ± 0.20	1.22 ± 0.36	0.012

A significant (p<0.050 or p<0.010) differences were noticed between all semen and sperm parameters between normal volunteers and patients except for the pH and concentrations of the leukocytes and protamine 2 (p>0.050).

Sperm's concentrations, motility, and semen volumes were significantly higher (p<0.010) in normal volunteers (80.2 ±27.5, 37.1 ± 14.7, 3.9 ± 1.4) compare to that of patients (61.4 ± 31.1, 27.4 ± 12.0, 3.2 ± 1.7). Similar were obtained for sperm vitality (Eosin) and the sperm membrane integrity (HOS), (45.2 ± 16.9, 53.4 ± 15.8, vs. 34.7 ± 17.3, 45.1 ± 20.8, p<0.010 respectively. However, chromatin condensation (positive CMA_3), P1 and P1/P2 ratios of the patient's sperms (32.9 ± 8.2, 416.30 ± 101.70, 1.22 ± 0.36) were lower than that of normal volunteers (23.3 ± 7.0, 378.20 ± 100.00, 1.10 ± 0.20). Moreover, DNA fragmentation (Tunel) was significantly higher (p<0.010) in patients (14.2 ± 5.6), in comparison to volunteers group (9.6 ± 5.4). Likewise, the mean percentage of morphologically normal spermatozoa was significantly higher (p<0.010) in volunteers than that in patients group (45.9 ± 13.9 vs. 29.4 ± 12.7). In contrast, MDA, ROS, Cotinine, and 8-OHdG concentrations in seminal plasma were significantly higher (p<0.010) in patients in comparison to volunteers group (Table 11).

4-2-1- Normal Volunteers

The correlation coefficients for sperm and seminal plasma characteristics for normal volunteers were illustrated in tables 12a; 12b and 12c.

Table 12a. Correlation coefficient of sperm parameters of normal volunteer samples (n=50)

		Count (mill/ml)	Motility (%motile)	Sperm vitality (Eosin) (%)	Membrane integrity (HOS) (%)%	Chromatin condensation (positive CMA_3)(%)	DNA Fragmentation (positive TUNEL) (%)	Morphologically Normal spermatozoa (%)	Protamine 1 (ng/ 10^6 sperm)	Protamine 2 (ng/ 10^6 sperm)	P1/P2 Ratios
Count (mill/ml)	r =	1.000	0.144	-0.209	-0.178	-0.138	-0.011	0.225	0.193	0.053	0.184
	p =	.	0.319	0.145	0.216	0.339	0.940	0.116	0.180	0.716	0.202
Motility (%motile)	r =	0.144	1.000	0.361**	0.366**	-0.563**	-0.541**	0.548**	0.101	0.153	-0.237
	p =	0.319	.	0.010	0.009	0.000	0.000	0.000	0.487	0.289	0.098
Sperm vitality (Eosin) (%)	r =	-0.209	0.361**	1.000	0.600**	-0.272	-0.147	0.031	0.015	0.069	-0.179
	p =	0.145	0.010	.	0.000	0.050	0.309	0.832	0.919	0.632	0.213
Membrane integrity (HOS) (%)	r =	-0.178	0.366**	0.600**	1.000	-0.314*	-0.289*	0.179	0.173	0.314*	-0.193
	p =	0.216	0.009	0.000	.	0.026	0.042	0.213	0.228	0.026	0.180
Chromatin condensation (positive CMA_3) (%)	r =	-0.138	-0.563**	-0.272*	-0.314*	1.000	0.589**	-0.469**	-0.091	-0.097	0.128
	p =	0.339	0.000	0.050	0.026	.	0.000	0.001	0.530	0.505	0.375
DNA Fragmentation (positive TUNEL) (%)	r =	-0.011	-0.541**	-0.147	-0.289*	0.589**	1.000	-0.560**	-0.053	-0.161	0.296*
	p =	0.940	0.000	0.309	0.042	0.000	.	0.000	0.716	0.265	0.037
Morphologically Normal spermatozoa (%)	r =	0.225	0.548**	0.031	0.179	-0.469**	-0.560**	1.000	0.038	0.114	-0.240
	p =	0.116	0.000	0.832	0.213	0.001	0.000	.	0.796	0.432	0.094
Protamine 1 (ng/ 10^6 sperm)	r =	0.193	0.101	0.015	0.173	-0.091	-0.053	0.038	1.000	0.699**	0.481**
	p =	0.180	0.487	0.919	0.228	0.530	0.716	0.796	.	0.000	0.000
Protamine 2 (ng/ 10^6 sperm)	r =	0.053	0.153	0.069	0.314*	-0.097	-0.161	0.114	0.699**	1.000	-0.214
	p =	0.716	0.289	0.632	0.026	0.505	0.265	0.432	0.000	.	0.136
P1/P2 Ratios	r =	0.184	-0.237	-0.179	-0.193	0.128	0.296*	-0.240	0.481**	-0.214	1.000
	p =	0.202	0.098	0.213	0.180	0.375	0.037	0.094	0.000	0.136	.

Analysis of the semen samples of the normal volunteers showed that (Table 12a) sperm motility was significantly positive correlations ($p<0.010$) with sperm vitality (Eosin-test) ($r=0.362$), sperm membrane integrity (HOS-test), ($r=0.366$), and morphologically normal sperm (Fig.19). In contrast, motility showed significant negative correlations ($p<0.010$) with chromatin condensation, (positive CMA_3-test), DNA fragmentation (Tunel-test), ($r=-0.458$, $r=-0.406$ respectively). P1/P2 ratios showed correlations but not significant ($r=-0.237$, $p=0.098$) with motility. Besides, perm vitality showed positive significant correlation with sperm membrane integrity ($r=0.600$, $p<0.010$) and a negative significant difference with chromatin condensation ($r=-0.272$, $p<0.050$). In addition, a significant negative correlations ($p<0.050$) were found between sperm membrane integrity with non- condensed chromatin and DNA fragmentation ($r=-0.314$; $r=-0.289$ respectively). Moreover, positive significance correlation between sperm vitality with P2 concentration ($r=0.314$, $p<0.050$) was shown. Figure 20 illustrated the correlation between sperm non- condensed chromatin and DNA fragmentation, while Figure 21 showed the negative coefficient correlation with morphologically normal sperm.

Sperm DNA integrity was correlated negatively with morphologically normal sperm ($r=-0.506$, $p<0.010$), and correlated significantly with P1/P2 ratios ($r=0.296$, $p<0.050$). P1 concentration showed a positive correlations ($p<0.010$) with P2 concentrations and P1/P2 ratios (Table 12a).

Table 12b. Correlation coefficient of semen parameters of normal volunteer samples (n=50)

		Volume (ml)	pH	Leukocytes (%)	Malondialdehyde (MDA) (µM) (N=113)	Reactive oxygen species (ROS) (µmol/l) (N=113)	Cotinine (ng/ml) (N=113)	8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)
Volume (ml)	r =	1.000	-0.037	-0.206	-0.140	-0.296*	-0.256	-0.250
	p =	.	0.798	0.151	0.334	0.037	0.072	0.079
pH	r =	-0.037	1.000	-0.107	-0.055	0.030	-0.022	-0.065
	p =	1.000	.	0.459	0.705	0.837	0.879	0.655
Leukocytes (%)	r =	-0.206	-0.107	1.000	0.149	0.022	-0.173	-0.048
	p =	0.151	0.459	.	0.303	0.882	0.229	0.739
Malondialdehyde (MDA) (µM) (N=113)	r =	-0.140	-0.055	0.149	1.000	0.527**	0.614**	0.557**
	p =	0.334	0.705	0.303	.	0.000	0.000	0.000
Reactive oxygen species (ROS) (µmol/l) (N=113)	r =	-0.296*	0.030	0.022	0.527**	1.000	0.673**	0.611**
	p =	0.037	0.837	0.882	0.000	.	0.000	0.000
Cotinine (ng/ml) (N=113)	r =	-0.256	-0.022	-0.173	0.614**	0.673**	1.000	0.752**
	p =	0.072	0.879	0.229	0.000	0.000	.	0.000
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)	r =	-0.250	-0.065	-0.048	0.557**	0.611**	0.752**	1.000
	p =	0.079	0.655	0.739	0.000	0.000	0.000	.

In table 12b, normal volunteers semen volumes (ml) correlated significantly negative with concentration of ROS (µmol/l), (r=-0.296, p<0.050) and non-significantly with concentrations of cotinine (ng/ml) and 8-OHdG (ng/ml) (r=-0.256, r=-0.250; p=0.072, p=0.079 respectively). The pH and the concentration of leukocytes showed no significant correlations. A positive significant correlations (p<0.010) were showed between the concentrations of MDA (µM) with ROS (µmol/l), (r=0.527), cotinine (ng/ml) (r=0.614) and 8-OHdG (ng/ml) (r=0.577). The same results showed between ROS with cotinine and 8-OHdG (r=0.673; r=0.611 respectively. In addition cotinine and 8-OHdG showed positive significant correlation (r=0.752, p<0.010).

Table 12c. Correlation coefficient of semen parameters of normal volunteer samples (n=50)

		Count (mill/ml)	Motility (%motile)	Sperm vitality (Eosin) (%)	Membrane integrity (HOS) (%)	Chromatin condensation (positive CMA_3) (%)	DNA Fragmentation (positive TUNEL) (%)	Morphologically Normal spermatozoa (%)	Protamine 1 (ng/ 10^6 sperm)	Protamine 2 (ng/ 10^6 sperm)	P1/P2 Ratios
Volume (ml)	r =	0.291*	0.346*	-0.013	0.006	-0.165	-0.111	0.387**	0.206	0.126	0.040
	p =	0.040	0.014	0.928	0.968	0.252	0.444	0.005	0.151	0.382	0.781
pH	r =	-0.263	-0.158	-0.093	-0.034	0.172	0.021	-0.055	-0.156	-0.146	0.012
	p =	0.065	0.272	0.523	0.814	0.234	-0.112	-0.029	0.280	0.311	0.934
Leukocytes (%)	r =	-0.131	-0.202	-0.085	-0.117	0.019	-0.112	-0.029	-0.078	-0.092	-0.026
	p =	0.365	0.159	0.555	0.418	0.897	0.439	0.843	0.588	0.526	0.858
Malondialdehyde (MDA) (µM) (N=113)	r =	0.090	-0.580**	-0.040	-0.093	0.527**	0.536**	-0.455**	0.146	0.075	0.281*
	p =	0.536	0.000	0.785	0.522	0.000	0.000	0.001	0.311	0.605	0.048
Reactive oxygen species (ROS) (µmol/l) (N=113)	r =	0.101	-0.632**	-0.248	-0.196	0.636**	0.577**	-0.555**	0.020	-0.030	0.262*
	p =	0.486	0.000	0.083	0.173	0.000	0.000	0.000	0.889	0.834	0.046
Cotinine (ng/ml) (N=113)	r =	-0.035	-0.584**	-0.255	-0.300*	0.559**	0.657**	-0.565**	0.125	0.045	0.315*
	p =	0.811	0.000	0.074	0.034	0.000	0.000	0.000	0.386	0.755	0.026
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)	r =	-0.091	-0.667**	-0.283*	-0.303*	0.588**	0.694**	-0.573**	0.034	-0.119	0.337*
	p =	0.528	0.000	0.047	0.032	0.000	0.000	0.000	0.814	0.410	0.017

Table 12c demonstrated that the four seminal plasm parameters MDA (µM), ROS (µmol/l), (cotinine (ng/ml), and 8-OHdG (ng/ml) concentrations were correlated significantly negative (p<0.010) with motility. A negative significant correlation was observed between sperm vitality

and 8-OHdG (r=-0.283, p=0.047), while low significance noteced with ROS and cotinine (r=-0.248, p=0.083; r=-0.255, p=0.074). Moreover, significant negative correlatios (p<0.050) were found between sperm membrane integrity with cotinine and 8-OHdG (r=-0.300, r= -0.303 respectively). In contrast, a positive correlations (p<0.010) were found between the seminal plasma parameters and non-condensed chromatin and DNA fragmentation. P1/P2 ratios also showed a significant correlation (p<0.050) with the four parameters (Table 12c).

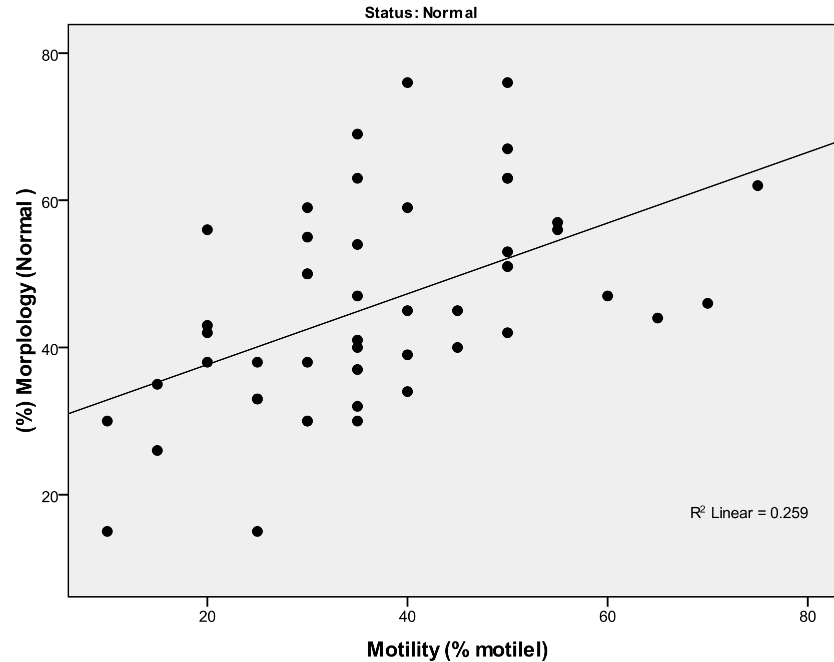


Figure 19: Scatter plot of correlation between sperm motility (%) and the mean percentage of morphologically normal spermatozoa of volunteers. A significant positive correlation was indicated (r=0.548; p<0.010).

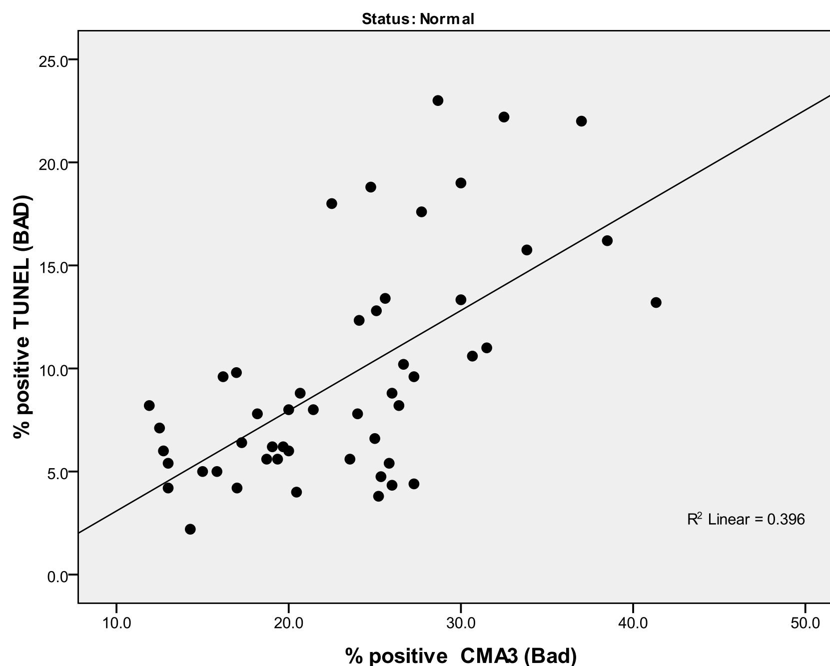


Figure 20: Scatter plot of correlation between the percentages of sperm chromatin condensation evaluated by CMA_3 and sperm DNA fragmentation (TUNEL) of volunteers. A significant positive correlation (r=0.598, p<0.010) was found.

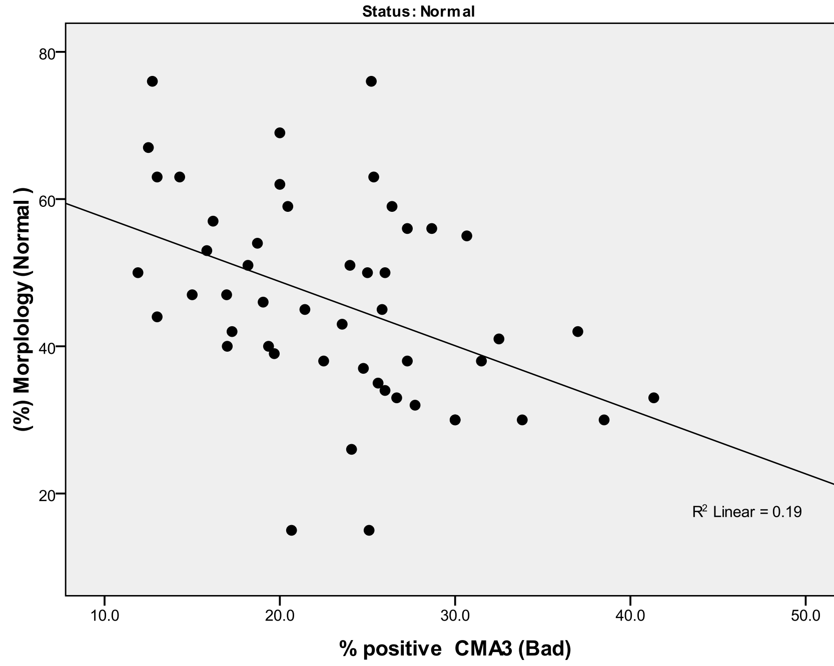


Figure 21: Scatter plot of correlation between the percentage of sperm chromatin condensation (CMA_3) and the mean percentage of morphologically normal spermatozoa of volunteers. A significantly negative correlation ($r=-0.469$, $p<0.010$) was found.

4-2-2-Patients

Table 13a, b, and c illustrated the correlation coefficient of patient's sperm and semen parameters.

Table 13a. Correlation coefficient of sperm parameters of patient samples (n=116)

		Count (mill/ml)	Motility (% motile)	Sperm vitality (Eosin) (%)	Membrane integrity (HOS) (%)%	Chromatin condensation (positive CMA_3) (%)	DNA Fragmentation (positive TUNEL) (%)	Morphologically Normal spermatozoa (%)	Protamine 1 (ng/ 10^6 sperm)	Protamine 2 (ng/ 10^6 sperm)	P1/P2 Ratios
Count (mill/ml)	r =	1.000	0.096	0.191*	0.139	-0.208*	0.021	0.115	0.203*	0.160	-0.063
	p =	.	0.305	0.040	0.136	0.025	0.825	0.220	0.029	0.086	0.500
Motility (% motile)	r =	0.096	1.000	0.501**	0.417**	-0.260**	-0.219*	0.299**	0.031	0.082	-0.138
	p =	0.305	.	0.000	0.000	0.005	0.018	0.001	0.738	0.380	0.140
Sperm vitality (Eosin) (%)	r =	0.191*	0.501**	1.000	0.559**	-0.226*	-0.162	0.134	0.011	-0.010	-0.040
	p =	0.040	0.000	.	0.000	0.015	0.082	0.152	0.907	0.918	0.668
Membrane integrity (HOS) (%)	r =	0.139	0.417**	0.559**	1.000	-0.342**	-0.266**	0.377**	0.121	0.132	-0.107
	p =	0.136	0.000	0.000	.	0.000	0.004	0.000	0.196	0.157	0.255
Chromatin condensation (positive CMA_3) (%)	r =	-0.208*	-0.260**	-0.226*	-0.342**	1.000	0.213*	-0.323**	-0.167	-0.208*	0.182
	p =	0.025	0.005	0.015	0.000	.	0.022	0.000	0.074	0.025	0.051
DNA Fragmentation (positive TUNEL) (%)	r =	0.021	-0.219*	-0.162	-0.266**	0.213*	1.000	-0.356**	-0.136	-0.035	0.062
	p =	0.825	0.018	0.082	0.004	0.022	.	0.000	0.146	0.711	0.508
Morphologically Normal spermatozoa (%)	r =	0.115	0.299**	0.134	0.377**	-0.323**	-0.356**	1.000	-0.080	-0.004	-0.154
	p =	0.220	0.001	0.152	0.000	0.000	0.000	.	0.395	0.962	0.100
Protamine 1 (ng/ 10^6 sperm)	r =	0.203*	0.031	0.011	0.121	-0.167	-0.136	-0.080	1.000	0.623**	0.105
	p =	0.029	0.738	0.907	0.196	0.074	0.146	0.395	.	0.000	0.261
Protamine 2 (ng/ 10^6 sperm)	r =	0.160	0.082	-0.010	0.132	-0.208*	-0.035	-0.004	0.623**	1.000	-0.638**
	p =	0.086	0.380	0.918	0.157	0.025	0.711	0.962	0.000	.	0.000
P1/P2 Ratios	r =	-0.063	-0.138	-0.040	-0.107	0.182	0.062	-0.154	0.105	-0.638**	1.000
	p =	0.500	0.140	0.668	0.255	0.051	0.508	0.100	0.261	0.000	.

In Table 13a the concentration of sperms was shown to be correlated significantly ($p<0.050$) with sperm vitality and P1 concentration ($r=0.191$; $r=0.203$ respectively), and negatively with chromatin condensation ($r=-0.208$, $p<0.050$). However, the motility of the patient sperms was positively correlated with sperm vitality ($r=0.501$, $p<0.010$), membrane integrity ($r=0417$, $p<0.010$), and morphologically normal sperm ($r=0.299$, $p<0.010$). Besides a significantly negative correlations with chromatin condensation ($r=-0.260$, $p<0.010$) and DNA fragmentation ($r=-0.219$, $p<0.050$) were found. The sperm membrane integrity correlated negatively ($p<0.010$) with chromatin condensation and DNA fragmentation ($r=-0.342$, $r=-0.266$ respectively), whereas, a positive correlation with morphologically normal sperm ($r=0.377$, $p<0.010$) was observed. On the other hand, chromatin condensation (CMA_3) correlated positively with DNA integrity (Tunel) and significantly negative with P2 values ($r=0.213$, $p<0.050$; $r=-0.208$, $p<0.050$) and morphologically normal sperm ($r=-0.323$, $p<0.010$). DNA integrity (Tunel) in its return, showed a significant negative correlation with morphologically normal sperm ($r=-0.356$, $p<0.010$). In addition P1 correlated significantly positive with P2 ($r=0.623$, $p<0.010$). Whereas, significantly negative correlation was found between P2 and P1/P2 ratios ($r=-0.638$, $p<0.010$, Table 13a).

Table 13b. Correlation coefficient of semen parameters of patient samples (n=116)

		Volume (ml)	pH	Leukocytes (%)	Malondialdehyde (MDA) (µM) (N=113)	Reactive oxygen species (ROS) (µmol/l) (N=113)	Cotinine (ng/ml) (N=113)	8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)
Volume (ml)	r =	1.000	0.021	0.057	-0.116	0.029	-0.001	-0.090
	p =	.	0.825	0.546	0.363	0.819	0.995	0.485
pH	r =	0.021	1.000	-0.030	0.004	-0.076	0.057	-0.071
	p =	0.825	.	0.746	0.975	0.554	0.655	0.578
Leukocytes (%)	r =	0.057	-0.030	1.000	0.183	0.154	0.210	0.081
	p =	0.546	0.746	.	0.152	0.228	0.098	0.526
Malondialdehyde (MDA) (µM) (N=113)	r =	-0.116	0.004	0.183	1.000	0.595**	0.619**	0.590**
	p =	0.363	0.975	0.152	.	0.000	0.000	0.000
Reactive oxygen species (ROS) (µmol/l) (N=113)	r =	0.029	-0.076	0.154	0.595**	1.000	0.733**	0.502**
	p =	0.819	0.554	0.228	0.000	.	0.000	0.000
Cotinine (ng/ml) (N=113)	r =	-0.001	0.057	0.210	0.619**	0.733**	1.000	0.761**
	p =	0.995	0.655	0.098	0.000	0.000	.	0.000
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)	r =	-0.090	-0.071	0.081	0.590**	0.502**	0.761**	1.000
	p =	0.485	0.578	0.526	0.000	0.000	0.000	.

Table 13b: No significant correlations were detected between the volume of the semen, the pH, and the concentration of leukocytes with other parameters. However, a significantly positive correlations ($p<0.010$) were found between MDA levels and ROS ($r=0.595$), cotinine ($r=0.619$) and 8-OHdG ($r=0.590$) in seminal plasma. Moreover a significantly positive correlations ($p<0.010$) were observed between ROS with cotinine and 8-OHdG ($r=0.733$, $r=0.562$, respectively). Similar correlation was found between cotinine and 8-OHdG ($r=0.761$, $p<0.010$).

Table 13c. Correlation coefficient of sperm and semen parameters of patient samples (n=116)

		Count (mill/ml)	Motility (% motile)	Sperm vitality (Eosin) (%)	Membrane integrity (HOS) (%)	Chromatin condensation (positive CMA_3) (%)	DNA Fragmentation (positive TUNEL) (%)	Morphologically Normal spermatozoa (%)	Protamine 1 (ng/ 10^6 sperm)	Protamine 2 (ng/ 10^6 sperm)	P1/P2 Ratios
Volume (ml)	r =	-0.107	-0.049	0.038	0.048	-0.081	-0.044	0.069	0.152	0.123	-0.019
	p =	0.252	0.600	0.683	0.611	0.387	0.635	0.460	0.104	0.190	0.840
pH	r =	-0.151	-0.076	-0.025	-0.005	0.023	0.049	-0.008	-0.069	-0.034	-0.044
	p =	0.106	0.418	0.792	0.954	0.809	0.604	0.930	0.459	0.716	0.637
Leukocytes (%)	r =	0.057	0.051	0.061	-0.114	0.113	0.060	0.015	-0.041	-0.129	0.157
	p =	0.544	0.586	0.518	0.223	0.226	0.521	0.873	0.664	0.169	0.093
Malondialdehyde (MDA) (µM) (N=113)	r =	-0.076	-0.249*	-0.239	-0.340**	0.351**	0.517**	-0.428**	-0.034	-0.189	0.345**
	p =	0.552	0.049	0.060	0.006	0.005	0.000	0.000	0.789	0.137	0.006
Reactive oxygen species (ROS) (µmol/l) (N=113)	r =	-0.183	-0.248*	-0.133	-0.280*	0.510**	0.402**	-0.371**	-0.122	-0.299*	0.368**
	p =	0.151	0.050	0.299	0.027	0.000	0.001	0.003	0.342	0.017	0.003
Cotinine (ng/ml) (N=113)	r =	-0.102	-0.208	-0.207	-0.276*	0.451**	0.500**	-0.514**	-0.049	-0.268*	0.411**
	p =	0.426	0.102	0.104	0.028	0.000	0.000	0.000	0.702	0.034	0.001
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)	r =	-0.145	-0.346**	-0.305*	-0.233*	0.432**	0.497**	-0.445**	-0.161	-0.301*	0.374**
	p =	0.255	0.006	0.015	0.046	0.000	0.000	0.000	0.206	0.017	0.003

Table 13c illustrated an inverse correlation ($p<0.050$) between motility and concentrations of MDA and ROS ($r=-0.249$; $r=-0.248$ respectively). In addition motility was significantly reduced as concentration of 8-OHdG increase ($r=-0.346$, $p<0.010$). Sperm vitality significantly reduced as concentration of 8-OHdG increase ($r=-0.305$, $p<0.050$). Similar an inverse significant correlation was shown between concentration of MDA, ROS, cotinine and 8-OHdG in seminal plasma and sperm membrane integrity (Table 13c). In contrast a significantly positive correlations ($p<0.010$)

were found between the MDA, ROS, cotinine, and 8-OHdG in seminal plasma and chromatin condensation (bad sperm), as well as with high DNA fragmentation. The concentrations of MDA, ROS, cotinine, and 8-OHdG showed inverse effects on Protamine 2 (r=-0.189, r=-0.299, r=-0.268, r=-0.301 respectively). In contrast, P1/P2 ratios of spermatozoa showed a positive correlations ($p<0.010$) with seminal plasma parameters.

4-3- Non-smokers versus Smokers

4-3-1- All participants

Table 14. Comparison of semen and sperm parameters for all participants (normal and patients) dividing into nonsmokers and smokers.

Parameters	All Participants	Non Smokers	Smokers	p-value
Samples	166	94	72	
Age (year)	34.1 ± 7.8	33.3 ± 7.7	35.2 ± 7.9	0.165
Volume (ml)	3.4 ± 1.6	3.6 ± 1.6	3.1 ± 1.6	0.027
pH	8.7 ± 0.4	8.7 ± 0.4	8.7 ± 0.4	0.927
Count (mill/ml)	67.1 ± 31.2	68.4 ± 31.2	65.4 ± 31.5	0.641
Motility (% motile)	30.3 ± 13.6	35.8 ± 14.6	23.4 ± 8.0	0.000
Sperm vitality (Eosin) (%)	37.8 ± 17.8	41.2 ± 17.3	33.5 ± 17.6	0.005
Membrane integrity (HOS) (%)%	47.6 ± 19.8	54.1 ± 18.4	39.2 ± 18.4	0.000
Leukocytes (%)	2.5 ± 1.5	2.4 ± 0.6	2.8 ± 2.1	0.036
Chromatin condensation (positive CMA_3) (%)	30.0 ± 9.0	26.4 ± 8.0	34.6 ± 8.1	0.000
DNA Fragmentation (positive TUNEL) (%)	12.8 ± 5.9	9.6 ± 4.3	16.8 ± 5.1	0.000
Morphologically Normal spermatozoa (%)	36.8 ± 15.2	41.4 ± 13.7	26.6 ± 12.0	0.000
Malondialdehyde (MDA) (μM)	7.21 ± 1.77 (n=113)	6.10 ± 1.18 (n=62)	8.51 ± 1.43 (n=51)	0.000
Reactive oxygen species (ROS) (μmol/l)	87.80 ± 50.85 (n=113)	52.89 ± 28.48 (n=62)	128.75 ± 39.46 (n=51)	0.000
Cotinine (ng/ml)	41.86 ± 58.18 (n=113)	2.34 ± 2.20 (n=62)	88.22 ± 58.05 (n=51)	0.000
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml)	1.59 ± 1.47 (n=113)	0.71 ± 0.53 (n=62)	2.63 ± 1.54 (n=51)	0.000
Protamine 1 (P1) (ng/10^6 sperm)	404.81 ± 1024.37	402.63 ± 93.96	407.60 ± 128.80	0.804
Protamine 2 (P2) (ng/10^6 sperm)	358.69 ± 105.08	376.81 ± 97.36	335.60 ± 110.60	0.030
P1/P2 ratio	1.18 ± 0.33	1.09 ± 0.18	1.30 ± 0.42	0.000

The distribution of all participants into non-smokers (n=94) and smokers (n=72) groups was reported in figure 22, while table 14 reported the semen and sperm characteristics. Significant differences ($p<0.050$) were found in volume of the semen sample, number of leukocytes and concentration of P2 between the two groups. A significant higher differences were found between other investigated parameters ($p<0.010$), except for age, pH, count of the sperms and the concentration of P1 which showed no statistical differences ($p>0.050$) between the two groups.

The seminal plasma concentrations of MDA, ROS, cotinine and 8-OHdG were significantly higher in smokers group (8.51 ± 1.43, 128.75 ± 39.46, 88.22 ± 58.05, and 2.63 ± 1.54) in comparison to that of non-smokers group (6.10 ± 1.18, 52.89 ± 28.48, 2.34 ± 2.20, and 0.71 ± 0.53).

Moreover, the mean percentage of chromatin condensed spermatozoa was significantly lower ($p<0.010$) in smokers (bad sperms =34.6 ± 8.1%) in comparison to the value of the non-smokers (bad sperms =26.4 ± 8.0%). Whereas, the mean percentage of DNA fragmentation of smokers was higher than that of non-smokers (16.8 ± 5.1 vs. 9.6 ± 4.3, $p<0.010$). Also, P1/P2 ratios were significantly ($p<0.010$) higher in smokers (1.30 ± 0.42) than non-smokers (1.09 ± 0.18) (Figure 23). There were no significant correlations between the pH, the concentration of sperms, and P1 concentrations among the two groups

Figure 22. The distribution of all samples (n=166) into non-smokers (n=94) and smokers (n=72).

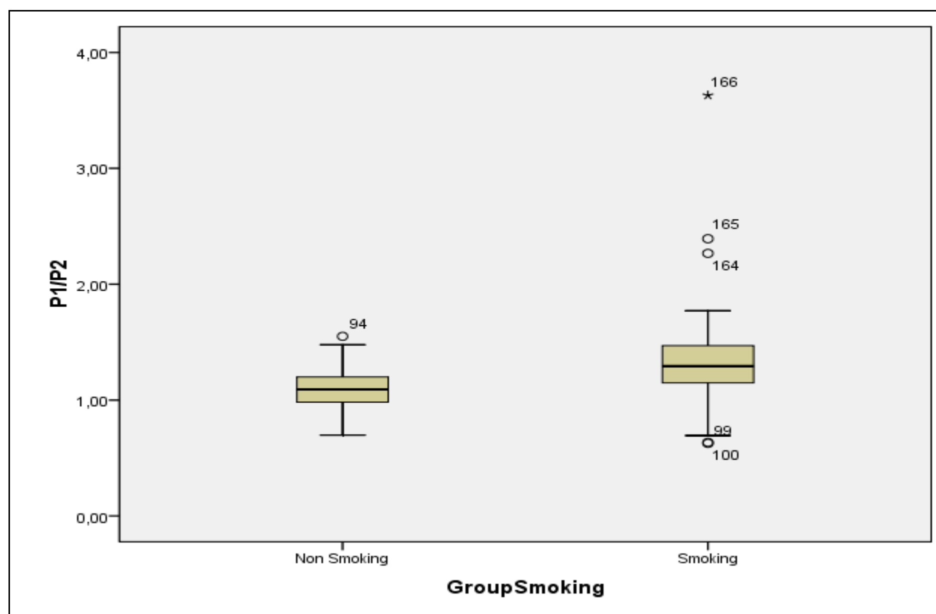


Figure 23. Box-plots showing the mean, median, and range of P1/P2 of both smokers and non-smokers. P1/P2 was significantly higher in smokers in comparison to non-smokers group (1.30 ± 0.42 VS 1.09 ± 0.18; p<0.010).

4-3-2- Normal volunteers

Table 15. Comparison of semen analysis parameters for all normal participants including nonsmokers and smokers.

Parameters	All Normal	Non Smokers	Smokers	p-value
Samples	50	31	19	
Age (year)	25.5 ± 4.5	25.7 ± 5.2	25.2 ± 3.4	0.944
Volume (ml)	3.9 ± 1.4	4.1 ± 1.5	3.4 ± 1.0	0.062
pH	8.6 ± 0.3	8.6 ± 0.3	8.6 ± 0.2	0.787
Count (mill/ml)	80.2 ±27.5	81.0 ± 27.6	78.9 ± 28.2	0.956
Motility (% motile)	37.1 ± 14.7	44.7 ± 12.5	24.7 ± 8.2	0.000
Sperm vitality (Eosin) (%)	45.2 ± 16.9	47.6 ± 15.3	41.3 ± 19.1	0.165
Membrane integrity (HOS) (%)%	53.4 ± 15.8	56.6 ± 14.5	48.2 ± 16.8	0.080
Leukocytes (%)	2.4 ± 0.5	2.5 ± 0.5	2.4 ± 0.6	0.963
Chromatin condensation (positive CMA_3) (%)	23.3 ± 7.0	19.6 ± 4.8	29.4 ± 5.5	0.000
DNA Fragmentation (positive TUNEL) (%)	9.6 ± 5.4	6.1 ± 1.8	15.1 ± 4.5	0.000
Morphologically Normal spermatozoa (%)	45.9 ± 13.9	52.9 ± 10.8	34.4 ± 10.3	0.000
Malondialdehyde (MDA) (µM)	6.45 ± 1.43	5.71 ± 1.17	7.66 ± 0.9	0.000
Reactive oxygen species (ROS) (µmol/l)	67.6 ± 42.2	40.47 ± 20.45	111.82 ± 28.66	0.000
Cotinine (ng/ml)	27.30 ± 40.17	2.02 ± 2.03	68.55 ± 38.58	0.000
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml)	1.15 ± 0.82	0.61 ± 0.40	2.02 ± 0.51	0.000
Protamine 1 (P1) (ng/10^6 sperm)	378.20 ± 100.00	367.60 ± 80.00	395.60 ± 125.90	0.743
Protamine 2 (P2) (ng/10^6 sperm)	347.30 ± 77.30	352.9 ± 69.3	338.10 ± 90.20	0.682
P1/P2 ratio	1.10 ± 0.20	1.04 ± 0.11	1.19 ± 0.27	0.016

Table 15 showed that concentrations of MDA, ROS, cotinine and 8-OHdG in seminal plasma of normal volunteers smokers were significantly higher (p<0.010) in comparison to that of the non-smokers (7.66 ± 0.9, 111.82 ± 28.66, 68.55 ± 38.58, 2.02 ± 0.51 vs. 5.71 ± 1.17, 40.47 ± 20.45, 2.02 ± 2.03, 0.61 ± 0.40 respectively). In contrast, the mean percentages of motility, and morphologically normal spermatozoa were significantly lower (p<0.010) in smokers (24.7 ± 8.2, 34.4 ± 10.3 respectively), in comparison to non-smokers (44.7 ± 12.5, 52.9 ± 10.8 respectively), while P1/P2 ratios were higher in smokers than in non-smokers (1.19 ± 0.27 vs. 1.04 ± 0.11, p<0.010). In contrast, a significantly lower chromatin condensed spermatozoa and significantly higher DNA fragmentation was found in smokers in comparison to non-smokers (Table 15). Other parameters showed a non-significant correlations.

Table 16a, b demonstrated the correlation coefficients for sperm and seminal plasma characteristics for smokers group of normal volunteers.

Table 16a. Correlation coefficient of sperm parameters of smoker samples of normal volunteers (n=19)

		Count (mill/ml)	Motility (%motile)	Sperm vitality (Eosin) (%)	Membrane integrity (HOS) (%)%	Chromatin condensation (positive CMA_3) (%)	DNA Fragmentation (positive TUNEL) (%)	Morphologically Normal spermatozoa (%)	Protamine 1 (ng/ 10^6 sperm)	Protamine 2 (ng/ 10^6 sperm)	P1/P2 Ratios
Count (mill/ml)	r =	1.000	0.017	-0.565*	-0.548*	0.142	0.356	0.338	0.126	-0.133	0.187
	p =	.	0.945	0.012	0.015	0.563	0.135	0.157	0.607	0.587	0.444
Motility (%motile)	r =	0.017	1.000	0.060	0.058	0.111	0.392	0.169	0.259	0.313	0.014
	p =	0.945	.	0.808	0.815	0.650	0.097	0.490	0.285	0.192	0.954
Sperm vitality (Eosin) (%)	r =	-0.565*	0.060	1.000	0.725**	-0.347	-0.273	-0.285	-0.004	0.230	-0.334
	p =	0.012	0.808	.	0.000	0.146	0.257	0.237	0.989	0.343	0.163
Membrane integrity (HOS) (%)	r =	-0.548*	0.058	0.725**	1.000	-0.198	-0.343	-0.225	0.117	0.402	-0.368
	p =	0.015	0.815	0.000	.	0.416	0.150	0.355	0.633	0.088	0.121
Chromatin condensation (positive CMA_3) (%)	r =	0.142	0.111	-0.347	-0.198	1.000	0.278	0.271	-0.269	-0.125	-0.076
	p =	0.563	0.650	0.146	0.416	.	0.249	0.261	0.265	0.611	0.756
DANN Fragmentation (positive TUNEL) (%)	r =	0.356	0.392	-0.273	-0.343	0.278	1.000	0.347	-0.100	-0.351	0.307
	p =	0.135	0.097	0.257	0.150	0.249	.	0.145	0.684	0.141	0.201
Morphologically Normal spermatozoa (%)	r =	0.338	0.169	-0.285	-0.225	0.271	0.347	1.000	-0.173	-0.042	-0.139
	p =	0.157	0.490	0.237	0.355	0.261	0.145	.	0.478	0.866	0.571
Protamine 1 (ng/ 10^6 sperm)	r =	0.126	0.259	-0.004	0.117	-0.269	-0.100	-0.173	1.000	0.574*	0.463*
	p =	0.607	0.285	0.989	0.633	0.265	0.684	0.478	.	0.010	0.046
Protamine 2 (ng/ 10^6 sperm)	r =	-0.133	0.313	0.230	0.402	-0.125	-0.351	-0.042	0.574*	1.000	-0.370
	p =	0.587	0.192	0.343	0.088	0.611	0.141	0.866	0.010	.	0.119
P1/P2 Ratios	r =	0.187	0.014	-0.334	-0.368	-0.076	0.307	-0.139	0.463*	-0.370	1.000
	p =	0.444	0.954	0.163	0.121	0.756	0.201	0.571	0.046	0.119	.

In table 16a, the concentration of the sperms was correlated significantly (p<0.050) negative with sperm vitality and membrane integrity (r=-0.565; r=-0.548 respectively). Whereas, the sperm vitality was correlated significantly positive with membrane integrity of the sperms (r=0.725, p<0.010). Other parameters showed a correlations but not significant.

Table 16b. Correlation coefficient of semen parameters of smoker samples of volunteers (n=19)

		Volume (ml)	pH	Leukocytes (%)	Malondialdehyde (MDA) (µM) (N=113)	Reactive oxygen species (ROS) (µmol/l) (N=113)	Cotinine (ng/ml) (N=113)	8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)
Volume (ml)	r =	1.000	0.081	-0.007	0.232	-0.010	-0.355	-0.008
	p =	.	0.742	0.977	0.339	0.967	0.136	0.972
pH	r =	0.081	1.000	-0.519*	-0.145	0.129	0.181	0.071
	p =	0.742	.	0.023	0.554	0.600	0.458	0.772
Leukocytes (%)	r =	-0.007	-0.519*	1.000	0.356	-0.027	-0.498*	-0.319
	p =	0.977	0.023	.	0.135	0.914	0.030	0.182
Malondialdehyde (MDA) (µM) (N=113)	r =	0.232	-0.145	0.356	1.000	0.193	-0.515*	-0.528*
	p =	0.339	0.554	0.135	.	0.428	0.024	0.020
Reactive oxygen species (ROS) (µmol/l) (N=113)	r =	-0.010	0.129	-0.027	0.193	1.000	-0.171	-0.283
	p =	0.967	0.600	0.914	0.428	.	0.483	0.241
Cotinine (ng/ml) (N=113)	r =	-0.355	0.181	-0.498*	-0.515*	-0.171	1.000	0.389
	p =	0.136	0.458	0.030	0.024	0.483	.	0.099
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)	r =	-0.008	0.071	-0.319	-0.528*	-0.283	0.389	1.000
	p =	0.972	0.772	0.182	0.020	0.241	0.099	.

Table 16b showed a significantly negative correlations between the pH with leukocytes concentration (r=-0.519, p<0.050) and concentrations of leukocytes with cotinine (r=-0.498, p<0.050). Whereas, the concentration of MDA showed a significant negative correlation with cotinine and 8-OHdG (r=-0.515, r=-0.578; p<0.050 respectively).

4-3-3-Patients

Table 17. Comparison of semen analysis parameters for all patients participating in the study, including nonsmokers and smokers.

Parameters	All Patients	Non Smokers	Smokers	p-value
Samples	116	63	53	
Age (year)	37.9 ± 5.7	37.1 ± 5.7	38.8 ± 5.6	0.185
Volume (ml)	3.2 ± 1.7	3.4 ± 1.6	3.1 ± 1.8	0.169
pH	8.7 ± 0.4	8.7 ± 0.4	8.7 ± 0.4	0.923
Count (mill/ml)	61.4 ± 31.1	62.1 ± 31.1	60.6 ± 31.4	0.904
Motility (% motile)	27.4 ± 12.0	31.3 ± 13.6	23.0 ± 8.0	0.001
Sperm vitality (Eosin) (%)	34.7 ± 17.3	38.1 ± 17.5	30.7 ± 16.4	0.030
Membrane integrity (HOS) (%)%	45.1 ± 20.8	52.9 ± 20.1	36.1 ± 18.1	0.000
Leukocytes (%)	2.6 ± 1.7	2.3 ± 0.6	2.9 ± 2.4	0.014
Chromatin condensation (positive CMA_3) (%)	32.9 ± 8.2	29.8 ± 7.1	36.4 ± 8.1	0.000
DNA Fragmentation (positive TUNEL) (%)	14.2 ± 5.6	11.3 ± 4.2	17.4 ± 5.3	0.000
Morphologically Normal spermatozoa (%)	29.4 ± 12.7	36.1 ± 9.0	23.4 ± 10.1	0.000
Malondialdehyde (MDA) (μM)	7.82 ± 1.79 (n=63)	6.51 ± 1.06 (n=31)	9.01 ± 1.46 (n=32)	0.000
Reactive oxygen species (ROS) (μmol/l)	103.85 ± 5.68 (n=63)	65.74 ± 30.16 (n=31)	138.49 ± 41.85 (n=32)	0.000
Cotinine (ng/ml)	53.42 ± 67.33 (n=63)	2.68 ± 2.36 (n=310	99.55 ± 64.58 (n=32)	0.000
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml)	1.95 ± 1.76 (n=63)	0.81 ± 0.63 (n=31)	2.99 ± 1.82 (n=32)	0.000
Protamine 1 (P1) (ng/10^6 sperm)	416.30 ± 101.70	420.2 ± 95.8	411.80 ± 108.80	0.750
Protamine 2 (P2) (ng/10^6 sperm)	363.60 ± 114.9	388.8 ± 107.2	334.7 ± 117.7	0.030
P1/P2 ratio	1.22 ± 0.36	1.11 ± 0.20	1.34 ± 0.46	0.000

In the group of patients (n=116, Table 17), the mean numbers of morphologically normal spermatozoa, motility, and membrane integrity were significantly lower (p<0.010) in smokers (23.4 ± 10.1, 23.0 ± 8.0, 36.1 ± 18.1) in comparison to non-smokers (36.1 ± 9.0, 31.3 ± 13.6, 52.9 ± 20.1). Whereas, the mean percentage of chromatin condensation (CMA_3), and DNA fragmentation (Tunel) were significantly lower in non-smokers than that of smokers (bad sperms: 29.8 ± 7.1, 11.3 ± 4.2 vs. 36.4 ± 8.1, 17.4 ± 5.3 respectively, p<0.010).

Protamine 2 was significantly higher in non-smokers (388.8 ± 107.2) than smokers (334.7 ± 117.7, p<0.050), on the opposite side, P1/P2 ratios of non-smokers were significant lower than in smokers (1.11 ± 0.20, 1.34 ± 0.46, p<0.010). Whereas, P1 showed no significant difference between the two groups (420.2 ± 95.8, 411.80 ± 108.80, p<0.050).

Furthermore, smokers levels of MDA (9.01 ± 1.46), ROS (138.49 ± 41.85), cotinine (99.55 ± 64.58), and 8-OHdG (2.99 ± 1.82) were significantly higher than those values observed in non-smokers group (6.51 ± 1.06, 65.74 ± 30.16, 2.68 ± 2.36, 0.81 ± 0.63, p<0.010). A significant differences (p<0.051) were demonstrated between the non-smokers versus smokers groups for the sperm vitality (38.1 ± 17.5, 30.7 ± 16.4), and leukocytes concentration (2.3 ± 0.6, 2.9 ± 2.4). Other parameters showed a non-significant correlation.

Table 18a, b demonstrated the correlation coefficients for sperm and seminal plasma characteristics for smokers group of patients (n=53)

Table 18a. Correlation coefficient of sperm parameters of smoker patients samples (n=53)

		Count (mill/ml)	Motility (%motile)	Sperm vitality (Eosin) (%)	Membrane integrity (HOS) (%)%	Chromatin condensation (positive CMA_3) (%)	DNA Fragmentation (positive TUNEL) (%)	Morphologically Normal spermatozoa (%)	Protamine 1 (ng/ 10^6 perm)	Protamine 2 (ng/ 10^6 sperm)	P1/P2 Ratios
Count (mill/ml)	r =	1.000	0.185	0.501^{**}	0.399^{**}	-0.320^{*}	0.084	-0.042	0.326^{*}	0.116	0.015
	p =	.	0.184	0.000	0.003	0.019	0.548	0.763	0.017	0.407	0.916
Motility (%motile)	r =	0.185	1.000	0.454^{**}	0.130	-0.067	0.040	-0.039	0.081	0.176	-0.179
	p =	0.184	.	0.001	0.353	0.634	0.776	0.780	0.564	0.208	0.199
Sperm vitality (Eosin) (%)	r =	0.501^{**}	0.454^{**}	1.000	0.526^{**}	-0.120	-0.046	-0.167	0.240	0.249	-0.151
	p =	0.000	0.001	.	0.000	0.391	0.741	0.231	0.083	0.072	0.282
Membrane integrity (HOS) (%)	r =	0.399^{**}	0.130	0.526^{**}	1.000	-0.175	0.033	0.021	0.164	0.174	-0.180
	p =	0.003	0.353	0.000	.	0.211	0.814	0.881	0.241	0.213	0.198
Chromatin condensation (positive CMA_3) (%)	r =	-0.320^{*}	-0.067	-0.120	-0.175	1.000	0.011	-0.062	-0.263	-0.150	0.042
	p =	0.019	0.634	0.391	0.211	.	0.940	0.657	0.057	0.284	0.768
DNA Fragmentation (positive TUNEL) (%)	r =	0.084	0.040	-0.046	0.033	0.011	1.000	-0.248	-0.122	-0.039	0.051
	p =	0.548	0.776	0.741	0.814	0.940	.	0.074	0.384	0.782	0.716
Morphologically Normal spermatozoa (%)	r =	-0.042	-0.039	-0.167	0.021	-0.062	-0.248	1.000	-0.114	-0.115	-0.002
	p =	0.763	0.780	0.231	0.881	0.657	0.074	.	0.416	0.410	0.991
Protamine 1 (ng/ 10^6 sperm)	r =	0.326^{*}	0.081	0.240	0.164	-0.263	-0.122	-0.114	1.000	0.554^{**}	0.072
	p =	0.017	0.564	0.083	0.241	0.057	0.384	0.416	.	0.000	0.610
Protamine 2 (ng/ 10^6 sperm)	r =	0.116	0.176	0.249	0.174	-0.150	-0.039	-0.115	0.554^{**}	1.000	-0.686^{**}
	p =	0.407	0.208	0.072	0.213	0.284	0.782	0.410	0.000	.	0.000
P1/P2 Ratios	r =	0.015	-0.179	-0.151	-0.180	0.042	0.051	-0.002	0.072	-0.686^{**}	1.000
	p =	0.916	0.199	0.282	0.198	0.768	0.716	0.991	0.610	0.000	.

Table 18b. Correlation coefficient of semen parameters of smoker patients samples (n=53)

		Volume (ml)	pH	Leukocytes (%)	Malondialdehyde (MDA) (µM) (N=113)	Reactive oxygen species (ROS) (µmol/l) (N=113)	Cotinine (ng/ml) (N=113)	8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)
Volume (ml)	r =	1.000	-0.183	0.044	-0.081	0.005	0.123	0.002
	p =	.	0.191	0.752	0.658	0.979	0.504	0.991
pH	r =	-0.183	1.000	0.007	0.067	-0.274	0.259	-0.032
	p =	0.191	.	0.961	0.716	0.129	0.152	0.863
Leukocytes (%)	r =	0.044	0.007	1.000	-0.194	0.064	0.246	-0.140
	p =	0.752	0.961	.	0.288	0.730	0.175	0.444
Malondialdehyde (MDA) (µM) (N=113)	r =	-0.081	0.067	-0.194	1.000	0.007	-0.052	0.285
	p =	0.658	0.716	0.288	.	0.972	0.779	0.114
Reactive oxygen species (ROS) (µmol/l) (N=113)	r =	0.005	-0.274	0.064	0.007	1.000	0.069	-0.254
	p =	0.979	0.129	0.730	0.972	.	0.709	0.160
Cotinine (ng/ml) (N=113)	r =	0.123	0.259	0.246	-0.052	0.069	1.000	0.099
	p =	0.504	0.152	0.175	0.779	0.709	.	0.589
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=113)	r =	0.002	-0.032	-0.140	0.285	-0.254	0.099	1.000
	p =	0.991	0.863	0.444	0.114	0.160	0.589	.

In table 18a, the concentration of sperms correlated significantly with sperm vitality (r=0.501, p<0.010), membrane integrity (r=0.399, p<0.010), and with P1 (r=0.326, p<0.050). Besides, it was correlated significantly negative with chromatin condensation (r=-0.320, p<0.050).

A significant correlations (p<0.010) were detected between sperm viability with sperm motility and membrane integrity (r=0.454, r=0.526 respectively). Others, a significant correlation was found between P1 and P2 (r=0.574, p<0.010) and between P2 with P1/P2 ratios (r=0.686, p<0.010). Other semen parameters (Table 18b) showed no significant correlations within the group.

4-3-4- Normal smokers versus patient smokers

Table 19. Comparison of semen analysis parameters for normal smokers and patients smokers.

Parameters	Normal Smokers	Patients Smokers	p-value
Samples	19	53	
Age (year)	25.2 ± 3.4	38.8 ± 5.6	0.000
Volume (ml)	3.4 ± 1.0	3.1 ± 1.8	0.128
pH	8.6 ± 0.2	8.7 ± 0.4	0272
Count (mill/ml)	78.9 ± 28.2	60.6 ± 31.4	0.020
Motility (% motile)	24.7 ± 8.2	23.0 ± 8.0	0.401
Sperm vitality (Eosin) (%)	41.3 ± 19.1	30.7 ± 16.4	0.041
Membrane integrity (HOS) (%)%	48.2 ± 16.8	36.1 ± 18.1	0.024
Leukocytes (%)	2.4 ± 0.6	2.9 ± 2.4	0.305
Chromatin condensation (positive CMA_3) (%)	29.4 ± 5.5	36.4 ± 8.1	0.000
DNA Fragmentation (positive TUNEL) (%)	15.1 ± 4.5	17.4 ± 5.3	0.094
Morphologically Normal spermatozoa (%)	34.4 ± 10.3	23.4 ± 10.1	0.000
Malondialdehyde (MDA) (µM)	7.66 ± 0.90	9.01 ± 1.46 (n=33)	0.000
Reactive oxygen species (ROS) (µmol/l)	111.82 ± 28.66	138.49 ± 41.85 (n=33)	0.047
Cotinine (ng/ml)	68.55 ± 38.58	99.55 ± 64.58 (n=33)	0.066
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml)	2.02 ± 0.51	2.99 ± 1.82 (n=33)	0.094
Protamine 1 (P1) (ng/10^6 sperm)	395.60 ± 125.90	411.80 ± 108.80	0.348
Protamine 2 (P2) (ng/10^6 sperm)	338.10 ± 90.20	334.7 ± 117.7	0.934
P1/P2 ratio	1.19 ± 0.27	1.34 ± 0.46	0.335

Table 19 demonstrated the standard semen parameters in both normal smoker and patient smoker groups. Sperm count, motility, vitality, membrane integrity, and morphologically normal sperm were significantly higher in volunteer’s smokers than in patients smokers, the results were as follow: for volunteer’s smokers (78.9 ± 28.2, 24.7 ± 8.2, 41.3 ± 19.1, 48.2 ± 16.8, 34.4 ± 10.3) and the corresponding results in patients smokers were (60.6 ± 31.4, 23.0 ± 8.0, 30.7 ± 16.4, 36.1 ± 18.1, 23.4 ± 10.1). Chromatin condensation of volunteers smokers was significantly higher than patient smokers (bad sperms: 29.4 ± 5.5 vs. 36.4 ± 8.1, p<0.010), besides, sperm DNA fragmentation. P1, P2, P1/P2 ratios showed no significant difference (p>0.050).

Semen parameters MDA and ROS were significant higher in patients group in comparison to volunteers group (9.01 ± 1.46, 138.49 ± 41.85 vs. 7.66 ± 0.90, 111.82 ± 28.66, p<0.010, p<0.050 respectively). Besides low significant difference were observed for cotinine and 8-OHdG between the two groups.

4-4- Protamines Quantification

The mean ± standard deviation of P1, P2, and P1/P2 ratios for all participants were (404.81 ± 1024.37, 358.69 ± 105.08, 1.18 ± 0.33 respectively)

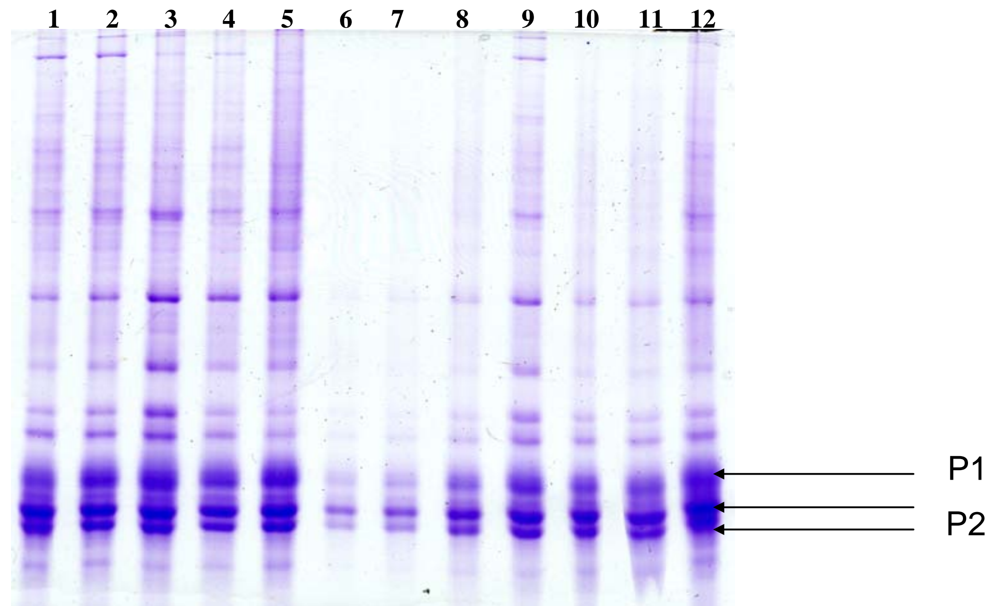


Figure 24. Analysis of protamine 1 (P1), protamine 2 (P2).
Proteins extracted from spermatozoa, separated on a polyacrylamide-acetic acid-urea gel and stained with Coomassie Blue. Lanes 6–9 correspond to increasing amounts of a human protamine standard (0.50, 1.00, 1.50 and 2.00 µg) included in each lane. Lane 10 correspond to control sample. Lanes 1-5, 11-12 correspond to different sperm samples from patients. (Each lane loaded with 10 µl of protein extracts)

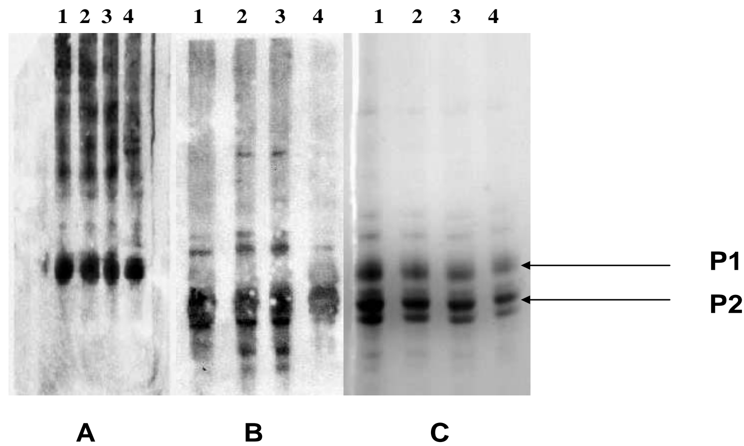


Figure 25. Analysis of protamine 1 (P1), protamine 2 (P2).
(**A**) Western blot, corresponding to a replica of the gel shown in **C**, using an antibody specific to the P1.
(**B**) Western blot, corresponding to a replica of the gel shown in **C**, using an antibody specific to the P2.
(**C**) Proteins extracted from spermatozoa, separated on a polyacrylamide-acetic acid-urea gel and stained with Coomassie Blue. Lanes 1–4 correspond to increasing amounts of a human protamine standard (0.50, 1.00, 1.50 and 2.00 µg) included in each gel.

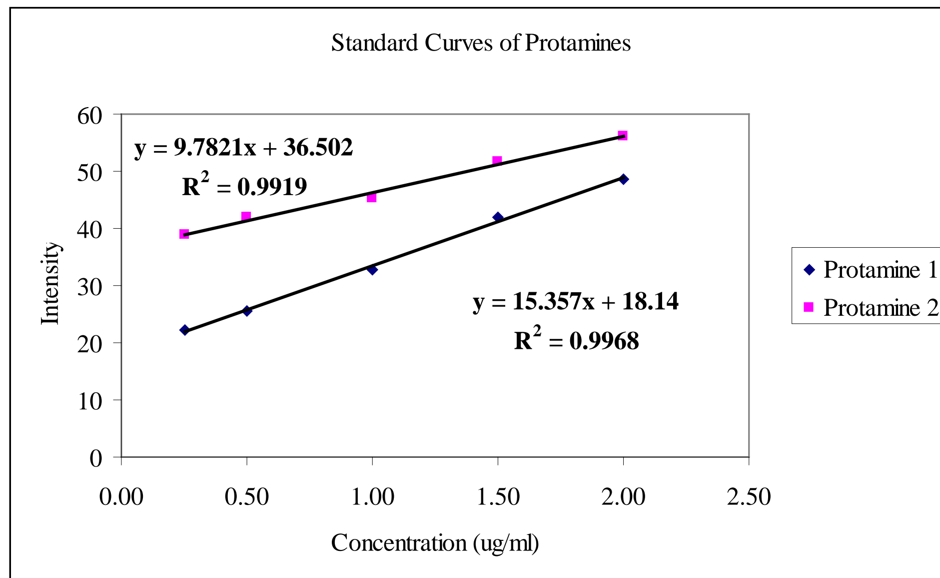


Figure 26. Representative standard curves used to calculate the concentrations of P1 and P2. The loaded concentrations were (0.25, 0.50, 1.00, 1.50, and 2.00 µg)

4-4-1- Volunteers versus Patients

Table 20. Compare the mean ± standard deviation of the P1, P2, and P1/P2 ratios between normal volunteers and patient samples

Parameters	Normal	Patients	p-value
Samples	50	116	
Protamine 1 (ng/ 10^6 sperm)	378.20 ± 100.00	416.30 ± 101.70	0.007
Protamine 2 (ng/ 10^6 sperm)	347.30 ± 77.30	363.60 ± 114.9	0.424
P1/P2 ratios	1.10 ± 0.20	1.22 ± 0.36	0.012

Protamine quantification revealed (Table 20, Fig. 27) that the mean P1 concentrations in the sperms of normal volunteers was significantly lower ($p<0.010$) than that in patients, they were 378.20 ± 100.00 vs. 416.30 ± 101.70 ng/10^6 sperm (Fig. 28). However, P2 concentrations in volunteers were 347.30 ± 77.30 and the corresponding values in patients were 363.60 ± 114.9 ng/10^6 sperm, ($p>0.050$, Fig. 29). The P1/P2 ratios were significantly higher in patients (1.22 ± 0.36) than that in normal (1.10 ± 0.20, $p<0.050$, Fig. 30).

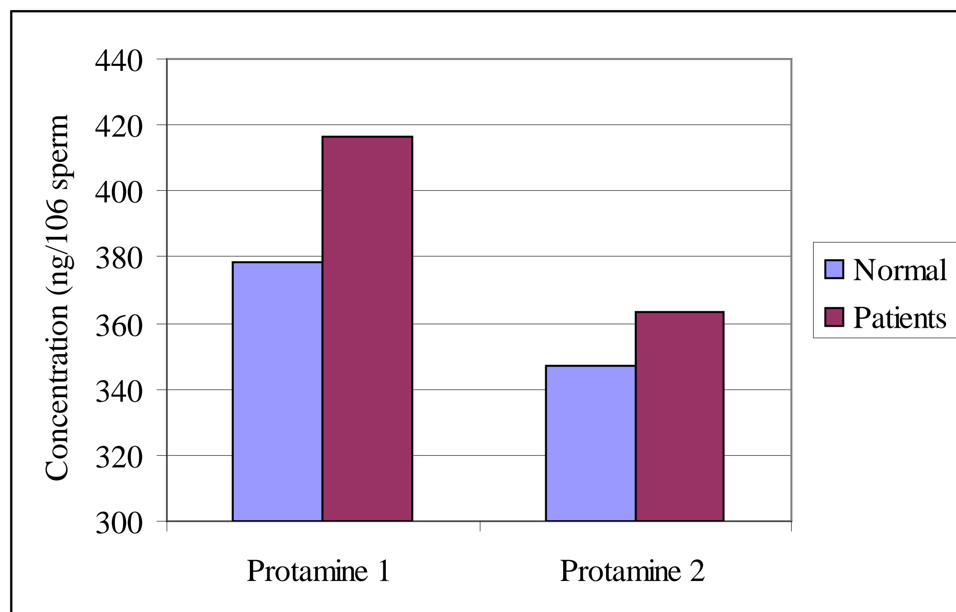


Figure 27. P1 and P2 content in the sperms of volunteers and patients.

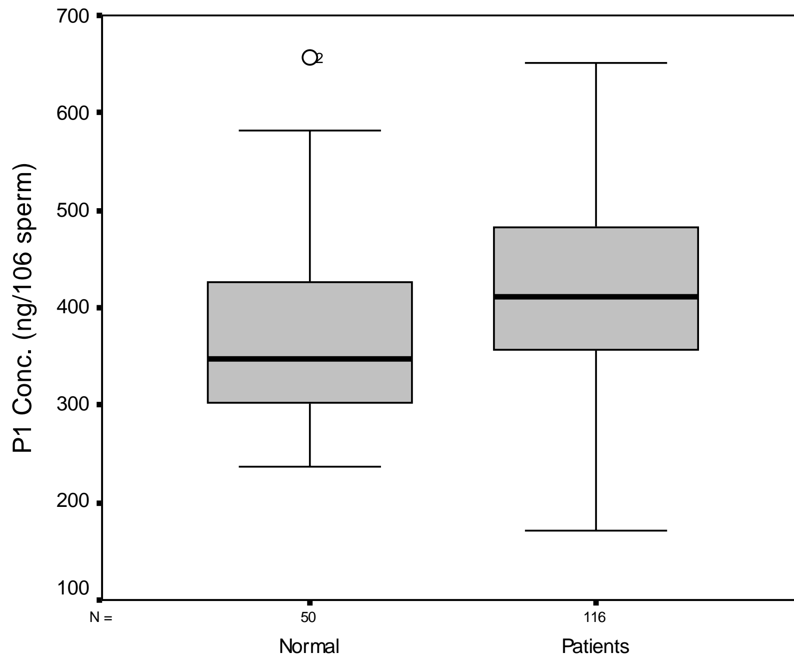


Figure 28. Box-plots showing the mean, median, and range of P1 of both normal and patients. P1 was significantly higher in patients group in comparison to normal group (416.30 ± 101.70, 378.20 ± 100.00 ng/10^6 sperm, $p<0.010$).

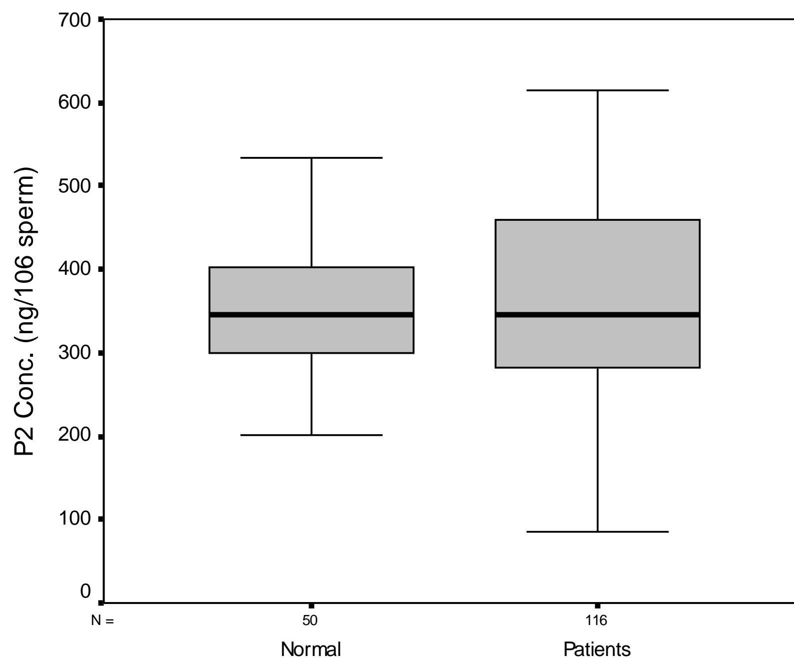


Figure 29. Box-plots showing the mean, median, and range of P2 of both normal and patients. P2 concentration showed no significant difference between patients group in comparison to normal group, it was higher in patients (363.60 ± 114.9) than normal (347.30 ± 77.30) ng/10^6 sperm, $p<0.010$.

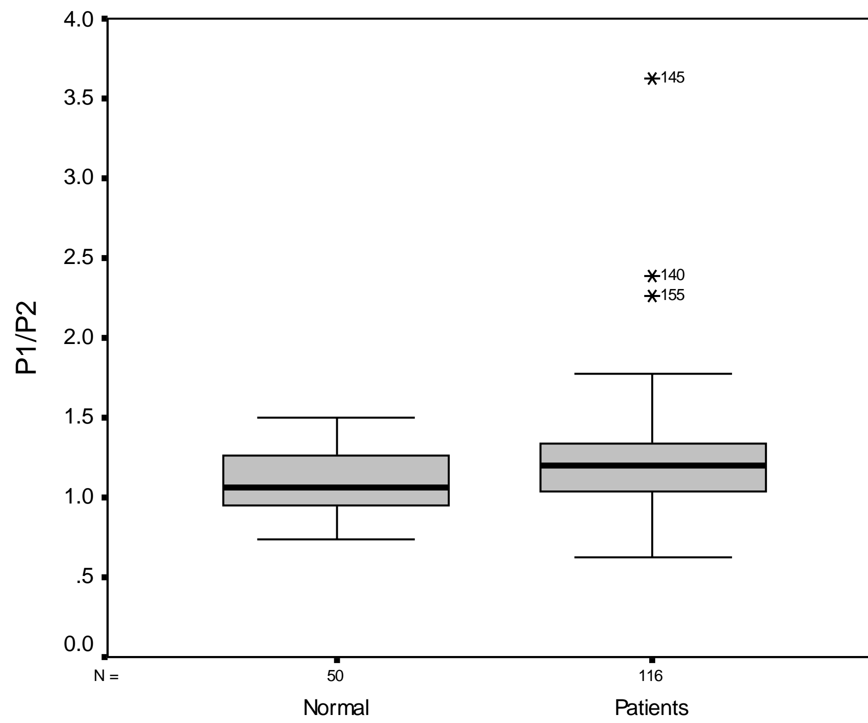


Figure 30. Box-plots showing the mean, median, and range of P1/P2 ratios of both normal and patients. P1/P2 ratio was significantly higher in patients group in comparison to normal group (1.22 ± 0.36, 1.10 ± 0.20, $p<0.010$).

4-4-2- Non-smokers versus smokers

Table 21. Compare the mean ± standard deviation of the P1, P2, and P1/P2 ratios between all participant non-smokers and smokers samples.

Parameters	Non Smokers	Smokers	p-value
Samples	94	72	
Protamine 1 (ng/10^6 sperm)	402.63 ± 93.96	407.60 ± 128.80	0.804
Protamine 2 (ng/10^6 sperm)	376.81 ± 97.36	335.60 ± 110.60	0.030
P1/P2 ratios	1.09 ± 0.18	1.30 ± 0.42	0.000

Table 21 demonstrated the concentrations of P1, P2, and P1/P2 ratios for all non-smokers (n=94) and all smokers (n=72). P1 concentrations in both groups non-smoker and smokers were closely similar (402.63 ± 93.96; 407.60 ± 128.80, $p>0.050$ respectively). On the contrary, P2 concentrations in non-smokers group (376.81 ± 97.36) were significantly higher ($p<0.050$) than in smokers group (335.60 ± 110.60). Highly significance difference was demonstrated in P1/P2 ratios for both groups, where it was (1.09 ± 0.18) for non-smokers group and (1.30 ± 0.42) for smokers group, ($p<0.010$), (Fig. 31).

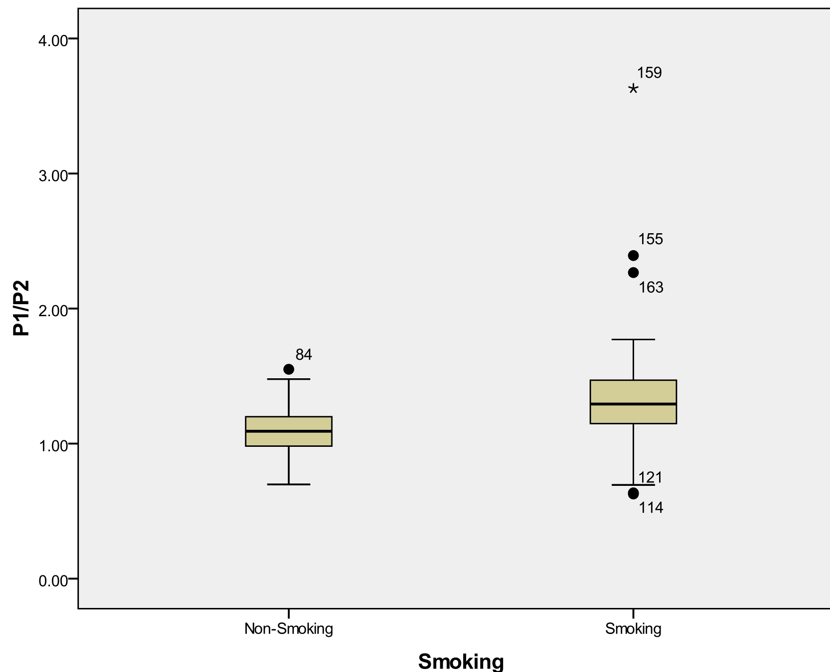


Figure 31. Box-plots showing the mean, median, and range of P1/P2 ratios of both all smokers and all non-smokers groups. P1/P2 ratio was significantly higher in smokers group in comparison to non-smokers group (1.30 ± 0.42, 1.09 ± 0.18, $p<0.010$).

4-4-2-1-Volunteers non-smokers versus smokers

Table 22. Compare the mean ± standard deviation of the P1, P2, and P1/P2 ratios between volunteer's non-smokers and smokers samples.

Parameters	All Volunteers	Non Smokers	Smokers	p-value
Samples	50	31	19	
Protamine 1 (ng/10^6 sperm)	378.20 ± 100.00	367.60 ± 80.00	395.60 ± 125.90	0.743
Protamine 2 (ng/10^6 sperm)	347.30 ± 77.30	352.90 ± 69.30	338.10 ± 90.20	0.682
P1/P2	1.10 ± 0.20	1.04 ± 0.11	1.19 ± 0.27	0.016

Neither P1 nor P2 showed a significant difference (p>0.050) between non-smokers (367.60 ± 80.00, 352.9 ± 69.3) and smokers (395.60 ± 125.90, 338.10 ± 90.20) in volunteers group. Whereas, a significant increase in P1/P2 ratios of smokers compared to non-smokers were observed (1.19 ± 0.27 vs. 1.04 ± 0.11, p<0.050) (Table 22, Fig. 32).

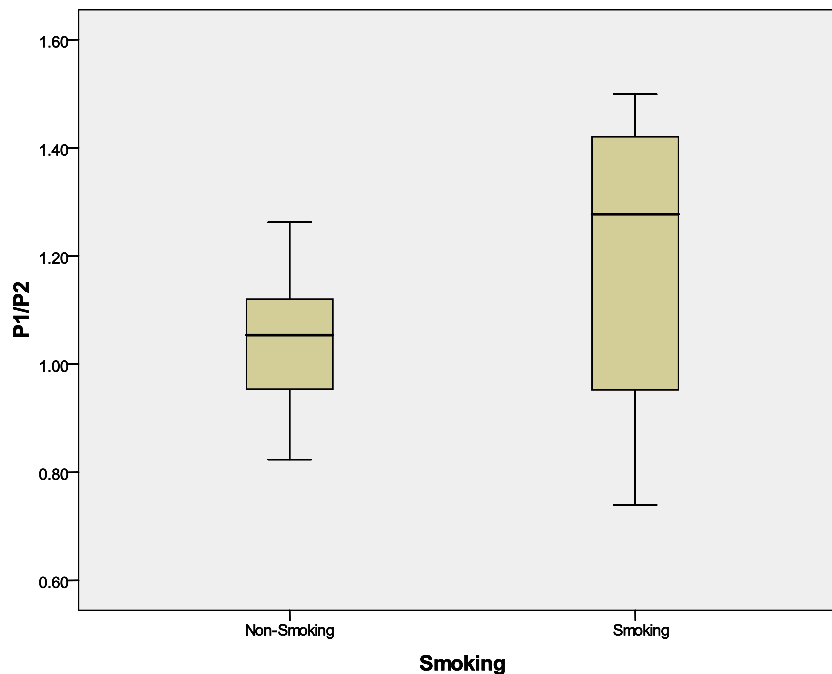


Figure 32. Box-plots showing the mean, median, and range of P1/P2 ratios of both smokers and non-smokers of volunteers. P1/P2 ratio was significant higher in smokers in comparison to non-smokers (1.19 ± 0.27, 1.04 ± 0.11, p<0.050).

4-4-2-2-Patients non-smokers versus smokers

Table 23. Compare the mean ± standard deviation of the P1, P2, and P1/P2 ratios between patient's non-smokers and smokers samples.

Parameters	All Patients	Non Smokers	Smokers	p-value
No. Samples	116	63	53	
Protamine 1 (ng/10^6 sperm)	416.30 ± 101.70	420.2 ± 95.8	411.80 ± 108.80	0.750
Protamine 2 (ng/10^6 sperm)	363.60 ± 114.9	388.8 ± 107.2	334.7 ± 117.7	0.030
P1/P2	1.22 ± 0.36	1.11 ± 0.20	1.34 ± 0.46	0.000

Table 23 illustrated the concentrations of P1, P2, and P1/P2 ratios of all patients undergoing ICSI therapy, including non-smokers and smokers. P1 concentrations were closely similar ($p>0.050$) in smokers (411.80 ± 108.80) and non-smokers (420.2 ± 95.8), whereas, P2 concentration and P1/P2 ratios showed a significant difference between smokers (334.7 ± 117.7; 1.34 ± 0.46) and non-smokers (388.8 ± 107.2; 1.11 ± 0.20). (Table 23; Fig. 33).

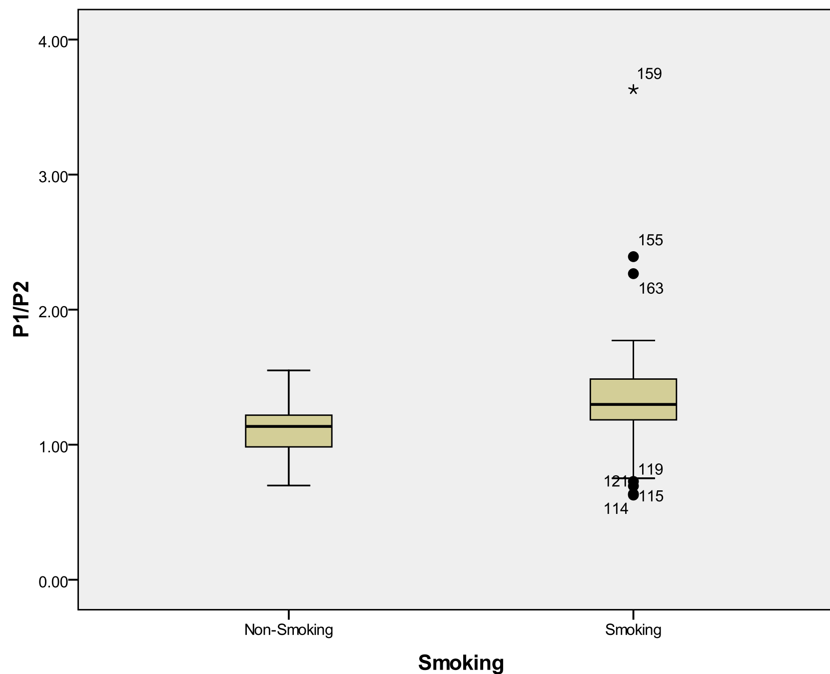


Figure 33. Box-plots showing the mean, median, and range of P1/P2 ratios of both smokers and non-smokers of patients. P1/P2 ratio was significant higher in smokers in comparison to non-smokers (1.34 ± 0.46, 1.11 ± 0.20, $p<0.010$).

4-4-2-3- Patients smokers versus volunteers smokers

Table 24. Compare the mean ± standard deviation of the P1, P2, and P1/P2 ratios between all normal volunteers' smokers and patients' smoker's samples.

Parameters	Volunteers Smokers	Patients Smokers	p-value
No. Samples	19	53	
Protamine 1 (ng/ 10^6 sperm)	395.60 ± 125.90	411.80 ± 108.80	0.348
Protamine 2 (ng/ 10^6 sperm)	338.10 ± 90.20	334.7 ± 117.7	0.934
P1/P2	1.19 ± 0.27	1.34 ± 0.46	0.335

P1 levels in patients smokers were higher volunteers smokers (411.80 ± 108.80, vs. 395.60 ± 125.90, p>0.050), in contrast, P2 levels were higher in volunteers smokers in comparison to patients smokers (338.10 ± 90.20 vs. 334.7 ± 117.7, p>0.050) and P1/P2 ratios were non-significant higher (p>0.050) in patients smokers (1.34 ± 0.46) than volunteers (1.19 ± 0.27) (Table 24).

4-4-3- P1/P2 ratio grouping

Depending on the values of P1/P2 ratios the participants were divided into three groups; low P1/P2 ratio (<0.90), normal P1/P2 ratio (0.90 – 1.10), and high P1/P2 ratio (>1.10). The P1/P2 ratios for all participants found to be (1.18 ± 0.33), and for volunteers and patients were (1.10 ± 0.20 and 1.22 ± 0.36 respectively).

Table 25. Distribution of all participants, volunteers and patients according to the P1/P2 ratios values

Parameters	All Participants (n=166)	Volunteers (n=50)	Patients (n=116)	p-value
Total P1/P2	1.18 ± 0.33	1.10 ± 0.20	1.22 ± 0.36	0.012
Low P1/P2 (< 0.90)	15.1% (6)	12.0% (6)	16.4% (19)	0.000
Normal P1/P2 (0.90-1.10)	21.7% (36)	42.0% (21)	12.9% (15)	
High P1/P2 (> 1.10)	63.3% (105)	46.0% (23)	70.7% (82)	

All participant samples were divided into the three P1/P2 ratios groups (low, normal, and high). By comparing P1/P2 ratios of the volunteers and patients samples distributed within these groups, the Chi square values showed a significant difference (p<0.010), (Table 25). These results revealed that 70.7 % (82/116) of the patients have high P1/P2 ratio, 16.4 % (19/116) have low ratio and 12.9 % (15/116) have normal ratios. Meanwhile, 46.0% (23/50) of the volunteers have high ratios, 12.0% (6/50) have low ratios and 42.0% (21/50) have normal ratios.

These results indicated that in volunteers group 58.0% revealed protamine abnormality (high and low P1/P2 ratios) and remaining 42.0% were normal. In contrast, in patients underwent ICSI therapy the corresponding values were 87.1% and 12.9% respectively. In addition, these results indicated that 78.4% of all participants have abnormal P1/P2 ratios while only 21.3% have normal ratios.

Table 26. Distribution of volunteer's non-smokers and smokers according to the P1/P2 ratios values

Parameters	All volunteers samples (n=50)	Non smokers (n=31)	Smokers (n=19)	p-value
Total P1/P2	1.10 ± 0.20	1.04 ± 0.11	1.19 ± 0.27	0.016
Low P1/P2 (< 0.90)	12.0% (6)	6.5% (2)	21.1% (4)	0.002
Normal P1/P2 (0.90-1.10)	42.0% (21)	61.2% (19)	10.5% (2)	
High P1/P2 (> 1.10)	46.0% (23)	32.3% (10)	68.4% (13)	

Furthermore, evaluation of the smoking effect (Table 26) for volunteers within P1/P2 ratio groups revealed a significant difference ($p<0.010$) between the non-smokers and smokers groups. Smokers group showed that 68.4%, (13/19) are within high P1/P2 ratio, 21.1%, (4/19) within low P1/P2 ratio and 10.5%, (2/19) normal P1/P2 ratio versus (32.3%, 10/31) of the non-smokers within high P1/P2 ratio, (6.5%, 2/31) within low P1/P2 ratio (6.5%, 2/31) and (61.3%, 19/31) within normal P1/P2 ratio. It was shown that 89.5% of smokers revealed abnormal P1/P2 ratios (low and high), whereas, in non-smokers only 38.8% presented abnormal ratios.

Table 27. Distribution of patient's non-smokers and smokers according to the P1/P2 ratios values

Parameters	All patients samples (n=116)	Non smokers (n=63)	Smokers (n=53)	p-value
Total P1/P2	1.22 ± 0.36	1.11 ± 0.20	1.34 ± 0.46	0.000
Low P1/P2 (< 0.90)	16.4% (19)	17.5% (11)	15.1% (8)	
Normal P1/P2 (0.90-1.10)	12.9% (15)	23.8% (15)	0.0% (0)	0.000
High P1/P2 (> 1.10)	70.7% (82)	58.7% (37)	84.9% (45)	

Furthermore, in table 27, patients group (n=116) was divided into two groups: The first group (smokers) 100% protamine abnormality was found. While 76.2% showed such abnormality in the second group (non-smokers).

Table 28. Distribution of all participants' non-smokers and smokers according to the P1/P2 ratios values

Parameters	All samples (n=166)	Non smokers (n=94)	Smokers (n=72)	p-value
Total P1/P2	1.18 ± 0.33	1.09 ± 0.18	1.30 ± 0.42	0.000
Low P1/P2 (< 0.90)	15.1% (6)	13.8% (13)	16.7% (12)	
Normal P1/P2 (0.90-1.10)	21.7% (36)	36.2% (34)	2.8% (2)	
High P1/P2 (> 1.10)	63.3% (105)	50.0% (47)	63.3% (58)	0.000

The participants underwent in the present study divided into non-smokers (n=94) and smokers (n=72) (Table 28). A highly significant difference ($p<0.010$) was found by comparing the all non-smokers groups with that of smokers. By analyzing the data the results were as follow: 97.4% of smokers showed protamine abnormality, besides, 63.8% of non-smokers were found to have abnormal protamination.

4-4-3-1- Relationship between P1/P2 ratios and seminal parameters

4-4-3-1-1- All participants

Table 29. Comparison of semen analysis parameters between all participant samples (n=166). According to the P1/P2 ratios distribution.

Parameters	Low P1/P2 (< 0.90) 1	Normal P1/P2 (0.90-1.10) 2	High P1/P2 (> 1.10) 3	p-value
Samples	25	37	104	
Age (year)	34.2 ± 8.1	31.2 ± 8.3	35.2 ± 7.4	0.049 $^{2-3}$
Volume (ml)	3.3 ± 1.7	3.6 ± 1.7	3.4 ± 1.6	0.658
pH	8.7 ± 0.4	8.7 ± 0.3	8.7 ± 0.4	0.820
Count (mill/ml)	63.0 ± 32.4	73.1 ± 32.2	65.9 ± 30.6	0.406
Motility (% motile)	31.0 ± 16.3	35.8 ± 13.5	28.2 ± 12.5	0.027 $^{2-3}$
Sperm vitality (Eosin) (%)	38.8 ± 17.9	39.6 ± 15.7	37.0 ± 18.6	0.664
Membrane integrity (HOS) (%)%	49.8 ± 21.4	52.0 ± 17.6	41.5 ± 20.0	0.192
Leukocytes (%)	2.9 ± 3.6	2.5 ± 0.5	2.4 ± 0.6	0.347
Chromatin condensation (positive CMA_3) (%)	30.5 ± 6.9	24.8 ± 7.9	31.7 ± 9.2	0.000 $^{1-2,2-3}$
DNA Fragmentation (positive TUNEL) (%)	14.7 ± 4.6	8.5 ± 4.1	13.8 ± 6.0	0.000 $^{1-2,2-3}$
Morphologically Normal spermatozoa (%)	33.4 ± 11.9	48.1 ± 13.0	31.7 ± 14.0	0.000 $^{1-2,2-3}$
Malondialdehyde (MDA) (μM)	7.17 ± 1.66 (n=14)	6.31 ± 1.26 (n=33)	7.76 ± 1.78 (n=66)	0.000 $^{1-2,2-3}$
Reactive oxygen species (ROS) (μmol/l)	87.05 ± 33.00 (n=14)	49.80 ± 26.10 (n=33)	106.96 ±52.87 (n=66)	0.000 $^{1-2,2-3}$
Cotinine (ng/ml)	36.11 ± 43.41 (n=14)	7.44 ± 23.34 (n=33)	60.30 ± 65.06 (n=66)	0.000 $^{1-2,2-3}$
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml)	2.03 ± 1.63 (n=14)	0.70 ± 0.55 (n=33)	1.95 ± 1.57 (n=66)	0.000 $^{1-2,2-3}$
Protamine 1 (P1) (ng/10^6 sperm)	371.31 ± 75.73	373.14 ± 78.12	424.13 ± 110.94	0.009 $^{1-3,2-3}$
Protamine 2 (P2) (ng/10^6 sperm)	480.79 ± 91.55	370.55 ± 71.07	325.12 ± 95.72	0.000 $^{1-2,1-3,2-3}$
P1/P2 ratio	0.77 ± 0.07	1.01 ± 0.05	1.35 ± 0.30	0.000 $^{1-2,1-3,2-3}$

Table 29 illustrated the means ± standard deviations and the p-values of the semen and sperm parameters of the 166 semen samples. According to P1/P2 ratio groups, samples were divided into three group's low, normal and high groups (section 4-4-3). A correlation between these parameters within the P1/P2 ratios was detected. Motility showed significant difference (p<0.050) between the normal and high groups. Whereas, a significant differences (p<0.010) detected for sperm's chromatin condensation, DNA fragmentation, morphologically normal sperm and P1 between normal group with low and high groups. Similarly, the seminal plasma parameters MDA, ROS, cotinine, and 8-OHdG showed significance differences (p<0.010) between normal with low and high groups. A significant differences (p<0.010) were found within all groups P2 and P1/P2 ratios. Figures 34 to 44 illustrated these correlation differences.

Other parameters showed that: volume of the semen in low and high P1/P2 ratios was lower than that of normal P1/P2 ratio (34.2 ± 8.1, 3.4 ± 1.6 and 3.6 ± 1.7, for low, high and normal, p>0.050 respectively). Sperm concentrations were reduced in low and high group compared to normal (63.0 ± 32.4. 65.9 ± 30.6, 73.1 ± 32.2, p>0.050 respectively). Sperm vitality was almost the same in the three groups while membrane integrity was higher in normal group participants (52.0 ± 17.6) than that low and high P1/P2 ratios (49.8 ± 21.4, 41.5 ± 20.0). These results revealed that participants with normal P1/P2 ratios (0.90-1.10) have sperm and seminal plasma parameters better than that of those within low (<0.90) and high (>1.10) P1/P2 ratios. These results indicated an inverse effect of abnormal protamination on sperm and semen parameters.

4-4-3-1-2- Smokers

The comparison between means ± standard deviations for the sperm and semen parameters for all smokers (n=72, normal and patients smokers) within P1/P2 ratios showed that smokers with normal P1/P2 ratios have sperm's vitality, membrane integrity and morphologically normal sperm higher than that of those with low and high ratios. Similarly, chromatin condensation and DNA integrity were higher in smokers with normal P1/P2 ratios.

The concentrations of seminal plasma parameters MDA, ROS, and 8-OHdG of the smokers with normal P1/P2 ratios (7.40 ± 0.10, 101.63 ± 19.55, 1.81 ± 0.16 respectively) were lower than those with low P1/P2 ratios (8.11 ± 1.13, 105.59 ± 20.98, 2, 83 ± 1.70 respectively) and those with high P1/P2 ratios (8.66 ± 1.51, 132.92 ± 40.25, 2.67 ± 1.56 respectively). Whereas cotinine levels for normal, low and high ratios were (91.09 ± 49.27, 61.00 ± 23.47, 95.14 ± 59.86 respectively). These results showed a higher oxidative stress in samples of smokers with abnormal P1/P2 ratios, which reflect the negative effect of smoking on sperm and semen parameters. Moreover, protamines levels showed that P1, P2, and P1/P2 ratio for smokers with normal P1/P2 ratios were (304.76 ± 5.37, 320.14 ± 10.31, 0.95 ± 0.01), while that for those with low P1/P2 ratios showed an increase in P1 levels (343.50 ± 50.80) and P2 levels (462.02 ± 76.13) and low P1/P2 ratio (0.75 ± 0.07). Smokers with high P1/P2 ratios results were (425.61 ± 117.97, 310.71 ± 101.44, 1.43 ± 0.73) respectively. These protamine results showed that smokers with low ratio have higher P2 level than those with normal ratio, whereas higher P1 level and lower P2 level observed in smokers with high level. These results indicated an over expression of P1 and under expression of P2 within the abnormal ratios groups.

Protamines evaluation in volunteers smoker's showed that those with low P1/P2 ratios have similar P1 (304.32 ± 51.14) level, higher P2 level (398.69 ± 63.49) and lower P1/P2 ratio (0.76 ± 0.02) in comparison to those with normal P1/P2 ratios (304.76 ± 5.37, 320.14 ± 10.31, 0.95 ± 0.01). On the contrary, volunteers smokers with high P1/P2 ratios showed a higher P1 level (437.72 ± 130.54), similar P2 level (322.16 ± 98.10), and higher ratio (1.36 ± 0.10). These findings revealed that over expression of P2 and P1 are responsible for the protamination abnormality (low and high P1/P2 ratio).

Furthermore, none of the patient's smokers showed normal P1/P2 ratio. Smokers with Low P1/P2 ratios P1, P2, and P1/P2 ratio was (363.04 ± 40.23, 493.68 ± 62.78, 0.74 ± 0.09) and those with high P1/P2 ratios was (422.11 ± 115.43, 307.39 ± 103.23, 1.45 ± 0.42). Over production of P2 and P1 were indicated in patients' smokers with low and high P1/P2 ratios respectively.

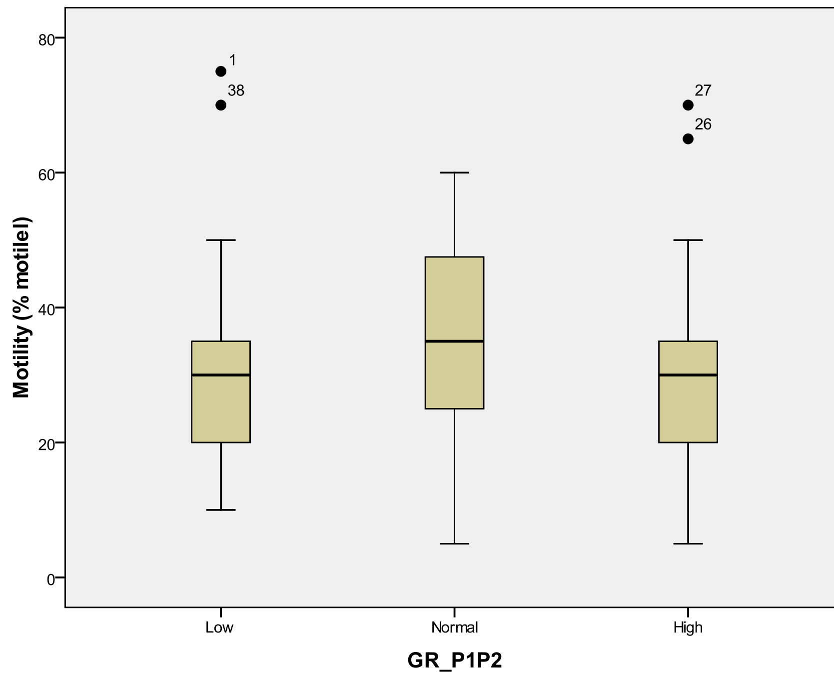


Figure 34. Box-plots showing the mean, median, and range of the sperm motility compared between the P1/P2 ratios groups (low, normal, and high) for all participant samples. A significant difference was found between normal and high groups of P1/P2 ratios (35.8 ± 13.5, 28.2 ± 12.5, p<0.050).

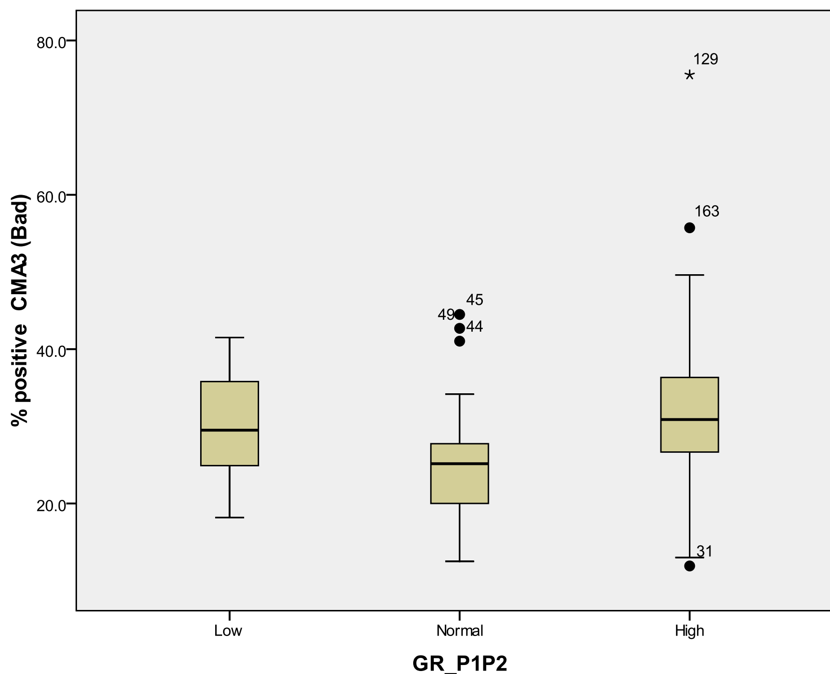


Figure 35. Box-plots showing the mean, median, and range of the mean percentage of chromatin condensation (positive CMA_3) compared between the P1/P2 ratios groups (low, normal, and high) for all participant samples. A significant differences between normal with low and high groups of P1/P2 ratios were found (24.8 ± 7.9, 30.5 ± 6.9, 31.7 ± 9.2, p<0.010).

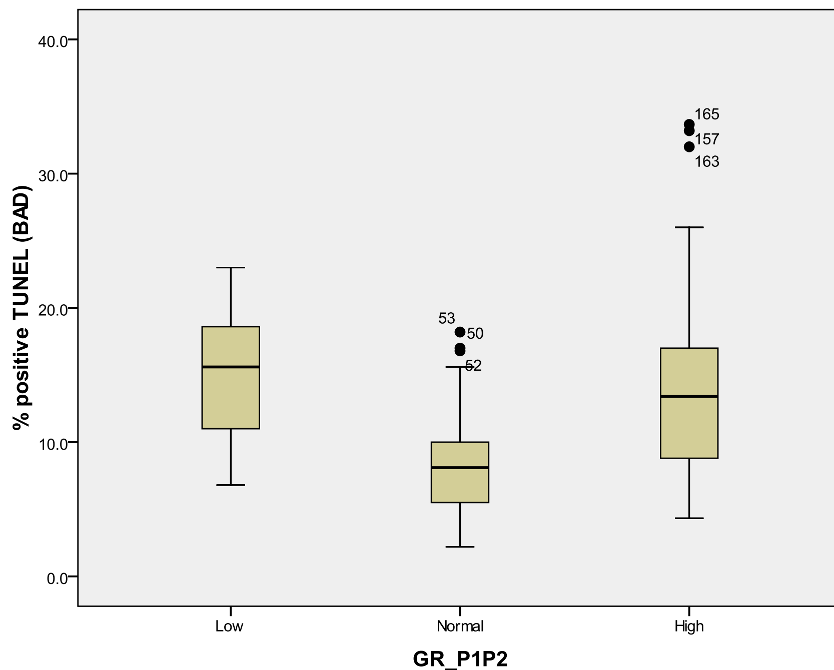


Figure 36. Box-plots showing the mean, median, and range of the mean percentages of DNA fragmentation compared between the P1/P2 ratios groups (low, normal, and high) for all participant samples. A significant differences between normal with low and high groups of P1/P2 ratios (8.5 ± 4.1, 14.7 ± 4.6, 13.8 ± 6.0, p<0.010) were observed.

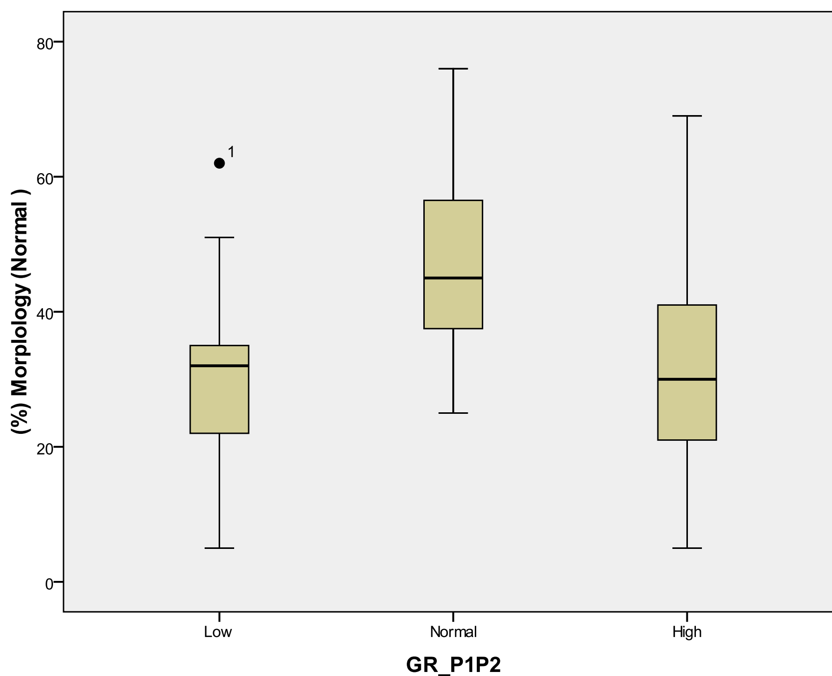


Figure 37. Box-plots showing the mean, median, and range percentages of the morphologically normal spermatozoa compared between the P1/P2 ratios groups (low, normal, and high) for all participant samples. A significant differences between normal with low and high groups of P1/P2 ratios were observed (48.1 ± 13.0, 33.4 ± 11.9, 31.7 ± 14.0, p<0.010).

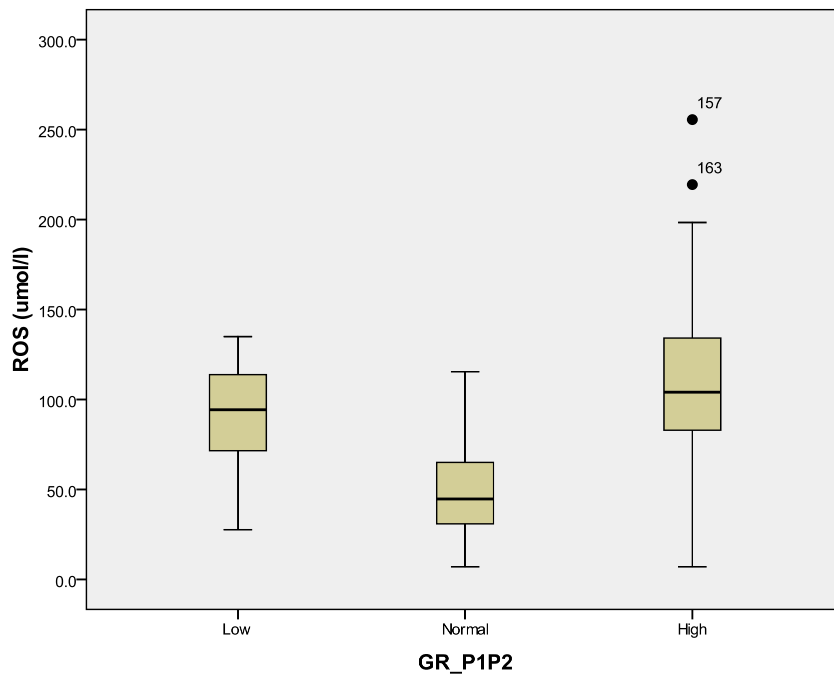


Figure 38. Box-plots showing the mean, median, and range of the ROS (µmol/l) concentrations in seminal plasma compared between the P1/P2 ratios groups (low, normal, and high) for all participant samples. A significant differences between normal with low and high groups of P1/P2 ratios were observed (49.80 ± 26.10, 87.05 ± 33.00, 106.96 ±52.87, p<0.010).

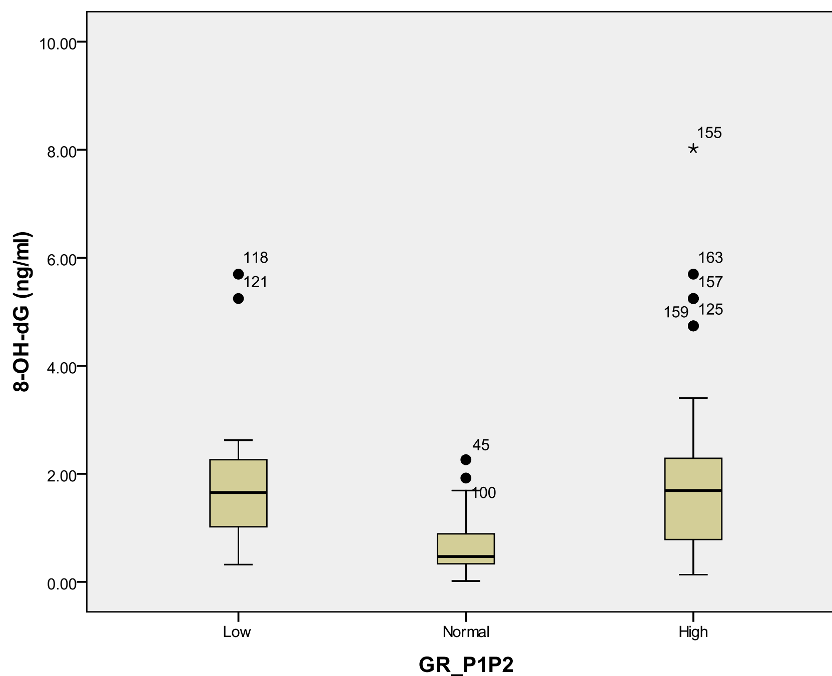


Figure 39. Box-plots showing the mean, median, and range of the 8-OHdG (ng/ml) concentrations in seminal plasma compared between the P1/P2 ratios groups (low, normal, and high) for all participant samples. A significant differences between normal with low and high groups of P1/P2 ratios were observed (0.70 ± 0.55, 2.03 ± 1.63, 1.95 ± 1.57, p<0.010).

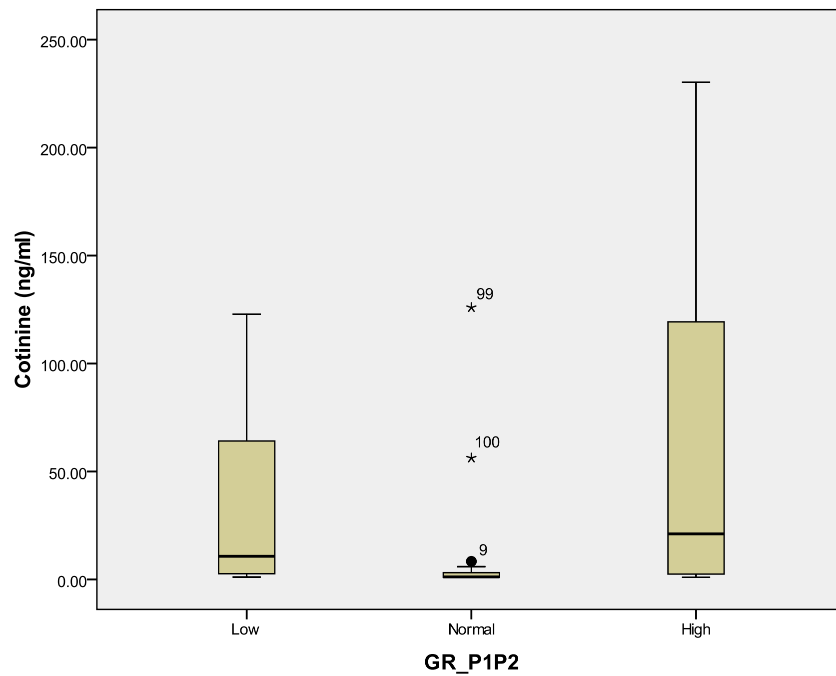


Figure 40. Box-plots showing the mean, median, and range of the Cotinine (ng/ml) concentrations in seminal plasma compared between the P1/P2 ratios groups (low, normal, and high) for all participant samples. A significant differences between normal with low and high groups of P1/P2 ratios were found (7.44 ± 23.34, 36.11 ± 43.41, 60.30 ± 65.06, $p<0.010$).

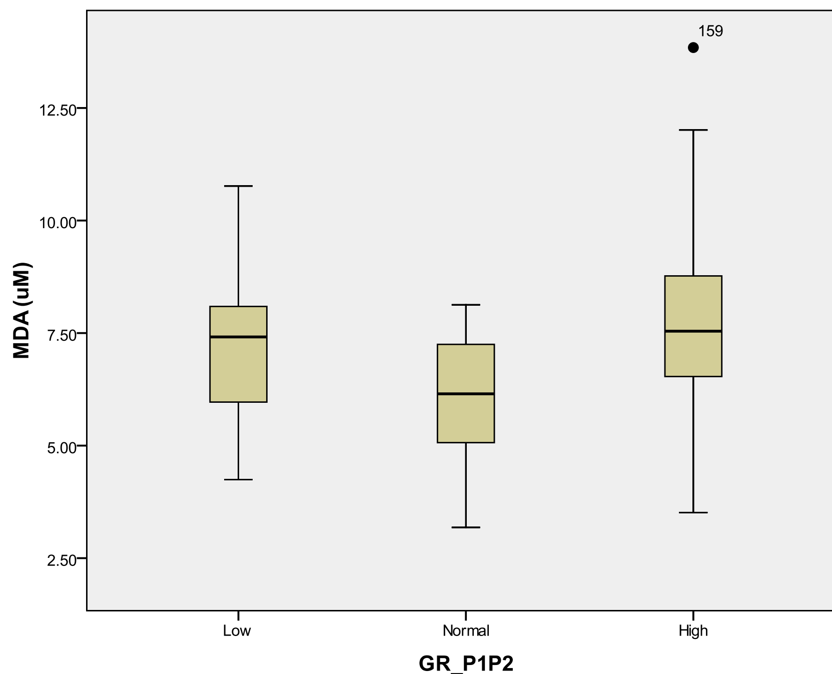


Figure 41. Box-plots showing the mean, median, and range of the MDA (µM) concentrations in seminal plasma compared between the P1/P2 ratios groups (low, normal, and high) for all participant samples. A significant differences between normal with low and high groups of P1/P2 ratios were detected (6.31 ± 1.26, 7.17 ± 1.66, 7.76 ± 1.78, $p<0.010$).

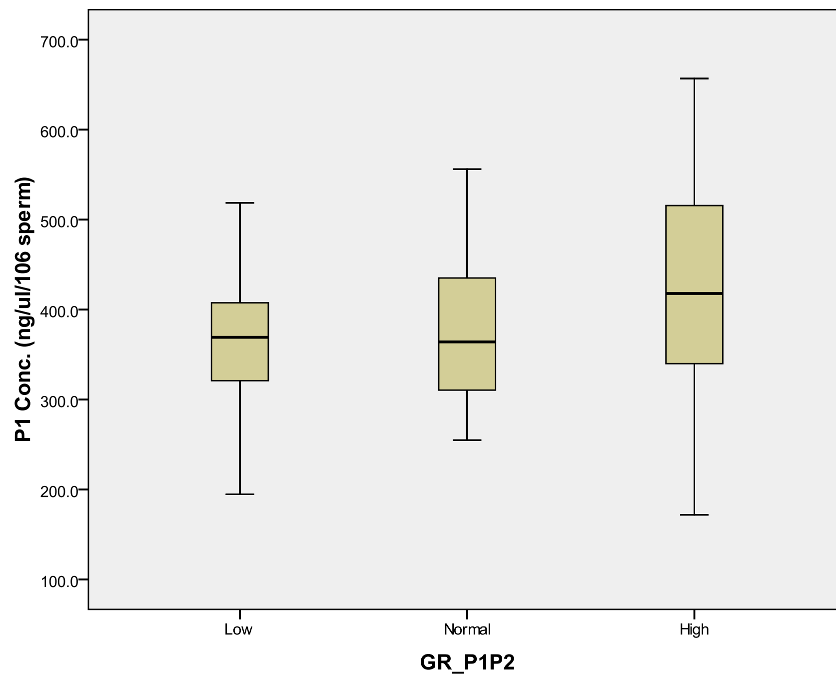


Figure 42. Box-plots showing the mean, median, and range of the P1 (ng/10^6 sperm) concentrations of sperm compared between the P1/P2 ratios groups (low, normal, and high) for all participant samples. A significant difference between normal and high groups of P1/P2 ratios was found (373.14 ± 78.12, 424.13 ± 110.94, p<0.010).

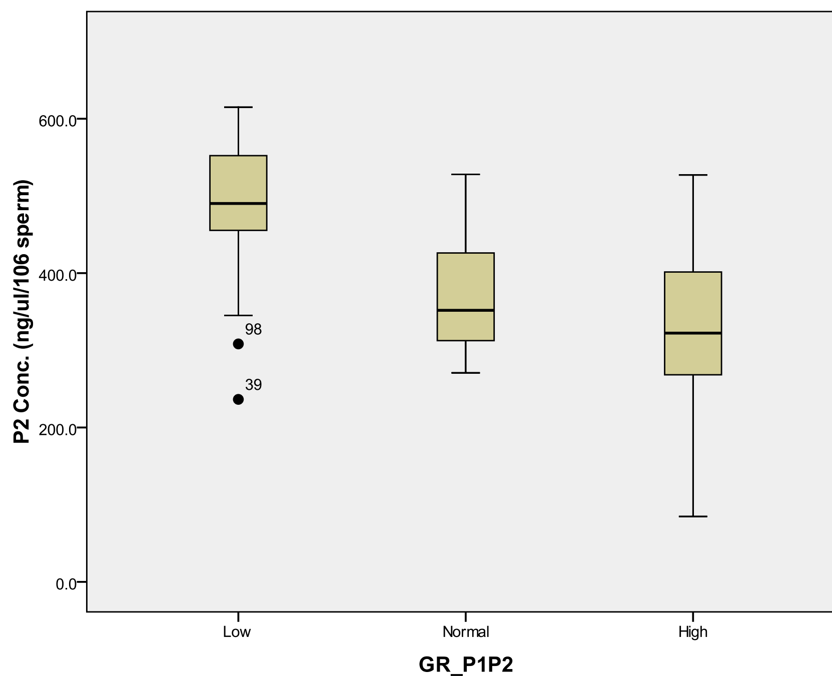


Figure 43. Box-plots showing the mean, median, and range of the P2 (ng/10^6 sperm) concentrations of sperm compared between the P1/P2 ratios groups (low, normal, and high) for all participant samples. A significant differences between the three groups of P1/P2 ratios were found (370.55 ± 71.07, 480.79 ± 91.55, 325.12 ± 95.72, p<0.010).

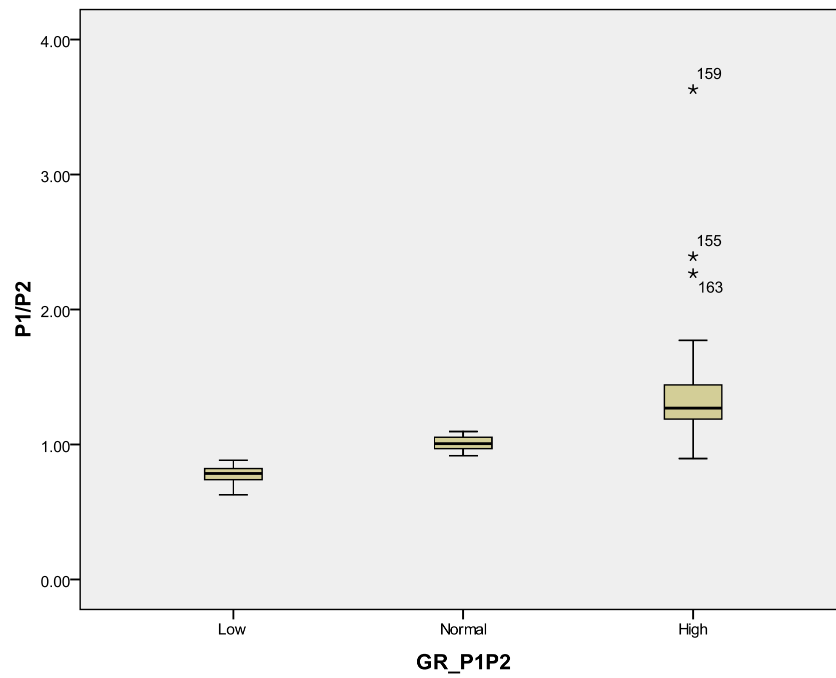


Figure 44. Box-plots showing the mean, median, and range of the P1/P2 ratios compared between the P1/P2 ratios groups (low, normal, and high) for all participant samples. A significant difference between the three groups of P1/P2 ratios were found (0.77 ± 0.07, 1.01 ± 0.05, 325.12 ± 95.72, $p<0.010$).

4-5- Clinical Data

27 women with average age 35.2 ± 4.4 years underwent ICSI therapy. The mean numbers of retrieved, fertilized, and transferred oocytes were 8.0 ± 4.4, 4.6 ± 3.9, and 2.2 ± 0.8 respectively. The rate of fertilization was 57.4 ± 26.9 %, the rate of pregnancy per embryo was 10.34 % (no. of pregnancies divided by no. of transferred embryos, 6/58), and the ongoing pregnancy rate was 22.22%.

Table 30. Correlation coefficient of semen and sperm parameters of all participant samples with fertilization rate and pregnancy of women underwent ICSI (n=27)

		Fertilization (%)	Pregnancy
Volume (ml)	r =	0.063	0.295
	p =	0.754	0.135
pH	r =	0.113	0.298
	p =	0.573	0.131
Count (mill/ml)	r =	0.509**	-0.300
	p =	0.007	0.128
Motility (% motile)	r =	0.275	0.275
	p =	0.165	0.165
Sperm vitality (Eosin) (%)	r =	0.267	0.110
	p =	0.178	0.586
Membrane integrity (HOS) (%)	r =	0.140	0.191
	p =	0.488	0.340
Leukocytes %	r =	0.292	0.000
	p =	0.139	1.000
Chromatin condensation (positive CMA_3) (%)	r =	-0.450*	0.160
	p =	0.019	0.425
DNA Fragmentation (positive TUNEL) (%)	r =	0.055	0.080
	p =	0.784	0.691
Morphologically Normal spermatozoa (%)	r =	-0.044	-0.084
	p =	0.892	0.796
Malondialdehyde (MDA) (μM) (N=12)	r =	-0.234	0.168
	p =	0.464	0.603
Reactive oxygen species (ROS) (μmol/l (N=12)	r =	-0.208	0.000
	p =	0.516	1.000
Cotinine (ng/ml) (N=12)	r =	-0.138	-0.028
	p =	0.669	0.931
8-hydroxy-2-deoxyguanisine (8-OH-dG) (ng/ml) (N=12)	r =	-0.164	-0.038
	p =	0.413	0.911
Protamine 1 (ng/ 10^6 sperm)	r =	-0.154	0.194
	p =	0.433	0.331
Protamine 2 (ng/ 10^6 sperm)	r =	-0.231	-0.092
	p =	0.246	0.650
P1/P2 Ratios	r =	0.128	0.172
	p =	0.526	0.392
Fertilization rate (%)	r =	1.000	-0.241
	p =	.	0.225
Pregnancy rate	r =	-0.241	1.000
	p =	0.225	.

Table 30 illustrated the correlation coefficients of the fertilization rates (%) and pregnancy rates of the women underwent ICSI with male sperm and semen parameters. Fertilization rates showed a highly positive correlations with the concentration of sperms (r=0.509, p<0.010, Fig. 45), and negative significant correlation with non-condensed chromatin (r=-0.450, p<0.050, Fig. 46). A non-significant negative (p>0.050) correlations were observed with morphologically normal sperm,

oxidative biomarkers (MDA, ROS, 8-OHdG), cotinine P1, and P2. On the contrary, a positive correlations were detected with other parameters. Meanwhile, pregnancy rates showed negative but non-significant correlations with morphologically normal sperm, cotinine and 8-OHdG, whereas, positive but also non-significant correlations observed with other sperm and semen parameters.

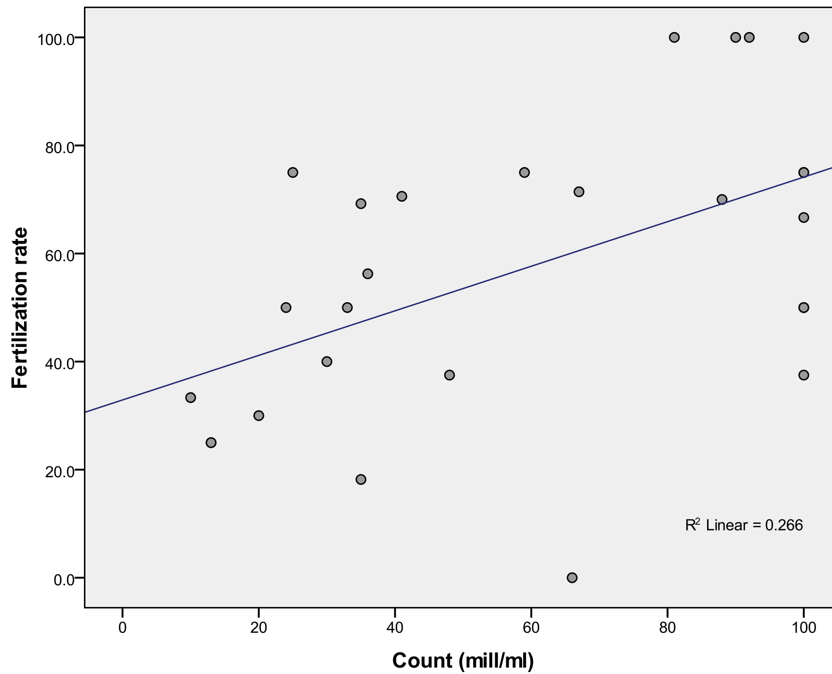


Figure 45: Scatter plot of correlation between the concentrations of sperms (mill. /ml) and the oocytes fertilization rate (%). A significant positive correlation was found (r=0.509, p<0.010).

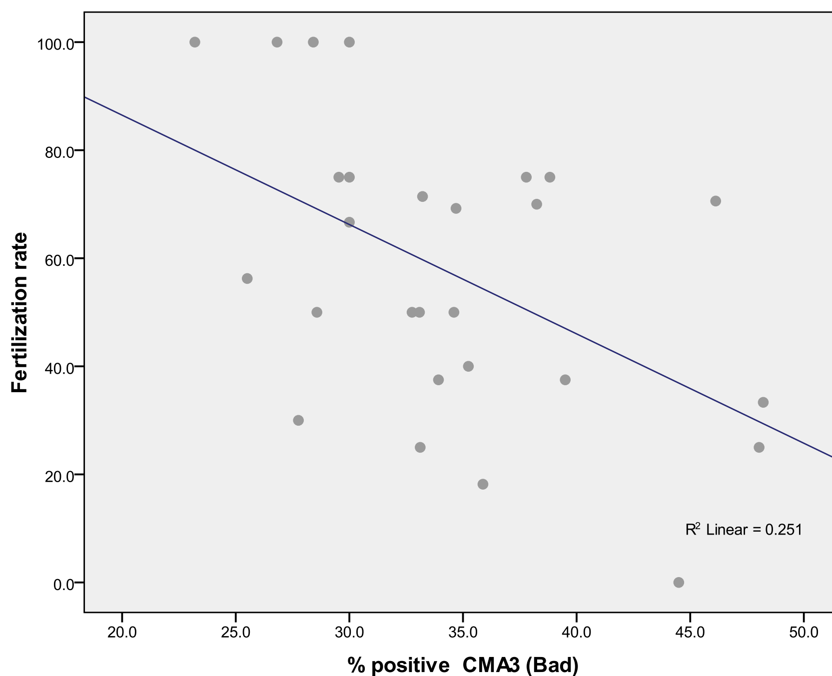


Figure 46: Scatter plot of correlation between mean percentage of sperm chromatin condensation (CMA_3) and the oocytes fertilization rate (%). A significant negative correlation was found (r=-0.450, p<0.010).

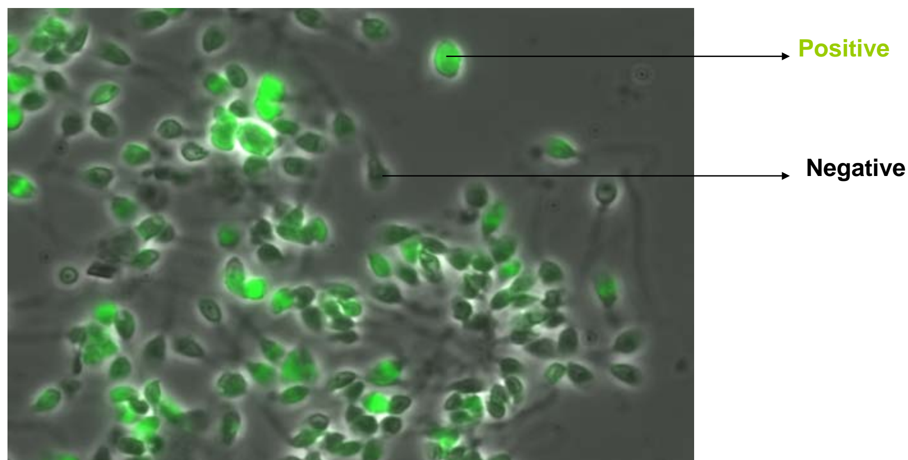


Figure 47: Fluorescence assay of sperm chromatin condensation using Chromomycin A_3 (CMA_3).
Condensed sperm chromatin showed dull green (good sperm) and Non-condensed sperm
chromatin showed bright green (bad sperm).

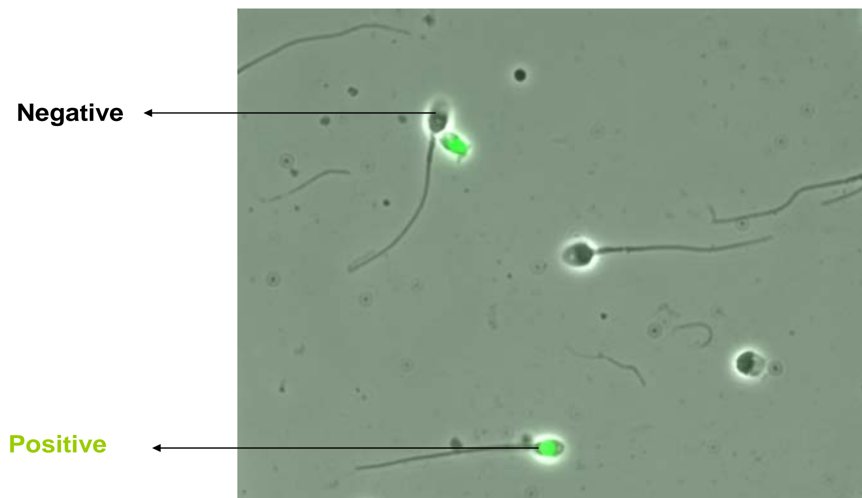


Figure 48: Fluorescence assay of sperm DNA fragmentation using TUNEL.
Sperms with fragmented DNA showed bright green and Sperms with non-fragmented DNA
Showed dull green.

5-Discussion

Male-factor infertility accounts for up to half of all cases of infertility (Moriss *et al.,* 2002) and affects one in twenty in the general population (McLachlan and de Kretser, 2001). Accumulating evidence now suggesting that reactive oxygen species (ROS) mediated damage to sperm is significantly contributing pathology in 30-80% of cases (Iwasaki and Gagnon, 1992; Shekarriz *et al.,* 1995; Agarwal *et al.,* 2006). Jones *et al.,* (1979) reported that ROS –induced peroxidation of sperm membrane decreases its flexibility and therefore tail motion. Besides direct ROS damage to mitochondrial, decreasing energy availability may impede sperm motility (de Laminarde *et al.,* 1994) resulting in less sperm reaching the oocytes for fertilization (Whittington *et al.,* 1999; Kao *et al.,* 2008).

Evaluation of male-factor infertility is becoming more important and informative as new diagnostic techniques and therapeutic options become available (Kao *et al.,* 2008). DNA damage may result from aberrant chromatin packaging during spermiogenesis (Zini and Libman, 2006), defective apoptosis before ejaculation (Moustafa *et al.,* 2004) or excessive production of ROS in the ejaculate (Aitken and Krausz, 2001). In addition, extra testicular factors such as age (Schmid *et al.,* 2007), drugs (Stahl *et al.,* 2006), cigarette smoking (Gaur *et al.,* 2007), genital tract inflammation (Ozmen *et al.,* 2007), hormonal factors (Ozmen *et al.,* 2007), varicocele and testicular hyperthermia (Naser-Esfahani *et al.,* 2009) may be reasons for DNA damage.

In addition ROS have the ability to directly damage sperm DNA by attacking the purine and pyrimidine bases and the deoxyribose backbone. Infertile men often exhibit deficit protamination, leaving the sperm DNA particularly vulnerable to ROS attack (Oliva, 2006).

Several investigators (reviewed by Tremellen *et al.,* 2008) have now found a link between oxidative stress and sperm DNA damage using various techniques such as sperm chromatin structure assay (SCSA), terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) and 8-hydroxydeoyguanosine (8-OHdG). Smoking results in a 48% increase in seminal plasma leukocytes concentration and a 107% increase in seminal ROS levels (Saleh *et al.,* 2002a), and decreasing seminal plasma antioxidants (Fraga *et al.,* 1996; Mostafa *et al.,* 2006), placing their sperm at additional risk of oxidative damage. In the present study, the link between smoking and oxidative stress and their effect on DNA strand breaks, DNA protamination, and other sperm parameters were analyzed. In addition, the consequences of these parameters on the ICSI results were investigated.

5-1- Sperm DNA Integrity and Semen Parameters (CMA_3 and TUNEL)

While male infertility has been traditionally evaluated by sperm density, motility and morphology, sperm DNA quality has been recognized as one of the most important determinants of male reproductive potential (Lewis and Aitken, 2005; Ozmen *et al.,* 2007; Tarozzi *et al.,* 2007). The analysis of sperm DNA integrity is being used with increasing frequency as independent parameters of semen quality as it offers more accurate diagnostic and prognostic information than the traditional parameters of semen evaluation. There is now some evidence to suggest that markers of sperm DNA integrity may help differentiate fertile from infertile men, but the clinical value of sperm DNA integrity testing remains to be defined (Spano *et al.,* 2000; Zini *et al.,* 2001). Different studies reported either weak or no correlation between conventional semen parameters and sperm DNA damage. Other studies reported that spermatozoa from patients with abnormal sperm count, morphology and motility showed an increase of DNA damage (Erenpreiss *et al.,* 2006). A substantial number of researchers have reported a negative relationship between the degree of DNA damage and the fertilization rate, embryo cleavage rate, implantation rate, pregnancy rate, and live birth of rate of offspring (Host *et al.,* 2000a; Morris *et al.,* 2002; Benchaib *et al.,* 2003; Agarwal and Allamaneini, 2004; Agarwal *et al.,* 2005). Recently a meta-study carried by Evenson and Wixon, 2008 have now confirm the link between sperm DNA damage and failure to achieve natural

conception. However, a few researchers showed no correlations between sperm DNA damage and pregnancy (Gandini *et al.,* 2004; Huang *et al.,* 2005).

The spermatozoa of subfertile males revealed structural changes in their DNA organization such as epigenetic alterations, single or double DNA strand breaks, wrong number of chromosomes and/or chromosome Y microdeletions (reviewed by Seli and Sakkas, 2005).

In particular, abnormalities in protamination make the sperm DNA susceptible to DNA damage especially by oxidative stress (Ozmen *et al.,* 2007). Evidence has been presented suggesting that defects in protamination may therefore be a cause of sperm DNA damage (Naser-Esfahani *et al.,* 2005; Aoki *et al.,* 2006b; Torregrosa *et al.,* 2006; Carrell *et al.,* 2007; Tarrozi., *et al.,* 2009). Both sperm DNA damage and protamine deficiency are related to infertility (reviewed by Oliva, 2006).

However, DNA damage occurred under the effect of endogenous or exogenous factors. During late spermiogenesis, DNA nicks and ligations are induced by the topoisomerase II (topo II) in order to facilitate protamination (McPherson and Longo, 1993a, b). Marcon and Boissoneault (2004) have suggested that DNA damage may be the result of incorrect repair of transient DNA nicks that are introduced during spermiogenesis (the nicks are introduced to relieve the torsional strain on the DNA helix during the exchange of histones to protamines). A second mechanism proposed for DNA damage is DNA nicks through apoptosis (Gorczyca *et al.,* 1993).

Protamine deficiency occurred during late stages of spermiogenesis may decrease the fertilization rate (Manicardi *et al.,* 1995). Histone-protamine exchange is a late spermiogenesis event, along with acrosome formation, membrane remodeling and other significant morphological and biochemical events that are necessary for normal sperm function (Nasr-Esfahani *et al.,* 2004a). Premature chromosomal condensation in spermatozoa, induced by protamine deficiency may be another cause for decreased fertilization rate in protamine-deficient spermatozoa after ICSI. The percentage of premature chromosomal condensation has been reported to be higher in semen samples with high CMA_3 positivity compared to those with low CMA_3 positivity (Nasr-Esfahani *et al.,* 2004b). Moreover, around 60% of the variations in DNA damage can be accounted for by the quality of protamination as measured by chromomycin CMA_3 (Aitken, 2007)

Evaluation of protamine deficiency by chromomycin A_3 (CMA_3), and evaluating of DNA fragmentation by TUNEL had been applied to configure the relationship between protamine and DNA integrity. A correlation between under-expression of protamines, DNA damage and lack of viability was detected using many techniques (TUNEL assay, CMA_3 and Aniline Blue staining assays) (Manicardi *et al.,* 1995; Hammadeh *et al.,* 2001; Aoki *et al.,* 2006b).

Moreover, abnormal sperm chromatin packaging evaluated by CMA_3 staining has been correlated with the presence of DNA damage, decreased sperm penetration, the absence of sperm decondensation within the oocyte and IVF and ICSI failure (Esterhuizen *et al.,* 2002; Razavi *et al.,* 2003). It also reported that a sperm DNA fragmentation index (DFI) (predictive threshold of 27 %, detected by SCSA), is necessary to obtain a successful pregnancy both by IVF and ICSI (Larson *et al.,* 2000; Larson-Cook *et al.,* 2003).

In the current study, significant differences were found between the normal volunteers and patients samples regarding the non-condensed chromatin (23.3 ± 7.0 vs. 32.9 ± 8.2, $p<0.010$), and DNA damage (9.6 ± 5.4 vs. 14.2 ± 5.6, $p<0.010$). Besides, abnormal protamination evaluated by chromatin condensation test for all participant samples showed significant negative correlations ($p<0.010$) with sperm concentration, motility, vitality, membrane integrity and morphology (Fig. 9, Table 10a). Moreover a significant positive correlation was observed between non-condensed chromatin (CMA_3–assay) and sperm DNA fragmentation, as assessed by TUNEL assay ($p< 0.010$, Fig. 8). There was also a negative correlation between TUNEL and other sperm parameters; motility, vitality, membrane integrity, and morphologically normal sperm, and sperm concentration (Table 10a).

In volunteers samples (Table 12a), chromatin condensation was negatively correlated with sperm concentrations (r=-0.138, p>0.050), sperm motility (r=-0.563, p<0.010), morphologically normal sperm (r=-0.469, p<0.010, Fig 21), sperm vitality (r=-0.272, p<0.050) and membrane integrity (r=-0.314, p<0.050). Besides, DNA fragmentation was correlated negatively with sperm concentration and vitality (r=-.011; r=-0.147, p>0.050), and showed significantly negative correlation with membrane integrity (r=-0.289, p<0.050), sperm motility (r=-0.514, p<0.010) and morphologically normal sperm (r=-0.560, p<0.010). Figure (20) showed significantly positive correlation between non-condensed chromatin and DNA fragmentation (r=598, p<0.010).

Similarly, patients samples analysis (Table 13a) of both chromatin condensation (CMA_3) and DNA fragmentation (Tunel) showed significantly negative correlations with other sperm parameters; concentration, vitality, motility, membrane integrity and morphologically normal sperm. Whereas, non-condensed chromatin was significantly positive with DNA fragmentation (r=213, p<0.050).

Results in this study are in accordance with that of Tarrozi *et al.,* (2009), who found significant negative correlations (p<0.050) between CMA_3 positivity and sperm concentration, motility, and morphology. They showed a significant positive correlation between CMA_3 positivity and sperm DNA fragmentation. The present data are in accordance with results of other previous studies analyzing the relationship between abnormal protamination and traditional sperm parameters (Carrell and Liu, 2001; Mengual *et al.,* 2003; Aoki *et al.,* 2005a; Torregrosa *et al.,* 2006; Borini *et al.,* 2006; Tarrozi., *et al.,* 2009), and between under-protamination and sperm DNA fragmentation (Manicardi *et al.,* 1995; Aoki *et al.,* 2005b, 2006b; Torregrosa *et al.,* 2006; Tarrozi., *et al.,* 2009). However, a connection between abnormal protamination and diminished semen quality parameters is reasonable depending on whether the regulation of protamine exchange is linked to a broader control of spermatogenesis (Carrell *et al.,* 2007). Two main hypotheses had been discussed by Carrell and colleagues about this topic: (i) protamines may act as a ‘checkpoint’ during spermatogenesis, and abnormal protamine expression leads to an increased level of apoptosis that results in diminished semen quality; or (ii) abnormal protamine expression may be indicative of a general alteration of spermatogenesis, possibly due to an abnormal function of a transcriptional or translational regulator. The strong link between abnormal protamination and sperm DNA fragmentation can also account for the important function of protamines in the protection of the paternal genome from chemical and physical damage, conferring a high level of compactness to the sperm nucleus (Barone *et al.,* 1994; Braun, 2001); so a potential consequence of under-protamination may be the greater susceptibility to DNA damage.

Moreover, this study demonstrated that chromatin condensation (CMA_3 positivity) showed significantly negative correlation with fertilization rates (p<0.050, Fig. 46). Therefore, the influence of sperm protamine abnormality seems to be a good prognostic tool for predicting ICSI fertilization. The concentrations of P1, and P2 (Table 21) also showed negative correlation but were non-significant with the fertilization rate. This result indicates that abnormal protamination effects on assisted reproduction outcomes are related to the effect of protamines on spermiogenesis. Carrell *et al.,* also postulated that the selection of morphologically normal sperm for treatment decreases the risk of using a sperm with less condense chromatin (Carrell *et al.,* 2007). However, these results are related to that of previous studies using the same kind of protamine detection (Carrell and Liu, 2001; Aoki *et al.,* 2005b).

The recommended values of sperm DNA fragmentation to discriminate between good and poor prognosis patients show a high degree of variability (reviewed in Tesarik *et al.,* 2006). The data of sperm DNA fragmentation assay (TUNEL) correlated with fertilization rate and pregnancy rate. This finding indicated that DNA fragmentation test is not a prognostic tool for predicting ICSI fertilization, which may be due to the low number of cases. This result is in conflict with some published data (Saleh *et al.,* 2003a; Boe-Hansen *et al.,* 2006; Evenson and Wixon, 2006a), but agreed with others who found no relationship between DNA fragmentation and reproductive outcome in either IVF or ICSI patients (Gandini *et al.,* 2004; Huang *et al.,* 2005). Also similar are the results of Borini *et al.* (2006), and Tarrozi., *et al,* (2009), who found that a DNA fragmentation

test is not a prognostic tool for IVF outcome. To overcome these conflicted findings, it is important to consider the following factors; the techniques used applied for DNA fragmentation tests (SCSA, TUNEL, comet assay, or in-situ nick translation), the testing models applied (microscopy or flow cytometry), type of samples (treated or raw samples), or others. For example; the predictive value of DNA damage test decreases when applying the test after treating the cell by density gradient centrifugation (Sakkas *et al.,* 2000; Tomlinson *et al.,* 2001; O'Connell *et al.,* 2003; Seli and Sakkas, 2005). The effect of DNA damage on the reproductive outcomes affected by other variables like the status of both male and female (maternal age, presence of pathological conditions, ability of the oocytes to repair the sperm DNA damage, which is related to maternal age) (Menezo, 2006). The small but statistically significant association between sperm DNA integrity test results and pregnancy in IVF and ICSI cycles is not strong enough to provide a clinical indication for routine use of these tests in infertility evaluation of men. It is possible that yet to be determined subgroups of infertile couples may benefit from sperm DNA integrity testing (Collins *et al.,* 2008).

Sperm DNA fragmentation is affected by many factors; it may be a result of oxidative stress resulted from high concentration of reactive oxygen species produced by the sperm mitochondria, leukocytes and or others. Abortive apoptosis also may affect (Aitken and De Iuliis, 2007).

5-2-Oxidative stress

Reactive oxygen species (ROS) are produced by the defective spermatozoa, sperm mitochondria and leukocytes that protect against pathogenic germs (Vernet *et al.,* 2001; Zini and Libman, 2006). Other ROS-generating systems are the enzymes nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX5) that is potentially capable of generating ROS in the presence of calcium and NADPH (Aitken *et al.,* 1997; Banfi *et al.,* 2001), lipoxygenase (Oliw and Sprecher, 1989), tumor-associated cell surface NADH oxidase (tNOX) (Morré and Morré, 2003) and cytochrome P450 reductase (Baker *et al.,* 2004).

Recently, the over-production of ROS in the male reproductive tract has become a real concern. Oxidative stress is resulted from an imbalance between the productions of ROS and the antioxidant capacity (Sharma and Agarwal, 1996). Oxidative damage to spermatozoa, induced by excessive production of free oxidants or impairment of the native antioxidant mechanisms, has been identified as a parameter of male subfertility (Sikka *et al.,* 1995). Many studies showed that oxidative stress appears to be the major cause of DNA damage in the male germ line (Saleh *et al.,* 2003a; Aitken *et al.,* 2003a). However, physiological levels of ROS are necessary for protein activities and for sperm proliferation, differentiation and function (Gagnon *et al.,* 1991; Aitken, 1999). H_2O_2 and $^{\cdot}O_2^{-}$ promote sperm capacitation and acrosome reaction, as well as hyperactivation and oocyte fusion (de Lamirande and Gagnon, 1995; Zini *et al.,* 1996). Besides, hydrogen peroxide and superoxide anion are necessary for controlling the tyrosine phosphorylation events, which is important for sperm capacitation (Aitken *et al.,* 1991; Aitken *et al.,* 1998a).

A strong positive correlation exists between immature spermatozoa and ROS production, which in turn negatively affects the sperm quality (Said *et al.,* 2004). The excessive production of ROS that exceeds critical levels can overwhelm all the antioxidant defense systems of spermatozoa and seminal plasma causing oxidative stress (de Lamirande *et al.,* 1997).

In addition, ROS generation by defective spermatozoa can be increased by the removal of seminal plasma, which has endogenous antioxidant mechanisms during sperm preparation for ART. Centrifugation of sperm during preparation also plays a role in rapid production of ROS (Gagnon *et al.,* 1991) and may enhance the generation of ROS 2-5 fold above baseline, within 5 minutes, which can damage sperm membrane and DNA. Oxidative stress has also been correlated with high frequencies of single and double DNA strand breaks (Twigg *et al.,* 1998a: b; Aitken and Krausz, 2001). Sperm are particularly susceptible to oxidative stress due to the high content of unsaturated fatty acids in their membranes, as well as their limited stores of antioxidant enzymes (Bakera and Aitken, 2005).

Tight DNA packaging and high levels of antioxidants present in seminal plasma protects the sperm from oxidative stress (Twigg *et al.,* 1998b). Infertile men with poor sperm motility and morphology have increased DNA fragmentation compared with individuals with normal semen parameters (Lopes *et al.,* 1998; Irvine *et al.,* 2000; Zini *et al.,* 2001). Oxidative stress is suggested as the main factor responsible for DNA damage in ejaculated sperm (Sakkas *et al.,* 1999b; Greco *et al.,* 2005; Evenson and Wixon, 2006a). Sperm DNA damage has been associated with high levels of ROS (approximately 25% of infertile men have high levels of semen ROS (Zini *et al.,* 1993; Irvine *et al.,* 2000).

5-2-1- Oxidative Stress and Semen Parameters

ROS are related to poor sperm parameters. Head of the sperm is rich in polyunsaturated fatty acids that make up approximately 40 % of the lipids in the sperm head, which are important for membrane fluidity, sperm motility, capacitation, and sperm binding to the egg zona pellucida. These polyunsaturated fatty acids are extremely susceptible to oxidative damage. Lipid peroxidation under the effect of high ROS concentrations will impair the motility of the sperm (Armstrong *et al.,* 1999).

Negative correlations were shown between ROS concentration in seminal plasma and sperm vitality, membrane integrity, sperm density, chromatin condensation, and DNA single stand breaks in both IVF and ICSI groups (Hammadeh *et al.,* 2008a). Furthermore, it has been shown that ROS can damage DNA by causing deletions, mutations, and other lethal genetic effects (Tominga *et al.,* 2004). Damage of the sperm DNA by free radicals induces activation of the poly (ADP ribose) synthetase enzyme. This splits NAD^+ to aid the repair of DNA. Depletion of the NAD^+ concentration will disrupt the function of the cells and cause cell death. The relative proportion of ROS–producing immature sperm was directly correlated with nuclear DNA damage value in mature sperm and inversely correlated with recovery of motile, mature sperm (Aitken *et al.,* 1998b).

The data from this study showed significant differences between the sperm parameters of normal volunteers and patients (Table 11). A sperm's concentrations (80.2 ± 27.5 vs. 61.4 ± 31.1, $p<0.010$), motility (37.1 ± 14.7% vs. 27.4 ± 12.0%, $p<0.010$), semen volume (3.9 ± 1.4 vs. 3.2 ± 1.7, $p<0.010$), sperm vitality (Eosin) (45.2 ± 16.9% vs. 34.7 ± 17.3%, $p<0.010$), sperm membrane integrity (HOS) (53.4 ± 15.8% vs. 45.1 ± 20.8%, $p<0.010$), the non-condensed chromatin (23.3 ± 7.0% vs. 32.9 ± 8.2%, $p<0.010$), DNA damage (9.6 ± 5.4% vs. 14.2 ± 5.6%, $p<0.010$), and morphologically normal sperm (45.9 ± 13.9% vs. 29.4 ± 12.7%, $p<0.010$). Furthermore, the seminal plasma concentrations of oxidative biomarkers MDA, ROS, Cotinine, and 8-OHdG of patients were higher than normal volunteers; MDA (6.45 ± 1.43 vs. 7.82 ± 1.79, $p<0.010$), ROS (67.6 ± 42.2 vs. 103.85 ± 5.68, $p<0.010$), cotinine (27.30 ± 40.17 vs. 53.42 ± 67.33, $p<0.050$), and 8-OHdG (1.15 ± 0.82 vs. 1.95 ± 1.75, $p<0.050$).

For both groups (volunteers and patients) (Table 10c) concentrations of oxidative biomarkers MDA, ROS, and 8-OHdG in seminal plasma showed significantly negative correlations ($p<0.010$) with sperm motility (r=-0.513, r=-0.537, r=-0.532), vitality (r=-0.309, r=-0. 298, r=-0.373), membrane integrity (r= -0.362, r= -0.343, r=-0.335), and morphologically normal sperm (r=-0.514, r=-0.547, r=-0.513). In contrast, concentrations of MDA, ROS, and 8-OHdG in seminal plasma showed significant positive correlations ($p<0.010$) with non-condensed chromatin (r=0.555, 0.629, and 0.545, respectively), and sperm DNA fragmentation (r=0.624, r=0.630, r=0.600, respectively).

The data of normal volunteers group (Table 12c) demonstrated that the mean percentage value of sperm motility showed significantly negative correlation ($p<0.010$) with seminal plasma concentrations of MDA (r=-0.580), ROS (r=-0.632), and 8-OHdG (r=-667). A significant negative correlation was found between 8-OHdG with sperm vitality (r=-0.283, $p<0.050$), and sperm membrane integrity (r=-0.303, $p<0.050$). A low significance correlation noticed between sperm vitality and ROS (r=-0.248, $p=0.083$). Whereas, positive correlations ($p<0.010$) were found

between the MDA, ROS, and 8-OHdG with non-chromatin condensation (r=0.527, 0.636, 0.588 respectively) and with DNA fragmentation (r=0.536, 0.577, and 0.694 respectively).

In the patients groups (Table 13c), the motility of the sperm was significantly decreased as the concentration of oxidative biomarkers MDA, ROS and 8-OHdG increase. An inverse significant correlation was found between sperm vitality and concentration of 8-OH-dG ($p<0.050$). Also, sperm membrane integrity significantly decreased ($p<0.010$) as concentration of MDA, ROS, and 8-OHdG increased. Whereas, significant positive correlations ($p<0.010$) were found between MDA, ROS, and 8-OHdG concentrations with non-condensed chromatin and DNA fragmentation.

Our study showed similarities with that conducted by Moein *et al.,* (2007). They showed that the level of ROS in seminal fluid of infertile men was significantly higher than fertile donors.

These results are in agreement with that of Hammadeh *et al.,* (2006), who studied the effect of ROS on seminal plasma and sperm parameters of male partners of patients who underwent IVF/ICSI therapy. They found that increase ROS concentration in seminal plasma correlated negatively with sperm vitality, morphology, DNA fragmentation, and sperm concentrations. Moreover, from the same group, they demonstrated a negative correlation between ROS concentration in seminal plasma and sperm vitality, membrane integrity, sperm density, chromatin condensation, and DNA single stand breaks in both IVF and ICSI groups (Hammadeh *et al.,* 2008a). Many other studies reported a correlation between sperm concentration and increased production of ROS in patients (Henkel and Schill, 1998). Also, these results are in accordance with those (Zorn *et al.,* 2003, Aziz *et al.,* 2004) who found that ROS concentration in seminal fluid was negatively correlated with progressive motility, and normal morphology.

It was reported that overproduction of ROS in semen of infertile males may cause abnormal and immature spermatozoal morphology, motility and concentration (Gyun and Edward *et al.,* 2006). Moreover, a negative correlation was found between levels of ROS production in semen and percentage of normal spermatozoa form (Ollero *et al.,* 2001). ROS causes a decrease in sperm motility as a result of lipid peroxidation and loss of membrane PUFA particularly docosahexanoic acid (Connor *et al.,* 1998). Lipid peroxidation plays a significant role in disrupting sperm functions and semen quality especially sperm motility and morphology and may account for some cases of male infertility.

Overproduction of ROS in semen, mainly by leukocytes and abnormal spermatozoa, especially high concentrations of hydrogen peroxide that induced lipid peroxidation and result in cell death could be a cause for infertility (de Lamirande and Gagnon, 1995). Also, increased ROS production by spermatozoa is associated with a decreased mitochondrial membrane potential (MMP). The patients with abnormal semen parameters had a significantly lower MMP (Wang *et al.,* 2003). The same group also indicated that oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male-factor infertility. High levels of cytochrome c, and caspases 9 and 3, had been detected in those patients.

The results related to lipid peroxidation (MDA) induced by ROS are in agreement with many previous studies (Geva *et al*. 1996; Fraczek *et al*. 2001). The same compatible results with that of Hsieh *et al., et al.,* (2006) showed that seminal MDA concentrations were negatively correlated with sperm concentration and motility, and might provide a simple and useful tool in predicting sperm parameters. In addition, Nabil *et al.,* (2008), found that ROS-induced lipid peroxidation induces significant sperm membrane damage and markedly influences sperm motility and morphology, and may account for male infertility.

These findings suggested that the lipid peroxidation effect (MDA activity), might compromise the sperm viability. So, increased MDA level might represent the pathologic lipid peroxidation of spermatozoa membrane and the following inhibition of sperm motility and viability (Hsieh *et al.,* 2006).

In addition, 8-OHdG concentration effects on semen and sperm parameters in our data are in convenience with that of Kao *et al.,* (2008) who found a significantly higher levels of 8-OHdG in spermatozoa with poor motility and with asthenozoospermia and oligospermia. Besides, a significant negative correlation was found between sperm motility and 8-OHdG.

Oxidized deoxynucleoside, 8-OHdG, in human sperm DNA had been detected as a biomarker of oxidative modification (Loft *et al.,* 2003). If not repaired, 8-OHdG modifications in DNA are mutagenic and may cause embryonic loss, malformations, or childhood cancers (Aitken and Baker, 2006). The mutagenic potential of 8-OHdG is reflected in its miscoding properties. Oxidative attack on dGTP creates 8-OHdGTP. This damaged 8-OHdGTP is incorporated at sites opposite to A, giving rise to A:T/G:C transversions (Loft *et al.,* 2003; Nakabeppu *et al.,* 2006)

In this study the oxidative stress biomarkers (MDA, ROS, and 8-OHdG) and fertilization rate showed a negative correlation ($r=-0.234$, $r=-0.208$, $r=-0.164$ respectively, $p>0.050$). These results indicated that oxidative stress inversely affects the quality of sperm that may affect the (concentration, motility, morphology, and DNA integrity), (discussed above) and hence its fertilization capacity, which in turn may lead to decrease pregnancy rate.

5-3- Smoking and infertility

Cigarette smoking become a serious health and social problem, and also presents challenging for the physicians. Smoking has been established as a number one preventable cause of death and diseases in the world (Zavos *et al.,* 1998). Cigarette smoke contains a large number of hazardous substances, including nicotine, carbon monoxide and recognized carcinogens and mutagens such as radioactive polonium, benzopyrene, dimethylbenzanthracene, dimethylnitrosamine, naphthalene and methanphthalene (Zavos *et al.,* 1998; Wong *et al.,* 2000). These substances may be found in seminal plasma of smokers via various modes of diffusion and active transport (Zavos *et al.,* 1998). Therefore, smoking negatively affects male fertility, but this relationship remains controversial.

Despite the high prevalence of cigarette smoking in the general population, our knowledge of its impact on male reproductive function is still very limited. In a meta-analysis, smoking was only found to have an adverse effect on sperm concentration and motility in healthy volunteers and sperm donors, but not in infertility patients (Vine *et al.,* 1994). Vine, (1996) suggested that the effects of cigarette smoking on sperm parameters would be more important in the general population than in infertile patients.

Moreover, some studies found that tobacco adversely affects sperm quality in variables such as concentration, motility and normal morphology (Handelsman *et al.,* 1984; Sofikitis *et al.,* 1995; Mak *et al.,* 2000). Other studies with many samples found no relationship between cigarette smoking and sperm quality, although they were conducted on men with fertility problems (Dikshit *et al.,* 1987; Trummer *et al.,* 2002). Several studies have observed that cigarette smoking has an effect on the semen quality, especially in those who are heavy smokers or who have been smoking for many years (Zahng *et al.,* 2000; Kunzle et *al.,* 2003).

5-3-1- Smoking and oxidative damage

5-3-1-1- Smoking and ROS

Smoking was associated with a 107% increase in reactive oxygen species (ROS) levels and a 10-point decrease in ROS-Total antioxidant capacity (ROS-TAC) scores (Saleh *et al.,* 2002a). A significant reduction of ROS-TAC scores associated with smoking can be attributed to a significant increase in seminal ROS levels, which is related to the significant increase in leukocyte concentration in the semen of infertile smokers (Close *et al.,* 1990).

Some studies have reported that the association between man smoking and semen quality was

stronger in healthy men than in the infertile population (Vine 1996; Zinaman et *al.,* 2000). It should be emphasized that cigarette smoke has been associated with increased frequency of aneuploidy in sperm (Twigg *et al.,* 1998a, b), lower seminal plasma antioxidant levels and increased oxidative damage to DNA (Fraga *et al.,* 1996; Shen *et al.,* 1997). Agarwal *et al.,* (2006) reported that high ROS may serve as a marker of male-factor infertility. Furthermore, it has been shown that ROS can damage DNA by causing deletions, mutations, and other lethal genetic effects (Tominga *et al.,* 2004).

The results from this work showed that the ROS concentrations in seminal plasma of all smokers (n=72), volunteers smokers (n=19), and patients (n=53) smokers (128.75 ± 39.46, 111.82 ± 28.66, 138.49 ± 41.85) were significantly higher (p<0.010) than that in non-smokers (52.89 ± 28.48, 40.47 ± 20.45, 65.74 ± 30.16). The concentrations of ROS in all participants samples were negatively correlated with other sperm parameters (Table 10c); semen volume (r=-0.176, p=0.062), sperm's concentration (r=-0.133, p=0.160) motility (r= -0.537, p<0.010), vitality (r=-0.298, p<0.010), membrane integrity (r=-0.343, p<0.010) and morphologically normal sperm (r=-0.547, p<0.010), whereas, positive correlations (p<0.010) were observed with sperm non-condensed chromatin and DNA damage (r=0.629, r=0.630 respectively).

Similarly high levels of seminal oxidative stress have been correlated with sperm impairment of sperm metabolism, motility, and fertilizing capacity (Armstrong *et al.,* 1998).

Several mechanisms had been proposed to explain the effect of smoking on semen quality. Oxidative stress induced ROS that has destructive effects on sperm quality and function. Smoking may increase oxidative stress by: (i) Smoking itself contains high levels of ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydrogen radicals ($OH^\bullet$) (Saleh *et al.,* 2002a; Kunzle *et al.,* (2003). H_2O_2 directly affects sperm functions critical at fertilization process in a dose- and time-dependent fashion. Low concentrations maintain capacitation; whereas, high concentrations have deleterious effects, as determined by the end points of the capacitation process. These effects are probably dependent on modifications of plasma membrane and intracellular homeostasis by the oxidative process (Oehninger *et al.,* 1995), (ii) smoking metabolites may induce an inflammatory reaction in the male reproductive tract that cause formation of leukocytes that can generate high levels of ROS (Saleh *et al.,* 2002a, b). Oxidative stress induced by leukocytes had a damage effect on the polyunsaturated fatty acids of sperm phospholipids which may result, among the other effects, in decreased membrane fluidity (Zalata *et al.,* 1998), (iii) smoking metabolites may affect spermatogenesis. Excess of free radical generation frequently involves an error in spermiogenesis resulting in the release of spermatozoa from the germinal epithelium exhibiting abnormally high levels of cytoplasmic retention. Redundant cytoplasm contains enzymes that fuel further generation of ROS by the spermatozoa's plasma membrane redox systems (Saleh *et al.,* 2002a; Aitken and Sawyer 2003).

ROS cause oxidative damage to normal sperm DNA, proteins and lipids that may be related to sperm abnormalities (Aitken and Baker, 2006). High levels of polyunsaturated fatty acids in the plasma membrane of the sperm and the low level of antioxidants in its cytoplasm make it susceptible to damage by ROS impaired motility of the sperm (Agarwal and Prabakaran, 2005; Aitken and Baker, 2006). Sperm DNA damage also induced by excessive ROS may enhance sperm germ cells apoptosis causing decrease in the concentration of sperms (Agarwal and Allamaneni, 2004). Studies correlated ROS with impaired motility (Agarwal *et al.,* 1994a, b; Armstrong *et al.,* 1999; Agarwal and Allamaneni, 2004). Many studies proposed that the effect of smoking on sperm parameters may have had a dose-dependent effect, and high levels of cigarette smoking were positively related to decreased sperm parameter quality (Wang *et al.,* 2001; Martini *et al.,* 2004; Pasqualotto *et al.,* 2004; Colagar *et al.,* 2007). This might be due to the toxic components of smoke.

Reactive oxygen species status may be used as an important indicator for clinical evaluation and treatment of male-factor infertility. Recently, Agarwal *et al.,* (2006) reported that high ROS may serve as a marker of male-factor infertility.

5-3-1-2- Cotinine and sperm parameters

Cotinine has been reported to have pharmacological activity. It interferes with the release of brain neurotransmitters and affects enzymes involved in the synthesis of estrogen and testosterone, decreases vascular resistance and blood pressure in animals, and has been reported to have effects on cognitive performance and to modify nicotine withdrawal symptoms in abstinent smokers (Zevin *et al.,* 2000).

Cotinine is the major metabolite of nicotine, which is the major psychoactive substance found in cigarette smoke (Zenzes *et al.,* 1996). Cotinine had been used as a specific biomarker of cigarette smoking (Hulka, 1991). Besides, cotinine with a half-life in sperm of 5–7 days is a better indicator of long-term exposure to cigarette smoke than urinary metabolites index (Jarvis *et al.,* 1988). Cotinine may decrease male fertility by inhibiting density, reducing total progressively motile sperm count, and increasing the percentage of sperm with abnormal morphology (Chen and Kuo 2007). A previous research found nicotine and cotinine in the seminal plasma of smokers and suggested that other harmful components of tobacco smoke would pass through the blood–testis barrier (Vine *et al.,* 1993).

In the current study, seminal plasma cotinine levels were found to be significantly higher ($p<0.010$) in smokers than non-smokers for all participants (88.22 ± 58.05 vs. 2.34 ± 2.20), volunteers (68.55 ± 38.58 vs. 2.02 ± 2.03), and patients (99.55 ± 64.58, 2.68 ± 2.36).

Furthermore, in all participants samples (Table 10c) an inverse correlations were detected between concentrations of cotinine with other sperm parameters; semen volume ($r=-0.151$, $p=0.110$), sperm's concentration ($r=-0.137$, $p=0.148$), motility ($r=-0.489$, $p<0.010$), vitality ($r=-0.322$, $p<0.010$), membrane integrity ($r=-0.555$, $p<0.010$) and morphologically normal sperm ($r=-0.555$, $p<0.010$), whereas, a significant positive correlations ($p<0.010$) were observed with sperm non-condensed chromatin and DNA damage ($r=0.549$, $r=0.629$ respectively). These results demonstrate that cigarette smoking is the main contributor to semen cotinine.

These findings are in according with that of Chen and Kuo (2007). They demonstrated a relation between semen cotinine concentrations and sperm quality that was also consistent with other reports. Pacifici *et al.,* (1993) found that total motility of spermatozoa was showed significantly negative correlation with concentrations of cotinine and hydroxycotinine. Zavos *et al.,* (1998) found that smoking affected sperm viability and had a strong detrimental effect on motility of spermatozoa. Besides, Wong *et al.,* (2000) found a negative correlation between cotinine concentration and morphologically normal sperm. In addition, cotinine concentration impaired sperm motility, membrane function, and their ability to undergo capacitation (Sofikitis *et al.,* 1995). These results also in agreement with previous work of Hammadeh *et al.* (2008b).

Moreover, in this study, negative correlations were recorded between cotinine concentrations and fertilization and pregnancy rates ($r=-0.164$, $r=-0.028$ respectively, $p>0.050$). This result demonstrated an inverse effect of cotinine on the quality of sperm (concentration, motility, morphology, and DNA integrity), discussed above, that will affect the fertilization pregnacy rates. These results also consistent with the work of Hammadeh *et al.* (2008b).

5-3-1-3- Smoking cigarette and MDA levels

Peroxidation of polyunsaturated fatty acids has been implicated in a wide variety of pathological conditions including infertility, cardiac and cerebral ischaemic-reperfusion injury, and inflammatory joint diseases amongst others (Sanocka and Korpisz, 2004). The human sperm cell membrane is particularly susceptible to oxidation by ROS and other oxidative agents such as smoking metabolities due to the existence of high concentration of polyunsaturated fatty acids (PUFA) in these membranes (Tavilani *et al.,* 2005; Cocuzza *et al.,* 2007). Lipid peroxidation damage of the plasma membrane of spermatozoa plays an important role in the mechanism of male

infertility (Khosrowbeygi and Zarghami, 2007). However, the toxic lipid peroxides are known to cause various impairments of the sperm cell, such as membrane damage and decrease in motility (Alvarez *et al.,* 1987; Aitken *et al.,* 1993). The most popular (but not the most important) product of lipid peroxidation is malondialdehyde (MDA).

This work reported that seminal plasma concentrations of MDA were significantly lower in all participant's non-smokers compared to that of smokers (6.10 ± 1.18 vs. 8.51 ± 1.43, p<0.010 respectively). Similar significant lower levels (p<0.010) were found in volunteers (5.71 ± 1.17) and patients (6.51 ± 1.06) non-smokers groups in comparison to that of volunteers smokers (7.66 ± 0.9) and patients smokers (9.01 ± 1.46). Moreover, this study showed that MDA was negatively correlated with sperm's concentration (r=-0.069, p>0.050), semen volume (r=-0.205, p<0.050), sperm motility (r=-0.513, P<0.010), viability (r=-0.309, p<0.010), membrane integrity (-0.362, p<0.010), and morphologically normal sperm (r=-0.514, p<0.010). In addition, MDA showed significantly positive correlations (p<0.010) with non-chromatin condensation (r=0.555) and DNA fragmentation (r=624).

These findings are in agreement with that of Elsaeid *et al.,* (2006) who showed a significantly negative correlation of MDA with sperm concentration (r=-0.621, p < 0.001), motility (r=-0.572, p<0.005), and percentage of normal forms (r=-0.447, p<0.05). Similarly, Saraniya *et al.,* (2008) found a significant correlation between MDA concentrations and sperm motility and concentration in subfertile patients compared to normal. Besides, Nakamura *et al.,* (2002) found that subfertile patients showed significantly higher concentrations of malondialdehyde than fertile controls. There was no significant difference between the levels of malondialdehyde in the seminal plasma of patients with normozoospermia and asthenozoospermia.

Moreover, Nabil *et al.,* (2008), reported that lipid peroxidation plays a significant role in disrupting sperm functions and semen quality especially sperm motility and morphology and may account for some cases of male infertility. Furthermore, Tavilani *et al.,* (2008), observed a higher content of lipid peroxidation product malondialdehyde (MDA) in spermatozoa of asthenozoospermic compared with normozoospermic samples (p<0.05). Although, they found that the difference in MDA of seminal plasma in both groups was not significant.

5-3-1-4 Smoking cigarette and 8-OHdG levels

Smoking may increase oxidative stress. ROS including superoxide radical, hydrogen peroxidase, and hydroxyl radical, can cause several types of DNA lesions, such as chemical modifications to the bases or to 2-deoyribose moieties, chain breaks, adducts, and crosslinks. Hydroxyl radical can add to guanine and adinine at position 4, 5, or 8 in the purine ring, generating a multitude of products. One of the most abundant lesions is 8-oxo-7, 8 dihydroguanine (8-oxoG), also called 8-Hydroxy guanine (8-ODdG), which frequently mispairs with adenine. (8-ODdG) is the most commonly used biomarker of DNA oxidation. It has been estimated that several thousand 8-OHdG lesions may form daily in a mammalian cell, representing >5% of all oxidative lesions.

It was reported that sperm DNA damage is closely related to male infertility, and 8-OHdG is a sensitive marker of oxidative DNA damage caused by ROS in human sperm (Shen and Ong 2000). The levels of 8-OHdG were detected in serum and urine. They were correlated with smoking (Loft *et al.,* 1992), cancer (Toyokuni *et al.,* 1995), aging (Halliwell *et al.,* 1998), and diabetes mellitus (Leinonen *et al.,* 1997). Elevation levels of 8-OHdG in sperm DNA had been demonstrated by Shen *et al.,* (1997), and related to male infertility (Kodama *et al.,* 1997; Shen *et al.,* 1999). Fraga *et al.,* (1996) reported that smoking, which depletes antioxidants (a-tocopherol and ascorbate) and increases oxidative stress, induced a significant increase in 8-OHdG of sperm DNA.

In this study we observed that the concentrations of 8-OHdG in seminal plasma of all participants non-smokers, volunteers and patients non-smokers (0.71 ± 0.53, 0.61 ± 0.40, 2.99 ± 1.82) were significantly lower (p<0.010) than that of the smokers of the three groups (2.63 ± 1.54, 2.02 ± 0.51,

2.99 ± 1.82). In addition, the levels of 8-OHdG in all samples showed significant negative correlation with sperm concentration (r=-0.169, p=0.073), semen volume (r=-0.193, p<0.050), sperm motility (r=-0.532, p<0.010), vitality (r=-0.373, p<0.010), membrane integrity (r=-0.335, p<0.010) and morphologically normal sperm (r=-0.513, p<0.010). Whereas, significant positive (p<0.010) correlations were found with sperm DNA integrity (r=0.600) and non-condensed chromatin (r=0.545).

These data are consistent with that of Ni *et al.,* (1997) who reported an association between 8-OHdG with sperm motility, morphology and density. Other studies by Nakamura *et al.,* (2002) reported that the levels of 8-OHdG in the seminal plasma of subfertile patients with normozoospermia and those with asthenozoospermia were significantly higher than those of fertile controls (p<0.050), and Inoue *et al.,* (2003) demonstrated that the serum levels of 8-OHdG of smokers was higher than that of non-smokers. Besides, Kao *et al.,* (2008) demonstrated a significant negative correlation between 8-OHdG with motility.

The increased levels of 8-OHdG in seminal plasma of smokers in our data suggest that sperm dysfunction caused by smoking induced oxidative stress extends to the DNA levels.

5-3-2- Smoking and Semen Parameters

The production and function of healthy normal spermatozoa was affected by the number of cigarettes smoked per day, the years of smoking and the level of nicotine by-products present in the body fluids, which correlate negatively with semen and sperm quantity and quality (Chia *et al.,* 1994a; b). While some studies demonstrated a positive correlation between male smoking and diminished sperm parameters (concentration, volume, motility and morphology), (Mak *et al.,* 2000; Taszarek *et al.,* 2005; Agarwal *et al.,* 2005; Mostafa *et al.,* 2006; Colagar *et al.,* 2007), others showed no significant correlation between smoking and semen parameters (Trummer *et al.,* 2002; Sobreiro *et al.,* 2005; Sepaniak *et al.,* 2006). Pasqualotto *et al.,* (2005) found the same non-significant results except for the volume of the semen. Moreover, there was not a trend to an alternation of these conventional parameters (concentration, volume, motility and morphology) in smokers, as had been suggested by others (Wong *et al.,* 2000).

The present study (Table 14) showed that all smokers (n=72) have a significantly lower volume of semen (3.1 ± 1.6 vs. 3.6 ± 1.6, p<0.050), and sperm's concentrations (65.4 ± 31.5 vs. 68.4 ± 31.2, p=0.641), motility (23.3 ± 8.0 vs. 35.8 ± 14.6, p<0.010), viability (33.5 ± 17.6 vs. 41.2 ± 17.3, p<0.010), membrane integrity (39.2 ± 18.4 vs. 54.1 ± 18.4, p<0.010), morphologically normal sperm (26.6 ± 12.0 vs. 41.4 ± 13.7, p<0.010), chromatin condensation (% bad sperm) (34.6 ± 8.1 vs. 26.4 ± 8.0, p<0.010), and DNA integrity (% bad sperm) (16.8 ± 5.1 vs. 9.6 ± 4.3, p<0.010) than non-smokers. On the contrary, the concentrations of oxidative biomarkers MDA, ROS, cotinine and 8-OHdG were found to be significantly higher (p<0.010) in smokers than non-smokers; (6.10 ± 1.18 vs. 8.51 ± 1.43), (52.89 ± 28.48 vs. 128.75 ± 39.46), (2.34 ± 2.20 vs. 88.22 ± 58.05), and (0.71 ± 0.53 vs. 2.63 ± 1.54) respectively.

Besides, non-smokers volunteers (Table 15) showed a significant higher (p<0.010) sperm motility (44.7 ± 12.5, vs. 24.7 ± 8.2), and morphologically normal sperm (52.9 ± 10.8 vs. 34.4 ± 10.3) than smokers. In contrast, smokers showed significantly higher (p<0.010) non-condensed chromatin (29.4 ± 5.5 vs.19.6 ± 4.8), and DNA fragmentation (15.1 ± 4.5 vs. 6.1 ± 1.8) than non-smokers. The concentrations of seminal plasma parameters; ROS, cotinine and 8-OHdG were significantly higher (p<0.010) in smokers (7.66 ± 0.9, 111.82 ± 28.66, 68.55 ± 38.58, 2.02 ± 0.51 respectively) in comparison to non-smokers (5.71 ± 1.17, 40.47 ± 20.45, 2.02 ± 2.03, 0.61 ± 0.40 respectively).

In Table 17, non-smokers of patients group showed higher sperm vitality (38.1 ± 17.5 vs. 30.7 ± 16.4, p<0.050), membrane integrity (52.9 ± 20.1 vs. 36.1 ± 18.1 p<0.010), sperm motility (31.3 ± 13.6 vs. 23.0 ± 8.0, p<0.010), and morphologically normal sperm (36.1 ± 9.0 vs. 23.4 ± 10.1, p<0.010) than that of smokers in the same group; whereas, smokers showed higher percentage of

non-chromatin condensation (36.4 ± 8.1vs. 29.8 ± 7.1, p<0.010), and DNA fragmentation (17.4 ± 5.3 vs. 11.3 ± 4.2, p<0.010) in comparison to non-smokers. On the other hand, patients non-smokers showed significantly lower (p<0.010) seminal plasma concentrations of MDA (6.51 ± 1.06 vs. 9.01 ± 1.46), ROS (65.74 ± 30.16 vs. 138.49 ± 41.85), cotinine (2.68 ± 2.36 vs. 99.55 ± 64.58), and 8-OHdG (0.81 ± 0.63 vs. 2.99 ± 1.82) than smokers.

Furthermore, the data resulted from comparing volunteers smokers and patients smokers (Table 19) demonstrated that volunteers smokers have higher semen volume (3.4 ± 1.0 vs. 3.1 ± 1.8, p=0.128), sperm motility (24.7 ± 8.2 vs. 23.0 ± 8.0, p=0.401), sperm concentration (78.9 ± 28.2 vs. 60.6 ± 31.4, p<0.050), sperm vitality (41.3 ± 19.1 vs. 30.7 ± 16.4, p<0.050), sperm membrane integrity (48.2 ± 16.8 vs. 36.1 ± 18.1, p<0.050), and morphologically normal sperm (34.4 ± 10.3 vs. 23.4 ± 10.1, p<0.010) than patients smokers. On the other hand, sperm non-condensed chromatin (36.4 ± 8.1 vs. 29.4 ± 5.5, p<0.010), and DNA fragmentation (17.4 ± 5.3 vs. 15.1 ± 4.5, p=0.094) were higher in patients smokers than volunteers smokers. In addition, the concentrations of MDA, ROS, cotinine and 8-OhdG were significantly higher in patients smokers than volunteers smokers; (7.66 ± 0.90 vs. 9.01 ± 1.46, p<0.010), (111.82 ± 28.66 vs. 138.49 ± 41.85, p<0.050), (99.55 ± 64.58 vs. 68.55 ± 38.58, p=0.066), and (2.99 ± 1.82 vs. 2.02 ± 0.51, p=0.094) respectively.

These results reported that smoking has negative effects on sperm and semen parameters (concentration, motility, morphology, vitality, membrane integrity, chromatin condensation, DNA integrity, MDA, ROS, cotinine, and 8-OHdG). These results were in accordance with other studies that showed a deleterious effect of smoking on sperm quality. Omu *et al.,* (1998) found that non-smokers and light smokers had better sperm parameters than heavy smokers as indicated by the sperm count (p<0.030), active motility (p<0.030), asthenozoospermia (p<0.050), and positive hypo-osmotic swelling (p<0.050) tests. Also, the number of smokers with higher titers of circulating anti-sperm antibodies was larger than that of non-smokers (p< 0.010). Gauer *et al.,* (2007) demonstrated a negative correlation of smoking with sperm motility. Colagar *et al.,* (2007) showed a reverse effect of smoking on sperm quality. Moreover, many other previous studies found an inverse relationship between smoking and sperm parameters (Vine *et al.,* 1996; Saleh *et al.,* 2002a, Kuntzle *et al.,* 2003). However, these results conflict with others that had found no association between smoking and sperm quality and function (Voget *et al.,* 1986; Dikshit *et al.,* 1987).

5-3-3- Smoking and Semen volume

The results of the current study showed significant differences (p<0.050) between the semen volume of non-smokers (n=94) (3.6 ± 1.6) and smokers (n=72) (3.1 ± 1.6). This result is in accordance with that of Saaranen *et al.,* (1987), who found that there was an effect of smoking on semen volume and specifically, semen volume of heavy smokers that was significantly lower than that of non-smokers. Other study by Holzki *et al.,* (1991) showed that smokers had significantly smaller semen volumes compared to non-smokers. Although, a dose dependent deleterious effect of tobacco on semen volume and sperm counts was found (Osser *et al.,* 1992).

Cigarette smoking effect on semen volume was reported by Chia *et al.,* (1998), who reported significantly negative differences in semen volume between smokers and non-smokers. These results were supported by Zhang *et al.,* (2000), who performed a study on infertile males in Shandong Province, China. Zhang *et al.* concluded that cigarette smoking had a detrimental effect on semen volume of smoking infertile males, compared to non-smoking infertile and control fertile ones. While the concentration, motility and morphology of the sperm appeared not affected by smoking, semen volume showed a decline in smokers compared to non-smokers (Pasqualotto *et al.,* 2005). Similar effect of smoking on semen volume was reported by (Ramlau-Hansen *et al.,* 2006).

5-3-4- Smoking and Sperm motility

Many studies had reported that progressive motility of sperms were higher in heavy smoker than light smokers (Dikshit *et al.,* 1987; Goverde *et al.,* 1995; Vine *et al.,* 1996; Ozgur *et al.,* 2003). The possible explanations for this enhancement of rapidly progressive motility due to heavy smoking in comparison to light smoking is a definite number of cigarettes per day that may constitute a threshold for the enhancement of rapid progressive motility in smokers. Some researchers have found a significant correlation between smoking and decreased sperm motility or morphology and lower sperm density (Merino *et al.,* 1998; Zenzes, 2000; Evans *et al.,* 2004).

The present study showed that sperm motility of all smokers was significantly ($p<0.010$) lower than that of all non-smokers (23.4 ± 8.0 vs. 35.8 ± 14.6 respectively). Smoker volunteers samples analysis showed a significant ($p<0.010$) decrease in motility comparing with that of non-smokers (24.7 ± 8.2 vs. 44.7 ± 12.5 respectively). The same results were detected for patients smokers and non-smokers (23.0 ± 8.0 vs. 31.3 ± 13.6 respectively, $p<0.010$)

In Table 10a, motility showed significant negative correlations ($p<0.010$) with non-condensed chromatin, DNA fragmentation, ($r=-0.458$, $r=-0.406$, respectively), and significant positive correlations ($p<0.010$) demonstrated with sperm vitality ($r=0.494$), sperm membrane integrity ($r=0.446$), and morphologically normal sperm (%) ($r=0.479$). On the other hand, motility (Table 10c) showed significant negative correlations ($p<0.010$) with seminal plasma concentrations of MDA (μM), ROS (μmol/l) (Fig. 14), cotinine (ng/ml), and 8-OH-dG (ng/ml) ($r=-0.513$, -0.537, -0.489, and -0.532 respectively)

These results were in consistent with other studies. Omu *et al.,* (1998) found that motility of sperms of smokers was lower than non-smokers, Jedrzejczak *et al.,* (2004) reported that smokers had significantly fewer motile spermatozoa, and Gaur *et al.,* (2007) indicated that asthenozoospermia, the most common semen variable in their study, can be an early indicator of reduction in quality of semen, as seen in light smokers. In addition, heavy smoking produces teratozoospermia, which further reduces semen quality. Oligozoospermia may be due to factors other than smoking. Lower sperm motility, concentration and morphology had been shown to be lower in smokers than non-smokers (Colagar *et al.,* 2007).

Previous studies reported that cigarette smoking effects on sperm motility, concentration and morphology were related with poor sperm quality (Close *et al.,* 1990; Sofikitis *et al.,* 1995; Vine *et al.,* 1996; Saleh *et al.,* 2002a, Kuntzle *et al.,* 2003), although, Richthoff *et al.,* (2007) found that smoking status did not affect sperm density or motility.

5-3-5- Smoking and Sperm Morphology

Abnormal sperm morphology is related to sperm head abnormalities, and may involve the acrosome, nucleus and post acrosomal region (Zamboni, 1987). Increased quantities of ROS have been shown to be detrimental to the DNA of spermatozoa, thus producing a negative effect on the viability and morphology of spermatozoa (Koksal *et al.,* 2003).

The present study indicated that the mean numbers of morphologically normal sperm were significant lower ($p<0.010$) in smokers compared to non-smokers ($26.6 \pm 12.0\%$ vs. $41.4 \pm 13.7\%$ respectively). Similarly, like-wise were found within the smokers and non-smokers of volunteers group (34.4 ± 10.3 vs. 52.9 ± 10.8) and patients group (23.4 ± 10.1 vs. 36.1 ± 9.0). Moreover, the mean percentage of morphologically normal sperm correlated significantly positive ($p<0.010$) with sperm concentration, motility, viability and membrane integrity. Whereas, significant negative correlations were demonstrated ($p<0.010$) with non-condensed chromatin (CMA_3 positive), membrane integrity, and seminal plasma concentrations of MDA, ROS, cotinine, and 8-OHdG.

These data are in agreement with other studies that demonstrated lower percentage of normal sperm morphology found in semen samples of smokers compared to non-smokers (Osser *et al.,* 1992; Chia *et al.,* 1994a: b). Kunzle *et al.,* (2003) and Ozgur *et al.,* (2003) found that cigarette smoking significantly reduced the mean percentage of normal sperm morphology, ejaculate volume and sperm vitality. In addition, heavy smoking produces teratozoospermia, which further reduces semen quality (Gaur *et al.,* 2007).

5-3-6- Smoking and DNA damage

The real influence of tobacco on male fertility remains controversial and several mechanisms have been proposed such as: impairment of spermatogenesis (Weisberg, 1985), induction of ultrastructural abnormalities (Zavos *et al.,* 1998), and apoptosis (Gandini *et al.,* 2000; Sakkas *et al.,* 2002).

Several studies attempt to find a correlation between the tobacco consumption and an alteration of sperm quality (Oldereid *et al.,* 1989; Vine, 1996). Many other studies showed the effect of smoking on conventional sperm parameters such as sperm density (Vine *et al.,* 1994), motility, viability, and morphologically normal sperm (Zavos *et al.,* 1998), and nuclear integrity (Potts *et al.,* 1999).

Indeed, cigarette smoking is linked to significantly increased levels of seminal reactive oxygen species (ROS), which are responsible for an oxidative stress (Saleh *et al.,* 2002a). This is why smoking may induce alterations of the sperm plasma membrane and a high degree of DNA fragmentation (Church and Pryor, 1985). In contrast, others have found no significant correlation between smoking and sperm parameters (Vogt *et al.,* 1986; Vine, 1996; Trummer *et al.,* 2002; Sepaniak *et al.,* 2006).

Studies have found that children of fathers who smoked cigarettes pre-conceptually have a higher risk of developing childhood cancers (Ji *et al.,* 1997). These studies suggest that there may be a link between sperm DNA damage and the subsequent development of childhood diseases.

The results of this study by using the TUNEL and CMA_3 assay for sperm DNA integrity showed a statistically significant difference ($p < 0.010$) between smokers (n=72) and non-smokers (n=94) (16.8 ± 5.1 versus 9.6 ± 4.3 by TUNEL), (34.6 ± 8.1 versus 26.4 ± 8.0 by CMA_3). A similar effect was observed in the healthy volunteers group (Table 15). In the patients group the results were (17.4 ± 5.3 versus 11.3 ± 4.2 by TUNEL) and (36.4 ± 8.1 versus 29.8 ± 7.1 by CMA_3). For both groups of volunteers smokers and patients smokers, a low difference was found in DNA fragmentation (TUNEL) ($p>0.050$) and a significant difference in chromatin condensation (CMA_3) ($p<0.010$).

These results are in close agreement with other reports (Sofikitis *et al.,* 1995) which showed that single-strand-to double-strand breaks DNA spermatozoa were significantly higher in smokers. Potts *et al.,* (1999) and Zenses, (2000) showed previously a link between DNA damage and smoking. Whereas, Sergerie *et al.,* (2000) showed the opposite, that there is no relationship between smoking and DNA fragmentation. Recently, Sepaniak *et al.,* (2006) showed a positive correlation ($p<0.010$) between cigarette smoking and sperm DNA fragmentation among smoker patients seeking for infertility counseling. A previous study from our group showed that the sperm of smokers have higher DNA fragmentation than the sperm of non-smokers (Hammadeh *et al.,* 2008b).

In the present study, it was difficult to find a significant correlation between DNA integrity and other sperm parameters in the normal and patients smokers group ($p>0.050$). The same observations were made by Sepaniak *et al.,* (2006) and Henkel *et al.,* (2004). Similarly, DNA fragmentations evaluated by SCSA were not statistically different whether the conventional sperm parameters were normal or abnormal in a population of infertile men (Saleh *et al.,* 2002b). In addition, other researchers showed no correlation between sperm DNA damage and pregnancy (Gandini *et al.,* 2004; Huang *et al.,* 2005). Borini *et al.,* (2006), which showed inverse and direct

correlation between sperm DNA damage and pregnancy and pregnancy loss rates, is also explained by paternal genome anomalies blocking the correct embryo development. While Irvine *et al.,* (2000) found a significant higher fragmentations in DNA from infertile men in compared to fertile men.

These current findings support previous studies and suggest that DNA fragmentation rate is an independent factor as regard to conventional parameters, and cannot be suspected by the means of standard sperm evaluation. DNA fragmentation measurement may explain some cases of otherwise unexplained infertility. In addition to the above-discussed diagnostic interest of the sperm DNA fragmentation rate, most of the authors (Lopes *et al.,* 1998; Zini *et al.,* (2001) agree to consider this parameter as a prognostic factor in the probability of a successful pregnancy.

5-4- Protamines Quantifications

Protamines are small, highly arginine-rich, nuclear proteins that in the haploid phase of spermatogenesis are required for sperm head condensation and associated transcriptional silencing (Sassone-Corsi, 2002). Human sperm nuclei contain considerably fewer protamines (approximately 85%) than those of bull, stallion, hamster and mouse (Gatewood *et al.,* 1987; Bench *et al.,* 1996). In contrast to the bull, cat, and ram, whose spermatozoa contain only one type of protamine (P1) , human and mouse spermatozoa contain a second type of protamine (P2), which is deficient in cysteine residues (Corzett *et al.,* 2002). Therefore, human sperm chromatin is less regularly compacted and frequently contains DNA strand breaks (Sakkas *et al.,* 1999b, Irvine *et al.,* 2000). The histone-bound DNA sequences are less tightly compacted, and it is thought that these DNA sequences and/or genes may be involved in fertilization and early embryo development (Gatewood *et al.,* 1987; Gineitis *et al.,* 2000).

Similar levels of P1 and P2 were shown in spermatozoa from normozoospermic men (Balhorn *et al.,* 1988). Numerous studies, however, demonstrated that male infertility is associated with an abnormal histone to protamine ratio (Zhang *et al.,* 2006) and an aberrant P1/P2 ratio at both the protein level in ejaculated spermatozoa (Balhorn *et al.,* 1988; Belokopytova *et al.,* 1993) and the mRNA level in testicular spermatids (Steger *et al.,* 2003).

5-4-1- P1 and P2 Concentrations

Protamines are considered a good marker of sperm nuclear maturity since they are replacing the histones in the last stage of spermatogenesis. Many functions of protamines had been proposed by Oliva and Dixon, (1991): (i) facilitate the movement of the sperm cell: the spermatozoa with the most compact and hydrodynamic nucleus would move faster and may fertilize the oocyte first; (ii) protection of the DNA from chemical and physical damage, such as ROS, nucleases, mutagens or other factors potentially present in the internal or in the external media; (iii) confer an epigenetic mark on some regions of the sperm genome, influencing its reactivation upon fertilization; (iv) removing proteins and transcription factors from the spermatid, allowing a proper post-fertilization genetic reprogramming; (v) protamines could be part of a checkpoint during spermiogenesis and (vi) they could have a role in the fertilized oocyte, allowing the synchronization of the cell cycle between the oocyte in metaphase II phase and spermatozoa in G1 (Braun, 2001).

Findings from this work showed that protamines in spermatozoa were higher in patients than in normal volunteers. P1 concentration in patients was significantly higher (<0.010) in comparison to the values observed in semen samples of healthy volunteers (416.30 ± 101.70 vs. 378.20 ± 100.00), whereas, the P2 concentration was almost similar in both groups (363.60 ± 114.9 vs. 347.30 ± 77.30, p>0.050). Besides, the ratios of P1/P2 of patients was significantly higher than that of healthy volunteers (1.22 ± 0.36, 1.10 ± 0.20, p<0.050) (Table 20).

The results of this study showing showed different percentages of P1 and P2 between men who underwent ICSI therapy (subfertile) and those healthy fertile volunteers. Higher amounts of

protamines in patients samples were observed. One reason for these discrepancies might be the technical procedures mainly the measurement of the intensity of the P1 and P2 bands. Moreover, patients group members were chosen randomly from men who came to the IVF unit. Many of them exhibited a normal sperm parameters including distribution of protamines. On the contrary, many of the volunteers showed abnormal P1/P2 ratios.

In addition, attention has been focused on the possible role of cigarette smoking and protamines concentrations and ratios in spermatozoa taking together, the results of present study (Table 21) suggest a negative biological effect of smoking on spermatozoa chromatin condensation and protamines distribution. Although, the results from this work showed different percentages of protamine P1 and P2 and P1/P2 ratios between ICSI patients and volunteers (Table 20; Figures 27-30). The results in Table 21 showed that concentration of P1 and P1/P2 ratios (Figure 31) were higher in smokers in comparison to non-smokers. P2 was significantly lower in smokers (335.60 ± 110.60 vs. 376.81 ± 97.36, $p<0.05$).

Health volunteers smokers and non-smokers revealed similar results (Table 22) P1; (395.60 ± 125.90 vs. 367.60 ± 80.00, p=0.743), P2: (338.10 ± 90.20 vs. 352.90 ± 69.30, p=0.682) and P1/P2 ratio: (1.19 ± 0.27 vs. 1.04 ± 0.11, $p<0.05$). Remarkably, the concentrations in patients of ICSI of P1 and P2 levels were higher in non-smokers than levels measured in smokers (Table 23). In contrast, P1/P2 ratio was significantly higher in smokers (1.34 ± 0.46 vs. 1.11 ± 0.20, $p<0.010$) (Figure 33).

These results support the hypothesis that subfertility in general and cigarette smoking in particular, increase the sperm susceptibility to chromatin abnormalities and fragmentations. These results are in agreement with other reports (Balhorn *et al.,* 1988; Chevaillier *et al.,* 1990; de Yebra *et al.,* 1993; 1998; Carrell and Liu, 2001; Aoki *et al.,* 2005a) in which they revealed an abnormal elevation of P1/P2 ratio of infertile male populations, and proposed that reduction of P2 expression is the mean factor responsible for P1/P2 ratios in infertile men. In the since de Yebra *et al.,* (1993) and Carrell and Liu, (2001), showing complete absence of P2 in infertile men.

P2 deregulation is responsible for many cases of abnormal P1/P2 ratios; the reason is that P2 deregulation occurs more frequently than P1 deregulation. The gene of P2 is more recently derived than that of P1and highly variable in mammals (Lewis *et al.,* 2003). Support for this data comes from the regulatory mechanisms responsible for P2 expression that are more susceptible to variation than those for P1 expression, and about 90% of cases with high P1/P2 ratio exhibited P2 under-expression. Besides, P1 deregulation proposed as a cause of aberrant P1/P2 ratios with P2 deregulation in infertile men, no defined mechanism explains the over expression of one protamines by under-expression of other (Aoki *et al.,* 2005a). Steger *et al.,* (2008) demonstrated a significant difference in the P1/P2 ratios among infertile "1:1.7 in ejaculates" and fertile "1:1 in ejaculates" men. Both P1 and P2 change between fertile and infertile men but P2 changes more than P1, suggesting different mRNA stabilities for the two molecules. In another study conducted by the same group, Steger *et al.* (2003) did not find differences in P2 transcript levels in spermatozoa isolated from men with impaired spermatogenesis compared to men with normal spermatogenesis. However, they observed significantly decreased P1 transcript contents in the group of patients with impaired smermatogenesis.

The etiology of protamines deregulation remains unclear; it could be due to mutations in the genes of P1, P2, or any of the accessory proteins such as transition proteins 1 and 2, serine/arginine protein specific kinase 1, and Ca^{2+}/calmodulin dependent protein kinase IV, these proteins demonstrated to play a critical role in protamine expression, processing, and function (Papoutsopoulou *et al.,* 1999; Wu *et al.,* 2000; Cho *et al.,* 2001; 2003; Zhao *et al.,* 2004).

Defects in the regulation process during transcription and translation could be another possible cause of irregular P1/P2 ratios. The human sperm P1 and P2 genes exist in single chromatin domain and their regulation is regulated by the same upstream regulatory elements. Transcriptional

regulation may be a cause of P1/P2 ratio abnormalities (Johnson *et al.,* 1988; De Jonckheere *et al.,* 1994; Nelsen and Kraawetz, 1994). Furthermore, defects in translation regulation may be another cause of abnormal P1/P2 ratios (Aoki and Carrell, 2003). Defects in post-translational process of protamines may end with deregulated P1/P2 ratios (de Yebra *et al.,* 1998; Carrell and Liu, 2001).

Significantly aberrant protamine ratios and a higher Bcl2 content in testes biopsy and ejaculates of infertile men were compared to controls, suggesting that these molecules may be useful biomarkers for predicting male infertility (Steger *et al.,* 2008).

Both P1 and P2 are necessary for proper chromatin condensation. Animal models demonstrated by Cho *et al.,* (2001) and (2003) showed that insufficiency of P1 or P2 causes infertility in mice and P2 deficiency cause damage of the sperm DNA and embryo death. However, no such studies exist for humans.

Smoking may affect protamination inversely by preventing proper binding of P1 and/or P2 with DNA. Several chemical agents have been proposed to induce chromatin damage of spermatozoa. Smoking increases the levels of oxidative stress by increasing the levels of ROS. Smoking is considered as a source of ROS (Knutzle *et al.,* 2003), or enhancing the production of ROS by leukocytes that induced by inflammatory reaction in reproductive tract under the effect of smoking (Saleh *et al.,* 2002a). One may speculate that under oxidative stress an oxidative damage to sperm DNA, proteins and lipids that may be related to sperm abnormalities (Aitken and Baker, 2006). This damage of the DNA may prevent binding of protamines to DNA properly. Protamines like other proteins in the body may be denatured directly by smoking components, this denaturation may cause abnormalities in protamination that make the sperm DNA more susceptible to DNA damage (Carrell *et al.,* 2007). Moreover, smoking may affect the levels of protamine through affecting the spermatogenesis process. Excess production of free radicals by smoking may be involved in errors in spermiogenesis resulting in abnormalities in protamination.

In the light of the increasing body of evidence, DNA packaging and DNA damage had been correlated with abnormal chromatin packaging (Razavi *et al.,* 2003, Aoki *et al.,* 2006b) that might be related to poor protamination. Tarozzi *et al.,* (2009) found a highly negative correlation ($p<0.010$) between abnormal protamination (CMA_3) and DNA fragmentation (Tunel).

5-4-2- Protamines and DNA integrity

Chromatin integrity is crucial for maintaining DNA packaging and reproductive potential of spermatozoa. Approximately, 15% of infertile men have been reported to exhibit deficient protamine packaging (Carrell and Liu, 2001). Poor or aberrant protamination of sperm DNA during spermatogenesis is clearly a major factor defining the susceptibility of sperm chromatin to damage (Nasr-Esfahani *et al.,* 2005; Carrell *et al.,* 2007).

Protamines are critical for proper sperm chromatin packaging (Balhorn *et al.,* 2000). Protamines play a crucial role in sperm chromatin condensation and protection of the paternal genome from DNA damage (Leberge and Boissonneault, 2005; Aoki *et al.,* 2005b; Kempisty *et al.,* 2006). It was proposed that protamine deficiency may lead to DNA damage accumulation in sperm (Evenson *et al.,* 2002; Seli and Sakkas, 2005), morphological abnormalities, and initiation of apoptotic pathway, inactivating mitochondria and decreasing sperm motility (Miyagawa *et al.,* 2005). Sperm DNA damage appeared to be detrimental to fertility in humans and was linked to lower embryo quality (Virant-Klun *et al.,* 2002), blastulation rates (Seli and Sakkas, 2005), and *in vitro* fertilization (IVF) pregnancy rates (Bungum *et al.,* 2004; Virro *et al.,* 2004).

Therefore, it would be helpful to be able to identify with certainty, whether a particular DNA damage related to protamination defects. In addition, previous studies attempted to correlate DNA damage with abnormal protamination using indirect measurements of protamine levels using the

chromomycin A_3 (CMA_3) fluorochrome. Results of these studies indicated that protamine deficiency is related to increasing levels of DNA damage (Bianchi *et al.,* 1993; Manicardi *et al.,* 1995; Borini *et al.,* 2006; Tarrozi., *et al.,* 2009).

Evidences from studies using sophisticated atomic force microscopy and proton-induced X-ray emission spectroscopy, provided insights into the details of protamine-DNA binding interactions (Hud *et al.,* 1994; Bench *et al.,* 1996; Balhorn *et al.,* 2000). Binding of protamines with DNA tends to form characteristic clusters within species (Corzett *et al.,* 2002). Disruption of P1/P2 ratio may disrupt this characteristic protamine-DNA binding, possibly influencing secondary chromatin structure. Additionally, based on amino acid sequencing, P2 requires a longer stretch of DNA (approximately 15 bp) than P1 (approximately 11 bp) for proper chromatin incorporation (Bench *et al.,* 1996). This may provide evidence for abnormal chromatin packaging when P2 is less abundant than P1, as is the case in patients with high P1/P2 ratios (Aoki *et al.,* 2005b). Physical size constraints dictate that there would be overall disturbances in the amount of protamine bound to the DNA, thereby reducing the overall number of disulphide cross-links.

In the present study, direct quantitative measurements of sperm protamine concentrations using electrophoresis revealed that P1, and P2 inversely correlated with DNA fragmentation. The concentrations of P1 and P2 in all participant samples (n=166) (Table 10a), volunteers samples (Table 12a) and patients samples (Table 13a) showed a negative correlation ($p>0.050$) with non-condensed chromatin (CMA_3 positive) and DNA fragmentation (Tunel). The P1/P2 ratios showed significant positive ($p<0.01$) correlations with DNA fragmentation in all participants and healthy volunteers samples. The levels of DNA fragmentation assessed by Tunel in patients samples (Table 11) were significantly higher than healthy volunteers ($p<0.010$). These data are in agreement with those obtained using CMA_3 staining which indicated that protamine deficiency is significantly related to increases in DNA damage (refer to section 5-1).

These results support previous study by Aoki *et al.,* 2005b who found that sperm P1 and P2 concentrations inversely correlate with DNA fragmentation. Moreover, the results of this work indicate that protamines play an important and critical role against sperm DNA damage. These results are supported by findings of Cho *et al.,* (2001) and (2003) who reported that protamine deficiency is a causative agent for DNA damage and infertility in mice. The presence of cysteine in protamines structure facilitate the formation of cross-bridges not only within the protamines but also between protamines and DNA via disulfide bonds that increase the stability of sperm chromatin (Fuentes-Mascorro *et al.,* 2000). Besides, sperm premature chromatin decondensation resulted as protamine-deficient human sperm used for ICSI, which indicated a less stable sperm chromatin in these patients (Nasr-Esfahani *et al.,* 2004b).

There is some evidence that links high DNA fragmentation with diminished sperm DNA integrity (Aoki *et al.,* 2005b), decreased protamine content (Nasr-Esfahani *et al.,* 2005) and lower IVF and ICSI rates (Evenson and Wixon, 2006a).

Protamines P1/P2 ratio appears to be critical for chromatin stability. Although the P1/P2 ratio varies between genera, within a species it is highly conserved (Corzett *et al.,* 2002). The results of this study indicated that spermatozoa with high P1/P2 ratios correlated significantly with elevated DNA fragmentation (Tables 10a; 12a; 13a) and these findings are in agreement with other previous investigators (Chevaillier *et al.,* 1987; Balhorn *et al.,* 1988; Belokopytova *et al.,* 1993; de Yebra *et al.,* 1993, 1998; Carrell and Liu, 2001; Aoki and Carrell, 2003; Aoki *et al.,* 2005a, b) who have also found a relationship between abnormal protamine expression, DNA fragmentation and male infertility.

Aoki *et al.,* (2005b) demonstrated three reasons for considering evaluation of the P1/P2 ratio as a valuable clinical diagnostic test: first, the highly correlation between P1/P2 ratio with sperm penetration ability, count, morphology and motility. Second, protamine content appears to be critical for chromatin integrity which is important for proper embryogenessis (Manicardi *et al.,*

1998; Agarwal and Allamaneni, 2004). Third, human sperm protamines may be of utmost clinical significance even though they do not impair IVF/ICSI pregnancy rates. It is now clear that in a study by Paldi, (2003), it was found that proper chromatin structure is critical for methylation of imprinted genes.

However, chromatin structure aberrations and abnormalities in the P1/P2 ratio may themselves be unrelated and may simply reflect generalized problems during spermiogenesis. Further studies are critical for our understanding of these proposed chromatin structure changes in patients with aberrant P1/P2 ratios.

5-4-3- P1/P2 Content and Sperm Quality

Many studies had detected a relation between abnormal protamine expression and male infertility (de Yebra *et al.,* 1993; 1998; Carrell and Liu, 2001; Mengual *et al.,* 2003). Animal studies reported that protamine or transition protein haplo-insufficiencies resulted in infertility, abnormal chromatin packaging, DNA damage, and altered sperm morphology (Cho *et al.,* 2001; Zhao *et al.,* 2004; Suganuma *et al.,* 2005).

The finding of the current study showed that P1/P2 ratios for all participants (n=166) (Table 10a) was correlated negatively with sperm concentration (r=-.055, p>0.050) and sperm viability (r=-0.139, p>0.050). Significant negative correlations were found with sperm membrane integrity (r=-0.168, p<0.050), sperm motility (r=-0.235, p<0.010) and morphologically normal sperm (r=-0.253, p<0.010). Sperm non-condensed chromatin and DNA fragmentation showed significantly positive correlations (p<0.010) with P1/P2 ratios (r=0.264, r=0.337 respectively). In contrast, P1/P2 ratios in patients samples were higher than in volunteers samples (1.22 ± 0.36 vs. 1.10 ± 0.20, p<0.05 respectively).

In healthy volunteers samples P1/P2 ratios (Table 12a) were correlated (p>0.050) negatively with sperm motility, viability, membrane integrity and morphologically normal sperm. A significant correlation was found with DNA fragmentation (r=0.296, p<0.050). Likewise, in patients samples (Table 13a) negative (p>0.05) correlations were detected with sperm concentrations, viability, membrane integrity and morphologically normal sperm, and positive correlations with non-condensed chromatin and DNA fragmentation.

Furthermore, in the present investigation the P1/P2 ratios (Table 14) were significantly higher in all smokers group (n=72) in comparison to all non-smokers (n=94) (1.30 ± 0.42, vs. 1.09 ± 0.18, p<0.010). Nevertheless, in volunteers samples (Table 15) a significant difference (p<0.050) was found in the P1/P2 ratios between healthy volunteers smokers (1.19 ± 0.27) and healthy volunteers non-smokers (1.04 ± 0.11). Similarly, in subfertile patients samples (Table 17) P1/P2 ratios of smokers was significant higher (p<0.010) than non-smokers (1.11 ± 0.20, 1.34 ± 0.46 respectively).

According to these finding, there seem to be that abnormalities in P1/P2 ratios affect the sperm quality and function. These results are in close agreement with other reports (Aoki *et al.,* 2005a,b) who showed that in patients with abnormal P1/P2 ratio the mean percentage of sperm count, motility, viability, sperm penetration capacity, and abnormal sperm morphology decreased and the mean percentage of DNA fragmentation increased (Aoki *et al.,* 2006b). These results are consistent with the published literatures (de Yebra *et al.,* 1993; 1998; Carrell and Liu, 2001; Mengual *et al.,* 2003). Recently, Hammoud *et al.,* (2009) found that patients with abnormal P1/P2 ratios had less normal sperm heads and more tapered sperm when compared to patients with a normal protamine ratio although these differences were not significantly different among the group with abnormal and those with normal P1/P2 ratio groups.

5-4-4- P1/P2 Content and Semen Parameters

When sperm is passing from the testis to the epididymis, a further stabilization of nuclear structure is achieved by thiol-oxidation of the cysteine-rich protamines. In mouse and man cauda epididymis, 95% of SH groups are converted into -S-S- bridges (Seligman *et al*., 1994). Protamine thiol oxidation has been linked to the stability of the DNA (Kosower *et al*., 1992).

It was shown that oxidative stress can damage sperm DNA by causing deletions, mutations, and other lethal genetic effects (Tominga *et al.,* 2004). Many studies have reported a connection between oxidative stress and DNA damage (Irvine *et al.,* 2000; Mustafa *et al.,* 2004).

Our results showed P1/P2 ratios that were significantly positive ($p<0.010$) with the concentrations of the oxidative biomarkers; MDA, ROS (Fig. 18), cotinine, and 8-OHdG ($r=0.384$, $r=0.410$, $r=0.411$, r=0393 respectively) for all participants underwent this study. Moreover, by dividing the participants into two groups, a healthy volunteers and subfertile patients who underwent ICSI therapy, significant correlations were found between P1/P2 ratios and oxidative stress markers in seminal plasma (MDA, ROS, Cotinine, and 8-OHdG) in both groups.

The current data suggest that the observed alterations in protamines P1/P2 ratios in spermatozoa are the negative biological effect of oxidative stress on sperm DNA.

Oxidative stresses affect DNA, proteins, and lipids in spermatozoa and increase the sperm abnormalities (Aitken and Baker, 2006). The damage of the nucleus proteins caused by oxidative stress and free radicals may prevent the binding of protamines to sperm DNA. Accordingly, Aoki *et al.,* (2006b) pointed out those abnormalities in protamination making the sperm DNA more susceptible to DNA damage. Denaturation of protamines may be resulting from oxidative stress compounds directly. Moreover, oxidative stress may affect the levels of protamine through affecting the spermatogenesis process.

As proteins comprise the major non-water component of cells, they are also the major intracellular ROS targets (for reviews see Davies, 2005). Under conditions of oxidative stress, the amount of ROS generated is expected to be greater than under normal redox regulation conditions and may lead to oxidation of specific proteins.

Proteins are one of the prime targets for oxidative damage (Jung *et al.,* 2007), and cysteine residues are particularly sensitive to oxidation because the thiol group (-SH) in cysteine can be oxidized (Eaton, 2006). Redox ratios disturbances in plasma, increasing vulnerability of thiol groups to oxidative damage (Kemp *et al.,* 2008). Due to the fact that protamines are rich in cysteine, which is rich in –SH group, they are susceptible to oxidation, and high levels of these oxidative stress components may affect the formation of inter- and intra-disulfide bonds, resulting in less compaction of the sperm chromatin and high incidence of DNA damage. Proportion between P1/P2 ratios indicated that P2 is more vulnerable to oxidative stress.

5-4-5- P1/P2 Content and Fertilization and Pregnancy Rates

Normal sperm nuclear composition is essential to maintain sperm DNA integrity (Chao *et al.,* 2001; 2003), as any disruption will likely have a profound effect on the integrity of sperm DNA and its fertilization potential. Abnormalities in sperm protamination are a common defect in male infertility, indicating that the proper functioning of protamines is essential for successful egg-sperm fertilization process (Nasr-Esfahani *et al.,* 2008). In addition, Sakkas *et al.,* (1999b), reported that a high percentage of injected or fail fertilized oocytes contain condense sperm or sperm with premature chromosomal condensation. The ratios of sperm P1 to P2 is important since it is directly related to fertilization and pregnancy rate. Individual sperm cells that display the lowest protamine levels show diminished viability and increased susceptibility to DNA damage (Aoki *et al.,* 2006a; b)

The result of this study shows that P1, P2 and P1/P2 ratio concentrations (Table 30) correlated none significantly with the fertilization rate and pregnancy rate. This non-significant correlation may be due to low number of cases involved in this study. These results indicate that protamine deficiency or alterations in protamination are associated with male infertility. These results are supported by a study conducted by Steger *et al.,* (2008) who reported that protamine ratios exhibited a highly significant difference between patients and controls. However, no significant correlations could be demonstrated with the fertilization and pregnancy rates. This is in contrast with IVF rates that have been reported to be significantly reduced in patients with abnormally low and high P1/P2 ratios (Aoki *et al.,* 2006b). Recently, de Mateo *et al.,* (2009) reported that Pre-P2/P2 and P1/P2 ratios are positively associated with the pregnancy rate and P1/P2 ratio is positively associated with the proportion of embryos obtained by IVF, but not by ICSI.

Carrell *et al.,* (2007) postulated that the selection of morphologically normal sperm for treatment decreases the risk of using a sperm with less condensed chromatin. However, these results are related to that of previous studies using the same kind of protamine detection (Carrell and Liu, 2001; Aoki *et al.,* 2005b).

The influences of the damaged or decondensed sperm chromatin in the fertilization and subsequent development of the embryo are not clear. It has been proposed that sperm with normal morphology are important up to the fertilization step, while the DNA integrity becomes the most important sperm parameter related to the establishment and continuation of a pregnancy (Tomlinson *et al.,* 2001). It is clear that poor chromatin condensation and possible DNA damage may also contribute to failure of sperm decondensation after ICSI and subsequently result in fertilization failure (Bianchi *et al.,* 1996). It is possible that protamine 2 acts after the fertilization process as initiating factor for pronucleus formation and the correct development of the embryo (Corzett *et al.,* 2002).

More studies involving a large number of subjects, together with multivariate logistic regression analysis including all semen variables and female factors, are needed to determine the impact of sperm protamination on the outcome of IVF/ICSI procedures.

5-4-6- P1/P2 ratio Grouping

Protamines 1 and 2 are the most abundant of the nuclear proteins in the sperm nucleus that package the human male genome (Bianchi *et al.,* 1992; Lewis *et al.,* 2003). The protamine P2 content in the nucleus of normal sperm cells is similar to that of protamine P1 (P1/P2 ratio almost equal 1) and ratios outside of 0.8 to 1.2 appears to indicate abnormal expression (Balhorn *et al.,* 1988; Corzett *et al.,* 2002). The P1/P2 ratio has been considered by many authors as evidence of nuclear maturity (Belokopytova *et al.,* 1993; Colleu *et al.,* 1996). However, it remains unknown whether the differences in the P1/P2 ratio among independent samples are due to an overall variation in the levels of protamine P2 common to all sperm types present in the ejaculate or whether, instead, the differences are due to the mixed sperm populations. A reduction in P2 protamine content has been reported in different studies in infertile patients (Torregrosa *et al.,* 2006).

De Yebra *et al.,* (1993) have demonstrated that infertile men have a higher degree of variability in the relative sperm histone to total nuclear protein ratio. 1n addition, de Yebra and Oliva (1993) were the first to notice with biochemical methods that over a range of infertile patients higher P1/P2 ratios correlated with higher histone levels. An excess of nuclear histones may result in poorer chromatin compaction due to incomplete protamination and an increased susceptibility to DNA damage (Cho *et al.,* 2001; 2003; Aoki *et al.,* 2005b; 2006b).

It was shown that only 20% of spermatozoa of fertile men possess human testis specific histone 2B, suggesting the presence of different sperm population in human ejaculate (Zalensky *et al.,* 2002). Moreover, two distinct human testis/sperm –specific H2B variants (hTSH2B/H2BF WT) have been cloned and characterized (Zalensky *et al.,* 2002; Churikove *et al.,* 2004a). These isoform variants may not be uniformly distributed throughout the sperm population.

Many studies reported that high and low P1/P2 ratios were associated with altered seminal parameters in infertile patients (Balhorn *et al.,* 1988; de Yebra *et al.,* 1993; Mengual *et al.,* 2003; Aoki *et al.,* 2005a).

In this study 25 samples (15.1%) were identified with abnormally low P1/P2 ratios, 36 samples (21.7%) with normal P1/P2 ratios and 105 samples (63.3%) with abnormally high P1/P2 ration. In addition, results indicated that 58.0% of the healthy volunteers group revealed protamine abnormality (high and low P1/P2 ratios) and the remaining 42.0% were normal. In contrast, the patients group underwent ICSI therapy and the corresponding values were 87.1% and 12.9% respectively.

Furthermore, by grouping all participants (Tables 26 and 27) into smokers group (n=72) and non-smokers group (n=94). 70 samples (97.2%) of smokers showed abnormal P1/P2 ratios comparing to 60 samples (63.8%) of non-smokers. In addition, it was shown that 89.5% of volunteers smokers revealed abnormal P1/P2 ratios (low and high), whereas, in non-smokers only 38.8% presented abnormal ratios. Besides, patients smokers showed 100% protamine abnormality (no normal P1/P2 ratio detected), while 76.2% showed such abnormality in the non-smokers group.

All these findings indicated that most of the participants in this study had high P1/P2 ratio (63.3 %, 105/166) and 80.5%, (58/72) of the smokers have high P1/P2 ratios. This indicated that under-expression of P2 is the reason for the majority of the cases.

In a study by Aoki *et al.,* 37 patients were identified with abnormally low P1/P2 ratios, 99 patients with normal P1/P2 ratios and 13 patients with abnormally high P1/P2 ration (Aoki *et al.,* 2005b). There was also a significant increase in the incidence of DNA fragmentation in patients with diminished levels of either P1 or P2. These data highlight a relationship between human sperm protamine content and increased levels of DNA fragmentation. The precise mechanism that is responsible for P1/P2 ratios deregulation in infertile males are unknown (Aoki *et al.,* 2006b), although studies have shown decreased levels of P1 mRNA in testes from infertile men (Steger *et al.,* 2003).

The present results suggest that those infertile (patients and smokers) with lower or higher P1/ P2 ratios may also have poor sperm DNA integrity and enhanced susceptibility to DNA damage and consequently reduced fertility *in vivo* and *in vitro* (Hammadeh. *et al.,* 1996; Spano *et al.,* 2000; Zini *et al.,* 2001; Morris *et al.,* 2002; Benchaib *et al.,* 2003, Bungum., *et al.,* 2004; Hammadeh *et al.,* 2008).

Sperm protamine deficiency (partial or complete) is observed in a subgroup of infertile men. It is noteworthy that altered P1/P2 ratios and the absence of P2 are associated with human male fertility problems (Balhorn *et al.,* 1988; de Yebra *et al.,* 1993, 1998a; Bench *et al.,* 1998; Carrell and Liu 2001; Mengual *et al.,* 2003). Mengual *et al.,* (2003) have reported that the sperm nuclear P1/P2 ratio in the subgroup of men with asthenospermia was not significantly different from that of the fertile men. However, the P1 to P2 ratio was significantly higher in the subgroup of men with oligospermia compared to the fertile controls. In addition, when the ratio of P1 to P2 in normospermic human samples is around 1 (Balhorn *et al.,* 1988), is evaluated in oligospermic subjects, often a shortage of P2 is found in combination with the presence of P2 precursor proteins (de Yebra *et al.,* 1998).

5-4-7- P1/P2 ratios and seminal characteristics

Numerous reports have indicated abnormalities in the nucleus of ejaculated sperms (Sakkas *et al.,* 2000; Ollero *et al.,* 2001; Tomlinson *et al.,* 2001). Sperms DNA fragmentation have been correlated with non-chromatin condensation (Gorczyca *et al.,* 1993; Borini *et al.,* 2006; Tarrozi., *et al.,* 2009). Also, abnormalities in protamination have been correlated with nicking of DNA (Bianchi *et al.,* 1993). Marcon and Boissoneault (2004) have suggested that DNA damage may be

the result of incorrect repair of transient DNA nicks that are introduced during spermiogenesis. These nicks may cause improper replacement of histones by protamines which may have resulted in low P1/P2 ratios. Different mechanisms have been proposed to explain the presence of these abnormalities in human ejaculate. First Sakkas *et al.,* (2002) had proposed that some sperm could have escaped programmed cell death (apoptosis), that could be linked to defects in remodeling of the cytoplasm during spermiogenesis. A second proposed mechanism was that damaged DNA could be resulting from defective protamine deposition during nuclear remodeling during spermiogenesis (Ollero *et al.,* 2001). Ollero *et al.,* (2001) also proposed that the coexistence of both mature and immature sperm during transportation from the seminiferous tubules to the epididymis could result in oxidative DNA damage of mature sperm. Our findings could be consistent with these proposed mechanisms.

Sperm DNA damage has been associated with protamine deficiency (Cho *et al.,* 2001; Aoki *et al.,* 2005b; 2006b). Infertile men have an increased sperm histone to protamine ratio when compared with fertile controls, and an important subset of infertile men (5%–15%), but not of fertile men, possesses a complete protamine deficiency (Zhang *et al.,* 2006). Protamine deficiency (absolute or relative) can potentially result in defective chromatin compaction and in an increased susceptibility to DNA damage (Aoki *et al.,* 2005b).

The result of this work showed that within the low, normal and high P1/P2 ratios, the distributions of protamines were varied (Table 29; Figures 34-44). Comparing to the levels of P1, P2, and P1/P2/ ratio of normal P1/P2 ratios group (373.14 ± 78.12, 370.55 ± 71.07, 1.01 ± 0.05), the low P1/P2 ratio (0.77 ± 0.07) group showed similar level of P1 (371.31 ± 75.73) and over-expression of P2 (480.79 ± 91.55) levels, whereas, high P1/P2 ratio (1.35 ± 0.30) group showed over-expression of P1 (424.13 ± 110.94) and under-expression of P2 (325.12 ± 95.72). These results indicated that P2 over-expression was the cause of low P1/P2 ratio, while in high P1/P2 ratio both over-expression of P1 and under-expression of P2 were the causes. The levels of oxidative stress markers (MDA, ROS, and 8-OHdG) and smoking marker (cotinine) levels were significantly higher ($p<0.010$) in low and high P1/P2 ratios groups compared to normal ratio group. The levels of these markers were higher in high P1/P2 ratio than low P1/P2 ratio groups except for 8-OHdG that was almost similar.

These results demonstrated that oxidative stress and smoking affected the integrity of the sperm DNA and the protamines levels could be explained by 1) oxidative stress and smoking may affect the protamines proteins by disturbing the formation of inter- and intra- disulphide bonds between the protamines and DNA or within the protamines themselves, 2) oxidative stress and smoking may affect the sperm DNA directly causing genetic effects (Tominga *et al.,* 2004) that might affect the binding of protamines with DNA properly, and 3) oxidative stress and smoking may affect the levels of protamine through affecting the spermatogenesis process, where excess production of free radicals from cigarette smoke or environmental pollutants, may be involved in errors in spermiogenesis resulting in abnormalities in protamination and DNA damage.

Moreover, these findings could be confirmed on two levels; 1) the results of studying the chromatin condensation and DNA integrity within this work showed significantly higher ($p<0.010$) difference in the levels of non-condensed chromatin and DNA fragmentation in low P1/P2 ratio group (30.5 ± 6.9, 14.7 ± 4.6 respectively) and high P1/P2 ratio group (31.7 ± 9.2, 13.8 ± 6.0 respectively) comparing to normal P1/P2 ratio group (24.8 ± 7.9, 8.5 ± 4.1 respectively); and 2) these findings are in agreement with numerous previous studies that found a correlation between DNA integrity and protamination (Bianchi *et al.,* 1993; Manicardi *et al.,* 1995; Razavi *et al.,* 2003; Naser-Esfahani *et al.,* 2005; Aoki *et al.,* 2005 b; 2006; Torregrosa *et al.,* 2006; Borini *et al.,* 2006; Carrell *et al.,* 2007; Tarrozi., *et al.,* 2009).

The elevation in high P1/P2 ratios within patients and volunteers indicated that P2 under-expression accounts for the majority of cases with high ratios. This is in agreement with the results of De Yebra *et al.,* (1998b) who reported that the increased P1/P2 ratio could be due to a decrease in the P2 content caused by deficient processing of this protamine. A number of reports have noted

abnormal P1/P2 ratios in the sperm of infertile human males, suggesting that the relative amounts of each protamine is important for proper spermatid differentiation (Balhorn *et al.,* 1988; Belokopytova *et al.,* 1993; Steger *et al.,* 2003; Aoki *et al.,* 2005a , 2006a; Carrell *et al.,* 2007).

In addition, high P1/P2 ratios were correlated with pre-P2 detected in patients samples. This is consistent with the hypothesis that states that incomplete processing of pre-P2 may result in lower levels of mature P2 and therefore an increased P1/P2 ratio (Bench *et al.,* 1998; de Yebra *et al.,* 1998; Torregrosa *et al.,* 2006; de Mateo *et al.,* 2009). Torregrosa *et al.,* (2006) found an inverse correlation between Pre P2/P2 ratio with sperm count, normal morphology and motility. They suggested that positive correlations between TUNEL –positive sperm and Pre –P2 at low pre2/P2 ratios link between deficient protamine processing and decreased DNA integrity.

Inefficient sperm chromatin packaging in sperm of patients with low P1/P2 ratio may be due to P2 over-expression or/and P1 under-expression (Aoki *et al.,* 2005a). Zhang *et al.,* (2006) found that the low P1/P2 ratios patients have high contents of H2B histones. These data suggest that those infertile men with high sperm nuclear histone H2B to protamine ratio have a defect in spermiogenesis (the later stage of spermatogenesis), as this is the specific step in spermatogenesis where the final assembly of sperm proteins (displacement of histones by transition proteins and then by protamines) occurs. Recently, Hammoud *et al.,* (2009) had found that a decrease in P1/P2 ratio corresponded with an increase in the amount of histones retained. Moreover, de Mateo *et al.,* (2009) demonstrated that Pre-P2/P2 and P1/P2 ratios are positively associated with the pregnancy rate. The potential explanation for this correlation could be that Pre-P2/P2 ratio also correlates with P1/P2 ratio and that P1/P2 ratio correlates with pregnancy outcome. Thus, a low pre-P2/P2 ratio could be a marker for a low P1/P2 ratio.

5-4-8- Relationship between Protamines and Smoking

The real influence of tobacco on male fertility remains controversial. Many studies have been conducted to examine the relationship between cigarette smoking and male infertility; however, the exact molecular mechanisms are not well understood in most cases (Arabi and Mosthaghi, 2005). Cigarette smoking may be associated with subfertility in men and may result in decreased sperm concentration, lower sperm motility, and a reduced percentage of morphologically normal sperm (Sofikitis *et al.,* 1995). Smoking may damage the chromatin structure and produce endogenous DNA strand breaks in human sperm (Zenezes, 2000). In fact, levels of DNA damage tend to be higher in smokers (Saleh *et al.,* 2002a).

The findings of this study showed that total amounts of protamines in all non-smokers were higher than smokers (779.44 vs. 743.20 respectively). P1 concentrations in non-smokers and smokers were almost equal (402.63 ± 93.96 vs. 407.60 ± 128.80, $p>0.05$), whereas, P2 concentrations in non-smokers were significantly higher ($p<0.050$) than that of smokers (376.81 ± 97.36 vs. 335.60 ± 110.60 respectively). The data from volunteers group showed that P2 concentrations in smokers were lower than in non-smokers (338.10 ± 90.20 vs. 352.9 ± 69.3). The same trend was found within the patients group (334.7 ± 117.7 vs. 388.8 ± 107.2). These data also showed that P2 concentration in smokers of patients was lower than that of volunteers smokers. Besides, the P1/P2 ratios of smokers in all smokers, volunteers and patients were significantly higher ($p<0.010$) than non-smokers, (1.30 ± 0.42, 1.19 ± 0.27, 1.34 ± 0.46 for smokers respectively) and (1.09 ± 0.18, 1.04 ± 0.11, 1.11 ± 0.20 for non-smokers respectively).

These findings indicated that the causative agent for higher P1/P2 ratios in smokers was the under-expression of P2 and suggest that smoking may affect P2 expression greater than P1. This is the first study conducted to evaluate the effect of smoking on protamination process.

Moreover, smoking may affect protamines themselves directly or their binding with DNA or DNA directly through increasing the oxidative stress. Smoking itself may be a source of ROS or inducing oxidative stress through its metabolites that may induce an inflammatory reaction, which resulted in

leukocytes production considered a source of ROS. Smoking toxic metabolites may also affect the spermatogenesis process directly.

Many studies proposed that P2 under-expression was the reason for elevation of P1/P2 ratio (Balhorn *et al.,* 1988; de Yebra *et al.,* 1993; 1998; Mengual *et al.,* 2003; Aoki *et al.,* 2005a), but none of them study the effect of smoking on protamines. Studying the evolution of protamines by Lewis *et al.,* (2003) revealed that P2 gene is more recently derived than P1 and highly variable within the mammalian gen. Also, incomplete post-transitional process of protamines may result in under-expression of P2 and an increase in P2-precursors (de Yebra *et al.,* 1998; Carrell and Liu, 2001).

Conclusion

The data from this study indicated that abnormalities in sperm chromatin condensation evaluated by CMA_3 and DNA integrity evaluated by TUNEL were related to abnormalities in sperm parameters, and also affected fertilization and pregnancy rates. This work showed the possibility of using CMA_3 and TUNEL tests as prognostic tools for IVF/ICSI patients.

Oxidative stress induced lipid peroxidation that in turn induces significant sperm membrane damage and DNA damage, which remarkably influenced sperm quality (concentration, motility, morphology, viability, membrane integrity, chromatin condensation, DNA integrity). It is quite probable that such a deleterious effect may account for some cases of male infertility and evaluation of oxidative stress biomarkers (ROS, MDA, and 8-OHdG), and may be a part of infertile male workup in the near future. Also, oxidative stress inversely affects fertilization and pregnancy rates. Reactive oxygen species status may be used as an important indicator for clinical evaluation and treatment of malefactor infertility.

In this study smokers showed high levels of oxidative stress biomarkers and decreased sperm quality compared to non-smokers. This study also demonstrated that cigarette smoking impairs sperm function by reducing the quality and fertilizing capacity. This is probably because of high levels to toxic components of smoking that cause to increase of seminal plasma free radicals and oxidative stress.

The high level of smoking biomarker cotinineas found in this work may decrease male fertility by reducing sperm quality and inversely affect the percentage of fertilization and pregnancy rates.

Quantification of the protamines revealed that most of the cases were of high P1/P2 ratio and the majority was due to under-expression of P2. Results also showed that aberrant P1/P2 ratios are associated with reduced sperm quality and percentages of fertilization and pregnancy rates. Protamine concentrations (P1 and P2), were inversely correlate with the DNA fragmentation and non-condensed chromatin. Evaluation of the effects of smoking on protamines demonstrated a reduction in protamines concentrations and elevation of P1/P2 ratio in smokers compared to non-smokers.

Given the potential adverse effects of smoking on fertility, physicians should advise infertile patients who smoke cigarettes to quit smoking. Additional studies with large number of subjects are needed to confirm the effect of smoking on protamines and clarifying the potential mechanism behind this effect.

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