**DEPARTMENT OF OBSTETRICS, GYNAECOLOGY & REPRODUCTIVE MEDICINE**
**FACULTY OF MEDICINE OF THE SAARLAND UNIVERSITY HOMBURG/SAAR**

# The impact of cigarettes smoking on DNA integrity and sperm protein (histone, transition protein and protamines) alterations

Dissertation in partial fulfillment of the requirement for the award the degree of ***Doctor Rerum Medicinae (Dr. rer. med.)*** conferred by the Faculty of Medicine of the Saarland University, Germany

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# Abbreviations

| | |
|---|---|
| AB | Aniline blue |
| ADP | Adenosine diphosphate |
| APS | Ammonium persulphate |
| ART | Artificial reproductive technologies |
| AU-PAGE | Acid-Urea Polyacrylamide Gel Electrophoresis |
| cAMP | cyclic adenosine monophosphate |
| CENP-A | centromeric protein A |
| CMA3 | Chromomycin A3 |
| CRE | (cAMP) response element |
| CTAB | Hexadecyltrimethylammonium bromide |
| DFI | DNA fragmentation index |
| DNA | Deoxyribonucleic acid |
| DSB | Double strand break |
| DTT | Dithiothreitol |
| W | Water |
| EDTA | Ethylenediaminetetraacetic acid |
| Gn-HCl | Guanidine hydrochloride |
| H 4 | Histone H 4 |
| H1 | Linker Histone |
| H2A | Histone H2A |
| H2A.Bbd | H2A Barr body-deficient |
| H2B | Histone H2B |
| H2Bfwt | H2B family member W testis-specific |
| H3 | Histone H3 |

# Abbreviations

| | |
|---|---|
| HILS1 | H1-like protein in spermatids 1 |
| HOS | Hypo osmotic solution |
| hTSH2B | human testis-specific H2B |
| ICSI | Intracytoplasmic sperm injection |
| IVF | In vitro fertilization |
| LPO | Lipid peroxidation |
| MDA | Malondialdehyde |
| MG | Methyl green |
| mH2A | macro H2A |
| $Na_2HPO_4$ | Disodium hydrogenphosphate |
| NaCl | Sodium chloride |
| OAT | Oligoasthenoteratozoospermic |
| OS | Oxidative stress |
| P1 | Protamine 1 |
| P2 | Protamine 2 |
| PBS | Phosphate buffer saline |
| PMSF | Penylmethylsulfonyl fluoride |
| PUFA | Poly unsaturated fatty acid |
| ROS | Reactive oxygen species |
| RT | Room temperature |
| TBA | Thiobarbituric acid |
| TCA | Trichloroacetic acid |
| TEM | Transmission electron microscopy |
| TEMED | Tetramethylethylenediamine |
| TH2B | Testis H2B |
| TP 2 | Transition protein 2 |

# Abbreviations

| | |
|---|---|
| TP1 | Transition protein 1 |
| TPs | Transition proteins |
| TUNEL | Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick- end labeling |
| WHO | World health organization |
| β-MSH | β- Mercaptoethanol |

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# 1-Summary

## 1-1- Abstract

Sperm chromatin is supremely tightly compacted by virtue of the unique associations between the DNA and sperm nuclear proteins. The compaction of human sperm chromatin is the result of the replacement of ~85% of histones by protamines. Appropriate histone retention during sperm chromatin condensation plays critical roles in the process of fertilization and early embryo development. Infertile men possess a higher proportion of spermatozoa with an increased histone to protamine ratio than fertile controls.

Male infertility is a common and complex problem affecting 5% men. Oxidative stress induced by reactive oxygen species (ROS) has recently been proposed as one of the major causes for human male infertility. Cigarette smoking is one of the causes of increased ROS production in seminal fluid of infertile men. Smoking is strongly associated with abnormalities in histone - transition protein - protamine transition and with alteration of protamine expression in human spermatozoa. A proper histone to protamine ratio is crucial for sperm chromatin maturity and DNA integrity. The purpose of this study is to determine the effect of cigarette smoking on nuclear proteins of spermatozoa and on other sperm parameters, sperm chromatin condensation and DNA fragmentation.

In this study, I found a significant difference between samples from smokers and non-smokers samples in sperm concentrations, sperm motility, vitality, membrane integrity, sperm maturity (aniline blue), protamine deficiency (CMA3) and DNA fragmentation.

In addition, smoking has an effect on histones (H2A, H2B, and H3), transition protein (TP1), protamine (P2) concentration and the P1/P2 ratio. There was a significant lower of histone H2A, H2B and H3 concentration in non-smokers in comparison to smokers. Transition protein TP1 concentration in smokers was significantly higher than of those of non-smokers. Protamine P2 concentration in non-smokers was significantly higher than those of smokers. In contrast, the P1/P2 ratio was significantly higher in smokers in comparison with non-smokers. These findings indicated that the causative agent for higher P1/P2 ratio in smokers was the under-expression of protamine P2.

The oxidative stress biomarker (MDA) was significantly higher in smokers than in non-smokers. In addition, the oxidative stress biomarkers (MDA) of all samples showed

significantly negative correlations with sperm concentration, sperm motility, vitality and membrane integrity. In contrast, significant positive correlations between oxidative stress biomarker (MDA) and protamine deficiency (CMA3), histone H2B and transition protein TP1 were observed. Furthermore, the seminal plasma smoking marker cotinine levels was found significantly higher in samples from smokers than non-smokers. In general, significant negative correlations were found between smoking marker cotinine levels in seminal plasma with sperm concentration, sperm motility, vitality and membrane integrity. It was positively correlated with sperm maturity (aniline blue), sperm protamine deficiency (CMA3) and DNA fragmentation.

The present study showed high levels of oxidative stress biomarkers and decreased sperm quality in smokers in comparison to non-smokers. This study also observed that cigarette smoking impairs sperm quality. This is probably the consequence of high levels of toxic components in smoke, such as cotinine that cause an increase of seminal plasma free radicals and oxidative stress.

## 1-2- Zusammanfassung

Die Chromatinstruktur der Spermien ist äußerst dicht verpackt aufgrund der einzigartigen Assoziation zwischen DNA und Kernproteinen. Die Verdichtung des menschlichen Chromatins in den Spermien erfolgt durch den Austausch von ungefähr 85% der Histone durch Protamine. Die restlichen Histone spielen während der Chromatinkondensation in den Spermien eine entscheidende Rolle im Prozess der Befruchtung und der frühen Entwicklung des Embryos. Unfruchtbare Männer besitzen einen höheren Anteil an Spermien mit einem erhöhten Histon zu Protamin-Verhältnis als fertile Kontrollen.

Männliche Unfruchtbarkeit ist ein häufiges und komplexes Problem, was 5% der Männer betrifft. Es wurde kürzlich vorgeschlagen, dass oxidativer Stress, induziert durch reaktive, freie Sauerstoffradikale (ROS), einer der wichtigsten Ursachen für die männliche Infertilität ist. Das Rauchen von Zigaretten ist eine Ursache für erhöhte ROS-Produktion in der Samenflüssigkeit von unfruchtbaren Männern. Rauchen ist stark assoziiert mit Veränderungen in den Kern-Protein Verhältnissen (Histon, Transition Protein, Protamin) und mit Veränderung der Protaminexpression in menschlichen Spermatozoen. Ein richtiges Histon-Protamin-Verhältnis ist für die Spermienchromatinreife und die DNA-Integrität von entscheidender Bedeutung. Das Ziel dieser Studie ist es, die Wirkung von Zigarettenrauchen auf die Kernproteine der Spermatozoen und auf weitere Spermienparameter, Chromatinkondensation in den Spermien und DNA Fragmentierung zu bestimmen.

In dieser Studie habe ich einen signifikanten Unterschied zwischen Rauchern und Nichtrauchern gefunden im Hinblick auf die Konzentration, Motilität und Vitalität der Spermien, sowie der Unversehrtheit der Membran, der Spermienreife (Anilinblau), des Protaminmangels (CMA3) und der DNA Fragmentation. Zusätzlich hat das Rauchen einen Einfluß auf die Histone (H2A, H2B und H3), auf das Transition Protein (TP1), die Protamin 2 (P2) Konzentration und auf das P1/P2 Verhältnis. Es gab eine signifikante Reduktion von Histon H2A, H2B und H3 beim Vergleich von Nichtrauchern und Rauchern. Die Konzentration der Transition Protein TP1 ist bei Rauchern signifikant höher. Die Konzentration von Protamin P2 ist signifikant höher bei Nichtrauchern im Vergleich zu Rauchern. Im Unterschied dazu, war das P1/P2 Verhältnis bei Rauchern

höher, als in der Nichtraucher Gruppe. Diese Ergebnisse lassen die Vermutung zu, dass das höhere P1/P2 Verhältnis bei den Rauchern die Unterexpression von Protamin P2 zu Grunde liegt.

Der oxidative Stress Biomarker (MDA) war signifikant höher bei Rauchern als bei Nichtrauchern. Zusätzlich zeigte der oxidative Stress Biomarker (MDA) aller Proben eine signifikant negative Korrelation zur Konzentration, Motilität und Vitalität der Spermien, ebenso zur Intaktheit der Membran. Im Gegensatz dazu konnte eine signifikant positive Korrelation zwischen dem oxidativen Stress Biomarker (MDA) und dem Protamin Verlust (CMA3), dem Histon H2B und dem Transition Protein TP1 beobachtet werden. Weiterhin wurde gefunden, dass die Konzentration des Rauchermarkers Cotinin im Seminalplasma bei Rauchern signifikant höher war. Im Allgemeinen konnte eine negative Korrelation zwischen dem Rauchermarker Cotinin im Seminalplasma und der Konzentration, Motilität und Vitalität der Spermien, sowie der Unversehrtheit der Membran gefunden werden. Dies war positiv korreliert mit der Spermienreife (Anilinblau), dem Verlust von Protamin (CMA3) und der DNA Fragmentierung.

Die vorliegende Arbeit zeigt hohe Konzentrationen des oxidativen Stress Biomarkers und abnehmende Spermienqualität beim Vergleich zwischen Rauchern und Nichtrauchern. In dieser Arbeit konnte ebenso gezeigt werden, dass Zigarettenrauchen die Qualität der Spermien reduziert. Die Beeinträchtigung der Spermienqualität könnte aufgrund der toxischen Substanzen im Zigarettenrauch zurückzuführen sein. Cotinin, als Beispiel für eine toxische Komponente, erhöht die freien Radikale im Seminalplasma und verursacht dadurch die Erhöhung von oxidativem Stress und könnte damit zu einer Verschlechterung der Spermienqualität beitragen.

# 2- Introduction

## 2-1- Sperm chromatin structure

Sperm cells are highly specialized delivery vehicles for the chromatin cargo in sexual reproductive animals, which are composed of DNA and their associated proteins (Boe-Hansen *et al*., 2006; Bungum *et al*., 2007; Cebesoy *et al*., 2006). Sperm cells are remarkably different from somatic cells in their chromatin structure. During spermatogenesis, the majority of histones replace transition proteins and protamine (small highly basic proteins bound to the sperm DNA) (Oliva, 2006; Balhorn, 2007). As a result, sperm chromatin is highly compacted during transit of spermatozoa through the epididymis. Protamines build a cross-link by the formation of disulfide bridges and produce more compacted structures (Balhorn, 2007; Hammoud *et al*., 2009a). Human sperm contain two protamines, protamine 1 (P1) and protamine 2 (P2), both are expressed in roughly similar quantities with a mean P1/P2 ratio of approximately 1.0 (Carrell and Liu, 2001; Oliva, 2006). Human sperm nuclei contain approximately 85% protamines in their nucleoprotein component, which is considerably less than those of a bull, stallion, hamster and mouse (Wykes and Krawetz, 2003). Human sperm chromatin, therefore, is less regularly compacted and frequently contains DNA strand breaks (Aoki *et al*., 2006a). Sperm chromatin is tightly packaged by protamines, while up to 15% of the histones remain in the mature human spermatozoa (Wykes and Krawetz, 2003). These retained histones within the sperm nucleus possibly contribute to the sperm function (Hammoud *et al*., 2009b).

Histone proteins consist of the linker histone (H1) and four core histones (H2A, H2B, H3, H4), which form a nucleosome DNA of around 147 base pairs which is wrapped around the nucleosome (Davey *et al*., 2002). Histones are loosely bound to DNA sequences, which considered that these DNA sequences involve in fertilization and early embryonic development (Rajender *et al*., 2011). The retained histones play a role in telomere DNA recognition (Gineitis *et al*., 2000). Also, the histone: protamine ratio in fertile men is significantly higher than in infertile men (Steger *et al*., 2000; Oliva, 2006). An excess of nuclear histones (> 15%) results in low chromatin compaction and subsequent increased

susceptibility to toxicants. During spermiogenesis some of them can have an impact on germ cells (Codrington *et al*., 2004).

The complexity of histones increases due to the existence of sequence variants and a high degree of post-translational modifications (PTMs) (Paull *et al*., 2000). All histones in differentiating germline cells are subject of a number of modifications including lysine acetylation, lysine/arginine methylation, serine/threonine phosphorylation, lysine ubiquitination, lysine sumoylation and poly-adenosine diphosphate ribose (ADP) - ribosylation of glutamic acid (Fig. 1) (Spotswood and Turner, 2002; Freitas *et al*., 2004; Miller *et al*., 2010).

Nucleosome particles can be modified in the composition, structure and location by chromatin remodeling complexes that introduce post-translational modifications to the core histones (Jenuwein and Allis, 2001; Peterson and Laniel, 2004) and leads to nucleosome removal and reassembly (Cosgrove *et al*., 2004; Korber *et al*., 2004). In addition, the chromatin fiber can be modified by the incorporation of histone variants (Malik and Henikoff, 2003; Henikoff *et al*., 2004). Histones and their variants (Henikoff, 2008; Rando and Chang, 2009) are classified according to Ausió *et al*., (2001) into two main family, homomorphous and heteromorphous, this classification is determined by the amino acid sequence length which are different to the main canonical isoforms (occasionally genes are expressed at the S phase of the cell cycle) (Lewis *et al*., 2003a). Homomorphous variants involve changes only in a few amino acid (i.e. H2A.1 and H2A.2; H3.1, H3.2, and H3.3), while heteromorphous variants include changes that affect the larger portions of histone molecules (i.e. H2A. X, H2A. Z, macro H2A (mH2A), H2A Barr body-deficient (H2A.Bbd) and centromeric protein A (CENP-A)} (West and Bonner, 1980; Pehrson and Fried, 1992; Chadwick and Willard, 2001a; Chadwick and Willard, 2001b; Talbert and Henikoff, 2010).

In mammals, eleven different linker histone variants have been identified: Seven somatic variants: (H1.1 - H1.5, H10 and H1x) (Ausió and Abbott, 2004; Happel *et al*., 2005; Albig *et al*., 1997; Parseghian *et al*., 1994), three spermatogenic variants: H1t (Seyedin and Kistler, 1980), H1T2 (Martianov *et al*., 2005), H1-like protein in spermatids 1(HILS1) (Yan *et al*., 2003; Iguchi *et al*., 2003) and an oocyte-specific H1 (Tanaka *et al*., 2003b).

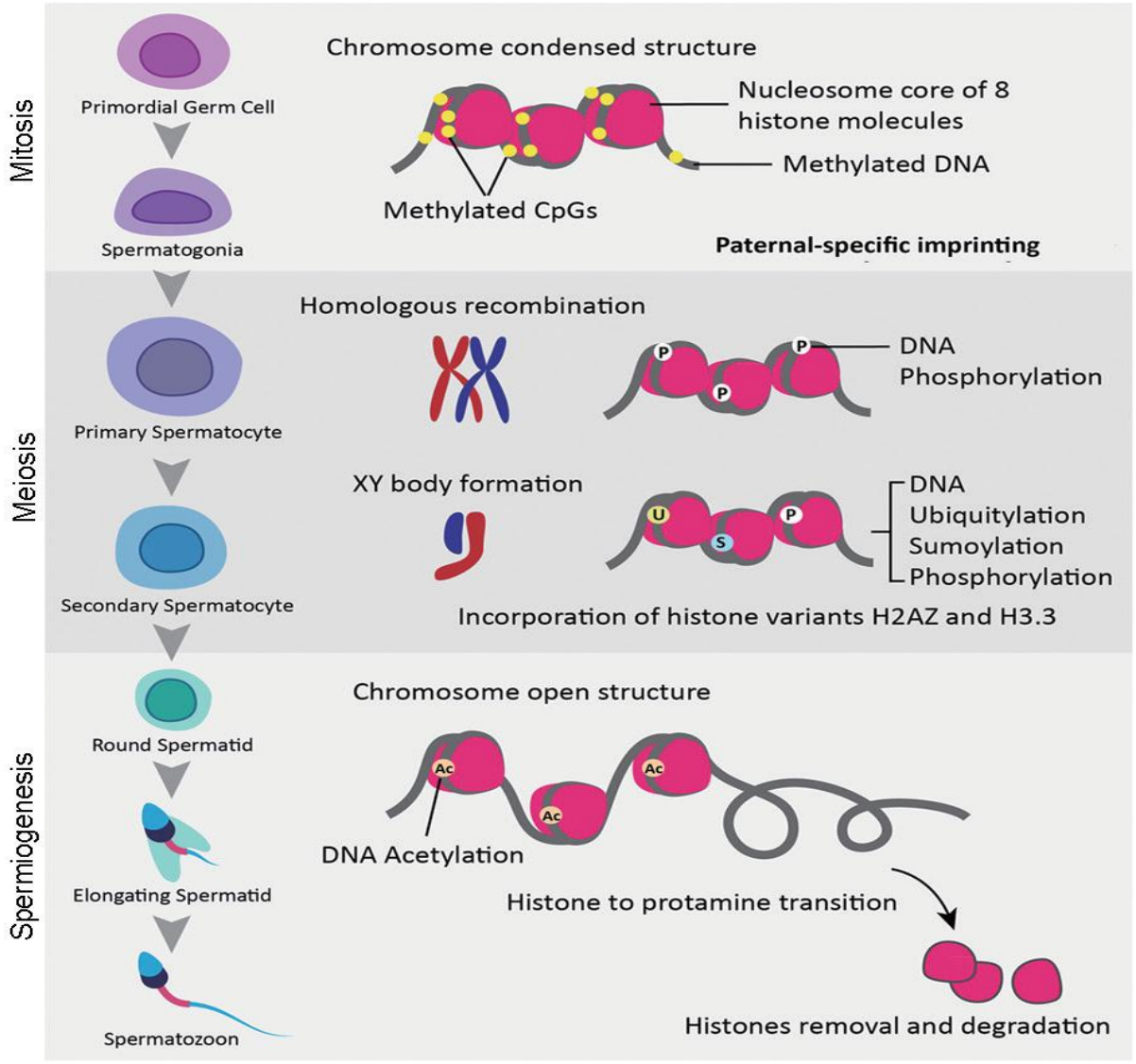


**Figure 1:** Epigenetic modifications occurring during spermatogenesis. DNA methylation occurs in mitotic germ cells, setting up the paternal specific imprints. Phosphorylation occurs in meiotic cells, assisting in both recombination and XY body formation. Ubiquitylation, sumoylation, and incorporation of the H2AZ and H3.3 variants are all involved in XY body formation. During spermiogenesis, hyperacetylation occurs to assist in the histone‑protamine transition (Ding *et al*., 2015).

Histone H2A is the core histone with the greatest number of variants, and this showed crucial functions in gene expression and nuclear dynamics (Ausió and Abbott, 2002). The histone H2A family has many variants such as histone H2A X, histone H2AZ (West and Bonner, 1980), histone H2A Bbd and histone MacroH2A (Chadwick and Willard, 2001b).

In contrast to H2A and H3 histones, histone H2B and particularly histone H4 exhibit a low level of amino acid sequence variability; they play an important role in chromatin compaction through gametogenesis (Pusarla and Bhargava, 2005). Histone H2B variant documented in the sperm of vertebrate (Churikov *et al*., 2004a), invertebrate organisms (Poccia, 1995) and plant male gametes (Ueda and Tanaka, 1995; Ueda *et al*., 2000). Furthermore, in humans, three testis-specific variants have been classified to (TH2B) (Shires *et al*., 1976), human testis-specific H2B (hTSH2B) (Zalensky *et al*., 2002) and histone H2B family member W testis-specific (H2Bfwt) (Churikov *et al*., 2004a).

Histone H3 family comprises five main members in mammals, including centromere-specific centromere protein (CENP)-A, H3.1, H3.2, H3.3, which are identified as replication-independent histones that are expressed throughout the whole cell cycle. Additionally, histone H3t was detected in testis (Govin *et al*., 2005; Szenker *et al*., 2011).

Histone H4 is the most highly conserved histone. It makes extensive contacts with the other three core histones in the nucleosome core particle and is thus constrained in its sequence variability (Akhmanova *et al*., 1996; Malik and Henikoff, 2003). However, Bhasin *et al*., (2006) reported that there is an identical sequence of histone H4 genes which are constitutively expressed throughout the cell cycle (Bhasin *et al*., 2006).

The transformation of spermatids into spermatozoa (spermiogenesis) involves dramatic changes in chromatin structure and function that occur in all cell types (Yu *et al*., 2000). During the late stage of spermiogenesis, the nucleus elongates, transcription ceases, histones mostly disappear, and the chromatin seems as smooth fibers and then becomes highly condensed (Balhorn, 2007). In many animal and plant species, chromatin condensation is facilitated by protamines, highly basic nuclear proteins (Oliva and Dixon, 1991). In mammals, histones are replaced by a group of arginine- and lysine- rich proteins called transition protein (TP) that bind strongly to DNA, then transition protein (TP) are replaced by protamines (Meistrich *et al*., 2003). Transition proteins are simpler than histones and more complicated than protamines (Zhao *et al*., 2004b). Despite the presence of at least four transition proteins in humans, transitional proteins 1, 2 (TP1, 2) are the major ones (Meistrich *et al*., 2003).

Transition proteins consist of 90% basic chromatin proteins in condensing spermatids (Meistrich *et al*., 2003). They seem to be vital, but evidence has been limited to *in vitro* studies indicating possible roles for the transition proteins (TPs) in some remodeling processes such as: first, transition proteins can condense DNA, transition protein TP2 are considered most effective than transition protein TP1 in DNA condensation (Levesque *et al*., 1998; Brewer *et al*., 2002). Second, both transition proteins (TPs) have been proposed to be alignment factors for DNA strands. Transition protein TP1 is implicated in the DNA strand break repair (Caron *et al*., 2001; Boissonneault, 2002). Third, the potential roles of transition proteins in removing histones and in the deposition of the protamines (Wouters-Tyrou *et al*., 1998; Meistrich *et al*., 2003).

The relationship between transition proteins expression in spermatids and fertility remains unclear. In mutant mice lacking both transition proteins, histone displacement and protamine deposition proceed relatively normal, while chromatin condensation is irregular in all spermatids, and many spermatids displayed DNA fragmentation (Zhao *et al*., 2004a). Zhao *et al*. (2004a) postulating that transition proteins are essential for chromatin integrity preservation and completing of its condensation. Surprisingly, all mice with transition protein TP1 deficiency were found to be infertile (Yu *et al*., 2000) or displayed major sperm defects such as poor motility and tail abnormalities (Shirley *et al*., 2004; Zhao *et al*., 2004a; Suganuma *et al*., 2005) which resemble those observed in infertile men. Moreover, Jedrzejczak *et al*. (2007) investigated that the mRNA for transition proteins (TP1, 2) has been detected at a higher level in spermatozoa of asthenozoospermic men, in comparison to normozoospermic. However, Mitchell *et al*. (2007) was worked on transition protein TP1 mRNA, and they found that its expression was similar in non-pregnant and pregnant couples, suggesting that it is not a critical factor in embryo development. Furthermore, they suggested that cellular transition protein TP1 mRNA in spermatids from infertile men was a consistent predictive indicator for the existence of spermatids/spermatozoa in testis biopsies.

Transition protein TP1 is a small basic protein (6.2 kDa) of about 54 amino acid, rich in arginine (20%) and lysine (20%), distributed uniformly, without any cysteine (Dadoune, 2003). The level of transition protein TP1 is about 2.5 fold higher than that of transition

protein TP2 (Yu *et al*., 2000). The transition protein TP1 has played a critical role for DNA destabilizing properties, probably due to the existence of dual tyrosine residues lined by basic amino acids (Schumacher *et al*., 1998). *In vitro* studies have shown that the possible functions of transition protein TP1 include, relaxation of the DNA in nucleosomal core particles, decreasing the melting temperature of DNA, and stimulation of the DNA-relaxing activity of topoisomerase I (Singh and Rao, 1988; Akama *et al*., 1998, 1999; Meistrich *et al*., 2003). Finally, transition protein TP1 has been found in vitro to stimulate repair of single-strand DNA breaks (Caron *et al*., 2001). Transition protein TP1 is possibly more important in humans than in mice for spermiogenesis. The mutation in the recognition site of the cyclic adenosine monophosphate (cAMP) response element (CRE) transcription factor of the promoter region of transition protein TP1 is thought to be a cause of male infertility (Miyagawa *et al*., 2005).

Transition protein TP2 is a protein with 13 kDa (117-138 amino acids), it consist of serine (22%), arginine (14%), proline (13%), lysine (9%) and cysteine (5%) basic residues (Dadoune, 2003). Transition protein TP2 is found on the same chromosome with the protamine 2 (P2) genes (Meetei *et al*., 2002), which suggests that they arose by gene duplication and might have retained common functions (Meistrich *et al*., 2003). On the other hand, transition protein TP1 was found on a separate chromosome (Heidaran *et al*., 1989).

Transition protein TP2 has a highly basic C-terminal domain and contains two putative zinc fingers in the amino-terminal region (Meetei *et al*., 2000; Zhao *et al*., 2001), which is essential for the CpG islands recognition in the genome (Pradeepa and Rao, 2007). Opposite to transition protein TP1, transition protein TP2 can increase the compaction of DNA in nucleosome cores, consequently, increasing the melting temperature of DNA. It has been suggested to be a DNA-condensing protein (Baskaran and Rao, 1990). Pradeepa and Rao, (2007) demonstrated that transition protein TP1 is a DNA melting protein, while transition protein TP2 is a DNA condensing protein.

Protamines are small (5-8 kDa) and highly basic proteins related to nuclear DNA in the spermatozoa, which is responsible for the DNA packaging in a very compact manner (Fig. 2) (Lewis *et al*., 2003b; Balhorn, 2007). They are positively charged proteins with a central

anchoring domain rich in arginine residues (about 55 to 79% of the amino acid residues) that neutralize the negative phosphodiester backbone of the DNA (Erenpreiss *et al*., 2006). Moreover, they contain several cysteines which increase the stability on sperm chromatin by intermolecular disulfide cross-links (Balhorn, 2007). *In vitro*, sperm DNA de-condensation cannot be done without reducing reagents (Balhorn *et al*., 1991), and formation of these inter and intra molecular disulphide bonds between cysteine residues, firmly stabilizing the nucleoprotamine structure in the sperm nucleus, and the folding of different protamine domains (Vilfan *et al*., 2004). It has been proposed that zinc ions contribute to DNA–protamine packaging by linking protamines with zinc bridges (Björndahl and Kvist, 2010). However, the function of this higher compact structure remains unclear. Several concepts elucidate why sperm display such a specialized chromatin structure; 1) Spermatozoa paternal genome condensation create more condensed and hydrodynamic nuclei. These allow faster sperm movement and more oocyte fertilization efficiency; 2) Removing of transcription factors and other proteins from the spermatid in addition to spermatid competition cause a blank paternal genetic message, which cause loss of epigenetic information, therefore allowing oocyte reprogramming; 3) Paternal genome imprinting during spermatogenesis. Protamines themselves could confer an epigenetic mark on some regions of the sperm genome, affecting its reactivation upon fertilization (Braun, 2001; Oliva, 2006; Balhorn, 2007).

The main difference between protamine 1 (P1) and protamine 2 (P2) is that, protamine P1 is translated as a 50 amino acids mature protein, whilst protamine P2 contains at first 103 amino acids and then undergoes N-terminus cleavage to forms a mature protein with 57 amino acids (Aoki and Carrell, 2003). Unlike protamine P1, P2 is a zinc finger protein with one Cys2-His2 motif (Bal *et al*., 2001). Although the arginine content of both proteins is similar, protamine P2 is slightly more basic due to its higher histidine and lysine content. Protamine P2 proteins are shown only in some mammals including human and mouse (Balhorn *et al*., 1999; Dahm, 2005; Yoshii *et al*., 2005) whereas, protamine P1 is invariably present in all species of mammals studied (Carrell and Liu, 2001; Corzett *et al*., 2002; Yoshii *et al*., 2005). Protamine P1 in some species have more basic and conserved functions, while protamine P2 display more accessory functions in assisted reproduction outcome (Aoki *et al*., 2005b).

Protamine P1 constitutes in human sperm nucleus is similar to the protamine P2 also P1/P2 ratio of approximately 1.0. Variations in the ratio are unusual in fertile men and mostly dominant in infertile men (Balhorn *et al*., 1999; Carrell and Liu, 2001; Corzett *et al*., 2002; Oliva, 2006; Carrell *et al*., 2007). Perturbation of this ratio is characterized by poor semen quality (counts, motility, and morphology), sperm functional ability, increases DNA damage (Carrell and Liu, 2001; Mengual *et al*., 2003; Aoki *et al*., 2005a), and decreases fertility (Carrell and Liu, 2001; Aoki *et al*., 2005a).

Sperm DNA fragmentation is correlated significantly with low P1/P2 ratio compared with normal and high P1/P2 ratio (Aoki *et al*., 2005b). Therefore, patients who under-expressed protamine P1, P2 or both protamine P1 and P2 had significantly elevated levels of DNA fragmentation in comparison to patients who expressed protamine P1 and protamine P2 normally (Li *et al*., 2008).

## 2-2- The nucleohistone – nucleotransition protein – nucleoprotamine transition

The formation of male germ cells includes a series of mitosis, meiosis, changes in cytoplasmic structure, topological rearrangements, and alteration in transcription (Yamauchi *et al*., 2011). Following spermatogenesis, histone- protamine transition takes place during spermiogenesis where histones become hyperacetylated and replaced by transition proteins and finally, they are replaced by protamines leading to highly condensed insoluble and stable chromatin (Fig. 2) (Hales *et al*., 2011). By that, it protects the sperm genome from epigenetic modification during spermiogenesis (Balhorn, 2007). Furthermore, it removes transcription factors and proteins to help resetting the imprinting code in the oocyte (Oliva, 2006).

Histone variants must first be incorporated (Govin *et al*., 2007) and histone H4 must be hyperacetylated (Sonnack *et al*., 2002). Hyperacetylation of histones points to loosening of chromatin structure. Loose protein structure stimulates DNA strand breaks caused by topoisomerase enzyme, and facilitates separation of histones, and their replacement by transition proteins (TPs) and protamines (Sonnack *et al*., 2002; Rousseaux *et al*., 2011).

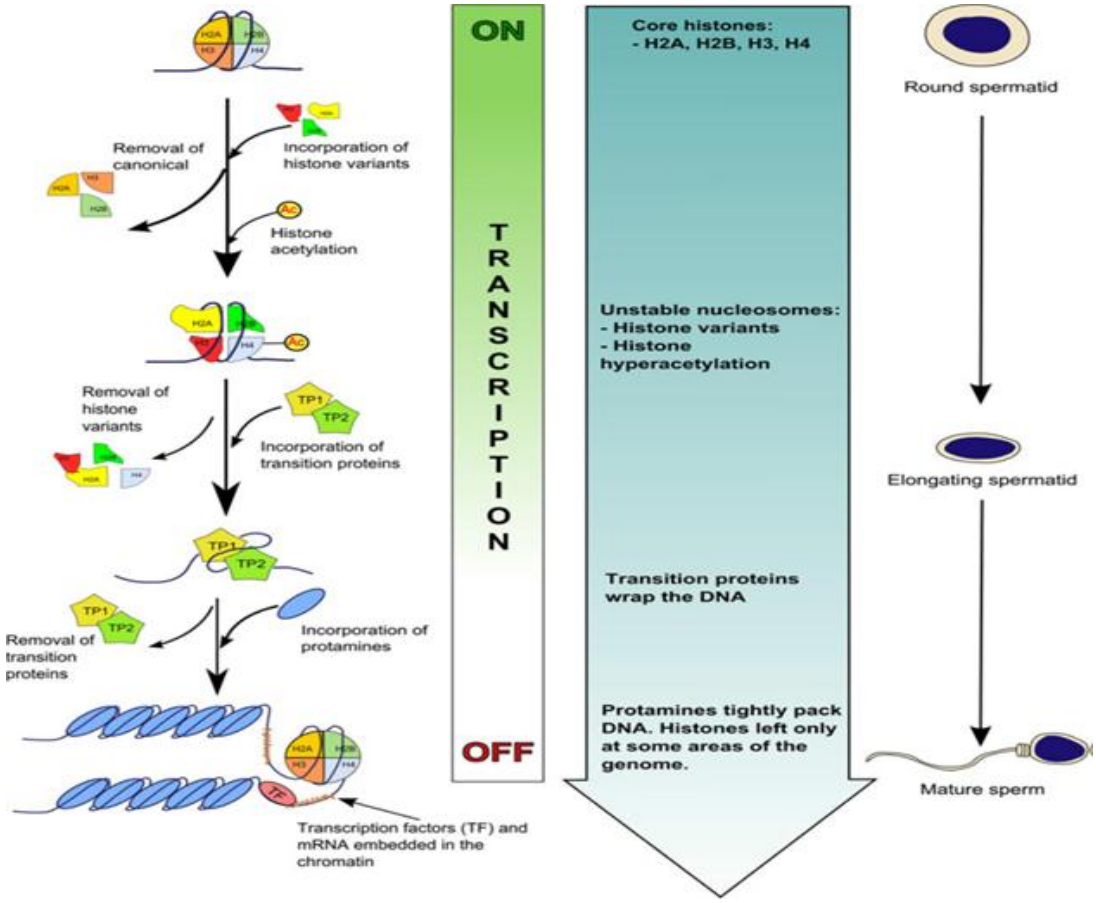


**Figure 2:** Major chromatin changes during spermiogenesis. In order to achieve a sperm-like chromatin state, the round spermatid which enters the spermiogenesis process undergoes a series of chromatin remodelling events. First, canonical core histones packing the chromatin in the round spermatid can be replaced by histone variants, which together with global histone acetylation, leads to instability of the nucleosome structure. Subsequently, transition proteins are incorporated in place of unstable nucleosomes in the elongating spermatid. Finally, transition proteins are replaced with protamines. The mature sperm chromatin is mainly composed of protamines, with interspersed histones and with tightly associated mRNAs and transcription factors. All these processes are occurring in parallel with the cessation of transcription—round spermatids are transcriptionally active, whereas no transcription is detected in the mature sperm (Teperek and Miyamoto, 2013).

## 2-3- Cigarette smoking

Cigarette smoking is a broadly identified health hazard and a principal cause of mortality (Colagar *et al*., 2007), but still people continue to smoke cigarettes on a regular basis (Künzle *et al*., 2003). The world health organization (WHO) has reported that approximately one-third of the world's populations older than 15 years are smokers (1999).

The higher prevalence of smoking is observed among young men during fertility years. Globally, 46% of smokers are aged between 20 to 39 years (Agarwal and Said, 2003).

Cigarette smoke contains known mutagens and carcinogens. Cigarette smoking increases oxidative damage, DNA fragmentation, mutations and chromosome abnormalities, and heritable mutations in sperm (DeMarini, 2004; Marchetti *et al*., 2011). Moreover, paternal exposure to secondhand smoke may have reproductive consequences (Marchetti *et al*., 2011), and the hypothesis has been raised that tobacco consumption may have unfavorable effects on male reproduction (Blanco-Muñoz, 2012).

Cigarette smoking is the main source of hazardous effects on semen quality and specifically, reduced sperm density and motility and abnormal morphology (Künzle *et al*., 2003; Hammadeh *et al*., 2010; Practice Committee of the American Society for Reproductive Medicine, 2012), also reported that cigarette smoking lead to a decrease in fertilizing activity of the sperm (Zitzmann *et al*., 2003; Practice Committee of the American Society for Reproductive Medicine, 2012). Moreover, smoking may have a negative impact on the smoker's offsprings and may result in poor quality embryos or development of childhood cancers (Sepaniak *et al*., 2004). It is suggested that smoking causes an increase of leukocyte derived reactive oxygen species production, which has adverse effects on mature sperm (Henkel, 2005; Mohamed *et al*., 2011; Sankako *et al*., 2012).

Cigarette smoking is responsible for the generation of toxins that interact directly or indirectly with gametes affecting their reduction in sperm count and motility and increase in some morphologically abnormal sperm cells (Sepaniak *et al*., 2006).

Cotinine is considered the main degradation product of nicotine (Binnie *et al*., 2004). It can be detected easily in human body fluids, such as urine, saliva (Zenzes *et al*., 1996; Binnie *et al*., 2004), and seminal plasma (Hammadeh *et al*., 2010). It has been reported to have to be more stable than nicotine; and it has been used as a specific biomarker of cigarette smoking (Hammadeh *et al*., 2010). In addition, cotinine with a half-life in sperm of 5-7 days is considered a more reliable indicator than urinary metabolite index for long-term cigarette smokers (Jarvis *et al*., 1988).

Cotinine in the cigarette smoke can disrupt the testicular microcirculation and cause DNA or chromosomal damage in germinal cells (Zenzes, 2000). However, cotinine decreased male fertility by: inhibiting semen concentration; reducing total progressively motile sperm count, sperm viability; and increasing the percentage of sperm with abnormal morphology (Hammadeh *et al*., 2010).

In addition, Sofikitis *et al*., (2000) had demonstrated that, ICSI treatment rather than IVF is a better a way of therapy for male smokers with high seminal plasma cotinine concentration. Moreover, some previous work showed an inverse correlation between cotinine concentration and some parameters such as sperm concentration, motility, vitality, morphology, membrane integrity and DNA fragmentation (TUNEL) (Hamad *et al*., 2014). Also, they found that smoking had negative effects (Oxidative stress) on the final stage of spermatogenesis, where interchanging occurs between histones and protamines, smoking is considered as a reason for under expression of protamines ending with high levels of histone to protamines ratio (Hamad *et al*., 2014).

Successful fertilization required normal sperm genetic material and it is essential in embryo and fetal development which lead to healthy offspring (Andrabi, 2007). Sperm DNA contributes half of the offsprings genomic material and abnormal DNA can lead to failures in the reproductive process (Agarwal and Said, 2009). Damage to sperm DNA can occur at any step of the whole spermatogenesis (Erenpreiss *et al*., 2006).

The precise sources of sperm DNA damage have not been fully elucidated yet, and several different studies have been proposed to explain DNA anomalities in the ejaculated human spermatozoa: the first study reported that the DNA anomalities result from chromatin structural abnormalities causing altered tertiary chromatin configuration (Sakkas *et al*., 1999a). Then, the second study demonstrated that, oxidative stress (OS) which generate overproduction of reactive oxygen species (ROS) can be the main source of DNA anomalities (Aitken and Krausz, 2001; Agarwal *et al*., 2005). The last group proposes that the presence of endogenous nicks is characteristic of programmed cell death (apoptosis); aiming to the functional elimination of possibly defective germ cells from the genetic pool (Agarwal *et al*., 2005).

Apoptosis is a mechanism of cellular death which depends on a mechanism that stimulates a series of biochemical, cellular, morphological alterations, leading to the suicide of spermatozoa (Huszar *et al*., 1997). Apoptosis appears to play two primary roles during spermatogenesis: first, limitation in the population of germ cells to a number that can be supported by sustentacular sertoli cells, thus guaranteeing normal spermatogenesis; second, it has been proposed that apoptosis can play a critical role in the selective depletion of abnormal germ cells, i.e., histone-protamine substitution, DNA remodeling and also cytoplasmic and membrane anomalities (Sakkas *et al*., 1999b). Moustafa *et al*. (2004) found that there is a significant correlation between DNA fragmentation index and apoptosis in spermatozoa of infertile men.

Oxygen, which is essential for normal biological functions. Reactive oxygen species (ROS) at low levels play a major role in sperm maturation and normal physiological functions such as acrosome reaction, and sperm-oocyte fusion (Wang *et al*., 2003; Agarwal *et al*., 2008). While under pathological conditions, the uncontrolled production of ROS exceeds the antioxidant capacity of the seminal plasma, causes sperm damage and may lead to selective cell death (apoptosis) (Wang *et al*., 2003; Henkel, 2011; Trussell, 2013). However, ROS might damage DNA through modification or deletions of all bases, chromosomal rearrangement, DNA cross-linkages, single and double strand DNA breaks, and gene mutations (Aitken and Krausz, 2001; Spiropoulos *et al*., 2002; Sharma *et al*., 2004).

Oxidative stress (OS) can lead to infertility and sperm damage by several pathways (Fig. 3) (Agarwal *et al*., 2005). Semen of about 25 - 40% of infertile male patients has high ROS levels and oxidative stress (Agarwal *et al*., 2006). There was an association between OS and the excessive production of ROS with impaired sperm motility, concentration, and morphology. The presence of ROS in patients with varicocele revealed a negative relationship between ROS and sperm concentration (Aitken and Baker, 2013). However, hydrogen peroxide is the major ROS producer in human spermatozoa. Moderately elevated concentrations of hydrogen peroxide diffuse across the plasma membrane into the spermatozoa and lead to sperm immobilization, mostly through intracellular ATP depletion, and leading to axonemal proteins damage (Sikka, 2004; Agarwal *et al*., 2005;

Saalu, 2013). Also there was a higher incidence of relation between abnormal sperm morphology and ROS (Saleh *et al*., 2002c). Abnormal sperm morphologically and immature sperm can lead to even higher levels of ROS production during sperm migration, and consequently, result in ROS-related damage in mature sperm (Ollero *et al*., 2001).

Apoptosis is initiated in spermatozoa when high levels of ROS damage the mitochondrial membranes, and cytochrome-c proteins are released (Agarwal *et al*., 2008). This activates caspases 9 and 3, which both play important roles in apoptosis (Said *et al*., 2004). Moustafa *et al*., (2004) also investigated that infertile people had increased ROS levels in their seminal plasma correlated with a rise in percentage of apoptosis compared to normal healthy donors (Moustafa *et al*., 2004). increase in levels of caspases and cytochrome-c have been correlated with increasing of sperm DNA damage levels including both single and double-stranded DNA strand breaks (Said *et al*., 2004).

Several studies reported that there is a positive correlation between the percentage of DNA damage (TUNEL positive spermatozoa) and abnormal sperm a parameters, such as sperm concentration, motility, vitality, membrane integrity, in humans and rodent models (Spano *et al*., 2005; Delbes *et al*., 2007, Hammadeh *et al*., 2008; Hammadeh *et al*., 2010; Hamad *et al*., 2014).

If sperm DNA damage resulted as an adverse effect of ROS, then a relationship with sperm motility could be expected (Erenpreiss *et al*., 2002). This is due to the effect of ROS on the lipid peroxidation of sperm membranes, rich with unsaturated fatty acids (Erenpreiss *et al*., 2006). Furthermore, the percentage of DNA present in the comet tail has been correlated with age, abnormal sperm morphology and low sperm count and motility (Morris *et al*., 2002). Saleh *et al*. (2003) demonstrated that the sperm DNA fragmentation index (DFI) negatively correlated with sperm concentration, percentage motility and normal sperm morphological forms.

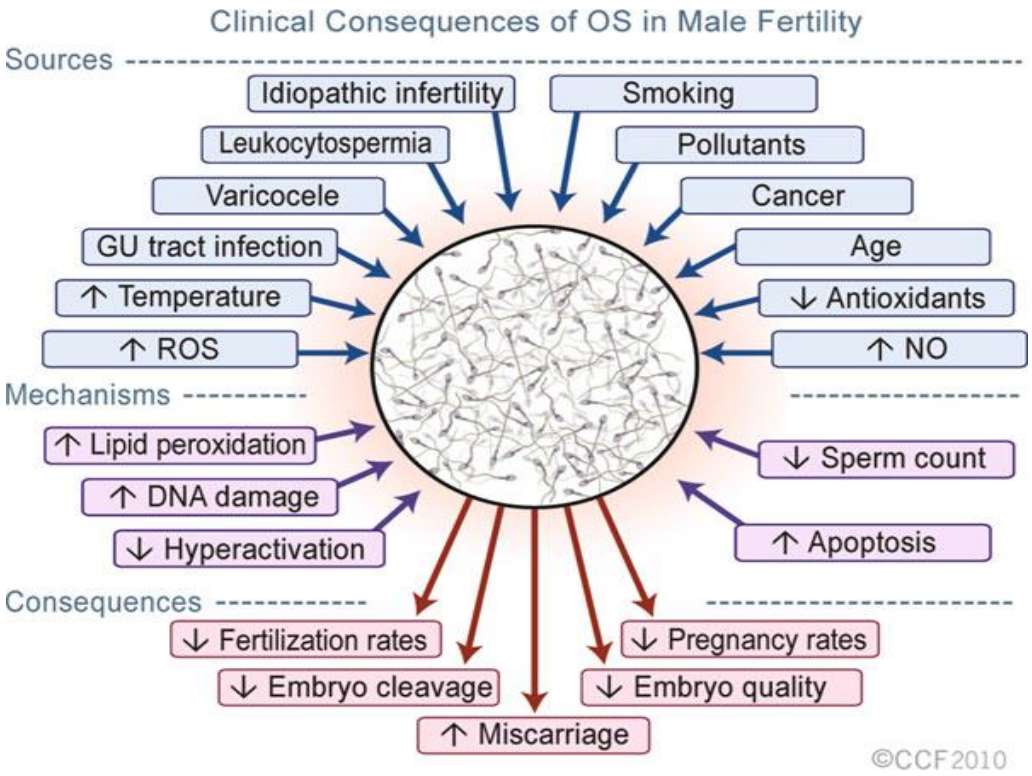


**Figure 3:** the general sources, mechanisms, and consequences of oxidative stress (OS) on male fertility are summarized. Clinical conditions related to OS include idiopathic infertility, leukocytospermia, Varicocele, genitourinary tract infection, environmental and lifestyle factors. OS acts through several mechanisms which lead to subfertility, such as lipid peroxidation, DNA damage, and apoptosis. OS can lead to several consequences related to male fertility, both in an in vivo and in vitro setting (Said *et al*., 2012).

## 2-4- Aim of the study:

The aim of this study was to examine the impact of cigarette smoking on nuclear sperm alteration, histone (H2A, H2B, and H3), transition protein (TP1) and protamine (P1, P2). Furthermore, these parameters should be correlated with other semen parameters such as DNA integrity, DNA fragmentation and sperm count, motility, vitality and sperm membrane integrity.

# 3- Materials and Methods

## 3-1- Materials

### 3-1-1- Semen samples

Semen samples from heavy smokers (≥ 20 cigarettes/day) and non-smokers (control group) enrolled in this study were collected from male partners of couples facing infertility who came to the assisted reproduction and andrology laboratory at the department of obstetrics and gynecology, Saarland University, Homburg/Saar were included in this study. Information regarding body weight, height, smoking, consumption of alcohol and occupational exposures was obtained from a questionnaire. Patients who had surgeries of their reproductive system or who had a history of sub- or infertility were excluded. Also, patients with history of conditions such as injury to testes, varicocele, hydrocele, undescended testis or its corrective surgery, vasectomy-reversal surgery or history of any chronic illness such as tuberculosis and mumps or pyospermia, haemospermia or chronic urinary tract infection. This stringent selection was done to exclude as many known co-existing factors as possible from the study groups, since we aimed to study the impact of cigarette smoking on specific aspects of sperm characteristics.

### 3-1-2- Chemicals and laboratory materials

| | |
|---|---|
| Acetic acid | Fluka, Neu-Ulm, Germany |
| Acetone | Merck, Darmstadt, Germany |
| Acrylamide-Stock solution:<br>Rotiphorese Gel 30<br>Rotiphorese Gel 40 | Carl Roth GmbH, Karlsruhe, Germany |
| Ammonium persulphate (APS) | Sigma, München, Germany |
| Aniline Blue | Sigma, München, Germany |
| Bovine-IgG | Bio-Rad, München, Germany |
| Bradford Protein Assay Kit | Bio-Rad, München, Germany |
| Chromomycin | Sigma, München, Germany |

| | |
|---|---|
| Coomassie Blue Stain<br>Brilliant R 250<br>Brilliant G 250 | Serva, Heidelberg, Germany |
| Disodium hydrogenphosphat | Merck, Darmstadt, Germany |
| Dithiothreitol (DTT) | Sigma, München, Germany |
| Dry milk powder | J.M. Galber Saliter GmbH & Co.KG, Obergünzburg, Germany |
| Eosin Y stain | Sigma, München, Germany |
| Ethanol | Merck, Darmstadt, Germany |
| Ethylene diamine tetraacetic acid disodium salt (EDTA) | Sigma, München, Germany |
| Glutaraldehyde | Sigma, München, Germany |
| Glycerol | Fluka, Neu-Ulm, Germany |
| Guanidine hydrochloride | Sigma, München, Germany |
| HAM's F10 medium PAN | Biotech, Germany |
| Hexadecyltrimethylammonium bromide (CTAB) | Sigma, München, Germany |
| Hydrochloric acid | Merck, Darmstadt, Germany |
| Lumi-Light Western Blotting Substrate | Roche, Mannheim, Germany |
| ß-Mercaptoethanol | Merck, Darmstadt, Germany |
| Methanol | Merck, Darmstadt, Germany |
| Methyl green | Sigma, München, Germany |
| Paraformaldehyde | Merck, Darmstadt, Germany |
| Phenylmethylsulfonyl fluoride (PMSF) | Sigma, München, Germany |
| Potassium acetate | Sigma, München, Germany |

| | |
|---|---|
| Potassium chloride | Merck, Darmstadt, Germany |
| Potassium dihydrogenphosphate | Merck, Darmstadt, Germany |
| Pure sperm gradient | Nidacon International AB, Sweden |
| PVDF membrane | Roche, Mannheim, Germany |
| Sodium chloride | Merck, Darmstadt, Germany |
| tri-Sodium citrate: 2 H2O | Merck, Darmstadt, Germany |
| Sodium iodoacetate | Sigma, München, Germany |
| Sucrose | Sigma, München, Germany |
| N,N,N/,N/- Tetramethylendiamine (TEMED) | GA Healthcare, Freiburg |
| 1,1,3,3-Tetramethoxypropane (Malonaldehyde bis (dimethyl acetal) MDA) | Sigma-Aldrich, Germany |
| Thiobarbituric acid (TBA) | Merck, Darmstadt, Germany |
| Trichloroacetic acid (TCA) | Sigma, München, Germany |
| Tris(hydroxymethyl)aminomethane (Tris) | Sigma, München, Germany |
| Triton X-100 | Sigma, München, Germany |
| Tween 20 | Serva, Heidelberg, Germany |
| Urea | Sigma, München, Germany |

### 3-1-3- Buffers and Solutions

| | |
|---|---|
| 1. Ammonium persulphate (APS) solution | 10 % (w/v) Ammonium persulphate |
| 2. Aniline blue solution | 2.5% (w/v) aniline blue |

| | |
|---|---|
| | 2% (v/v) acetic acid |
| 3. Blocking buffer | PBS, pH 7.4 |
| | 0.1% (v/v) Tween 20 |
| | 5% (w/v) Nonfat-dry milk |
| 4. CMA3 solution | 0.25 gm/ml |
| | PBS, pH 7.4 |
| | 10 mmol $MgCl_2$ |
| 5. Coomassie blue stain | 0.2% Brilliant R250 |
| | 0.01% Brilliant G250 |
| | 50% methanol |
| | 10% acetic acid |
| | 40% water |
| 6. Decondensation buffer 1 | 6 M Guanidine hydrochloride (Gn-HCl) |
| | 575 mM Ditheothreitol (DTT) |
| 7. Decondensation buffer 2 | 522 mM Sodium iodoacetate |
| 8. Denaturing solution | 0.5 N HCl (Hydrochloric acid) |
| 9- Destaining buffer | 20 % methanol |
| | 10 % acetic acid |
| | 70% water |
| 10. Electrophoresis buffer | 5% (v/v) Acetic acid |
| | 95% water |
| 11. Fixation solution for CMA3 | 3 X Methanol |
| | 1 X Glacial acetic acid |

| | |
|---|---|
| 12. Fixation solution for TUNEL | 4% Paraformaldehyde |
| | PBS, pH 7.4 |
| 13. Hypo osmotic solution (HOS) | 150 mmol/l Fructose (13.5 g/L) |
| | 150 mmol/l Sodium citrate:2 $H_2O$ (7.35 g/L) |
| 14. Loading buffer | 0.375 M Potassium acetate, pH 4.0 |
| | 15% Sucrose |
| | 0.05% Methyl green (MG) |
| 15. Phosphate buffer saline (PBS), pH 7.4 | 137 mM Sodium chloride (NaCl) |
| | 2.7 mM Potassium chloride (KCl) |
| | 8 mM Disodium hydrogenphosphate ($Na_2HPO_4$) |
| | 1.5 mM Potassium dihydrogen phosphate ($KH_2PO_4$) |
| 16. Permeability solution | 0.10% Triton X-100 |
| | 0.10 M Sodium citrate, pH 6.0 |
| 17. Precipitating solution | 100% Trichloroacetic acid (TCA) |
| 18. Thiobarbituric acid - Trichloroacetic acid (TBA–TCA) Extraction reagent | 15% (w/v) TCA (Trichloroacetic acid) |
| | 0.375% (w/v) TBA (Thiobarbituric acid) |
| | 0.25 N HCl (Hydrochloric acid) |
| 19. Transfer buffer | 0.0009 N Acetic acid |
| 20. Washing buffer 1 | mmol/l (1 mM) Phenylmethylsulfonyl fluoride (PMSF) |
| 21. Washing buffer 2 | 100 mM Tris, pH (8.0) (Tris(hydroxymethyl)aminomethane, |
| | 1 mM phenylmethylsulfonyl fluoride |
| | 20 mM EDTA (Ethylenediaminetetraacetic |

| | |
|---|---|
| | acid, EDTA). |
| 22. Washing buffer 3 | 1% β- Mercaptoethanol (β-MSH) |
| | Acetone |
| 23. Washing buffer 4 | PBS, pH 7.4 |
| | 0.1% (v/v) Tween 20 |
| | 1% (w/v) Non-fat dry milk |
| 24. Washing buffer 5 | PBS, pH 7.4 |
| | 0.1% (v/v) Tween 20 |

### 3-1-4- Apparatus and Instruments

| | |
|---|---|
| Automatic Pipettes | Eppendorf, Hamburg, Germany |
| Bio-Rad microplate reader 3550-UV | Bio-Rad, München, Germany |
| Electrophoresis chamber | Home Made |
| Enhance chemilumenscence (ECL) system | Bio-Rad, Germany |
| Eppendorf 5414C table centrifuge | Eppendorf, Hamburg, Germany |
| pH-Meter: pH537 | Carl Roth GmbH & Co, Karlsruhe, Germany |
| Light microscope | Olympus |
| Fluorescence microscope | Zeiss, Oberkochen, Germany |
| Macklar counting chamber | Macklar Co. |
| Magnetic stirrer | Eppendorf, Hamburg, Germany |
| Magnetic bars | Eppendorf, Hamburg, Germany |
| Power supply | Amersham Bioscience, Freiburg, |

| | |
|---|---|
| | Germany |
| Simple beam photometer | Amersham Bioscience, Freiburg, Germany |
| Ultrasonic bath transonic 460 | Elma GmbH, Singen, Germany |
| Vortex | Elma GmbH, Singen, Germany |

### 3-1-5- Antibodies

#### 3-1-5-1- Primary antibodies

| | |
|---|---|
| Protamine 1 (Hup 1N) | Mouse polyclonal antibody (Gift from Prof Dr Rod Balhorn, Lawrence Livermore National Laboratory, USA) |
| Protamine 2 (Hup 2B) | Mouse polyclonal antibody (Gift from Prof Dr Rod Balhorn, Lawrence Livermore National Laboratory, USA) |
| Histone 2 A (H2A) | Mouse monoclonal antibody (Cell signaling technology, Germany). |
| Histone 3 (H3) | Rabbit monoclonal antibody (Cell signaling technology, Germany). |
| Histone 2B (H2B) | Rabbit polyclonal antibody (Thermo scientific, USA). |
| Transition protein 1 (TP1) | Mouse monoclonal antibody (Sigma aldrich, Germany). |

#### 3-1-5-2- Secondary antibody

| | |
|---|---|
| Western blot | Horseradish peroxidase conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany). |
| Western blot | Horseradish peroxidase conjugated goat anti- rabbit antibody (Dianova, Hamburg, Germany). |

### 3-1-5-3- Standard Antibody

| | |
|---|---|
| Histone 2A (H2A) | (Cell signaling technology, Germany). |
| Histone 2 B (H2B) | (Cell signaling technology, Germany). |
| Histone 3 ( H3) | (Cell signaling technology, Germany). |

### 3-1-6- ELISA Kits

| **Kit** | **Company** |
|---|---|
| Cotinine ELISA | Calbiotech, CA, USA |
| *In situ* cell death detection kit, Fluorescein (TUNEL) | Roche diagnostics GmbH, Mannheim, Germany |

## 3-2- Methods

### 3-2-1- Ejaculates and sperm preparation

Semen samples were collected by masturbation. Samples were collected in sterile containers and allowed to liquefy at 37$^{o}$C for 30 min and processed immediately after complete liquefaction, according to world health organization (WHO, 2010). Briefly, semen samples were examined for volume, pH, leukocytes, sperm concentration, motility, vitality, membrane integrity, and morphology and the presence of agglutination, according to WHO guidelines (WHO, 2010). After liquefaction each specimen was prepared by discontinuous pure sperm gradient (Nidacon international AB, Sweden) by layering 1 ml of different concentration of pure sperm solution 80%, and 40% in a sterilized tube, beginning at the bottom of the tube with that of 80% density.

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

2- Centrifugation of the samples was done at room temperature (RT) for 25 minutes at 250xg.

3- After centrifugation, the supernatant (seminal plasma) was immediately separated, and examined before storage to rule out the presence of spermatozoa in the supernatant. Aliquot of seminal plasma was stored at -80°C until use.

4- The 40% layer was aspirated with a Pasteur pipette and discarded.

5- The final fraction that contains motile spermatozoa was transferred into a new tube and washed by adding 4-5 ml HAM's F10 medium using 10 min of centrifugation at 250xg at room temperature.

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

7- Aliquot containing 20 - 40 X $10^6$ sperm were transferred into an Eppendorf tube and centrifugated for 10 min at 800xg at room temperature, then the pellet were stored at – 80°C until use for sperm nuclear protein extraction.

8- The rest of the sperms was kept at – 80°C.

### 3-2-2- Assessment of sperm vitality (eosin-nigrosin-test)

Sperm vitality was assessed in wet mount smears after supravital staining with aqueous eosin-nigrosin as follows. One drop of semen was mixed on a slide with one drop of 0.5% aqueous yellowish eosin solution and one drop of nigrosin (10% in water) and covered with a cover slip. After 1–2 min the spermatozoa which stained red (dead spermatozoa) can be distinguished from the unstained spermatozoa (live spermatozoa). Nigrosine was used as a counter-stain to facilitate visualization of the unstained live spermatozoa. On each slide 100 spermatozoa from each semen sample were evaluated.

### 3-2-3- Assessment of sperm membrane integrity (hypo osmotic swelling test) (hos-test)

Hypo-osmotic swelling test (hos-test) was used for the assessment of the membrane integrity of spermatozoa.

A 100 µl sample of sperm suspension was added to 1 ml of hypoosmotic solution (equal parts of 150 mmol/l fructose and 150 mmol/l sodium citrate solutions), followed by 60 min incubation at 37°C. After incubation, a minimum of 200 spermatozoa were examined per slide under the light microscope and the percentage of spermatozoa that showed typical tail abnormalities (curly tail) indicative of swelling were calculated.

### 3-2-4- Assessment of protamine deficiency (aniline blue) of spermatozoa

Protamine deficiency was measured by aniline blue (AB) staining as previously described by Hammadeh *et al*. (2001). Briefly, after sperm preparation, 5 µl of the prepared spermatozoa were spread onto glass slides, and allowed to dry. The smears were fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min. Slides were then stained with 5% aqueous aniline blue mixed with 4% acetic acid (pH 3.5) for 5 min; 200 sperm cells per slide were evaluated and the percentage of unstained sperm heads was calculated. Three classes of staining intensities were distinguished: unstained, completely or partially stained.

### 3-2-5- Assessment of protamine deficiency (chromomycin A3, CMA3) of spermatozoa

Protamine deficiency was measured also by chromomycin A3 (CMA3) staining as previously described by Hammadeh *et al*. (2010). Briefly, semen aliquots were washed in Dulbecco's $Ca^{2+}$ – $Mg^{2+}$ free phosphate buffer saline (PBS) and centrifuged at 250xg for 10 min. The spermatozoa were washed, fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) at 48ºC for 5 min and then spread on clean slides. The CMA3 (Sigma, St. Louis, MO, USA) was dissolved in Mcilvaine buffer (pH 7.0) supplemented with 10 mmol/l $MgCl_2$ (17 ml of 0.1 mmol/l citric acid mixed with 83 ml of 0.2 mol/l $Na_2HPO_4$ and 10 mmol/l $MgCl_2$) to a concentration of 0.25 mg/ml. Each slide was treated for 20 min with 100 ml of CMA3 solution in the dark.

Fluorochrome was examined using a Zeiss photomicroscope III using a combination of exciter dichromic barrier filter of BP 436/10: FT 580: LP 470. A total of 200 spermatozoa were evaluated on each slide. Evaluation of CMA3 staining is done by distinguishing spermatozoa that stain bright yellow (CMA3 positive) from those staining dull yellow (CMA3 negative).

### 3-2-6- DNA fragmentation analysis (TUNEL assay)

DNA fragmentation was assessed using the terminal deoxyribonucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay as previously described (Hammadeh *et al*., 2010). The TUNEL assay was performed using the *in situ* Cell Death Detection Kit

according to the manufacturer's guidelines (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, semen samples were smeared on microscopic slides, air dried then fixed in 4% paraformaldehyde in PBS, pH 7.4 and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, pH 6.0. Fragmented DNA was detected by the TUNEL assay kit following the manufacturer's guidelines.

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

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

### 3-2-7- Protamine quantification

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

**A- Preparation of protein standard curve**

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

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

- 750 µl of stock A in 1 ml W, this will give a concentration of 7.5 µg/ml (solution B)

- 500 µl of stock A in 1 ml W, this will give a concentration of 5 µg/ml (solution C)

- 250 µl of stock A in 1 ml W, this will give a concentration of 2.5 µg/ml (solution D)

- 150 µl of stock A in 1 ml W, this will give a concentration of 1.5 µg/ml (solution E)

- 100 µl of stock A in 1 ml W, this will give a concentration of 1 µg/ml (solution F)

**B- Determination of protein concentration**

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

1- Reaction mixtures were prepared as following (table 1):

**Table 1:** Reaction mixtures used for preparing the protein standard curve.

| T.T. No. | Volume of concentration (µl) | $H_2O$ (µl) | Protein assay reagent (µl) | Final volume (µl) |
|---|---|---|---|---|
| 1 | 1 µl stock F (1 µg/ml) | 799 | 200 | 1000 |
| 2 | 1 µl stock E (2 µg/ml) | 799 | 200 | 1000 |
| 3 | 1 µl stock D (3 µg/ml) | 799 | 200 | 1000 |
| 4 | 1 µl stock C (5 µg/ml) | 799 | 200 | 1000 |
| 5 | 1 µl stock B (7 µg/ml) | 799 | 200 | 1000 |
| 6 | 1 µl stock A (10 µg/ml) | 799 | 200 | 1000 |
| 7 | 1.5 µl stock A (15 µg/ml) | 798.5 | 200 | 1000 |
| 8 | 2 µl stock A (20 µg/ml) | 798 | 200 | 1000 |
| 9 | 2.5 µl stock A (25 µg/ml) | 797.5 | 200 | 1000 |

Note: Analysis was done in triplicate

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

3- The absorbance was measured against water at a wave length of 595 nm.

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

**3-2-7-2- Protein extraction**

Sperm nuclear proteins extraction of each sample was performed by using an established protein extraction protocol described by Hammadeh *et al*. (2010). Briefly, $40 \times 10^6$ spermatozoa were washed using wash buffer I [1 mM (mmol/l) of phenylmethylsulfonyl fluoride (PMSF) in water] and centrifuged at RT for 5 min at 800×g. Pellets were

resuspended in 100 µl of wash buffer 2 [20 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 1 mM PMSF in 0.1 M Tris (pH 8.0)] and 100 µl of the decondensation buffer 1 (6 M guanidine and 575 mM DTT) was then added and mixed. Afterwards, 200 µl of decondensation buffer 2 (522 mM sodium iodoacetate) was added and mixed further. After incubation in the dark for 30 min at RT, each sample was mixed again with 1 ml of ice cold ethanol at - 20°C for 1 min, then centrifuged at 13,400xg for 10 min at 4°C and the supernatant was discarded. Each pellet was then resuspended with 0.8 ml of denaturing solution (0.5 M HCl), and incubated for 15 min at 37°C before centrifugation at 13,400xg for 10 min at 4°C. The supernatant was transferred to another tube containing 200 µl of 100% trichloroacetic acid (TCA) to precipitate the nuclear proteins. After incubation on ice for 3 min, the mixture was centrifuged at 13,400xg for 10 min at 4°C. The supernatant was discarded and the pellet washed twice with 1 ml of wash buffer 3 containing 1% ß-mercaptoethanol in acetone. The final pellet was air dried at 4°C overnight and stored at -20°C.

A human protamine standard and a quality control sample were prepared and applied to ensure that protamine quantification procedure are valid and give reproducible results as previously shown by Hammadeh *et al*. (2010).

### 3-2-7-3- Production of a protamine and transition proteins standard

Human protamine or transition protein standards were prepared as described by Hammadeh *et al.* (2010). Sperm samples of 20 known fertile donors were pooled to give a total of 1 x $10^9$ sperm.

1- The semen samples were centrifuged at 800xg for 10 minutes at RT to remove the seminal plasma.

2- The sperm were washed with PBS and then placed into Eppendorf tubes each containing 1 x $10^8$ sperm, centrifuged at 800xg for 10 minutes at RT and the supernatant was discarded.

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

4- The samples then centrifuged at 800xg for 5 min at RT then the supernatant removed.

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

5- The pellets washed with 0.25 ml of washing buffer 2 (20 mM EDTA, 1 mM PMSF in 0.1 M Tris (pH 8.0)) then centrifuged at 800xg for 5 min at RT then discarded the supernatant.

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

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

7- Each sample of the extracted protamines was suspended in 50 µl water.

8- All samples were collected in one tube.

NOTE: If a precipitate was present, add 100 µl of water until the precipitate is completely diluted.

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

10- The protamine extract was analyzed by acid-urea polyacrylamide gel electrophoresis to determine the ratio of P1 and P2 (refer to section 3-2-7-6 below). Bands of P1 and P2 can be seen in Figure 17.

11- The transition protein 1 (TP1) extract was analyzed by acid-urea polyacrylamide gel electrophoresis to determine the TP1. Bands of TP1 can be seen in Figure 30.

12- The intensity of the P1, P2 and TP1 bands were used to draw the standard curve and to calculate the linear equation and the $r2$ value of the regression curve (Figs. 14 B, 14 C, 29 B, respectively).

### 3-2-7-4- Production of histone protein standard:

The protein concentration (1 mg/ml) was used to prepare the standard curve for histones H2A, H2B, and H3:

1. Histone H2A standard curve (0.5, 0.75, 1.00, 1.50 µg/µl).
2. Histone H2B standard curve (0.5, 1.00, 1.50, 2.00 µg/µl).
3. Histone H3 standard curve (0.5, 1.00, 1.50, 2.00 µg/µl).

Recombinant histone proteins were loaded on 15% acidic acid urea polyacrylamide gel and stained with Western blot. The histone dilutions were analyzed by acid-urea polyacrylamide gel electrophoresis to determine the histone H3 (Fig. 15), histone H2A (Fig. 16) and histone H2B (Fig. 17). The intensity of the histone H2A, H3 and H2B bands were used to draw the standard curve and to calculate the linear equation and the *r*2 value of the regression curve (Figs. 11, 12, 13, respectively).

### 3-2-7-5- Protein extraction control sample:

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

1- Aliquots of 40 $x10^6$ sperm were prepared as described for sample preparation and stored at – 80°C.

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

### 3-2-7-6- Acid-urea polyacrylamide gel electrophoresis (AU-PAGE):

Protamine extracts were tested by using acetic acid-urea polyacrylamide gel electrophoresis (AU-PAGE) method as described by Hammadeh *et al*. (2010) with some modifications.

### 3-2-7-6-1- AU-PAG preparation:

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

2- The resolving gel (15%) was prepared and filled in about 10 cm of the space (the glass plates).

3- 150 µl of N-butanol were added on top of the resolving gel to avoid air bubbles.

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

5- The comb was placed into the stacking gel.

6- After the gel was polymerized it was kept in moist atmosphere in a refrigerator until use.

For resolving gel preparation we used an acrylamide solution with 40% (w/v) acrylamide and 0.8% (w/v) N,N′-methylenebisacrylamide, while for the stacking gel we used an acrylamide solution with 30% (w/v) acrylamide and 0.8% (w/v) N,N′-methylenebisacrylamide and 0.375 M potassium acetate (pH 4.0). For both gels we used 2.5 M urea and 43% acetic acid. In addition, for polymerization we used 10% (w/v) ammonium persulphate for both and 0.5% and 2% (w/v) TEMED for resolving and stacking gels, respectively. Table (2) summarizes the volumes of solutions used for preparing both gels.

**Table 2:** Solution volumes used for preparing polyacrylamide gels.

| Resolving gel | 15% |
|---|---|
| 40% polyacrylamide gel (19:1) | 4 ml |
| 10 M Urea | 2 ml (final concentration 2.4M) |
| 43% Acetic acid | 1 ml |
| 10% Ammonium persulphate | 160 µl (freshly prepared) |
| TEMED | 40 µl |
| Water | 1.4 ml |

| | |
|---|---|
| Stacking gel | 7.5% |
| 30% polyacrylamide gel (19:1) | 625 µl |
| 10 M Urea | 625 µl |
| 43% Acetic acid | 625 µl |
| 10% Ammonium persulphate | 50 µl |
| 3 M Potassium acetate (pH 4.0) | 312 µl |
| Water | 275 µl |

**3-2-7-6-2- Electrophoresis**

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

**A-Pre-electrophoresis**

1- The AU-polyacrylamide gel was electrophoresed before loading the samples.
2- The electrophoresis conditions were, 25 mA for 30 minutes (reverse polarity was used).
3- The running buffer was replaced by a fresh buffer before the next step.

**B-Slow run electrophoresis**

1- The extracted nuclear proteins were solubilized in 80 µl sample loading buffer (0.375 M potassium acetate (pH 4.0), 15% sucrose, and 0.05% methyl green).
2- Lanes of each gel loaded with 10 µl of each sample, control sample and human protein standards.
3- The electrophoresis conditions were 25 mA for 15 minutes (reverse polarity was used).

**C-Fast run electrophoresis**

For fast run electrophoresis the same gels with the same running buffer were used but the electrophoresis conditions were changed to 25 mA for 50 min (reverse polarity was used).

**3-2-7-6-3- Staining**

Gels were stained with Coomassie blue.

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

2- The stacking gel was cut away

3- The gel was transferred to a container containing Coomassie blue for 20 minutes at RT with shaking.

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

5- The gels were distained using distaining buffer (20% methanol, 10% acetic acid and 70% water) over night with shaking at RT.

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

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

8- The intensity of the bands corresponding to P1 and P2 were quantified.

9- P1 and P2 quantities were calculated against the standard curve generated from the human protamine standard as described above in section (3-2-7-3) (Fig. 17).

**3-2-8- Western blot:**

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

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

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

3- The transfer conditions were 150 mA, for overnight (reverse polarity was used)

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

5- Membranes were washed with washing buffer 4 (1x PBS, pH 7.4, 0.1% (v/v) Tween 20, 1% (w/v) non-fat dry milk) three times, each for 7 minutes with shaking at RT.

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

- One membrane was incubated with a Hup 1N (anti-protamine 1) specific primary antibody diluted 1:100000
- The second membrane was incubated with a Hup 2B (anti-protamine 2) specific primary antibody diluted 1:500000
- One membrane was incubated with an anti-histone 2A primary antibody diluted 1:1000
- One membrane was incubated with an anti-histone 2B primary antibody diluted 1:250
- One membrane was incubated with an anti-histone 3 primary antibody diluted 1:2000
- One membrane was incubated with an anti-transition protein 1 primary antibody diluted 1:1000

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

8- Membranes were incubated with the horseradish peroxidase-conjugated secondary antibody diluted in washing buffer 5 for 1 hour at RT with shaking.

A- Horseradish peroxidase-conjugated goat anti-mouse secondary antibody diluted 1:10000.

B- Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody diluted 1:30000.

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

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

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

### 3-2-9- Lipid peroxidation assay in seminal plasma (measurement of malondialdehyde, MDA)

The level of lipid peroxidation (LPO) in the biological samples (serum, sperm, seminal plasma and follicular fluid) was measured by determining the MDA production, using a modified thiobarbituric acid (TBA) according to the method of Hammadeh *et al*. (2010). Briefly, 250 ml of seminal plasma were added to 2.0 ml of the TBA–TCA extraction reagent [15% (w/v) TCA, 0.375% (w/v) TBA in 0.25 N HCl (hydrochloric acid)]. The mixture was then heated in a water bath at 95ºC for 30 min. After cooling, samples were centrifuged at 800xg for 10 min at RT and the absorbance was measured at 535 nm against a blank. The concentration of MDA (mM) was calculated from the equation of the plotted standard curve.

For standard curve preparation, the same procedures were applied using a 250 ml sample of each standard concentration in duplicate.

**Standard Solutions**

A malondialdehyde standard was prepared by modifying the method described by Sangalli *et al*. (2003). Briefly, a 10 mM stock solution (A) was prepared by mixing 25.97 µl of 1, 1, 3, 3-tetramethoxypropane (6 M) with 1 M hydrochloric acid at 37ºC 30 minutes. This solution was then further diluted with water (W) to prepare various working standard solutions as following:

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

Standard solutions were stored at 4ºC.

### 3-2-10- Cotinine measurement

The levels of cotinine in seminal plasma were measured using the calbiotec cotinine direct ELISA kit (Calbiotech, CA, USA) which is designed to detect cotinine in serum, urine and other fluids.

**Principles of the test**

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

**Performance characteristics accuracy**

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

**Assay Procedures**

Assay procedures were applied according to the manufacturer's guidelines. Briefly, standards, controls and seminal plasma were pipetted into selected wells in duplicate, the enzyme conjugate was added to each well and the plates were incubated for 60 min in the dark at RT. After washing with water, the substrate reagent was added to each well. After a 30 min incubation at RT in the dark, stop solution was added and absorbance was read at 450 nm. The concentration of cotinine was calculated against the standard curve generated from the standards (0.0, 5.0, 10, 25, 50, 100 ng/ml) applied in the kit in the same plate

(absorbance for cotinine standards (vertical axis) versus cotinine standards concentrations (horizontal axis)). Samples with concentrations higher than 100 ng/ml were diluted and measured once more.

### 3-2-11- Statistical analysis

Data analysis was performed using GraphPad prism 5 program. Data were expressed as mean ± SD and range. The relationship between histone (H2A, H2B, H3), transition protein 1 (TP1), protamines 1 and 2 (P1, P2), P1/P2 ratio, ROS, cotinine, DNA integrity, chromatin condensation, sperm viability, membrane integrity, concentration, motility and seminal volume were analyzed using nonparametric correlation (Spearman's test). Furthermore, semen characteristics, ROS, and cotinine concentrations were analyzed in seminal plasma for smokers and non-smokers and their effect on nuclear sperm protein (histones, transition proteins and protamines) were analyzed using parametric and non-parametric methods. The Mann-Whitney was used for non–paired data. The results were presented by the mean ± SD values. Spearman's test was used to calculate the coefficient of correlations between variables. They were considered statistically significant when $P < 0.050$ and highly significant when $P < 0.010$.

# 4- Results

## 4-1- Sperm nuclear proteins

### 4-1-1- sperm parameters

Spermatozoa and seminal parameters were measured in 75 patients (45 smokers (age (year) = 25 – 55) and 30 non-smoker controls (age (year) = 24 – 48). The first question that came to mind was, does the age of the patients (smokers and non-smokers) correlate with semen quality? To answer this question, the age was similar for both groups (33.87 ± 0.93 years; 35.30 ± 1.21 years). There were no relationship between the age of the patients and the sperm parameters (Fig. 4).

In order to analyze whether there is any relationship between cigarette smoking and sperm parameters? Samples were collected in sterile containers and allowed to liquefy at 37ºC for 30 min and then processed immediately. The seminal parameters for smokers and non-smokers were determined by measuring the semen volume, sperm concentration, sperm motility, sperm vitality, and sperm membrane integrity. Smoking affects sperm parameters negatively and decrease sperm parameter. Such as, sperm concentrations (mill./ml) and the mean percentage of motile spermatozoa were significantly higher (P = 0.000) in non-smokers (84.27 ± 6.01 mill./ml, 46.83 ± 4.12%) in comparison to smokers (51.87 ± 5.05 mill./ml, 29.67 ± 2.47% respectively) (Figs. 5 and 6). Figure 4 showed that the ejaculate volume were not significantly different (P > 0.05) for both smokers and non- smokers (3.09 ± 0.19 ml; 3.20 ± 0.24 ml respectively).

Sperm vitality was assessed in wet mount smears after supravital staining with aqueous eosin-nigrosin, as described in material and methods (section, 3-2-2). The mean percentage of vital sperm is significantly lower (P = 0.000) in smokers (41.04 ± 2.49%) than that of non-smokers (59.00 ± 3.33%) (Fig. 7). The membrane integrity of spermatozoa was quantified by hypo-osmotic swelling test (hos-test), as described in material and methods (section, 3-2-3). Membrane integrity of spermatozoa is significantly lower in smokers (59.24 ± 1.79%) in comparison to non-smokers (71.30 ± 3.16%; P = 0.000) (Fig. 8).

The levels of cotinine in seminal plasma were measured using the Calbiotec cotinine direct ELISA kit, as described in material and methods (section, 3-2-10). The cotinine concentration was significantly higher in smokers in comparison to non-smokers (60.44 ± 5.29 vs. 2.01 ± 0.65, P = 0.0001) (Fig. 9).

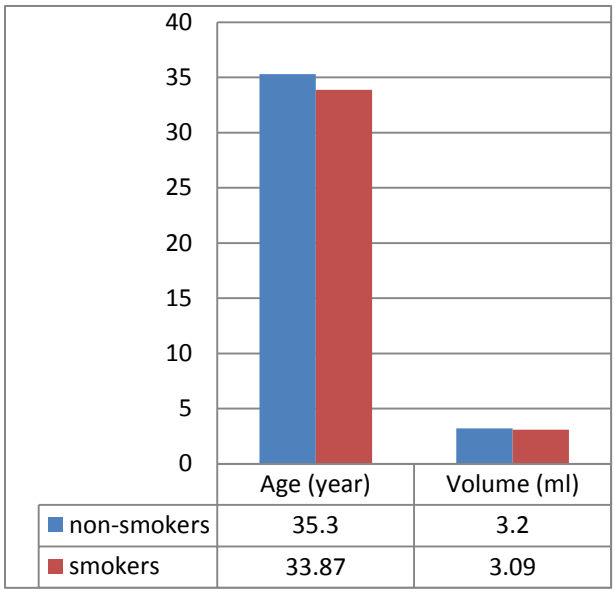


**Figure 4:** Age (year) of patients and semen volume (ml) of both smokers and non-smokers patients. Box-plots showing the mean, median and range of age of patients and semen volume of both smokers and non-smokers. Age of patients and semen volume were no significantly between smokers and non-smokers (33.87 ± 0.93, 3.09 ± 0.19 vs. 35.30 ± 1.21, 3.20 ± 0.24, P > 0.05).

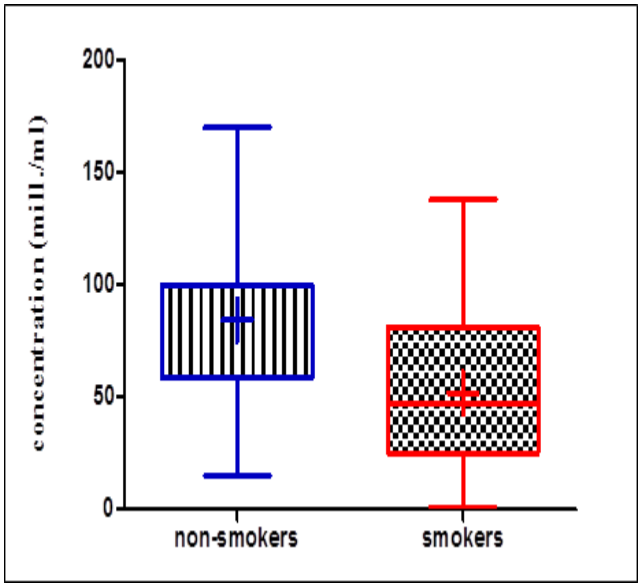


**Figure 5:** Concentration of sperm from smokers and non-smokers. Box plots showing the mean, median and range of sperm concentration of both smokers and non-smokers. Sperm concentration was significantly lower in smokers in comparison to non-smokers (51.87 ± 5.05 vs. 84.27 ± 6.01, P = 0.0001).

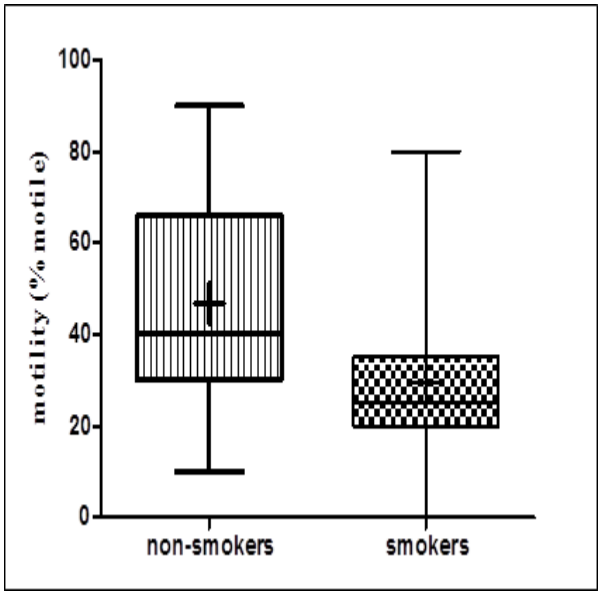


**Figure 6:** Motility of sperm from smokers and non-smokers. Box plots showing the mean, median and range of sperm motility of both smokers and non-smokers. Sperm motility was significantly lower for smokers in comparison to non-smokers (29.67 ± 2.47 vs. 46.83 ± 4.12, P = 0.0004).

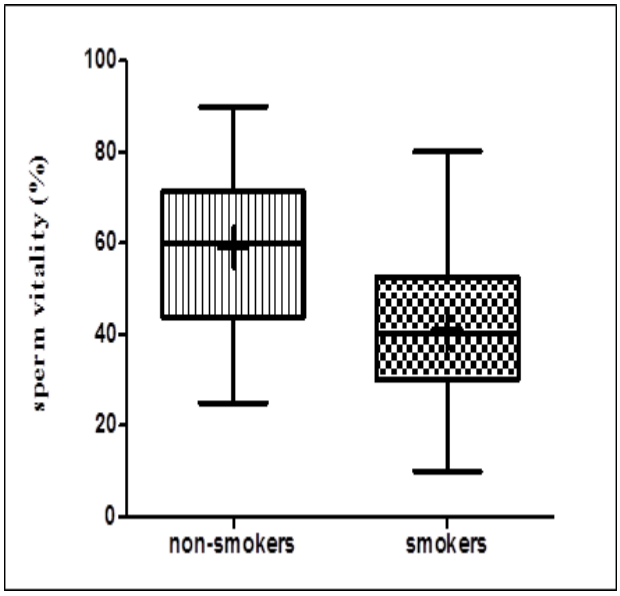


**Figure 7:** Vitality (eosin test) of sperm from smokers and non-smokers. Box plots showing the mean, median and range of sperm vitality of both smokers and non-smokers. Sperm vitality was significantly lower for smokers in comparison to non-smokers (41.04 ± 2.49 vs. 59.00 ± 3.33, P = 0.0001).

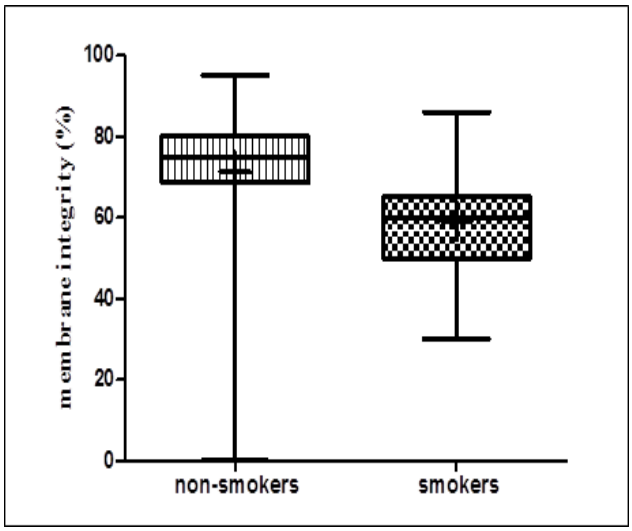


**Figure 8:** Sperm membrane integrity (hos test) of both smokers and non-smokers. Box plots showing the mean, median and range of sperm membrane integrity of both smokers and non-smokers. Sperm membrane integrity was significantly lower for smokers in comparison to non-smokers (59.24 ± 1.79 vs.71.30 ± 3.16, P = 0.0001).

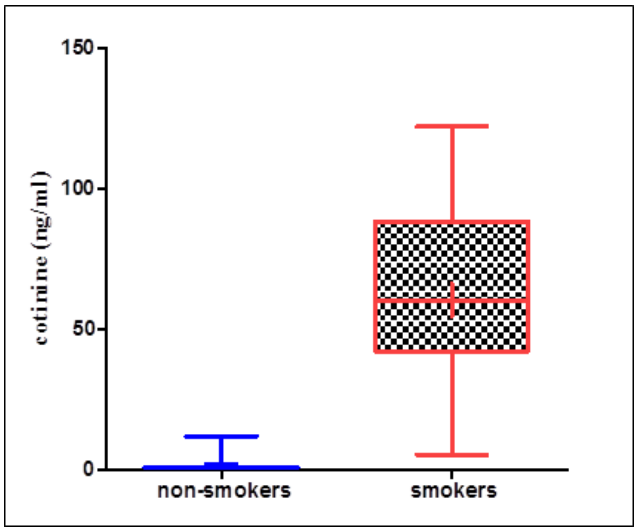


**Figure 9:** Cotinine concentrations in the sperm of both smokers and non-smokers. Box plots showing the mean, median and range of cotinine of both smokers and non-smokers. Cotinine concentrations were significantly higher for smokers in comparison to non-smokers (60.44 ± 5.29 vs. 2.01 ± 0.65, P = 0.0001).

In the next step we asked whether histone retention differs between smokers and non-smokers? Sperm immaturity was measured indirectly by aniline blue (AB) staining as described in material and methods (section, 3-2-4). Histone retention was significantly higher in smokers in comparison to non-smokers. In Figure 10 showed that the mean percentage in the aniline blue test of smokers were significantly higher ($P < 0.01$) compared to that of the non-smokers ($27.47 \pm 1.40$ vs. $19.77 \pm 1.76$).

We next asked whether protamination differs between smokers and non-smokers? Protamine deficiency was measured indirectly also by chromomycin A3 (CMA3) staining as described in material and methods (section 3-2-5). Spermatozoa in smokers showed a chromatin deficiency. Figure 10 showed that the mean percentages of CMA3 test of smokers were significantly higher ($P < 0.01$) compared to that of the non-smokers ($36.91 \pm 1.87$ vs. $20.95 \pm 1.35$).

The next question was whether smoking might damage DNA in spermatozoa? DNA fragmentation was assessed using the terminal deoxyribonucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay as described in material and methods (section 3-2-6). According to the results in this study smoking has a detrimental effect on DNA integrity cause a single and double strand break of spermatozoa. In Figure 10 showed that the mean percentage of DNA fragmentation of smokers were significantly higher ($P < 0.01$) in comparison to that of the non-smokers ($25.29 \pm 1.14$ vs. $12.47 \pm 1.41$).

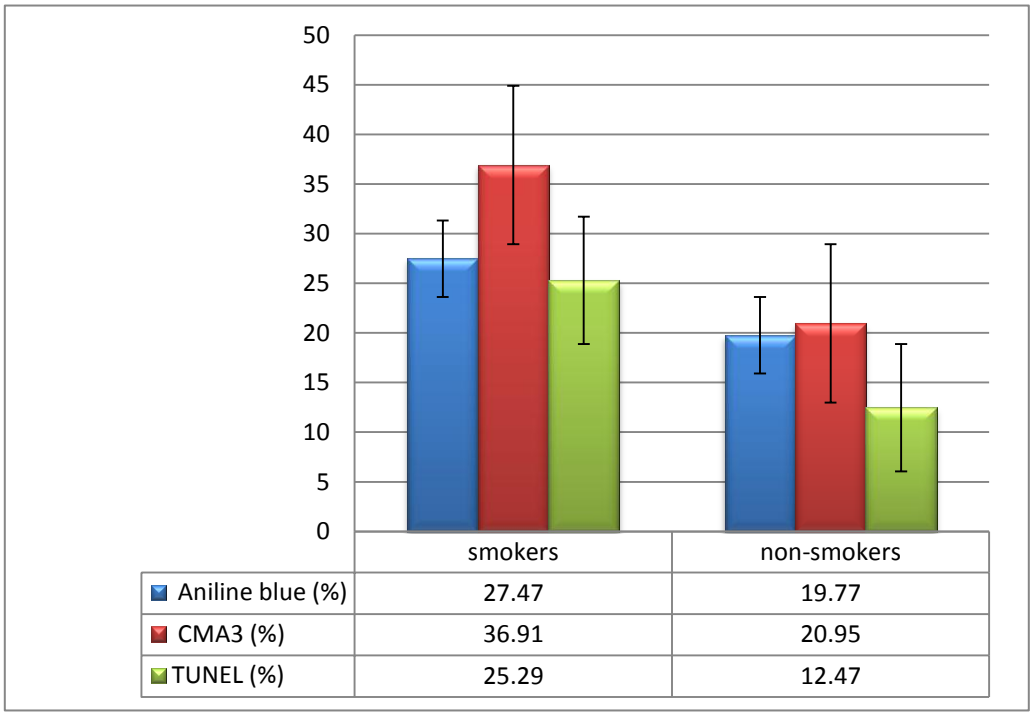


**Figure 10:** Sperm immaturity (aniline blue) (%), protamine deficiency (CMA3) (%), DNA fragmentation (TUNEL) (%) in the sperm of smokers and non-smokers.

### 4-1- 2- Histone and protamine

In order to quantity protein concentration by acetic acid-urea polyacrylamide gel electrophoresis I first had to measure proteins in a gel and to scan the protein band in order to generate a standard curve. Sperm nuclear protein standards (H2A, H2B, H3, P1 and P2) were analyzed by acid-urea polyacrylamide gel electrophoresis, as described in material and methods (section, 3-2-7-3 and 3-2-7-4). This is a pilot study and a standard value for sperm protein contents was not available in the literature. Therefore, a histone H2A standard was prepared as described in material and methods (section 3-2-7-4). The histone H2A protein concentration was determined by using the RC DC protein assay kit and a serial dilution of standards (0.50, 0.75, 1.00 and 1.50 µg) were loaded in the polyacrylamide-acetic acid-urea gel and a standard regression curve was formulated based on the protein concentrations and corresponding densitometry reading from individual bands (Fig.11 A). A minimum R2 regression value of 0.90 was obtained for the gel (Fig.11 B).

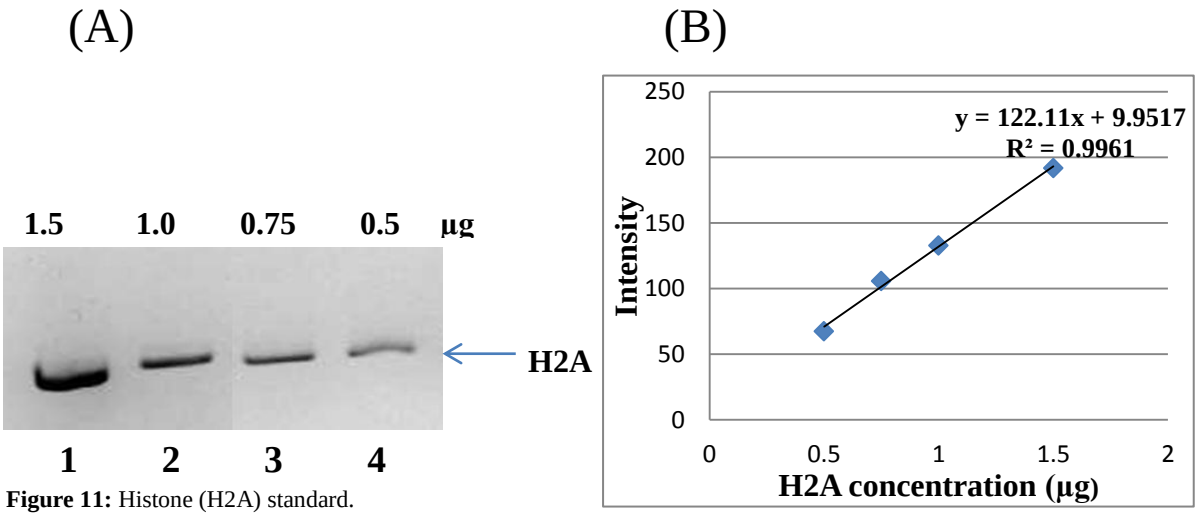


**Figure 11:** Histone (H2A) standard.
**(A)-** Western blot analysis of recombinant histone H2A, Lane 1 – 4 correspond to decreasing amounts of a human histone H2A standard included in each lane.
**(B)-** Standard curve of histone (H2A) (0.50, 0.75, 1.00, and 1.50 µg).

Also, a histone H3 standard curve was prepared as described in material and methods (section 3-2-7-4). It was determined by using the RC DC protein assay kit and a serial dilution of standards (0.50, 1.00, 1.50 and 2.00 µg) were loaded in the polyacrylamide-acetic acid-urea gel and a standard regression curve was formulated based on the protein concentrations and corresponding densitometry reading from individual bands (Fig.12 A). A minimum R2 regression value of 0.90 was obtained for the gel (Fig.12 B)

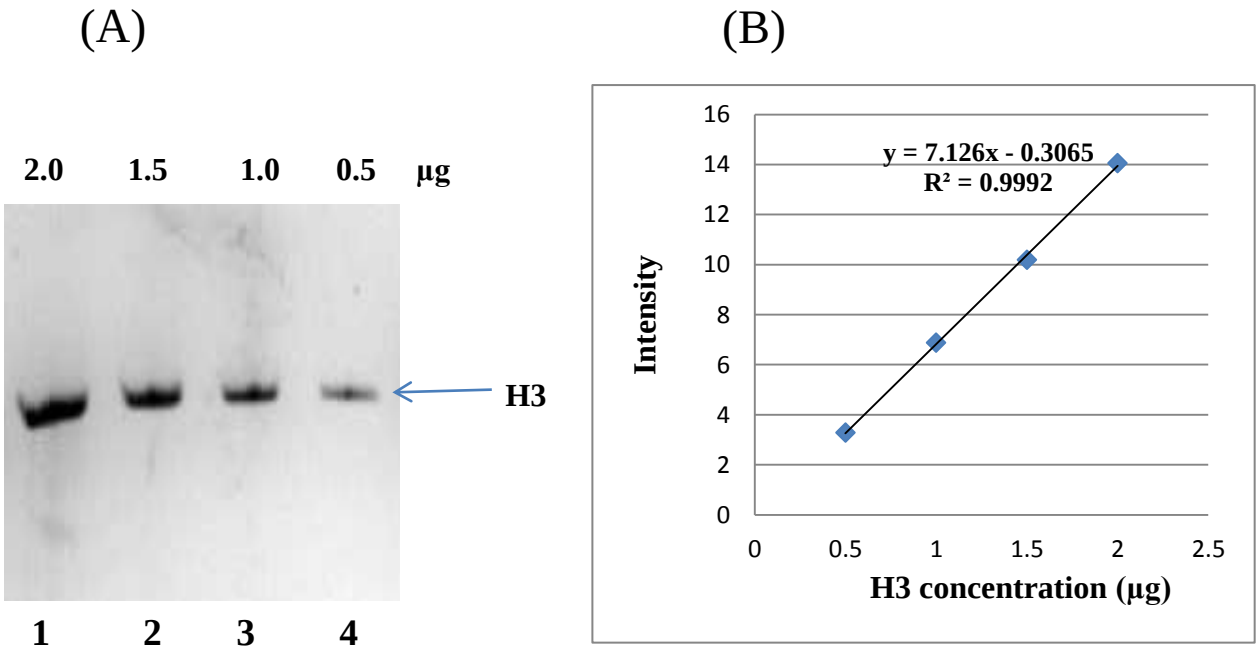


**Figure 12:** Histone (H3) standard.
**(A)** – Western Blot analysis of recombinant histone H3. Lane 1 – 4 correspond to decreasing amounts of histone H3 standard included in each lane**.**
**(B)** - Standard curve of histone 3 (0.50, 1.00, 1.50 and 2.00 µg).

A histone H2B standard was prepared as described in material and methods (section 3-2-7-4). A standard regression curve was formulated based on the protein concentrations and corresponding densitometry reading from individual bands (Fig.13 A). A minimum R2 regression value of 0.90 was obtained for the gel (Fig.13 B).

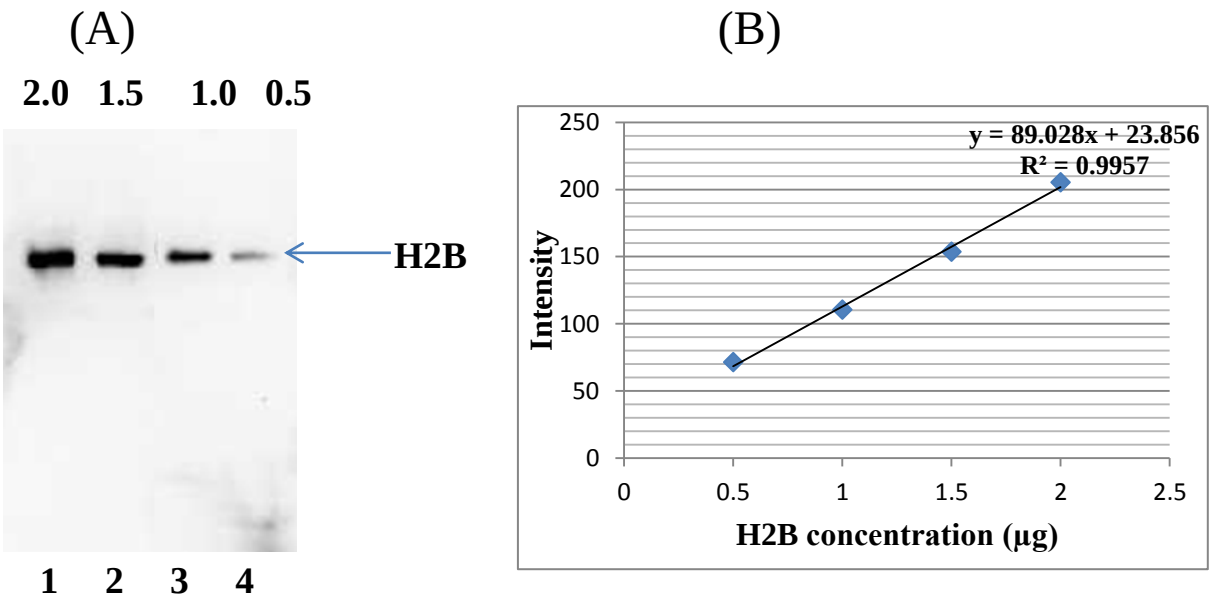


**Figure 13**: Histone H2B standard.
**(A)-** Western Blot analysis of recombinant histone H2B. Lane 1 – 4 correspond to decreasing amounts of histone H2B standard included in each lane.
**(B)-** Standard curve of histone H2B (0.50, 1.00, 1.50 and 2.00 µg)

In addition, a human protamine standard was prepared as described in material and methods (section 3-2-7-3). A standard regression curve was formulated based on the protein concentrations and corresponding densitometry reading from individual bands (Fig. 14 A). A minimum R2 regression value of 0.90 was obtained for the gel (Fig. 14 B, C).

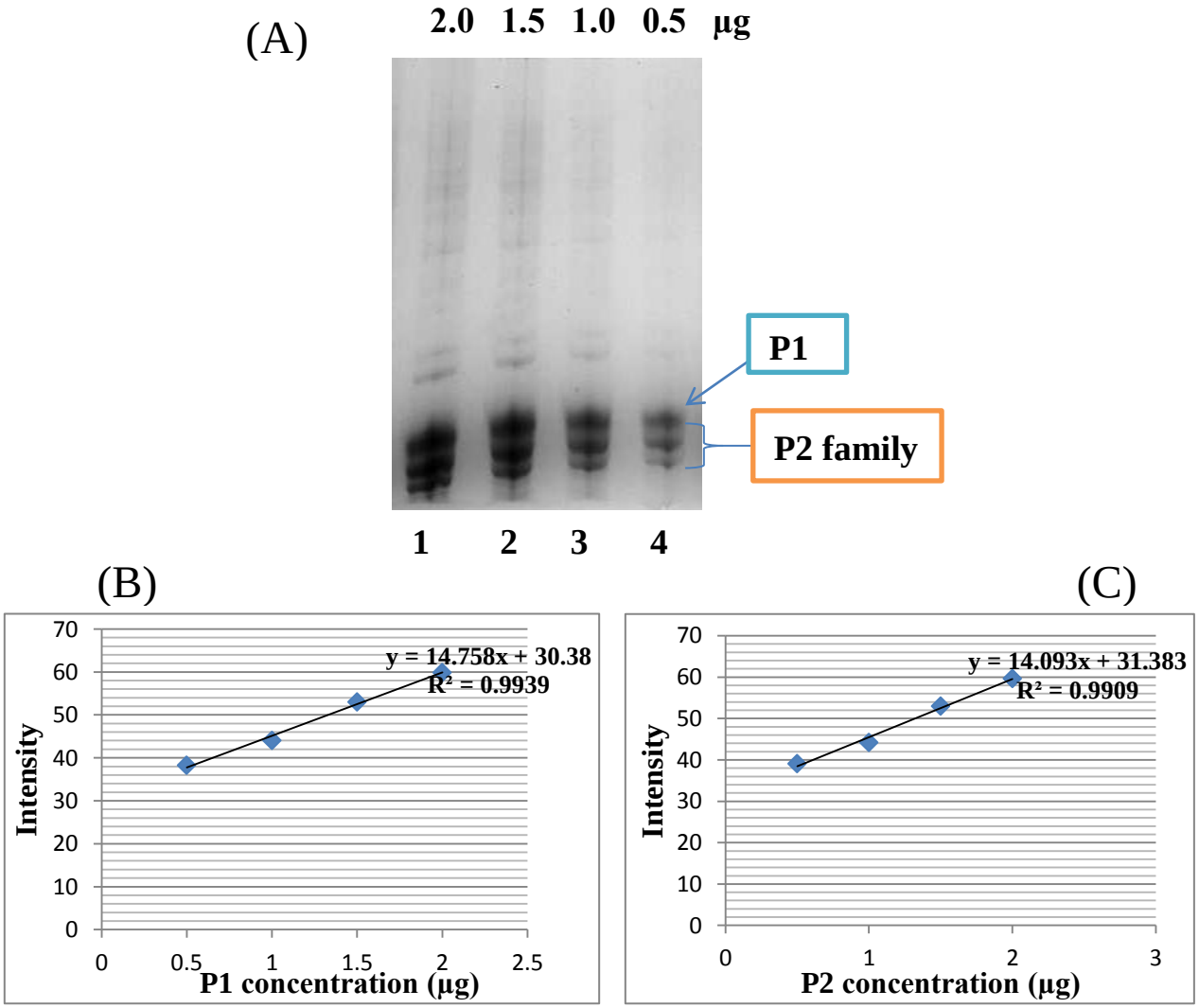


**Figure 14:** Protamine (P1 and P2) standard.
**(A)-** Coomassie blue analysis of recombinant protamine (P1 and P2). Lane 1 – 4 correspond to decreasing amounts of protamine P1 and P2 standard included in each lane.
**(B)-** Standard curve of protamine 1 (0.50, 1.00, 1.50 and 2.00 µg).
**(C)-** Standard curve of protamine 2 (0.50, 1.00, 1.50 and 2.00 µg).

Now, I could address the question whether there are histone concentration (H3, H2A and H2B) differences between smokers and non-smokers. Figure 15, 16 and 17 are acetic acid-urea polyacrylamide gels of H3, H2A and H2B, respectively. There are only examples of a series of Western Blots of all sperm samples. I found, that semen samples from smokers possessed significantly higher levels of spermatozoa nuclear histone H2B, H2A and H3 concentration than non-smoker. Figure 15 illustrates the increase of histone H3 protein concentration in sperm of smokers in comparison with non-smokers. Also, Figure 16 demonstrates that the concentration of histone H2A protein in sperm of smokers was higher than non-smokers. Furthermore, Figure 17 higher concentrations of histone H2B protein in sperm of smokers were found when compared with non-smokers. This study was first attempt to correlate cigarette smoking with histones levels in smokers and non-smokers.

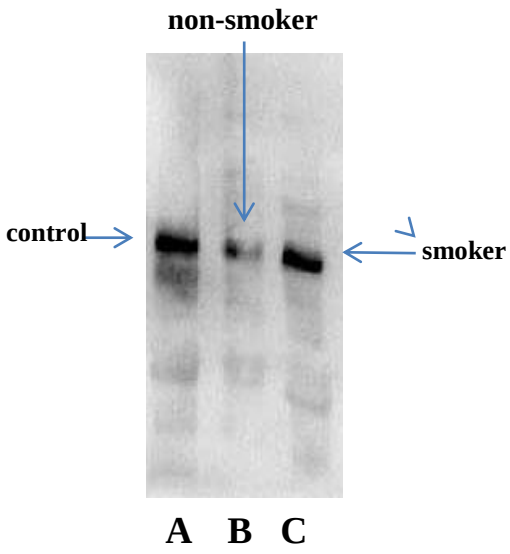


**Figure 15:** Analysis of sperm nuclear proteins; Histone (H3). (A) H3 control. (B) Western blot for histone H3 in sperm of nonsmokers and (C) Western blot for histone H3 in sperm of smoker, using an antibody specific for histone H3 (rabbit monoclonal antibody, cell signaling technology).

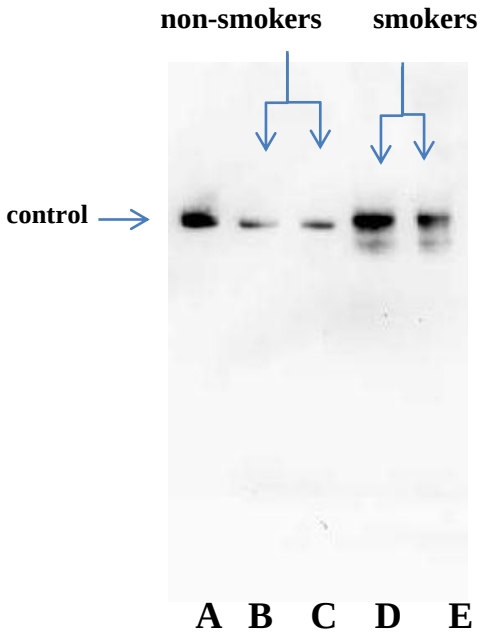


**Figure 16:** Analysis of sperm nuclear proteins; histone (H2A). (A) H2A control. (B, C) Western blot for histone H2A in sperm of non-smokers and (D, E) Western blot for histone H2A in sperm of smokers, using an antibody specific for histone H2A (mouse monoclonal antibody, cell signaling technology).

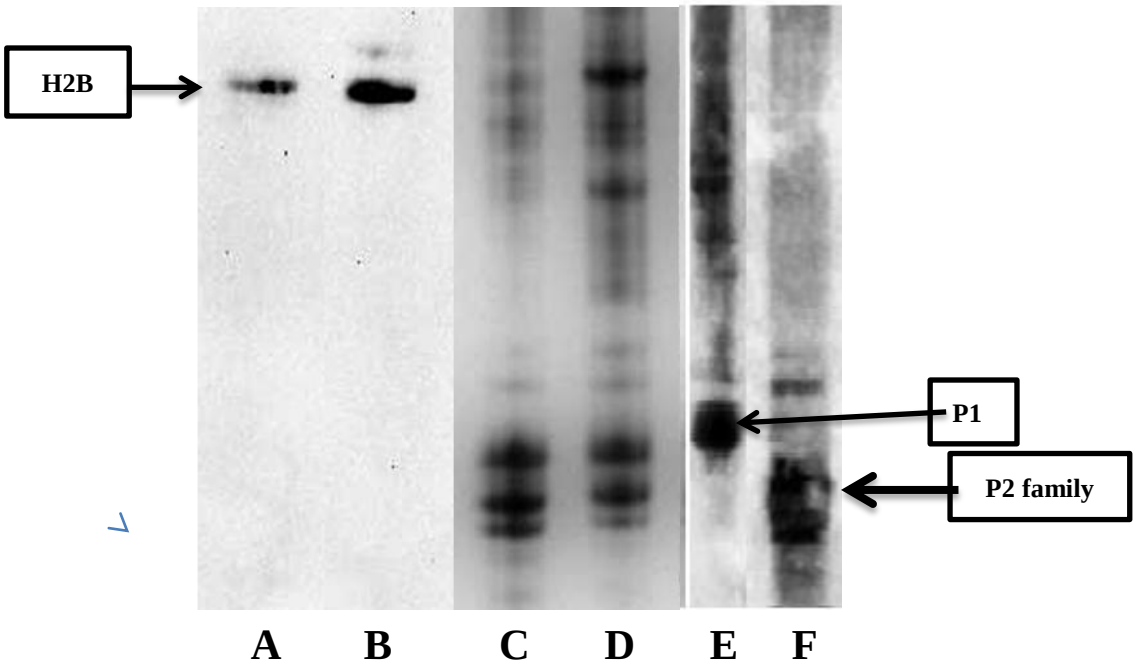


**Figure 17:** Analysis of sperm nuclear proteins; histone H2B, protamine 1 (P1), and protamine 2 (P2). (A) Western blot of sperm protein of non-smokers, corresponding to a replica of the gel shown in C, using an antibody specific for histone H2B, (B) Western blot of sperm proteins of smokers, corresponding to a replica of the gel shown in D, using an antibody specific for histone H2B (rabbit polyclonal antibody, thermo scientific), (C) Proteins extracted of sperm of nonsmoker, separated on an acetic acid-urea polyacrylamide gel and stained with Coomassie blue, (D) Proteins extracted from the sperm of smokers, separated on an acetic acid-urea polyacrylamide gel and stained with Coomassie blue, (E) Western blot for protamine P1, using an antibody specific for protamine P1 (mouse polyclonal antibody, lawrence livermore national laboratory), (F) Western blot for protamine P2, using an antibody specific for protamine P2 (mouse polyclonal antibody, lawrence livermore national laboratory).

Sperm nuclear protein concentrations (H2A, H2B, H3, P1 and P2) of all patients were analyzed by acid-urea polyacrylamide gel electrophoresis, as described in material and methods (section, 3-2-7-6). Now, these data allows the analysis of sperm nuclear protein concentration for smokers and non-smokers. According to these results shown in Table 3, smoking has an adverse effect on sperm nuclear protein. Such as, the mean concentration of histone H2A, H2B and H3 in the sperm of smokers were significantly higher ($P < 0.05$) in comparison to that of non-smokers (219.8 ± 16.35, 292.27 ± 58.24, 225.6 ± 19.76 vs. 156.4 ± 12.6, 109.1 ± 43.70, 151.2 ± 24.06, respectively) (Table 3). In addition, the P1/P2

ratio was higher in smokers than in non-smokers (1.21 ± 0.03 vs. 1.04 ± 0.02, P < 0.05) (Table 3). While, the mean of protamine P2 concentration was significantly lower (P < 0.05) in smokers than in non-smokers (354.9 ± 11.28 vs. 412.8 ± 16.24) (Table 3). In contrast, the protamine P1 concentration showed no significant difference between smokers and non-smokers group (425.6 ± 14.56 vs. 412.8 ± 16.24, P > 0.05) (Table 3).

**Table 3:** Comparison of protein analysis parameters for all participants including smokers and non-smokers.

| **Parameters** | **All Samples** | **Smokers** | **Non-smokers** | ***P-value*** |
|---|---|---|---|---|
| **H2A (ng/$10^6$ sperm)** | 194.5 ± 11.54 | 219.8 ± 16.35 | 156.4 ± 12.6 | **0.004** |
| **H2B (ng/$10^6$ sperm)** | 227.81 ± 103.07 | 292.27 ± 58.24 | 109.1 ± 43.70 | **0.000** |
| **H3 (ng/$10^6$ sperm)** | 150.7 ± 16.36 | 225.6 ± 19.76 | 151.2 ± 24.06 | **0.019** |
| **P1 (ng/$10^6$ sperm)** | 425.7 ± 10.82 | 425.6 ± 14.56 | 425.8 ± 16.26 | 0.875 |
| **P2 (ng/$10^6$ sperm)** | 378.1 ± 9.87 | 354.9 ± 11.28 | 412.8 ± 16.24 | **0.011** |
| **P1/P2 ratio** | 1.14 ± 0.02 | 1.21 ± 0.03 | 1.04 ± 0.02 | **0.000** |

Bold data in the tables represent the values that show significantly between parameters
P < 0.05 significant. P < 0.001 highly significant.

Furthermore, we considered the relation between protamine deficiency, DNA fragmentation, malondialdehyde and cotinine concentration with other semen parameters such as semen volume, sperm concentration, motility, vitality and sperm membrane integrity. In order to find correlations between sperm nuclear protein (protamine deficiency and DNA), malondialdehyde and cotinine concentration in seminal plasma and semen sperm parameters. To answer this question, table 4 illustrated the correlation coefficients between all parameters of sperm quality of all participant (n = 75). A significantly negative correlation was found between protamine deficiency (aniline blue) and sperm concentration (r = -0.281, P = 0.015), sperm motility (r = -0.415, P = 0.0002), sperm vitality (eosin test) (r = -0.366, P = 0.001), and sperm membrane integrity (hos test) (r = -0.282, P = 0.014). In addition, a significantly negative correlation was found for protamine deficiency (CMA3 test) with sperm concentration, and sperm vitality (r = -0.356, P =

0.008; r = -0.400, P = 0.002, respectively). Also a negative correlation, but non-significant, was found for protamine deficiency (CMA3 test) with sperm motility and sperm membrane integrity (r = -249, P = 0.069; r = -0.239, P = 0.081, respectively). Sperm DNA fragmentation showed significant negative correlations ($P < 0.05$) with sperm vitality (r = -0.338), and sperm membrane integrity (r = -0.294), but a non-significant ($P > 0.05$) negative correlation was shown for sperm DNA fragmentation, sperm concentration (r = -0.203) and sperm motility(r = -0.207). In addition, the concentrations of MDA (µM) showed negative a significant correlation with sperm count (r = - 0.461, P = 0.0001), motility (r = -0.309, P = 0.023), vitality (r = -0.279, P = 0.041), and membrane integrity (r = -0.463, P = 0.0001). Furthermore, significantly negative correlation was demonstrated between cotinine concentration with sperm concentration, sperm motility, sperm vitality and sperm membrane integrity (r = -0.301, P = 0.027; r = -0.334, P = 0.014; r = -0.430, P = 0.001; r = -0.485, P = 0.000, respectively).

**Table 4:** Correlation coefficient of sperm parameters of all participant (n=75).

| samples | | protamine deficiency (AB) | protamine deficiency (CMA3) | DNA fragmentation (TUNEL) | malondialdehyde (MDA) | cotinine |
|---|---|---|---|---|---|---|
| **age** | R | 0.316 | 0.023 | -0.008 | -0.182 | -0.129 |
| | P | 0.005 | 0.868 | 0.951 | 0.189 | 0.353 |
| **volume** | R | -0.012 | -0.091 | -0.005 | 0.18 | -0.149 |
| | P | 0.918 | 0.512 | 0.969 | 0.900 | 0.283 |
| **count** | R | **-0.281** | **-0.356** | -0.203 | **-0.461** | **-0.301** |
| | P | **0.015**$^{**}$ | **0.008**$^{**}$ | 0.139 | **0.000**$^{***}$ | **0.027**$^{*}$ |
| **motility** | R | **-0.415** | -0.249 | -0.207 | **-0.309** | **-0.334** |
| | P | **0.0002**$^{***}$ | 0.069 | 0.132 | **0.023**$^{*}$ | **0.014**$^{*}$ |
| **sperm vitality (eosin)** | R | **-0.366** | **-0.400** | **-0.338** | **-0.279** | **-0.430** |
| | P | **0.001**$^{**}$ | **0.002**$^{**}$ | **0.012**$^{*}$ | **0.041**$^{*}$ | **0.001**$^{**}$ |
| **membrane integrity (hos)** | R | **-0.282** | -0.239 | **-0.294** | **-0.463** | **-0.485** |
| | P | **0.014**$^{*}$ | 0.081 | **0.030**$^{*}$ | **0.000**$^{***}$ | **0.000**$^{***}$ |

Bold data in the tables represent the values that show significantly between parameters
R represent spearman rank correlation
P represent P-value
*represent significant and P-value (0.05 > P > 0.001)
***represent highly significant and P-value p < 0.001

Table 5 illustrates the relation between protamine deficiency (aniline blue and CMA3 test) with DNA fragmentation (TUNEL test) and cotinine concentration. It shows that protamine deficiency (aniline blue) positively correlated with cotinine concentration (r = 0.304, P = 0.025), while a non-significant positive correlation between protamine deficiency (aniline blue test) with protamine deficiency (CMA3 test) and DNA

fragmentation (TUNEL test) (r = 0.207, P = 0.132; r = 0.240, P = 0.080, respectively) was found. Moreover, protamine deficiency (CMA3 test) correlated significantly (P < 0.05) with DNA fragmentation (TUNEL test) (r = 0.859), and cotinine concentration (r = 0.716). There was a non-significant positive correlation between protamine deficiency (CMA3 test) and protamine deficiency (aniline blue test) (r = 0.207, P = 0.132). The DNA fragmentation (TUNEL test) showed a significant positive correlation with protamine deficiency (CMA3 test) and cotinine concentration (r = 0.859, P = 0.0001; r = 0.667, P = 0.0001 respectively). While a non-significant positive correlation was found between DNA fragmentation (TUNEL test) and protamine deficiency (aniline blue test) (r = 0.240, P = 0.080).

**Table 5:** Correlation coefficient of sperm parameters of all participant (n = 75).

| **parameters** | | **protamine deficiency (AB)** | **protamine deficiency (CMA3)** | **DNA fragmentation (TUNEL)** | **cotinine** |
|---|---|---|---|---|---|
| **protamine deficiency (AB)** | R | 1.00 | 0.207 | 0.240 | **0.304** |
| | P | - | 0.132 | 0.080 | **0.025*** |
| **protamine deficiency (CMA3)** | R | 0.207 | 1.00 | **0.859** | **0.716** |
| | P | 0.132 | - | **0.0001***** | **0.0001***** |
| **DNA fragmentation (TUNEL)** | R | 0.240 | **0.859** | 1.00 | **0.667** |
| | P | 0.080 | **0.0001***** | - | **0.0001***** |
| **cotinine** | R | **0.304** | **0.716** | **0.667** | 1.00 |
| | P | **0.025*** | **0.0001***** | **0.0001***** | - |

Bold data in the tables represent the values that show significantly between parameters
R represent spearman rank correlation, P represent P-value
*represent significant and P-value (0.05 > P > 0.001)
***represent highly significant and P-value P < 0.001

Also, the relationship between sperm nuclear proteins (histone and protamine) with protamine deficiency (aniline blue stain and CMA3 stain), DNA fragmentation (TUNEL assay) and cotinine concentration of spermatozoa has been demonstrated in Table 6 which shows the correlation coefficient of sperm parameters of all participant (n = 75). There was a significant positive correlation between protamine deficiency (aniline blue test) with histone H2A, H3, and the P1/P2 ratio ($r = 0.551$, $P = 0.0001$; $r = 0.431$, $P = 0.0001$; $r = 0.354$, $P = 0.001$ respectively) (Fig.18, 19, 21). But a non-significant negative correlation for protamine deficiency (aniline blue test) to protamine P2 ($r = -0.147$, $P = 0.205$) was found (Fig. 20).

Moreover, Table 6 shows that the protamine deficiency (CMA3 test) ($P < 0.05$) positively correlated with histone H2A ($r = 0.327$), H3 ($r = 0.307$), and P1/P2 ratio ($r = 0.450$). While a non-significant negative correlation between protamine deficiency (CMA3 test) and protamine P2 ($r = -0.264$, $P = 0.053$) was found. Likewise, a significant positive correlation ($P < 0.05$) was found for DNA fragmentation (TUNEL test) and histone H2A ($r = 0.313$), histone H3 ($r = 0.296$) (Figs. 22, 23) and the P1/P2 ratio ($r = 0.327$) (Table 6). There was a negative correlation between DNA fragmentation (TUNEL test) and protamine P2 ($r = -0.199$, $P = 0.147$) (Table 6), which mean that the sperm is not completely condensed and is fragile for other noxis. In contrast, there was no significant correlation between DNA fragmentations (TUNEL test) and protamine P1 level ($r = 0.039$, $P = 0.778$) (Table 6). However, cotinine concentration showed a significant positive correlation with histone H2A ($r = 0.322$, $P = 0.017$) (Fig. 24) and the P1/P2 ratio ($r = 306$, $P = 0.024$) (Table 6). There was a significant negative correlation between cotinine concentration and protamine P2 ($r = -.287$, $P = 0.035$). There was no significant ($P > 0.05$) correlation observed for cotinine concentration and histone H3 and protamine P1 ($r = 0.221$, $r = -0.027$ respectively). In addition, Figures 25 and 26 demonstrate that the levels of histone H2B were significantly positively correlated with levels of MDA (µM) ($r = 0.607$, $P = 0.0001$) and the smoking marker cotinine ($r = 0.592$; $P = 0.001$).

**Table 6:** Correlation coefficient of sperm parameters of all participant (n = 75).

| parameters | | protamine deficiency (AB) | protamine deficiency (CMA3) | DNA fragmentation (TUNEL) | cotinine |
|---|---|---|---|---|---|
| **H2A** | R | **0.551** | **0.327** | **0.313** | **0.322** |
| | P | **0.000**$^{***}$ | **0.015**$^{*}$ | **0.021**$^{*}$ | **0.017**$^{*}$ |
| **H3** | R | **0.431** | **0.307** | **0.296** | 0.221 |
| | P | **0.0001**$^{***}$ | **0.023**$^{*}$ | **0.029**$^{*}$ | 0.108 |
| **P1** | R | 0.094 | 0.022 | 0.039 | -0.027 |
| | P | 0.420 | 0.872 | 0.778 | 0.845 |
| **P2** | R | -0.147 | -0.264 | -0.199 | **-0.287** |
| | P | 0.205 | 0.053 | 0.147 | **0.035*** |
| **P1/P2 ratio** | R | **0.354** | **0.450** | **0.327** | **0.306** |
| | P | **0.001**** | **0.000***** | **0.015*** | **0.024*** |

Bold data in the tables represent the values that show significantly between parameters
R represent spearman rank correlation
P represent P-value
*represent significant and P-value (0.05 > P > 0.001)
***represent highly significant and P-value P < 0.001

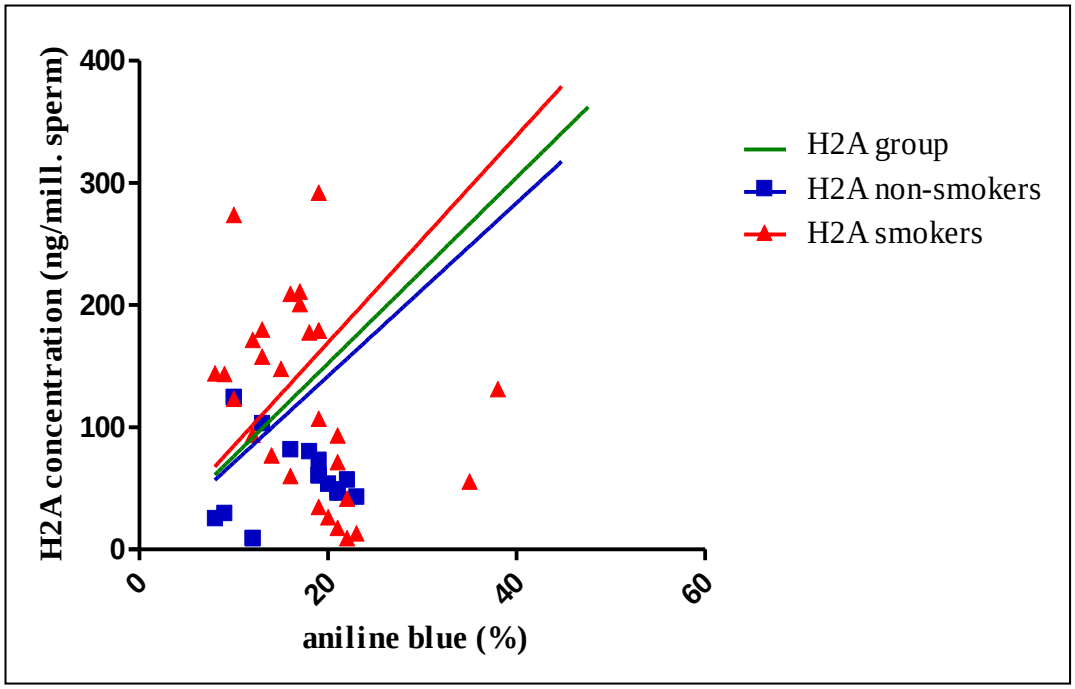


**Figure 18:** Correlation between protamine deficiency (aniline blue) and histone H2A concentration (ng/mill.sperm). Scatter plot of correlation between protamine deficiency (aniline blue) and histone H2A concentration in the spermatozoa of smokers, non-smokers and total population. In both groups, significant positive correlation were demonstrated (P = 0.0001).

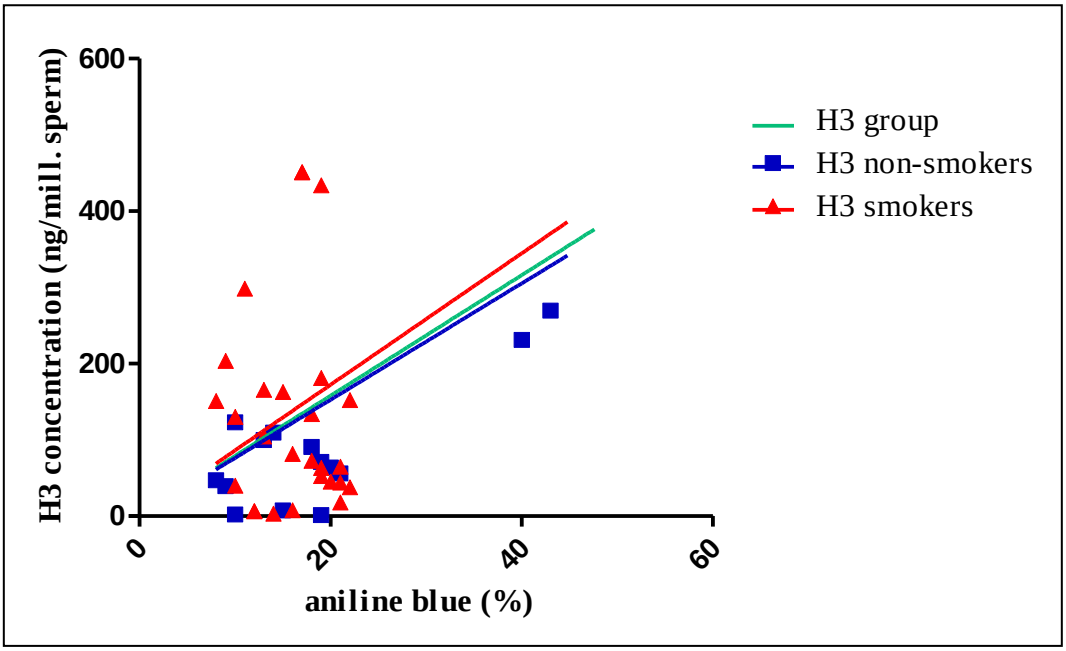


**Figure 19:** Correlation between protamine deficiency (aniline blue test) and histone H3 concentration (ng/mill. sperm). Scatter plot of correlation between protamine deficiency (aniline blue test) and histone H3 concentration (ng/mill.sperm) in the spermatozoa of smokers, non-smokers and total population. In both groups, significant positive correlation were demonstrated (P = 0.0001).

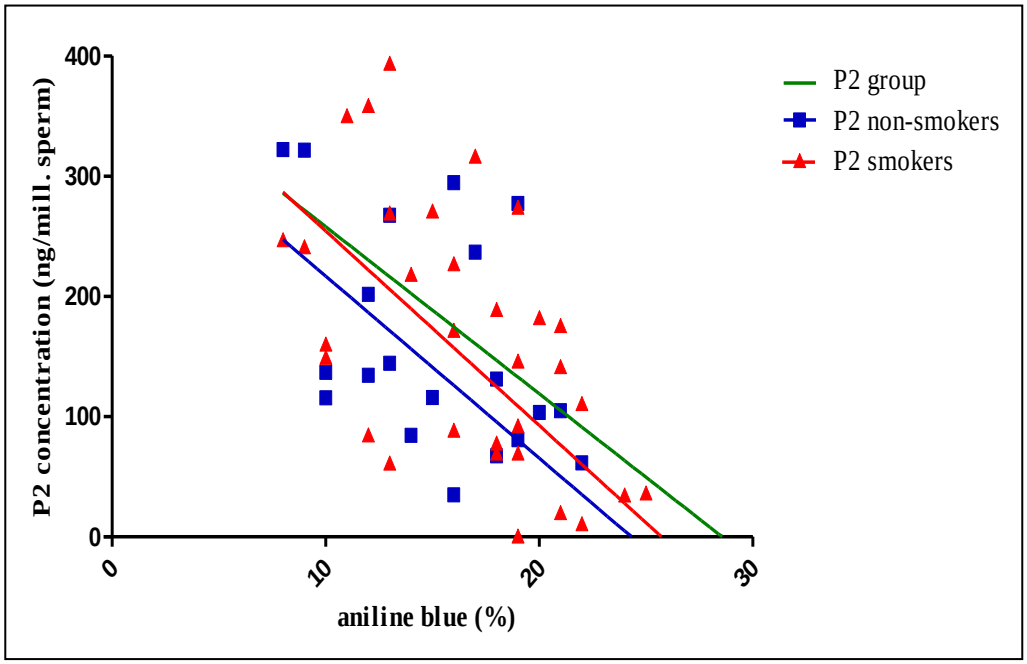


**Figure 20:** Correlation between protamine deficiency (aniline blue test) and protamine P2 concentration (ng/mill. sperm). Scatter plot of the correlation between protamine deficiency (aniline blue test) and protamine P2 concentration (ng/mill. sperm) in the spermatozoa of smokers, non-smokers and total population. In both groups, non-significant negative correlation were demonstrated (r = -0.147, P = 0.205).

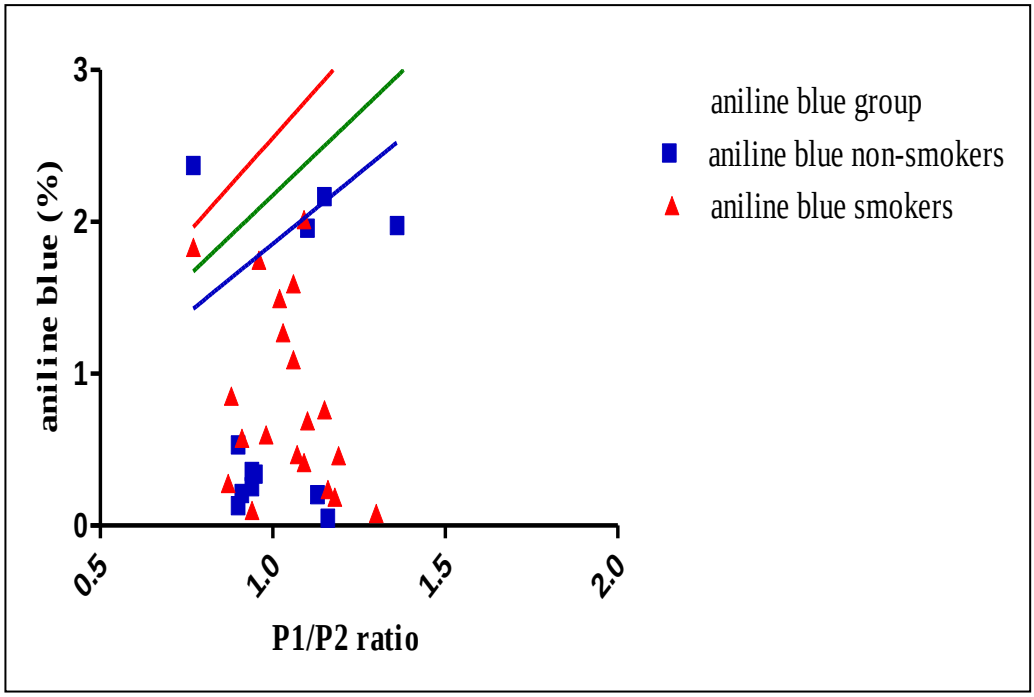


**Figure 21:** Correlation between protamine deficiency (aniline blue test) and the P1/P2 ratio. Scatter plot of the correlation between protamine deficiency (aniline blue test) and the P1/P2 ratio in the spermatozoa of smokers, non-smokers and total population. In both groups, significant positive correlation were demonstrated (P = 0.017).

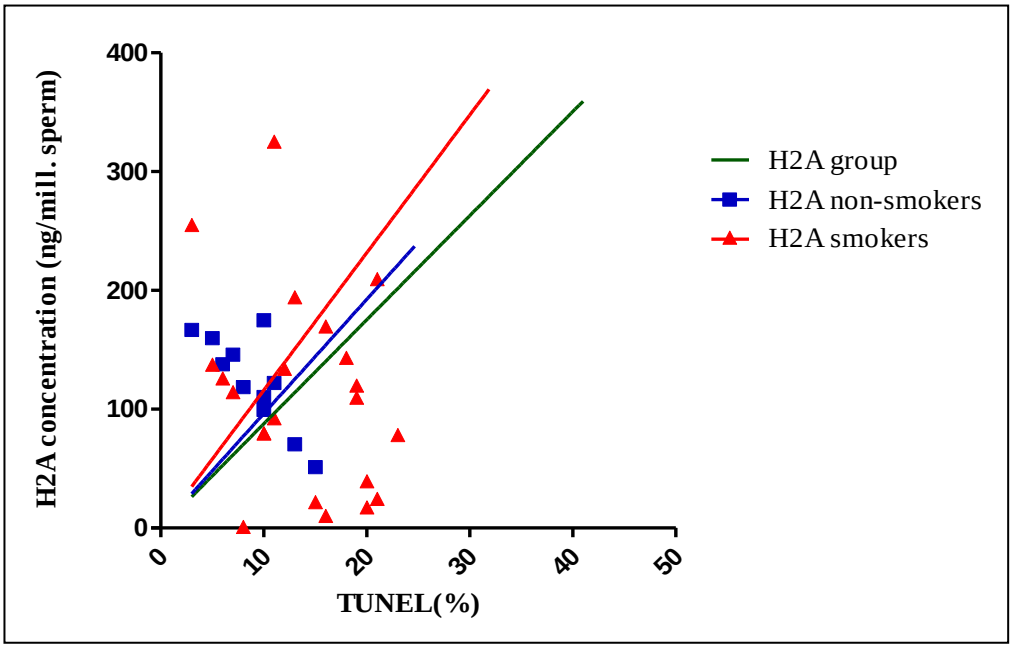


**Figure 22:** Correlation between DNA fragmentation (TUNEL test) and histone H2A concentration (ng/mill. sperm). Scatter plot of the correlation between DNA fragmentation and histone H2A concentration (ng/mill. sperm) in the spermatozoa of smokers, non-smokers and total population. In both groups, significant positive correlation were demonstrated (P = 0.021).

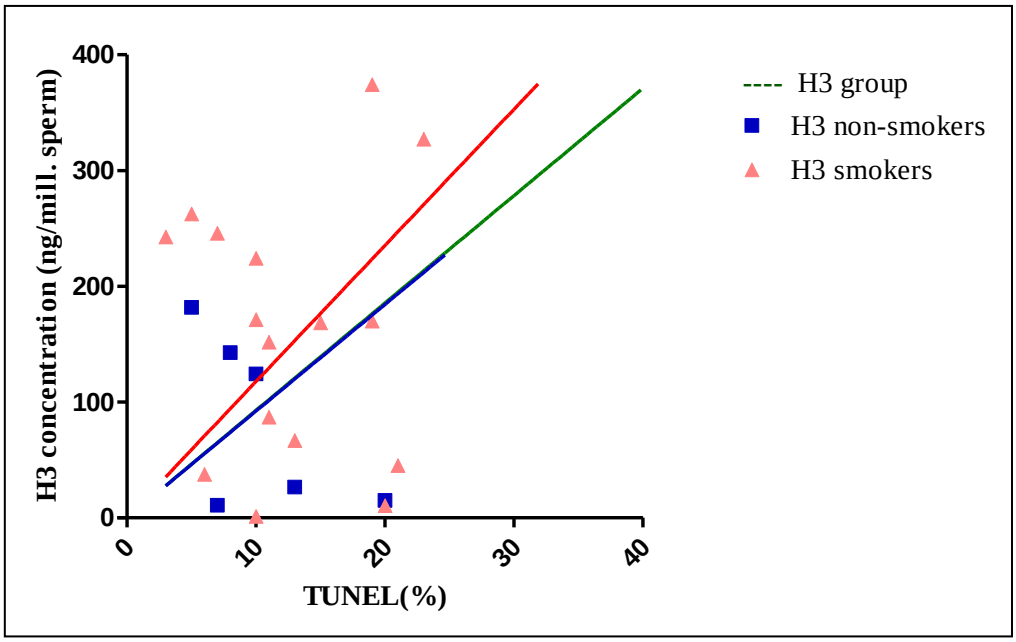


**Figure 23:** Correlation between DNA fragmentation (TUNEL test) and histone H3 concentration (ng/mill. sperm). Scatter plot of the correlation between DNA fragmentation and histone H3 concentration (ng/mill. sperm) in the spermatozoa of smokers, non-smokers and total population. In both groups, significant positive correlation were demonstrated (P = 0.029).

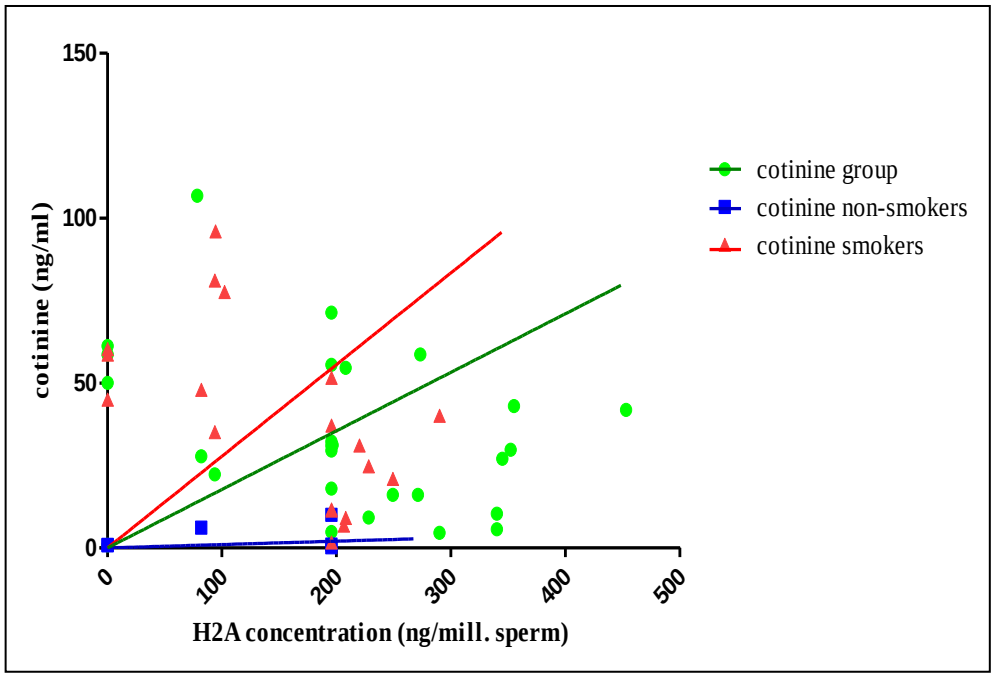


**Figure 24:** Correlation between histone H2A concentration (ng/mill. sperm) and cotinine concentration. Scatter plot of the correlation between histone H2A concentration (ng/mill. sperm) and cotinine concentration in the spermatozoa of smokers, non-smokers and total population. In both groups, significant positive correlation were demonstrated (P = 0.017).

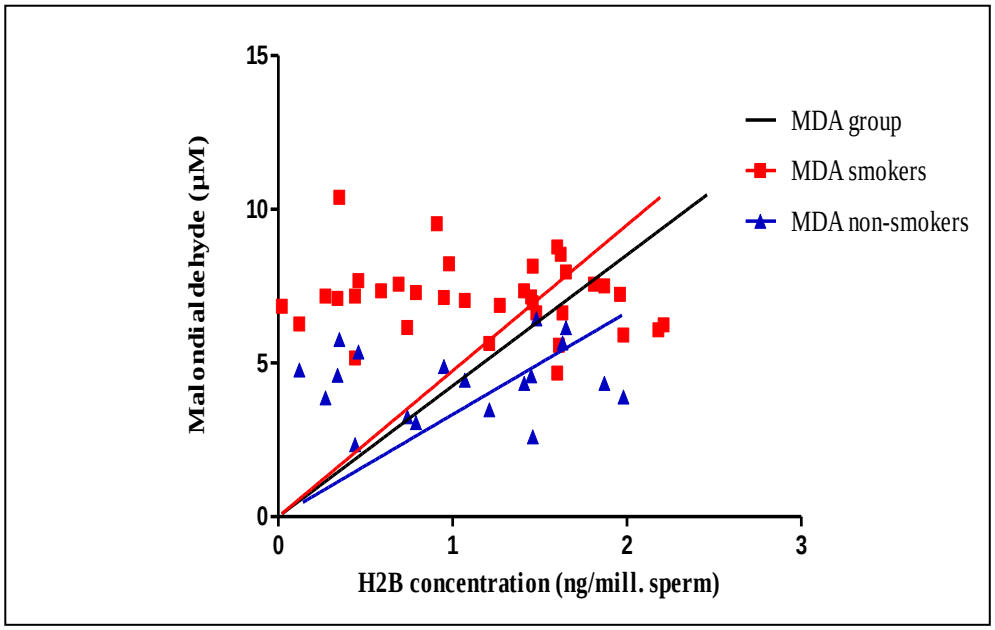


**Figure 25**: Scatter plot of the correlation between malondialdehyde concentration (µM) in seminal plasma and the concentration of histone H2B concentration (ng/mill. sperm) in the sperm of smokers, non-smokers and total population. In both groups, significant correlations were illustrated (P = 0.0001).

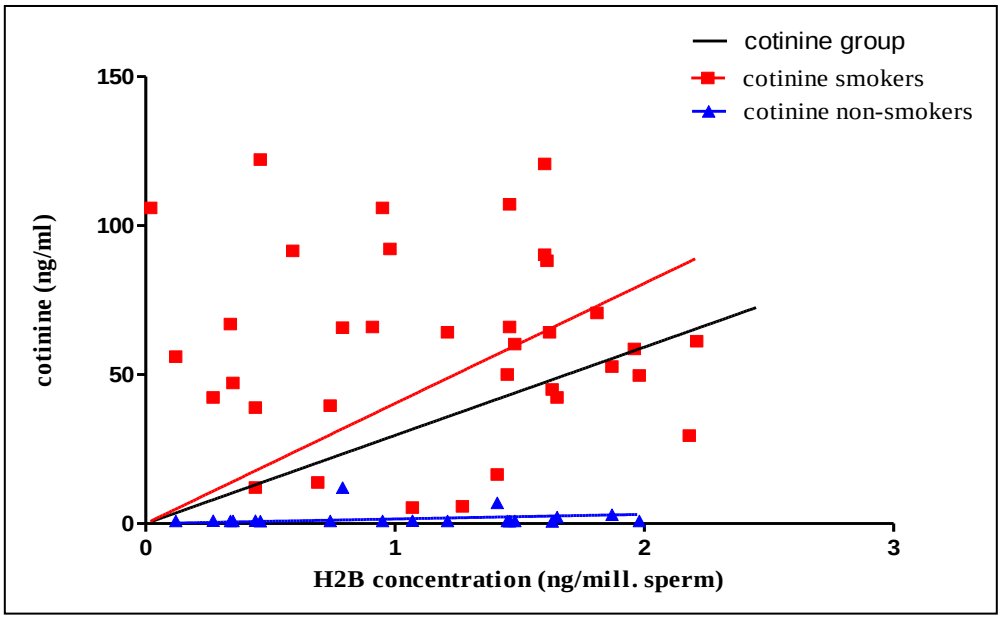


**Figure 26**: Scatter plot of the correlation between cotinine levels (ng/ml) in seminal plasma of smokers and non-smokers and the concentration of histone H2B (ng/mill. sperm) in the sperm of smokers, non-smoker and total population. In both groups, significant correlations were illustrated (r = 0.607; P = 0.0001).

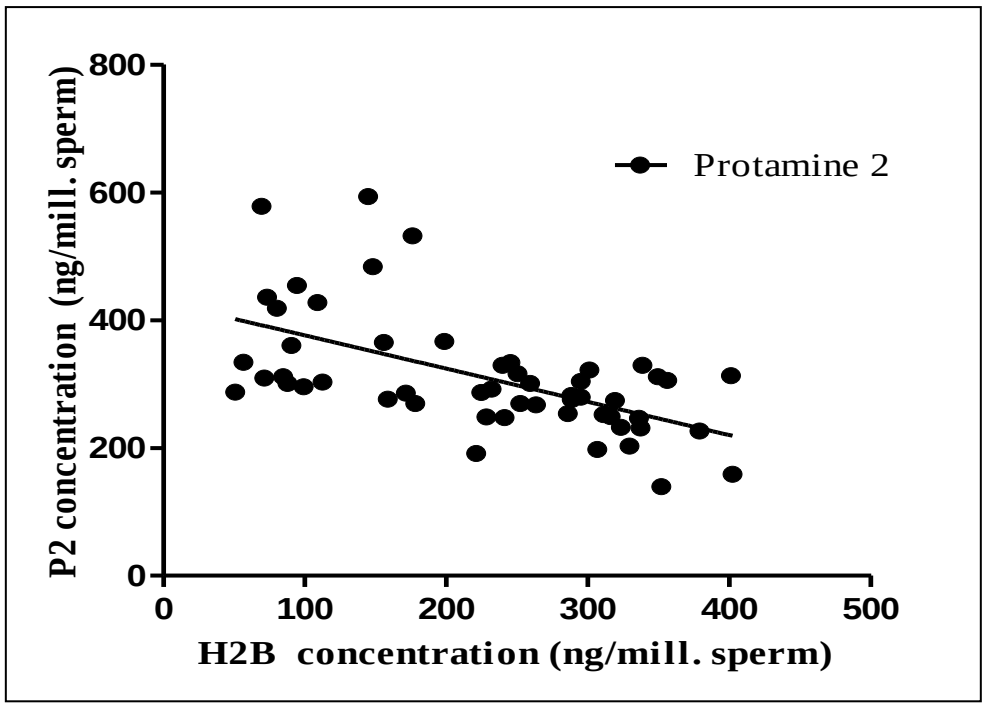


**Figure 27**: Scatter plot of the correlation between concentrations of protamine 2 (ng/mill. sperm) and the concentration of histone H2B (ng/mill. sperm) in the sperm of all participants (smokers and non-smokers). In both groups, significant negative correlations were illustrated (r = -0. 537, P = 0.000).

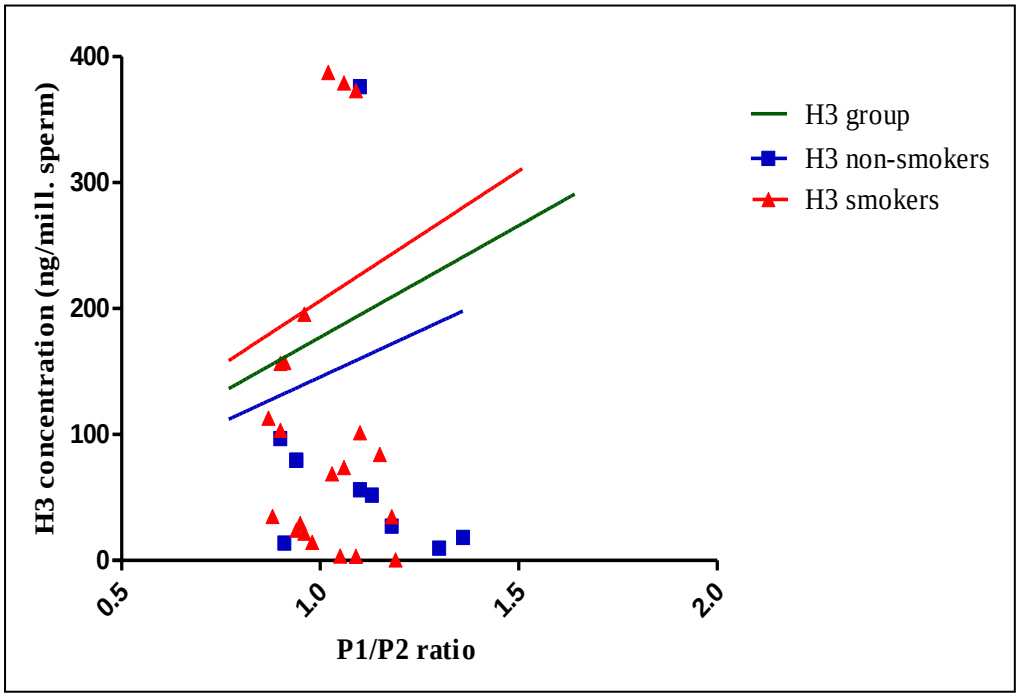


**Figure 28:** Correlation between histone H3 concentration (ng/mill. sperm) and the P1/P2 ratio. Scatter plot of the correlation between histone H3 concentration and the P1/P2 ratio in the spermatozoa of smokers, non-smokers and total population. In both groups, significant positive correlation were demonstrated (P = 0.004).

It was important to investigate the correlation between sperm nuclear protein concentrations (histone and protamine) and other semen parameters such as semen volume, sperm count, sperm motility, sperm vitality and sperm membrane integrity because the sperm protein levels could be used as a predicative factor for sperm analysis and subsequent fertility. Thus, I have addressed the question whether there is a correlation between sperm nuclear protein (histone and protamine) contents and semen sperm parameters. To answer this question, Table 7 demonstrated that there are adverse correlations ($P < 0.05$) between histone H2A and sperm motility, sperm vitality (eosin test), and sperm membrane integrity (hos test) ($r = -0.389$, $r = -0.496$, $r = -0.232$ respectively), while non-significant ($P > 0.05$) negative correlations were found for histone H2A and sperm count ($r = -0.215$). A significant ($P < 0.05$) negative correlation was observed for histone H2B and sperm count and sperm membrane integrity (hos test) ($r = -0.403$, $r = -0.456$ respectively). In addition, significant negative correlations ($P < 0.05$) were found between histone H3 and semen volume, sperm count, sperm motility, sperm vitality (eosin test), and sperm membrane integrity (hos test) ($r = -0.353$, $r = -0.371$, $r = -$

0.297, r = -0.455, r = -0.384 respectively). Likewise, a significant negative correlation (P < 0.05) was found for the P1/P2 ratio and sperm vitality (eosin test) and sperm membrane integrity (hos test) (r = -0.290, r = -0.233 respectively). A significant correlation was shown for protamine P2 and sperm membrane integrity (hos test) (r = 0.256, P = 0.026). While other parameters showed a non-significant correlation.

**Table 7:** Correlation coefficient of sperm parameters of all participant (n = 75).

| parameters | | H2A | H2B | H3 | P1 | P2 | P1/P2 ratio |
|---|---|---|---|---|---|---|---|
| **age** | R | 0.141 | **-0.307** | 0.057 | 0.164 | 0.165 | -0.038 |
| | P | 0.229 | **0.024**$^{*}$ | 0.622 | 0.157 | 0.154 | 0.740 |
| **volume** | R | -0.078 | -0.006 | **-0.353** | -0.131 | -0.062 | -0.066 |
| | P | 0.501 | 0.965 | **0.002**$^{**}$ | 0.261 | 0.592 | 0.573 |
| **count** | R | -0.215 | **-0.403** | **-0.371** | -0.017 | 0.065 | -0.204 |
| | P | 0.064 | **0.003**$^{**}$ | **0.001**$^{**}$ | 0.878 | 0.574 | 0.078 |
| **motility** | R | **-0.389** | -0.213 | **-0.297** | -0.013 | 0.022 | -0.148 |
| | P | **0.000**$^{***}$ | 0.122 | **0.009**$^{**}$ | 0.909 | 0.846 | 0.203 |
| **sperm vitality (eosin)** | R | **-0.496** | -0.199 | **-0.455** | -0.131 | 0.054 | **-0.290** |
| | P | **0.000**$^{***}$ | 0.148 | **0.000**$^{***}$ | 0.261 | 0.642 | **0.011**$^{*}$ |
| **membrane integrity (hos)** | R | **-0.232** | **-0.456** | **-0.384** | 0.090 | **0.256** | **-0.233** |
| | P | **0.044**$^{*}$ | **0.001**$^{**}$ | **0.000**$^{***}$ | 0.44 | **0.026*** | **0.04**$^{*}$ |

Bold data in the tables represent the values that show significantly between parameters
R represent spearman rank correlation
P represent P-value
*represent significant and P-value (0.05 > P > 0.001)
***represent highly significant and P-value P < 0.001

Table 8 showed the correlation coefficient of sperm parameters of all participant (n = 75). Such as, histone H2A correlated significantly positive with histone H3, (r = 0.272, P = 0.018). In contrast, a negative correlation but non-significant (P > 0.05) was observed for histone H2A and protamine P1, P2, (r = -0.021, r = -0.157). A significant positive correlation, however, was found for histone H2B and histone H2A and the P1/P2 ratio (r = 0.278, P = 0.014, vs. r = 0.644, P = 0.000 respectively). While, a significant negative correlation was observed for H2B and P2 (r = -0.537, P = 0.000) (Fig. 27). Besides, histone H3 correlated significantly (P < 0.05) positive with histone H2A (Table 8) and the P1/P2 ratio (Fig. 28) (r = 0.272, r = 0.344 respectively). Moreover, there was a significantly (P < 0.05) positive correlation between protamine P1 with protamine P2, and the P1/P2 ratio, (r = 0.774, r = 0.314). Also, protamine P2 and the P1/P2 ratio showed a significant negative correlation (r = -0.304, P = 0.008).

**Table 8:** Correlation coefficient of sperm parameters of all participants (n = 75).

| parameters | | H2A | H2B | H3 | P1 | P2 | P1/P2 ratio |
|---|---|---|---|---|---|---|---|
| **H2A** | R | 1.00 | **0.278** | **0.272** | -0.021 | -0.157 | 0.157 |
| | P | - | **0.014*** | **0.018*** | 0.854 | 0.178 | 0.176 |
| **H2B** | R | **0.278** | 1.00 | 0.222 | 0.134 | **-0.537** | **0.644** |
| | p | **0.014*** | - | 0.106 | 0.336 | **0.000***** | **0.000***** |
| **H3** | R | **0.272** | 0.222 | 1.00 | 0.124 | -0.098 | **0.344** |
| | P | **0.018*** | 0.106 | - | 0.287 | 0.399 | **0.002**** |
| **P1** | R | -0.021 | 0.134 | 0.124 | 1.00 | **0.774** | **0.314** |
| | P | 0.854 | 0.336 | 0.287 | - | **0.000***** | **0.006**** |
| **P2** | R | -0.157 | **-0.537** | -0.098 | **0.774** | 1.00 | **-0.304** |
| | P | 0.178 | **0.000***** | 0.399 | **0.000***** | - | **0.008**** |
| **P1/P2 ratio** | R | 0.157 | **0.644** | **0.344** | **0.314** | **-0.304** | 1.00 |
| | P | 0.176 | **0.000***** | **0.002**** | **0.006**** | **0.008**** | - |

Bold data in the tables represent the values that show significantly between parameters
R represent spearman rank correlation
P represent P-value
*represent significant and P-value (0.05 > P > 0.001)
***represent highly significant and P-value P < 0.001

## 4-2- Transition protein 1 (TP1)

### 4-2-1- Sperm parameters

In the next step I wanted to know whether the amount of the transition nuclear protein TP1 can be used as a quality marker for sperm and whether this parameter would correlate to other quality markers of sperm. For these analyses I have used another group of smokers and non-smokers whose sperm parameters had to be analyzed first. All samples (18 from non-smokers and 27 from heavy smokers >20 cigarettes/day) were obtained by masturbation and collected into sterile containers. After liquefaction, semen samples for smokers and non-smokers were subjected to a conventional light microscopic semen analysis at room temperature (RT) for volume, viscosity, leucocytes, sperm count, motility, vitality and sperm membrane integrity. The question that came to mind were 1) does the age of the patients (smokers and non-smokers) correlate with semen quality? And 2) is there any relationship between cigarette smoking and sperm parameters? To answer the first question, the age of the patients showed no significant difference ($P > 0.05$) between smokers and non-smokers (Table 9). But, smoking affects sperm parameters negatively and decrease sperm parameter. Such as, the sperm concentrations (mill./ml) and the mean percentage of motile spermatozoa were significantly higher ($P < 0.01$) in non-smokers (74.61 ± 7.29 mill/ml, 42.22 ± 5.15) in comparison to smokers (43.19 ± 5.76 mill/ml, 25.56 ± 2.48% respectively) (Table 9). Table 9 also shows that the sperm of smokers had a significantly higher leucocyte concentration (2.85 ± 0.08 leucocytes/ml) than those of non-smokers (2.33 ± 0.16 leucocytes/ml). Sperm vitality was assessed in wet mount smears after supravital staining with aqueous eosin-nigrosin, as described in material and methods (section, 3-2-2). The mean percentage of vital sperm is significantly lower ($P < 0.01$) in smokers (40.37 ± 3.20%) than that of non-smokers (56.67 ± 3.77%) (Table 9). The membrane integrity of spermatozoa was measured by hypo-osmotic swelling test (hos-test), as described in material and methods (section, 3-2-3). Membrane integrity of spermatozoa is significantly lower in smokers (56.30 ± 2.07%) in comparison to non-smokers (72.22 ± 2.03%; $P < 0.01$) (Table 9).

The levels of cotinine in seminal plasma were measured using the Calbiotec cotinine direct ELISA kit, as described in material and methods (section, 3-2-10). The cotinine

concentration was significantly higher ($P < 0.01$) in smokers in comparison to non-smokers ($62.53 \pm 6.15$ vs. $2.06 \pm 0.68$) (Table 9).

CMA3 test is a fluorochrome used to detect sperms with low condensation chromatin due to protamine deficiency which is correlated with the extent of nicked DNA. Table 9 showed that the mean percentage of protamine deficiency (CMA3 test) of smokers were significantly higher ($P < 0.01$) in comparison to that of non-smokers ($36.96 \pm 2.33\%$ vs. $21.06 \pm 1.42\%$).

Malondialdehyde is a final product of lipid peroxidation (LPO) which was measured through a modified thiobarbituric acid (TBA) assay as described in material and methods (section 3-2-9). Table 9 showed that the concentration of MDA (μM) ($6.99 \pm 0.22$) was significantly ($P < 0.01$) higher in seminal plasma of smokers than for non-smokers ($4.39 \pm 0.27$) (Table 9).

**Table 9:** Sperm and seminal plasma parameters of smokers and non-smokers.

| parameters | all patients (n=45) | smokers (n=27) | non-smokers (n=18) | P-value |
|---|---|---|---|---|
| **Age (years)** | 32.84 ± 0.96 | 31.44 ± 1.23 | 34.94 ± 1.47 | 0.062 |
| **volume (mL)** | 2.86 ± 0.19 | 2.65 ± 0.22 | 3.19 ± 0.34 | 0.194 |
| **count (mill./ml)** | 55.76 ± 5.03 | 43.19 ± 5.76 | 74.61 ± 7.29 | **0.001** |
| **motility (%motile)** | 32.22 ± 2.79 | 25.56 ± 2.48 | 42.22 ± 5.15 | **0.003** |
| **sperm vitality (eosin) (%)** | 46.89 ± 2.69 | 40.37 ± 3.20 | 56.67 ± 3.77 | **0.002** |
| **membrane integrity (hos) (%)** | 62.67 ± 1.88 | 56.30 ± 2.07 | 72.22 ± 2.03 | **0.000** |
| **malondialdehyde (MDA) (µM)** | 5.95 ± 0.25 | 6.99 ± 0.22 | 4.39 ± 0.27 | **0.000** |
| **cotinine (ng/ml)** | 38.35 ± 5.78 | 62.53 ± 6.15 | 2.06 ± 0.68 | **0.000** |
| **leucocytes (leucocytes/ml)** | 2.64 ± 0.09 | 2.85 ± 0.08 | 2.33 ± 0.16 | **0.002** |
| **protamine deficiency (CMA3) (%)** | 30.60 ± 1.90 | 36.96 ± 2.33 | 21.06 ± 1.42 | **0.000** |
| **protamine 1(P1) (ng/mill. sperm)** | 393.1 ± 12.49 | 395.0 ± 16.20 | 389.4 ± 20.16 | 0.586 |
| **protamine 2 (P2) (ng/mill. sperm)** | 353.2 ± 12.13 | 336.5 ± 15.99 | 378.3 ± 17.43 | 0.059 |
| **P1/P2 ratio** | 1.13 ± 0.03 | 1.20 ± 0.03 | 1.02 ± 0.02 | **0.000** |
| **transition protein 1(TP1) (ng/mill. sperm)** | 93.63 ± 10.18 | 117.1 ± 13.72 | 58.48 ± 10.84 | **0.001** |

Bold data in the tables represent the values that show significantly between parameters
$P < 0.05$ significant. $P < 0.001$ highly significant.

### 4-2-2- Sperm nuclear protein

In order quantity the transition protein TP1 content in sperm from smokers and non-smokers it was first necessary to generate a standard curve for TP1 based on increase of concentration of TP1. A human transition protein 1 (TP1) standard was analyzed by acid-urea polyacrylamide gel electrophoresis, as described in material and methods (section, 3-

2-7-3). A standard value for transition protein TP1 was not available in the literature. Therefore, a standard curve has been established from 20 normal semen samples. A standard regression curve was formulated based on the protein concentrations and corresponding densitometry reading from individual bands (Fig. 29 A). A minimum R2 regression value of 0.90 was obtained for the gel (Fig. 29 B).

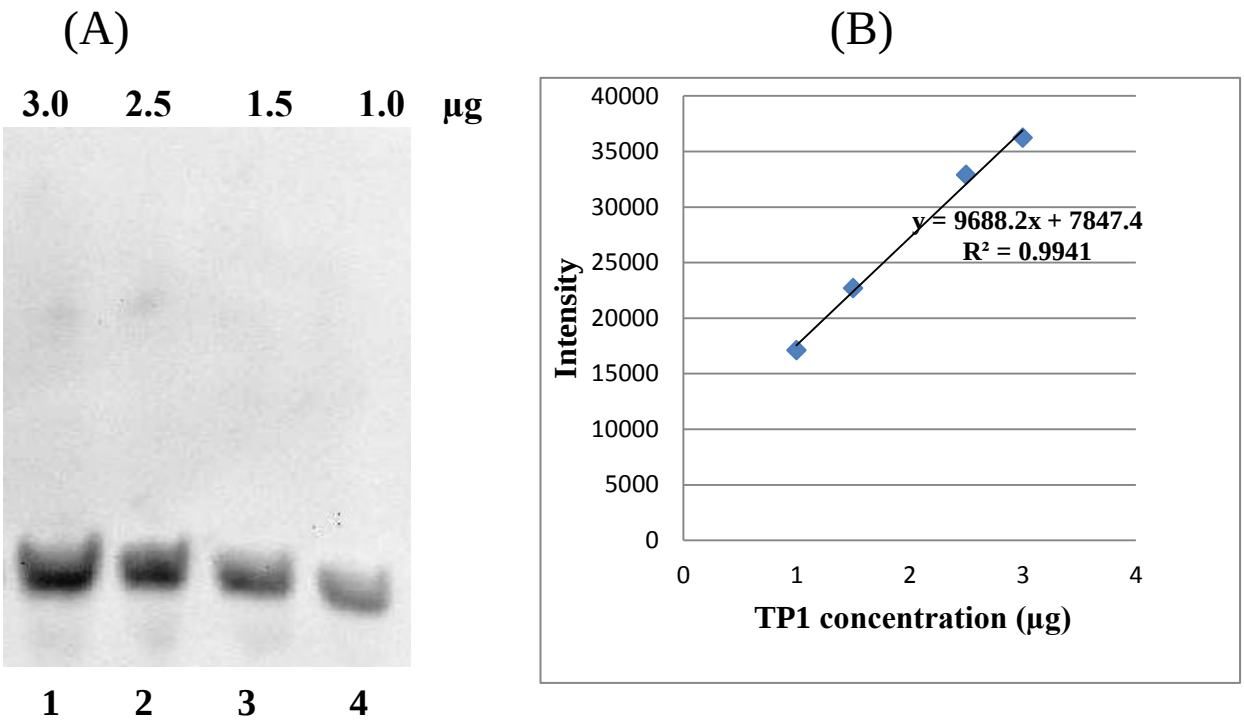


**Figure 29:** Transition protein TP1 standard.
**(A)-** Western blot analysis of recombinant transition protein TP1. Lane 1 – 4 correspond to decreasing amounts of transition protein TP1 standard included in each lane.
**(B)-** Standard curve of transition protein TP1 (1.00, 1.50, 2.50, 3.00 µg).

Based on the standard curve how I could analyze all samples form smokers and non-smokers and quantity the protein amount, Figure 30 shows an acetic acid-urea polyacrylamide gel electrophoresis of transition protein TP1. This is only an example of series of Western Blot of all sperm samples. I found that semen samples from smokers possessed higher levels of spermatozoa nuclear transition protein (TP1) than non-smokers. Figure 31 illustrates the increase in the concentration of transition protein TP1 in sperm of smokers in comparison to non-smokers.

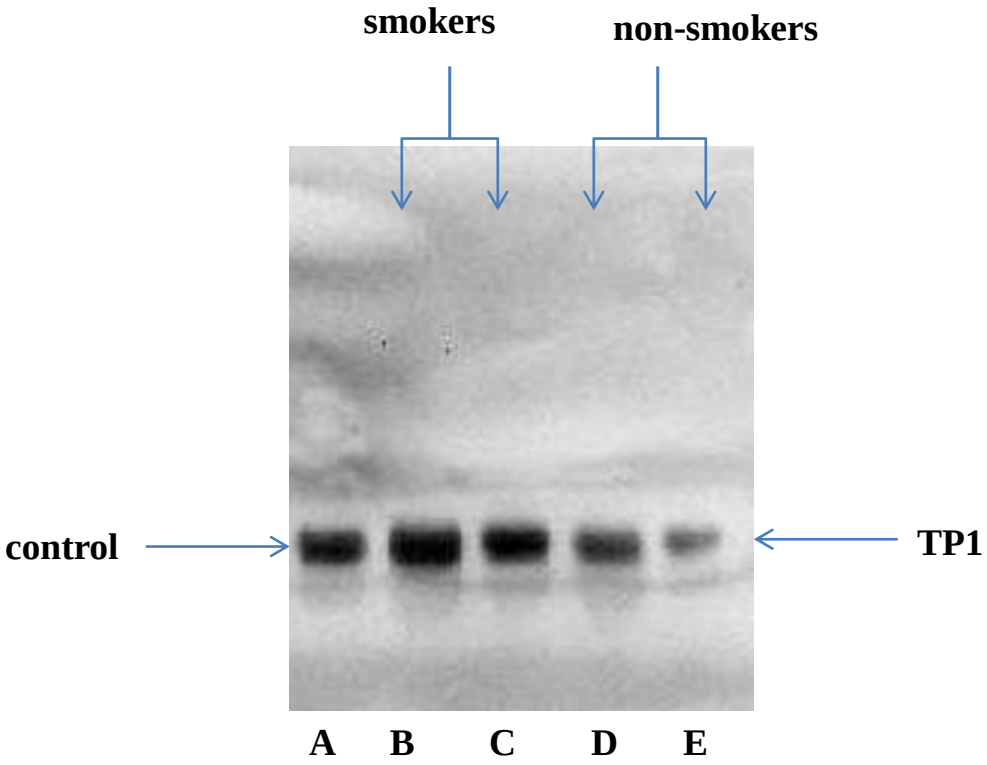


**Figure 30:** Analysis of sperm nuclear proteins; transition protein 1 (TP1). (A) Transition protein TP1 control. (B, C) Western blot for transition protein TP1 in sperm of smokers and (D, E) Western blot for transition protein TP1 in sperm of non-smoker, using an antibody specific for transition protein TP1 (mouse monoclonal antibody, sigma aldrich).

Sperm nuclear proteins concentration (transition protein and protamine) of all patients were analyzed by acid-urea polyacrylamide gel electrophoresis, as described in material and methods (section 3-2-7-6). Bands were quantified by densitometry. Are there sperm nuclear protein concentration (transition protein and protamine) difference between smokers and non-smokers? According to the results in the present study, smoking has an adverse effect on sperm nuclear protein. Figure 31 shows that the levels of transition protein TP1 in the sperm of smokers (117.1 ± 13.72) were significantly higher (P = 0.001) than that of non-smokers (58.48 ± 10.84), while there was no significant difference (P > 0.05) shown between the levels of protamine P1 and P2 in smokers and non-smokers (395.0 ± 16.20, 336.5 ± 15.99 vs. 389.4 ± 20.16, 378.3 ± 17.43, respectively) (Fig. 31).

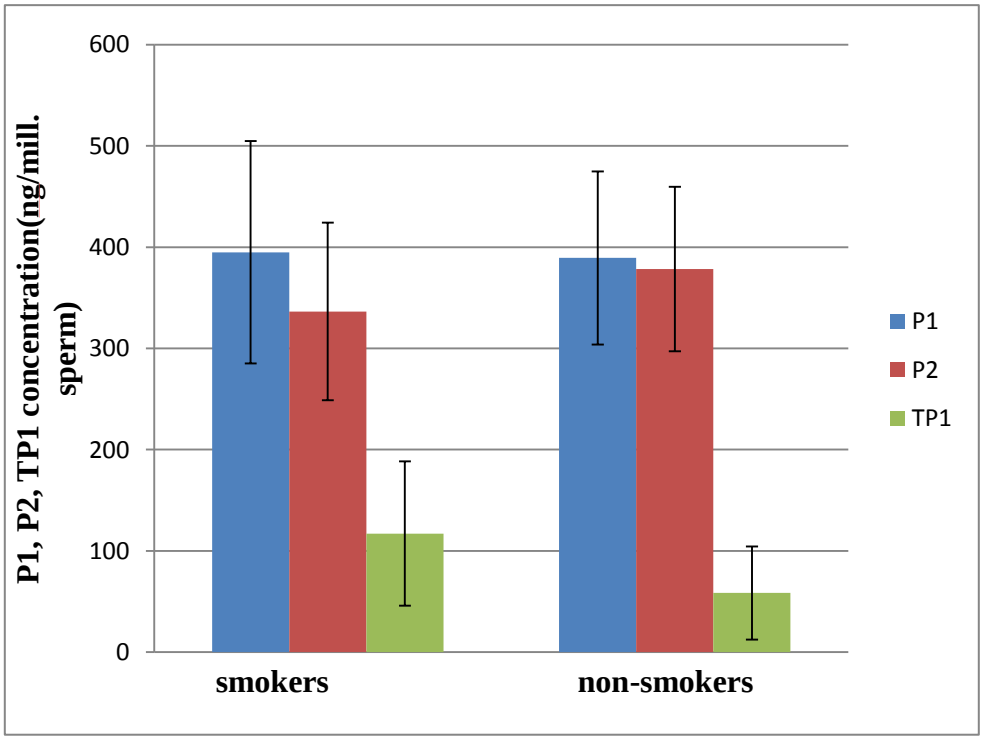


**Figure 31:** Transition protein 1(TP1), protamine 1, protamine 2 contents in the sperm of smokers and non-smokers. Protamine P1 in the sperm of smokers and nonsmokers (395.4 ± 16.20 vs. 389.4 ± 20.16, P > 0.05). Protamine P2 in the sperm of smokers and non-smokers (336.5 ± 15.99 vs. 378.3 ± 17.43, P > 0.05). Transition protein TP1 in the sperm of smokers and non-smokers (117.1 ± 13.72 vs. 58.48 ± 10.84, P < 0.05).

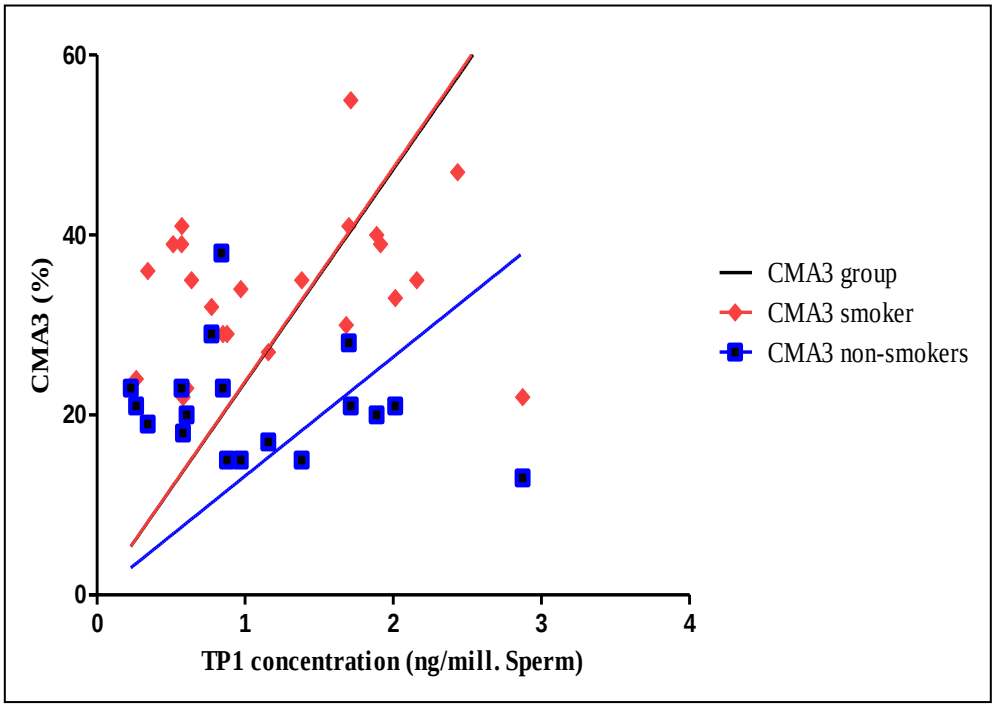


**Figure 32:** Correlation between protamine deficiency (CMA3) and transition protein TP1 concentrations. Scatter plot of the correlation between protamine deficiency (CMA3) of smokers and non-smokers and the concentration of transition protein TP1 (ng/mill. sperm) in the spermatozoa of smokers, non-smokers and total population. In both groups, significant correlation were illustrated (r = 0.481, P = 0.0008).

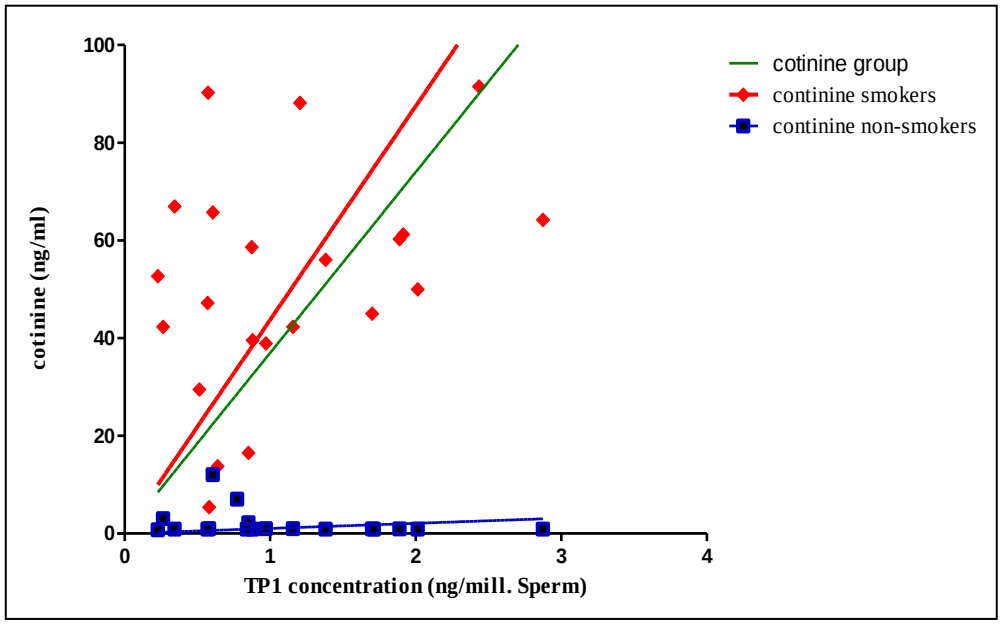


**Figure 33:** Correlation between cotinine and transition protein TP1 concentrations. Scatter plot of the correlation between cotinine levels (ng/ml) in seminal plasma of smokers and non-smokers and the concentration of transition protein TP1 (ng/mill. sperm) in the spermatozoa of smokers, non-smokers and total population. In both groups, significant correlation were illustrated (r = 0.544, P = 0.0001).

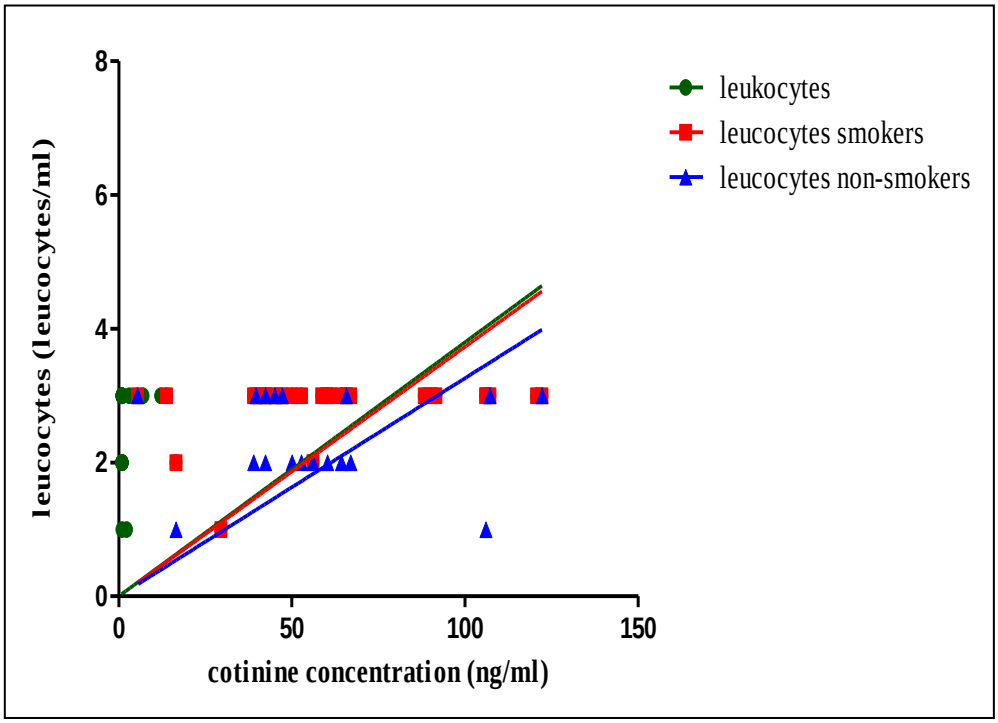


**Figure 34:** Correlation between cotinine and leucocytes concentrations. Scatter plot of the correlation between cotinine levels (ng/ml) in seminal plasma of smokers and non-smokers and the concentration of leucocytes (leucocyte/ml) in the spermatozoa of smokers, non-smokers and total population. In both groups, significant correlation were illustrated (r = 0.521, P = 0.0002).

Furthermore, another question that came in mind was, are there any correlations between sperm nuclear protein (protamine deficiency and DNA), malondialdehyde and cotinine concentration in seminal plasma and semen sperm parameters? To answer this question, table 10 illustrated that the protamine deficiency, malondialdehyde, cotinine concentration and the sperm nuclear protein concentrations (transition protein and protamine) had relationship with other semen parameters such as semen volume, sperm concentration, motility, vitality and sperm membrane integrity. For example, the concentrations of MDA (µM) indicated negative significant correlations with sperm count (r = -0.534, P = 0.0002), motility (r = -0.509, P = 0.0004), vitality (r = -0.431, P = 0.003) and membrane integrity (r = -0.597, P = 0.0001). Similarly, protamine deficiency (CMA3 test) were found to correlate negative with sperm count (r = -0.586, P = 0.0001), motility (r = -0.470, P = 0.001), vitality (r = -0.560, P = 0.0001) and membrane integrity (r = -0.435, P = 0.002). Moreover, cotinine showed a negative significant correlation with sperm count (r = -0.301, P = 0.044), motility (r = -0.454, P = 0.001), vitality (r = -0.561, P = 0.0001) and membrane

integrity (r = -0.547, P = 0.0001) (Table 10). While, Figure 34 reported that positive significant correlation between cotinine level with leucocytes concentration (leucocyte/ml) (r = 0.521, P = 0.0002).

In addition, the mean levels of transition protein TP1 showed significant negative correlations with sperm motility (r = -0.533, P = 0.0002), sperm vitality (r = -0.504, P = 0.0004) and membrane integrity (hos-test) (r = -0.491, P = 0.0006), while negative but non-significant correlations were (P > 0.05) found with sperm counts (Table 10).

The P1/P2 ratio demonstrated a significant negative correlations with sperm motility (r = -0.393, P = 0.007), and sperm vitality (r = -0.361, P = 0.014) and membrane integrity (r = -0.315, P = 0.034). While negative but no significant correlations were (P > 0.05) found with sperm concentration and sperm volume (r = -0.251, P = 0.095; r = -0.217, P = 0.150 respectively) (Table 10).

**Table 10:** Correlation of sperm and seminal plasma parameters of all samples (n = 45)

| parameters | | malondialdehyde (MDA) | cotinine | protamine deficiency (CMA3) | P1 | P2 | P1/P2 ratio | TP1 |
|---|---|---|---|---|---|---|---|---|
| **age** | R | -0.140 | -0.127 | 0.089 | 0.165 | 0.263 | -0.164 | 0.111 |
| | P | 0.358 | 0.404 | 0.558 | 0.278 | 0.08 | 0.281 | 0.465 |
| **volume** | R | -0.102 | -0.243 | -0.116 | -0.151 | 0.001 | -0.217 | -0.129 |
| | P | 0.504 | 0.106 | 0.447 | 0.321 | 0.994 | 0.150 | 0.397 |
| **count** | R | **-0.534** | **-0.301** | **-0.586** | -0.135 | -0.015 | -0.251 | -0.174 |
| | P | **0.000**$^{**}$ | **0.044**$^{*}$ | **0.000**$^{***}$ | 0.374 | 0.919 | 0.095 | 0.251 |
| **motility** | R | **-0.509** | **-0.454** | **-0.470** | -0.241 | 0.026 | **-0.393** | **-0.533** |
| | P | **0.000**$^{**}$ | **0.001**$^{**}$ | **0.001**$^{**}$ | 0.111 | 0.861 | **0.007**** | **0.000**$^{***}$ |
| **sperm vitality (eosin)** | R | **-0.431** | **-0.561** | **-0.560** | -0.270 | 0.016 | **-0.361** | **-0.504** |
| | P | **0.003**$^{**}$ | **0.000**$^{***}$ | **0.000**$^{***}$ | 0.073 | 0.911 | **0.014*** | **0.000**$^{***}$ |
| **membrane integrity (hos)** | R | **-0.597** | **-0.547** | **-0.435** | 0.095 | **0.352** | **-0.315** | **-0491** |
| | P | **0.000**$^{**}$ | **0.000**$^{***}$ | **0.002**$^{**}$ | 0.531 | **0.017**$^{*}$ | **0.034**$^{*}$ | **0.000**$^{***}$ |

Bold data in the tables represent the values that show significantly between parameters
R represent spearman rank correlation
P represent P-value
*represent significant and P-value (0.05 > P > 0.001)
***represent highly significant and P-value P < 0.001

In order to find out whether the sperm protein levels could be used as a predicative factor for sperm analysis and consequently fertility it was important to investigate the correlation among sperm nuclear protein concentrations (transition protein and protamine). To answer this question, Table 11 shows correlation of sperm and seminal plasma parameters of all samples (n = 45). The levels of transition protein TP1 were significantly positive correlated with the levels of protamine deficiency (CMA 3) (r = 0.481, P = 0.0008) (Fig. 30), oxidative stress (MDA) (r = 0.310, P = 0.038) (Table 11), and the smoking marker cotinine (r = 0.544, P = 0.0001) (Fig. 33). Also, the levels of transition protein TP1 showed

significant positive correlation with the P1/P2 ratio (r = 0.568, P = 0.000) (Table 11). In contrast, the levels of transition protein TP1 were negative correlated but not significant with levels of protamine P2 (r = -0.242, P = 0.109) (Table 11).

Moreover, significant positive correlations were found for the P1/P2 ratio and that of protamine deficiency (CMA3 test) (r = 0.503, P = 0.000), cotinine (r = 0.577, P = 0.000) and MDA concentration (r = 0.298, P = 0.046) (Table 11). In addition, the P1/P2 ratios demonstrated significantly ($P < 0.05$) positive with concentration of protamine P1 (r = 0.333). In contrast, the levels of P1/P2 ratio was significantly negatively correlated with the concentration of protamine P2 (r = -0.350, P = 0.018) (Table 11).

**Table 11:** Correlation of sperm and seminal plasma parameters of all samples (n = 45)

| parameters | | Malondial-dehyde (MDA) | cotinine | protamine deficiency (CMA3) | P1 | P2 | P1/P2 ratio | TP1 |
|---|---|---|---|---|---|---|---|---|
| **Malondial-dehyde (MDA)** | R | 1.000 | **0.630** | **0.611** | 0.184 | -0.069 | **0.298** | **0.310** |
| | P | - | **0.000**$^{***}$ | **0.000**$^{***}$ | 0.227 | 0.648 | **0.046*** | **0.038**$^{*}$ |
| **cotinine** | R | **0.630** | 1.000 | **0.699** | 0.246 | -0.167 | **0.577** | **0.544** |
| | P | **0.000**$^{***}$ | - | **0.000**$^{***}$ | 0.102 | 0.272 | **0.000***** | **0.000**$^{***}$ |
| **protamine deficiency (CMA3)** | R | **0.611** | **0.699** | 1.000 | **0.372** | 0.03 | **0.503** | **0.481** |
| | P | **0.000**$^{***}$ | **0.000**$^{***}$ | - | **0.011*** | 0.834 | **0.000***** | **0.000**$^{***}$ |
| **P1** | R | 0.184 | 0.246 | **0.372** | 1.000 | **0.72** | **0.333** | 0.182 |
| | P | 0.227 | 0.102 | **0.011*** | - | **0.000***** | **0.025*** | 0.231 |
| **P2** | R | -0.069 | -0.167 | 0.03 | **0.720** | 1.000 | **-0.350** | -0.242 |
| | P | 0.648 | 0.272 | 0.834 | **0.000***** | - | **0.018*** | 0.109 |
| **P1/P2 ratio** | R | **0.298** | **0.577** | **0.503** | **0.333** | **-0.350** | 1.000 | **0.568** |
| | P | **0.046*** | **0.000***** | **0.000***** | **0.025*** | **0.018*** | - | **0.000***** |
| **TP1** | R | **0.310** | **0.544** | **0.481** | 0.182 | -0.242 | **0.568** | 1.000 |
| | P | **0.038**$^{*}$ | **0.000**$^{***}$ | **0.000**$^{**}$ | 0.231 | 0.109 | **0.000***** | - |

Bold data in the tables represent the values that show significantly between parameters
R represent spearman rank correlation
P represent P-value
*represent significant and P-value (0.05 > P > 0.001)
***represent highly significant and p value P < 0.001

Results demonstrate that smoking has a negative impact on sperm maturity, which mean that smoking affect the histone to protamine exchange in spermatozoa and more histone and transition protein remain in sperm. Furthermore, sperm still not mature as protamine does not replace the histone to protamine completely (protamine deficiency).

To conclude, sperm nuclear protein (histone, transition protein and protamine) levels can be measured by acetic acid-urea gel electrophoresis as a predictive indicator for sperm

immaturity. Therefore, it could be recommended that, the patients should quit smoking at least 3 months before undergoing in assisted reproductive therapy, as the sperm production from spermatogonia to matures sperm in human take almost 72 days.

# 5. Discussion

## 5-1- Histone (H2B, H2A and H3):

### 5-1-1- Effect of smoking on semen quality

In recent decade studies about fertility and it relation to lifestyle and exposure to toxic environmental substances have received particular attention. Moreover, men seem more likely to suffer from these effects than women (Skakkebaek et al., 2006). The generation of reactive oxygen species (ROS) can be formed by several lifestyle factors; such as excessive smoking and alcohol consumption; and environmental factors such as toxins and radiation (Esteves, 2002; Choudhary *et al*., 2010; Gharagozloo and Aitken, 2011).

The impact of cigarette smoking on male fertility and sperm parameters remains a highly controversial issue. Smoking is recognized as a possible risk factor that impact on male fertility, sperm parameters and reproductive outcomes. A consistent number of studies have reported the deleterious effects of cigarette smoking on male fertility due to its toxic constituents; their findings proposed a substantial adverse effect on sperm production, count, motility, and morphology (Weber *et al*., 2002; Ramlau-Hansen *et al*., 2007; Hammadeh *et al*., 2010; Fawzy *et al*., 2011, Hamad *et al*., 2014). Even though, there is no definite agreement about the effects of cigarette smoking on these parameters (Anderson *et al*., 2010).

Therefore, in the present study I have analyzed the effect of smoking on sperm quality. I have shown that smokers have lower sperm count, motility, vitality, and membrane integrity than non-smokers. There was no statistically significant differences between smokers and non-smokers with regard to age or ejaculate volume. These findings are similar to studies by Hammadeh *et al*. (2010) and Künzle *et al*. (2003). They found that cigarette smoking was related to a decrease in sperm count, motility and normal morphology. Also, Gaur *et al*. (2007) found that motility was reduced in smokers. Ramlau-Hansen *et al*. (2007) demonstrated an inverse dose-response relation between smoking and semen volume, sperm count and motility. Similar, Colagar *et al*., (2007) found a deleterious effect of smoking on sperm quality. Furthermore, toxic metabolites from smoking also may affect the spermatogenesis process directly (Barratt *et al*., 2010). In a

retrospective study Collodel *et al*. (2010) evaluated the effects of cigarette smoking on sperm quality in a group of men with idiopathic infertility. The semen parameters of 2 groups of men with idiopathic infertility, smokers (n = 118) and non-smokers (n = 153), were compared. Infertile smokers and non-smokers observed similar sperm parameters, although sperm motility and sperm morphology assessed by transmission electron microscopy (TEM) analysis, values in both groups were significantly impaired in comparison to controls. Furthermore, smokers were classified as heavy (> or = 20 cigarettes/day), moderate (> 10 and < 20 cigarettes/day), or mild (> or = 1 and < or = 10 cigarettes/day) smokers. By comparison between these classes of smokers, sperm concentration in heavy smokers was significantly lower than that showed in mild smoker and non-smoker groups. Fawzy *et al*. (2011) found that Shish (water pipe) and cigarette smoking has reproductive toxic effects on semen parameters and hormone levels, they found that smoking caused a decrease in the sperm count, motility and percentage in sperms with normal morphology. Recently, Davar *et al*. (2012) found that semen parameters (morphology, motility and concentration) were lower in smokers compared to those of non-smokers. Similarly, Meri *et al*. (2013) concluded that cigarette smoking has a deleterious impact on some of the seminal fluid indicators (morphology, motility and leukocyte count) which in turn may result in male subfertility. In addition, Zhang *et al*. (2013) reported the impact of cigarette smoking on sperm parameters and detection of leukocytes within the semen in Northeastern Chinese idiopathic infertile men. They found that smokers had a significant decrease in semen volumes, sperm vitality and rapid progressive motility; moreover, smokers had a significant increase in the levels of semen leukocytes and immotile sperms. Whereas, pH and sperm concentration were not statistically significant in comparison with non- smoker controls.

In contrast, some studies found no correlation between cigarette smoking and seminal quality (Trummer *et al*., 2002; Chohan and Badawy, 2010). Another study conducted by de Jong *et al*. (2012), concluded that cigarette smoking and alcohol consumption by an asthenozoospermic and fertile populations did not appear significantly affect sperm parameters such as sperm count, volume, morphology, and motility, or pregnancy outcome.

In the present study, I found a significant increase in the mean percentages of sperm quality (sperm concentration, motility, sperm vitality, and sperm membrane integrity) in smokers in comparison with non-smokers.

**5-1-2- Effect of smoking on DNA fragmentation, and sperm protamine deficiency.**

The tobacco primary alkaloid, nicotine is an oxidant agent capable of causing sperm chromatin decondensation and DNA fragmentation (Benoff and Gilbert, 2001). Abnormality in sperm chromatin condensation can take place at several levels including 1) histone/ protamine replacement, 2) a lack of protamines, 3) epididymal maturation, and 4) maintenance of chromatin stability during ejaculation (Kazerooni *et al*., 2009). Determine of protamine deficiency by chromomycin A3 test, and evaluating of DNA fragmentation by TUNEL had been applied to find the relationship between protamine deficiency and DNA integrity (Aoki *et al*., 2006c; Hammadeh *et al*., 2001, 2010).

CMA3 is a fluorochrome used to detect cells with low condensation chromatin due to protamine deficiency which is correlated with the extent of nicked DNA (Franken *et al*., 1999). Protamines bind to the minor groove of the GC-rich sequence of DNA (Balhorn, 1982). The fluorochrome CMA3 forms an ion coordinated dimer, which binds to this minor groove of the GC-rich DNA sequence mediated by a single divalent metal ion, such as $Mg^{2+}$ (Bianchi *et al*., 1996; Hou *et al*., 2004; Simões *et al*., 2009). In mature spermatozoa, where DNA is compacted and stabilized by protamines, the GC-rich minor grooves are masked by the protamines arginine-rich sequence, and therefore, they are non-accessible to CMA3 binding. CMA3 staining is considered as a sign for protamine deficiency (Tavalaee *et al*., 2010; Fathi *et al*., 2011).

The role of proteins is not limited to protamines, which are the most abundant chromatin proteins in sperm cells (Jenkins *et al*., 2011). Histones also play a critical role for sperm chromatin condensation. Aniline blue (AB) staining is used for sperm chromatin condensation observation (Agarwal *et al*., 2009). This staining technique depends on the lysine residues with AB detection which is an indicator of an extra histone remaining bound to the sperm DNA (Dadoune *et al*., 1988; Nanassy and Carrell, 2008; Jenkins *et al*., 2011). Hammadeh *et al*., (2001) used AB staining to detect the value of sperm chromatin

condensation analysis in the male fertility assessment. The TUNEL test is a fluorescence staining used for direct detection of DNA damage in mammalian spermatozoa (Gorczyca *et al*., 1993). This TUNEL assay has usually been used to distinguish between fertile controls and infertile men (Tunc *et al*., 2009).

My results were similar to many studies reported a positive correlation between DNA fragmentation and abnormal protamination using indirect measurements of protamine deficiency using the chromomycin (Manicardi *et al*., 1995; Borini *et al*., 2006; Tarozzi., *et al*., 2009; Hammadeh *et al*., 2008, 2010; Hamad *et al*., 2014).

Another studies also investigated that, defects in protamination can be as a consequence of sperm DNA damage (Nasr-Esfahani *et al*., 2004a; Aoki *et al*., 2006c; Torregrosa *et al*., 2006; Carrell *et al*., 2007). Both sperm DNA damage and protamine deficiency are correlated with infertility (Oliva, 2006). Some studies related to male smoking, have suggested that a severe DNA damage could be a cause of infertility. For examples, Sepaniak *et al*. (2006) reported a positive correlation between cigarette smoking and sperm DNA fragmentation among smokers seeking for infertility counseling. Viloria *et al*. (2007) also reported that an increased rate of DNA fragmentation in smokers was found in pre and post-swim-up samples. In addition, Elshal *et al*. (2009) found that cigarette smoking of idiopathic infertile men caused a high percentage of DNA fragmentation index (DFI %). These findings showed that cigarette smoking may have deleterious effects on sperm nuclear DNA. Recently, Selit *et al*. (2013) pointed to an increasing percentage of abnormal sperm DNA and RNA in fertile smokers compared with fertile non-smokers; they found a similar result when they compared oligoasthenoteratozoospermia (OAT) smokers with OAT non-smokers. Increased percentage of sperm DNA and RNA damage was shown for fertile heavy smokers compared with fertile light smokers and in OAT heavy smokers compared with OAT light smokers. In addition, Talebi *et al*. (2012) investigated the relationship between sperm DNA integrity and sperm protamine deficiency determined by cytochemical assays, traditional sperm parameters, and recurrent spontaneous abortion. They found that the recurrent spontaneous abortion group had less chromatin condensation and poorer DNA integrity than fertile men with no history of recurrent spontaneous abortion. Micale *et al*., (2013) showed however, that in mice exposed to cigarette smoking,

oxidative stress and the resulting DNA damage are main sources of mice to be high susceptible in their early in life (Micale *et al*., 2013).

In the present study, the mean percentage of protamine deficiency and DNA fragmentation in smokers were significantly higher than in non-smokers. In addition, a significant positive correlation was found between DNA fragmentations (TUNEL test) and protamine deficiency (CMA3 test). Thus, tobacco smokers were linked to an increase in DNA fragmentation and protamine deficiency of sperm, which may lead to an increase in male infertility.

### 5-1-3- Correlation between DNA fragmentation, sperm protamine deficiency with semen parameters and sperm proteins.

Different studies reported that spermatozoa from patients with abnormal sperm quality (sperm count, motility, vitality and morphology) had increased levels of DNA damage (Gandini *et al*., 2000; Giwercman *et al*., 2003; Benchaib *et al*., 2003; Erenpreiss *et al*., 2006; Wyrobek *et al*., 2006). The spermatozoa of subfertility males revealed structural changes in their DNA organization such as epigenetic alterations, single or double DNA strand breaks and a wrong number of chromosomes and Y chromosome microdeletions (Seli and Sakkas, 2005). Also, Hammoud *et al*. (2011) reported that histone retention was the main reason for different histones localizations in the fertile men sperm cells in comparison with their infertile counterparts.

My present data showed a significant negative correlation for protamine deficiency (aniline blue) with sperm concentration, sperm motility, sperm vitality and sperm membrane integrity. But, a non-significant negative correlation for protamine deficiency (aniline blue) and P 2 concentration. In contrast, sperm protamine deficiency (aniline blue) was found significant correlated with histone H2A concentration, histone H3 concentration and P1/P2 ratio. In addition, a significant negative correlation was found for protamine deficiency (CMA3 test) with sperm concentration, and sperm vitality. Also, a non-significant negative correlation was found for protamine deficiency (CMA3 test) and sperm motility and sperm membrane integrity. A non-significant negative correlation was found for protamine deficiency (CMA3 test) and protamine P2 concentration. While, protamine deficiency

(CMA3 stain) was observed significantly positive correlated with histone H2A concentration, histone H3 concentration and P1/P2 ratio. Sperm DNA fragmentation is significantly negative correlated with sperm vitality, and sperm membrane integrity. Non-significant negative correlation was however, shown for sperm DNA fragmentation and sperm concentration, sperm motility and P2 concentration. In contrast, sperm DNA fragmentation (TUNEL test) was observed significantly positive correlated with histone H2A concentration, histone H3 concentration and P1/P2 ratio.

These results are in accordance with that of Franken *et al*. (1999) who reported a significantly higher percentage of aniline blue positive spermatozoa between teratozoospermia and normozoospermic. Moreover, Paradowska *et al*. (2012) showed that subfertile men with increased levels of spermatozoa stained with aniline blue, displayed qualitative alterations of histones related to acetylation sites in their sperm cells. De Oliveira *et al*. (2013) found an overall abnormal chromatin condensation associated with abnormal retention or accessibility of sperm histones, and a correlation with bull fertility. Zhang *et al*. (2006) and Zini *et al*. (2008) showed that the anomalities in the histones proportion and protamines within a spermatozoon correlated with DNA instability, and thus with vulnerability to damage (Zhang *et al*., 2006, Zini *et al*., 2008). Hammoud *et al*. (2009b) demonstrated significant negative correlation between P1/P2 ratio and the histone expression level in the various layers of a density gradient preparation.

Tarozzi *et al*. (2009) found a significant, negative correlation ($P < 0.050$) between CMA3 positivity and sperm concentration, motility and morphology. They showed a significant positive correlation between CMA3 positivity and sperm DNA fragmentation. Kazerooni *et al*. (2009) found a negative correlation for sperm morphology and protamine deficiency and a negative correlation of sperm progressive motility with protamine deficiency.

A numbers of researchers have reported a positive correlation between DNA fragmentation and abnormal seminal parameters, such as sperm concentration, motility and sperm morphology in humans and rodent models (Morris *et al*., 2002; Trisini *et al*., 2004; Spano *et al*., 2005; Delbes *et al*., 2007). Schmid *et al*. (2004) investigated infertility and oligozoospermia were correlated with structural chromosomal abnormalities (FISH for chromosome 1). Wyrobek *et al*. (2006) and Sergerie *et al*. (2007) demonstrated that men

with asthenozoospermia have significantly more sperm with DNA fragmented, and those with teratozoospermia have a higher degree of DNA damage than men with normal morphology. However, Saxena *et al*. (2008) found that a poor-quality semen is often due to chromatin packed too loosely or damaged DNA. Nanassy and Carrell (2008) also reported that DNA fragmentation was significantly higher in teratozoospermia men compared with normozoospermic men. Smit *et al*. (2010) reported a negative correlation of DNA fragmentation and sperm concentration, progressive motility, normal morphology and sperm vitality. García-Ferreyra *et al*., (2014) showed a group with DFI ≥ 30 % has poorer results of standard semen parameters; concentration, motility, morphology and vitality of sperm.

Here, I found a relationship between nuclear sperm proteins (histone and protamine) and sperm quality (sperm concentration, motility, vitality and sperm membrane integrity). In addition protamine deficiency was linked to DNA fragmentation. I concluded that abnormal retention of histone concentration in human sperm lead to abnormality of sperm chromatin condensation, and it was correlated with DNA fragmentation. Also, I found a relation between higher levels of histones retention and abnormality of chromatin condensation, which is believed to have an impact on the sperm quality (sperm concentration, motility, vitality, and sperm membrane integrity).

### 5-1-4- Effect of smoking on histone, protamine concentration and P1/P2 ratio

About15% of the nuclear sperm proteins remain as histones (Wykes and Krawetz, 2003). Many histone isoforms (H2A, H2B, H3 and H4) and isoform variants were found in mature human spermatozoa (Gatewood *et al*., 1987). The concentration of these isoform variants very in human spermatozoa indicating the presence of different populations in the human semen (Zalensky *et al*., 2002).

These histones may play an essential role for proper sperm physiology and early embryonic development (Gardiner-Garden *et al*., 1998; Gineitis *et al*., 2000; Govin *et al*., 2005). Histone protein also plays a crucial role in gene promoter site. Whole gene families, which are essential for spermiogenesis and early events of fertilization, are preferentially related to histones human spermatozoa (Ostermeier *et al*., 2005; Hammoud *et al*., 2009a;

Arpanahi *et al*., 2009). Churikov *et al*., (2004a) suggested that the H2B variant (H2BFWT) play a role in early chromatin remodeling at fertilization.

In addition to H2B, H3 and its variant replacement of H3.3 have been demonstrated to be related to gene stimulation (Mito *et al*., 2005). Specific post translational modifications (PTMs) within H3 are located in the sperm genome regions that are active in early embryonic development and spermatogenesis (Brykczynska *et al*., 2010, Steilmann *et al*., 2011). The histone H2A families play a crucial role in a number of cellular processes including chromatin structure organization in somatic and germinal cells, DNA replication and gene transcription (Eirín-López *et al*., 2005; Abbott *et al*., 2005).

Zini *et al*. (2008) reported that semen samples from asthenoteratozoospermia infertile men possess a higher percentage of spermatozoa with diffuse sperm nuclear histone H2B staining than sperm from fertile men and that the diffuse nuclear histone H2B staining is inversely related to both sperm motility and sperm concentration. Also, they suggested a positive correlation between sperm DNA damage and abnormally high histone H2B levels.

Alterations in the P1/P2 ratio are not only associated with sperm quality, but also related to decreasing in embryo quality and IVF outcome in comparison with infertile patients who had a normal P1/P2 ratio (Oliva, 2006; Aoki *et al*., 2006b; Carrell *et al*., 2007). The P1/P2 ratio in fertile men lies close to 1.0 (Balhorn *et al*., 1999; Carrell and Liu, 2001; Corzett *et al*., 2002), and ranges from 0.8 to 1.2 (Balhorn *et al*., 1999; Carrell and Liu, 2001). Any change of this ratio, in either direction, leads to poor semen quality, DNA damage upregulation, and fertility downregulation (Aoki *et al*., 2006b). Abnormal levels of specific nucleoproteins such as protamines (Simon *et al*., 2011) and histones (Zhang *et al*., 2006) in spermatozoa have been found to be associated with human subfertility.

In the present study, I have shown the level of histones H2B, H2A and H3 in smokers were significantly higher than in non-smokers. In addition, P1/P2 ratio was higher in smokers than in non-smokers. A significant negative correlation was observed for the histone H2A concentration with sperm motility, sperm vitality and sperm membrane integrity. Also negative correlation but not significant between the histone H2A concentration and protamine P1 concentration and protamine P2 concentration were found. In contrast,

significant positive correlation was found between histone H2A concentration with histone H2B concentration and histone H3 concentration, besides non-significant positive correlation between histone H2A concentrations and P1/P2 ratio. However, the present study has shown a significant negative correlation between the histone H2B concentration and sperm count and sperm membrane integrity, also a significantly negative correlation was found for histone H2B concentration and protamine P2 concentration. In contrast, the histone H2B concentration significantly positive correlated and the P1/P2 ratio was found.

Moreover, I have found a significant negative correlation of the histone H3 concentration with sperm volume, sperm count, sperm motility, sperm vitality and sperm membrane integrity. Also, a negative correlation, but not significant, was shown for the histone H3 concentration and protamine P2 concentration. In contrast, a significant positive correlation was observed for the histone H3 concentration and the P1/P2 ratio.

This study is the first attempt to correlate cigarette smoking with histone levels in patients. There was a significant increase in levels of histone H2B, H2A and H3 from smokers semen samples when compared to non-smokers controls.

These findings revealed that smokers with high sperm nuclear histones H2B, H2A, and H3 concentration had a spermiogenesis defect, in which histones were replaced by transition proteins and finally by protamines. Moreover, my data support the perception that sperm with relative increase in histones concentration H2B, H2A and H3 consequently, decrease protamine concentration particularly, protamine P2, which may have increase the P1/P2 ratio. Also, I suggested that, these changes in the spermiogenesis indicates sperm chromatin immaturity, which cause abnormal sperm quality that may have an impact on infertility.

**5-1-5- Effect of oxidative stress on the histone, protamine and P1/P2 ratio.**

One of the main causative agents of male infertility is oxidative stress (OS). High levels of reactive oxygen species (ROS) have an impact on sperm quality (motility, concentration, and morphology). Increased ROS levels have been found in infertile smoking people and concomitantly with a decrease in the level of antioxidants in seminal plasma (Soares and Melo, 2008; Pasqualotto *et al*., 2008). These parameters are the most important predictors

of an individual's potential to produce viable sperm (Kao *et al*., 2008). Oxidative damage of the sperm DNA leading to sperm abnormalities was associated with ROS (Aitken and Baker, 2004). Furthermore, Horak *et al*. (2003) showed that cigarette smoke oxidants are believed to damage sperm DNA and smokers have more damage in DNA in their sperm than non-smokers.

Moreover, some sperm DNA damages resulted from oxidative stress including; mutations, chromosome deletions, strand breaks (Agarwal *et al*., 2003; Tominga *et al*., 2004), and induction of apoptosis that ended with single- and double-stranded DNA breaks (Agarwal and Allamaneni, 2004). High ROS levels negatively affect the sperm plasma membrane integrity, which is essential for sperm motility and fertilization capacity (Velando *et al*., 2008). Also, ROS causes sperm damage by inducing lipid peroxidation (LPO) of the high levels of polyunsaturated fatty acids in the sperm membrane (Dandekar *et al*., 2002; Agarwal *et al*., 2005).

Lipid peroxidation (LPO) resulted from ROS is related to oxidation of sperm plasma membrane polyunsaturated fatty acid (PUFA) (Fraczek *et al*., 2007). This will affect sperm plasma membrane fluidity followed by loss of the ability for oocyte fusion and fertilization (Mammoto *et al*., 1996).

Malondialdehyde (MDA) is a lipid peroxidation product of polyunsaturated fatty acids that was used for lipid peroxidation diagnosis and analysis of etiology of male infertility (Laudat *et al*., 2002).

In the present study, I found that MDA concentration had a significant negative correlation with sperm count, motility, vitality and membrane integrity. In addition, MDA concentrations were positively correlated with levels of histone H2B in smokers. This result reflects the negative effect of lipid peroxidation in the replacement process of histones by protamines, which supports a study presented by Hammadeh *et al*. (2010) in which they identified significant negative associations of MDA levels with different sperm parameters and a positive relationship with the P1/P2 ratios in smokers compared to non-smokers.

### 5-1-6- Effect of cotinine on semen parameters, and histones, protamines and the P1/P2 ratio

Cotinine is the main metabolite of nicotine, the major psychoactive substance in cigarette smoke (Zenzes *et al*., 1996). It was detected in seminal plasma and used as smoking biomarker (Hammadeh *et al*., 2010). The mechanisms through which cigarette smoking might affect semen quality is based on the direct effects of toxic materials in cigarette smoke such as nicotine, carbon monoxide, carcinogens and mutagens, such as radioactive polonium, cadmium, benzo(a)pyrene, and others. These materials might inversely impact male and female gametes and embryos (Zenzes, 2000; Jurasovic *et al*., 2004; Kumosani *et al*., 2008; Thompson and Bannigan, 2008). Toxic constituents found in cigarette smoke is a reason for several negative effects in the male reproductive system by affecting seminal fluid components, volume and its liquefaction time (Gaur *et al*., 2007). Nicotine and its metabolites; cotinine and trans-3-hydroxycotinine can cross the blood-testis barrier, making seminal plasma of smokers a toxic environment for spermatozoa (Sepaniak *et al*., 2006).

The present study revealed significant higher concentrations of cotinine (smoking marker) in smokers seminal plasma in comparison to non-smokers. In our analysis, there was an inverse significant relation between cotinine and studied sperm parameters, count, motility, vitality and membrane integrity. In contrast, a significant positive correlation was observed for cotinine concentration and protamine deficiency, and DNA fragmentation.

These findings are similar to that found by Chen and Kuo (2007). They found a relation between semen cotinine concentrations and sperm quality, which was also consistent with other reports. Pacifici *et al*. (1993) demonstrated that the total motility of spermatozoa significantly negatively correlated with concentrations of cotinine and hydroxy cotinine. Zavos *et al*. (1998) investigated that smoking affected sperm viability and had a strong detrimental effect on motility of spermatozoa. Wong *et al*. (2000) showed a negative correlation between cotinine concentration and morphologically normal sperm. High cotinine concentration impaired sperm motility, membrane function, and their ability to undergo capacitation (Sofikitis *et al*., 2000). These results also were supported by Hammadeh *et al*. (2010), who showed that the cotinine concentration in seminal plasma

correlated negatively with semen parameters (count, motility, vitality and membrane integrity) and P2 level. There was a positive correlation with cotinine concentration and the P1/P2 ratio.

In the present study I have also found the cotinine concentration were directly significantly positive correlated with levels of the histone H2B, H2A, and the P1/P2 ratio. A significant negative correlation was found for the cotinine concentration and protamine P2 concentration.

These data demonstrate the adverse effect of cotinine concentration on the final stage of spermatogenesis, where replacement occurred between histones and protamines, suggesting that cotinine caused downregulation of protamines expression particularly, for protamine P2. This is possibly due to the non-simultaneous protamines gene expression, which had a possible impact on down-regulation of P2 expression more than P1. Subsequently, this causes a high P1/P2 ratio.

## 5-2- Transition protein 1 (TP1)

### 5-2-1- Correlation between leucocyte level and cotinine concentration

Leukocytes are the primary source of reactive oxygen species in the human ejaculate and increased leukocytes may affect fertility by the reactive oxygen species formation (Shen *et al*., 1999). Cigarette smoking has been related to an increase in leucocyte level in semen up to a 48% and an increase in the levels of seminal ROS in the body to about 107% (Saleh *et al*., 2002a), which was possibly responsible for a decrease in sperm production, an increase in sperm oxidative stress, and LPO (Linschooten *et al*., 2011; Fariello *et al*., 2009). Toxic metabolites in smoke may also have a direct affect in the spermatogenesis process (Barratt *et al*., 2010).

A similar effect has been shown in the present study, where an increase in the leucocyte content in smokers compared to non-smokers was observed. In addition, cotinine levels significant positive correlated with the levels of leucocytes.

These findings are similar with that found by Zhang *et al*. (2013) who reported that the concentration of seminal leukocytes was significantly higher in smoking groups. With an

increasing degree of smoking, seminal leukocytes steadily increased (mild, moderate and heavy smokers, respectively). Trummer *et al*. (2002), investigated a significant increase in seminal fluid leukocyte count in smokers compared to non-smokers.

Many researchers showed that leukocytospermia impaired some parameters including decreased concentration, and motility, acrosomal damage, abnormal morphology (Pasqualotto *et al*., 2000; Aziz *et al*., 2004; Lackner *et al*., 2010). In contrast, other studies demonstrated no significant relationship between leukocytospermia and decreased sperm function or parameters (Tomlinson *et al*., 1993; Aitken *et al*., 1994).

I found a relation between cotinine concentration and an increase in leucocytes levels in the semen, which is possibly due to the increase of malonyldialdehyde (MDA) concentration in the seminal plasma for sperm, which may caused an increase in male infertility.

**5-2-2- Cotinine and sperm parameters**

Some researchers have reported a negative effect of cotinine and nicotine amount on male fertility like sperm concentration, sperm motility and sperm morphology (Vine *et al*., 1996; Zitzmann *et al*., 2003; Hassa *et al*., 2007; Chen and Kuo, 2007).

The present study suggested an adverse biological effect of cotinine on spermatozoa and semen parameters, and there is a significantly higher concentration of cotinine (smoking marker) in smokers than in non-smokers. Besides, there was a significant inverse relationship between cotinine and sperm count, motility, vitality and sperm membrane integrity.

These results in agreement with Hammadeh *et al*. (2008, 2010), they found that negative correlation between cotinine concentration and sperm count, motility, viability, and morphology. Kim *et al*. (2008) reported a negative effect of nicotine concentration on the progressive motility in mouse sperm. Pasqualotto *et al*. (2008) and El-Melegy and Ali (2011) showed that the nicotine concentration was associated with inhibition of progressive sperm motility. Oyeyipo *et al*. (2011) have shown adverse effects of nicotine concentration on sperm count, motility, viability and fertility potentials in rats.

In the present study, I found a positive correlation for cotinine concentration and the levels of TP1, and the P1/P2 ratios. This was the first attempt to study the relation between cotinine concentration and transition protein levels (TP1) in patients. These data demonstrated the adverse effect of cotinine concentration in the final stage of spermatogenesis, in which histones are replaced by transition proteins and finally by protamines. I found that high levels of transition protein TP1 concentration correlated with abnormal chromatin condensation, which is believed to have an impact on the sperm quality (sperm concentration, motility, vitality, and sperm membrane integrity).

### 5-2-3- Smoking cigarette and MDA levels

Malondialdehyde (MDA) is one of the lipid peroxidation products in seminal plasma (Shang *et al*., 2004). Toxic lipid peroxides had a contrary effect on sperm parameters and possibly play a significant role in the male infertility etiology. Malondialdehyde (MDA) is an index for lipid peroxidation, which is more likely a diagnostic tool for the infertility analysis (Tavilani *et al*., 2005; 2008).

In the present study, an increase in malonyldialdehyde (MDA) content in smokers compared to non-smokers was found. A significantly negative correlation for MDA concentration in seminal plasma and sperm concentration, vitality, motility and membrane integrity was observed. Also, MDA concentrations significantly positive correlation with cotinine concentration. These results are in accordance with Hsieh *et al*. (2006) who reported that an increasing MDA level might represent the pathologic lipid peroxidation of spermatozoa membrane and the following inhibition of sperm motility and viability. Nabil *et al*. (2008) also found a correlation for lipid peroxidation and sperm functions and semen quality such as sperm motility and morphology, and may account for male infertility. Saraniya *et al*. (2008) reported a significantly higher level of MDA in seminal plasma of abnormal semen compared to healthy sperm. Also, they found that the percentage of non-motile spermatozoa were significantly positive correlated with the MDA concentration. Sperm counts showed a significant negative correlation with MDA level of seminal plasma (Saraniya *et al*., 2008). Also, Fariello *et al*. (2012) showed a lower level of lipid peroxidation in the control group which increased steadily according to the degree of smoking.

The direct correlation between levels of cotinine and MDA reported in the present study suggests higher levels of MDA as a result from oxidative stress formed by smoking and this assumption is supported by previous reports (Mostafa *et al*., 2006). These results were also supported by other reports (Tavilani *et al*., 2005; Hsieh *et al*., 2006; Abdallah *et al*., 2009) which have shown that high MDA levels correlate with other decreased sperm parameters such as concentration and motility.

In the present study, I found a negative correlation between MDA and P2 levels. A significant positive correlation for MDA concentration and the P1/P2 ratios was found. The inverse relationship between MDA and sperm chromatin protamination indicates that MDA had an effect on P2 more than on P1. These results are in agreement with Hammadeh *et al*. (2010), who found a significant negative correlation for MDA levels and sperm parameters and positive relationship with P1/P2 ratios in samples from smokers compared with those of non-smokers, which reflects the negative effect of MDA on the replacement of histones by protamines at the last step of spermatogenesis.

In the present study, I also found that the MDA concentrations were positively correlated with levels of transition protein TP1. Here, I found a relationship between MDA concentration and transition protein levels (TP1) in patients. This reflects the negative effect of lipid peroxidation on the process of TP1 transition protein replacement by protamines.

### 5-2-4- Protamine deficiency (CMA3 test) and semen parameters

Protamine deficiency, which occurs during late stages of spermiogenesis, may decrease the fertilization rate (Manicardi *et al*., 1995). The histone-protamine exchange is a late spermiogenesis event, along with acrosome formation, membrane remodeling and other significant morphological and biochemical events that are necessary for normal sperm function (Nasr-Esfahani *et al*., 2004a). The percentage of premature chromosomal condensation has been reported to be higher in semen samples with high CMA3 positivity compared to those with low CMA3 positivity (Nasr-Esfahani *et al*., 2004b).

Evaluation of protamine deficiency by chromomycin A3 (CMA3) had been applied to configure the relationship between protamine and DNA integrity (Aoki *et al*., 2006c; Aoki

*et al*., 2005a; Nili *et al*., 2009). A correlation between under-expression of protamines, DNA damage and lack of viability was detected using many different techniques (TUNEL assay, CMA3 and aniline blue staining assays) (Manicardi *et al*., 1995; Hammadeh *et al*., 2001; Aoki *et al*., 2006b).

In the present study, significant differences were found for smokers and non-smokers samples regarding the protamine deficiency. Protamine deficiency showed a significant negative correlation with sperm concentration, motility, vitality, and sperm membrane integrity. A significant positive correlation was confirmed for protamine deficiency and MDA concentration, cotinine concentration, protamine P1 concentration, the P1/P1 ratio and transition protein TP1 concentration.

The present data are in accordance with results of other previous studies analyzing the relationship between protamine deficiency and traditional sperm parameters (Carrell and Liu, 2001; Mengual *et al*., 2003; Aoki *et al*., 2005a; Torregrosa *et al*., 2006; Borini *et al*., 2006). Tarozzi *et al*. (2009), found a significant negative correlation for protamine deficiency and sperm concentration, motility, and morphology. They showed a significant positive correlation for protamine deficiency and sperm DNA fragmentation. Lakpour *et al*. (2012) found a relationship for standard sperm parameters (count, motility, and morphology) and protamine deficiency and sperm chromatin damage. Also, they have observed a significant positive correlation between sperm chromatin damage and protamine deficiency. Utsuno *et al*. (2014) reported that protamine deficiency was significantly lower in spermatozoa with normal head morphology than in those with abnormal head morphology.

In this study, there was a significant increase in the mean percentages of protamine deficiency in smokers in comparison with non-smokers. Tobacco smoking was linked to an increase in sperm protamine deficiency, such deficiency considered as one of the major causes of sperm immaturity or sperm chromatin damage, which leads to an increase in male infertility.

### 5-2-5- Protamine P1, P2 content and P1/P2 ratio and sperm quality

The human sperm DNA is extremely condensed in the head of sperm by both P1 and P2 (Tanaka *et al*., 2003a). It has been observed that the nuclear condensation disturbances might cause male infertility. The sperm protamine deficiency has been reported in some infertile men, suggesting that errors in chromatin packaging can cause infertility (Tanaka *et al*., 2003a; Iguchi *et al*., 2006).

The present study has shown that the concentration of protamine P2 was lower in smokers than in non-smokers but not significant. In contrast, the P1/P2 ratio was significantly higher in the smokers. The P1/P2 ratio demonstrated a significantly positive correlation with concentration of protamine P1. In contrast, the levels of the P1/P2 ratio were significantly negatively correlated with concentration of protamine P2. Also, the P1/P2 ratio demonstrated a significant negative correlation with sperm motility, and sperm vitality and membrane integrity. No significant correlations were found for sperm concentration and sperm volume.

These results are in agreement with Aoki *et al*. (2005a, b) who showed that in patients with abnormal P1/P2 ratio there was a decreased mean percentage of sperm count, motility, viability, sperm penetration capacity, and abnormal sperm morphology, and the average percentage of DNA fragmentation was increased. By proteomic approaches Carrell *et al*. (2007) found an alteration in the P1, P2 family or histone expression were related to male infertility. Hammoud *et al*. (2009a) found that patients with abnormal P1/P2 ratios had less normal sperm heads and more tapered sperm when compared to patients with a standard protamine ratio although these differences were not significantly different among the group with abnormal and those with standard P1/P2 ratio groups.

These results show a negative effect of smoking on the process of protamination. Accordingly, I found that P1/P2 ratios in sperm abnormalities had possible effects on sperm quality and function, these abnormalities of protamination were considered as one of the causes of sperm chromatin packaging abnormalities.

### 5-2-6- TP1 content and sperm quality

Transition nuclear proteins (TPs) are main nuclear proteins that replace histones, leading to subsequent replacement by protamines during human spermiogenesis (Meistrich *et al*., 2003). They postulated that transition protein TPs are essential for chromatin integrity preservation and completion of its condensation (Steger *et al*., 1998). A report from Yu *et al*., (2000) suggests that transition protein TP1 plays an important role in nuclear formation during spermiogenesis in mice. Transition protein TP1 is possibly more important for spermiogenesis in humans than in mice. Mutations in the recognition site of the cyclic adenosine monophosphate (cAMP) response element (CRE) in the promoter region of transition protein TP1 is thought to be a cause of male sterility (Miyagawa *et al*., 2005). Xu *et al*. (2013) reported that exposure of mice to cigarette smoking also leads to epigenetic alterations in testis (Xu *et al*., 2013), which have been associated with male infertility (Rajender *et al*., 2011).

The possibility of an impact of smoking on fertility and the health of offsprings has been a matter of debate recently. Therefore, this study was performed to evaluate the effect of smoking on transition protein 1 (TP1) contents and other indicators of spermatozoa in smokers and non-smokers. In the present study, I found a negative biological effect of cigarette smoking on TP1 and other nuclear proteins in spermatozoa. The semen samples from smokers possessed significantly higher levels of spermatozoa nuclear proteins such as transition protein 1 (TP1) in comparison to non-smokers. In addition, transition protein 1 (TP1) concentration for all participant samples showed significant negative correlations with sperm motility, vitality, and sperm membrane integrity. But, a non-significant negative correlation for transition protein TP1 and protamine P2 was found. In contrast, significant positive correlation for transition protein TP1 and the P1/P2 ratio was observed.

This study was the first attempt to correlate cigarette smoking with transition protein TP1 levels in patients. I found significantly higher levels of transition protein TP1 in semen samples from smokers in comparison to non-smokers.

These findings revealed that smokers with high transition protein TP1 concentration had a spermiogenesis defect, in which histones are replacing by transition proteins and finally by

protamines. My data support the perception that sperm with relative increase in transition protein concentration TP1 and consequently decrease in protamine concentration, can cause an increase in the P1/P2 ratio. I suggest this change in the spermiogenesis indicates higher immature sperm in semen.

## 5-3- Conclusion

In this study, abnormalities in sperm protamine deficiency and DNA fragmentation related to abnormalities in sperm parameters (concentration, motility, vitality, and membrane integrity) were analyzed.

Smokers have high levels of oxidative stress biomarkers (MDA) and decreased sperm quality compared to non-smokers. Cigarette smoking impairs sperm function by reducing the sperm quality (concentration, motility, vitality, membrane integrity, chromatin condensation, and DNA fragmentation). Also, the high level of smoking biomarker cotinine had an impact on the reduction of sperm quality.

Also, in the present study I found a relationship between sperm nuclear proteins (histone, transition protein and protamines) with protamine deficiency (Aniline blue stain and CMA3 stain) and DNA fragmentation (TUNEL assay) of spermatozoa. Investigation of the effects of smoking on sperm nuclear proteins (histone, transition protein and protamines), related an increase in histone H2A, H2B, H3 and transition protein TP1 concentration. However, there was a reduction in protamines concentration mainly, protamine P2. Results also showed a high P1/P2 ratio which was due to under-expression of protamine P2. I found a relationship for the P1/P2 ratio and sperm parameter (concentration, motility, vitality, membrane integrity, protamine deficiency, and DNA fragmentation).

These results show adverse effects of smoking on sperm quality. An infertile patient who smoke should be advised to quit smoking before undergoing in assisted reproductive treatment.

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# 7- Publication/Acknowledgement

## 7-1- Publication

1- Hamad MF, Shelko N, Kartarius S, Montenarh M and Hammadeh MH (2014). Impact of cigarette smoking on histone (H2B) to protamine ratio in human spermatozoa and its relation to sperm parameters. Andrology, 2: 666–677.

2- Montenarh M, Shelko N and Hammadeh ME (2014). Rauchen schädigt Erbgut in Spermien und vermindert die Fruchtbarkeit von Männern. Magazin forschung Universitat des Saarlands: 4-6.

3- Shelko N, Hamad MF, Montenarh M and Hammadeh ME (2016). The influence of cigarette smoking on sperm quality and sperm membrane. Current Women`s Health Reviews USA, in press

4- Shelko N, Hamad MF, Montenarh M and Hammadeh ME (2016) The structure of human sperm transition proteins of normo- and subnormospermia and its relationship with DNA integrity and conventional sperm parameters. Human reproduction, 31: 137. (Abstract)

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