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“Impact of Alcohol and Cigarette smoking on sperm quality and their influence on male infertility„

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Abstract

To be able to swim through the reproductive tracts and fertilize the egg, the spermatozoa DNA go through cellular and nuclear changes during spermatogenesis. One of these changes is chromatin repackaging so that 15% of sperm DNA will remain compacted with histone proteins and 85% compacted with protamines. Beside its importance for the fertilization process, chromatin condensation inactivates most of the paternal genes to protect the paternal genome from external damages.

Two lifestyle habits that threaten human health are tobacco smoking and alcohol consumption. Different studies have demonstrated the negative effects of these two factors on male reproductive health.

The purposes of the present study were to investigate the impact of cigarrete smoking, acohol intake on semen parameters determined by standard parameters (WHO, 2010), sperm DNA maturity assessed by Chromomycine staining CMA3, Sperm DNA fragmentation evaluated by TUNEL Assay and secondly to determine whether tobacco smoking or alcohol consumption is more harmful fur sperm quality by comparing semen parameters, DNA maturityand DNA fragmentation betweein smokes group and Alcohol consumer group among infertile men.

According to the number and volume of cigarettes smoked in one day and in a year the duration of cigarettes smoked, the participants were categorized to heavy-smoker participants (n=48) patients who smoke one pack/day or more at least for ten years or two pack/ day at least for five years, and non-smokers (n=70) who did not smoke.

On the other hand, the measurement used for alcohol intake was estimated by the units of alcohol consumed: 1 alcohol consumption unit was taken and considered as follow: 100 millilitres (ml) wine, and beer one unit equals 200ml, 30 ml of whisky or either vodka. According to alcohol consumption, patients were divided into two groups: non-drinkers (n=41) and heavy alcohol consumers (> 7 units/week) (n=52).

All of participant in this study underwent semen analysis according to the 5th WHO criteria. Semen parameters, including semen volume, sperm concentration, total sperm count, motility (A+B) and morphology, Sperm maturity (CMA 3) and DNA fragmentation, TUNEL –Test) were investigated.

Cigaratte smoking and Alcohol consumption were assessed by questionnaire. Cigarette smoking (≥20 cigarette/per day for 10 years) whereas heavy drinkers (≥9 units/week).

The present study demonstrates that smoking adversely affects sperms (volume, density, membrane, and sperm DNA integrity) in men attending assisted reproduction programs. The semen parameters were significantly higher in nonsmokers than in smokers. The total motility (PR+NP) in smokers was 24.27 ± 31.32 % vs. 37.86 ± 14.00% ($p < 0.0001$). The same was observed for sperm vitality (36.2 ± 18.56% vs. 42.9 ± 17.74%; $p = 0.035$), membrane integrity (41.6 ± 18.6% vs. 56.2 ± 18.6 %; $p = 0.0001$), and morphologically normal sperm (28.8 ± 11.8% vs. 44.13.85%; $p < 0.0001$).

By comparing the level of protamine deficiency and sDF between smokers and non-smokers, the results showed that protamine deficiency was significantly higher in smoker in comparison to non –smoker group (33.27 ± 8.6% vs. 26.00 ± 8.28%; $p<0.0001$) and the sDF also was significantly higher in smokers (15.55 ± 3.33%) in comparison to non-smokers group (8.91 ± 4.15% $p < 0.0001$).

By comparing the sperm parameters between the drinker (n=52) and non drinker group (n=41), the data showed that sperm count (53.519 ± 32.67 mill/ml), total sperm motility (23.75 ±10.750%), sperm vitality (34.62 ± 16.652), membrane functional integrity (45.96 ± 17.99%) and morphologically normal spermatozoa (27.06 ± 13.136%), were significantly lower in drinker group than in non-drinker group (73.244 ± 30.5219mill/ml; 35.00 ± 19.17%; 45.24 ± 18.47%; 58.54 ± 18.345% and 35.95 ± 11.97% respectively).

However, Protamin deficiency CMA3+ (37.03 ± 9.75%) and Sperm DNA fragmentation sDF (22.37 ± 7.60%) were significantly higher in drinker in comparison to non-drinker (24.76 ± 7.44% and 11.98 ± 5.17%; $p <0.0001$respectively).

By comparing the deterioration effect of smoking and drinking groups, no significant difference in the semen analysis parameters was observed between the smoker and drinker groups (semen volume 3.20 ±1.43 (ml), semen count 65.8 ±31. 32(mill/ml)), Total motility (24. 27 ± 8. 18%), Sperm vitality (36.15 ±18. 57%), functional integrity (41.6 ± 18.6) and the mean percentage of morphologically normal spermatozoa (28.77 ± 11.82% vs. 2.81± 1.56 (ml), (53.51±32.67 (mill/Ml), 23.75±1.75 (%), 34.62± 16.7(%), 45. 96±17.9% and 27.1±13.13 respectively). However, Protamine deficiency was significantly higher in the drinker group in comparison to the smokers (37.03 ± 9.75 vs. 33.27 ± 8.56, $p=0.02$). Similarly, the sDF was

significantly higher in the drinker in comparison to the smoker group (22.37 ± 7.602 vs. 15.55 ± 3.33, $p < 0.0001$).

These findings suggest that cigarette smoking and heavy alcohol intake deteriorate sperm parameters in men seeking infertility treatment. However, alcohole consumption deteriorates sperm maturity (CMA3) and damage DNA integrity significantly higher than cigarette smoking.

Zusammenfassung

Um durch die Fortpflanzungsorgane schwimmen und die Eizelle befruchten zu können, durchläuft die Spermatozoen-DNA während der Spermatogenese zelluläre und nukleare Veränderungen. Eine dieser Änderungen ist die Neuverpackung des Chromatins, so dass 15 % der Spermien-DNA mit Histonproteinen und 85 % mit Protaminen kompaktiert bleiben. Neben seiner Bedeutung für den Befruchtungsprozess inaktiviert die Chromatinkondensation die meisten väterlichen Gene, um das väterliche Genom vor äußeren Schäden zu schützen.

Zwei Lebensgewohnheiten, die die menschliche Gesundheit bedrohen, sind Tabakrauchen und Alkoholkonsum. Verschiedene Studien haben die negativen Auswirkungen dieser beiden Faktoren auf die männliche reproduktive Gesundheit gezeigt.

Die Zwecke der vorliegenden Studie waren die Bestimmung des Einflusses des Zigarettenrauchens bzw. des Alkoholkonsums auf die durch Standardverfahren bestimmten Samenparameter (WHO 2010), die mittels Chromomycin-Färbung CMA3 bewertete Spermien-DNA-Reife und die durch den TUNEL-Assay bewertete Spermien-DNA – Fragmentierung. Es soll festgestellt werden, ob Tabakrauchen oder Alkoholkonsum schädlicher für die Spermienqualität ist, indem Samenparameter, DNA-Reife und DNA-Fragmentierung zwischen der Rauchergruppe und der Alkoholkonsumentengruppe bei unfruchtbaren Männern verglichen werden.

Zweihundertelf (n=211) Männer im gebärfähigen Alter, die eine Fruchtbarkeitsbehandlung im Prince Rashid Ben Al Hassan Military Hospital in der jordanischen Stadt Irbid aufsuchten, wurden in diese Studie eingeschlossen.

Nach Anzahl und Menge der an einem Tag gerauchten Zigaretten und in einem Jahr nach der Dauer der gerauchten Zigaretten wurden die Teilnehmer in stark rauchende Teilnehmer (n=48) Patienten eingeteilt, die mindestens zehn Jahre lang eine Packung/Tag oder mehr rauchten oder Zweierpackungen/Tag mindestens für fünf Jahre und Nichtraucher (n=70), die nicht geraucht haben.

Andererseits wurde das Maß für den Alkoholkonsum anhand der Einheiten des konsumierten Alkohols geschätzt: 1 Einheit Alkoholkonsum wurde genommen und wie folgt betrachtet: 100 Milliliter (ml) Wein und Bier eine Einheit entspricht 200 ml, 30 ml Whisky oder whisk entweder Wodka. Nach dem Alkoholkonsum wurden die Patienten in zwei Gruppen

eingeteilt: Nichttrinker (n=41) und starke Alkoholkonsumenten (> 7 Einheiten/Woche) (n=52).

Alle Teilnehmer dieser Studie wurden einer Samenanalyse nach den 5. WHO-Kriterien unterzogen. Es wurden Samenparameter wie Samenvolumen, Spermienkonzentration, Gesamtspermienzahl, Motilität (A+B) und Morphologie, Spermienreife (CMA 3) und DNA-Fragmentierung, TUNEL-Test) untersucht. Zigarettenrauchen und Alkoholkonsum wurden per Fragebogen erfasst. Zigarettenrauchen (≥20 Zigaretten/Tag für 10 Jahre), während starke Trinker (≥9 Einheiten/Woche).

Die vorliegende Studie zeigt, dass Rauchen die Spermien (Volumen, Dichte, Membran und Spermien-DNA-Integrität) bei Männern, die an Programmen zur assistierten Reproduktion teilnehmen, nachteilig beeinflusst. Die Samenparameter waren bei Nichtrauchern signifikant höher als bei Rauchern. Die Gesamtmotilität (PR+NP) bei Rauchern betrug 24,27 ± 31,32 % vs. 37,86 ± 14,00 % (p < 0,0001). Das gleiche wurde für die Spermienvitalität (36,2 ± 18,56 % vs. 42,9 ± 17,74 %; p = 0,035), die Membranintegrität (41,6 ± 18,6 % vs. 56,2 ± 18,6 %; p = 0,0001) und morphologisch normale Spermien (28,8 ± 11.8%) beobachtet 11,8 % vs. 44,13,85 %; p < 0,0001).

Durch den Vergleich des Ausmaßes von Protaminmangel und sDF zwischen Rauchern und Nichtrauchern zeigten die Ergebnisse, dass der Protaminmangel bei Rauchern im Vergleich zur Nichtrauchergruppe signifikant höher war (33,27 ± 8,6% vs. 26,00 ± 8,28 %; p<0,0001) und der sDF war auch bei Rauchern (15,55 ± 3,33%) signifikant höher im Vergleich zur Nichtrauchergruppe (8,91 ± 4,15% p < 0,0001).

Durch den Vergleich der Spermienparameter zwischen der Trinker- (n=52) und der Nichttrinkergruppe (n=41) zeigten die Daten, dass die Spermienzahl (53,519 ± 32,67 Mio (34,62 ± 16,652), Membranfunktionsintegrität (45,96 ± 17,99 %) und morphologisch normale Spermatozoen (27,06 ± 13,136 %) waren in der Trinkergruppe signifikant niedriger als in der Nichttrinkergruppe (73,244 ± 30,5219 Mio./ml; 35,00 ± 19,17 %). ; 45,24 ± 18,47 %; 58,54 ± 18,345% bzw. 35,95 ± 11,97%).

Allerdings waren der Protaminmangel CMA3+ (37,03 ± 9,75 %) und die Spermien-DNA-Fragmentierung sDF (22,37 ± 7,60 %) bei Trinkern signifikant höher im Vergleich zu Nichttrinkern (24,76 ± 7,44 % und 11,98 ± 5,17 %; p <0,0001).

Beim Vergleich der Verschlechterungswirkung von Raucher- und Trinkergruppen wurde kein signifikanter Unterschied in den Samenanalyseparametern zwischen den Raucher- und Trinkergruppen beobachtet (Samenvolumen 3,20 ± 1,43 (ml), Samenzahl 65,8 ± 31,32 (Mill/ml)) , Gesamtmotilität (24, 27 ± 8, 18 %), Spermienvitalität (36,15 ± 18, 57 %), funktionelle Integrität (41,6 ± 18,6) und der mittlere Prozentsatz morphologisch normaler Spermatozoen (28,77 ± 11,82 % vs. 2,81 ± 1,56 1. (ml), (53,51 ± 32,67 (Mill/ml), 23,75 ± 1,75 (%), 34,62 ± 16,7 (%), 45, 96 ± 17,9 % bzw. 27,1 ± 13,13). Trinkergruppe im Vergleich zu den Rauchern (37,03 ± 9,75 vs. 33,27 ± 8,56, p=0,02) Ebenso war der sDF in der Trinkergruppe im Vergleich zur Rauchergruppe signifikant höher (22,37 ± 7,602 vs. 15,55 ± 3,33, $p < 0,0001$).

Diese Ergebnisse deuten darauf hin, dass Zigarettenrauchen und starker Alkoholkonsum die Spermienparameter bei Männern, die eine Unfruchtbarkeitsbehandlung suchen, beeinträchtigen. Alkoholkonsum verschlechtert jedoch die Spermienreife (CMA3) und schädigt die DNA-Integrität deutlich stärker als das Rauchen von Zigaretten.

LIST OF ABBREVIATIONS

ART	Assisted Reproductive Technique
CMA3	Chromomycin A3
CMA3+	CMA3 positivity
DFI	DNA fragmentation index
DNA	Deoxyribonucleic acid
DSBs	double strand breaks DNA
FSH	Follicle-stimulating hormone
GnRH	Gonadotrophin-releasing hormone
H1	Linker histone H1
H1.T	Histone H1-like protein
H1.t	Testis-specific histone H1
H2A	Histone H2A
H2B	Histone H2B
H2BFWT	H2B histone family, member W, testis-specific
H3	Histone H3
H4	Histone H4
HILS1	Spermatid-specific linker histone H1-like protein
ICSI	Intracytoplasmic sperm injection
IUI	Intrauterine Insemination
IVF	In Vitro Fertilization
LH	Luteinizing hormone
MDA	Malondialdehyde
Mg	Microgram
NP	Non-progressive motility
P1	Protamine 1
P2	Protamine 2
PR	Progressive motility
PR + NP	Total motility
RNPs	Ribonucleoproteins
ROS	Reactive Oxygen species
SCB	Sertoli cell barrier
SCSA	Sperm chromatin structure assay

sDF	Sperm DNA fragmentation
S-S	Disulfide bridges
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WHO	World Health Organization

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1. Introduction

Over the years, infertility has become a major global problem. Almost 48.5 million couples throughout the world are facing this issue (Mascarenhas, 2012; Inhorn, 2015). Infertility is described as a disease characterised by a failure to conceive after regular unprotected intercourse of one year and is used interchangeably with the term "subfertility" (Zegers-Hochschild, 2017).

Approximately 15% of couples worldwide are subfertile, with male infertility contributing to around half of the cases (Pasqualotto et al., 2007). It is estimated that 1 in 20 men in the general population is subfertile (Schowell et al., 2014). More than 600,000 cycles of Intracytoplasmic sperm injection (ICSI) and in vitro fertilisation (IVF) are performed each year in North America and Europe alone (Centers for Disease Control and Prevention, 2017; Calhaz-Jorge et al., 2017). Unfortunately, the success rate is found to be very low. In 2015, approximately one of three (~33.3%) live births in the United States resulted from assisted reproductive technology (ART) cycles (Centers for Disease Control and Prevention, ASRM, 2015).

ICSI and IVF have greatly helped infertile and subfertile couples conceive. The success of these technologies and techniques depends on the sperm quality parameters (Chemes & Rawe, 2003). Male-related factors contribute to 50% of infertile cases (Agarwal et al., 2015). However, 30% of male infertility is yet to be explained and, thus, classified as idiopathic (Naz & Kamal, 2017).

Several studies reveal a wide variation in the estimation of the occurrence of male infertility (estimated to be 5%–35%), thus showing real differences between populations in terms of the following factors: quality of primary health care, environment, occupation, exposure to toxicants responsible for infertility, age, being overweight and obesity, climate conditions, educational status, recreational use of or constant exposure to drugs, and genetic and epigenetic factors (Aitken, 2020).

That tobacco smoking is one of the lifestyle factors associated with male infertility is still under debate. However, several studies suggest a strong connection between smoking and altered semen parameters (NICE, 2013; NCCWCH, 2004). Further investigation is also needed to determine how a moderate exposure to Cd and Pb (heavy metals found in cigarettes) affects the male reproductive and endocrine functions, although a few studies

suggest that these metals decrease human semen significantly, thus impairing male fertility (Pant et al., 2014; Sengupta et al., 2017).

A smoking habit in males also has an adverse effect on pregnancy outcomes among IVF patients (Cinar et al., 2014). An association between cigarette smoking and altered ICSI and IVF outcomes was reported (Zitzmann et al., 2003). In a study by Klonoff-Cohen et al. (2001), the number of retrieved oocytes decreased by almost 46% in smokers; the males were active smokers, and the females were passive smokers. In addition, a decrease in live birth rates was noticed in 166 couples seeking pregnancy using ART (Fuentes et al., 2010).

Alcohol consumption is another factor that influences male fertility. Vital side effects depend on the amount consumed. However, the limit beyond which alcohol starts affecting male reproductive functions is still unknown. Together, drinking alcohol and smoking may be responsible for causing infertility (Martini et al., 2004; Joo et al., 2012).

No specific evidence of the harmful effects of alcohol and smoking together on semen parameters and fertility outcomes has been observed (Gaur et al., 2010; Petraglia et al., 2013). However, the degradation of sperm quality is positively associated with alcohol or cigarette consumption.

The contradictions in the literature concerning the deleterious effects of tobacco smoking and alcohol consumption on male fertility encouraged us to explore the correlations between smoking, alcohol intake, and sperm parameters in fertile and subfertile men and to find out which habit causes more damage.

The aim of evaluating infertile males is to identify the precise reason behind male infertility, which can help in making therapeutic decisions regarding the issue (Bach et al., 2018).

1.1.Spermatogenesis

Spermatogenesis refers to the proliferation and maturation of males' germ cells from diploid spermatogonia to mature haploid sperm cells through meiotic division. The whole sequence is controlled epigenetically, and disruption at any of the stages involved may possibly result in infertility (Das et al., 2017).

Spermatogenesis starts with cell lines of germ originating from primary germ cells (PGCs), commonly called gonocytes (Donovan & de Miguel, 2003). In males, PGC development

occurs in the endoderm cells close to the end the third week of embryonic development and then move or migrate towards the genital tract by the fifth week, where the existence of the Y chromosome in males plays a vital and key role in the proliferation, development, and transformation of males' genital tracts into primary sexual organs (Boe-Hansen et al., 2006; Looijenga et al., 2007). During mitosis, spermatogonia halt during the cell cycle at the G3 phase and remain dormant until birth (Sasaki et al., 2016). After birth, spermatogenesis starts with the mitotic proliferation of spermatogonia type A (Brinster et al., 1994; de Kretser et al., 1998).

The initial cycle of spermatogenesis in males takes roughly 16 days to complete, and it takes about 4.6 cycles for the development and differentiation to produce adult sperm cells (Galdon et al., 2016), approximately 74 days in total (Pieri et al., 2017; Xu et al., 2016).

At the beginning of spermatogenesis, spermatogonia differentiate via mitosis to primary spermatocytes (44 chromosomes, XX or XY). These spermatocytes undergo meiosis I and II stage that produce haploids spermatids (22, X or Y). Later, the spermatids undergo a series of morphological changes (head, midpiece, and tail), and their chromatin structure and function change (Balhorn, 2018). In fact, there are imprinted genes in the male genome, epigenetic changes in the DNA, and nucleoproteins that edit the chromatins to make them ready for the control of the embryonic growth and development (Canovas & Ross, 2016). Then, step by step, the chromatins will be genetically silenced in the spermatozoa (Ren et al., 2017).

Leydig and Sertoli cells are both important in regulating the whole course of spermatogenesis. In addition, they provide support to germ cells and play a key role in regulating the fate of these cells through several mechanisms and factors. These factors include hormonal, several pro- and anti-apoptotic agents, and energy substrates, such as lactate, especially by spermatids and spermatocytes (Jutte et al., 1981, 1982).

During spermiogenesis, spermatids, which are round haploids at this stage, turns into a flagellated and highly condensed form known as spermatozoa. Then, these spermatozoa differentiate and mature to achieve motility. Sperm nuclear DNA integrity is important in enabling the sperm to function properly in early embryogenesis (Coward & Wells, 2013).

Sertoli cells provide germ cells with mitogens, sources of energy and differentiation factors. They are also protective factors against other toxic agents (Russell et al., 1993). Previous studies showed that Sertoli cells are one of the main targets of various toxicants that harm

normal testicular functions (Boekelheide, 2000). Some of these toxicants include a group of plasticisers. Their commonly used form is known as phthalates, which are present abundantly in the toys of children and in soft plastics commonly used in healthcare products. They have been shown to play a role in the testicular dysgenesis syndrome in rats (Fisher et al., 2003). It has been speculated that exposure to environmental toxicants, referred to as disrupters of the endocrine system, potentially disrupts physiological hormonal events and balance (Skakkebaek et al., 2001).

1.2. Hormonal pathway regulating spermatogenesis

The complete spermatogenesis is a lengthy, multipart, and finely tuned process. The regulation requires and gets distinguished in two stages (Duan et al., 2016; Garolla et al., 2017).

The hypothalamus that secretes the gonadotrophin-releasing hormone (Gn-RH) which provokes adenohypophysis to excrete hormones like follicle stimulating hormone (FSH) and luteinising hormone (LH). The LH stimulates the Leydig cells to release and produce testosterone. The FSH function is to assist Sertoli cells in supporting the gem cells during the different phases of spermatogenesis (Figure 1). Besides FSH and LH, other hormones, such as prolactin and growth hormone, play crucial roles during spermatogenesis (Sharma & Agarwal, 2011).

As a response to FSH stimulation, Sertoli cells produce the peptide hormones, such as inhibin B, into circulation. Inhibin B has a major effect on inhibiting FSH hormone secretion. However, its role as an autocrine factor on Sertoli cells must still be studied. Measurements of levels of serum inhibin B are clinically indicative of and used to assess the presence and functioning of Sertoli cells during childhood. On the other hand, the serum inhibin B levels in adults correlate with the presence of germ cells (de Kretser et al., 2004).

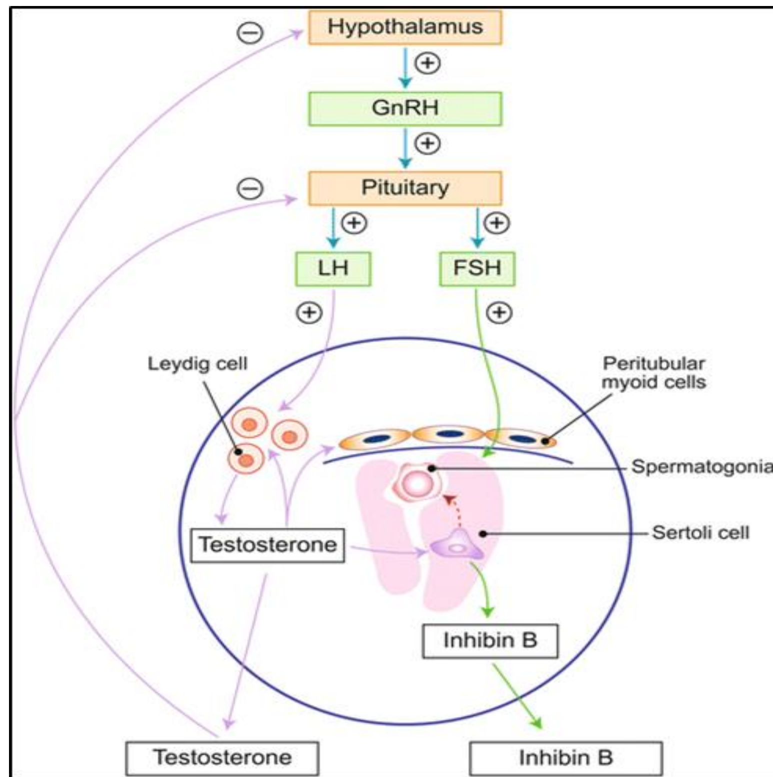

Figure 1: Gonadotropin and steroid hormone control of spermatogenesis [adapted from Mitchell et al. (2017)].

1.3. Chromatin remodelling during spermiogenesis

DNA in the sperm is confined to nucleus because of interaction with protamines, thus contributing to sperm cell maturation, as reported by Gunes et al. (2015).

To obtain a hydrodynamic sperm head and to protect the paternal genome from any modifications in the male or female reproductive tracts, the human sperm DNA, in early spermiogenic phases, undergoes major cellular and nuclear changes (Ward & Coffey, 1991).

The first phase in spermiogenesis is the Golgi phase. This phase is characterised by the formation of polarity in spermatids. Golgi apparatus differentiate to acrosome responsible for the synthesis of proteolytic enzyme. The distal centriole gives raise to the axoneme, and the Mitochondria move to the midpiece. Next, the other centriole pair elongates to form flagellum (Russell et al., 1993).

Further, the chromatin in the elongated nucleus becomes ten times more compact than the chromatin in the nucleus of a somatic cell through progressive modifications (Braun, 2001; Figure 2).

So, in early spermiogenic phases, major chromatin packaging takes place. The nucleosome-bound DNA configuration will first be destabilised by hyperacetylation of the canonical histones, which will neutralise the positive charge of lysine, reducing their affinity for DNA. Double and single DNA strand breaks by DNA topoisomerase II (topo II), in turn, reduce the tension of the DNA (McPherson & Longo, 1993; Laberge & Boissonneault, 2005).

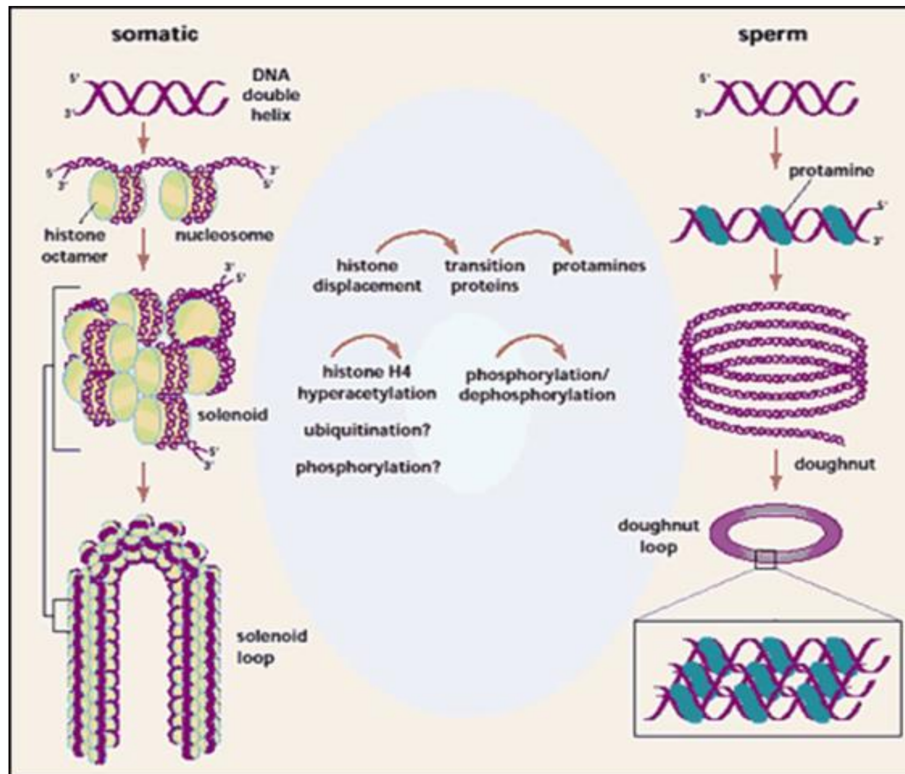

Figure 2: The difference in the chromatin packaging between a somatic cell and a spermatozoon. The chromatin changes from a solenoid loop structure (nucleohistone) into a toroid structure (nucleoprotamine). Post-translational modifications of the proteins facilitated the histone-protamine transition: acetylation, ubiquitination, and phosphorylation of histone H4, and phosphorylation and dephosphorylation of the transition proteins [adapted from Braun (2001)].

"Canonical histones" which are core histone proteins (H2B, H2A, H3, and H4) and a linker histone protein (H1) will be replaced by testis-specific histones. These histones are called "histone variants" and start to appear during different stages of the spermatozoan formation, and they are found in lower quantities during the cell cycle (Cheema & Ausió, 2015; Figure 3). Among these histones, there are H2A variants, which are major players, like H2A.Bbd (González-Romero et al., 2008), H2B variants like H2BFWT (Churikov et al., 2004a), H3 variants like H3.T (Witt et al., 1996), and H1 variants like H1.T and H1.T2 (Tanaka et al., 2005). Some of these histone variants are present only in masculine germ cells (Talbert & Hanikoff, 2010).

Churikov et al. (2004b) demonstrated that the TH3 histone variant is shown in spermatogonia, TH2A and TH2B, prior to meiosis and combined with the chromatin of spermatocytes. Moreover, there are post-translational modifications of histones that are also important for the good progress of the spermiogenesis (Carrell, 2012; Godmann et al., 2007). During the elongated spermatid phase, H3 and H4 are acetylated, and consequently, the interactions between these histones and the sperm DNA are relaxed, especially in the regulatory regions of genes that contribute to embryonic development (Nair et al., 2008; Sonnack et al., 2002). H4 methylation increases during the differentiation of the spermatogonia to spermatid (Luense et al., 2016) but decreases in the elongated spermatids (Sonnack et al., 2002). H3 histone is methylated in the round spermatids, and this methylation has previously been demonstrated to mark the repressed genes on an evolutionary regulatory site (Godmann et al., 2007), which are important for both gamete differentiation and embryo development (Khalil et al., 2004; Hammoud et al., 2009).

It has been previously reported that 10%–15% of canonical histones and their variants remain bound to DNA in mature human spermatozoa (Bench et al., 1996; Carrell & Hammoud, 2009; Gatewood et al., 1990; Wykes & Krawetz, 2003). This part of genomemainly concerns the transcription sites of genes in sperm that are important for the preservation of the paternal genome epigenetics for their later expression during early embryonic development (Carrell & Hammoud, 2009; Ihara et al., 2014). The regulatory sequences (Castillo et al., 2014; Brykczynska et al., 2010), microRNA clusters, transcription factors, paternally imprinted genes (Hammoud et al., 2009), the centromeric and telomeric DNA (Zalenskaya & Zalensky, 2004), retroposons (Pittoggi et al., 1999), matrix-associated regions (Ward, 2009), and genes that produce rRNA are transcribed at the final stages of spermatogenesis (Sillaste et al., 2017).

At the mid-stage of spermatid formation, the changes in chromatin structure will be more obvious when transition proteins TP2 (13 kDa) appears in step 1, and TP1 (6.2 kDa) appears in step 3 are formed and get deposed (Steger et al., 1998) (Figure 3).

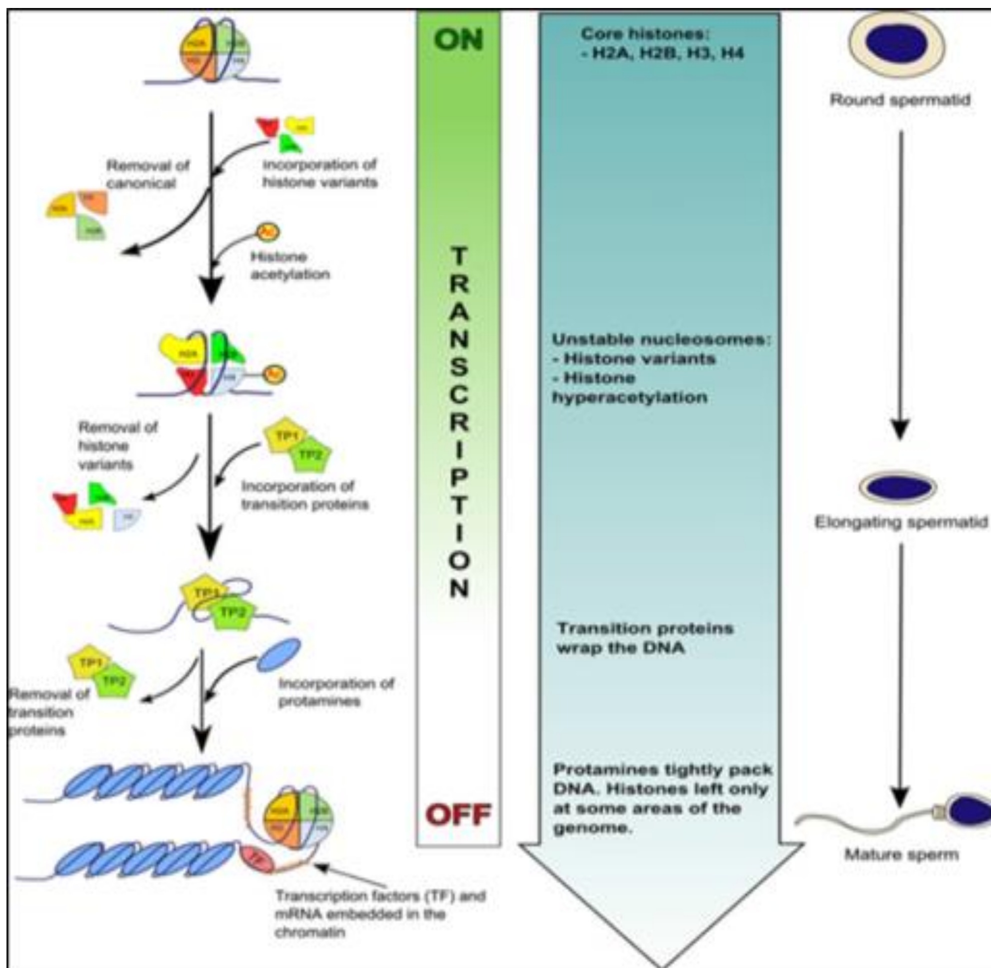

Figure 3: Major chromatin structure remodelling events during spermiogenesis [adapted from Teperek & Miyamoto (2013)].

Pradeepa and Rao (2007) reported that TP1 plays the main role in the destabilisation of the nucleosome structure and the initiation of gene transcription termination when TP2 is attached to CG-rich sequence by zinc fingers. Also, it is important for the chromatin condensation progress (Zhao et al., 2001). It has also been shown that TP1 facilitates the repair mechanism of the DNA strand alterations (Caron et al., 2001).

These proteins are attached to the DNA only for a short period of time. Therefore, they are modified in arginine and lysine residues by methylation, acetylation, and phosphorylation to lose their ability to attach to the sperm DNA (Nikhil et al., 2015). Moreover, the transition proteins have been shown to be important not only for the chromatin condensation procedure but also for the DNA damage repair caused during the histones' replacement (Boissonneault, 2002). The Transition proteins are then replaced by highly basic proteins: protamines 1 and 2 in the late spermatid stage (Figure 3). Each of the protamines is encoded each by a single gene (PRM1 for P1 and PRM2 for P2) located in a cluster of genes beside the TNP2 gene.

They are located on chromosome 16 (16p13.13) (Oliva, 2006; Martins & Krawetz, 2007). The messenger RNA of these proteins keeped silence and undergoes translation after the transition of histones to transition proteins. This step is important to ensure a good process of chromatin repackaging (chromatin decondensation) after the intrusion of sperm cells into the cytoplasm of the oocyte (Hecht, 1989; Kleene & Flynn, 1987; Lee et al., 1995) (Figure 4).

The sperm protamine 1 (P1) (51 AA) is the first to be synthesised as a mature form of protein (Queralt et al., 1995; Green et al., 1994). Protamine 2 (101 residues) is created as a precursor protein and undergoes cleavage by proteolysis after its deposition onto sperm DNA to eliminate short fragments of the peptide (Aoki et al., 2005). Both proteins are reported to be expressed and present in equal ratio and quantity (P1/P2 ratio almost equals one) (Brewer et al., 2002). Further, Nanassy et al. (2011) suggested a clinical value of the protamine ratio between 0.54 and 1.43 for a fertile normozoospermic man.

These proteins have a characteristic constitution, mainly composed of arginine (48%) and cysteine residues (Balhorn, 1989, 2007; Oliva & Castillo, 2011). These amino acids are highly positive charged which leads to a highly ordered nucleo-protamine complex in combination with negatively charged DNA (Oliva & Castillo, 2011) (Figure 4).

The cysteine residues are responsible for the formation of inter- and intra-protamine disulphide bridges (S-S), which stabilises the nucleo-protamine complex (Lewis et al., 2005; Vilfan et al., 2004; Balhorn, 2018). Zinc is abundant in human sperm nuclei (Morisawa & Mohri, 1972), and it is likely to get trapped by the S-S (Bedford et al., 1973). The Cys2/His2 motif of P2 is a zinc finger domain, which leads to the appearance of zinc bridges responsible for the high stabilisation of the chromatin in the mature spermatozoa and in the cessation of transcription until the fertilisation (Bianchi et al., 1993; Björndahl & Kvist, 2010).

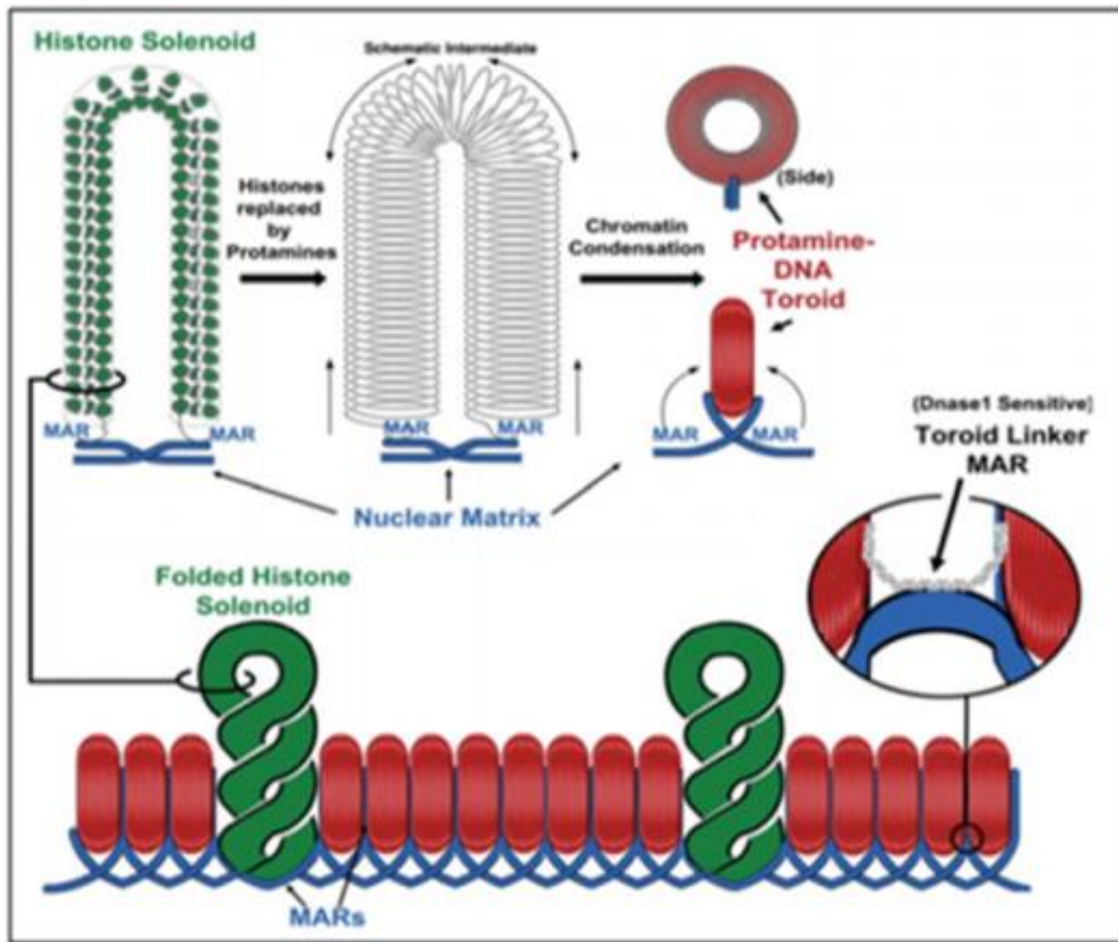

Figure 4: The sperm chromatin condensation: from a solenoid structure to a toroid structure [adapted from Ward (2009)].

1.4.Infertility in relation to oxidative stress

Various reasons are responsible for male factor infertility. These reasons range from physiological and environmental factors, genetic causes, endocrine and immunological conditions to infections or obstruction in the male reproductive tract (Wald, 2005; Ghuman & Ramalingam, 2018). Genetic causes are also responsible for about 15% of cases of male infertility and are classified into two major groups: either chromosomal aberrations or other forms of gene mutations (Güney et al., 2012; Stouffs et al., 2014). Genetic diseases are responsible for about 14% of cases of males with azoospermia and 2% of cases of males with oligozoospermia (Mafra et al., 2011; Pylyp et al., 2013; Figure 5).

Various genetic diseases associated with male infertility are multiple sclerosis, pituitary adenoma, cystic fibrosis, diabetes, hypopituitarism, chromosomal abnormalities like Klinefelter syndrome, cancers like prostate cancer and testicular cancer, tumours of the spinal cord, endocrine problems like thyroid disease, and infections like UTI (Bach et al., 2018;

Walsh et al., 2010). However, the exact etiology is unknown; therefore, labelling them as idiopathic infertility (Agarwal & Sekhon, 2011).

Several other factors, such as adipositas, influence fertility (Fode et al., 2012). Wearing restrictive and tight-fitting clothing or underwear results in increased scrotum and testicular overall temperature and heat (Jung & Schuppe, 2006; Harlev et al., 2015). Smoking cigarette, consuming too much ethanol in the form of alcohol, using steroids, such as anabolic ones, excessively (Naz & Kamal, 2017), and amplified and prolonged exposure to environmental toxicants and pesticides (Jensen et al., 2006; Walczak–Jedrzejowska et al., 2013) are considered responsible for male infertility (Schulte et al., 2010).

Multiple studies reported on the main etiological factor in infertility: oxidative stress (OS), which results in sperm quality impairment and DNA damage (Agarwal et al., 2017; Tremblay et al., 2018).

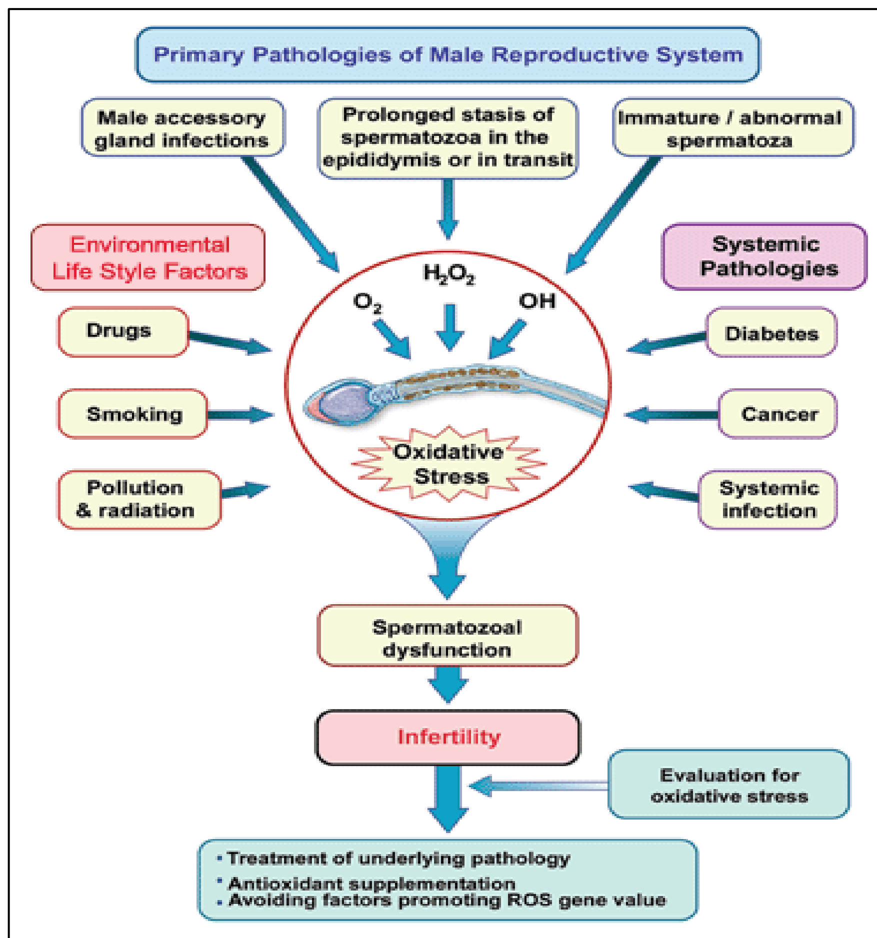

Figure 5: Relationship of infertility with male reproductive system and its primary pathologies and oxidative stress [adapted from Esteves and Agarwal (2011)].

OS, as mentioned earlier, is a common pathological factor observed in about half (30%–80%) of all male infertility cases (Agarwal et al., 2006). OS can be the result of multiple factors, such as an unhealthy lifestyle, smoking habits and alcohol abuse, and exposure to other environmental toxicants or pollutants.

High quantities of oxygen species, such as reactive oxygene species (ROS), or nitrogen species, such as RNS, can be due to previously mentioned factors or impaired ROS/RNS clearance as decrease antioxidant levels and concentrations may result in overall OS. ROS and RNS are different forms of reactive radical or nonradical forms and derivatives of oxygen and nitrogen, respectively (Powers et al., 2011). The high and elevated values of ROS/RNS in the semen of males can be due to the existence and presence of leukocytes in the male seminal plasma and the presence of mitochondria in the spermatozoa. As reported earlier, leukocytes present in the seminal plasma generate and produce 1,000 times more ROS/RNS than those in the spermatozoa (Tremellen, 2008; Figure 6).

Besides, the production of ROS takes place at various intracellular spaces like that in complex I and complex III of the mitochondrial inner membrane during the electron transport chain process (Quinlan et al., 2013; Holmström & Finkel, 2014).

Therefore, OS is defined as the process where there is disproportion in the quantities between the generation and production levels of ROS and the capacity of available antioxidants to scavenge them, which ultimately leads to redox (Agarwal et al., 2014).

ROS plays a significant physiological role; besides their harmful effects, they are also essential to the regulation of cell signalling pathways, enzymatic activities and their pathways, and immune defences (Di Meo et al., 2016). ROS is also crucial for the normal functioning of sperm cells. Some of these functions include the compaction of chromatin in maturing stages of spermatozoa during its epididymal transit. For the capacitation process, acrosomal reaction, hyperactivation, and sperm-oocyte fusion, a delicate balance between reduction and oxidation is necessary and required (Wright et al., 2014; Du Plessis et al., 2015).

On the contrary, a high production and high levels of ROS result in the peroxidation of lipids (LPO), DNA (deoxyribonucleic acid) damage, and ultimately, induction of the apoptotic process (Agarwal et al., 2003; Sharma et al., 2004), which has been reported earlier to have a drastic and adverse effect on total sperm concentration (Agarwal et al., 2014; Takeshima et al., 2017), overall motility (Yumura et al., 2017), and morphology (Aziz et al., 2004; Yumura et al., 2009; Figure 6).

However, numerous studies and research have confirmed that a supraphysiological level of ROS causes reduced sperm motility (Kao et al., 2008), a decline in sperm's overall fertilising ability (Mostafa et al., 20015), and as peroxidation of lipids that affects the sperm membrane integrity (Agarwal and Said, 2003). All of these factors are important processes and steps that affect sperm quality and its function directly. Dutta et al. (2019) demonstrated that higher levels and values of ROS cause lipid peroxidation, affecting the integrity of the membrane as well, cause sperm DNA fragmentation and apoptosis of germ cells, and ultimately result in altered male fertility.

Prolonged exposure to ROS causing defects within the mid-piece of the sperm has been reported as well (Pasqualotto et al., 2000). The formation and development of DNA lesions due to ROS cause sperm instability and lead to an increase in negative effects, such as increased sperm DNA fragmentation levels (Santiso et al., 2010). The cell membrane is

composed largely of lipid molecules (unsaturated fatty acids), which facilitate its oxidation through the process of LPO due to the production and the presence of high and elevated levels of ROS (Walczak-Jedrzejowska et al., 2013).

DNA damage caused by ROS can also increase the incidence of germ cell apoptosis, resulting in an overall reduction of the sperm concentration, thus affecting the semen parameters and quality and causing male infertility (Agarwal et al., 2003). ROS generally affects all forms of biological compounds, like carbohydrates, lipids, proteins, and nucleic acids within cells. It can also alter the physical and chemical properties of proteins (Luddi et al., 2016). A significant high correlation has been reported between the levels of ROS and abnormal parameters, such as head problems, acrosomal deformities of structure and function, structural midpiece anomalies of the sperm, problem of cytoplasmic droplets, and major tail defects and problems (Kobayashi & Suda, 2012).

ROS also leads to the activation of caspase by disrupting mitochondrial membranes, which further leads to apoptosis. The apoptotic pathways also involve the release of cytochrome c, augmenting the levels of ROS, DNA damage, and apoptosis. DNA bases are also susceptible to damage from OS, resulting in some base pair modifications and strand breaks and hindering chromatin cross-linking processes. OS is reported to be a major factor in causing damage to the DNA in germ line cells (Agarwal et al., 2005; Thomson et al., 2009).

Damage to the DNA of the male germ line contributes not only to infertility but also to miscarriages and birth defects in future offspring (Aitken & Baker, 2009).

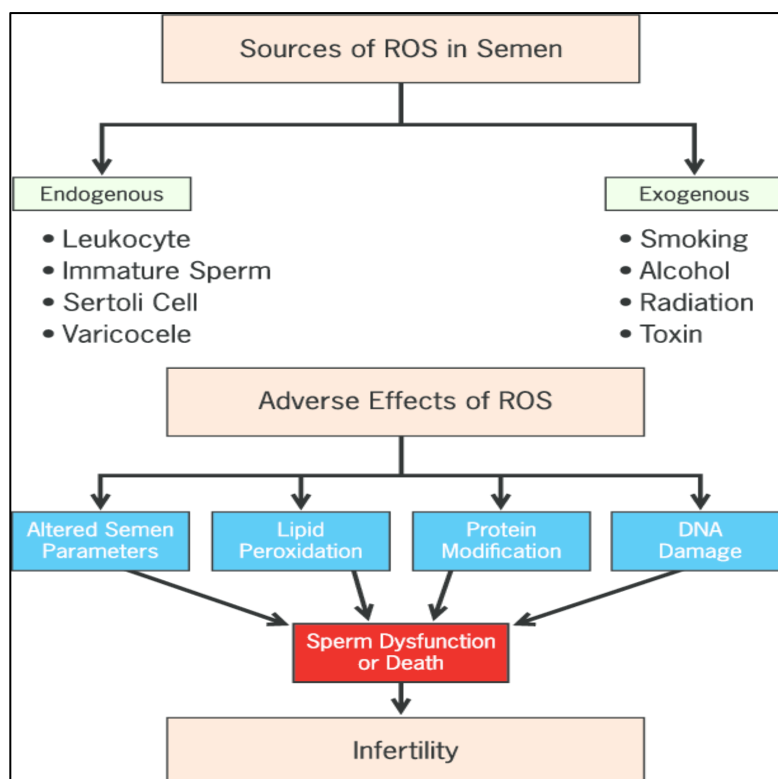

Figure 6: Reactive oxygen species sources and their effect on biological systems and infertility.

1.4.1. Reactive oxygen species endogenous sources

During metabolism, the ROS generates normally at low concentration, which is needed in various cellular functioning (de Lamirande et al., 1997). About 0.2%–2% of the total oxygen absorbed and consumed by the cell is converted or either emitted as ROS (Balaban et al., 2005). Genomic instability can also be caused by ROS accumulation and OS. It also results in the buildup of these misfolded proteins in the cell and causes alteration to multiple processes, like proteostasis, autophagy, and mitochondrial functions (Liochev et al., 2013; Sala et al., 2016). The endogenous ROS is formed mainly by leukocytes, such as neutrophils and macrophages, and by immature forms of spermatozoa, while other exogenous sources of ROS include smoking different forms of tobacco, industrial compounds, and ethanol or alcohol consumption (Murphy et al., 2011).

1.4.1.1. Leukocytes

Under certain circumstances, leukocytes, particularly neutrophils, can generate high levels of ROS. The production of these high levels of ROS plays a very significant and important role in the overall body and defence mechanism against any foreign bodies and infections (Walczak-Jedrzejowska et al., 2013).

ROS is also important in the integral mechanism of body defence whereby neutrophils destroy pathogens and help defend against infection. This, in turn, provides evidence and markers, which links high levels of seminal leukocytes to increased levels of OS, gradually resulting in male infertility (Tremellen, 2008).

Polymorphonuclear leukocytes (50%–60%) and macrophages (20%–30%) are peroxidase-positive leukocytes (Saleh et al., 2003). In prostate and seminal vesicles, these leukocytes are present in large quantities. Infection activates various stimuli for the production of ROS, so leukocytes can release up to 100 folds more ROS than they do under normal conditions without the stimuli. Infectionalso increases the NADPH production and levels by activating the hexose monophosphate shunt (Lavranos et al., 2012; Agarwal et al., 2003). Further, an increase in the levels of proinflammatory markers and factors like cytokines and interleukin (IL-8) and low quantities of antioxidants, such as superoxide dismutase (SOD), can cause a respiratory burst. ROS in elevated quantities can cause damage to the sperm if the seminal concentration of leukocyte is high, like in leukocytospermia (Lu et al., 2010). The presence of peroxidase-positive cells > 1 mill/ml in male semen is set as a quantitative limit by the World Health Organization (WHO, 2010).

Several studies suggest a correlation between alterations of sperm function and seminal plasma with multifactor abnormalities, such as elevated levels of ROS, IL-8, and IL-6 and tumour necrosis factor. All of these factors increase the levels of LPO in the membranes of the male sperm cell (Lavranos et al., 2012; Nandipati et al., 2005). The important sources of OS in the human body and cells are environmental exposure to contaminants, rays in the form of radiation, and chemical compounds, such as anti-cancerous medication and drugs, tobacco products, and alcohol (Sosa et al., 2013). In vivo nicotine, especially, is an oxidative agent that induces double-strand DNA breaks in the DNA of the sperm cells (Arabi, 2004). A 48% increase in seminal leukocyte levels and a 100% increase in ROS levels have been reported among smokers compared with the nonsmoker group (Saleh & Agarwal, 2002). The values of DNA fragmentation (DFI) in sperm cells have been compared between infertile smokers and infertile nonsmokers (37.66% vs. 14.51%, $P < 0.001$, respectively; Elshal et al., 2009).

1.4.1.2. Infections

Inflammatory reactions of male reproductive tract and ducts are considered as an exogenous source of ROS (Gonzales et al., 2004). In an earlier study, a high production of ROS in chronic inflammation with a nonbacterial cause was reported (D'agata et **al., 1990).** Mazzilli et al. (1994) demonstrated that the production of superoxide anion was elevated in patients whose sperm culture tested positive for aerobic bacteria. Also, products of cytokines can increase the levels of ROS generation in polymorphonuclear leukocytes (Zhang et al., 2013). Increased ROS production has been noticed during virus infection as well. When the cell is invaded by a virus, it distorts the normal cellular functions, disrupting the overall ROS system (Nabel & Baltimore, 1987). Besides, a strong correlation between the inflammation of the male genital system and infertility has been found and reported (Ochsendor, 1999). ROS production in the urogenital system is dangerous for sperm cells because ROS can target and affect them for a long period and because they have very low antioxidant protection (Nicopoullos et al., 2004; Frodsham et al., 2006).

Infections are found to be responsible for up to 15% of male infertility cases (Moretti et al., 2009). Chlamydia and gonorrhoea are among the main bacterial infections (Zeyad et al., 2018). Sexually transmitted diseases (STDs), specifically bacteria, usually transmitted during intercourse, can also affect sperm quality and the overall parameters. Reports have shown that around 33% of men get affected due to this reason (Moretti et al., 2009).

1.4.1.3. Immature spermatozoa

In human spermatozoa, hydrogen peroxide (H_2O_2) is the main form of ROS. The excessive production of ROS by different sources, like abnormal spermatozoa or leukocytes, has been linked to male infertility (Sharma & Agawal, 1996). The main sources of ROS reported by another study are either immature or abnormal spermatozoa or leukocytes (Lobascio et al., 2015).

During the complex process of spermatogenesis, developing spermatozoa prepare for fertilisation and shed their cytoplasm. However, an excess amount of cytoplasm (ERC) was retained by immature spermatozoa.

ERC activates the system of NADPH through the process of the hexose monophosphate shunt, which is consumed by the spermatozoa as a major source of electrons, which also leads to the ROS generation (Rengan et al., 2012). ERC affects sperm parameters, such as overall

motility, the structure of the sperm cell, and its fertilisation capability, which may cause male infertility (Saalu et al., 2010). High levels of ROS have also been observed in morphologically abnormal spermatozoa (Tomlinson et al., 1992; Figure 7).

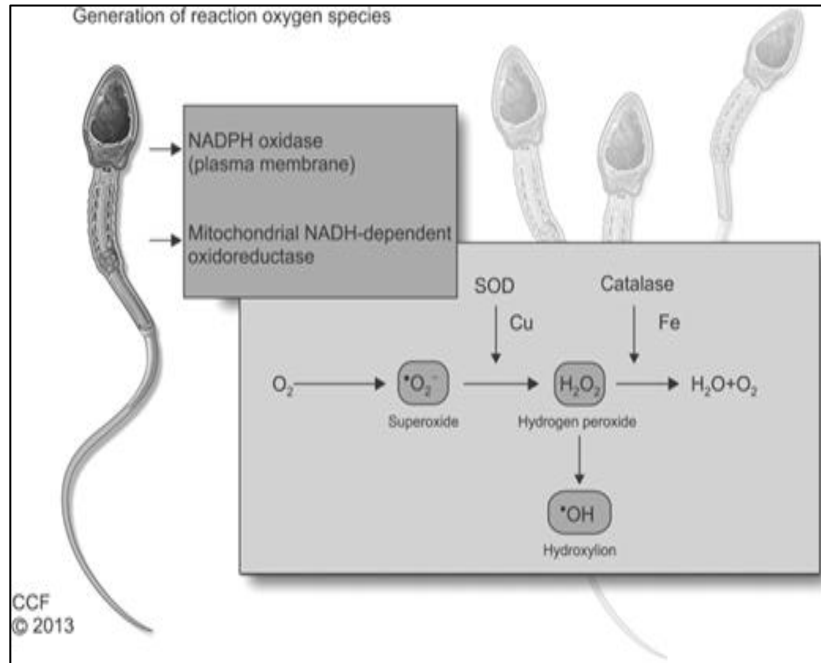

Figure 7: Generation of ROS. NADPH (nicotinamide adenine dinucleotide phosphate) and NADH (nicotinamide adenine dinucleotide), Cu and SOD [Agarwal et al. (2014)].

1.4.1.4. Varicocele

Veins that get abnormally dilated in the pampiniform plexus near and around the spermatic cord are known as varicoceles. Varicoceles are also sometimes responsible for infertility as they have been detected in 40% of infertile males (Will et al., 2011). Seminal ROS levels have been reported to be directly associated with varicoceles and their grade (Shiraishi et al., 2012). Ha et al. (2011) reported that ROS also possesses the capacity to damage the blood-testis barrier between Sertoli cells. Therefore, a high level of ROS is harmful in patients with varicoceles (Ha et al., 2011). Fisher et al. (2003) stated that sperm cells from such patients showed high cytoplasmic droplets, causing the production of ROS in higher levels. Thus, it is obvious that a high level of ROS is proportionally related to the high grade of varicoceles (Fisher et al., 2003).

Several studies suggested a link between varicoceles and decreased antioxidant levels in men (Agarwal et al., 2003), leading to high levels of ROS (Meucci et al., 2003). Patients with varicoceles have more spermatozoa with a high degree of abnormal condensation of the chromatin than fertile control males (Talebi et al., 2008). Towards the spermatozoa, NO and

superoxide released and produced by monocytes form peroxynitrite and have, in turn, more negative effects. NO is a lipophilic molecule and has a tendency to have cytotoxic effects on sperm cells adjacent to it (Santoro et al., 2001).

A meta-analysis demonstrated a direct relationship between a high degree of DNA fragmentation (SDF) and the grade of varicoceles in male patients irrespective of fertility status and the situation of the individuals (Zini & Dohle, 2011).

Another meta-analysis showed a significantly raised SDF value of 9.84% in patients with varicoceles compared with healthy controls (Wang et al., 2012). There are further reports on the impact and consequence of varicoceles on sperm quality or parameters and specifically on SDF and pregnancy outcomes. SDF reduction by 3.37% was reported after varicocele ectopy (Wang et al., 2012). After varicocelectomy, lower SDF values and a higher chance of conception either naturally or with the help of assisted reproduction were observed (Smit et al., 2013). Conception and live birth rates in the ICSI cycle also increased after varicocele treatment (Esteves et al., 2016).

OS has been a main concern and major cause of male infertility. In response to varicocele, the testis responds differently as the scrotal area temperature increases either by testicular hypoxic condition, adrenal metabolite backflow, or the change and increase in the production of vasodilators, including compounds like nitric oxide (Shiraishi et al., 2012).

1.4.2. ROS and its exogenous sources

Several factors, such as lifestyle, excessive increase in levels of environmental pollutants, alcohol consumption, tobacco smoking, e-cigarettes and vaping, physical trauma, and different kinds of stress, are considered few among the major exogenous factors and reasons for ROS production (Rakhit et al., 2013; Barazani et al., 2014).

Water and air pollution are considered major ROS exogenous sources, as well as exposure to heavy metals, radiation, and several types of drugs (Agarwal et al., 2016). Besides, regular consumption of alcohol leads to ROS production in excessive quantities, resulting in the peroxidation of lipids and in reduced SOD activity and GSH levels (Kovacic, 2005). During ART procedures, ROS can be produced from spermatozoa, oocyte, embryos, culture media, and oxygen concentration in the incubator; these are known as endogenous sources (Agarwal et al., 2014; Figure 7).

1.4.2.1. Alcohol consumption

Alcohol is a broad term. The common form used is known as ethyl alcohol or ethanol (EtOH). It promotes the excessive production of ROS. These ROS interact with macromolecules of the cell producing aldehydes in major forms, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), which are most involved in ROS production. It is also reported that these aldehydes and ROS directly interact with both proteins and DNA, ultimately leading to transcription-repression of genes and affecting their regulation as well. In fact, the impact of ROS and aldehydes appears to serve as a key factor in these alterations and defects, partially affirming that antioxidant intake helps prevent EtOH-induced cellular alterations and repression (Byun, 2018). In the metabolism pathway of alcohol, chemicals like NADH and acetaldehyde are generated. These NADH surges the activity of respiratory chains inside the mitochondria, while acetaldehyde interacts with proteins and lipids to produce ROS (Agarwal et al., 2005; Saleh & Agarwal, 2002).

Alcohol is the most studied dietary factors as potential disruptors of fertility. However, the results are inconsistent. Various studies showed the deleterious effects of alcohol (Eggert et al., 2004; Tolstrup et al., 2003), whereas others demonstrated no association (Hatch et al., 2012; Peck et al., 2010; Mikkelsen et al., 2016; Spinelli et al., 1997; Hassan et al., 2004). The evidence from couples undergoing ART or any of the infertility treatments remains controversial (Abadia et al., 2017). A study on 8,344 healthy male subjects reported that consuming alcohol in moderate quantities correlated with higher testosterone levels, but semen parameters didn’t show any change (Jensen et al., 2014).

Chronic alcohol consumption showed impact on both semen quality and its parameters and on the levels of reproductive male hormones (Muthusami & Chinnaswamy, 2005). High levels of ethanol administration decreased testicular steroidogenesis by disrupting testosterone synthesis and by decreasing the antioxidant enzyme activity, resulting in an OS increase (Maneesh et al., 2005). The nucleous and plasma membrane of spermatozoa abnormalities have also been reported with the increase in alcohol consumption (Sharma et al., 2004). An experimental study showed a correlation between ethanol consumption and increased chromatin abnormalities in sperm cells (Talebi et al., 2011).

1.4.2.2. Cigarette smoking

Worldwide tobacco product consumption directly contributes to multiple health problems, and around six million die annually because of it. Roughly 600,000 people also die each year from passive smoking, which is from exposure to second-hand smoke (Low & Binns, 2013). Currently, smoking contributes to the death of around 1 in 10 adults worldwide, and according to the WHO statistical data, the number of people who smoke is increasing, especially in developing countries despite various attempts by governments to ban or decrease the sale or use of tobacco and its products (Ng et al., 2014).

Deaths due to tobacco consumption worldwide are among the preventable types. Specifically, tobacco cigarettes are known to contain around 4,000 different toxins and chemical compounds that are hazardous in nature, including alkaloids, nitrosamines, and many other inorganic molecules. These toxins and chemicals cause disproportionate levels of ROS generation and antioxidant levels in the semen of tobacco smokers (Lavranos et al., 2012). This imbalance (OS) has many effects on the semen quality and its parameters. Studies have linked smoking to a 48% increase in leukocyte concentrations in seminal plasma and a significant increase of 107% in total ROS levels (Saleh et al., 2002). It decreases sperm parameters, such as total motility and morphology (Saleh & Agarwal, 2002). It is also a key factor in producing high levels of ROS through lipid peroxidation (Künzle et al., 2003). Dai et al. (2015) also reported that tobacco smoking negatively affects sperm parameters, such as volume, concentration, motility, morphology, and viability, leading to male infertility (Dai et al., 2015).

Levels of antioxidants, such as vitamins E and C, are decreased among smokers, increasing the OS and damaging sperm cells (Esteves et al., 2002). This was also confirmed by a study in which a significant increase in the levels of 8-OHdG was noted in the male seminal plasma due to tobacco smoking and was considered as marker of the oxidative damage (Dietrich et al., 2003; Mohammedi et al., 2013).

Another study performed on tobacco smokers and its analysis revealed that higher levels of heavy metals, such as cadmium and lead, in serum and in semen increase the production of ROS, reducing sperm motility (Kiziler et al., 2007).

Moreover, cigarette smoking increases inflammatory reactions, resulting in increased levels of leukocytes in the testicles (Majo et al., 2001; Jorsaraei et al., 2008). Fragmentation of the

sperm DNA, axonemal damage, and decreased concentrations of sperm cells have also been observed among smokers (Rubes et al., 2005; Zavos et al., 1998; Sun et al., 1997).. Some other chemicals that are present in tobacco smoke and cause damage to the cells are tar, nicotine, CO, hydrocarbons, such as polycyclic aromatic hydrocarbons, some radioactive compounds, and toxic heavy metals. All of these chemicals and compounds directly or indirectly contribute to male infertility (Halmenschlager et al., 2009). Tobacco smokes either directly or indirectly enhances OS—directly through the mechanism of producing reactive oxygen radicals and indirectly through the process of reducing antioxidant levels, downregulating the antioxidant defence mechanisms (Shiels et al., 2009; Trummer et al., 2002).

Some of the studies also indicated that tobacco smoking has a major effect on both types of cells (Leydig & Sertoli) cells. It can cause a change in levels of PRL (Halmenschlager et al., 2009), E2, FSH, testosterone, LH, and SHBG in plasma (Shiels et al., 2009; Trummer et al., 2002).

Besides altering semen parameters, smoking also affects glands, such as pituitary, thyroid, and adrenal glands. These effects were observed in both fertile and infertile males (Kapoor & Jones, 2005). ART procedure outcomes have also been observed to be lower in couples undergoing IVF and ICSI treatment when the male partners are smokers (NCCWCH, 2004; NICE, 2013).

1.4.2.3. Radiation

Radiation on humans has significant effects clinically. Exposure to radiation causes an increase in chromosomal aberrations and abnormalities (Martin et al., 1986).

The male organ most sensitive to radiation exposure is the testis, including the germinal epithelium and spermatogonia (Fischbein, 1997; Xu et al., 2008). Several studies concerning male reproductive health reported that mobile phone radiation increased ROS production in semen samples and caused impaired parameters (Agarwal et al., 2008; Aitken et al., 2005). Further, the duration of exposure correlated with impaired semen quality and parameters, such as concentration, total motility, and morphological aspect (Agarwal et al., 2008). Another study demonstrated that radiation types, such as electromagnetic energy and radiation, can induce ROS production, which can damage the DNA of spermatozoa. The duration of radiation exposure affects sperm parameters as well (De Iuliis et al., 2009).

Another study states that radio frequency electromagnetic wave (RF-EMR) causes DNA fragmentation (De Iuliis et al., 2013). The conclusion of these studies confirmed the impact of radiations on multiple factors related to male fertility. In addition, radiofrequency electromagnetic waves (RF-EMR) disrupt normal cellular and organelle functions (Lavranos et al., 2012).

Studies revealed that radiation from cell phones and related equipments, microwave ovens, laptops, or even Wi-Fi produces has deleterious effects on the testes' functions, which may damage the sperm quality and increase damage to the DNA, causing micronuclei formation and leading towards genomic instability, protein kinases, other hormones, and antioxidant enzyme disruption. RF-EMF can also induce OS with an increased level of ROS, which, as described earlier, has negative effects on sperm quality and may lead to infertility (Kesari et al., 2018).

1.4.2.4. Toxins

Toxins possess a potential negative on the structure and the function of the sperm cell (Esfandiari et al., 2002). Phthalates are found in both domestic and industrial products (Pant et al., 2008; Latini et al., 2006). They harm spermatogenesis and damage the DNA of the sperm cell (Kasahara et al., 2002). Occupational exposure to toxins, such as heavy metals, including cadmium, chromium, lead, manganese, and mercury, impairs semen concentration and other semen parameters (Jurasović et al., 2004).

Furthermore, levels of polychlorinated biphenyls (PCBs) and semen quality have been found to be correlated. Mitochondrial dysfunction can be caused by the loss of intracellular molecules, such as ATP, increasing ROS production and decreasing sperm motility (Jiang et al., 2017). These toxins can also bring estrogenic or anti-androgenic effects to an altered state of the hypothalamic-pituitary-gonadal axis. This damages the sperm and its DNA. These toxins have also been reported to cause epigenetic changes to the sperm (Mima et al., 2018).

1.4.2.5. Obesity

Obesity has been linked to various health issues (Lastra et al., 2006). According to WHO, obesity is classified as someone with a body mass index (BMI) greater than 30, and an overweight person is someone with a BMI of 25 (Sikaris, 2004). Obesity has physiological, psychological, and economical effects irrespective of the cultural and ethnic status (Zhang et al., 2020). Obesity can also lead to the production of superoxide from multiple pathways, like

from oxidases of NADPH (NOX), oxidative phosphorylation, protein kinase C (PKC) activation, glyceraldehyde auto-oxidation, and polyol and hexosamine pathways (Savini et al., 2013; Serra et al., 2012). ROS has also been reported to affect body weight by interacting with the hypothalamic neurons, which are normally involved in the control of satiety and hunger behaviour (Horvath et al., 2009). Obesity has been linked to the systemic induction of OS, which is associated with the disruptive and irregular production of adipokines, resulting in metabolic problems (Esposito et al., 2006).

Various studies have shown that fat- and carbohydrate-rich diet increases OS and has a role in inflammation in people already suffering from obesity (Patel et al., 2007). Different studies reported that OS plays a major role in the pathogenesis of obesity and can lead to infertility (Savini et al., 2013). Suboptimal semen quality and disrupted hormonal balance in obese people are due to ROS and OS overproduction (Kashou et al., 2012). The OS in such patients may be further promoted by the dysregulation of adipocytokine and high levels of ROS (Furukawa et al., 2004). High saturated fat intake has also been reported to be associated with decreased sperm concentration, linking obesity with male infertility (Tsai et al., 2013). A large meta-analysis reported that sperm morphology was disrupted among obese males (Campbell et al., 2015).

Spermatogenesis is further affected by the increased ROS production and testicular temperature, which has the tendency to denature the required enzymes (Hjollund et al., 2000). Decreased sperm concentrations are also correlated with the increased scrotal skin temperature (Hjollund et al., 2000).

1.4.2.6. Diabetes

A metabolic disorder, such as diabetes mellitus, is characterised by high blood sugar levels and is linked to intense and higher levels of free radicals along with reduced and antioxidant capabilities. All these factors lead to macro- and microvascular complications (Bashan et al., 2009).

The mechanism through which OS works and sometimes expedites the complications of diabetes is still under research. Atli et al. (2004) pointed out that there was an increase in OS in patients suffering from type 2 diabetes (T2D), which can be comparatively balanced or tackled by an improved antioxidant defence system. For the first time, Baynes et al. (1991) linked ROS elevation to diabetes mellitus. ROS levels increased in diabetic patients, and

antioxidant defence capacity was impaired (Bloch-Damti et al., 2005). However, the overgeneration of superoxide in mitochondria in hyperglycaemic patients (Valko et al., 2007) resulted in OS (Bloch-Damti et al., 2005). Agbaje et al. (2007) showed that sperm cells had more fragmented DNA in men with diabetes. Further, studies demonstrated that sperm chromatin DNA integrity in diabetic patients was affected (Alves et al., 2013; Mangoli et al., 2013). Spermatozoa's quality was also impaired in these patients (Talebi et al., 2014; Vignera et al., 2012).

1.4.2.7. Aging

The progressive loss of tissue and organ functions is associated with age. Aerobic cells in the body produce reactive oxygen and nitrogen species (RONS), and they have a huge impact on ageing and diseases associated with it (Venkataraman et al., 2013). Drastic decreases in semen parameter have been observed in males above the age of 40 years (Katib et al., 2014; Ramasamy et al., 2015). In addition, the increased deterioration of the DNA of sperm has been reported to correlate with increasing age in fertile males (Wyrobek et al., 2006; Moskovtsev et al., 2006). Moreover, single- or double-strand breaks among nuclear DNA, modification of bases (8-oxo guanine, 8-oxo-7, 8-dihydro- 2′deoxyguanosine), in addition, to cytosine glycol and to thymine glycol has been registered (Jacob et al., 2013).

The total sperm volume, vitality, motility, concentration, morphological abnormality, and overall poor sperm kinematics have been observed and increase with age (more than 40 years). Oxidative damage appears to increase with age (Jacob et al., 2013). Thus, changes in sperm parameters may be related to ROS production in elderly patients (Veron et al., 2018; Desai et al., 2017).

In addition, the process of ageing predominantly includes loss of telomere caps, oxidative damage to the subcellular structures, and overall damage to the DNA from oxidation due to free radicals. These species include ROS, nitrogen species (RONS), reactive aldehyde species, transition metal intermediates, and products of advanced glycation end (AGE) (Singh et al., 2001; Jacob et al., 2013).

Accoding to Harman's free radical theory of ageing, collective oxidative DNA damage and other cellular components and tissue over a period of time cause ageing and ultimately lead to death (Adams et al., 2015). Among the elderly, low cognitive capabilities were reported to be

associated with high levels of inflammatory cytokines, which increase the levels of OS markers (i.e., MDA, GSH-Px, and PC; Baierle et al., 2015).

1.4.2.8. Physical exercise

Personal routines and habits affect one's quality of life and may expose a person to various risk factors and diseases. For example, a physical activity or exercise can cause adverse effects, like extreme exertion leading to the production of large amounts of ROS, which then causes OS (Peake et al., 2007). ROS production has been examined during and after exercise, and the results have shown that excessive exercise leads to ROS production and OS, which in turn give rise to infertility (Cooper et al., 2010; Nikolaidis & Jamurtas, 2009; Berryman, 2010).

However, ROS production has some supportive functions, such as in muscle metabolism, homeostasis, and the adaptive response to exercise (Pattwell, 2004; Powers et al., 2011).

Extreme physical activity increases oxygen consumption, energy demand, and consequently, ROS production to meet the energy demands of the body for carrying out various activities (Gomes et al., 2017). However, ROS produced because of intense exercise is important in cell signalling. This elevated production may not only pose a risk to the health of the person but also represent a signalling adaptation response to exercise (Silva, 2015).

Various studies have shown that acute exercise-induced OS contributes to proteinuria in untrained rats in a post-exercise scenario (Gündüz & Şentürk, 2003). Maximal bicycle workouts and exercise have also led to DNA strand breaks (Moller et al., 2001).

It has been reported in various studies that strenuous forms of exercise for at least half an hour increases oxidant production in the muscles, limiting a person's overall performance and causing ROS production (Reid, 2016).

Furthermore, it has been suggested that ROS may be produced and generated enzymatically by extra-mitochondria in contracting muscle, including NADPH oxidase (NOX) (Sakellariou et al., 2013) and xanthine oxidase (Powers et al., 2016).

Exposure to cold and heat results in stress, which is acute in nature, depends on the presence of causative factors, and is removed with the removal of the aetiological factor. Similarly,

stress that occurs due to physical activities or even due to complete immobilisation is considered acute (Rahal et al., 2009).

1.4.2.9. Psychological stresses

A person having mental stress is exposed to many hazards. Sperm quality is decreased by psychological stress, which enhances ROS production inside seminal plasma by diminishing the capacity of antioxidants (Fenster et al., 1997). The presence of psychological stress distorts the function of gonadotrophins, which are important hormones regulating many functions within the body (Eskiocak et al., 2006). Psychological stress increases the serum level of cortisol (in human), causing apoptosis in Leyding cells (Gao et al., 2002).

In a meta-analysis including 29 different studies, Jiménez-Fernández et al. (2015, 2021) reported that different OS markers, such as MDA and others like total nitrites, were higher in patients with depression disorders, whereas various antioxidants types, like uric acid, zinc, or antioxidant-enhancing enzymes, such as SOD, catalase (CAT), and glutathione peroxidase (GPX), were lower in comparison to healthy controls. They suggest that OS plays a role in depression and that antidepressant activity may be mediated by improving the antioxidant function (Jiménez-Fernández et al., 2015, 2021).

It was demonstrated that associated directly with higher OS, MDA levels ($P < 0.001$), lower antioxidant uric acid ($p = 0.06$; $p = 0.030$) and zinc levels ($P < 0.0001$), and higher SOD levels were reported as well ($n = 902$; SMD <0.62; 95% CI, 0.07–1.17; $P < 0.028$). However, changes in the levels of total nitrites, CAT and GPX, were found to be nonsignificant.

Treatment of patients using antidepressant medication significantly reduced the Hamilton depression rating scale scores (SMD = 2.65; 95% CI, 1.13–4.15; $P = 0.00065$), reduced MDA levels (4 studies; $n = 194$; SMD = −1.45; 95% CI, −2.43 to −0.47; $P = 0.004$), and increased uric acid levels (3 studies; $n = 212$; SMD = 0.76; 95% CI, 0.03–1.49; $P = 0.040$) and zinc levels (3 studies; $n = 65$; SMD = 1.22; 95% CI, 0.40–2.04, $P = 0.004$) without differences in MDA levels ($P = 0.60$), uric acid ($P = 0.10$), and zinc ($P = 0.163$) compared to the normal and healthy control group.

1.5. Relationship between cigarette smoking and male fertility alterations

Tobacco consumption is another factor that negatively affects human health. Despite efforts worldwide to make people aware of its consequences on health, there are still 1.1 billion

smokers worldwide, with majority of them suffering from negative effects produced by tobacco (WHO, 2018).

Cigarette smoke has many hazardous substances, including tar, nicotine (addictive compound), carbon monoxide, and heavy metals (e.g., cadmium and lead). The more smokers are exposed to these, the more they are at increased or immediate risk of developing various conditions, including infertility (Dai et al., 2015).

Cigarette smoke contains around >7,000 chemicals, such as nitrosamines, which are tobacco-specific [e.g., nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone], polycyclic aromatic hydrocarbons, and further different volatile organic compounds, such as benzene (CDC, 2010). These chemical substances could cause damage in almost all the human organs, for example, in the lungs, heart, circulatory system, immune system, and male and female reproductive systems (Drope et al., 2018).

Cigarette smoke and tobacco usage are considered to be among the main factors causing infertility. Smoking results in leucocytospermia, as discussed previously, and is considered as a major endogenous source of ROS. Tobacco smoke contains various hazardous and endogenous substances that result in increased ROS generation and production. The level of ROS increases in semen, and the spermatozoa become more prone to the risk of OS. As a result, the function of sperms gets impaired, thus affecting male fertility (Harlev et al., 2015). However, the mechanism by which smoking affects the quality of sperms is not yet fully explained.

Many studies were previously conducted to explain the adverse effects of smoking on different sperm parameters. In a meta-analysis involving regular male smokers from 26 countries, the results showed that smoking was responsible for reducing the overall sperm quality, count, and other parameters in fertile and nonfertile males (Li et al., 2011). The study also revealed that among male smokers, the sperm count declined to 13%–17% compared to the sperm count among nonsmokers (Vine et al., 1994).

The total decline in semen parameters was found to be more prominent in heavy smokers who smoke >20 cigarettes/day, in the moderate group (10–20 cigarettes/day), and in the mild smoker group (1–10 cigarettes/day).

Thus, the sperm quality and the chance of developing infertility are directly related to the number of cigarettes smoked. The negative effect of cigarette smoking was shown to be higher in infertile male patients than in the general population (Sharma et al., 2016).

Another meta-analysis including 13.317 men was conducted to analyse the effect of smoking on sperm quality, showing that cotinine was responsible for breaking down testosterone hormones (Zhao et al., 2016). Also, paternal smoking is a factor that may result in a number of abnormalities in the offspring.

It has also been reported that pre-conception paternal tobacco smoking increases the chances and risk of multiple forms of morbidities in the foetus and offspring, which could be mediated through epigenetic modifications (Jenkins et al., 2017).

Male tobacco smokers showed a tendency towards increased alteration levels of global methylation patterns genome-wide among their sperm DNA compared with those who never smoked (Jenkins et al., 2017).

Mothers who smoked during the pregnancy or lactation period could potentially cause harmful effects on male children. The changes that occur as a result of maternal smoking during gestation and lactation include decreased number of germ cells, damage to the DNA inside the germ cells, and the formation of defective sperms in male offspring (Sobinoff et al., 2014).

Paternal smoking may decrease the success rate of ART outcomes (Kovac et al., 2015). Moreover, male smoking habits could influence the outcomes and clinical pregnancy rate per intrauterine insemination (IUI) cycle, thus posing a threat to fertility (Thijssen et al., 2017). However, smoking cessation by male partners reduces the risk of ART failure by 4% (Vanegas et al., 2017).

1.6. Alcohol intake and male fertility alterations

The consumption of alcohol affects the male reproductive function by altering the entire HPG axis (hypothalamus–pituitary–gonadal). The production and distribution of GnRH, FSH, LH, and testosterone is affected by alcohol intake, and Leydig and Sertoli cells functions get altered as well. Thus, alcohol intake impairs the spermatozoa production, shape (morphology of sperm), and sperm maturation (Emanuele & Emanuele, 1998).

Several studies have shown the mechanism by which alcohol interferes with spermatozoa production. Alcohol affects GnRH by various mechanisms, which include distorting its cleavage from its precursor forms and interfering with the movement of protein kinase, C15 proteins, which is required for GnRH to stimulate the production and release of FSH and LH from the pituitary gland (Uddin et al., 1996; Kim et al., 2003). Eventually, the endocrine balance is disrupted, and consequently, the semen parameters are negatively affected (Salonen & Huhtaniemi, 1990).

The levels of various hormones in the body, such as FSH, LH, and testosterone, are affected by chronic alcohol consumption, which negatively affects the interactions between the neural and endocrine systems (Emanuele & Emanuele, 2001; Maneesh et al., 2006).

It is evident from several studies of animals and humans that increased alcohol consumption eventually increases the level of a hormone called estradiol, which stimulates the production and release of beta-endorphin (Emanuele & Emanuele, 2001). Various experimental studies have shown that the chronic use of alcohol affects semen total parameters, its quality, and the levels of male reproductive hormones (Pajarinen et al., 1996; Muthusami et al., 2005). Studies involving heavy drinkers and nondrinkers have shown that in heavy drinkers, spermatogenesis is arrested either partially or completely; it also results in the Sertoli-cell-only syndrome (Pajarinen et al., 1994).

Sertoli cells tend to be more affected by chronic and prolonged alcohol intake (Zhu et al., 1997). Prolonged alcohol intake causes Sertoli, Leydig, and germ cell atrophy (testicular atrophy) and decreases the size of seminiferous tubules and their lumen, leading to apoptosis of Sertoli cells and, consequently, male infertility (Zhu et al., 1997).

Studies on various infertile couples made it evident that teratozoospermia was found in around 63% of moderate alcohol drinkers (40–80 gm/day) and in 72% of heavy alcohol drinkers (who drank more than 80 gm/day). These studies suggest that alcohol consumption plays a very significant and complex role in male infertility (La Vignera et al., 2012). In a study conducted by Gaur et al. (2010), around 64% of the participants were oligozoospermic, suggesting the damage of the testes in response to regular alcohol consumption (Gaur et al., 2010). Similarly, oligozoospermia was significantly higher in men with extreme alcohol consumption than those in the control group (Zhang et al., 2012). Therefore, alcohol intake

does play a pivotal role in causing infertility, but the threshold amount has not yet been determined.

One study also addressed and reported the dose-dependent response and relationship between recent alcohol consumption (during the last five days) and semen parameters among young Danish men (n = 347). They found that semen characteristics decline with increasing alcohol consumption, but not in a dose-dependent way. Also, the estradiol/testosterone ratio increased proportional to alcohol intake (Hansen et al., 2012).

1.7.Purpose

The aim of this research and study was to investigate the effect of alcohol consumption and tobacco smoking on sperm parameters determined according to the WHO laboratory manual (pH, sperm volume, count, total motility, morphologically normal spermatozoa, vitality, and membrane functional integrity) in male partners of couples undergoing ART.

Moreover, to determine whether alcohol intake or cigarette smoke causes more deterioration to sperm DNA maturity (CMA3) positivity.and sperm DNA fragmentation (sDF).

2. Materials and Methods

2.1. Study Population

It is a cross-sectional study performed and conducted at the Laboratory facility of Reproductive Medicine, Department of Obstetrics and Gynaecology, at the Saarland University Hospital, Germany. Semen samples were collected from Prince Rashid Ben Al Hassan Military Hospital, located in Irbid city of Jordan and the samples were approved to be used, based on the approval received from the Medical Services Human Research Committee approval having an approval number of (8/2018) and all participants were given a written consent before they were getting included in this study. Two hundred and eleven men in reproductive age with primary infertility were included in this study. After excluding the infertility reasons from and caused by female factors and in this study women younger or less than 40 years of age with normal menstrual cycles were included only, also with normal ovulation, and uterine cavity.

Moreover, the criteria that were kept to include the patients visiting for treatment, were as follow, males who never or did not suffered from or have any form of cryptorchidism, or have received any sort of present or past cancer or chemotherapy treatment and medication, further genetic or inherited and acquired abnormalities and anomalies such as Klinefelter's syndrome patients or having microdeletions at Y-chromosome, hypogonadotropic hypogonadism (hormonal disorder), any other drug abuse, having varicocele of any grade, and/or recent fever episode. Each patient included in the study had a physical evaluation and examination.

Repeat participants or Males who did not provide complete data and information about smoking habits and further about alcohol consumption were excluded. According to the number and volume of cigarettes smoked in one day and in a year the duration of cigarettes smoked, the participants were categorized to heavy-smoker participants (n=48) patients who consumers or smokes cigarette more than one pack/day for 10 years at least or two pack/ day for at least 5 years, and non-smokers (n=70) who did not smoke.

On the other hand, the measurement used for alcohol intake and consumption was estimated by the unites of alcohol consumed: 1 alcohol consumption unit was taken and considered as follow: 100 milli litre (ml) wine, and beer one unit equals to 200ml, 30 ml of whisky or either vodka. Two groups were studied: no alcohol consumers (n=41) and heavy alcohol consumers (n=52) drinking > 7 unites / week.

2.2. Methods

2.2.1. Reproduction and Andrology laboratory materials

Reagent or chemical	**Company**
Combur 2 Test LN	Roche, Switzerland
Eosin G (Sperm staining)	Merck, Germany
Immersion Oil	Merck, Germany
PureSperm 100 (Sperm preperation)	Nidacon international, Sweden
Sodium chloride (NaCl) 0,9%	B.Braun, Germany
1 ml insulin Syringe sterile U-40	BD Medical, USA
Biosphere Filter tips (10-20-200-100 ml)	Sarstedt, Germany
Centrifuge tube (15 ml)	Vitrolife, Sweden
Coverslips	R. Langenbrinck, Germany
Handling micropipettes	Origio, Denmark
Handling pipette for assisted reproduction	MTG Medical, Germany
Microscope Slides	R. Langenbrinck, Germany
Pipettes	Eppendorf, Germany
centrifuge bench-top	Sigma-Aldrich, Germany.
light microscope (Binocular)	Olympus, Japan.
CO2 incubator (C200)	Labotect, Germany
Incubator C16	Labotect, Germany
Makler Counting Chamber	Origio, Germany
Vortex-Genie 2	Scientific industries, USA.

2.2.2. During experimental part Reagents, chemicals, kits, and equipment

Reagent or chemical used.	**Manufacturer**
Ethanol (Absolute)	Merck, Germany.
Chromomycin A3 (CMA3)	Merck, Germany
DAPI(4′,6-Diamidine-2′-phenylindole dihydrochloride).	Merck, Germany
Methanol	
Nuclease-free water	
Paraformaldehyde (PFA) 4% in PBS	Merck, Germany
Phosphate buffer saline (PBS)	Qiagen, Germany
Sodium citrate	Morphisto, Germany
Tris-Acetate-EDTA buffer (TAE)	Sigma-Aldrich, Germany
Tris-EDTA (TE)	Merck, Germany
In-Situ cell death detection kit, Fluorescein (TUNEL)	Sigma-Aldrich, Germany
Fluorescence Microscope	
Light Microscope	Roche Diagnostics, Germany.

2.2.3. Collection of the Sample's Procedure

All Semen samples from the patients were collected, through the process of masturbation, after a period of sexual abstinence for minimum of three days. The container for the collection and storage of specimen was kept till the analysis on the heating stage or incubated at (37°C temprature) for minimum of 30 minutes to 1 hour for liquefaction process. Then, macroscopic examination was performed which included analysis such as (ejaculate appearance of the sample, its viscosity, and its pH further analysing volume) and during microscopic analysis (The total spermatozoa count and concentration, its total motility along with form of motility, vitality, also the aggregation, and morphology of the semen were observed and analysed) all of the evaluations was done according and by guidance of the WHO laboratory manual (WHO, 2010).

Table 1: Semen values according to the World health organization in 2010

Parameters	Reference value (Unit)
Semen samples volume	1.5 ml
Sperm concentration (10^6 per ml)	15 x 10^6/ml
Total motility (PR + NP)	40%
Progressive motility (PR)	32%
Vitality	58%
Sperm normal morphology	4%

*NP: non-progressive motility, *PR: progressive motility.

To examine sperm morphology, protamine deficiency and sperm DNA fragmentation, 4 smears were prepared using 20 µl of ejaculate. After the semen has liquified (>30 minutes), the specimen was good mixed before pipetting the aliquot onto the slide and then mixed again before preparing the next slide. The Slides air dried for 24hours.

Papanicolaou staining was performed to analyze and evaluate the sperm total morphology according to WHO (2010) strict criteria. After Slides preparation they were analyzed and evaluated for the morphological normal spermatozoa percentages and their head, midpiece, and flagellum defects with the aid of a bright-field microscope (Zeiss, Jena, Germany) under magnification power of 1,000x and included minimum evaluation of 200 spermatozoa.

All semen samples were later subjected to the process of purification to remove the somatic cells in the sample and other debris as well, the process was as follow: all of the semen samples from the patients were loaded onto 45%–90% “two-layer” discontinuous Pure-sperm gradients by (Nidacon International) and then subject to centrifugation at 500g at room temperature at least for 20 min, which completely separates normal sperm cells from other cells like of lymphocytes, epithelial cells, and also from abnormal or immature sperm cells, also from bacterial cells, and seminal fluid.

The supernatant was then discarded and by leaving behind only the sperm pellet at the bottom of the 95% fraction and further layered with sperm washing medium which was pre-incubated before and centrifuged again for another 500-x g for the time period of 10 min. The supernatant this time again was discarded leaving the pellet of sperm cells behind settled in the bottom of the tube. A desired and calculated volume and concentration of sperm washing media or solution was added to sperm pellet to make 1 ml final sperm suspension. then it was kept and stored at - 80ºC temperature for later use and for analysis.

2.2.4. Sperm Cells Vitality Assessment (Eosin-Nigrosin-analysis)

Sperm vitality test and analysis was performed and then analyzed on wet mount smears after using the supravital staining with aqueous eosin-nigrosin process as defined as follows. One drop of the semen sample was mixed on a fresh slide with one drop of 0.5% aqueous yellowish color eosin solution and one drop of nigrosin (10% in water) and covered with the help of cover slip. After analyzing again after time of 1–2 minutes the spermatozoa or sperm cells which stained red (indicated dead sperm cells) can be easily distinguished from theones who left unstained which were representing live sperm cells or spermatozoa. Nigrosine was further used to counterstain to facilitate visualization of the unstained live sperm cells. On each slide arround 100 sperm cells from each slide were analyzed and evaluated.

2.2.5. Assessment of Sperm Membrane Integrity (Hos-Test)

For the analysis of membrane integrity of the sperm cells, hos (hypo-osmotic swelling)-testwas used, total of 100 µl semen sample from the sperm suspension was added to hypoosmotic solution of 1 ml (containg equal parts of 150 mmol/l fructose and total of 150 mmol/l sodium citrate solutions), then after addition it was followed by a 60 min period of incubation at 37 ºC temprature. After this incubation period, a minimum number of 200 sperm cells were examined and analyzed for each slide with the help of light microscope and further

the percentage and number of spermatozoa that showed abnormalities like in tail part such as curly tail is an indicative of changes like swelling in it, these changes were noted and calculated.

2.2.6. Chromomycine (CMA3) staining: Protamine deficiency assessment

Proteins like protamine deficiency was measured in the sperm cells, by using chromomycin A3 (CMA3) staining method as previously presented by Hammadeh et al. (2010). Described as, semen sample aliquots were subject to washing in Dulbecco's Ca^{2+} – Mg^{2+} free phosphate buffer saline (PBS) and centrifuged at the speed of 250xg for 10 minutes. The sperm cells were then subjected to washing, then fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) at 48ºC for the period of 5 min and then spread on fresh clean slides. The CMA3 (Sigma, St. Louis, MO, USA) was then dissolved in Mcilvaine buffer (pH 7.0) supplemented with 10 mmol/l MgCl2 (17 ml of 0.1 mmol/l citric acid mixed with 83 ml of 0.2 mol/l Na2HPO4 and 10 mmol/l MgCl2) to a total concentration of 0.25 mg/ml.

Each of these slides was further treated for period of 20 min with using 100 ml of CMA3 solution in th ecomplete dark environment. Fluorochrome was then analysed and examined using a Zeiss photomicroscope III using a combination of exciter dichromic barrier filter of BP 436/10: FT 580: LP 470. A total number of 200 sperm cells were analyzed and evaluated on each slide prepared. Evaluation of CMA3 staining is reported and done by distinguishing between spermatozoa that stain bright yellow considered as positive (CMA3 positive) from those staining dull yellow considered as CMA3 negative.

2.2.7. The Terminal deoxyribonucleotidyltransferase-mediated dUTP Nick-End Labelling assay (TUNEL)

TUNEL assay for Sperm DNA fragmentation was performed as described previously by Borini et al., 2006, using in situ Cell Death Detection Kit method according to the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany) guidelines.

All the semen samples from the patients were smeared on a microscopic slide, after smearing they all were subject to air dried then fixed in 4% of paraformaldehyde in PBS, at a pH of 7.4 and then permeabilized with 0.1% Triton X-100 in 0.1% of the sodium citrate, at pH of 6.0. DNA that was fragmented was detected with the help of the TUNEL assay kit following the manufacturer's guidelines. For evaluation, a total number of 500 spermatozoa were analyzed on each slide and noted, analysis was done by distinguishing spermatozoa that stained bright

green (TUNEL positive, indicating fragmented sperm DNA) from those stained dull green (TUNEL negative, representing intact DNA of the sperm cell). A Zeiss Photomicroscope III was used for the analysis and for fluorochrome evaluation via a combination of exciter dichromic barrier filter of BP 436/10: FT 580: LP 470. For quality control, a negative control was performed for each sample by using fluorescein-isothiocyanate-labelled dUTP without enzyme (Borini et al., 2006).

2.2.8. Statistical Analysis

For the statistical modelling and analysis of the data IBM SPSS (Windows software package version 24.0 SPSS Inc., USA) was used. After checking and application of the skewness test, Kurtosis test, Z-value, and Shapiro test, it has been revealed and demonstrated that the samples were not normally distributed. Thus, for the comparison of the quantitative variables between the heavy-smokers and non-smokers groups, another test such as the Mann-Whitney U-test was primarily used and for the determination of the correlation between the different studied parameters of the sperm, further Spearman test was further used and applied as well.

3. Results

3.1. The characteristic of the alcohol consumption study group

Table 2: Descriptive statistics of the studied parameters, for all the population in Alcohol consumption group (N= 93)

Parameters	Mean	SD	Range (Min-Max)
Age (Years)	35.74	6.542	24-50
PH	8.65	0.39	7.7-9.5
Semen volume (ml)	2.97	1.49	0.5-8.5
Sperm count (mill/ml)	62.21	33.07	1-100
Total motility (PR + NP %)	28.71	15.98	0-90
Sperm Vitality (%)	39.30	18.17	5-80
Functional integrity (%)	51.51	19.10	10-90
Morphologically normal spermatozoa (%)	30.98	13.33	4-58
CMA3 positivity (%)	31.62	10.68	13-66
Sperm DNA fragmentation (sDF)(%)	17.79	8.39	3-41

Mill/ml: million per ml, PR: progressive, NP: non-progressive, SD: standard deviation

The patients mean age was 35.74 ± 6.542 years. The sperm parameters: pH, semen volume (ml), sperm count (mil/ml), also total motility (PR + NP. %), sperm vitality (%), functional integrity (%), and morphologically normal spermatozoa (%) were (8.65 ± 0.39; 2.97 ± 1.49; 62.21 ± 33.07; 28.71± 15.98; 39.30 ± 18.17; 51.51± 19.10; 30.98 ± 13.33, respectively) (Table 2). By those patients, the protamine deficiency (CMA3 positivity) determined by the CMA3 staining was ranged between 13 and 66 % with a mean of 31.62 ± 10.688 %, and the sperm DNA fragmentation (sDF) determined by the TUNEL assay was in the range (3-41) with a mean of 17.79 ± 8.398 %.

Table 3: Comparison between the semen analysed parameters between drinkers and non-drinkers

Parameters	**Drinker (N=52) (M±SD)**	**Non-Drinker (N=41) (M±SD)**	**Significance (P-value)**
Age (Year)	35.19 ±7.055	36.44 ± 5.840	0.441
Semen volume (ml)	2.817±1.5688	3.171 ± 1.3828	0.118
pH	8.685 ±0.3770	8.620 ± 0.4094	0.369
Sperm count (mill/ml)	53.519 ± 32.6728	73.244 ± 30.5219	**0.002****
Total motility (PR+NP) (%)	23.75 ±10.750	35.00 ± 19.170	**0.001****
Sperm Vitality (%)	34.62 ± 16.652	45.24 ± 18.471	**0.009****
Functional integrity (%)	45.96 ± 17.988	58.54 ± 18.345	**0.001****
Normal sperm morphology (%)	27.06 ± 13.136	35.95 ± 11.969	**0.001****

M: mean**; N**: number; **SD**: standard deviation; **P-value is statistically high significant at the 0.01 level

By comparing the parameters between drinker (N=52) and non-drinker (N=41), we found that the count of the sperms, its total motility, also sperm vitality, functional integrity and normal morphology were significantly higher in the non-drinker group (p= 0.002, p=0.001, p=0.009, p=0.001 and p=0.001 respectively) (Table 3).

Table 4: Comparison of the grade of protamine deficiency in sperm DNA assessed by Chromomycine-A3 (CMA3 +) and sperm DNA fragmentation assessed using TUNEL-assay (sDF) between drinkers and non-drinkers

Parameters	**Drinker (N=52)** (M±SD)	**Non-Drinker (N=41)** (M±SD)	**Significance** (P-value)
Protamine deficiency (CMA3+) (%)	37.03 ± 9.753	24.76 ± 7.435	**<0.0001**[**]
Sperm DNA fragmentation sDF (%)	22.37 ± 7.602	11.98 ± 5.172	**<0.0001**[**]

M: mean**; N**: number; **SD**: standard deviation; **P-value is statistically high significant at the 0.01 level

The protamine deficiency was observed and showed a significantly lower deficiency values in non-drinker group in comparison to drinker group (24.76 ± 7.435 vs. 37.03 ± 9.753, p<0.0001). The sDF also showed to be significantly raised and higher in drinker in comparison to non-drinker (22.37 ± 7.60 vs. 11.98 ± 5.17 p<0.0001) (Table 4).

Table 5: Correlation between the investigated sperm parameters in non-drinker group (N=41)

		Total Semen volume (ml)	pH	Sperm count (mill/ml)	Total motility (PR+NP) (%)	Sperm Vitality (%)	Functional integrity (%)	Normal sperm morphology (%)
Age (Year)	r	-0.207	0.118	**-0.333***	-0.270	**-0.374***	-0.287	**-0.400****
	p	0.195	0.461	**0.033**	0.088	**0.016**	0.069	**0.010**
Semen volume (ml)	r	-	0.021	-0.055	-0.061	0.060	0.095	-0.015
	p	-	0.895	0.733	0.704	0.709	0.556	0.924
pH	r	0.021	-	-0.173	0.023	-0.037	-0.224	-0.186
	p	0.895	-	00.279	0.884	0.817	0.159	0.244
Sperm count (mill/ml)	r	-0.055	-0.173	-	0.242	0.120	0.077	**0.545****
	p	0.733	0.279	-	0.128	0.454	0.632	**0.0001**
Total motility (PR+NP) (%)	r	-0.061	0.023	0.242	-	**0.436****	**0.451****	0.238
	p	0.704	0.884	0.128	-	**0.004**	**0.003**	0.134
Sperm Vitality (%)	r	0.060	-0.037	0.120	**0.436****	-	**0.698****	0.277
	p	0.709	0.817	0.454	**0.004**	-	**0.0001**	0.080
Functional integrity (%)	r	0.095	-0.224	0.077	**0.451****	**0.698****	-	**0.356***
	p	0.556	0.159	0.632	**0.003**	**0.0001**	-	**0.022**
Normal sperm morphology (%)	r	-0.015	-0.186	**0.545****	0.238	0.277	**0.356***	-
	p	0.924	0.244	**0.0001**	0.134	0.080	**0.022**	-

** highly significant correlation at the 0.01 level, * significant Correlation at the 0.05 level.

In Non-drinker group, the age correlated negatively with sperm count (r=-0.333, p= 0.033), sperm vitality (r=-0.374, p= 0.016), and normal sperm morphology (r=-0.400, p=0.010) (Table 5).

A high positive correlation was found between the sperm count and the normal sperm morphology (r=0.545, p=0.0001). The functional integrity correlated highly positive with total motility (r=0.451, p=0.003), sperm vitality (r=0.698, p=0.0001), and normal sperm morphology (r=0.356, p=0.022) (Table 5).

Table 6: Correlation between the investigated sperm parameters, protamine deficiency (CMA3 positivity (CMA3+)) and fragmentation of the DNA of the sperm(sDF) (%) in non-drinker group (N=41)

		Protamine deficiency (CMA3+) (%)	Sperm DNA fragmentation sDF (%)
Age (Year)	r	0.193	0.154
	p	0.227	0.338
Semen volume (ml)	r	0.116	0.021
	p	0.470	0.895
pH	r	0.123	0.087
	p	0.443	0.589
Sperm count (mill/ml)	r	-0.235	-0.214
	p	0.139	0.180
Total motility (PR+NP) (%)	r	**-0.319***	-0.066
	p	**0.042**	0.683
Sperm Vitality (%)	r	**-0.495****	**-0.357***
	p	**0.001**	**0.022**
Functional integrity (%)	r	**-0.436****	-0.209
	p	**0.004**	0.190
Normal sperm morphology (%)	r	**-0.403****	**-0.313***
	p	**0.009**	**0.046**
Protamine deficiency (CMA3+) (%)	r	-	0.291
	p	-	0.065

**Correlation is high significant at the 0.01 level, *Correlation is significant at the 0.05 level

Table 6 shows and presented the correlations between the mean percentage of the different parameters of the sperm, the total protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) in non-drinkers group. The protamine deficiency (CMA3+) correlated negatively with total motility of the sperms (r= -0.319, p= 0.042), the sperm vitality (r= -0.495, p=0.001), functional integrity (r= -0.436, p= 0.004) and normal sperm morphology (r=-0.403, p=0.009). The sperm DNA fragmentation index (sDF) correlated negatively or inversely with the total sperm vitality (r=-0.357, p= 0.022) and normal sperm morphology (r=-0.313, p=0.046).

Table 7: Correlation between the investigated sperm parameters in drinker group (N=52)

		Semen volume (ml)	pH	Sperm count (mill/ml)	Total motility (PR+NP) (%)	Sperm Vitality (%)	Functional integrity (%)	Normal sperm morphology (%)
Age (Year)	r	0.098	0.077	**0.332***	-0.106	**-0.372****	-0.229	**-0.324***
	p	0.488	0.589	**0.016**	0.456	**0.007**	0.102	**0.019**
Semen volume (ml)	r	-	0.027	-0.013	0.060	0.029	0.024	-0.095
	p	-	0.851	0.928	0.671	0.841	0.868	0.503
pH	r	0.027	-	0.093	0.072	0.018	-0.150	-0.155
	p	0.851	-	0.513	0.610	0.900	0.287	0.273
Sperm count (mill/ml)	r	-0.013	0.093	-	**0.298***	0.229	0.210	-0.029
	p	0.928	0.513	-	**0.032**	0.0102	0.135	0.840
Total motility (PR+NP) (%)	r	0.060	0.072	**0.298***	-	**0.458****	0.231	**0.344***
	p	0.671	0.610	**0.032**	-	**0.001**	0.099	**0.012**
Sperm Vitality (%)	r	0.029	0.018	0.229	**0.458***	-	**0.617****	**0.451****
	p	0.841	0.900	0.102	**0.001**	-	**0.0001**	**0.001**
Functional integrity (%)	r	0.024	-0.150	0.210	0.231	**0.617****	-	**0.287***
	p	0.868	0.287	0.135	0.099	**0.0001**	-	**0.039**
Normal sperm morphology (%)	r	-0.095	-0.155	-0.029	**0.344***	**0.451****	**0.287***	-
	p	0.503	0.273	0.840	**0.012**	**0.001**	**0.039**	-

**Correlation is high significant at the 0.01 level, *Correlation is significant at the 0.05 level

In drinkers' group (Table 7), the age correlated positively or directly with the sperm count (r= 0.332, p= 0.016), but negatively with the sperm vitality (r=-0.372, p= 0.007) and the normal morphologically sperm (r= -0.324, p= 0.019). In addition, Total motility correlated positively with the sperm count (r=0.298, p=0.032), sperm vitality (r=0.458, p=0.001) and

normal sperm morphology (r=0.344, p=0.012). Besides, a high positive correlation is found between the sperm vitality and functional integrity (r=0.617, p=0.0001). Furthermore, the normal morphology showed and demonstrated a high positive correlation with the sperm vitality (r= 0.451, p=0.001) and a positive correlation, with the functional integrity (r= 0.287, p=0.039) (Table 7).

Table 8: Correlation between protamine deficiency (CMA3 positivity (CMA3+)) and fragmentation of the DNA of the sperm (sDF) (%) with the investigated sperm parameters in drinker group (N=52)

		Protamine deficiency (CMA3+) (%)	Sperm DNA fragmentation sDF (%)
Age (Year)	r	0.215	0.026
	p	0.126	0.857
Semen volume (ml)	r	-0.111	0.055
	p	0.433	0.701
pH	r	0.010	-0.048
	p	0.942	0.738
Sperm count (mill/ml)	r	**-0.359****	-0.178
	p	**0.009**	0.206
Total motility (PR+NP) (%)	r	**-0.442****	-0.058
	p	**0.001**	0.681
Sperm Vitality (%)	r	**-0.347***	-0.082
	p	**0.012**	0.564
Functional integrity (%)	r	-0.105	**0.289***
	p	0.459	**0.038**
Normal sperm morphology (%)	r	**-0.382****	-0.101
	p	**0.005**	0.477
Protamine deficiency (CMA3+) (%)	r	-	**0.402****
	p	-	**0.003**

**Correlation is high significant at the 0.01 level, *Correlation is significant at the 0.05 level

By studying the correlation between the protamine deficiency and the standard sperm parameters, we found that the $CMA3^+$ have a high negative correlation with the sperm count (r= -0.359, p=0.009), total motility (r= -0.442, p= 0.001) and the morphologically normal sperm (r=-0.382, p= 0.005). Besides, it correlates negatively with the sperm vitality (r= -0.347, p=0.012) (Table 8).

In the other hand, the sDF correlated positively with the sperm functional integrity (r=0.289, p=0.038) and with the protamine deficiency (r=0.402, p=0.003) (Figure 8) (Table 8).

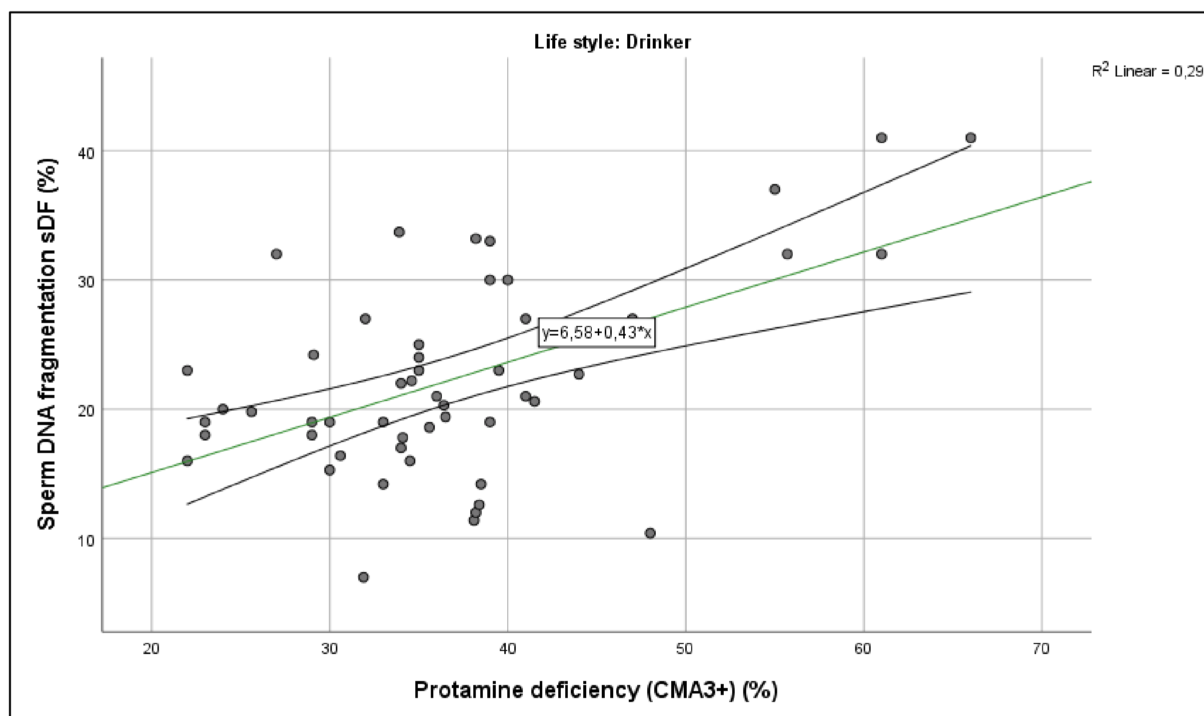

Figure 8: Correlation between protamine deficiency and sperm DNA fragmentation in group of drinker patients (r=0.402, p=0.003)

3.2. The characteristic of the Cigarette Smoking study group

Table 9: Descriptive statistic of studied parameters for all population in Cigarette Smoking study group (N=118)

Parameters	Mean	SD	Range (Min-Max)
Age (Year)	32.39	7.98	18.0 – 51.0
Semen volume (ml)	3.55	1.60	0.8 - 9.5
pH	8.65	0.37	7.9 - 9.5
Sperm count (mill/ml)	66.60	31.23	1.0 – 10.0
Total motility (PR+NP) (%)	32.33	13.69	5.0 – 75.0
Sperm Vitality (%)	40.13	18.31	1.0 – 80.0
Functional integrity (%)	50.25	19.83	5.0 – 90.0
Normal sperm morphology (%)	37.88	15.06	5.0 – 76.0
Protamine deficiency (CMA3+) (%)	28.95	9.09	12.0 – 76.0
Sperm DNA fragmentation sDF (%)	11.61	5.03	2.0 – 23.0

Mill/ml: million per ml, PR: progressive, NP: non-progressive, SD: standard deviation

Table 9 gave a brief description about the statistical analysis of all the sperm parameters, its protamine deficiency, and DNA fragmentation of the sperm cells. The means ± SD of the age was 32.39±7.98 years, the sperm volume was 3.33 ± 1.60 (ml), the pH was 8.65 ± 0.37, sperm count was 66.60 ± 31.23 (10^6/ml), total motility was 32.33 ± 13.69%, sperm vitality was 40.13±18.31%, functional integrity was 50.25 ± 19.83%, and normal spermatozoa morphology was 37.88 ± 15.06%.

The protamine deficiency ranged between 12 and 76% and had a mean value of 28.95 ± 9.09%. The sperm DNA fragmentation (sDF) ranged between 2 and 23% and had a mean of 11.61 ± 5.03% (Table 7). Based on their smoking status the participants were divided into a group of smokers of the tobacco and non-smokers: A group of non-smokers (n=70) and a group of smokers (n=48).

By doing comparison between these two groups (Table 10), we have found that the semen volume and sperm vitality were significantly raised and higher in the group of non-smokers in comparison to smokers (3.79 ± 1.67 vs. 3.20 ± 1.43, p=0.037; 42.86 ± 17.74 vs. 36.15 ± 18.57, p=0.035 respectively). The same was noticed for the total motility, functional integrity, and the percent of normal morphology sperm (p< 0.0001) (Table 9).

However, the CMA 3+ and the sDF were significantly higher in the group of smokers in comparison to smokers (33.27 ± 8.56 vs. 26.00 ± 8.28; 15.55 ± 3.33 vs. 8.91 ± 4.14 respectively, p< 0.0001) (Table 10).

Table 10: Comparison of the semen total parameters between smokers and non-smokers

Parameters	**Smoker (N=48)** (M±SD)	**Non-smoker (N=70)** (M±SD)	**Significance** (P-value)
Age (Year)	33.12 ± 8.21	31.89 ± 7.84	0.454
Semen volume (ml)	3.20 ± 1.43	3.79 ± 1.67	**0.037***
pH	8.64 ± 0.37	8.65 ± 0.37	0.913
Sperm count (mill/ml)	65.75 ± 31.32	67.18 ± 31.38	0.726
Total motility (PR+NP) (%)	24.27 ± 8.18	37.86 ± 14.00	**< 0.0001****
Sperm Vitality (%)	36.15 ± 18.57	42.86 ± 17.74	**0.035***
Functional integrity (%)	41.56 ± 18.57	56.21 ± 18.54	**< 0.0001****
Normal sperm morphology (%)	28.77 ± 11.82	44.13 ± 13.85	**<0.0001****

M: mean; **N**: number; **SD**: standard deviation; **P-value is statistically high significant at the 0.01 level, *P-value is statistically significant at the 0.05 level

Table 11: Comparison of the grade of protamine deficiency in sperm DNA assessed by Chromomycine-A3 (CMA3 +) and sperm DNA fragmentation assessed by TUNEL-assay (sDF) between smokers and non-smokers

Parameters	Smoker (N=48) (M±SD)	Non-smoker (N=70) (M±SD)	Significance (P-value)
Protamine deficiency (CMA3+) (%)	33.27 ± 8.561	26.00 ± 8.283	**< 0.0001****
Sperm DNA fragmentation sDF (%)	15.55 ± 3.334	8.91 ± 4.147	**< 0.0001****

M: mean**; N**: number; **SD**: standard deviation; **P-value is statistically high significant at the 0.01 level

Table 12: Correlation between the investigated sperm parameters in non-smokers group (N=70)

		Semen volume (ml)	pH	Sperm count (mill/ml)	Total motility (PR+NP) (%)	Sperm Vitality (%)	Functional integrity (%)	Normal sperm morphology (%)
Age (Year)	r	-0.174	0.203	**-0.396****	**-0.434****	**-0.292***	-0.147	**-0.442****
	p	0.151	0.091	**0.001**	**0.0001**	**0.014**	0.226	**0.0001**
Semen volume (ml)	r	-	-0.003	0.072	**0.284***	0.125	-0.060	0.199
	p	-	0.981	0.556	**0.017**	0.304	0.622	0.098
pH	r	-0.003	-	**-0.428****	-0.134	-0.045	0.131	-0.020
	p	0.981	-	**0.0001**	0.270	0.713	0.281	0.869
Sperm count (mill/ml)	r	0.072	**-0.428****	-	**0.371****	0.170	-0.008	**0.314****
	p	0.556	**0.0001**	-	**0.002**	0.159	0.945	**0.008**
Total motility (PR+NP) (%)	r	**0.284***	-0.134	**0.371****	-	**0.527****	**0.380****	**0.481****
	p	**0.017**	0.270	**0.002**	-	**0.0001**	**0.001**	**0.0001**
Sperm Vitality (%)	r	0.125	-0.045	0.170	**0.527****	-	**0.492****	**0.286***
	p	0.304	0.713	0.159	**0.0001**	-	**0.0001**	**0.016**
Functional integrity (%)	r	-0.060	0.131	-0.008	**0.380****	**0.492****	-	0.222
	p	0.622	0.281	0.945	**0.001**	**0.0001**	-	0.065
Normal sperm morphology (%)	r	0.199	-0.020	**0.314****	**0.481****	**0.286***	0.222	-
	p	0.098	0.869	**0.008**	**0.0001**	**0.016**	0.065	-

**Correlation is high significant at the 0.01 level, *Correlation is significant at the 0.05 level

In non-smokers, the age has a high negative correlation presented with respect to the sperm count (r=-0.396, p=0.001), total motility (r=-0.434, p=0.0001) and the percent of normal morphology spermatozoa (r=-0.442, p=0.0001) and a significant negative correlation (r=-0.292, p=0.014) (table 10). Moreover, the sperm count correlated negatively with the pH (r=-0.428, p=0.0001) but showed a high positive correlation with percent of normal morphology sperm (r=0.314, p=0.008) (Table 12).

The percent of total motile spermatozoa correlated highly significant with the sperm count (r=0.371, p=0.002), the sperm vitality (r= 0.527, p=0.0001), the functional integrity (r=0.380, p=0.0001) and the percent of normal morphology sperm (r=0.481, p=0.0001) (Table 12).

The sperm vitality correlated highly positive with the functional integrity (r=0.492, p=0.0001) and the percent of normal morphology spermatozoa (r=0.286, p=0.016) presented in Table 12.

Table 13: Correlation between the investigated parameters of the semen, the deficiency of protamine (CMA3 positivity (CMA3+)) and sperm DNA fragmentation (sDF) (%) among non-smoker group (N=70)

		Protamine deficiency (CMA3+) (%)	Sperm DNA fragmentation sDF (%)
Age (Year)	r	**0.446****	**0.484****
	p	**0.0001**	**0.0001**
Semen volume (ml)	r	-0.175	-0.023
	p	0.148	0.851
pH	r	**0.252***	0.154
	p	**0.035**	0.203
Sperm count (mill/ml)	r	**-0.391****	**-0.309****
	p	**0.001**	**0.009**
Total motility (PR+NP) (%)	r	**-0.409****	-0.276*
	p	**0.0001**	0.021
Sperm Vitality (%)	r	**-0.277***	-0.031
	p	**0.020**	0.796
Functional integrity (%)	r	-0.158	0.012
	p	0.190	0.920
Normal sperm morphology (%)	r	**-0.573****	**-0.452****
	p	**0.0001**	**0.0001**
Protamine deficiency (CMA3+) (%)	r	-	**0.451****
	p	-	**0.0001**

**Correlation is high significant at the 0.01 level, *Correlation is significant at the 0.05 level

Table 13 showed that the protamine deficiency in the non-smokers group correlated positively with the age (r=0.446, p=0.0001) and the pH (r=0.252, p=0.035) but negatively with the sperm count (r=-0.391, p=0.001), the total motility (r=-0.409, p=0.0001), the sperm vitality (r=-0.277, p=0.020) and the normal morphologically sperm (r=-0,537, p=0.0001). Like the CMA3+, the sperm DNA fragmentation correlated with the age positively (r=0.484, p=0.0001) but its correlation was found to be negatively with the sperm count (r=-0.309, p=0.009) and the percent of normal morphology sperm (r=-0.452, p=0.0001). Besides, a high positive association was shown between the CMA3+ and the sDF (r=0.451, p=0.0001) (Table 13).

Table 14: Correlation among the investigated sperm parameters in smokers' group (N=48)

		Semen volume (ml)	pH	Sperm count (mill/ml)	Total motility (PR+NP) (%)	Sperm Vitality (%)	Functional integrity (%)	Normal sperm morphology (%)
Age (Year)	r	-0.245	0.215	-0.168	-0.068	-0.128	**-0.294***	**-0.476****
	p	0.093	0.142	0.255	0.648	0.387	**0.042**	**0.001**
Semen volume (ml)	r	-	-0.162	0.204	-0.017	-0.053	-0.008	0.079
	p	-	0.271	0.163	0.910	0.719	0.957	0.591
pH	r	-0.162	-	0.018	-0.281	-0.196	-0.053	0.069
	p	0.271	-	0.901	0.053	0.181	0.719	0.641
Sperm count (mill/ml)	r	0.204	0.018	-	0.115	0.125	0.063	0.164
	p	0.163	0.901	-	0.436	0.396	0.668	0.264
Total motility (PR+NP) (%)	r	-0.017	-0.281	0.115	-	**0.313***	0.159	-0.158
	p	0.910	0.053	0.436	-	**0.030**	0.280	0.282
Sperm Vitality (%)	r	-0.053	-0.196	0.125	**0.313***	-	**0.557****	**-0.347***
	p	0.719	0.181	0.396	**0.030**	-	**0.0001**	**0.016**
Functional integrity (%)	r	-0.008	-0.053	0.063	0.159	**0.557****	-	0.033
	p	0.957	0.719	0.668	0.280	**0.0001**	-	0.826
Normal sperm morphology (%)	r	0.079	0.069	0.164	-0.158	**-0.347***	0.033	-
	p	0.591	0.641	0.264	0.282	**0.016**	0.826	-

**Correlation is significant at the 0.01 level, *Correlation is significant at the 0.05 level

The age in smokers group correlated negatively with the functional integrity r=-0.294, p=0.042) and the percent of normal morphologically sperm (r=-0.476, p=0.001) (Table 14). The sperm vitality correlated positively with the total motility percent (r=0.313, p=0.030), functional integrity (r=0.557, p=0.0001) but correlated negatively with the morphologically normal spermatozoa (r=-0.347, p=0.016) (Table 14).

In contradiction to non-smokers group, no correlation is observed between the CMA3+ and the sDF (Figeure 9). Besides, the CMA3+ correlated positively with the age (r=0.377, p=0.008) and negatively with the sperm count (r=-0.289, p=0.046). However, the sDF showed no correlation to any of the studied parameters mentioned in the table 15.

Table 15: Correlation between the investigated sperm parameters, protamine deficiency (CMA3 positivity (CMA3+)) and sDF (%) in smoker group (N=48)

		Protamine deficiency (CMA3+) (%)	Sperm DNA fragmentation sDF (%)
Age (Year)	r	**0.377****	0.117
	p	**0.008**	0.427
Semen volume (ml)	r	0.089	-0.158
	p	0.549	0.284
pH	r	-0.007	-0.050
	p	0.963	0.738
Sperm count (mill/ml)	r	-0.258	0.113
	p	0.076	0.443
Total motility (PR+NP) (%)	r	0.031	0.209
	p	0.835	0.154
Sperm Vitality (%)	r	**-0.289***	-0.044
	p	**0.046**	0.767
Functional integrity (%)	r	-0.259	-0.128
	p	0.075	0.386
Normal sperm morphology (%)	r	-0.090	-0.069
	p	0.543	0.643
Protamine deficiency (CMA3+) (%)	r	-	**0.099**
	p	-	**0.503**

**Correlation is significant at the 0.01 level, *Correlation is significant at the 0.05 level

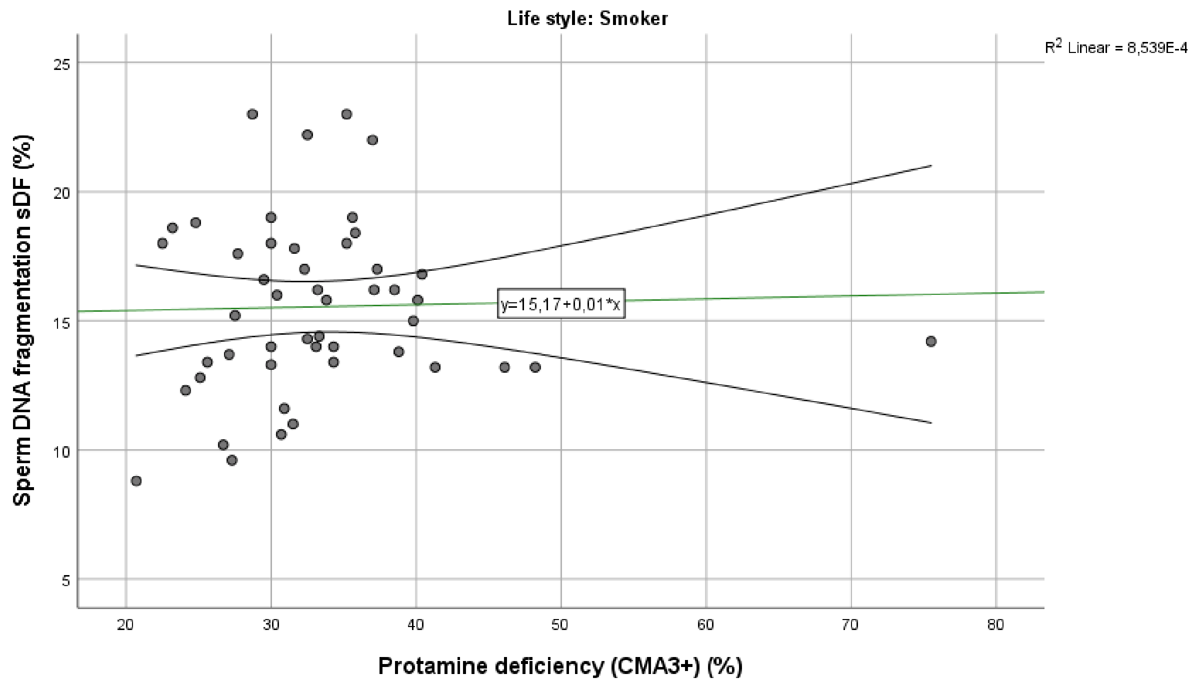

Figure 9: Correlation between protamine deficiency and sperm DNA fragmentation in group of smoker patients (**r=0.099, p=0.503**).

3.3. The characteristic of groups according to the lifestyle

Table 16: Descriptive statistic of studied parameters for all population in Lifestyle group (N= 100)

Parameters	Mean	SD	Range (Min-Max)
Age (Years)	34.20	7.66	21.0 – 5.0
PH	8.66	0.37	7.9 – 9.5
Semen volume (ml)	3.00	1.51	0.5 – 9.5
Sperm count (mill/ml)	59.39	32.45	10.0 – 100.0
Total motility (PR + NP %)	24.00	9.56	0 – 70.0
Sperm Vitality (%)	35.35	17.52	5.0 – 80.0
Functional integrity (%)	43.85	18.31	5.0 – 85.0
Morphologically normal spermatozoa (%)	27.88	12.48	5.0 – 58.0
CMA3 positivity (%)	35.22	9.34	21.0 – 76.0
Sperm DNA fragmentation (sDF)(%)	19.09	6.83	7.0 - 41.0

Mill/ml: million per ml, PR: progressive, NP: non-progressive, SD: standard deviation

The statistical analysis performed on the sperm different parameters such as protamine deficiency, sperm DNA fragmentation with respect to the lifestyle of population (N=100) was summarized in the table 14. The means ± SD of the age was 34.20±7.66 years, the pH was 8.66±0.37, the sperm volume was 3.00 ± 1.51 (ml), sperm count was 59.39 ± 32.45 (10^6/ml), total motility was 24.0 ± 9.51%, sperm vitality was 35.35±17.52%, functional integrity was 43.85 ± 18.31%, and normal spermatozoa morphology was 27.88 ± 12.48% (Table 16).

The protamine deficiency ranged between 21 and 76% and had a mean value of 35.22 ± 9.34%. The sperm DNA fragmentation (sDF) ranged between 7 and 41% and had a mean of 19.09 ± 6.83% (Table 16).

Table 17: Comparison of the semen analysis parameters between smokers group and drinkers group

Parameters	**Smoker (N=48) (M±SD)**	**Drinker (N=52) (M±SD)**	**Significance (P-value)**
Age (Year)	33.12 ± 8.21	35.19 ± 7.05	0.127
Semen volume (ml)	3.20 ± 1.43	2.81± 1.56	0.073
pH	8.64 ± 0.37	8.685 ± 0.37	0.479
Sperm count (mill/ml)	65.75± 31.32	53.51± 32.67	0.056
Total motility (PR+NP) (%)	24.27 ± 8.18	23.75 ± 1.750	0.470
Sperm Vitality (%)	36.15 ± 18.57	34.62 ± 16.65	0.835
Functional integrity (%)	41.56 ± 18.57	45.96 ± 17.98	0.127
Normal sperm morphology (%)	28.77 ± 11.82	27.06 ± 13.13	0.332

M: mean**; N**: number; **SD**: standard deviation

As illustrated in table 17, no significant difference was observed in the semen analysis parameters between the smoker and drinker groups.

However, the protamine deficiency was significantly higher in the drinker group in comparison to the smokers (37.03 ± 9.75 vs. 33.27 ± 8.56, p=0.02) (Table 18). The same for the sDF which was significantly higher in the drinker in comparison to the smoker (22.37 ± 7.602 vs. 15.55 ± 3.33, p < 0.0001) (Table 18).

Table 18: Comparison of the grade of protamine deficiency in sperm DNA assessed by Chromomycine-A3 (CMA3 +) and sperm DNA fragmentation assessed by TUNEL-assay (sDF) between smokers' group and drinkers' group

Parameters	Smoker (N=48) (M±SD)	Drinker (N=52) (M±SD)	Significance (P-value)
Protamine deficiency (CMA3+) (%)	33.27 ± 8.56	37.03 ± 9.75	0.020**
Sperm DNA fragmentation sDF (%)	15.55 ± 3.33	22.37 ± 7.60	< 0.0001**

M: mean**;** **N**: number; **SD**: standard deviation; **P-value is statistically high significant at the 0.01 level

4. Discussion

In the last decade specifically, infertility has become a global health problem not only for aged couples but also for couples in their reproductive ages (Fang et al., 2018). Different studies have shown that the number of couples with secondary infertility is increasing compared to couples with primary infertility diagnosis (Fang et al., 2018). Male infertility factors are subdivided into extrinsic ones, including environmental and lifestyle factors, and intrinsic ones, like congenital disorders (Wang et al., 2020)

In fact, different studies conducted showed that semen parameters may be affected by various lifestyle (Hammadeh et al., 2010; Jensen et al., 2014) and environmental (Boeri et al., 2019; Amor et al., 2021) factors. The issue concerning the relationship between tobacco smoking and the quality of semen still remains controversial. A different number of studies and research failed to establish the association between tobacco smoking and sperm parameters and quality (Ozgur et al., 2005; Kumar et al., 2014). On the contrary, some other studies pointed out that tobacco smoking acts as a risk factor for infertility (Amor et al., 2021; Hamad et al., 2014).

Therefore, in the first part of the study, we focused on how smoking adversely affects sperms (volume, density, functional or membrane integrity, and sperm DNA maturation in men attending assisted reproduction programs). The results indicated that the semen parameters were significantly higher in nonsmokers than in smokers. The total motility (PR+NP%) in smokers was 24.27 ± 31.32 % vs. 37.86 ± 14.00% ($p < 0.0001$). The same was observed for sperm vitality (36.2 ± 18.56% vs. 42.9 ± 17.74%; $p = 0.035$), membrane integrity (41.6 ± 18.6% vs. 56.2 ± 18.6%; $p = 0.0001$), and morphologically normal sperm (28.8 ± 11.8% vs. 44.13.85%; $p < 0.0001$). However, protamine deficiency (33.3 ± 8.6% vs. 26.0 ± 8.3%; $p<0.0001$) and DNA fragmentation (15.6 ± 3.3 vs. 8.9 ± 4.1%; $p<0.0001$) were significantly higher in smokers than in nonsmokers (Tables 8 and 9). However, it did not have any significant effect on the ejaculation volume (3.2 ± 0.37 ml vs. 3.79 ± 1.6 ml; $p = 0.913$) or sperm cell count (65.7 ± 31.32 vs 67.19 ± 31. 39 mill/ml) (Table 8). The current study shows that smoking causes sperm DNA damage, thus affecting its quality and eventually leading to infertility (Tables 8 and 9). These findings confirm earlier studies conducted by Hammadeh et al. (2010) and Amor et al. (2021).

The present results agree with those of several previous studies on the impacts of tobacco and cigarette smoking on human sperm cells (Aitken et al., 2014; Asare-Anane et al., 2016) and sperm parameters (Sharma et al., 2016; Mostafa et al., 2006).

Nicotine in low concentration (≤1 mmol) affects the motility of sperm. In higher amounts (≥1 mmol l), it decreases sperm viability (Oyeyipo et al., 2014; Bundhun et al., 2019). Cigarette smoke negatively influences the DNA integrity of spermatozoa, the protamination process (Hamad et al., 2014), and the spermatozoa DNA methylation patterns (Al Khaled et al., 2018; Hamad et al., 2018; Amor et al., 2021).

Cigarette smoking produces and promotes ROS production, as reported earlier (Kumar et al., 2015; Hammadeh et al., 2010; La Maestra et al., 2015; Perrin et al., 2011), causing OS. A cigarette's components affect the DNA by ROS (Opuwari & Henkel, 2016; Cui et al., 2016), sperm motility (Agarwal et al., 2003; Agarwal et al., 2015; Athayde et al., 2007), morphology (Aziz et al. 2004), and sperm DNA damage (Desaiet al., 2009; Agarwal et al., 2015; Hammadeh et al., 2010). They also cause apoptosis (Agarwal et al., 2005). It was suggested that smoking increases leukocyte levels in sperms by 48% compared to nonsmokers (Saleh et al., 2002). Smoking also elevates the DNA fragmentation index (37.66% vs. 14.51%, $P < 0.001$) (Elshal et al., 2008). Further, ROS production in smokers leads not only to OS but also to target protamine (Hammadeh et al., 2008). OS affects the sperm mitochondrial respiratory activity and nuclear DNA fragmentation (Ferramosca et al., 2013). Consequently, the motility of spermatozoa could be decreased by tobacco smoking. Extrinsic factors causing OS could damage the DNA of the sperm, which may result in several pathologies of the male reproductive system (Cho & Agarwal, 2017). An excessive production of ROS affects the endocrine function, leading to infertility (Darbandi et al., 2018).

OS affects sperm parameters, including total motility and overall sperm morphology in subgroups (Ramya et al., 2010; Oumaima et al., 2018; Dobrakowski et al., 2017; Bui et al., 2018), thus deteriorating fertilisation, implantation, pregnancy, and embryonic development (Osman et al., 2015; Simon et al., 2017). In addition to OS, nicotine (Haque et al., 2014) also damages the DNA of spermatozoa (Arabi, 2004). It also causes spermatozoa capacitation, causing infertility (Sofikitis et al., 2000).

Lead and cadmium are important constituents of tobacco smoking, causing mutagenesis and testicular degeneration (Jurasovic et al., 2004) and leading to male infertility (Wang et al.,

2016; de Angelis et al., 2017). However, the correlation between them remains controversial, as described by Pant et al. (2014). Also, a minimal exposure apparently has no effect (Pant et al., 2014). The findings of the present study demonstrated that sperm parameters were more affected in male smokers than in male nonsmokers (Tables 8 and 9).

In addition, male patients who were not smoking cigarettes (n = 70) showed that the mean % of the sperm DNA fragmentation (sDF) (8.9 ± 4.14%) showed significant positive correlation with the age of the patients (r = 0.0484; p < 0.0001), sperm count (r = -0.309; p < 0.009), total motility (r =-0.276; p < 0.021), morphologically normal spermatozoa (r = -452, p < 0.0001), and protamine deficiency (r = 0.451; p < 0.0001) (Table 11). In contrast, in the smoker group (n = 48), the mean percentage of sDF (15.55 ± 3.33%) showed that the semen volume, pH, sperm vitality, morphologically normal spermatozoa, and protamine deficiency (CMA3+) were negatively correlated with the age of the patients (r = 0.377, p < 0.008) and sperm vitality (r = -0. 289, p = 0.046) (Table 13).

Other studies have shown that the DNA of sperm cells of the smoker group have higher levels of fragmentation in male subjects in comparison to those of the nonsmoker group (Elshal et al., 2009; Andrabi., 2007; Hammadeh et al., 2010; Aydin et al., 2013). Smoking decreases the level of antioxidants in seminal plasma (Pasqualotto et al., 2008). Damage to the DNA may be idiopathic or due to excessive ROS production (Saleh et al., 2003). Abnormalities during the arrangement of chromatin may also cause infertility (Sakkas et al., 2000; Saleh et al., 2002; Spanò et al., 2005), affecting embryo development (Gannon et al., 2013; Simon et al., 2014). DNA damage may also be caused by apoptosis, DNA strand breakage, and any defect during the sperm maturation process (Sakkas &and Alvarez, 2010; Cho & Agarwal, 2017).

The epigenetic mechanism that leads to infertility is yet to be explained (Laqqan et al., 2017). Smoking may lead to infertility through methylation (Shenker et al., 2013; Al Khaled et al., 2018). In addition to smoking, alcohol consumption may affect the sperm quality (Condorelli et al., 2015) and the morphology of sperm reversibly (Hadi et al., 1987). Giving up alcohol intake may reverse the changes (Mokdad et al., 2004).

As shown by various studies, alcohol increases OS and consequently causes infertility (Yuksel et al., 2005). Loft et al. (2005) demonstrated that the breakage of DNA had no relation to the consumption of alcohol. However, Muthusami and Chinnasswamy (2005) showed that FSH, LH, E2, and testosterone levels decreased. One of the meta-analysis with

57 total studies (n = 29,914 participants) found a positive association between alcohol intake, semen volume, and morphology and motility of spermatozoa (Li et al., 2016). These findings were also supported by Rossi et al. (2011).

In the present study, a comparison between the alcohol drinker group (n = 52) and the nondrinker group (n = 41) showed that the mean sperm count (53.5±32.7 mill/ml), total motility (23.8±10.8%), sperm vitality (34.6±16.6%), membrane functional integrity (45.9±17.9%), morphologically normal spermatozoa (27.0±13.13%) were significantly lower in drinkers ($p < 0.001$) than in nondrinkers (73.2±30.5 mill/ml, 35.0±19.2%, 45.2±18.4%, 58.5±18.3%, 35.9±11.9%, $p < 0.001$, respectively) (Table 2). The chromatin deficiency (CMA3+) and sDF of the drinker group were significantly higher than those in the nondrinker group (37.0±9.7 and 22.4 ±7.6% vs. 24.8 ±7.4 and 11.9 ± 5.2%, respectively) (Table 3).

Also, the present study demonstrated that all investigated parameters differed significantly between alcohol drinkers and nondrinkers except in semen volume and pH values. These results were in accordance with other study results that reported that those who are regular drinkers have decreased volume and concentration of sperms (Kucheria et al., 1985) even for a few days (Hansen et al., 2012). Another meta-analysis involving 15 different cross-sectional studies suggests that regular alcohol consumption affects sperm parameters to a greater extent (Ricci et al., 2017). Boeri et al. (2019) reported a negative correlation between alcohol drinking and sperm parameters, but age and smoking may distort the result. In a cohort study (n = 258) by Dunphy et al. (1991) at an infertility treatment centre, 21% of the participants consumed around 1 unit per week of alcohol; 10% of these patients consumed 1–5 units per week; 23% consumed 6–10 units of alcohol per 7 days, 27% consumed alcohol in the range of 11–20 units per week, and 19% consumed 20 units of alcohol per week. This longitudinal study showed no association and link between alcohol consumption and infertility. Goverde et al. (1995) failed to find statistically significant differences between the volume of seminal fluid (4.16 ml vs. 3.363 ml), total sperm concentration (10.667.8 mill/ml vs. 8.965.8 mill/ml), and percentage of the motile form of spermatozoa (27.0% ± 6.5% vs. 25.5% ± 6.16%) between cases and controls with respect to the alcohol consumption pattern. They concluded that smoking and alcohol intake did not seem to play a pivotal role in decreasing semen quality. However, excessive alcohol intake may further decrease an already low percentage of sperm parameters.

NADH and acetaldehyde are produced through the alcohol metabolic pathway, leading to ROS production (Goverde et al., 1995; Agarwal et al., 2005) and affecting the DNA through apoptosis (Shiraishi et al., 2007). Alcohol affects GnRH, leading to an adverse impact on LH and FSH (Uddin et al., 1996; Kim et al., 2003) and decreasing testosterone levels (Emanuele & Emanuele, 2001; Maneesh et al., 2006), which destroy the endocrine balance and thus cause infertility (Salonen et al., 1990). A study involving alcohol consumers and a control group showed an increase in E2, FSH, and LH levels in the plasma (Maneesh et al., 2006). Also, higher 17-beta estradiol hormone levels and lower concentrations of testosterone affect the sperm quality (Muthusami et al., 2005).

In the nonalcohol drinker group (n = 41), the sperm count (mill/ml) showed significant negative correlation with age ($r = -0.333$; $p < 0.033$) and positive correlation with normal morphological values in ejaculate (r = 0. 545, $p < 0.001$). Total motility correlated positively with the overall spermatozoa vitality ($r = 436$; $p < 0.004$) and with the sperm membrane integrity or functional integrity ($r = 0.451$; $p < 0.003$). Semen vitality showed a correlation with the age of the patients ($r = 0.374$, $p < 0.016$) and with the functional integrity of sperm ($r = 698$; $p < 0.0001$). The functional integrity of sperm membranes significantly correlated with total sperm motility, sperm vitality, and morphologically normal spermatozoa ($r = 0.451$, $p < 0.003$; $r = 0698$, $p = 0.0001$; $r = 0.356$, $p = 0.022$, respectively). The mean percentage of morphologically normal spermatozoa correlated with the age of the patients ($r = -0.400$, $p < 0.010$), sperm count ($r = 0.545$; $p < 0.0001$), and spermatozoa's functional integrity ($r = 0.356$; $p < 0.022$) (Table 4).

Protamine deficiency (CMA3) demonstrated a significantly negative correlation with the total motility ($r = -0.319$, $p< 0.042$) and vitality ($r = -0.495$, $p < 0.001$) and positive correlation with the functional integrity ($r = 0.436$, $p < 0.004$) and with the morphologically normal spermatozoa ($r = 0.004$, $p < 0.009$). Further, sDF correlated negatively with the vitality of the sperm ($r = -0.357$, $p < 0.022$) and with sperm morphology ($r = -0.313$, $p < 0.046$) (Table 5).

In the alcohol drinker group, several positive correlations were found between the total sperm count and the age ($r = 0.332$, $p < 0.016$) and between the sperm count and the total motility ($r = 0.298$, $p < 0.032$) of the patients.

Total motility correlated positively with sperm count ($r = 0.298$, $p < 0.032$) and sperm vitality ($r = 0.458$, $p < 0.001$). The functional integrity of spermatozoa correlated with sperm vitality

($r = 0.617$, $p < 0.001$) and the mean percentage of morphologically normal spermatozoa ($r = 0.287$, $p < 0.039$). Sperm vitality correlated negatively with the age of the patients ($r = -0.372$, $p < 0.007$). However, total motility positively correlated with ($r = 0.458$, $p < 0.001$), functional integrity ($r = 617$, $p < 0.0001$) and morphologically normal spermatozoa ($r = 0.451$, $p < 0.046$) (Table 6).

The mean percentage of morphologically normal spermatozoa showed a significantly negative correlation with the age of the patients ($r =- 0.324$, $p < 0.019$) and positively correlated with total motility ($r = 0.344$, $p < 0.012$), sperm vitality ($r = 0.451$, $p < 0.001$), and functional integrity of the spermatozoa membrane ($r = 0.287$, $p = 0.039$) (Table 6). Protamine deficiency (CMA3+) has demonstrated a negative correlation with sperm count, concentration, total motility, sperm vitality, and morphologically normal spermatozoa (Table 7). As shown in Table 7, SDF (%) correlated with functional integrity and protamine deficiency ($r = 0.402$, $p < 0.003$). These findings confirm previous several studies showing a correlation between abnormal protamination (chromatin deficiency) and DNA strand breaks (Aoki et al., 2006; Carrell et al., 2007; Hammadeh et al., 2006).

The results of the present study also agree with previous studies conducted by Martini et al. (2004). Increased β-endorphin levels caused DNA fragmentation, resulting in degenerated spermatozoa (Anifandis et al., 2014). Alcohol and ethanol consumption negatively affected sperm parameters, like sperm count, DNA integrity, and sperm maturation (protamination) (Jana et al., 2010; Sansone et al., 2018).

ROS production may be enhanced by various lifestyles, advancement in technologies, environmental pollution, alcohol intake, smoking, and psychological stress (Rakhit et al., 2013; Barazani et al., 2014; Sullivan & Pfefferbaum, 2014). Obesity and dietary factors are also keys and important factors, as reported earlier (Mendiola et al., 2009; Li et al., 2011; Afeiche et al., 2013). Further, it has been shown that the risk of not achieving a live birth increased by 2.28 (1.08–4.80) to 8.32 (1.82–37.97) times in patients who drink alcohol (odds ratio: 55.49–45.64). Modifying drinking habits may increase ART outcomes (Klonoff-Cohen et al., 2005).

The 100 patients in this study were divided into heavy smokers (G.1 = 48) and alcohol drinkers (G.2 = 52). Sperm parameters in G.1 and G.2 were compared to determine whether smoking or alcohol consumption causes more deterioration in sperm parameters (Table 16).

By analysing the sperm quality in the two groups, we found that the age of the patients, semen volume, mean concentration of spermatozoa, total motility (PR+NP), sperm vitality, functional integrity, and mean percentage of morphologically normal spermatozoa were similar in both groups (33.12±8.21 years, 3.20±1.43 ml; 65.75±31.3 mill/ml, 24.27± 8.18%; 36.15±18.57%, 41.56±18.57, 28.8±11.8% vs. 35.19±7.1 years, 2.8±1.6 ml; 53.5±32.67 mill/ml; 23.75±1.7%; 34.6±16.6%; 45.9±17.9; 27.1±13.13%, respectively).

The findings in this study showed that smoking and alcohol intake have similar deleterious effects on the sperm parameters. Also, these results confirm previous studies' findings (Muthusami & Chinnaswamy, 2005; Martini et al., 2004; Gaur et al., 2010; Li et al., 2009). Various studies showed changes only in sperm morphology (Condorelli et al., 2015). Others showed contradictory results (Lopez et al., 2007; Povey et al., 2012; Hansen et al., 2012).

Furthermore, in the present study, the mean percentage of protamine deficiency (CMA3+) was significantly higher in the alcohol consumer group than in the heavy smoker patient group (37.0±9.75 vs. 33.3±8.6, $p = 0.020$). DNA fragmentation levels and the mean percentage of sDF in the alcohol consumer group (22.4±7.6%) were significantly higher ($p < 0.0001$) than in the heavy smoker group (15.6±3.3%) (Figure 18).

In the heavy tobacco smoker group (Figure 15), the mean percentage of protamine deficiency (CMA3+) showed no significant correlation with the DNA fragmentation of the sperm cells (SDF) ($r = 0.099$, $p < 0.503$). The alcohol consumer group (Figure 8) showed that DNA fragmentation SDF levels correlated significantly with the mean percentage of protamine deficiency ($r = 0.402$, $p < 0.003$). The present study suggests the adverse effects of cigarette smoking and alcohol consumption at various sperm parameters. Alcohol causes a peroxidation of lipids and consequently increases ROS production, protein degradation, and DNA fragmentation (Wu & Cerdebaum, 2003; Zorn et al., 2003).

ROS and nitrogen production affects the whole process of spermatogenesis (Agarwal & Allamaneni, 2004; Doshi et al., 2012). Exposure to high quantities and levels of ROS can generate various modified forms of DNA bases, causing mutagenicity as well as carcinogenicity (Soultanakis et al., 2000; Singh et al., 2011). Marselos and Vainio (1991) studied the effects of nicotine on sperm cells. Taken et al. (2016) and Ramgir and Abilash (2019) noted that nitrous oxide affected sperm motility. Decreased levels of GSH in spermatozoa also resulted in loss of sperm cell integrity (Bhardwaj et al., 2000). Heavy

alcohol consumption causes an increase in ROS generation, leading to infertility (Das & Vasudevan, 2007; Jensen et al., 2014a, 2014b). Moreover, smoking and alcohol consumption affect and suppress Nrf2 expression, which plays a major role in protecting against oxidative damage and stress (Elsamanoudy et al., 2017).

Alcohol intake decreases the sperm count and concentration (asthenozoospermia) and causes a progressive damage to sperm morphology, especially in the sperm head. So, the deterioration in sperm quality appears in direct proportion to the quantity of alcohol intake. However, moderate or heavy smoking affects the sperm motility and deteriorates sperm quality (Gaur et al., 2010).

It was demonstrated that in spermatozoa of infertile patients, a negatively significant ($p = 0.01$) association was observed between alcohol consumption (daily alcohol intake) and polycyclic aromatic hydrocarbon-DNA (which is an early indicator and marker for sperm genotoxicity). In addition, PAH-DNA adducts were negatively correlated with the mean percentage of morphological normal spermatozoa ($r = -0.18$, $p = 0.016$) and with the abnormalities of the neck of the spermatozoa ($r = -0.21$, $p < 0.009$) (Gaspari et al., 2003).

Therefore, patients should adopt lifestyle modifications, such as quitting smoking (Wright et al., 2014), losing weight through different methods, like diet, education, and exercise (Reis & Dias, 2012), and decreasing exposure to harmful toxins, like phthalate (Sedha et al., 2015). It was also previously reported that heavy alcohol consumption caused an elevation of scrotal temperature and testis, which increased the risk of infertility (Koch et al., 2004). A mild sustained increase in testicular and epididymal temperature leads to the production of morphologically abnormal spermatozoa (Ahmad et al., 2012).

Exposures to environmental toxins, like heavy metals and organic solvents, from cigarettes, alcohol, or other sources are able to induce toxicity even at low levels of exposure, increasing OS and the incidence of abnormal sperm production. As a result, the total sperm count decreases (Acharya et al., 2003).

Sengupta (2018) recommended few lifestyle changes from tobacco or cigarette smoking, ethanol or alcohol consumption, and the use of other illicit and recreational drugs, avoiding too much psychological stress, losing weight, and reducing caffeine intake to avoid their detrimental effect on male fertility and to bring improvement in the fertility outcome.

5. Conclusion

In the light of the present study, tobacco smoking and alcohol intake have deleterious effects on the sperm parameter. Separately or together, they negatively affect the sperm parameters, sperm maturation, and DNA integrity, but the total impact of these two lifestyle factors on sperm parameters is still controversial.

Chromomycin (CMA3) and TUNEL tests are useful and could be considered as supplementary tests to ART treatment to ensure a good prognosis for a patient. However, more advanced studies at the molecular level, like DNA methylation and gene polymorphism, are necessary to elucidate the harmful effect of tobacco and alcohol on sperm structure and quality.

Moreover, patients who like to undergo assisted reproductive treatment could be advised to avoid smoking and drinking alcohol at least three months before they start their ART therapy to improve their sperm quality and function.

6. References

Abadia L, Chiu YH, Williams PL, et al. (2017). The association between pre-treatment maternal alcohol and caffeine intake and outcomes of assisted reproduction in a prospectively followed cohort. Hum Reprod. 32(9):1846–1854,

Acharya UR, Acharya S, & Mishra M. (2003). Lead acetate induced cytotoxicity in male germinal cells of Swiss mice. Industrial health, 41(3): 291-294.

Adams L, Franco MC, Estevez AG. (2015). Reactive nitrogen species in cellular signaling. Exp Biol Med (Maywood). 240(6):711–717.

Afeiche M, Williams PL, Mendiola J, Gaskins AJ, Jørgensen N , Swan SH, Chavarro JE, (2013). Dairy food intake in relation to semen quality and reproductive hormone levels among physically active young men, Human Reproduction, 28(8): 2265–2275, https://doi.org/10.1093/humrep/det133

Agarwal A, Deepinder F, Sharma RK, Ranga G, Li J. (2008). Effect of cell phone usage on semen analysis in men attending infertility clinic: an observational study. Fertil Steril. 89(1): 124-128.

Agarwal A, Aditi Mulgund A, Hamada A, and Chyatte MR (2015). A unique view on male infertility around the globe. Reproductive Biology and Endocrinology. 13:37. 2-9 DOI 10.1186/s12958-015-0032-1

Agarwal A, Allamaneni SS. (2004). Role of free radicals in female reproductive diseases and assisted reproduction. Reprod Biomed Online 9(3): 338-47.

Agarwal A, Majzoub A, Esteves SC, Ko E, Ramasamy R, Zini A. (2016). Clinical utility of sperm DNA fragmentation testing: practice ecommendations based on clinical scenarios. Transl Androl Urol 5(6): 935-950.doi: 10.21037/ tau.2016.10.03

Agarwal A, Majzoub A. (2017). Laboratory tests for oxidative stress. Indian Journal of Urology 33(3):199-206.

Agarwal A, Prabakaran SA, Said TM. (2005) Prevention of oxidative stress injury to sperm. J Androl 26: 654-660.

Agarwal A, Prabakaran SA. (2005). Mechanism, measurement and prevention of oxidative stress in male reproductive physiology. Indian J Exp Biol. 43(11):963-74

Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG. (2006). Oxidative stress in an assisted reproductive techniques setting. Fertil Steril. 86: 503–512.

Agarwal A, Said TM. (2003). Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 9: 331-345.

Agarwal A, Said TM. (2004). Carnitines and male infertility. Reproductive BioMedicine Online (Reproductive Healthcare Limited). Reproductive Healthcare Limited, 8(4): 376-84

Agarwal A, Saleh RA, Bedaiwy MA. (2003). Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil. Steril. 79 (4): 829-843

Agarwal A, Sekhon LH. (2011). Oxidative stress and antioxidants for idiopathic oligoasthenoteratospermia: Is it justified? Indian J Urol. 27(1): 74-85.doi: 10.4103/0970-1591.78437. PMID: 21716893; PMCID: PMC3114591.

Agarwal A, Virk G, Ong C, du Plessis SS. (2014). Effect of oxidative stress on male reproduction. World J Mens Health. Apr; 32(1): 1-17.doi: 10.5534/wjmh.2014.32.1.1. Epub 2014 Apr 25. PMID: 24872947; PMCID: PMC4026229.

Agbaje I, Rogers D, McVicar C, McClure N, Atkinson A, Mallidis C, et al.(2007). Insulin dependant diabetes mellitus: implications for male reproductive function. Hum Reprod. 22: 1871-1877. [DOI:10.1093/humrep/dem077]

Ahmad G, Moinard N, Esquerré-Lamare C, et al. (2012). Mild induced testicular and epididymal hyperthermia alters sperm chromatin integrity in men. Fertil Steril 97: 546 -53.

Aitken RJ, Bennetts LE, Sawyer D, Wiklendt AM, King BV. (2005). Impact of radio frequency electromagnetic radiation on DNA integrity in the male germline. Int J Androl. 28:171–179. [PubMed] [Google Scholar]

Aitken RJ, Finnie JM, Muscio L, Whiting S, Connaughton HS, Kuczera L, Rothkirch TB, De Iuliis GN (2014). Potential importance of transition metals in the induction of DNA damage by sperm preparation media. Hum Reprod 29: 2136–2147.

Aitken RJ. (2020).The Male Is Significantly Implicated as the Cause of Unexplained Infertility. Semin Reprod Med. 38(1): 3-20.doi: 10.1055/s-0040-1718941. Epub 2020 Oct 21. PMID: 33086406.

Aitken RJ, Baker MA. (2020).The Role of Genetics and Oxidative Stress in the Etiology of Male Infertility-A Unifying Hypothesis? Front Endocrinol (Lausanne). 11:581-838. doi: 10.3389/fendo.2020.581838. PMID: 33101214; PMCID: PMC7554587

Alkhaled Y, Laqqan M, Tierling S, Lo Porto C, Amor H, Hammadeh ME. (2018). Impact of cigarette-smoking on sperm DNA methylation and its effect on sperm parameters. Andrologia. 2018 Jan 9.doi: 10.1111/and.12950. Epub ahead of print. PMID: 29315717.

Alves MG, Martins AD, Rato L, Moreira PI, Socorro S, Oliveira PF. (2013). Molecular mechanisms beyond glucose transport in diabetes-related male infertility. Biochim Biophys Acta 1832: 626–635

Amor H, Zeyad A, Hammadeh ME. (2021).Tobacco smoking and its impact on the expression level of sperm nuclear protein genes: H2BFWT, TNP1, TNP2, PRM1 and PRM2. Andrologia. 53(3): 1-11. https://doi.org/10.1111/and.13964

Amor H, Zeyad A, Bakry MS, Bosilah AMH et al. (2018). Protamine ratio as a predictor of the fertility potential of sperm by couples undergoing ICSI. International Journal of Women's Health and Reproduction Sciences, 6 (4): 400– 409.

Andrabi, S MH. (2007). Mammalian sperm chromatin structure and assessment of DNA fragmentation. Journal of assisted reproduction and genetics, 24(12): 561-569.

Anifandis G, Bounartzi T, Messini CI, Dafopoulos K, Sotiriou S, & Messinis I E. (2014). The impact of cigarette smoking and alcohol consumption on sperm parameters and sperm DNA fragmentation (SDF) measured by Halosperm®. Archives of gynecology and obstetrics, 290(4): 777-782.

Aoki V, Emery B, Liu L, and Carrell D. (2006a). Protamine levels vary between individual sperm cells of infertile human males and correlate with viability and DNA integrity. J Androl; 27: 890–898.]

Aoki VW, Liu L, Carrell DT. (2005). Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. Hum Reprod. 20 (5):1298-1306.

Arabi M. (2004). Nicotinic infertility: assessing DNA and plasma membrane integrity of human spermatozoa. Andrologia, 36(5): 305-310. https://doi.org/10.1111/j.1439-0272.2004.00623.x.

Asare-Anane H, Bannison SB, Ofori EK, Ateko RO, Bawah AT, Amanquah SD, Oppong SY, Gandau BB, Ziem JB. (2016). Tobacco smoking is associated with decreased semen quality. Reprod Health. 13(1): 90.

Athayde KS, Cocuzza M, Agarwal A, Krajcir N, Lucon AM, Srougi M, Hallak J. (2007). Development of normal reference values for seminal reactive oxygen species and their correlation with leukocytes and semen parameters in a fertile population. J Androl. 28(4): 613-20.doi: 10.2164/jandrol.106.001966. Epub 2007 Apr 4. PMID: 17409462

Atli T, Keven K, Avci A, Kutlay S, et al. (2004). Oxidative stress and antioxidant status in elderly diabetes mellitus and glucose intolerance patients. Archives of gerontology and geriatrics, 39(3): 269-275.

Aydin MS, Senturk GE, & Ercan F. (2013). Cryopreservation increases DNA fragmentation in spermatozoa of smokers. Acta histochemica, 115(4): 394-400.

Aziz N, Saleh RA, Sharma RK, Lewis-Jones I, Esfandiari N, Thomas AJ Jr, Agarwal A.(2004). Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. Fertil Steril. 81(2): 349-54.doi: 10.1016/j.fertnstert.2003.06.026. PMID:

Bach PV, Patel N, Najari BB et al (2018). Changes in practice patterns in male infertility cases in the United States: The trend toward subspecialization. Fertil Steril. 110(1):76-82.

Bach PV, Schlegel PN. (2018). Male Infertility. In: Strauss J.F., Barbieri R.L. III, Gargiulo A.R., editors. Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management. 8th ed. Elsevier; Amsterdam, the Netherlands: 582–593.e2.

Baierle M, Nascimento SN, Moro AM, et al. (2015). Relationship betweeninflammation and oxidative stress and cognitive decline in the institutionalized elderly. Oxid Med Cell Longev. 1-12. 804198.

Balaban RS, Nemoto S, Finkel T (2005). Mitochondria, oxidants, and aging. Cell 120, 483–495.

Balhorn R. (2007) the protamine family of sperm nuclear proteins. Genome Biol 8, 227 https://doi.org/10.1186/gb-2007-8-9-227

Balhorn R. (1989). Mammalian protamines; structures and molec- ular interactions. In: Molecular Biology of Chromosome Function (ed. K. W. Adolp), 366-395. Springer Verlag, New York.

Balhorn R. (2018). Sperm Chromatin: An Overview. In: Zini A, Agarwal A. (eds). A Clinician's Guide to Sperm DNA and Chromatin Damage. Springer, Cham. https://doi.org/10.1007/978-3-319-71815-6_1

Barazani Y, Katz BF, Nagler HM, Stember DS. (2014). Lifestyle, environment, and male reproductive health. Urol Clin North Am 41(1):55-66.

Bashan N, Kovsan J, Kachko I, Ovadia H, Rudich A. (2009). Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species. Physiol Rev. 89(1): 27-71.doi: 10.1152/physrev.00014.2008. PMID: 19126754.

Baynes JW. (1991). Role of oxidative stress in development of complications in diabetes. Diabetes 40: 405–412.

Bedford JM, Bent MJ, Calvin H (1973). Variations in the structural character and stability of the nuclear chromatin in morphologically normal human spermatozoa. J Reprod Fertil 33:19–29

Bench GS, Friz AM, Corzett MH, Morse D H Balhorn R. (1996). DNA andtotal protamine masses in individual sperm from fertile mammalian subjects. Cytometry: The Journal of the International Society for Analytical Cytology, 23(4): 263-271.

Berryman DE, List EO, Palmer AJ et al. (2010). Two-year body composition analyses of long-lived GHR null mice. J Gerontol A Biol Sci Med Sci. 65: 31–40.

Bhardwaj A, Verma A, Majumdar S, Khanduja KL. (2000). Status of vitamin E and reduced glutathione in semen of oligozoospermic and azoospermic patients. Asian J Androl. 2(3): 225-8.

Bhasin S. (2007). Approach to the infertile man. J Clin Endocrinol Metab. 92(6):1995-2004.

Bianchi, PG, Manicardi, G.C., Bizzaro, D. et aL (1993). Effect of DNA protaminanon on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. Biol Reprod 49: 1638-1643.

Björndahl L, Kvist U. (2010). Human sperm chromatin stabilization: a proposed model including zinc bridges. Mol Hum Reprod. 16(1): 23-9.doi: 10.1093/molehr/gap099. Epub 2009 Nov 20. PMID: 19933313.

Bloch-Damti A, Bashan N. (2005). Proposed mechanisms for the induction of insulin resistance by oxidative stress. Antioxid Redox Signal 7: 1553–1567.

Boe-Hansen GB, Fedder J, Ersboll AK, Christensen P. (2006). The sperm chromatin structure assay as a diagnostic tool in the human fertility clinic. Human Reproduction 21 (6):1576-82.

Boekelheide K, Fleming SL, Johnson KJ, Patel SR, Schoenfeld HA (2000). Role of Sertoli cells in injury-associated testicular germ cell apoptosis. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.) (2000). 225: 105-15. PMID 11044252 DOI: 10.1111/J.1525-1373.2000.22513.X

Boeri L, Capogrosso P, Ventimiglia E, Pederzoli F, Cazzaniga W, Chierigo F, et al. (2019). Heavy cigarette smoking and alcohol consumption are associated with impaired sperm parameters in primary infertile men. Asian J. Androl. 21 478–485. 10.4103/aja.aja_110_18 [PMC free article] [PubMed] [CrossRef] [Google Scholar]

Boissonneault G. (2002). Chromatin remodeling during spermiogenesis: a possible role for the transition proteins in DNA strand break repair. FEBS Lett. 2002 Mar 13; 514(2-3):111-4.doi: 10.1016/s0014-5793(02)02380-3. PMID: 11943135

Braun, RE. (2001) Packaging paternal chromosomes with protamine. Nature genetics, 28(1), 10.

Brewer L, Corzett M, Balhorn R. (2002). Condensation of DNA by spermatid basic nuclear proteins. J Biol Chem. 277(41): 38895-38900

Brinster RL, Zimmermann JW. (1994). Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci. 91 (24):11298–11302. doi:10.1073/pnas.91.24.11298

Brykczynska U, Hisano M, Erkek S, Ramos L, Oakeley E J, Roloff, T. et al. (2010). Repressive and active histone methylation mark distinct promoters inhuman and mouse spermatozoa. Nature structural & molecular biology 17(6): 679.

Bui AD, Sharma R, Henkel R, Agarwal A. (2018). Reactive oxygen species impact on sperm DNA and its role in male infertility. Andrologia e13012. Wiley onlinelibrary.com/journal/and | 1 of 10 https://doi.org/10.1111/and.13012

Bui AD, Sharma R, Henkel R, Agarwal A. (2018). Reactive oxygen species impact on sperm DNA and its role in male infertility. Andrologia 50(8):e13012.doi: 10.1111/and.13012. Epub 2018 Apr 11. PMID: 29644708.

Bundhun P K, Janoo G, Bhurtu A, Teeluck A R, et al. (2019). Tobacco smoking and semen quality in infertile males: a systematic review and meta-analysis. BMC Public Health, 19(1), 1-11.

Byun EH, Kim K, Kim YE, Cho EJ, Min HS, Lee JH, Cho G, Yoon WJ, Kim BC, Ahn DH, Park W. (2018). Comparison study of antioxidantactivity and neuroprotective effects of barley sprout leaf, root, and stem ethanol extracts. J. Korean Soc. Food Sci. Nutr. 47: 973-980.

Calhaz-Jorge C, De Geyter C, Kupka MS, de Mouzon J, Erb K, Mocanu E, Motrenko T, Scaravelli G, Wyns C, Goossens V. (2017). Assisted reproductive technology in Europe, 2013: results generated from European registers by ESHRE. Hum Reprod. 32(10):1957–1973.

Campbell JM, Lane M, Owens JA, and Bakos HW. (2015). Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis. Reprod. Biomed. Online 31: 593–604. doi: 10.1016/j.rbmo.2015.07.012

Canovas S, & Ross PJ. (2016). Epigenetics in preimplantation mammalian development. Theriogenology, 86(1): 69-79.

Caron N, Veilleux S and Boissonneault G (2001). Stimulation of DNA repair by the spermatidal TP1 protein Molecular Reproduction and Development 58: 437-443

Carrell D., Emery B., Hammoud S. (2007). Altered protamine expression and diminished spermatogenesis: what is the link?. Hum Reprod Update 13: 313–327 [Google Scholar]

Carrell DT, Hammoud SS. (2009). The human sperm epigenome and its potential role in embryonic development. MHR: Basic science of reproductive medicine, 16(1): 37-47.

Carrell DT. (2012) Epigenetics of the male gamete, Fertil Steril. 97: 267-274.

Castillo J, Amaral A, Azpiazu R, Vavouri T, Estanyol JM, Ballescà JL & Oliva R. (2014). Genomic and proteomic dissection and characterization of the human sperm chromatin. Molecular human reproduction 20 (11): 1041-1053.

Centers for Disease Control and Prevention (US); National Center for Chronic Disease Prevention and Health Promotion (US); Office on Smoking and Health (US). How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General. Atlanta (GA): Centers for Disease Control and Prevention (US); 2010. PMID: 21452462.

Centers for Disease Control and Prevention ASfRM, Society for Assisted Reproductive Technology. 2015 Assisted Reproductive Technology Fertility Clinic Success Rates Report.. Atlanta (GA): US Dept of Health and Human Services; 2017. [Google Scholar]

Cheema M S, & Ausió J. (2015). The structural determinants behind the epigenetic role of histone variants. Genes, 6(3), 685-713.

Chemes EH, Rawe YV. (2003). Sperm pathology: a step beyond descriptive morphology. Origin, characterization and fertility potential of abnormal sperm phenotypes in infertile men. Hum Reprod Update. 9(5): 405-28.

Chia SE, Ong CN, Tsakok FM. (1994). Effects of cigarette smoking on human semen quality. Arch Androl. 33(3):163-8.doi: 10.3109/01485019408987820. PMID: 7857167.

Chia SE, Tay SK, Lim ST. (1998). What constitutes a normal seminal analysis? Semen parameters of 243 fertile men. Hum Reprod 13: 3394–8.Abstract/

Cho Ch-L, Agarwal A, Esteves SC. (2017). Clinical utility of sperm DNA fragmentation testing: concise practice recommendations. Transl Androl Urol 6 (Suppl 4): S501-S503

Churikov D, Zalenskaya IA, & Zalensky AO. (2004b). Male germline-specifichistones in mouse and man. Cytogenetic and genome research, 105(2-4): 203-214.

Cinar O, Dilbaz S, Terzioglu F, Karahalil B, Yücel C, et al. (2014). Does cigarette smoking really have detrimental effects on outcomes of IVF? Eur J Obstet Gynecol Reprod Biol; 174: 106-10.

Condorelli RA, Calogero AE, Vicari E, La Vignera S. (2015). Chronic consumption of alcohol and sperm parameters: our experience and the main evidences. Andrologia. 47:368–79

Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, Haugen TB, Kruger T, Wang C, Mbizvo MT, et al. (2010). World Health Organization reference values for human semen characteristics. Hum Reprod Update 16(3): 231–45.

Coward K, Wells D. Mammalian reproductive physiology. In text book of clinical Embryology edited by Coward, Kevin and Wells, Dagan,. Cambridge University Press. 2013, Online ISBN: 9781139192736 . DOI: https://doi.org/10.1017/CBO9781139192736.003.pp 1-132

Cui X, Jing X, Wu X, Wang Z, Li Q. (2016). Potential effect of smoking on semen quality through DNA damage and the downregulation of Chk1 in sperm. Mol Med Rep. 14: 753–61.

D'agata R, Vicari E, Moncada M, Sidoti G, Calogero A, Fornito M, et al. (1990). Generation of reactive oxygen species in subgroups of infertile men. Int J Androl 13: 344-351. [DOI:10.1111/j.1365-2605.1990.tb01042.x]

Dai JB, Wang ZX, Qiao ZD. (2015). The hazardous effects of tobacco smoking on male fertility. Asian J Androl 17: 954–960.

Darbandi M, Darbandi S, Agarwal A , Sengupta P, Durairajanayagam D, Henkel R and Sadegh MR. (2018). Reactive oxygen species and male reproductive hormones. Reproductive Biology and Endocrinology 16:87 https://doi.org/10.1186/s12958-018-0406-2

Das SK, Vasudevan DM. Alcohol-induced oxidative stress. Life Sci 2007; 81(3):177-87.

Das, L, Parbin S, Pradhan N, Kausar C, & Patra SK. (2017). Epigenetics of reproductive infertility. Front Biosci (Schol Ed), 9: 509-535.

de Angelis C, Galdiero M, Pivonello C, Salzano C, Gianfrilli D, Piscitelli P, Lenzi A, Colao A, Pivonello R. (2017). The environment and male reproduction: the effect of cadmium exposure on reproductive functions and its implication in fertility. Reprod Toxicol. 73:105–27. CAS PubMed Article Google Scholar

De Iuliis GN, Newey RJ, King BV, Aitken RJ. (2013). Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro [published correction appears in PLoS One 8(3). doi:10.1371/annotation/9a8a0172-3850-4059-b852-72c330769c1b]. PLoS One. 2009;4(7):e6446. Published 2009 Jul 31. doi:10.1371/journal.pone.0006446

De Iuliis GN, Newey RJ, King BV, Aitken RJ. (2009). Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. PLoS One. 4(7):e6446. doi: 10.1371/journal.pone.0006446. Erratum in: PLoS One. 2013;8(3). doi:10.1371/annotation/9a8a0172-3850-4059-b852-72c330769c1b. PMID: 19649291; PMCID: PMC2714176.

de Kretser DM (2004). Is Spermatogenic Damage Associated with Leydig Cell Dysfunction? The Journal of Clinical Endocrinology & Metabolism 89 (7): 3158–3160.doi: 10.1210/jc.2004-0741

de Kretser DM, Loveland KL, Meinhardt A, Simorangkir D, Wreford N. (1998). Spermatogenesis. Hum Reprod. 1998; 13(suppl 1):1–8. doi:10.1093/humrep/13.suppl_1.1

de Lamirande E, Leclerc P, Gagnon C (1997). Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Molecular Human Reproduction, (3): 175–194, https://doi.org/10.1093/molehr/3.3.175

Di Meo S, Reed TT, Venditti P, Victor VM. (2016). Role of ROS and RNS Sources in Physiological and Pathological Conditions. Harmful and Beneficial Role of ROS. Oxidative Medicine and Cellular Longevity. ID 1245049.

http://dx.doi.org/10.1155/2016/1245049. Hindawi Publishing Corporation

Desai NR, Kesari KK, Agarwal A. (2009). Pathophysiology of cell phone radiation: oxidative stress and carcinogenesis with focus on male reproductive system. Reprod Biol Endocrinol. 7:114. doi: 10.1186/1477-7827-7-114. PMID: 19849853; PMCID: PMC2776019.

Desai N, Ludgin J, Sharma R, Anirudh RK, & Agarwal A. (2017). Female and male gametogenesis. In Clinical reproductive medicine and surgery (pp. 19-45). Springer, Cham.

Dietrich M, Block G, Norkus EP, Hudes M, Traber MG, Cross CE, et al. (2003). Smoking and exposure to environmental tobacco smoke decrease some plasma antioxidants and increase γ-tocopherol in vivo after adjustment for dietary antioxidant intakes. Am J Clin Nutr. 77:160–166. [PubMed] [Google Scholar]

Dobrakowski M, Kasperczyk S, Horak S, Chyra-Jach D, Birkner E, Kasperczyk A. (2017). Oxidative stress and motility impairment in the semen of fertile males. Andrologia 49(10). doi: 10.1111/and.12783. Epub 2017 Mar 6. PMID: 28261836.

Donovan PJ, de Miguel MP. (2003). Turning germ cells into stem cells. Curr Opin Genet Dev. 13(5):463–471. doi:10.1016/j.gde.2003.08.010

Doshi SB, Khullar K, Sharma RK, Agarwal A. (2012). Role of reactive nitrogen species in male infertility. Reprod Biol Endocrinol. 10:109. [PMC free article] [PubMed] [Google Scholar].

Drope JM, Liber AC, Cahn Z, et al. (2018). Who's still smoking? Disparities in adult cigarette smoking prevalence in the United States. CA Cancer J Clin. 68: 106- 115.

Du Plessis SS, Agarwal A, Halabi J. et al. (2015). Contemporary evidence on the physiological role of reactive oxygen species in human sperm function. J Assist Reprod Genet 32: 509–520 https://doi.org/10.1007/s10815-014-0425-7

Duan P, Hu C, Quan C, Yu T, Zhou W, Yuan M, Shi Y, Yang K. (2016). 4-Nonylphenol induces apoptosis, autophagy and necrosis in Sertoli cells: Involvement of ROS-mediated

AMPK/AKT-mTOR and JNK pathways. Toxicology. 341-343:28-40. doi: 10.1016/j.tox.2016.01.004. Epub 2016 Jan 19. PMID: 26804764.

Dunphy BC, Barratt CL, Cooke ID. (1991). Male alcohol consumption and fecundity in couples attending an infertility clinic. Andrologia 23: 219–21.

Dutta S, Majzoub A, Agarwal A. (2019). Oxidative stress and sperm function: A systematic review on evaluation and management. Arab J Urol. 24; 17(2): 87-97. doi: 10.1080/2090598X.2019.1599624. PMID: 31285919; PMCID: PMC6600059.

Eggert J, Theobald H, Engfeldt P. (2004). Effects of alcohol consumption on female fertility during an 18-year period. Fertil Steril. 81: 379–83.

Elsamanoudy AZ, Dalia Shaalan D, Abo El-khair SM, Gaballah MA, State AF, Helaly AMN. (2017). NRF2 gene expression and DNA fragmentation markers as possible predictors of chronic smoking induced spermatozoa dysfunction in infertility with normal seminogram. Human Andrology 7 (4): 127-135.

Elshal MF, El-Sayed IH, Elsaied MA, El-Masry SA, Kumosani TA. (2009). Sperm head defects and disturbances in spermatozoal chromatin and DNA integrities in idiopathic infertile subjects: association with cigarette smoking. Clin Biochem. 42(7-8): 589-94. doi: 10.1016/j.clinbiochem.2008.11.012. Epub 2008 Dec 6. PMID: 19094977.

Emanuele MA and Emanuele NV. (1998). Alcohol's effects on male reproduction. Alcohol Health Res World; 22:195-218

Emanuele MA, Emanuele NV. (2001). Alcohol and the male reproductive system. Alcohol Res Health.; 25(4): 282-7. PMID: 11910706; PMCID: PMC6705705.

Esfandiari N, Saleh RA, Abdoos M, Rouzrokh A, Nazemian Z. (2002). Positive bacterial culture of semen from infertile men with asymptomatic leukocytospermia. Int J Fertil Womens Med. 47: 265–270.

Eskiocak S. et al. (2006). Effect of psychological stress on the L- arginine-nitric oxide pathway and semen quality. Braz. J. Med. Biol. Res. 39: 581–588

Esposito, K; Giugliano G, Scuderi,N, Giugliano D. (2006). Role of Adipokines in the Obesity–Inflammation Relationship: The Effect of Fat Removal, Plastic and Reconstructive Surgery 118 (4), 1048-1057 doi: 10.1097/01.prs.0000232281.49432.ce

Esteves SC, Agarwal A. (2011). Novel Concepts in Male Infertility. International Braz J Urol. 37 (1): 5-15, doi: 10.1590/S1677-55382011000700002

Esteves SC, Miyaoka R, Roque M, Agarwal A. (2016). Outcome of varicocele repair in men with nonobstructive azoospermia: systematic review and meta-analysis. Asian Journal of Andrology 18: 246–253. 2016 AJA, SIMM & SJTU. All rights reserved 1008-682X

Esteves SC. (2002). Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. Int Braz J Urol. 28: 484–485. [PubMed] [Google Scholar]

Fang YY, Wu Q J, Zhang T N, Wang T R, Shen ZQ, Jiao J, et al. (2018). Assessment of the development of assisted reproductive technology in Liaoning province of China, from 2012 to 2016. BMC Health Serv. Res. 18: 873. 10.1186/s12913-018-3585-9 [PMC free article] [PubMed] [CrossRef] [Google Scholar].

Fenster L, Katz DE, Wybrobek AJ, et al., (1997). Effects of Psychological Stress on Human Semen Quality. 1997: 18(2): 194-202.

Ferramosca A, Pinto Provenzano S, Montagna DD, Coppola L, Zara V. (2013). Oxidative stress negatively affects human sperm mitochondrial respiration. Urology (82): 78-83.

Fertility: Assessment and Treatment for People with Fertility Problems 2004, London, UK, RCOG Press http://www.ncbi.nlm.nih.gov/books/NBK45935/

Fischbein A, Zabludovsky N, Eltes F, Grischenko V, Bartoov B. (1997). Ultramorphological sperm characteristics in the risk assessment of health effects after radiation exposure among salvage workers in Chernobyl. Environ Health Perspect. 105:1445–9.

Fisher JS, Macpherson S, Marchetti N, & Sharpe R. M. (2003). Human 'testicular dysgenesis syndrome. a possible model using in-utero exposure of the rat to dibutyl phthalate. Human reproduction, 18(7): 1383-1394.

Fode M, Krogh-Jespersen SK, Brackett NL, OhlD A, Lynne CML, Sønksen J. (2012). Male sexual dysfunction and infertility associated with neurological disorders. Asian Journal of Andrology 14: 61–68. AJA, SIMM & SJTU. doi:10.1038/aja.2011.70;

Frodsham LC, Boag F, Barton S, Gilling-Smith C. (2006). Human immunodeficiency virus infection and fertility care in the United Kingdom: demand and supply. Fertil Steril. 85: 285-289. [DOI:10.1016/j.fertnstert.2005.07.1326

Fuentes A, Muñoz A, Barnhart K, Argüello B, Díaz M, et al. (2010). Recent cigarette smoking and assisted reproductive technologies outcome. Fertil Steril. 93: 89-95.

Furukawa S, Fujita T, Shimabukuro M, Iwaki M. et al. (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest. 114(12):1752. PubMedGoogle Scholar

Galdon G, Atala A, Sadri-Ardekani H. (2016). In vitro spermatogenesis: how far from clinical application? Curr Urol Rep. 17(7): 49. doi:10.1007/s11934-016-0605-3

Gannon AM, Stämpfli MR; Foster WG. (2013). Cigarette smoke exposure elicits increased autophagy and dysregulation of mitochondrial dynamics in murine granulosa cells. Biol. Reprod. 88, 63.

Gao HB, Tong MH, Hu YQ , Guo QSu, Ge R, Matthew MP, (2002). Glucocorticoid Induces Apoptosis in Rat Leydig Cells, Endocrinology 143 (1): 130–138, https://doi.org/10.1210/endo.143.1.8604.

Garolla A, Ghezzi M, Cosci I, et al. (2017). FSH treatment in infertile males candidate to assisted reproduction improved sperm DNA fragmentation and pregnancy rate. Endocrine. 56. (2): 416–425. doi:10.1007/s12020-016-1037-z

Gaspari L, Chang SS, ReginaM. Santella RM, Garte S, Pedotti P, Taioli E. (2003). Polycyclic aromatic hydrocarbon-DNA adducts in human sperm as a marker of DNA damage and infertility. Mutation Researchn 535(2):155-160. DOI: 10.1016/s1383-5718(02)00297-8 PMID: 12581533

Gatewood JM, Cook GR, Balhorn R, Schmid CW, & Bradbury EM. (1990). Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. Journal of Biological Chemistry 265(33): 20662-20666.

Gaur DS, Talekar MS, Pathak V. (2010). Alcohol intake and cigarette smoking: Impact of two major lifestyle factors on male fertility. Indian J Pathol Microbiol 53:35-40. ttps://www.ijpmonline.org/text.asp? 2010/53/1/35/59180

Ghuman N, Ramalingam M. (2018). Male infertility. Obstetrics, Gynaecology & Reproductive Medicine. 28 (1): 7-14. DOI: https://doi.org/10.1016/j.ogrm.2017.10.007

Gnoth C, Godehardt E, Frank-Herrmann P, et al. (2005). Definition and prevalence of subfertility and infertility, Human Reproduction 20 (5): 1144–1147, https://doi.org/10.1093/humrep/deh870

Godmann M, Auger V, Ferraroni-Aguiar V, Sauro AD et al., (2007). Dynamic regulation of histone H3 methylation at lysine 4 in mammalian spermatogenesis. Biology of reproduction, 77(5): 754-764

Gomes MJ, Martinez PF, Pagan LU, Damatto RL, et al (2017). Skeletal muscle aging: influence of oxidative stress and physical exercise. Oncotarge, (12): 20428-20440

Gonzales GF, Muñoz G, Sánchez R, Henkel R, et al. (2004). Update on the impact of Chlamydia trachomatis infection on male fertility. Andrologia 36(1):1-23. doi: 10.1046/j.0303-4569.2003.00594.x. PMID: 14871260.

González-Romero R, Méndez J, Ausió J & Eirín-López J M. (2008). Quickly evolving histones, nucleosome stability and chromatin folding: all about histone H2A. Bbd. Gene, 413(1): 1-7.

Goverde HJ, Dekker HS, Janssen HJ, Bastiaans BA, Rolland R, Zielhuis GA. (1995). Semen quality and frequency of smoking and alcohol consumption - an explorative study. Int J Fertil Menopausal Stud 40:135-138.

Green GR, Balhorn R, Poccia DL, Hecht NB. (1994). Synthesis and processing of mammalian protamines and transition proteins. Mol Reprod Dev. 37: 255–263.

Gündüz , Şentürk ÜK. (2003). The effect of reactive oxidant generation in acute exercise-induced proteinuria in trained and untrained rats. Eur J Appl Physiol 90: 526–532. https://doi.org/10.1007/s00421-003-0888-1

Gunes S, Al-Sadaan M, Agarwal A. (2015). Spermatogenesis, DNA damage and DNA repair mechanisms in male infertility. Reprod Biomed Online. 31(3): 309-19. doi: 10.1016/j.rbmo.2015.06.010. Epub 2015 Jun 19. PMID: 26206278.

Güney AI, Javadova D, Kurac D, Ulucan K, Koc G, Ergec D. (2012). Detection of Y chromosome microdeletions and mitochondrial DNA mutations in male infertility patients. Genetics and Molecular Research 11 (2): 1039-1048

Ha HK, Park HJ, Park NC. (2011). Expression of E-cadherin and α-catenin in a varicocele-induced infertility rat model. Asian J Androl 13: 470-475.

Hadi HA, Hill JA, Castillo RA. (1987). Alcohol and reproductive function: a review. Obstet Gynecol Surv. 42(2): 69-74. PMID: 3547207.

Halmenschlager G, Rossetto G, Müller Lara GM, Rhoden EL. (2009). Evaluation of the Effects of Cigarette Smoking on Testosterone Levels in Adult Men. The Journal of Sexual Medicine. 6 (6): 1763-1772. https://doi.org/10.1111/j.1743-6109.2009.01227.x.

Hamad MF, Dayyih WAA, Laqqan M, AlKhaled Y, Montenarh M, Hammadeh ME. (2018). The status of global DNA methylation in the spermatozoa of smokers and non-smokers. Reprod. Biomed. Online 37: 581–589. doi: 10.1016/j.rbmo.2018.08.016. PubMed Abstract | CrossRef Full Text | Google Scholar

Hamad MF, Shelko N, Kartarius S, Montenarh M, & Hammadeh M E (2014). Impact of cigarette smoking on histone (H2B) to protamine ratio in human spermatozoa and its relation to sperm parameters. Andrology 2(5): 666– 677. https://doi.org/10.1111/j.2047-2927.2014.00245.x.

Hammadeh ME, Al Hasani S, Rosenbaum P, Schmidt W, Fischer-Hammadeh C. (2008). Reactive oxygen species, total antioxidant concentration of seminal plasma and their effect on sperm parameters and outcome of IVF/ICSI patients, Arch Gynecol Obstet. 277: 515-526. 10.1007/s00404-007-0507-1

Hammadeh ME, Radwan M, Al-Hasani S, Micu R, Rosenbaum P, Lorenz M, Schmidt W. (2006). Comparison of reactive oxygen species concentration in seminal plasma and semen parametersof pregnant and non-pregnant after IVF/ICSI, Reprod Biomed Online, 13: 696-706. 10.1016/S1472-6483(10) 60661-X

Hammadeh ME, Hamad MF, Montenarh M, Fischer-Hammadeh C. (2010). Protamine contents and P1/P2 ratio in human spermatozoa from smokers and non-smokers. Hum Reprod. 25(11):2708-20. doi: 10.1093/humrep/deq226. Epub 2010 Sep 7. PMID: 20823111.

Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. (2009). Distinctive chromatin in human sperm packages genes for embryo development. Nature 460 (7254):473–478. [PubMed: 19525931]

Hansen LM, Thulstrup AM , Bondeb JP, Olsen J, LB, Ramlau-Hansen CH. (2012). Does last week's alcohol intake affect semen quality or reproductive hormones? A cross-sectional study among healthy young Danish. Reproductive Toxicology 34(3): 457-462.

Haque O, Vitale JA, Agarwal A, du Plessis SS. (2014). The effect of smoking on male infertility. In: Male Infertility. New York: Springer 19-30.

Harlev A, Agarwal A, Gunes SO, Shetty A, du Plessis SS. (2015). Smoking and male infertility: an evidence-based review. World J Mens Health 33:143– 60.

Hassan MAM, Killick SR. (2004). Negative lifestyle is associated with a significant reduction in fecundity. Fertil Steril. 81:384–92. [PubMed] [Google Scholar]

Hatch EE, Wise LA, Mikkelsen EM, et al. (2012). Caffeinated beverage and soda consumption and time to pregnancy. Epidemiology 23: 393–401.

Hecht NB. (1989). Molecular biology of structural chromosomal proteins of the mammalian testis. In KW Adolph (ed): "Molecular Biology of Chromosome Function." New York: Springer Verlag, pp 396- 420

Hjollund NHI, Bonde JPE, Jensen TK, Olsen J. (2000). Diurnal scrotal skin temperature and semen quality. Int J Androl 23: 309-318. [DOI:10.1046/j.1365-2605.2000.00245.x]

Holmström K, Finkel T. (2014). Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol 15: 411–421. https://doi.org/10.1038/nrm3801

Horvath TL, Andrews ZB, Diano S (2009). Fuel utilization by hypothalamic neurons: Roles for ROS. Trends Endocrinol. Metab. 20: 78–87

Ihara M, Meyer-Ficca, M, Leu NA. (2014). Paternal poly (ADP-ribose) metabolism modulates retention of inheritable sperm histones and early embryonic gene expression. PLoS genetics 10(5), e1004317.

Inhorn MC, Patrizio P. (2015). Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century. Hum Reprod Update. 21(4): 411-26. doi: 10.1093/humupd/dmv016. Epub 2015. 22. PMID: 25801630.

Jacob KD, Noren Hooten N, Trzeciak AR, Evans, MK. (2013). Markers of oxidant stress that is clinically relevant in aging and age-related disease. Mech. Ageing Dev. 134: 139–157.

Jana K, Samanta PK, De DK. (2010). Nicotine diminishes testicular gametogenesis, steroidogenesis, and steroidogenic acute regulatory protein expression in adult albino rats: possible influence on pituitary gonadotropins and alteration of testicular antioxidant status. Toxicol Sci. 116: 647-59.

Jenkins TG, James ER, Alonso DF, Hoidal JR, Murphy PJ, Hotaling JM, et al. (2017). Cigarette smoking significantly alters sperm DNA methylation patterns. Andrology 5:1089–99.

Jensen TK, Bonde JP, Joffe M., The influence of occupational exposure on male reproductive function, Occupational Medicine, 56(8): 2006, 544–553, https://doi.org/10.1093/occmed/kql116

Jensen TK, Gottschau M, Madsen JOB, et al (2014a) Habitual alcohol consumption associated with reduced semen quality and changes in reproductive hormones; a cross-sectional study among 1221 young Danish men. BMJ Open 4: e005462. doi: 10.1136/bmjopen-2014-005462

Jensen TK, Swan S, Jorgensen N, Toppari J, Redmon B, Punab M, et al. (2014b) Alcohol and male reproductive health: a cross-sectional study of 8344 healthy men from Europe and the USA. Hum Reprod 29:1801–9.

Jiang LG, Cheng LY, Kong SH, Yang Y, Shen YJ, et al. (2017). Toxic effects of polychlorinated biphenyls (Aroclor 1254) on human sperm motility. Asian Journal of Andrology 19: 561–566. www.asiaandro.com; www.ajandrology.com

Jiménez-Fernández S, Gurpegui M, Díaz-Atienza F, Pérez-Costillas L, Gerstenberg M, Correll CU. (2015). Oxidative stress and antioxidant parameters in patients with major depressive disorder compared to healthy controls before and after antidepressant treatment: results from a meta-analysis. J Clin Psychiatry. 1658-1667. doi: 10.4088/JCP.14r09179. PMID: 26579881.

Jiménez-Fernández S, Gurpegui M, Garrote-Rojas D, Gutiérrez-Rojas L, Carretero MD, Correll CU. (2021) Oxidative stress parameters and antioxidants in patients with bipolar disorder: Results from a meta-analysis comparing patients, including stratification by polarity and euthymic status, with healthy controls. Bipolar Disord. 23(2):117-129. doi: 10.1111/bdi.12980. Epub 2020 Sep 10. PMID: 32780547.

Joo KJ, Kwon YW, Myung SC, Kim Th. (2012). The Effects of Smoking and Alcohol Intake on Sperm Quality: Light and Transmission Electron Microscopy Findings. The Journal of International Medical Research. 40: 2327 – 2335

Jorsaraei SGA, Shibahara H. (2008). The in-vitro effects of nicotine, cotinine and leptin on sperm parameters analysed by CASA system. Iran J Reprod Med. 6:157–165.

Jung A, Schuppe HC. (2007). Influence of genital heat stress on semen quality in humans. Andrologia 39:203–215. [PubMed] [Google Scholar]

Jurasović J, Cvitković P, Pizent A, Colak B, Telisman S. (2004). Semen quality and reproductive endocrine function with regard to blood cadmium in Croatian male subjects. Biometals. 17:735–743. [PubMed] [Google Scholar]

Jutte NH, Grootegoed JA, Rommerts FFG, & Van der Molen H J. (1981). Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids. Reproduction, 62(2): 399-405.

Jutte NH, Jansen R, Grootegoed JA, RommertsFFG, et al. (1982). Regulation of survival of rat pachytene spermatocytes by lactate supply from Sertoli cells. Reproduction 65(2): 431-438

Kao SH, Chao HT, Chen HW, Hwang TIS, Liao TL, Wei YH. (2008). Increase of oxidative stress in human sperm with lower motility. Fertil Steril. 89(5):1183-1190. doi: 10.1016/j.fertnstert.2007.05.029. Epub 2007 Jul 31. PMID: 17669405.

Kapoor D, Jones TH. (2005). Smoking and hormones in health and endocrine disorders. Eur J Endocrinol. 152(4):491-9. doi: 10.1530/eje.1.01867. PMID: 15817903.

Kasahara E, Sato EF, Miyoshi M, Konaka R, Hiramoto K, Sasaki J, et al. (2002). Role of oxidative stress in germ cell apoptosis induced by di(2-ethylhexyl)phthalate. Biochem J. 365: 849–856. [PMC free article] [PubMed] [Google Scholar]

Kashou AH, du Plessis SS, Agarwal A. (2012). The Role of Obesity in ROS Generation and Male Infertility. In: Agarwal A., Aitken R., Alvarez J. (eds) Studies on Men's Health and Fertility. Oxidative Stress in Applied Basic Research and Clinical Practice. Humana Press. https://doi.org/10.1007/978-1-61779-776-7_26

Katib AA, Al-Hawsawi K, Motair W, Bawa AM. (2014). Secondary infertility and the aging male, overview. Central European Journal of Urology, 67: 184–188

Kesari KK, Agarwal A. & Henkel R. (2018). Radiations and male fertility. Reprod Biol Endocrinol 16: 118. https://doi.org/10.1186/s12958-018-0431-1

Khalil AM, Boyar FZ, & Driscoll DJ. (2004). Dynamic histone modifications mark sex chromosome inactivation and reactivation during mammalian spermatogenesis. Proceedings of the National Academy of Sciences, 101(47): 16583- 16587.

Kim JH, Kim HJ, Noha HS, Roh GS, Kang SS, et al. (2003). Suppression by ethanol of male reproductive activity. Brain Research 989 (1): 91-98

Kiziler AR, Aydemir B, Onaran I, Alici B, Ozkara H, Gulyasar T, Akyolcu MC. (2007). High levels of cadmium and lead in seminal fluid and blood of smoking men are associated with high oxidative stress and damage in infertile subjects. Biological trace element research. 120(1–3): 82–91. [PubMed: 17916958]

Kleene K C, Flynn J. (1987). Translation of mouse testis poly (a)+ mrnas for testis-specific protein, protamine 1, and the precursor for protamine 2. Developmental Biology 123: 125–135

Klonoff-Cohen H, Natarajan L, Marrs R, Yee B. (2001) Effects of female and male smoking on success rates of IVF and gamete intra-fallopian transfer. Hum Reprod 16: 1382-90

Klonoff-Cohen H. (2005). Female and male lifestyle habits and IVF: what is known and unknown. Hum Reprod Update 11(2):179-203

Kobayashi CI, Suda T. (2012). Regulation of reactive oxygen species in stem cells and cancer stem cells. J Cell Physiol 227: 421-430. [DOI:10.1002/jcp.22764]

Koch OR, Pani G, Borrello S, Colavitti R, Cravero A, Farrè S, Galeotti T. (2004). Oxidative stress and antioxidant defenses in ethanol-induced cell injury. Mol Aspects Med. 25(1-2):191-8. doi: 10.1016/j.mam.2004.02.019. PMID: 15051327.

Kovac JR, Khanna A, Lipshultz LI. (2015). The effects of cigarette smoking on male fertility. Postgrad Med 127: 338–41.

Kovacic P, Thurn LA. (2005) Cardiovascular Toxicity from the Perspective of Oxidative Stress, Electron Transfer, and Prevention by Antioxidants. Current Vascular Pharmacology 3 (2) 107-117

Kucheria K. Saxena R. Mohan D. (1985). Semen analysis in alcohol dependence syndrome. Andrologia 17: 558-563.

Kumar S, Murarka S, Mishra VV, Gautam AK. (2014). Environmental & lifestyle factors in deterioration of male reproductive health. Indian J Med Res 140(Suppl): S29–35.

Kumar SR, Davies SH, Weitzman M, Sherman S. (2015). A review of air quality, biological indicators and health effects of second-hand waterpipe smoke exposure. BMJ supplement. Tob Control; 24: i54–i59. doi:10.1136/tobaccocontrol-2014-052038

Künzle R, Mueller MD, Hänggi W, Birkhäuser MH, Drescher H, Bersinger NA. (2003). Semen quality of male smokers and nonsmokers in infertile couples. Fertil Steril. 79:287-91

La Maestra S, De Flora S, Micale RT. (2015). Effect of cigarette smoke on DNA damage, oxidative stress, and morphological alterations in mouse testis and spermatozoa. Int J Hyg Environ Health 218: 117–122.

Laberge RM, Boissonneault G. (2005). On the nature and origin of DNA strand breaks in elongating spermatids. Biol of Reprod 73: 289–296 [Google Scholar]

Laqqan M, Tierling S, Alkhaled Y, LoPorto C, Hammadeh ME. (2017). Alterations in sperm DNA methylation patterns of oligospermic males. Reprod Biol. 17(4): 396-400. doi: 10.1016/j.repbio.2017.10.007. Epub 2017 Nov 4. PMID: 29108863.

Lastra G, Manrique C, Sowers JR. (2006). Obesity, Cardiometabolic Syndrome, and Chronic Kidney Disease: The Weight of the Evidence. Advances in Chronic Kidney Disease. 13 (4), 365-373. https://doi.org/10.1053/j.ackd.2006.07.011

Latini G, Del Vecchio A, Massaro M, Verrotti A, De Felice C. (2006). Phthalate exposure and male infertility. Toxicology 226: 90–98. [PubMed] [Google Scholar]

La Vignera S, Condorelli RA, Balercia G, Vicari E, Calogero AE. (2013). Does alcohol have any effect on male reproductive function? A review of literature. Asian J Androl. 2013 Mar;15(2):221-5. doi: 10.1038/aja.2012.118. Epub 2012 Dec 31. PMID: 23274392; PMCID: PMC3739141.

Lavranos G, Balla M, Tzortzopoulou A, Syriou V, Angelopoulou R. (2012). Investigating ROS sources in male infertility: a common end for numerous pathways. Reprod Toxicol. 34: 298–307.

Lee K, Haugen HS, Clegg CH, Braun RE. (1995). Premature translation of protamine 1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice. Proc Natl Acad Sci U S A. 1995 Dec 19; 92(26):12451-5. doi: 10.1073/pnas.92.26.12451. PMID: 8618919; PMCID: PMC40375.

Lewis SE, and RJ, Aitken. (2005). DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell Tissue Res. 322: 33 – 41.

Li Y, Lin H, Ma M, Li L, Cai M, Zhou N, Han X, Bao H, Huang L, Zhu C, et al. (2009) Semen quality of 1346 healthy men, results from the Chongqing area of southwest China, Hum Reprod. 24: 459-469

Li Y, Lin H, Li Y, Cao J. (2011). Association between socio-psychobehavioral factors and male semen quality: systematic review and meta-analyses. Fertil Steril. 95:116–23.

Li Y, Wu J, Zhou W, & Gao E. (2016). Association between environmental exposure to cadmium and human semen quality. International journal of environmental health research, 26(2): 175-186.

Liochev SI. (2013). Reactive oxygen species and the free radical theory of aging. Free Radic Biol Med. 60:1-4. doi: 10.1016/j.freeradbiomed.02.011. Epub 2013 Feb 19. PMID: 23434764.

Looijenga LH. Pathogenesis of testicular germ cell tumors. In: Lau YF, CHan WY, editors. The Y Chromosome and Male Germ Cell Biology in Health and Diseases. World Scientific; 2007:263–288

Lopez Teijon M, Garcia F, Serra O, et al. (2007). Semen quality in a population of volunteers from the province of Barcelona. Reprod Biome online 15: 434–44.

Low WY, Binns C. (2013). Tobacco consumption: the Asia-Pacific region's major public health problem. Asia Pac J Public Health 25: 7S–9.

Lu JC, Huang YF, Lü NQ. (2010). WHO Laboratory Manual for the Examination and Processing of Human Semen: its applicability to andrology laboratories in China. Zhonghua Nan Ke Xue.16: 867–871.

Luddi A, Capaldo A, Focarelli R. et al. (2016). Antioxidants reduce oxidative stress in follicular fluid of aged women undergoing IVF. Reprod Biol Endocrinol 14, 57 https://doi.org/10.1186/s12958-016-0184-7

Luense LJ, Wang X, Schon SB, Weller A H, et al., (2016). Comprehensive analysis of histone post-translational modifications in mouse and human male germ cells. Epigenetics & chromatin 9(1), DOI 10.1186/s13072-016-0072-6

Ma A, Yang X, Wang Z, Shi D, Chen Y. (2008). Adult exposure to diethylstil bestrol induces spermatogenic cell apoptosis in vivo through increased oxidative stress in male hamster. Reprod Toxicol 25: 367-373.

Mafra FA, Christofolini DM, Bianco B, Gava MM, Glina S, Belangero SIN, Barbosa CP. (2011). Chromosomal and Molecular Abnormalities in a Group of Brazilian Infertile Men with Severe Oligozoospermia or Non-Obstructive Azoospermia Attending an Infertility Service. Int Braz J Urol. 37: 244-51. doi: 10.1590/S1677-55382011000200011

Majo J, Ghezzo H, Cosio M. (2001). Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. Eur Resp J.17: 946–953.

Maneesh M, Dutta S, Chakrabarti A, Vasudevan DM. (2006). Alcohol abuse-duration dependent decrease in plasma testosterone and antioxidants in males. Indian J Physiol Pharmacol. 50: 291–6.

Maneesh M, Jayalekshmi H, Dutta S, Chakrabarti A, Vasudevan DM. (2005). Effect of chronic ethanol administration on testicular antioxidant system and steroidogenic enzyme activity in rats. Indian J Exp Biol 43:445–9.

Mangoli E, Talebi AR, Anvari M, Pourentezari M. (2013). Effects of experimentally-induced diabetes on sperm parameters and chromatin quality in mice. Iran J Reprod Med; 11:53–60.

Marselos M, Vainio H, (1991). Carcinogenic properties of pharmaceutical agents enaluated in the IARC Monographs programme. Carcinogenesis 12: 1751- 66.

Martin RH, Hildebrand K, Yamamoto J, Rademaker A, et al (1986). An increased frequency of human sperm chromosomal abnormalities after radiotherapy. Mutat Res. 174: 219–25.

Martini AC, Molina RI, Estofán D, et al: (2004). Effects of alcohol and cigarette consumption on human seminal quality. Fertil Steril 82: 374 – 377.

Martins, RP, Krawetz, SA. (2007b). Decondensing the protamine domain for transcription. Proceedings of the National Academy of Sciences of the United States of America, 104(20), 8340–8345. https://doi.org/10.1073/pnas.0700076104

Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, & Stevens G A. (2012). National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. PLoS Med, 9(12), e1001356.

Mazzilli F, Rossi T, Marchesini M, Ronconi C, Dondero F. (1994). Superoxide anion in human semen related to seminal parameters and clinical aspects. Fertil Steril. 62(4): 862-868.

McPherson S, Longo F. (1993). Nicking of rat spermatid and spermatozoa DNA: possible involvement of DNA topoisomerase II. Dev Biol. 158: 122–130.

Mendiola J, Torres-Cantero A M, & Agarwal A. (2009). Lifestyle factors and male infertility: an evidence-based review. Arch Med Sci, 5(1A): S3-12.

Meucci E, Milardi D, Mordente A, Martorana GE, Giacchi E, De Marinis L, et al. (2003). Total antioxidant capacity in patients with varicoceles. Fertil Steril. 79: 1577-1583.

Mikkelsen EM, Riis AH, Wise LA, et al. (2016). Alcohol consumption and fecundability: prospective Danish cohort study. Bmj. 354: i4262. [PMC free article].

Mima M, Greenwald D & Ohlander S. (2018). Environmental Toxins and Male Fertility. Curr Urol Rep 19, 50. https://doi.org/10.1007/s11934-018-0804-1

Mitchell RT, O'Hara L, & Smith LB. (2017). Gonadotropin and Steroid Hormone Control of Spermatogonial Differentiation. In The Biology of Mammalian Spermatogonia (147-178). Springer, New York, NY.

Mohammedi S, Jalali M, Nikravesh MR, Fazel A, Ebrahimzadeh A, Gholamin M, et al. (2013). Effects of Vitamin-E treatment on CatSper genes expression and sperm quality in the testis of the aging mouse. Iran J Reprod Med. 11: 989–998. [PMC free article] [PubMed] [Google Scholar]

Mokdad AH, Marks JS, Stroup DF, Gerberding JL. (2004). Actual causes of death in the United States, 2000. JAMA. 10; 291(10):1238-45. doi: 10.1001/jama.291.10.1238. Erratum

in: JAMA. 2005 Jan 19; 293(3):298. Erratum in: JAMA. 2005 Jan 19; 293(3):293-4. PMID: 15010446.

Møller P, Loft S, Lundby C, Olsen NV. (2001). Acute hypoxia and hypoxic exercise induce DNA strand breaks and oxidative DNA damage in humans. FASEB J. 15(7):1181-6. doi: 10.1096/fj.00-0703com. PMID: 11344086.

Morisawa M. & Mohri H. (1972). Heavy metals and sperm motility. Expl Cell Res. 70, 311-316.

Moskovtsev SI, Willis J, Mullen JB. (2006). Age-related decline in sperm deoxyribonucleic acid integrity in patients evaluated for male infertility. Fertil Steril. 85: 496-9.

Mostafa T, Rashed LA, Osman I & Marawan M. (2015) Seminal plasma oxytocin and oxidative stress levels in infertile men with varicocele. Andrologia 47: 209– 213.

Mostafa T, Tawadrous G, Roaia M, Amer M, Kader R, Aziz A. (2006). Effect of smoking on seminal plasma ascorbic acid in infertile and fertile males. Andrologia. 38: 221–224.

Murphy MP, Holmgren A, Larsson NG, Halliwell B, Chang CJ, et al. (2011). Unraveling the biological roles of reactive oxygen species. Cell Metab 13: 361–6

Muthusami KR, Chinnaswamy P. (2005). Effect of chronic alcoholism on male fertility hormones and semen quality. Fertil Steril. 84: 919–24.

Nabel G, Baltimore D. (1987). An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature 326: 711–713. https://doi.org/10.1038/326711a0

Ng M, Freeman MK, Fleming TD, et al. (2014). Smoking prevalence and cigarette consumption in 187 countries, 1980-2012 J. Am. Med. Assoc., 311 (2): 183-192 https://doi.org/10.1001/jama.2013.284692

Nair M, Nagamori I, Sun P, Mishra D P. et al., (2008). Nuclear regulator Pygo2 controls spermiogenesis and histone H3 acetylation. Developmental Biology 320(2): 446-455.

Nanassy L, Liu L, Griffin J, Carrell DT. (2011). The clinical utility of the protamine 1/protamine 2 ratio in sperm. Protein Pept Letters.18:772–7.

Nandipati KC, Pasqualotto FF, Thomas AJ, Jr, Agarwal A. (2005). Relationship of interleukin-6 with semen characteristics and oxidative stress in vasectomy reversal patients. Andrologia 37:131–134. [PubMed] [Google Scholar]

National Collaborating Centre for Women's and Children's Health (NCC-WCH) (UK)(2004).

National Institute of Health and Clinical Excellence (NICE) clinical guidelineFertility: assessment and treatment for people with fertility problems, 2013 guidance.nice.org.uk/cg156

Naz M, Kamal M. (2017). Classification, causes, diagnosis and treatment of male infertility: a review. Oriental Pharmacy and Experimental Medicine: 17(2): 89-109. DOI: 10. 1007/s13596-017-0269-7

Nicopoullos JD, Almeida PA, Ramsay JW, Gilling-Smith C. (2004). The effect of human immunodeficiency virus on sperm parameters and the outcome of intrauterine insemination following sperm washing. Hum Reprod. 19: 2289-2297. [DOI:10.1093/humrep/deh426]

Nikhil G, Pradeepa MM, Anayat BU, Satyanarayana RM (2015) Mapping of post-translational modifications of transition proteins, TP1 and TP2 and identification of protein arginine methyl transferase 4 and lysine methyl transferase 7 as methyl transferase for TP2. J Biol Chem jbc. M114. 620443

Nikolaidis MG, Z .Jamurtas A. (2009). Blood as a reactive species generator and redox status regulator during exercise. Archives of Biochemistry and Biophysics. 490 (2): 77-84.

Ochsendorf FR. (1999). Infections in the male genital tract and reactive oxygen species. Human Reproduction Update (5) 399-420.

Oliva R. (2006). Protamines and male infertility. Hum Reprod Update; 12: 417–435.

Oliva R, & Castillo J. (2011). Sperm nucleoproteins. In Sperm chromatin (pp. 45– 60). Springer. Crossref Google Scholar

Opuwari CS, Henkel RR. (2016). An Update on Oxidative Damage to Spermatozoa and Oocytes. Biomed Res Int. 2016; 2016:9540142. doi: 10.1155/2016/9540142. Epub 2016 Jan 28. PMID: 26942204; PMCID: PMC4749785.

Osman A, Alsomait H, Seshadri S, El-Toukhy T, Khalaf Y. (2015). The effect of sperm DNA fragmentation on live birth rate after IVF or ICSI: a systematic review and meta-analysis. Reproductive Biomedicine Online 30 (2):120-127.

Oumaima A, Tesnim A, Zohra H, Amira S, et al. (2018). Investigation on the origin of sperm morphological defects: oxidative attacks, chromatin immaturity, and DNA fragmentation. Environmental Science and Pollution Research 25(14): 13775-13786.

Oyeyipo IP, Maartens PJ, du Plessis SS. (2014). In vitro effects of nicotine on human spermatozoa. Andrologia 46: 887–92

Ozgur K, Isikoglu M, Seleker M, Donmez L. (2005). Semen quality of smoking and non-smoking men in infertile couples in a Turkish population. Arch Gynecol Obstet. 271(2):109-12. doi: 10.1007/s00404-003-0572-z. Epub 2003 Dec 18. PMID: 14685893.

Pajarinen J, Karhunen PJ, Savolainen V, Lalu K, Penttilä A, Laippala P. (1996). Moderate alcohol consumption and disorders of human spermatogenesis. Alcohol Clin Exp Res 20: 332-7.

Pajarinen JT, Karhunen PJ. (1994). Spermatogenic arrest and 'Sertoli cell-only' syndrome– common alcohol-induced disorders of the human testis. Int J Androl. 17: 292–9.

Pant N, Kumar G, Upadhyay AD, Patel DK, Gupta YK, et al. (2014). Reproductive toxicity of lead, cadmium, and phthalate exposure in men. Environ Sci Pollut Res Int. 21: 11066–74.

Pant N, Shukla M, Patel DK, Shukla Y, Mathur N, Gupta YK, Saxena DK. (2008). Correlation of phthalate exposures with semen quality. Toxicol Appl Pharmacol 231(1):112–116. doi:10.1016/j.taap.2008.04.001

Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ, Agarwal A. (2000). Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. Fert. Steril, 73: 459-464

Pasqualotto FF, Umezu FM, Salvador M, Borges E, Jr, Sobreiro BP, Pasqualotto EB (2008). Effect of cigarette smoking on antioxidant levels and presence of leukocytospermia in infertile men: a prospective study. Fertil Steril, 90(2): 278-283.

Pasqualotto FF. (2007). Investigação e reprodução assistida no tratamento da infertilidade masculina. Rev Bras Ginecol Obstet. 29: 103-12.

Patel C, Ghanim H, Ravishankar S, Sia, CL et al. (2007). Prolonged reactive oxygen species generation and nuclear factor-kappaB activation after a high-fat, high-carbohydrate meal in the obese. J. Clin. Endocrinol. Metab. 92: 4476–4479.

Peake JM, Suzuki K, Coombes JS. (2007). The influence of antioxidant supplementation on markers of inflammation and the relationship to oxidative stress after exercise. J Nutr Biochem 18: 357-371. [DOI:10.1016/j.jnutbio.2006.10.005]

Peck JD, Leviton A, Cowan LD. (2010). A review of the epidemiologic evidence concerning the reproductive health effects of caffeine consumption: a 2000–2009 update. Food Chem Toxicol. 48: 2549–76.

Perrin J, Tassistro V, Mandon M, Grillo JM, Botta A, et al. (2011). Tobacco consumption and benzo(a)pyrene-diol-epoxide-DNA adducts in spermatozoa: in smokers, swim-up procedure selects spermatozoa with decreased DNA damage. Fertil Steril. 95: 2013–7

Petraglia F, Serour G, Chapron Ch. (2013). The changing prevalence of infertility. 123: S2: S4-S8. https://doi.org/10.1016/j.ijgo.2013.09.005. Management of Infertility Today: Proceedings of a Workshop sponsored by the Pontifical Academy for Life; February 24, 2012; Rome, Italy

Pieri NCG, Souza A, Mançanares ACF, et al. (2017). Immunolocalization of proteins in the spermatogenesis process of canine. Reprod Domest Anim. 52:170–176. doi:10.1111/rda.2017.52.issue-S2

Pittoggi C, Renzi L, Zaccagnini G, Cimini D, Degrassi F, Giordano R, & Spadafora C. (1999). A fraction of mouse sperm chromatin is organized in nucleosomal hypersensitive domains enriched in retroposon DNA. J Cell Sci, 112(20): 3537-3548.

Povey AC, Clyma J,-A, McNamee R, Moore HD, Baillie H, Pacey AA, Cherry NM. (2012). Participating Centres of Chaps-uk, Modifiable and non-modifiable risk factors for poor semen quality: a case-referent study, Human Reproduction 27 (9): 2799–2806, https://doi.org/10.1093/humrep/des183.

Powers SK, Ji LL, Kavazis AN, Jackson MJ. (2011). Reactive Oxygen Species: Impact on Skeletal Muscle. Comprehensive Physiology Compr Physiol 1: 941-969. https://doi.org/10.1002/cphy.c100054

Powers SK, Radak Z, Ji L. (2016). Exercise-induced oxidative stress: past, present and future. The Journal of Physiology. 594(18): 5081-5092

Pradeepa, MM and Rao, MR (2007). 'Chromatin remodeling during mammalian spermatogenesis: role of testis specific histone variants and transition proteins.' Society of Reproduction and Fertility supplement, 63. 1 - 10.

Pylyp LY, Spinenko LO, Verhoglyad NV, Zukin VD. (2013). Chromosomal abnormalities in patients with oligozoospermia and non-obstructive azoospermia. J Assist Reprod Genet. 30(5):729-32. doi: 10.1007/s10815-013-9990-4. Epub 2013 Apr 11. PMID: 23575767; PMCID: PMC3663966.

Queralt R, Adroer R, Oliva R. et al. (1995). Evolution of protamine P1 genes in mammals. J Mol Evol 40: 601– 607. https://doi.org/10.1007/BF00160507

Quinlan, Casey L, Perevoschikova, Irina V, et al (2013). The Determination and Analysis of Site-Specific Rates of Mitochondrial Reactive Oxygen Species Product. Methods in Enzymology 526: 189-217.

Rahal A, Singh V, Mehra D, Rajesh S, and Ahmad AH, (2009). “Prophylactic efficacy of Podophyllum hexandrum in alleviation of immobilization stress induced oxidative damage in rats,” Journal of Natural Products 2: 110–115,

Rakhit M, Gokul SR, Agarwal A, du Plessis SS. (2013). Antioxidant strategies to overcome OS in IVF-embryo transfer. In: Studies on Women’s Health. Editors: Agarwal, A., Aziz, N. and Rizk, B. Humana Press, Springer Science+Business Media, New York p. 237–262.

Ramasamy R, Chiba K, Butler P, Lamb DJ. (2015). Male biological clock: a critical analysis of advanced paternal age. Fertil Steril. 103: 1402–6

Ramgir SS and Abilash VG. (2019). Impact of Smoking and Alcohol Consumption on Oxidative Status in Male Infertility and Sperm Quality. Indian J Pharm Sci. 81(5): 933-945.

Ramya T, Man Mohan M, Devabrata S, Deoki N, (2010). Sperm function and seminal oxidative stress as tools to identify sperm pathologies in infertile men. Fertility and Sterility, 93(1): 297-300

Rao M, Zhao XL, Yang J, Hu SF, Lei H, Xia W, et al. (2015). Effect of transient scrotal hyperthermia on sperm parameters, seminal plasma biochemical markers, and oxidative stress in men. Asian J Androl. 17: 668–75.

Reid MB. (2016). Redox interventions to increase exercise performance. The journal of Physiology. 594(18): 5125- 5133

Reis LO, Dias FGF. (2012). Male Fertility, Obesity, and Bariatric Surgery. Reprod. Sci. 19, 778–785 https://doi.org/10.1177/1933719112440053

Ren X, Chen X, Wang Z, & Wang D. (2017). Is transcription in sperm stationary or dynamic? Journal of Reproduction and Development, 63(5): 439-443.

Rengan AK, Agarwal A, van der Linde M, du Plessis SS. (2012). An investigation of excess residual cytoplasm in human spermatozoa and its distinction from the cytoplasmic droplet. Reprod Biol Endocrinol. 10: 92. doi: 10.1186/1477-7827-10-92. PMID: 23159014; PMCID: PMC3551780.

Ricci E, Al Beitawi S, Cipriani S, Candiani M, Chiaffarino F, Vigano P, et al. (2017). Semen quality and alcohol intake: a systematic review and meta-analysis. Reprod Biomed Online 34: 38–47.

Rossi BV, Berry KF, Hornstein MD, Cramer DW, Ehrlich S, Missmer SA. (2011). Effect of alcohol consumption on in vitro fertilization. Obstet Gynecol. 117 (1):136–42.

Rubes J, Selevan SG, Evenson DP, Zudova D, Vozdova M, Zudova Z, et al. (2005). Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. Hum Reprod. 20: 2776– 2783.

Russell, L. D., Ettlin, R. A., Hikim, A. P. S., & Clegg, E. D. (1993). Histological and histopathological evaluation of the testis. International journal of andrology, 16 (1), 83-83.

Saalu LC. (2010). The incriminating role of reactive oxygen species in idiopathic male infertility: an evidence based evaluation. Pak J Biol Sci. 13: 413 – 422.

Sakellariou, G. K., Vasilaki, A., Palomero, J., Kayani, A., Zibrik, L., McArdle, A., et al. (2013). Studies of mitochondrial and nonmitochondrial sources implicate nicotinamide adenine dinucleotide phosphate oxidase(s) in the increased skeletal muscle superoxide

generation that occurs during contractile activity. Antioxid. Redox Signal. 18: 603–621. doi: 10.1089/ars. 2012.4623

Sakkas D, Alvarez, JG. (2010). Sperm DNA fragmentation: Mechanisms of origin, impact on reproductive outcome, and analysis. Fertility and Sterility 93(4): 1027–1036. https://doi.org/10.1016/j.fertnstert. 2009.10.046. Crossref CAS PubMed Web of Science®Google Scholar.

Sakkas D, Manicardi, GC, Tomlinson M. et al. (2000). The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. Hum Reprod., 15: 1112–1116.

Sala-Vila A, Navarro-Lerida I, Sanchez-Alvarez M, Bosch M, Calvo C, Lopez JA. et al. (2016). Interplay between hepatic mitochondria-associated membranes, lipid metabolism and caveolin-1 in mice. Sci. Rep. 6:27351. doi: 10.1038/srep27351

Saleh RA, Agarwal A, Sharma RK et al. (2003). Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. Fertil Steril 80:1431–1436

Saleh RA, Agarwal A, Sharma RK, Nelson DR, Thomas AJ. (2002). Effect of cigarette smoking on levels of seminal oxidative stress in infertile. Fertility and Sterility. 78 (3), 491-499. Presented at the 57th Annual Meeting of the American Society for Reproductive Medicine, Orlando, Florida, October 20–25, 2001.

Saleh RA, Agarwal A. (2002). Oxidative stress and male infertility: from research bench to clinical practice. J Androl. 23:737–752. [PubMed] [Google Scholar]

Salonen I, Huhtaniemi I, (1990). Effects of Chronic Ethanol Diet on Pituitary-Testicular Function of the Rat, Biology of Reproduction, 42 (1): 55–62, https://doi.org/10.1095/biolreprod42.1.55

Sansone A, Di Dato C, de Angelis C, Menafra D, Pozza C, Pivonello R, Isidori A, Gianfrilli D. (2018). Smoke, alcohol and drug addiction and male fertility. Reprod Biol Endocrinol. 16(1):3. doi: 10.1186/s12958-018-0320-7. PMID: 29334961; PMCID: PMC5769315.

Santiso R, Tamayo M, Gosalvez J, Meseguer M, et al., (2010). Simultaneous determination in situ of DNA fragmentation and 8-oxoguanine in human sperm. Fertility and Sterility 93(1): 314-318. doi:10.1016/j.fertnstert. 2009. 07. 969

Santoro G, Romeo C, Impellizzeri P, Ientile R, Cutroneo G, Trimarchi F, et al. (2001). Nitric oxide synthase patterns in normal and varicocele testis in adolescents. BJU Int 88: 967-973.

Savini I, Catani MV, Evangelista D, Gasperi V, Avigliano L. (2013). Obesity-Associated Oxidative Stress: Strategies Finalized to Improve Redox State. Int. J. Mol. Sci. 14, 10497-10538. https://doi.org/10.3390/ijms140510497

Schulte R, Ohl D, Sigman M, Smith G. (2010). Sperm DNA damage in male infertility: etiologies, assays, and outcomes. J Assist Reprod Genet: 27: 3–12.

Sedha S, Kumar S, Shukla H. (2015). Role of Oxidative Stress in Male Reproductive Dysfunctions with Reference to Phthalate Compounds.. Urology Journal 12(5):2304- 2316

Sengupta P, Agarwal A, Pogrebetskaya M, Roychoudhury S, Durairajanayagam D, Henkel R. (2018). Role of Withania somnifera (Ashwagandha) in the management of male infertility. Reprod Biomed Online. 36(3): 311–326. doi:10.1016/j. rbmo.11.007

Sengupta P, Dutta S, and Krajewska-Kulak E. (2017). The disappearing sperms: analysis of reports published between 1980 and 2015. Am. J. Mens. Health 11, 1279–1304. doi: 10.1177/1557988316643383

Serra D, Mera P, Malandrino MI, Mir JF, Herrero L. (2013). Mitochondrial fatty acid oxidation in obesity. Antioxid. Redox Signal. 19(3): 269-84. doi: 10.1089/ars.2012.4875. Epub 2012 Oct 5.

Sharma R, Harlev A, Agarwal A, Esteves SC. (2016). Cigarette Smoking and Semen Quality: A New Meta-analysis Examining the Effect of the 2010 World Health Organization Laboratory Methods for the Examination of Human Semen. Eur Urol. 70(4):635-645. doi: 10.1016/j.eururo.2016.04.010. Epub 2016 Apr 21. PMID: 27113031.

Sharma R, Said T, Agarwal A. (2004). Sperm DNA damage and its clinical relevance in assessing reproductive outcome. Asian J Androl. 6:139–148.

Sharma RK, Agarwal A. (1996). Role of reactive oxygen species in male infertility. Urology. 48: 835–850. [PubMed] [Google Scholar]

Shenker NS, Ueland PM, Polidoro S, et al. (2013). DNA methylation as a long-term biomarker of exposure to tobacco smoke. Epidemiology, 712-716.

Shiels MS, Rohrmann S, Andy Menke A, Selvin E, Crespo CJ, Rifai N, Dobs A, Feinleib M; Guallar E, Platz EA. (2009). Association of cigarette smoking, alcohol consumption and physical activity with sex steroid hormone levels in U.S. men. Cancer Causes Control. August 20(6): 877–886. doi:10.1007/s10552-009-9318-y.

Shiraishi K, Matsuyama H, Takihara H. (2012). Pathophysiology of varicocele in male infertility in the era of assisted reproductive technology. Int J Urol. 19: 538–550.

Shiraishi K, Naito K. (2007). Effects of 4-hydroxy-2-nonenal, a marker of oxidative stress, on spermatogenesis and expression of p53 protein in male infertility. J Urol. 178(3 Pt 1):1012–1017.

Showell MG, Mackenzie-Proctor R, Brown J, Yazdani A, Stankiewicz MT, Hart RJ. (2014). Antioxidants for male subfertility. Cochrane Database Syst Rev. (12):CD007411. doi: 10.1002/14651858.CD007411.pub3. Epub 2014 Dec 15. Update in: Cochrane Database Syst Rev. 2019 Mar 14;3:CD007411. PMID: 25504418.

Sikaris KA. (2004).The Clinical Biochemistry of Obesity. Clin Biochem Rev. l 25: 165-181.

Sillaste G, Kaplinski L, Meier R, Jaakma Ü, Eriste E, & Salumets A. (2017). A novel hypothesis for histone-to-protamine transition in Bos Taurus spermatozoa. Reproduction 153(3), 241-251.

Silva AN. da; LIMA, LCF. (2016). The association between physical exercise and Reactive Oxygen Species (ROS) production. Journal of Sports Medicine & Doping Studies 5: 1-7, Disponível em: http://www.sciencedirect.com/science/article/pii/S209525461300029X>. Acesso em: 26.

Simon L, Zini A, Dyachenko A, Ciampi A, Carrell DT A. (2017). Systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. Asian Journal of Andrology: 19 (1), 80.

Singh MP, Mishra M, Sharma, Shukla AK, Mudiam MKR, Patel DK, Ram KR, Schowdhuri DK. (2011). Genotoxicity and apoptosis in Drosophila melanogaster exposed to benzene, toluene and xylene: Attenuation by quercetin and curcumin. Toxicology and Applied Pharmacology. 253 (1), 15, 2011, 14-30

Singh R, Barden, A, Mori T, Beilin L. (2001). Advanced glycation end-products: A review. Diabetologia 44, 129–146.

Skakkebaek NE, Rajpert-De Meyts E and Main KM. (2001). Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. Hum Reprod; 16: 972–978.

Smit M, Romijn JC, Wildhagen MF, et al. (2013). Decreased sperm DNA fragmentation after surgical varicocelectomy is associated with increased pregnancy rate. J Urol 189: S146-50.

Sobinoff AP, Sutherland JM, Beckett EL, Stanger SJ, Johnson AG, Jarnicki AG, et al. (2014). Damaging legacy: maternal cigarette smoking has long-term consequences for male offspring fertility. Hum Reprod 29: 2719–35.

Sofikitis N, Takenaka M, Kanakas N, Papadopoulos H, Yamamoto Y, et al. (2000). Effects of cotinine on sperm motility, membrane function, and fertilizing capacity in vitro. Urol Res; 28: 370-5.

Sonnack V, Failing K, Bergmann M, & Steger K. (2002). Expression of hyperacetylated histone H4 during normal and impaired human spermatogenesis. Andrologia, 34(6): 384 - 390.

Sosa V, Moliné T, Somoza R, Paciucci R, Kondoh H, ME LL. (2013). Oxidative stress and cancer: an overview. Ageing Res Rev. 12(1): 376-390. doi:10.1016/j.arr.2012.10.004

Soultanakis RP, Melamede RJ, Bespalov IA, et al, (2000). Fluorescence detection of 8-oxoguanine in nuclear and mitochondrial DNA of cultured cells using a recombinant Fab and confocal scanning laser microscopy. Free Radic Biol Med. 28(6):987-98. doi: 10.1016/s0891-5849(00)00185-4. PMID: 10802231.

Southorn T. (2002). Great balls of fire and the vicious cycle: a study of the effects of cycling on male fertility. J. Fam. Plann. Reprod. Health Care. 28: 211-213

Spano M, Seli E, Bizzaro D, Manicardi GC, & Sakkas, D. (2005). The significance of sperm nuclear DNA strand breaks on reproductive outcome. Current Opinion in Obstetrics and Gynecology, 17(3), 255-260. https://doi.org/10.1097/01.gco.0000169102.77504.66. Crossref PubMed Web of Science®Google Scholar

Spinelli A, Figa-Talamanca I, Osborn J. (1997). Time to pregnancy and occupation in a group of Italian women. Int J Epidemiol. 26: 601–07. [PubMed] [Google Scholar]

Steger K, Klonisch TH, Gavenis K, et al., (1998). Expression of mRNA and protein of nucleoproteins during humanspermiogenesis Molecular Human Reproduction 4 10): 939–945, 1998

Stouffs K, Seneca S, Lissens W. (2014). Genetic causes of male infertilityCauses génétiques d'infertilité masculine. Annales d'Endocrinologie. 75 (2): 109-111

Sullivan EV, & Pfefferbaum A. (2014). Human imaging studies of brain circuitry disrupted by alcoholism. Neurobiology of Alcohol Dependence; Academic Press: San Diego, CA, USA, 131-151.

Sun JG, Jurisicova A, Casper RF., (1997). Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod. 56:602–607.

Taken K, Alp HH, Eryilmaz R, Donmez MI, Demir M, Gunes M, et al. (2017). Oxidative DNA damage to sperm cells and peripheral blood leukocytes in infertile men. Med Sci Monit 2016; 22:4289.

Takeshima T, Yumura Y, Kuroda S, Kawahara T, Uemura H, & Iwasaki A. (2017). Effect of density gradient centrifugation on reactive oxygenspecies in human semen, Systems Biology in Reproductive Medicine 2017: 63:3, 192-198, DOI: 10.1080/19396368.2017.1294214

Talbert PB, & Henikoff S. (2010). Histone variants—ancient wrap artists of the epigenome. Nature reviews Molecular cell biology, 11(4), 264

Talebi A, Moein M, Tabibnejad N, Ghasemzadeh J. (2008). Effect of varicocele on chromatin condensation and DNA integrity of ejaculated spermatozoa using cytochemical tests. Andrologia 40: 245-251.

Talebi AR, Mangoli E, Nahangi H, Anvari M, Pourentezari M, Halvaei I. (2014) Vitamin C attenuates detrimental effects of diabetes mellitus on sperm parameters, chromatin quality and rate of apoptosis in mice. European Journal of Obstetrics & Gynecology and Reproductive Biology. 181, 32-36

Talebi AR, Sarcheshmeh AA, Khalili MA, Tabibnejad N. (2011). Effects of ethanol consumption on chromatin condensation and DNA integrity of epididymal spermatozoa in rat. Alcohol. 45: 403–409.

Teperek M, & Miyamoto K. (2013). Nuclear reprogramming of sperm and somatic nuclei in eggs and oocytes. Reproductive medicine and biology, 12(4), 133-149.

Terquem A. and Dadoune JP. (1983) Aniline blue staining of human sperm chromatin: evaluation of nuclear maturation. In Andre, J. (ed.) The sperm cell. Martinus Nijhoff, London, pp. 249–252.

Thijssen A, Creemers A, Van der Elst W, Creemers E, Vandormael E, Dhont N, Ombelet W. (2017). Predictive factors influencing pregnancy rates after intrauterine insemination with frozen donor semen: a prospective cohort study. Reprod Biomed Online. Jun; 34 (6):590-597. doi: 10.1016/j.rbmo.2017.03.012. Epub 2017 Mar 23. PMID: 28396044.

Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang AM, Clark AM. (2009). Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod 24: 2061–70.

Tolstrup JS, Kjaer S, Holst C, Sharif H, Munk C, Osler M, et al. (2003). Alcohol use as predictor for infertility in a representative population of Danish women. Acta Obs Gynecol Scand. 82:744–9.

Tomlinson MJ, White A, Barratt CL, Bolton AE, Cooke ID. (1992). The removal of morphologically abnormal sperm forms by phagocytes: a positive role for seminal leukocytes. Hum Reprod. 7:517–522

Tremblay AR, Delbes G. (2018). In vitro study of doxorubicin-induced oxidative stress in spermatogonia and immature Sertoli cells. Toxicol. Appl. Pharmacol. 348:32 –42. doi: 10.1016/j.taap.2018.04.014.

Tremellen K. (2008). Oxidative stress and male infertility-a clinical perspective. Hum Reprod Update. 14(3):243-58. doi: 10.1093/humupd/dmn004. Epub 2008 Feb 14. PMID: 18281241.

Trummer H, Habermann H, Haas J, Pummer K. (2002). The impact of cigarette smoking on human semen parameters and hormones, Hum Reprod 17: 1554-1559)

Tsai WW, Niessen S, Goebel N, Yates JR, 3rd, Guccione E, Montminy M. (2013). PRMT5 modulates the metabolic response to fasting signals. Proc Natl Acad Sci U S A. 2013; 110:8870–8875.

Uddin S, Wilson T, Emanuele M.A, Williams D, Kelley MR, Emanuele N. (1996). Ethanol-Induced Alterations in the Posttranslational Processing, But Not Secretion of Luteinizing Hormone-Releasing Hormone In Vitro. Alcoholism. 20 (3): 556-560. https://doi.org/10.1111/j.1530-0277.1996.tb01092.x

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol; 39: 44-84.

Vanegas JC, Chavarro JE, Williams PL, Ford JB, Toth TL, Hauser R, et al. (2017). Discrete survival model analysis of a couple’s smoking pattern and outcomes of assisted reproduction. Fertil Res Pract; 3:5. https://doi.org/10.1186/s40738-017-0032-2.

Venkataraman K, Khurana S, Tai TC. (2013). Oxidative Stress in Aging-Matters of the Heart and Mind. Int. J. Mol. Sci. 14, 17897-17925; doi: 10.3390/ijms140917897

Verón GL, Tissera AD, Bello R, Beltramone F, Estofan G., et al. (2018). Impact of age, clinical conditions, and lifestyle on routine semen parameters and sperm kinematics. Fertility and Sterility, 110(1), 68– 75.

Vignera S, Condorelli R, Vicari E, D'Agata R, Calogero AE. (2012). Diabetes mellitus and sperm parameters. J Androl; 33: 145-153. [DOI:10.2164/jandrol.111.013193]

Vilfan ID, Conwell CC, Hud NV. (2004). Formation of native-like mammalian sperm cell chromatin with folded bull protamine, J Biol Chem, 279: 20088-20095)10.1074/jbc.M312777200

Vine MF, Margolin BH, Morrison HI, Hulka BS. (1994). Cigarette smoking and sperm density: a meta-analysis. Fertil Steril. 61: 35–43.

Walczak–Jedrzejowska R, Wolski JK, Slowikowska–Hilczer J. (2013). The role of oxidative stress and antioxidants in male fertility. Central European Journal of Urology 66(1) 60-67.

Wald M. (2005). Male infertility: Causes and cures. Sex. Reprod. Menopause 3:83–87. doi: 10.1016/j.sram.2005.09.006. [CrossRef] [Google Scholar]

Walsh TJ, Schembri M, Turek PJ, Chan JM, Carroll PR, Smith JF, Eisenberg (2010). Increased risk of high-grade prostate cancer among infertile men. Cancer 116: 2140–2147. doi: 10.1002/cncr.25075.

Wang X, Zhang J, Xu W, Huang Q, Liu L, Tian M, Xia Y, Zhang W, Shen H. (2016). Low-level environmental arsenic exposure correlates with unexplained male infertility risk. Sci Total Environ. 571:307–13.

Wang YJ, Zhang RQ, Lin YJ, et al. (2012). Relationship between varicocele and sperm DNA damage and the effect ofvaricocele repair: a meta-analysis. Reprod Biomed Online 25:307-14.

Wang Y, Ke CC, Chen YL, Lin YH., et al. (2020). Deficiency of the Tbc1d21 gene causes male infertility with morphological abnormalities of the sperm mitochondria and flagellum in mice. PLoS Genetics 16(9), e1009020.

Ward WS, Coffey DS. (1991). DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biol Reprod. 44: 569– 574.

Ward, W. S. (2009). Function of sperm chromatin structural elements in fertilization and development. MHR: Basic science of reproductive medicine, 16(1): 30-36.

Will MA, Swain J, Fode M, Sonksen J, Christman GM, Ohl D. (2011). The great debate: varicocele treatment and impact on fertility. Fertil Steril. 95:841–852. [PMC free article]

Witt O, Albig W & Doenecke D. (1996).Testis-specific expression of a novel human H3 histone gene. Experimental cell research 229(2). 301- 306.

World Health Organisation. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva: World Health Organization; 2010.

World Health Organization. (2018). Global Report on Trends in Prevalence of Tobacco Smoking 2000–2025 http://www.who.int/tobacco/publications/surveillance/ trends-tobacco-smoking-second-edition/en/

Wright C, Milne S, Leeson H. (2014). Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. Reproductive BioMedicine Online. 28(6): 684-703

Wu D, Cederbaum A. (2003). Alcohol, Oxidative Stress, and Free Radical Damage. Alcohol research health 27 (4): 277-284.

Wykes SM, and SA Krawetz (2003). The structural organization of sperm chromatin. J. Biol. Chem. 278:29471–29477.

Wyrobek AJ, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I,et al. (2006). Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. Proc Natl Acad Sci USA; 103: 9601–9606.

Xu H, Shen L, Chen X, et al. (2016). mTOR/P70S6K promotes spermatogonia proliferation and spermatogenesis in Sprague Dawley rats. Reprod Biomed Online. 32(2): 207–217. doi:10.1016/j. rbmo.2015.11.007

Yuksel N, UzbayI T, Karakilic H, Aki O E, Etik C, & Erbaş D. (2005). Increased serum nitrite/nitrate (NOx) and malondialdehyde (MDA) levels during alcohol withdrawal in alcoholic patients. Pharmacopsychiatry 38(02): 95-96.

Yumura Y, Iwasaki A, Saito K, Ogawa T and Hirokawa M. (2009). Effect of reactive oxygen species in semen on the pregnancy of the infertile couples. Int J Urol 16: 202–207.

Yumura Y, Takeshima T, Kawahara T, Sanjo H, Kuroda SN, Asai T, et al. (2017). Reactive oxygen species measured in the unprocessed semen samples of 715 infertile patients. Reprod Med Biol. 16(4): 354–363.

Zalenskaya I A, & Zalensky AO. (2004). Non-random positioning of chromosomes in human sperm nuclei. Chromosome Research, 12(2): 163-173.

Zavos PM, Correa JR, Karagounis CS, Ahparaki A, Phoroglou C, Hicks CL, et al. (1998). An electron microscope study of the axonemal ultrastructure in human spermatozoa from male smokers and nonsmokers. Fertil Steril. 69: 430–434.

Zegers-HochschildF, Adamson GD, Dyer S, Racowsky C, et al. (2017). The International Glossary on Infertility and Fertility Care, Human Reproduction, 32(9):1786–1801, https://doi.org/10.1093/humrep/dex234

Zhang L, Wang Z, Wang X, Chen Z, Shao L, Tian Y, Zheng C, et al., (2020). Prevalence of overweight and obesity in China: Results from a cross-sectional study of 441 thousand adults, 2012–2015 Obesity Research & Clinical Practice 14 (2)119-126. https://doi.org/10.1016/j.orcp.2020.02.005

Zhang ZB, Jiang YT, Yun X, Yang X, Wang RX, Dai RL, et al. (2012). Male infertility in Northeast China: a cytogenetic study of 135 patients with non-obstructive azoospermia and severe oligozoospermia. J Assist Reprod Genet; 29:83–7.

Zhang ZH, Zhu HB, Li LL, Yu Y, Zhang HG, Liu RZ. (2013). Decline of semen quality and increase of leukocytes with cigarette smoking in infertile men. Iran J Reprod Med. 11: 589-596.

Zhao J, Leung JY, Lin SL, Schooling CM. (2016). Cigarette smoking and testosterone in men and women: A systematic review and meta-analysis of observational studies. Prev Med 85:1–10.

Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, Deng JM, Arango N A, Terry NH and Weil MM (2001). Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice Molecular Cell Biology 21 7243–7255.

Zhu Q, Van Thiel DH, Gavaler JS. (1997). Effect of ethanol on rat Sertoli Cell function: Studies in vitro and in vivo. Alcohol Clin Exp Res 21:1409-17.

Zini A, Dohle G. (2011). Are varicoceles associated with increased deoxyribonucleic acid fragmentation? Fertil Steril. 96:1283-7.

Zitzmann M, Rolf C, Nordhoff V, Schräder G, Rickert-Föhring M, et al. (2003). Male smokers have a decreased success rate for in vitro fertilization and intracytoplasmic sperm injection. Fertil Steril 79 (3): 1550- 4.

Zorn B, Vidmar G, and Meden-Vrtovec H. (2003). Seminal reactive oxygen species as predictors of fertilization, embryo quality and pregnancy rates after conventional in vitro fertilization and intracytoplasmic sperm injection. International journal of andrology 26: 279–285

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Explanation

I expressly guarantee that I have written the work independently and without outside help, sources and resources other than those specified by me are not used and those from the used works verbatim or with regard to content taken individually according to edition (Edition and year of publication), volume and page of the work used recognizable done and the dissertation has not yet been given to a specialist at another or submitted to other university for review or otherwise for admission to Applied for a doctorate.