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The effect of reactive oxygen species and smoking on the spermatozoa quality and outcome of patients undergoing IVF/ICSI therapy.

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Submitted by
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Dedication

This work is dedicated to the person who supported me all through my life and never saved an effort to lead me to what I am now, my father ***Dr Marwan Jamil Al-Qatami***, PhD, ENT consultant and to the greatest women ever, my mother.

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1-Summery

Although the population is growing enormously in most of the developing countries, infertility remains the major problem, affecting about 15% of the couples. Impaired fertility of male partner is causative or contributory in approximately 50% of all couples unable to conceive spontaneously. During the last couple of decades considerable importance is being given to male-factor infertility. Impairment of sperm function may be due to biochemical, environmental or genetic reasons or interplay of all these factors.

The objectives of this study were to determine the difference between semen and spem quality of patients underwent IVF/ICSI therapy. Besides, to determin ROS, total antioxidant (TAS), of seminal plasma and other sperm characteristic parameters (count, morphology, HOS, maturity, strand break, and membrane integrity). Moreover, the relationship between smoking and cotinine concentration in seminal plasma on the sperm parameters, in one hand and the effect of these investigated parameters on IVF/ICSI in the others.

In the present study, 94 patients (57 IVF) and (37 ICSI) were included. The semen concentration of ROS, TAS, selenium, zink and sperm parameters like concentration, motility, vitality, membrane integrity (HOS-test) morphology, Chromatin condensation (Maturity, CMA3, DNA Fragmentation (Tunel-test, DNA denaturation (AO) were investigated and there effects on IVF/ICSI outcome were analysed. In addition, the semen parameters and cotinine concentrations in seminal plasma of 46 patients were investigated and spermatozoa quality and their influrence on IVF/ICSI outcome of heavy smokers (11) and non smokers (35) patients who underwent IVF, ICSI therapy were compared and evaluated. Semen samples of patients underwent IVF/ICSI therapy was allowed to liquefy and assessed according WHO (1999) guidelines except for morphology which was evaluated according to strict criteria Krüger et al (1986).

The seminal plasma on the top of the above layer of the two fraction 45% and 90% of PureSperm layer was withdrown after semen and gradient centrifugation and kept frozen at -80°C separately in two different tubes until the concentrations of ROS, TAS, cotinine were measured.. The pellet which contain morphologically normal spermatozoa was washed again, layered with 1 ml culture media and incubated at 37°C. The motile sperm was used for injection (ICSI) or insemination of oocytes (IVF).

Smears were made from each semen sample before (native) and after semen preparation (motile sperm in the supernatant) in order to evaluate the morphology, sperm maturity (Chromomycin CMA_3) and DNA stand break (Tunel assay) in addition to the count, motility, HOS-Test. Seminal plasma was examined for the concentrations of ROS, TAS and cotinine levels which were measured by the conventional colorometric assay using commercially available kits. ROS was measured by calorimetric assay for the quantitative determination of peroxide (Oxystat, kit Biomedical, Austria) and Semen antioxidant capacity was determined

with a photometer method based on the antioxidants inhibition of the absorbance of the radical cation 2.2`-azinobis (ABTS $^{+)}$. Cotinine concentration was measured with Bio Quant (Cotinine Direct ELISA) kit. DNA integrity was evaluated by Chromomycine (CMA_3-test) and DNA damage was assessed by Acridine orange staining and terminal deoxynucleotidyl transferase (TDT) mediated d UTP nick end labelling (TUNEL-test).

The sperm parameters and the concentration of ROS, TAS and cotinine in seminal plasma and other parameter (sperm concentration, morphology, maturity and Tunel results) was correlated with the IVF, ICSI outcome.

From the women side following date was collected: The age of the women, ovarian stimulation protocol, number of retrieved, mature, injected oocytes and the fertilization, cleavage and pregnancy rates.

Statistical analysis demonstrated significantly lower semen quality of ICSI in comparison to IVF patients. Significantly lower sperm density, vitality, motility and DNA integrity (CMA3) cleavage rate than those patients underwent ICSI therapy. Besides, semen volume, mean percentage of morphologically normal spermatozoa, DNA denaturation (AO) stand break and DNA fragmentation (TUNEL), ROS, TAS selenium and zinc concentaration in seminal plasma and fertilization rate were similar in ICSI patients in comparison to IVF patients.

Pregnancy rate was higher in patient underwent IVF in comparison to those who underwent ICSI therapy (31.5% vs. 29.7%; p=0.392). In IVF patients group 18 (18/ 57) pregnancies was achieved. Whereas only (11 women become pregnant (11/37). Only 40 women become transfer in IVF and 18 in ICSI groups, the other patients ended up with total fertilization or cleavage failure.

A negative non significant correlations were found between ROS concentration in seminal plasma and sperm concentration, motility, morphology, membrane and DNA integrity, DNA fragmentation, DNA denaturation, fertilization rate and pregnancy. Whereas, a positive correlations have been shown between TAS concentration in seminal plasma and Chromatin integrity, DNA denauration, pregnancy and zink, Ferritin, transferrin concentration. By comparing the smokers and non smokers, the following results have been shown:

In the smokers and non smokers patients groups, the sperm concentration, mean number of motil spermatozoa after sperm selection, morphologically normal spermatozoa, mean number cleaved and transferred oocytes and pregnancy rate were significantly higher in non-somoking patients group in comparison to smoking one. The other sperm parameters were similar in both groups. However, cotinine concentration was significantly higher in somkers than non-smokers. An inverse correlations were found between cotinine concentration and ejaculate volume, sperm concentration, motility, vitality, morphology membrane integrity, DNA fragmentation (TUNEL) and DNA denaturation, TAS concenrtration and fertilization. Whereras, a positive correlation was shown between cotinine and ROS concentration. In the

smoker group three patients become pregnant (27.3%) and 11 patients achieved pregnancy in the non-smoking group (34%; p=0.001).

ROS concentration in seminal plasma affect spermatozoa quality and consequently IVF/ICSI results. TAS concentration correlate positive with sperm parameters and IVF/ICSI outcome. Besides, cotinine concentration in seminal plasma has an advers effect on seminal plasma anti oxidants, sperm parameters and ART outcome and increase the production of ROS in seminal plasma. Therefore, it is recommended to quit smoking in childless patients who seeking ART therapy.

2-Zusammenfassung

Trotz des rapiden Bevölkerungswachstums in den Entwicklungsländern bleibt die Infertilität für manche Menschen ein Problem, 15% der Bevölkerung sind davon betroffen. Bei Paaren mit Kinderwunsch beträgt zur Zeit die männliche Infertilität 50%. In den letzten 20 Jahren wurde viel Forschung betrieben um das Problem der männlichen Infertilität zu bewältigen.
Der Dysfunktion der Spermien liegen folgende Ursachen zu Grunde; genetische Faktoren, Umwelteinflüsse, biochemische Stoffwechselreaktionen, es können auch Kombinationen dieser drei Faktoren eine Rolle spielen.
Ziel dieser Arbeit ist es einerseits die Unterschiede zwischen Samenflüssigkeit, sowie der Spermaqualität zwischen IVF-Patienten und ICSI-Patienten zu vergleichen. Insbesondere im Hinblick auf die Samenflüssigkeit sollen die freien Radikalen (ROS), die totalen Antioxidanten (TAS), Selen und Zink bestimmt werden. Im Hinblick auf die Spermaqualität allerdings sollen die Anzahl der Spermien im Ejakulat, der Reifegrad der Spermien, die Membranintegrität (HOS-Test), sowie die DNA-Strangbrüche, Fragmentationen untersucht werden.
In einer Untergruppe (46 Patienten) soll die Cotinine –Konzentration im Seminalplasma bestimmt werden. Diese Untergruppe konnte wiederum in 2 Bereiche geteilt werden; Raucher (11 Patienten) und Nichtrauchen (35 Patienten). Insgesamt soll die Auswirkung der Parameter auf die Spermaqualität einerseits, sowie als logische Konsequenz deren Auswirkung auf IVF-/ICSI-Patienten untersucht werden. Die Ejakulatproben von 94 Patienten (57 IVF und 37 ICSI) wurden nach den Kriterien der WHO (1999) untersucht, mit Ausnahme der Morphologie, welche nach den Krüger-Kriterien (1986) evaluiert wurde. Das Ejakulat wurde auf den PureSpermgradienten übertragen (45% und 90%).
Nach der Zentrifugation wurde der Seminalplasmaüberstand abgehoben und bei -80° bis zum Zeitpunkt der Untersuchung aufbewahrt. Das Sediment (Spermatozoen) wurde noch einmal gereinigt und für die routinemäßige Insemination, Injektion der Eizelle genutzt.
Es wurden mehrere Abstrichen von diesen Spermatozoen angefertigt, luftgetrocknet, fixiert und zur Beurteilung der Morphologie, der Chromatin-Condensation (Chromomycin CMA3), DNA-Denaturierung (Acridine-Orange AO), sowie der DNA-Strangbrüche (Tunel-Test).
Die Bestimmungen von ROS, TAS, Cotinine wurden mit einem kommerziellen Enzym Immun Assay (Alisa) durchgeführt.
Die Ergebnisse dieser Analyse wurden mit den Ergebnissen der Spermparameter korreliert, um den Einfluss dieser Faktoren auf die Spermaqualität festzustellen. Von der Frauenseite aus wurden die Stimulationsprotokolle, die gesammelten Eizellen, die Fertilitsationsrate, die Teilungsrate, sowie die Schwangerschaftsrate erhoben und evaluiert. Diese Ergebnisse wurden wiederum mit dem Ejakulatparameter verglichen.
Es wurde festgestellt dass die Spermaqualität (Anzahl der Spermien, Vitalität, Motilität,

CMA3) von ICSI-Patienten schlechter waren, als die der IVF-Patienten. Jedoch war das Spermavolumen, die Morphologie, AO, TUNEL, ROS, TAS, Selen- und Zinkkonzentrationen ähnlich, somit bestand kein signifikanter Unterschied.

Darüberhinaus konnte kein Unterschied zwischen der Fertilisationsrate, der Teilungsrate, sowie der Schwangerschaftsrate (31,5% vs. 29,7%, p=0.392) bei IVF- und ICSI-Patienten gefunden werden. In der IVF-Patientengruppe wurden 18 Schwangerschaften erzielt (18/57), in der ICSI-Patientengruppe wurden 11 (11/37) erreicht. Es ist hierbei zu erwähnen, dass nicht bei allen Frauen ein Transfer durchgeführt wurde. Bei dem IVF Programm bekamen 40 Frauen einen Transfer, bei dem ICSI Programm bekamen 18 Frauen einen Transfer.

Im weiteren Verlauf konnte eine negative Korrelation festgestellt werden zwischen ROS im Seminalplasma, der Spermkonzentration, Mortilität, Morphologie, Membran, DNA-Integrität, DNA-Strangbrüche, DNA-Denaturierung, Fertilisationsrate und der Schwangerschaftsrate.

Jedoch wurde eine positive Korrelation zwischen TAS im Seminalplasma und der Chromatinintegrität, der DNA-Denaturierung, der Zink-, Eisen-, Transferrinkonzentration und der Schwangerschaftsrate gezeigt.

Bezüglich der Raucher/ Nichtrauchergruppe war die Zahl der Spermakonzentration, der Spermamotilität nach Selektion, die Zahl morphologisch normaler Spermien, die Zahl der geteilten Eizellen, die Zahl der Transferierten, sowie die Schwangerschaftsrate bei Nichtraucher signifikant höher im Vergleich zur Rauchergruppe. Alle anderen Parameter ähnelten sich jedoch. Die Cotininkonzentration war in der Rauchergruppe signifikant höher im Vergleich zu der Nichtrauchergruppe.

Eine negative Korrelation konnte zwischen der Cotininkonzentration und dem Ejakulatvolumen, der Spermakonzentration, der Vitalität, der Morphologie, der Membran und DNA-Integrität, der DNA-Denaturierung und DNA-Strangbrüchen (TUNEL) festgestellt werden. Dennoch bestand eine positive Korrelation zwischen der Cotininkonzentraion und ROS im Seminalplasma. In der Rauchergruppe wurden 3 Schwangerschaften erzielt (27,3% Schwangerschaftsrate). In der Nichtrauchergruppe wurden 11 Schwangerschaften (34% Schwangerschaftsrate, p= 0.001) erzielt.

Die Konzentrationen von ROS und Cotinin im Seminalplasma haben einen negativen Einfluss auf die Spermaqualität und somit auch auf den Erfolg von IVF und ICSI. Im Gegensatz dazu konnte gezeigt werden dass TAS einen positiven Einfluss auf die Spermaqualität und somit auch auf den Erfolg von IVF und ICSI hat. Es ist daher zu empfehlen auf Nikotin zu verzichten, insbesondere bei kinderlosen Ehepaaren mit Kinderwunsch, welche im Verlauf sich einer Therapie mit IVF- und ICSI unterziehen.

3- Introduction

Infinity of infertility

Since the first appearance of humans on earth, infertility has been one of the most controversial medical and social issues. Some civilizations considered it to be a punishment, while others thought of it as an illness. Some blamed it on the woman; others could not explain it. This charge infertility has on the woman, had serious consequences on her social image and her psychological state (Morice et al., 1995). Additionally, physicians lack of knowledge of gonadal and sperm function, lead to mostly the female being considered to be the one responsible for infertility. It was not until the last decade that our knowledge of the human reproductive system has allowed us to determine that a very important parameter of a couples infertility is male infertility and more specifically, sperm malfunction (Hull et al., 1985). According to the report by WHO, 1975, infertility is a worldwide problem, affecting up to 10% of all couples and in some countries up to 30% (WHO Scientific group, 1975**).** It results from female disorders in about 30%, a male disorders in 30% and disorders in both 30%. No abnormalities are found in about 10% (Comhaire et al., 1987; Forti and Krausz , 1998).

Decreases in sperm concentrations, motility as well as morphological alterations are frequent findings in the semen analyses of these patients (Sigman et al., 1992). Many of these alterations have recently been linked to high levels of Reactive oxygen species (ROS) (Aitken , 1994, de Lamirande and Gagnon, 1994, Sharma and Agarwal, (1996) and decrease in total antioxidant capacity (TAC) of seminal plasma (Ochsendorf and Fuchs, 1993).

1-Reactive oxygen species an (ROS) and total antioxidants (TAS) effect on male infertility

1-1. Normal and impaired sperm function

Normal spermatozoa are those that successfully undergo a number of steps necessary for fertilization of the oocyte. The first step is the maturation of the spermatozoa, which is initiated in the male genital tract and is concluded with capacitation, the final step of maturation, occurring in the female genital tract. Fully mature spermatozoa must swim in the female reproductive system, reach the oocyte, undergo acrosome reaction (AR), penetrate the zona pellucidae and fuse with the oocyte pronucleus to form the zygote (Larson et al., 2000a ; b).

Failed attempts to conceive, after a period of two years, define infertility and the couple has to visit an expert to seek diagnosis and possible treatment (Hull et al., 1985). The male partner undergoes semen analysis and is evaluated according to the World Health Organization reference values. However, there are cases where the semen analysis indicates normal sperm, but the male is still infertile (WHO, 1999). This proves that there are parameters affecting sperm function that cannot be determined by routine semen analysis. Semen analysis includes assessment of seminal volume, spermatozoal motility, density, viability and morphology. Oxidative damage to spermatozoa, induced by excessive production of free radicals or impairment of the natural antioxidant mechanisms, has been identified as such a parameter (Sikka et al., 1995).

1.2. Free radicals origin

By definition, a free radical is any chemical compound with one or more unpaired electrons. The free radicals that have been associated with infertility are oxygen and oxygen-derived oxidants, namely, the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO^-) and hydroxyl radicals (OH^-) (Agarwal et al., 2005a; Bagchi and Puri, 1998). These oxidants are widely known as reactive oxygen species (ROS) and, due to the unpaired electron(s) tend to strongly react with other chemical compounds (Bagchi and Puri, 1998). More specifically, they seek stability by “stealing” electrons from nucleic acids, lipids and proteins, leading to the damage of cells and disease phenomena (Attaran et al., 2000; Pierce et al., 2004).

Reactive oxygen species are produced in the body by mitochondria, phagocytes, arachidonate pathways and other physiological processes, in which they act as vital signalling molecules. They are products of natural oxygen metabolism and represent approximately 1 to 2% of metabolized oxygen (Fulbert and Cals 1992). The balance between production and disposal of oxidant molecules is essential for tissue homeostasis. Increased rate of free radical production or decreased rate of removal leads to free radical accumulation and cellular damage (Berry and Kohn 1999). Additionally, their production is induced by external factors, such as cigarette smoke, radiation ultraviolet light and others (Bagchi and Puri, 1998). Reactive oxygen species have been associated with the pathology of numerous diseases, such as neurodegenerative diseases (Gibson and Huang, 2004), vascular disease (Madamanch et al., 2005; Halliwell., 1996), cancer, diabetes, periodontal diseases (Halliwell., 1996; Lantos et al., 1997; Moore et al., 1994; Pinzani et al., 1998) and of course, human infertility (Agarwal and Allamaneni., 2004; Agarwal et al., 2005b; Agarwal et al., 2003; Aitken and Baker., 2004; Baker and Aitken 2005; de Lammirande et al., 1997; Henkel., 2005; Kim and Parthasarathy., 1998; Sanocka and Kurpisz., 2004).

1.3. ROS and oxidative stress (OS)

The free radicals are a part of an organism's chemical reactions, they are necessary signaling molecules, as well as being vital for the maturation processes of several structures. Most free radicals in biology fit within the broader category of reactive oxygen species (ROS), which include not only oxygen–centred radicals such as superoxide anion radical (O_2^-), hydroxyl radical ($OH^{\cdot}$) or nitric oxide ($NO^{\cdot}$), but also some potentially dangerous non-radical derivatives of oxygen, such as H_2O_2 peroxynitrite anion ($ONOO^-$), and hypochlorous acid (HOCL) (Halliwell., 1993). The most common ROS that have potential implication in reproductive biology include superoxide (O^{-2}), anion, hydrogen peroxide (H^2O^2) peroxyl (ROO^-) and hydroxyl (OH-) radicals.

The free radical nitric oxide and (NO-) and peroxynitrite anion (ONOO-) also appear to play a significant role in reproduction and fertility. Oezezcan et al (1999) indicate that $ONOO^-$ might cause sperm dysfunction through an increase in lipid peroxidation, and total sulphydryl group depletion. The assumption that free radicals can influence male fertility has received substantial scientific support (Gagnon et al., 1991). Many reports have indicated that high levels of ROS are detected in semen samples of 25% to 40% of infertile men (Padron et al., 1997) however small controlled amounts of ROS are vital for spermatozoa to develop normally, and be capable of fertilization structures (Gagnon et al., 1991; Aitken; 1999; Aitken et al., 1997).

In addition, de Lamirande et al. and Zini et al. reported that H_2O_2 and O_2- promote sperm capacitation and acrosome reaction, and that H2O2 promotes hyperactivation and oocytes fusion (de Laminarde and Gagnon., 1993a; Zini et al., 1996). Hydrogen peroxide and superoxide anion are of great importance to spermatozoa. They are necessary for controlling the tyrosine phosphorylation events associated with sperm capacitation (Aitken., 1999; Aitken et al., 1998a).

1.4. ROS production by spermatozoa

ROS are also undoubtedly produced by spermatozoa (Aitken et al., 1992; Gavella and Lipovac., 1992; Gil-Guzman et al., 2001; Hendin et al., 1999), mainly through their mitochondrial system (Plante et al., 1994), as well as round cells during the spermatogenesis process and epithelial cells. In the human ejaculate ROS are mainly produced by leucocytes and marginal amounts by spermatozoa (Aitken and Baker 2002). The production of ROS in ejaculated spermatozoa is initiated in immature germ cells (Gil-Guzman et al., 2001) and continues in the epididymis when the surface of the spermatozoa is remodelled. When the mitochondrial capsule is assembled, chromatin undergoes condensation and motility is acquired for the capacitation of spermatozoa (Agnihorti et al., 1999; Fisher and Aitken.,

1997). However, superoxide (O_2^-) production by spermatozoa has been questioned (Richer and Ford. 2001) on the basis that no free radical signal can be detected by EPR spectroscopy. Electron leakage from complexes I and II of the mitochondrial transport chain has been proposed as a source of superoxide in male gametes (Vernet et al., 2001). Numerous studies have shown that human sperm exhibit the capacity to generate reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radicals (Aitken and Clarkson., 1987, Aitken et al., 1994). The production of ROS by human sperm is due to a membrane–bound NADH oxidase system (Aitken et al., 1989)

1.5. Spontaneous generation under aerobic conditions

Spermatozoa generate small amount of O_2^- and $NO^{\cdot}$ which are both tightly related to the cAMP pathway in the control of human sperm capacitation and protein tyrosine phosphorylation (Aitken et al., 1995; Belen-Herrero et al., 2000). Beckman et al., suggested that these two radicals could combine to form peroxy nitrite ($ONOO^-$) (Beckman et al., 1990). Peroxy nitrite is not a free radical because the unpaired electrons of NO and O_2^- have combined to form a new N-O bond in peroxy nitrite, but it is a strong one or two electron oxidant and nitrating agent (Herrero et al., 2001). Nevertheless, generating controlled low amounts of endogenous ROS by spermatozoa play a significant role in inducing sperm capacitation acrosome reaction and acquisition of sperm-fertilizing ability (Gagnon et al., 1991; de Laminarde and Gagnon., 1995).

1.6. Retained cytoplasm (RC)

The production of ROS is higher in spermatozoa that are either damaged or retain abnormal cytoplasmic inclusions (Gil-Guzman et al., 2001).

Radical production has also been detected in immature germ cells (Fisher and Aitken ., 1997). According to Iwasaki and Gagnon et al (1992), the capacity for ROS production is significantly enhanced in abnormal spermatozoa, and those sperm cells with retention of residual cytoplasm. Huszar and Vigue (1993) have found that morphological irregularities of sperm are significantly correlated with high CK activity. ROS production by spermatozoa has been associated with midpiece abnormalities, retained cytoplasm, cytoplasmic droplets and spermatozoa immaturity (Aitken et al., 1989; Gomez et al., 1996; Huszar and Vigue 1994).

The primary product of the immature spermatozoa system generating free radicals appears to be the superoxide anion ($O_2^{\cdot-}$), which secondarily dismutates to H_2O_2 through the catalytic

action of superoxide dismutase (SOD) (Aitken and Krausz., 2001; Alvarez et al., 1987). Moreover, the most prevalent ROS, hydrogen peroxide (H_2O_2) is synthesized from O_2 by mammalian spermatozoa (Tosic and Walton., 1946) by a two–stage reduction of superoxide ($O_2^{-)}$ by H^+ as the intermediate product (Holland et al., 1982). The retention of residual cytoplasm in the sperm midpiece after spermination has been associated with excessive production of ROS by spermatozoa (Gomez et al., 1996). The ROS levels are expected to rise at a faster pace and in greater intensity in sperm samples in the presence of cytoplasm residues. Morphometric analysis of the amount of residual cytoplasm present in the sperm midpiece has revealed significant correlations with the production of ROS (Gomez et al., 1996).

The enzyme gloucose-6-phosphat dehydrogenase (G6PD), which is over expressed in sperm residual cytoplasm, generates NADH, which in turn stimulates ROS formation (Aitken et al., 1997; Aitken et al., 1994; Gomez et al., 1996; Huszar and Vigue., 1994).

Sperm with cytoplasmic droplets show a higher cellular content of cytoplasm enzymes, including G6PD. This enzyme is responsible for the flux of glucose through the hexose monophosphate shunt and the associated generation of NADPH. It is theorized that the NADPH generated via this system serves as the major source of electrons responsible for the production of O_2^- by human spermatozoa. Therefore, the retention of residual cytoplasm creates a situation in which sufficient substrate would be available to support excessive NADPH–dependent ROS generation (Aitken et al., 1997; Baumber et al., 2000) Spermatozoa may generate ROS in two ways:

1- NADH-oxidase system at the level of sperm membrane level (Aitken et al., 1992) and

2- 2NADH-dependent oxido-reductase (diphorase) at the level of the mitochondria (Gavella and Lipovac., 1992). Besides, the biochemical markers of cytoplasm space, such as creatine kinase, are positively correlated with the induction of peroxidative damage (Huszar and Vigue., 1994; Gomez et al., 1996). Huszar and Vigue., (1994) have found a positive relationship between CK activity and the rate of lipid peroxidation, as measured by malondialdehyde (MDA) formation in sperm fractions. Hallak et al have found an inverse relationship between creatine kinase (CK) levels and sperm morphological forms and suggested that CK levels can be used as a reliable marker for sperm quality and fertilizing potential in subfertile men (Hallak et al., 2001).

Excessive ROS production by immature, morphologically abnormal spermatozoa with cytoplasmic residues such as those confronted in teratozoospermic semen specimens may

induce oxidative damage of mature spermatozoa during sperm migration from the seminiferous tubules to the epididymis and may be an important cause of male infertility (Sikka ., 2004). Immature spermatozoa are well-characterized source of ROS and a negative correlation between ROS production and semen quality has been documented (Gil-Guzman et al., 2001). The excessive generation of reactive oxygen species (ROS) by abnormal spermatozoa and by contaminating leukocytes (leukospermia) has been identified as one of the few defined aetiologies for male infertility.

Many reports pointed out that biochemical marker of the cytoplasmic space, such as creatin kinase, are positively correlated with the induction of peroxidative damage (Gomez et al., 1996; Huszar and Vigue., 1994). Only one third of ROS produced by spermatozoa is released extracellulary (Plante et al., 1994). In the case of oligozoospermic males whose spermatozoa generate particularly high levels of ROS, the source of cytotoxic oxygen radicals is frequently intracellular (Gomez et al., 1996). However, the main ROS-producing sources are immature spermatozoa, especially those with cytoplasmic droplets at the midpiece and leucocytes (Garrido et al., 2004).

1.7. ROS production by leukocytes

ROS are produced by leukocytes, which are present in the male reproductive system and in the ejaculate, as a result of their role in immunological defense against pathogenic germs (Tomlinson et al., 1992; Henkel 2005) Hence, their increased number in semen of men with conditions like spinal cord injuries is thought to be due to local immunological response.

Leukocytes are present throughout the male reproductive tract and are found in almost every human ejaculate (El-Demiry et al., 1987) The majority of leukocytes in semen are granulocytes (50-60%), followed by macrophages (20% to 30%) and T lymphocytes (2% to 5%) (WHO, 1999). Leukocytes play an important role in immune surveillance (Pudney and Anderson, 1993; Kiessling et al, 1995) and phagocyte clearance of abnormal sperm (Tomlison et al., 1992). However, WBCs in human semen are also capable of ROS generation (Aitken and West, 1990; Kessopoulou et al., 1992). Genital tract inflammation and an increased number of leukocytes in the ejaculate have been repeatedly associated with male subfertility and infertility (Plante et al., 1994; Wolff., 1995; Ochsendorf, 1999; Sharma et al., 2001). Leukocytes are a particularly important source of oxidative stress in the ejaculates of patients exhibiting leukocytospermia secondary to infection or as consequence of paraplegia (Sharma and Agarwal, 1996). Activated leukocytes can produce 100-fold higher amount of ROS than nonactivated leukocytes (Plante et al., 1994). Leukocytes may be activated in response to a variety of stimuli including inflammation and infection (Pasqualotto et al., 2000). Whittington and Ford (1999) demonstrated that infiltrating leukocytes are the predominant source of ROS production in unspecified sperm preparations. Sperm damage from ROS that is produced by leukocytes occurs if seminal leukocytes concentrations are

abnormally high like in leukocytospermia (Shekarriz et al., 1995), or by removing seminal plasma during sperm preparation for assisted reproduction (Ochsendorf ., 1999).

Leukocytospermia may induce alteration in sperm structure by means of excessive ROS production by activated granulocytes. It has been shown that leukocytospermia and excessive ROS levels are associated with an increase in chromatin alterations and DNA damage in sperm, as defined by the sperm chromatin structure assay (Alvarez et al., 2002) In patient samples that generated detectable ROS, the ability of the spermatozoa to retain motility for 24h after preparation on a 40/80% Percoll gradient was negatively correlated ($r=-0.310$, $p<0.05$) with basal ROS production (Whittington, et al., 1999).. Besides, ROS production was also related to the outcome of in vitro sperm mucus penetration tests. Wang et al. (2003) demonstrated that mitochondrial function is inhibited in spermatozoa of infertile men and is significantly correlated with the sperm concentration and the level of ROS production.

1.8. ROS and Male fertility: Physiological Association of ROS

1.8.1. Association with subfertility

Only in the case of excessive production of ROS or malfunction of the native antioxidant-production mechanisms, do the free oxidants cause problems, by putting the several tissues under oxidative stress (OS) (Agarwal and Said., 2003; Aitken., 2005; Aitken and Clarkson., 1987; Balercia et al., 2003). ROS have been widely associated with both the etiopathogenesis in female infertility (Agarwal et al., 2005a, b, c), as well as with male subfertility and impaired sperm function, but their exact role is still not clear.

It has been more than a decade that the possibility of ROS negatively affecting male infertility has been examined by scientists (Aitken and Clarkson., 1987; Gagnon et al., 1991) and there has been an increasing generation of scientific data supporting that view (Sharma et al., 1999). More than 60 years ago MacLeod and Ross et al., (1943) described the loss of sperm motility, when the latter is exposed to oxygen at $38^{o}C$ and 36 years later the first association of oxidative stress (OS) and impaired sperm function came from the University of Cambridge (Jones et al., 1979). Since then, numerous scientific publications with very interesting data have come to light, presenting the role of ROS in sperm physiology and morphology (Gomez et al 1996; Aziz et al., 2004), and subsequently, the reproductive outcome.

The plasma membrane of spermatozoa contains high amounts of polyunsaturated fatty acids (PUFA), making them highly susceptible to damage by OS (Alvarez and Storey., 1995; Lewis et al., 1995; Zalata et al., 2004). Furthermore, the concentration of scavenging enzymes, like superoxide dismutase (SOD) or glutathione peroxidase (Guerin et al., 2001), in spermatozoa

cytoplasm is very low, making the effect of OS more severe (de Laminarde and Gagnon1995; Lewis et al., 1995; Saleh and Agarwal. 2002; Saleh et al., 2002). Scientific data has been presented to support that ROS negatively affect sperm function by contributing to the occurrence of lipid peroxidation (Aitken et al 1989; Zalata et al., 2004; Griveau et al., 1995).

Increased production of ROS or impaired action of antioxidant mechanisms can lead to surplus of ROS and hence, OS. However, semen analysis of infertile men showed that increased levels of ROS are a result of over-production of ROS, rather than decreased enrichment of the seminal plasma with antioxidants (Zini et al., 1993). It is important to note that due to the existence of leukocytes in the semen, the ability of spermatozoa themselves to produce ROS, as well as other sources capable of producing ROS in semen, the effect of ROS on male fertility is more of a quantitative matter, irrespective of pathological or non-pathological conditions.

The increased production of ROS from immature spermatozoa has been demonstrated in recent studies, indicating a negative correlation between the percentage of normal spermatozoa and levels of ROS production in semen. These results were obtained after calculating ROS production in a ejaculated sperm gradient of immature and mature spermatozoa (based on WHO parameters (WHO., 1999), presenting high and low ROS production respectively, and immature germ cells, which also presented low production of reactive oxygen species (Ollero et al., 2001; Gil-Guzman et al., 2001). This excessive production of ROS from immature sperm could cause DNA damage in mature sperm within the male reproductive tract and, hence, account partly for male infertility.

The applied assisted reproduction techniques (ART), as their title indicates, aim at achieving fertilization that is not able to occur naturally. However, research studies have shown that repeated cycles of centrifugation in the process of sperm preparation can induce the production of reactive oxygen species by spermatozoa. This means that improving the motility of sperm through the routine preparation does not necessary mean that occasional DNA damage does not occur at the same time, endangering the outcome of fertilization (Agarwal et al., 1994; Lopes et al., 1998a).

1.8.2. Sperm motility and hyperactivation

ROS hydrogen peroxide in particular, plays a positive physiological role in sperm hyperactivation and capacitation. Low concentration of a NO–releasing compound, has been shown to be beneficial to the maintenance of post thaw human sperm motility and viability (Hellstrom et al., 1992).

Besides, ROS production was also related to the outcome of in vitro sperm mucus penetration tests. Unstimulated levels of ROS production showed a significant ($p<0.05$),

negative correlation with the number of progressively motile spermatozoa present in the mucus after 15 minutes (r=-0.379) and 60 (r=-0.362) min. (Whittington et al., 1999).

1.8.3. Sperm capacitation

The molecular basis of sperm capacitation is still unclear. The calcium uptake, an increase in cAMP concentration, a rise in intracellular pH, an efflux of cholesterol from the sperm plasma membrane (Yanagimachi, 1994; Visconti et al., 1998) and tyrosine phosphorylation of specific protein have been shown to occur during this process (Aitken et al., 1995; Leclerc et al., 1996). Capacitation of spermatozoa may thus occur by different ROS, but it takes place specifically by H_2O_2 following an increase in cAMP, the activation of protein kinase A, and downstream tyrosine kinase activation (Aitken et al., 1998b). It has also been demonstrated by many authors (Griveau et al., 1994; de Lamirande and Gagnon, 1995; Herrero et al., 1999) that reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot -}$) hydrogen peroxidase (H_2O_2) and nitric oxide ($NO^{\cdot}$) can induce sperm capacitation in vitro. Production of nitric oxide ($NO^{\cdot}$) by spermatozoa has also been reported (Herrero and Gagnon, 2001) and may serve as an additional oxidant source. NO has also been suggested to be involved in the capacitation of spermatozoa but only in the presence of H_2O_2 (Zini et al., 1996).

1.8.4. Acrosome reaction

Reactive oxygen species other than H_2O_2, such as nitric oxide and superoxide anion (O2-) , have been shown to promote sperm capacitation and acrosome reaction (Griveau et al., 1995; Zini et al., 1996). Stimulation of endogenous NADPH-dependent ROS generation in human sperm appears to regulate acrosome reaction via tyrosine phosphorylation (Leclerc et al., 1997).

1.8.5. Sperm-egg binding

Aitken et al. (1989) demonstrated that low level of ROS enhance the ability of human spermatozoa to bind zona pellucida, an effect that was reversed by the addition of vitamin E.

Treatment of human spermatozoa with low concentration of NO- releasing compounds like sodium nitroprosside (SNP; 10^{-7} -10^{-8} M) in the capacitating medium increased the number of spermatozoa bound to the hemizona (Sengoku et al., 1998).

1.8.6. Sperm egg fusion

The production of low concentrations of hydrogen peroxidase (H_2O_2 and O_2^{-}) by spermatozoa may have a functional role in the signalling events controlling capacitation and sperm-oocytes fusion (de Lamirande and Gagnon, 1993 a, b; Aitken et al., 1995, 1998).

However, poor sperm–oocytes fusion and in vitro fertilization experiments (Aitken et al.,

1994; 1995) and in standard IVF (de Geyter et al., 1992; Krausz et al., 1994; Sukcharon et al., 1995; 1996) are related to high ROS production.

Aitken and West., (1990) suggests that high failure rate of sperm-oocyte fusion bioassay can be related to increased generation of lipoperoxidase. In a subset of infertile patients sperm were refractory to the second messenger signal generated by calcium ionophore, excessively generated ROS and exhibited a high failure rate in sperm-oocytes fusion bioassays (Aitken and West., 1990).

1.8.7. ROS level and fertilization.

The redox status of human spermatozoa is likely to affect phosphorylation and adenosine triphosphate (ATP) generation with a profound influence on its fertilizing potential. However, one of the main features of sperm that might profoundly affect fertilization and subsequent development is chromatin structure (Sakkas et al., 1998). ROS generation during oxidative stress associated with the appearance of damage to the DNA especially in infertile patients, leading to high incidence of DNA strand breaks (Evenson et al., 2002; Agarwal and Said, 2003). High levels of seminal ROS also impaired the sperm fertilizing capacity by DNA damage and apoptosis (Agrawal and Saleh., 2002; Agrawal and Said.,2003). Under normal circumstances, spermatozoa with damaged DNA would not participate in the fertilization process because of collateral peroxidative damage to the sperm plasma membrane (Kodama et al., 1996).

1.8. 8. ROS level and cleavage (embryos) quality

The production of ROS may also be involved in bovine embryo development (Blondin et al., 1997). Zorn et al. (2003) found that high seminal plasma ROS levels are associated with impaired sperm fertilizing ability and lower pregnancy rates after IVF. In ICSI, a negative association of ROS with embryo development to the blastocyst stage has been observed.

1.8. 9. ROS level and pregnancy

After IVF, fertilization and pregnancy rates were negatively associated with ROS level. In ICSI, a negative association of ROS with embryo development to the blastocyst stage has been observed, and significant fewer ICSI derived embryos reached the morula-blastocyst stage on day 4 (Zorn et al., 2003).

1.9. ROS and Sperm DNA Damage: Pathology of ROS

Several studies have supported that oxidative stress caused by ROS production induces damage of the sperm DNA, even though sperm DNA appears to be more resistant than other types of cells, e.g. somatic cells (McKelvey-Martin et al., 1997) Specifically, sperm DNA is

protected by its advanced packaging on one hand (Fuentes-Mascorro et al., 2000) and the antioxidants of seminal plasma on the other (Twigg et al., 1998a; b). OS-induced DNA fragmentation, which is widely observed in spermatozoa of infertile men (Kodama et al., 1997; Sun et al., 1997), may not directly affect the fertilizing ability of spermatozoa, but it directly affects their contribution to normal embryonic development (Lewis and Aitken., 2005) and the mutational load of the embryo (Aitken ., 1999) (see figure 2). Evidence also supports that OS causes significant damage not only to the nuclear, but also to the mitochondrial DNA of human spermatozoa (Sawyer et al., 2003).

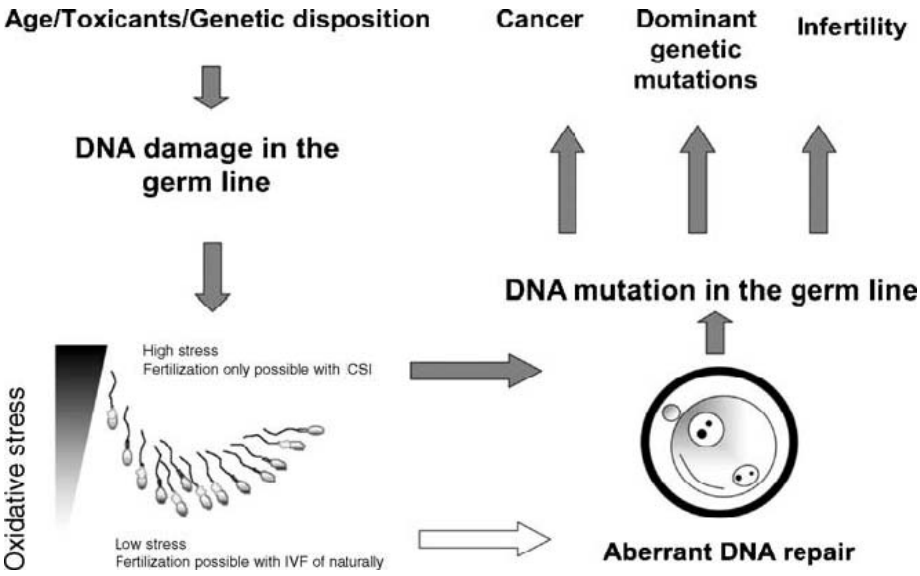

Figure 1. Schematic representation of the possible relationship between OS in the male germ line and abnormalities in the development of the embryo and the health and wellbeing of the offspring (Aitken and Baker., 2006).

Along their way from the testis to the oocyte, spermatozoa encounter many factors and conditions that can target their DNA and potentially damage it. Defective packaging of chromatin in the nucleus, apoptosis and ROS are the causes that attract the most scientific interest (Sakkas et al., 1999; Shen and Ong., 2000). Sperm DNA damage may also account for the loss of the ability to conceive naturally (Evenson et al., 1999; Spano et al., 2000) and for a number of cases of unexplained pregnancy loss (Carrell et al., 2003). As far as ARTs are concerned, their association with DNA damage has been widely discussed and examined in a variety of studies, and extensively reviewed by Agarwal and Allamaneni (Agarwal and Allamanani ., 2004).

1.9.1. Oxidative stress induced Apoptosis

The programmed death of eukaryotic cells, which occurs without inducing an inflammatory response, is called apoptosis (Majai et al., 2006). Apoptosis during spermatogenesis has been assessed, discussed and supported in several studies (Hikim et al., 1998; Kierzenbaum , 2001) and has been associated with male infertility (Hikim et al., 1998; Jurisicova et al., 1999**).** However, research on apoptosis in ejaculated spermatozoa seems to still be in its developing stages, with only a few studies discussing it (Sun et al., 1997; Gorczyca et al., 1993; Irvine et al., 2000), the latest primary article being that of Wang et al. (Wang et al., 2003). More specifically, the latter group reported positive correlation between the presence of apoptotic markers, due to OS-induced apoptosis, and spermatozoa DNA damage. OS-induced apoptosis in ejaculated spermatozoa has also been reviewed recently by Agarwal and Said (Agarwal and Said., 2005).

1.9.2. Role of NADPH and NADPH oxidase activity

Reactive oxygen species are the intermediate steps of oxygen reduction, namely, O_2-, H_2O_2 and OH are the products of reduction by one, two or three electrons respectively. Also, HO_2, the acid of superoxide anion, is another free radical with major contribution in the destructive lipid peroxidation process occurring in spermatozoa (Alvarez and storey., 1992). Exogenous molecules, like aromatic derivatives and iron complexes, with low molecular weight, can activate molecular oxygen, by catalyzing the electron transfer to it. They can also do that by activating the β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which theoretically exists in human spermatozoa and is believed to transfer electrons from NADPH to ground state oxygen, leading to the formation of the superoxide anion radical. The latter is then dismutated to hydrogen peroxide (H_2O_2), which is controlled by the antioxidant glutathione peroxidase. In case the function of the antioxidant is impaired for any reason, the spermatozoa experience hyperoxidation (oxidative stress status) (see figure 3) (Baker and Aitken ., 2005). Several other studies have supported the presence of NADPH oxidase-like activity, at the sperm plasma membrane, in human spermatozoa and the role of NADPH in O_2^- production and the subsequent consequences of the induced OS on the spermatozoa (Aitken et al., 1997; Fisher and Aitken ., 1997; Vernet et al., 2001; Lewis and Aitken 2005; Twigg et al., 1998a). The second ROS-generating system that has been proposed is a sperm diaphorase system, located in the middle piece and integrated into the mitochondrial

respiratory system of the spermatozoa (Aitken et al., 1997).

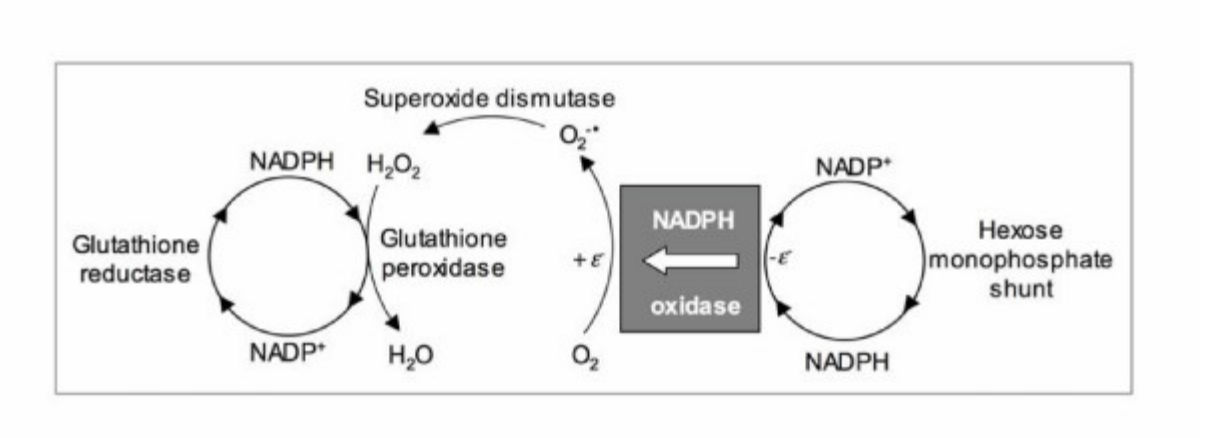

Figure 2. Schematic representation of NAD(P)H oxidase activity. This enzymes transfers electrons from NAD(P)H to ground state oxygen to create the superoxide anion radical. The latter then dismutates to hydrogen peroxide under the influence of superoxide dismutase. The hydrogen peroxide is predominantly scavenged by glutathione peroxidase, since human spermatozoa possess little catalase activity. Once this peroxidase activity is overwhelmed, a state of oxidative stress may be induced that disrupts the fertilizing capacity of the spermatozoa and the integrity of their DNA. Baker and Aitken *Reproductive Biology and Endocrinology* 2005 **3**:67 :10.1186/1477-7827-3-67

1.9.3. Leukocytospermia and male infertility

Apart from their role as members of the immune-defensive mechanism, leukocytes are also vital for the clearance of defective sperm via phagocytosis (Tomlinson et al., 1992). Leukocytospermia is a condition which, according to WHO guidelines (1999), is determined by the existence of a concentration greater than 1×10^6 /ml of peroxidase-positive leukocytes in semen and it is encountered in an average of 15% of infertile men (Sharma et al., 2001; Alvarez et al., 2002; Agarwal and said., 2003). Nevertheless, whether or not it is a pathological condition, leading to abnormal morphology and impaired sperm function is controversial among scientists.

Specifically, there are studies that found negative and other studies that found positive correlation of poor sperm quality/function, with increased white blood cell (WBC) concentration (leukocytospermia) (Wolff., 1995). More specifically, Curi et al. concluded that there is no positive correlation between leukocytospermia and impaired sperm motility asthenozoospermia (WHO., 1999). In another individual study, Tomlinson et al. could not find any association of leukocyte concentration neither with impaired sperm quality, nor with conception rates (Tomlinson et al., 1993). Also, no correlation was found between leukocyte counts, sperm density and motility, sperm antibodies and growth of micro-organisms, by(El-Demiry et al., 1987; Jeulin et al., 1989).

On the other hand, Yanushpolsky et al. (1996) presented a positive association of increased seminal granulocyte concentrations with abnormal semen parameters of statistical and clinical significance. In addition, Arata de Bellabarba et al. showed that increased WBC are a usual phenomenon in semen of infertile men and are associated with semen of poor quality parameters (Arata et al., 2000). Moreover, Lemkecher et al. supported the positive correlation, but also associated leukocytospermia with increased DNA fragmentation (Lemkecher et al., 2005). Aitken et al. also presented positive correlation between leukocytospermia and impaired sperm function, when leukocytes were introduced into the sperm mixture after preparation. The same correlation, when assessed during in vitro fertilization (IVF), was found to be negative (Aitken et al., 1994). In contrast, Vicino et al. showed that increases leukocyte concentration in semen can affect the results of both IVF and ICSI procedures (Vicino et al., 1999).

Apart from sperm quality, the effect of leukocytospermia on sperm morphology is also a highly controversial issue between researchers. Hence similarly, several studies have presented positive correlation between increased leukocyte concentration in semen and abnormal sperm morphology. Specifically, Berger et al. showed positive correlation between morphologically normal sperm and sperm penetration ability, in contrast to morphologically abnormal sperm due to increased leukocyte concentration (Berger et al., 1982). Eggert-Kruse et al. presented positive correlation between high rates of leukocytes of the round cells with increased morphological abnormality (Eggert-Kruse et al., 1992) and Yanushpolsky et al.(1996) presented statistically significant differences in sperm morphology and leukocyte concentration of double threshold according to the WHO criteria (Yanushpolsky et al. (1996). Moreover, Thomas et al. and Menkveld and Kruger presented positive correlation between WBCs in general and polymorphonuclear granulocytes (PMN) respectively, with morphological defects (Menkveld and Kruger., 1998; Thomas et al., 1997). Finally, two years ago, a primary article by Aziz et al. presented a highly significant negative correlation between leukocytospermia and impaired sperm structural integrity (Aziz et al., 2004).

On the contrary, two more studies presented a statistically insignificant association between increased concentrations of leukocytes and morphologically abnormal sperm (Fedder et al ., 1993; Van der Ven., 1987). To enhance the controversy concerning the role of leukocytes in abnormal sperm morphology, Kiessling et al. and Tomlinson et al. presented a positive correlation between increased leukocyte numbers and morphologically normal sperm, due to leukocytes' physiological function of abnormal-sperm phagocytosis (Tomlinson et al., 1992; Kiessling et al., 1995).

2-Antioxidants and male infertility

Types, role and origin

ROS are necessary for physiological functioning of spermatozoa, but they need to be controlled and their concentration maintained at a level that is not deleterious to the cells. This function is carried out by antioxidants (Young et al., 2001) (see Diagram 1), present in the seminal plasma (Henkel., 2005; Alvarez and Storey et al., 1983; Sies ., 1993; Sikka ., 1996; Zini et al., 2001a). The most common antioxidants, that protect spermatozoa from excess concentrations of ROS and OS-induced damage and altogether represent the total antioxidant capacity (TAC) of seminal plasma, are superoxide dismutases (SOD) (Alvarez et al., 1987) catalase (CAT) (Jeulin et al., 1989), the glutathione (GSH) peroxidase system with most discussed members selenium and selenoproteins like phospholipids hydroperoxide glutathione peroxidase (PHGPx) and the glutathione reductase system (Alvarez and Storey., 1989), vitamins A, C (Niki., 1991) and vitamin E (Chow ., 1991), glutathione (Kidd., 1997), spermin, thiols, urate (Gavella et al., 1997), albumin, taurine and hypotaurine (Alvarez and Storey., 1983), L-carnitine and zinc. Additionally, the possible protective antioxidant role of vitamins K and D has been reported in literature (Kodentsova et al., 1994). These antioxidants work together as a wide network of protection and many of them become radicals themselves while scavenging oxidants. The rest of the antioxidants in the network make sure that they are regenerated back to their original structure. For example, vitamin C and glutathione regenerate vitamin E. However, this antioxidant protection is not always strong enough to protect from OS. When the volume and distribution of the cytoplasm of spermatozoa is abnormal, antioxidant enzymes are unable to be hosted properly, and therefore their defensive, chain-breaking function is impaired (Aitken and Baker., 2002; Lewis et al., 1995).

Table 1. Some natural antioxidants.

Table 3. Some natural antioxidants.

Non-enzymatic antioxidant molecules		
Antioxidant molecule		Subcellular location
Ascorbate (vitamin C)		Plastid; apoplast; cytosol; vacuole
ß-Carotene		Plastid
Glutathione, reduced (GSH)		Plastid; mitochondrion; cytosol
Polyamines (e.g., putrescine, spermine)		Nucleus; plastid; mitochondrion; cytosol
α-Tocopherol (vitamin E)		Cell and plastid membranes
Zeaxanthin		Chloroplast
Antioxidant enzymes		
Enzyme	EC number	Subcellular location
Ascorbate peroxidase	1.11.1.11	Plastid stroma and membranes
Peroxidases (non-specific)	1.11.1.7	Cytosol; cell wall-bound
Catalase	1.11.1.6	Glyoxysome; peroxisome; cytosol; mitochondria
Superoxide dismutase (SOD)	1.15.1.1	Cytosol (Cu/ZnSOD); plastid (Cu/ZnSOD; FeSOD); mitochondrion (MnSOD); peroxisome
Dehydroascorbate reductase	1.8.5.1	Cytosol; plastid
Glutathione reductase	1.6.4.2	Mitochondrion; cytosol; plastid
Monodehydroascorbate reductase	1.6.5.4	Plastid stroma
Glutathione S-transferases	2.5.1.18	Cytosol; microsomal

Adopted from: Scandalios JG: oxidative Stress: molecular perception and transduction of signals triggering antioxidants gene defences. Braz J Med Biol Res 2005; 38: 995-1014

A number of reports have discussed the possible origin of antioxidants in semen, but our knowledge on that is still limited. Some of the studies supported the testicular origin of semen antioxidants, while others have presented evidence that the source of antioxidant activity is post-testicular. More specifically, in 1994, Bauche et al., presented evidence that SOD is of testicular origin, protecting it from deleterious ROS concentrations (1994). In 1998, Yeung et al. supported that the source of antioxidants is post-testicular, probably mainly the prostate and seminal vesicles (Yeung et al., 1998). Four years later, Zini et al. empowered that report, showing that antioxidants are post-testicular products and their probable role is the protection of ejaculated spermatozoa from OS such as that which occurs in the female reproductive tract (Zini et al.,2001b).

Association with Subfertility: A Remedy?

ROS increase or antioxidant deficit have been associated with a number of pathological conditions by several scientific studies. The protective action of antioxidants against the

deleterious effect of ROS on cellular lipids, proteins and DNA has been supported by several scientific studies (Sies., 1993). In this review, we examined the literature using PubMed and separated the publications into three groups, namely, studies discussing natural (enzymatic), synthetic (non-enzymatic) and both, respectively.

Antioxidants, in general, are free radical scavengers that suppress the formation of ROS and /or oppose their action. There are many antioxidants in seminal fluid; they can divide into two groups; Enzymatic like: Superoxide dismutase, catalase (Zini et al., 1993), and glutathione peroxidase (Alvarez and Storey. 1989) and nonenzymatic antioxidants, such as ascorbate, urate, alpha-tocopherol, pyruvate, glutathione, taurine, and hypotaurine (Saleh and Agarwal., 2002). Antioxidants that are present in the seminal fluid compensate for the deficiency in cytoplasmic enzyme in the spermatozoa (Donnelly et al., 1999). Spermatozoa themselves posses high concentration of thiol groups, as well as smaller amounts of ascorbic acid, alpha-tocopherol, uric acid and glutathione (Li., 1975; Lewis et al., 1997; Ochsendorf et al., 1998).

ROS production and total antioxidant capacity (TAC) can be used as a marker of oxidative stress in seminal fluid and is correlated with male infertility. Infertile men with male factor or idiopathic diagnoses had significantly lower ROS-TAC scores than controls (Sharma et al., 1999). Said et al (2005) suggested that abnormal sperm morphology combined with elevated ROS production may serve as a useful indicator of potential damage to sperm DNA Lisak et al (2004) examined the effect of oxidation of protein and lipids by a thermochemiluminescence (TCL) analyzer and showed that thermochemiluminescence indices in seminal plasma closely correlate with sperm characteristics among patients with sperm disturbances. Morphologically abnormal and immature spermatozoa that retain cytoplasmic residues in the mid piece can be separated by a double-density gradient procedure (Hall et al., 1995; Ollero et al., 2001; Gil-Guzman et al., 2001).

2.1. Enzymatic antioxidants

2.1.1. Superoxide dismutase (SOD)

Human sperm contains the enzyme system comprising glutathione peroxidase (GPX), glutathione reductase (GRD), and their substrates, glutathione (GSH) and glutathione disulfide (GSSG) (Alvarez et al., 1987; Alvarez and Storey ., 1989; Li ., 1975). This system functions as a defence against lipid peroxidation in human sperm by reducing formed lipid hydroperoxides, which are very reactive and damaging to the plasma membrane, and to hydroxylipids, which are essentially inert (Storey et al., 1998).

Superoxide dismutase (SOD), catalase, and glutathione peroxidase are antioxidants that convert superoxide (O_2) and peroxide (H_2O_2) radicals to form O_2 and H_2O.

2.1.2. Superoxide dismutase (SOD)

One family of the antioxidants used by organisms as a defence mechanism against oxidative damage, is in the family of superoxide dismutases (SOD) (***see*** figure 3) (Marklund., 1982; Marklund; 1984; McCord., 1969). One of the members of this family, namely, extracellular superoxide dismutase (EC-SOD), is a vital part of the defense against ROS-mediated tissue damage, and it is located in the extracellular matrix of tissues, hence, the name (Marklund et al., 1982., Marklund et al., 1986). SOD acts scavenges the superoxide anion, which is produced by a one-electron reduction of an oxygen molecule and initiates a radical chain reaction.

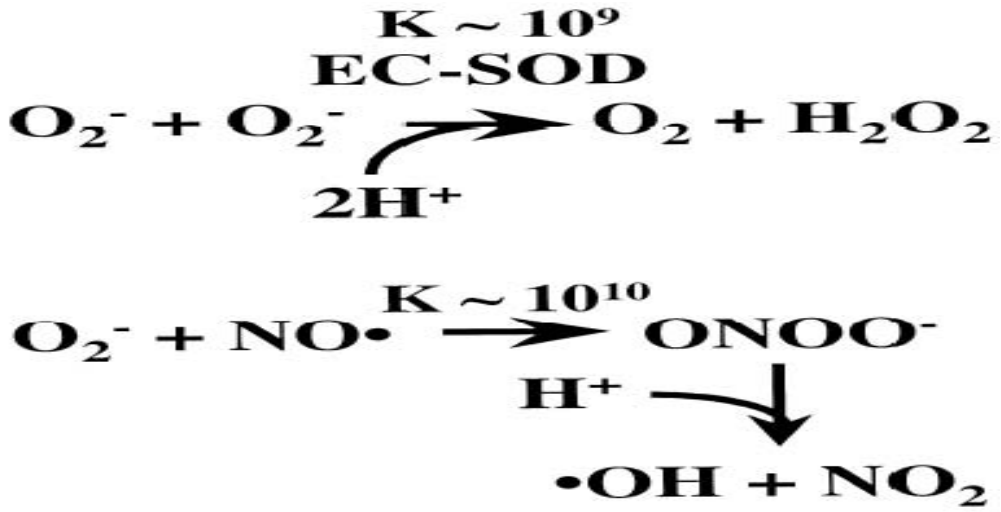

Figure 3. The enzymatic dismutation of superoxide anion by SOD, such as EC-SOD.

In addition, the reaction of superoxide with nitric oxide to produce peroxynitrite anion is depicted along with the products of peroxynitrite decay. (Adapted from (Oury et al., 1996)

In 2000, Potts et al., reported that seminal plasma contains antioxidants, whose role is to protect the spermatozoa from DNA damage and lipid peroxidation. Three years later, Lamond et al. presented evidence that adding SOD to the IVF media protects the sperm chromatin from breakdown (Lamond et al., 2003). Earlier, in 1992, Nonogaki et al. had shown that adding SOD to the culture medium protects the sperm viability and the development of the embryo *in vivo* and *in vitro* (Nonogaki et al., 1992).
The SOD antilipoperoxidative defence system in human sperm relies almost entirely on the activity of a single enzyme, the Cu/Zn isoform (Lasso et al., 1994).
SOD protects against spontaneous O_2 toxicity and lipid peroxidase (Blum and Fridovich ., 1985). SOD and catalase also remove (O_2^-) generated by NADH-oxidase in neutrophils and can play an important role in protecting spermatozoa during genitourinary inflammation (Baker et al., 1996).
The steady-state concentration of superoxide is under the control of extracellular SOD, the inhibition of which (by copper chelating) leads to the rapid disappearance of thiols (Miesel et al., 1993). A pivotal role of SOD in protection of testicular cells against heat stress–Induced apoptosis has been demonstrated in vivo and in vitro (Ikeda et al., 1999; Kumagai et al., 2002).

2.1.3. Catalase

Catalase is a well known antioxidant enzyme. Its localization is limited to peroxisome. Catalase activity has also been determined in human spermatozoa and seminal plasma of fertile and infertile males (Jeulin et al., 1989). However, a difference in the seminal catalase activity of asthenozoospermic and oligo-asthenozoospermic with hyperviscosity have been shown (Siciliano et al., 2001).

2.1.4. Protein and Sulfhydryl group (SH-group)

A sulfhydryl group (SH-group) plays an important role in sperm metabolism and the antioxidative defence. Nakamura et al., (2002) demonstrated that seminal plasma superoxide dismutase, catalyse, glutathione peroxidase and sulfhydryl group levels are significantly lower in infertile patients than those in controls, suggesting their relationship to male infertility.

2.1.5. Glutathione

Reduced glutathione is also part of the antioxidant system in the seminal fluid. Glutathione is a tripeptidyl molecule and present in either reduced (GSH) or the oxidized form (GSSG) by forming a disulfide bond between two molecules.

Lenzi et al found that glutathione injection (600mg/day, i. m.) for two months significantly improved the morphology and motility patterns of spermatozoa. Even the in vitro use of glutathione treatment increased the forward motility and migration from the pellet of spermatozoa obtained from leukospermia samples (Lenzi et al. 1992). Parinaud et al (1997) showed in vitro enhancement of sperm motility using a migration salt solution containing glucose and glutathione as an antioxidant..

2.1.6. L, N-Acetyl-Cysteine.

Glutathione (γ-glutamyl-cystenyl-glycine) is a natural, highly effective reducing agent. N – acetyl-cysteine is a potential regulator of germ-cell death. It is a well established inhibitor of physiological cell death in several systems. N-acetyl-L cysteine, a reducing substance, has been shown to improve sperm motility in vitro together with a decrease of ROS levels in infertile patients with high seminal level of ROS. L, N-Acetyl-Cysteine (0.1, 1 and 5 mg/ml) has a dose dependent effect in reducing ROS levels; the reduction was greater in patients with high levels of ROS than in those with low levels (Oeda et al., 1997). N-acetyl-cysteine and /or a mixture of essential fatty acid and natural vitamin A and E reduced levels of 8-hydroxy-2`-deoxyguanosine (8-OH-dG), which considered as a marker of oxidative stress-induced sperm DNA damage (Agarwal ., 2004)..

N, L Acetyl-L-Cysteine when given in concentration of 125, 100, 50 and 25 mmol/L suppressed germ cell death in a dose-dependent manner, However, more research is needed to validate its efficacy in vivo (Erkkilia et al., 1998)..

2.1.7.Glutathione peroxidase

Glutathione peroxidase and catalase activities are of prostatic and multi-glandular origin, respectively (Yeung et al 1998)..

Glutathione peroxidase, a selenium-containing antioxidant enzyme with glutathione as the electron donor, removes peroxyl radical from various peroxides including H_2O_2 to improve sperm motility. Minor alterations in sperm membranes in selected cases of dyspermia can be reversed by glutathione (GSH) therapy (Irvine , 1996). The presence of glutathione may prevent the accumulation of peroxynitrite ($ONOO^-$) to toxic levels and may convert $ONOO^-$ to secondary products with protective properties (Muijsers et al., 1997)..

Low level of NADH and glutathione, as a result of the increased activity of glutathione peroxidase to remove metabolites of membrane lipid peroxidation (LPO) will further affect cellular Ca_2^+ homeostasis (Alvares and Storey , 1989).

The selenium-containing enzyme glutathione peroxidase destroys peroxides before they can damage cell membrane and interacts synergistically with vitamin E (Wefers and Sies., 1988). Glutathione peroxidase has been measured in seminal plasma and correlated with male infertility (Giannattasio et al., 2002). Yeung et al (1998) studied the GPX, GSH reductase, and superoxide dismutase activities of normozoospermic patients and patients undergoing in vitro fertilization treatment and suggested that the origin of the GPX, GSH, reductase, superoxide dismutase is neither testicular nor epididymal..

Glutathione peroxidase has been shown to be able to maintain sperm motility, through scavenging of peroxyl radicals (ROO-) (Calvin et al. 1981) and also protect sperm from lipid peroxidation (Lenzi et al., 1994). Additionally, GSH has been shown to be a vital antioxidant mechanism maintaining normal sperm motility (Sikka 1996; Baumber et al., 2000) and the ability of sperm to undergo acrosome reaction (Baumber et al., 2000; Sikka 1996). In an earlier study, Lenzi et al. presented that glutathione therapy for 2 months has a statistically significant positive effect on sperm motility and morphology (Lenzi et al., 1994). Phospholipids hydroperoxid glutathione peroxidase is expressed at higher levels in rat testes than in any other tissue (Roveri et al 1994).

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is a unique intracellular enzyme. Its uniqueness lies in the fact that it has the ability to reduce intracellular membrane phospholipid hydroperoxides, hence, the name (Godeas et al., 1997). The highest activity of PHGPx has so far been measured in the testis, with a significant difference from that of other tissues, like brain and liver (Ursini et al., 1995; Maiorino et al., 2003). Due to the fact that sperm cells have high numbers of polyunsaturated fatty acids, they depend on PHGPx (together with other scavenging systems) to protect them from OS and lipid peroxidation (Lenzi et al., 2000) and that is the reason its concentration in spermatozoa is relatively high (Godeas et al., 1997).

2.1.8. Glutathione reductase

Glutathione reductase regenerates reduced GSH from its oxidized form (GSSG).

GSH has a likely role in sperm nucleus decondensation.

2.1.9 Alpha-glutamyl transpeptidase

Alpha-glutamyl transpeptidase is present in the midpiece and acrosomal region of spermatozoa and it may further regulate the GSH content of oocytes at the time of sperm penetration (Irvine, 1996).

2.2. Non-enzymatic antioxidants

Beside of enzymatic antioxidant system, there are numerous non-enzymatic low molecular mass antioxidants which are believed to be even more important scavengers than high molecular mass compounds (Zini et al., 1993).

2.2.1. Nitric oxide

Nitric oxide (NO) is synthesized from L-arginine by a family of enzymes known as the nitric oxide synthetases (NOS). NO is a molecule of great biological significance and has long been considered to play an important role in sperm physiology (Aitken and Fisher, 1994).

NO can act as a free radical scavenger, inactivating (Alvarez et al., 1987; McCall et al., 1989) and even inhibiting production of superoxide anion (O_2^{-}.)(Chancy et al., 1992), which causes lipid peroxidation, a process which leads to functional impairment of spermatozoa (Jeyendran et al., 1984). NO produced directly by spermatozoa and that constitutive NOS is present in two isoforms similar to those present in both endothelial (ecNOS) and in brain (bNOS) cells. Besides, spermatozoa from normozoospermic samples appeared to have greater amounts of NOS and higher amounts of NO production than those from asthenozoospermic samples (Lewis et al., 1997).

2.2.2. Albumin

Human serum albumin (HSA) present in culture media serves an antioxidants. Human serum albumin has a cystein residue in position 34 which is not involved in a disulfide bond and may exist in different oxidative states: as a fully reduced sulfhydryl group, as a mixed disulfide with e.g cystein, glutathione or homocystein or in a higher oxidative state like sulfonic acid. Serum albumin is discussed as a marker for systemic oxidative stress (Oettl et al., 2005).

Albumin is reported to exhibit an excellent ability to sustain sperm motility (Twigg et al., 1998b).

2.2.3. Vitamine C and E.

The most important antioxidant in seminal fluid seems to be vitamin C and vitamin E (Chow,

1991; Niki et al., 1991). Concentration of vitamin C in seminal plasma is 10 times greater than in blood plasma (364 vs. 40 micro mol/L) (Lewis et al., 1997). During nonenzymatic recycling pathway for vitamin E regeneration that occurs in membrane, vitamin C reduces chromanoxyl radicals to recycle vitamin E and eventually being itself consumed by the process (Packer, 1993). Vitamin C intake may reduce DNA strand breaks (Green et al., 1994) in human lymphocytes. Dietary supplementation of vitamin C protects human sperm from endogenous oxidative DNA damage (Fraga et al., 1991). Hughes et al. (1998; 1999) found that in vitro treatment of sperm with antioxidants (300 and 600μM ascorbic acid; 3 and 60μM alpha-tocopherol, and 400μM urate) reduce the magnitude of DNA damages as measured by comet assay.

Vitamin E is a term that encompasses a group of potent, lipid-soluble, chain-breaking antioxidants. Structural analyses have revealed that molecules having vitamin E antioxidant activity include four tocopherols (αβγδ) and four tocotrienols (αβγδ) (review Brigelius- Flohe, 1999). Vitamin E is known to readily reduce alkyl peroxy radicals of unsaturated lipids (Burton et al., 1983). It is a major chain-breaking antioxidant in the sperm membranes and it appears to have a dose-dependent protective effect (Bolli et al., 2002).

Vitamin E proved to be effective in preventing lipid peroxidation and other radical-driven oxidative events (Esterbauer et al., 1991). Vitamin E functions as a chain-breaking antioxidant that prevents the propagation of free radical reaction (Ingold et al., 1987; Kamal-Eldin and Appelqvist., 1996). Vitamin E prevents loss of spermatogenesis in males and the failure to retain zygote in female rats (Evans and Bishop, 1922). Many investigators have found a reduction in concentration of oxidized DNA (8-hydroxy-2 deoxyguanosine) in sperm after antioxidants supplementation (Kodama et al., 1997; Comhaire et al., 2000).

Vitamin E, inhibits lipoprrotein oxidation (LPO) in membranes by scavenging peroxyl ($RO^{\cdot}$) and alkoxyl ($ROO^{\cdot}$) radicals. However, the ability of alpha-tocopherol to maintain a steady-state rate of peroxl radical reduction in the plasma membrane depends on the recycling of α-tocopherol by external reducing agents such as ascorbate or thiols (Wefers and Sies, 1988).

Semen vitamin E levels were not increased after 3 months treatment with 400 mg vitamin E plus 500 mg vitamin C daily, although there was a marked increase in serum concentration (Whittington, 1997).

Ascorbic acid (vitamin C) is an important water-soluble antioxidant that reduces sulfhydryls, scavenger's free radicals, and protect against endogenous oxidative DNA damage (Fraga et al., 1991). Small doses of vitamin C (200 mg), have been shown to increase the seminal level of ascorbate in smokers from 5.6 to 13.1 mg/dl, which is similar to the level achieved (16.1 mg/dl) after 1000 mg of vitamin C (Dawson et al., 1992). In infertile patients with a high level of oxidative DNA damage in spermatozoa, even the combination of vitamin C and E with glutathione induced only a slight increase in sperm concentration (Kodama et al., 1997).

Administration of vitamin C (350 mg/d) and E (250 mg) together in vivo were not able to prevent DNA sperm damage occurring after ejaculation (Hughes et al., 1998). Vitamin C may become a pro-oxidant when free transition metals are present (Guerin et al., 2001; Yamamoto and Niki, 1988). In 2005, Greco et al., presented an interesting study, supporting the antioxidant role of vitamins C and E. More specifically, they detected a reduction in ejaculated spermatozoa DNA fragmentation, after treatment with a combination of the two vitamins. The interesting aspect of this study was that it was the first one to present the direct effect of antioxidant treatment on sperm DNA integrity in vivo (Alvarez and Storey ., 1995).

2.2.4. Urate

Ascorbate, urate and protein sulfhydryls are the major antioxidants present in seminal plasma, while GSH is practically undetectable (Yeung et al., 1998).

The proposed protective function of uric acid against free radicals in human blood (Becker, 1993) has not been sufficiently investigated in seminal plasma. Very few data could be found in the literature on its presence in seminal plasma (Persson et al., 1991) and on its antioxidative defence properties (Thiele et al., 1995). Gavella et al. (1997) found changes in total seminal plasma antioxidative capacity chiefly due to ascorbate and urate. Orally supplemented ascorbic acid is rapidly distributed in all body tissue, with the highest concentration in seminal vesicles (Mann and Lutwak-Mann, 1981; Dawson et al., 1990).

2.2.5. Selenium

Selenium, in the form of selenocysteine, functions as the catalytic centre in the active sites of at least 9 human enzymes, including 4 glutathione peroxidase antioxidant enzymes (Cohen and Takahashi, 1986; Zhang et al., 1989; Chu et al., 1996).

It is well known that, selenium is involved in male reproductive process. In maturing rats the testicular content of selenium increased greatly at the beginning of spermatogenesis (Behne etal., 1986). Selenium is also required for normal testicular development and spermatogenesis in rats (Behne et al., 1996). Rats with low selenium levels produced sperm with impaired motility and characteristic mid piece damage (Wu et al., 1996; Mc Coy and Weswig, 1969) indicating that selenium is necessary for normal sperm development.

The specific role of selenium in spermatogenesis appears to be related to phospholipids hydroperoxide glutathione peroxidase, which is expressed depending on the development state of spermatids (Maiorino et al., 1998), and seems to be converted into a structural component in the midpiece of mature spermatozoa (Maiorino et al., 1998).

Selenium deficiency is associated with impaired sperm motility, structural alteration of midpiece, and loss of flagellum (Brown and Burke, 1973). In mature spermatozoa, selenium is largely restricted to the mitochondeerial capsule, a keratin-like matrix that embeds the helix

of mitochondria in the sperm midpiece (Calvin et al., 1981). Phospholipids hydroperoxid glutathione peroxidase is expressed at high levels in rat testes than in any other tissue (Roveri et al., 1994).

Deficiencies of selenium or glutathione can lead to instability of mid–piece of spermatozoa, resulting in defective motility (Ursini et al., 1999; Hansen and Deguchi, 1996). Deficiencies of selenium or glutathione can lead to instability of the mid–piece of spermatozoa, resulting in defective motility (Hansen et al. 2001; Ursini et al., 1999).

2.2.6. Zinc

Zinc is an element whose importance in the biological systems is undisputed. Its importance can be understood by considering that when it is deficient, severe pathological consequences occur, like for example, in acrodermatitis enteropathica, a rare autosomal-recessive inheritable disease (Eggert-Kruse et al., 2002). As far as reproduction is concerned, this element has been shown to be highly important for conception, successful implantation and pregnancy outcome (Prasad ., 1995; Stephenson and Brackett ., 1999). Zinc is present in high concentrations in the seminal fluid and there is evidence that it may act in vivo as a scavenger of excessive O2- production by defective spermatozoa and/or leukocytes in semen after ejaculation (Gavella and Lipovac., 1998).

There is evidence that Zinc plays a vital role in the physiology of spermatozoa and spermatogenesis. Specifically, Bedwal and Bahuguna reported that this element decreases testicular weight and causes shrinkage of seminiferous tubules (Bedwal and Bahuguna, 1994). Its potential role in sperm production, viability and prevention of spermatozoa degradation and sperm membrane stabilization has also been supported (Lewis-Jones et al., 1996). After ejaculation, the present abnormal spermatozoa are sources of oxidants, namely, superoxide anions, which bind with Zinc and reduce its concentration in seminal plasma. Thus, Zinc is considered to be a vital antioxidant, guarding normal spermatozoa against superoxide anion-induced OS (Plante et al., 1994; Irvine ., 1996). Chia et al. supported that zinc concentration in seminal plasma is significantly correlated with sperm density, motility and viability (Chia et al., 2000).

The biological function of zinc and the characteristic features of zinc deficiency have been reviewed (Hidiroglou ., 1984; Prasad., 1985). Zinc is vital for spermatogenesis and for the development of primary and secondary sexual characteristics (Davies ., 1985). Experimental zinc deficiency in humans leads reversibly to reduced sperm count combined with reduced serum testosterone (Abbasi et al., 1980). There is a link between zinc deficiency and oligospermia (Piese., 1983).

The total zinc content of mammalian semen is high, 800-3000 µm/g of dry weight, and it has been demonstrated that zinc deficiency induces atrophy of the seminiferous tubules and

causes failure of spermatogenesis in rats (Stoltenberg et al., 1997). It has been suggested that the zinc ion exchange takes place between the epididymal epithelium and sperm cells as they pass along the epididymal duct and it might be significant for the maturation process of rat sperm cells during their passage through the epididymis (Fujimori et al., 1988; Stoltenberg et al., 1996).

3. Smoking and male infertility

Smoking is one of the most extensively experienced and potentially hazardous social habits throughout the world. It is more common among the male population compared to the female one. Cigarette smoke contains a number of substances such as nicotine, carbon monoxide, and recognized carcinogens and mutagens such as radio active polonium, cadmium, benzo(a) pyrene, dimethylbenz(a) anthracene, dimethylinitrosamine, naphthalene and metaphthalene (Ravenhott, 1982; Zavos, 1989 ; Zenzes 2000). Because of the fact that cigarette smoke contains mutagens and carcinogens, there have been concerns that smoking may have adverse effects on male reproduction. However, the influence of cigarette smoking on male fertility and sperm fecundity remains as highly controversial issue. Several studies have suggested association between semen characteristics and smoking, tobacco chewing and other hazardous chemicals. Active or passive smoking, or inhalation of cigarette smoking, lead to absorption of these agents through vasculature and blood-borne circulation throughout the body (Stillman et al., 1986).

There are various modes of diffusion and active transport through which Cigarette Smoking (CS) derived substances could end up in the Seminal Plasma (Zavos et al., 1998). Several investigations have been conducted to assess the relationship between cigarette smoking and male infertility (Calzada et al., 1992, Merino et al., 1998, Wong et al., 2000) but the exact molecular mechanism are not fully understood in most of the cases.

3.1. SMOKING AND SPERMATOGENESIS

The effect of nicotine on male fertility remains controversial and several mechanisms have been suggested as being responsible for the impairment of spermatogenesis (Weisberg, 1985), induction of ulrastructural abnormalities (Zavos et al., 1998) and apoptosis (Gandini et al., 2000, Sakkas et al., 2002).

It has been reported that nicotine and its soluble metabolite cotinine could be detectable in the seminal plasma of smokers; other harmful tobacco smoke compounds were showed to pass through the blood-testis barrier (Vine et al., 1993).

In habitual heavy smoking, long-term exposure to cigarette smoke, whose by-products move across the blood testis barrier and exist in high concentrations in the testis, may adversely affect the sperm function.

3.2. Nicotine induce apoptosis

The administration of nicotine, a key neuroactive component of cigarette smoke induces apoptotic cell death in various organs including brain, spleen, and thymus **(**Hakki et al .,2001).

Nicotine has received much attention for its interference with normal endocrine function, and it has been shown to cause testicular atrophy, gonadal dysfunction, and male factor infertility by triggering testicular cytotoxicity (Gocze and Freeman., 2000).

Lydig cells, which are located in the interstitial component of the mammalian testis, are responsible for the bulk of testosterone production in men (Iwabe et al., 2002); and it has been reported that nicotine suppresses the secretion of testosterone in adult male rats (Mio et al.,1992; Harrada et al., 1997). Leydig cell secretory deficiency, as well as defects in epididymal sperm maturation, in rats exposed to cigarette smoke, result in defects in the epididymal sperm maturation process and the sperm's capacity to penetrate the oocyte (Yamamoto et al., 1998). Therefore, destruction of leydig cell may cause testicular atophy, gonadal dysfunction, and male factor infertility. Kishimoto et al., (1998) reported that nicotine administration induces changes in gonadal functions and deficiency in sperm maturation and spermatogenesis and has a detrimental effect on the sperm fertilizing potential of the male rats. The production and function of healthy normal spermatozoa was affected by the number of cigarettes smoked per day, the years of smoking and the level of nicotine by-products present in the body fluids, which correlate negatively with semen and sperm quantity and quality (Zavos, 1989; Chi et al., 1994). In addition, smoking have been found to affect

accessory glands (e.g. prostate and seminal vesicles (Pakrashi and Chatterjeg, 1995) and seems to activate bone marrow. It is speculated that blood leucocytosis contribute to the chronic inflammation associated with cigarette smoking (Van Eeden and Hogg, 2000**).**

The mechanism however, which activates leucocytes in the semen of smokers is unclear. The level of leucocytes is elevated in smokers compared with non-smokers was also reported (Close et al., 1990). In a small study evaluating the ejaculates of 22 infertile smokers observed significantly elevated leucocytes in the peripheral blood of smokers (Parry et al., 1997**).** Leucocytes are the major source of Reactive Oxygen Species (ROS) in the ejaculate (Sharma and Agarwal, 1996). Cigarette smoking is also known to increase ROS levels through increased leukocyte generation. Infertile smokers are known to harbor increased levels of spermatid oxidative stress compared with infertile nonsmokers. This increase is associated with increased seminal leukocytes (Agarwal and Said, 2005).

Therefore, elevated leukocytes may by generate high levels of ROS in semen which may overwhelm the antioxidant strategies resulting in oxidative stress and impair fertility (Aitken et al., 1995).

ROS are harmful to sperm DNA and membrane phospholipid (Kim and Parthasarathy, 1998). Spermatozoa are particularly susceptible to damage induced by excessive ROS because their plasma membrane contains large quantities of poly unsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes (Sikka, 2001).

3.3. Smoking and semen characteristic

The effect of smoking on the semen quality is still a controversial issue. Merino et al. found that smoking have a negative effect on the parameters of sperm quality (Merino et al., 1998), In addition, a number of studies have shown a positive correlation between male smoking and decreased concentration and impaired motility and morphology (Evans et al., 1981, Handelsman et al., 1984, Mak et al., 2000, Sofikitis et al., 1995). There are also studies claiming a relation between cigarette smoking and decreased semen volume (Holzki et al ., 1991; Saarnen et al .,1987). While some studies did not find any effect on any of the sperm characteristics (Goverde et al., 1995 ; Osser et al., 1992). Howeve, The concentration of cotinine and hydroxycotinine in the seminal plasma is significantly correlated with total sperm motility of spermatozoa (Pacifici et al., 1993). There is a small but significant correlation between cotinine concentration in seminal plasma and abnormal sperm morphology, but not for other semen variables (Wong et al ., 2000). Cotinine concentration of 400-800ng/ml impair sperm motility, membrane function, and their ability to undergo capacitation (Sofikitis et al., 2000). In another study, it was demonstrated that semen samples from smokers have a significantly higher ratio of single-strand-to double-strand DNA spermatozoa (Sofikitis et al., 1995). Studies by Zenses (2000) and Zenses et al., (1999) have proposed that smoking

induces DNA damage in spermatozoa and in generated preimplantation embryos. Also, nicotine at a concenteration of 10mM for 24 hours resulted in the formation of definite fragmentation which could be seen via electrophoresis as a characteristic ladder pattern and 5mM nicotine treatment for 24 hours showed week intensity of DNA laddering (Khae-hawn et al., 2005). Moreover, Cigarette smoking is significantly correlated with increased levels of seminal oxidative stress, as evidenced by a significant reduction in Reactive Oxygen Species-Total Antioxidant Capacity score (ROS-TAC). To strengthen the above hypothesis, it should be emphasized that cigarette smoke has been associated with increased frequency of aneuploidy in sperm (Twigg et al., 1998a;b), lower seminal plasma antioxidant levels and increased oxidative damage to DNA (Fraga et al., 1996; Shen et al., 1997). It is concluded that certain life style factors, such as tobacco smoking, may reduce the antioxidant capacity of seminal plasma and impair the secretion of accessory sex glands (Depuydt et al., 1996; Klinefelterand Hess, 1998). The seminal plasma protects spermatozoa from excessive ROS by means of small molecular weight free radical scavengers, such as Ascorbate and Tocopheral, Uric acid and ROS-metabolizing enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Alvarez and Storey, 1989). ROS-TAC scores decrease as a result of an imbalance between levels of ROS and antioxidants in semen (Sharma et al., 1999).

Cigarette leads to imbalance between antioxidants and ROS mediated lipid peroxidation, which is reflected as impaired glutathione redox ratio (GSH/GSSG), with an inverse proportion of oxidative stress, and that protection could be provided by supplementing media with antioxidants, such as ascorbate and Trolox (a water soluble analog of vitamin E) **(**Arabi et al., 2003). In the testicular tissue there is a significant positive correlation between sperm DNA fragmentation and the level of ROS (Barroso et al., 2000; Henkel et al., 2003).

On the other hand, the existence of high quantities of Polyunsaturated Fatty Acids (PUFA) in the human spermatozoa, makes them very sensitive to ROS stimulation (Alvarez and Storey, 1995). The results of many studies (Alvarez et al., 1987 ; Arabi et al., 2003) in the field of peroxidation loss of fatty acids from phospholipids of human spermatozoa, indicate that nicotine has a potentially oxidant effect when added to sperm samples, acting by increasing the rate of Thiobarbituric Acid Reactive Substance, particularly Malondialdehyde (TBARS/MDA) generation significantly and dose-dependently. The end point of lipid peroxide process is the thiobarbituric acid-reacted MDA as an index of Lipoprotein (LPO) damage.

LPO also has a deleterious effect on the aspect of sperm viability. It also impairs plasma membrane ion exchange, which is necessary for maintening sperm movements (Rao et al., 1989). In a high proportion of infertility cases showing defective sperm function as a result of oxidative stress which leads to damage of sperm membrane (Aitken, 1994, Sharma and Agarwal, 1996).

It has been shown previously that oxidative damage to DNA or high intra testicular temperature (i.e., presence of varicocele) in rats or rabbits do not preclude the sperm head's ability to transform into male pronucleus. However, the derived embryo has lower capacity to implant (Sofikitis et al., 1996). Also, increased damage to DNA has been linked to an increase in early embryonic death (Sakkas et al., 1999). Therefore, exposure to cigarette smoke influences the sperm's capacity or epigenetic factor, but does not affect sperm capacity to fertilize oocyte (via ICSI) or to trigger early embryonic development. However, it can causes an impaired capacity for implantation of blastocyst (Ubaldi et al., 1999). They showed that some testicular defects have negative impact on the reproductive performance of testicular spermatozoa, resulting in a decreased embryonic potential for implantation, without any apparent effect on fertilization and early embryonic development. This is supported by previous studies, (Janny and Menezo, 1994; Sofikitis et al., 1998 ; Ubaldi et al., 1999) showing a parental contribution to embryonic capacity for implantation, resulting in a significantly lower number of alive offsprings per transferred embryo and normal blastocyst development rate, post ICSI.

In addition, paternal smoking has been associated with a significant increase in the percentage of spermatozoa with DNA damage and higher risk of birth defect and childhood cancers in the offspring (Magnani et al., 1990 ; Fraga et al., 1996; Sorahan et al ; 1997; Saleh et al 2002):

The purposes of current study were

The present prospective study aimed to evaluate the possible relationship between reactive oxygen species (ROS), total antioxidants (TAS) concentration in seminal plasma and semen quality among andrology referrals with normospermic and severe sperm impairment that underwent assisted reproduction treatment and in vitro fertilization (IVF/ICSI) outcome

Besides, the present work intends to evaluate the influence of Cotinine concentration in seminal plasma on sperm quality and their impact on the outcome of IVF/ICSI in term of fertilization, embryo development on day 2, embryo transfer and pregnancy rate.

4-Materials and Methods

4. 1. Materials

Semen samples were collected from unselected male partners of couples consulting for infertility at department of obstetrics and gynecology, university of Saarland/Homburg/Saar. Ejaculates were obtained in the early morning at the clinic (7.00–9.30 AM) after three days of sexual abstinence. The patients underwent either IVFor ICSI therapy according to the basic semen analysis (94 (57 IVF and 37 ICSI). All samples were collected by masturbation and allowed to liquefy for 30 min at room temperature. The samples were collected in separate polypropylene containers and assayed within 2h after collection. After liquefaction, the samples were analysed for concentration and motility according to recommendations of World Health Organization (World Health Organization, 1999)

The questionnaire was self-administered and designed to determine current smoking behaviour. Out of the 94 patient only 46 patients could be classified as heavy smokers or non smokers. Males who smoked >20 cigarettes day were enrolled in this study. They were divided into two groups: heavy makers (n=11) and nonsmokers (n=35). Semen concentrations of ROS, TAS, and cotinine and sperm quality were compared of these groups of patients. Cases with leukocytospermia or varicoceleor were excluded from this study because of their well-known high seminal ROS levels.

4.2. Methods

4.2.1 Basic sperm parameters evaluation

In all patients, a standard semen analysis was performed, assessing semen parameters, such as sperm count, concentration, and motility according to WHO criteria (World Health Organization, 1999). Morphology was evaluated according to strict criteria described by Krüger et al. (1987). Liquefied semen samples were layered on a 45% over 90% discontinuous density gradient PureSperm® gradients (Nidacon International AB, Sweden) and centrifuged at 500xg for 20 minutes at room temperature. The supernatant (seminal plasma) was immediately separated, and examined before storage to rule out the presence of spermatozoa in the supernatant. The seminal plasma was aliquot into storage ampoules and stored at -80°C until the assay was performed (within 3 months). Repeated assays on fresh and frozen seminal plasma samples from the same patient showed that sample storage

at -80°C did not significantly influence the evaluated parameters.
The pellet containing the normal spermatozoa was washed again, layered with 1 ml of culture media and incubated at 37 °C. The motile sperm was used for injection (ICSI) or insemination of oocytes (IVF).

4.2.2. Sperm vitality (Eosin test).

The Eosin test was used to evaluate the viability of spermatozoa before semen processing according to the method described by Eliasson and Treich (1971**).** Briefly, one drop of semen was mixed on a slide with 1 drop of 0.5% aqueous yellowish eosin solution and covered with a cover slide. After 1-2 minutes observation the spermatozoa which red stained (dead spermatozoa) can be distinguished from those unstained spermatozoa (alive spermatozoa). 100 sperm from each semen sample were evaluated per slide.

4.2.3 Sperm Morphology

Samples were spread on a plain glass slides, air dried for 24 hours and were stained using Papanicolaou staining and analysed. A total of 100 spermatozoa were scored per slide using bright field illumination and an oil immersion objective with a total magnification of x2000. At least ten high power fields from different areas of the slide were examined. The sperm morphology was classified according to the strict criteria described by Krüger et al (1987).

4.2.4. Sperm membrane integrity (Sperm Vitality (HOS-Test)

The HOS –test was performed according to the method described by Jeyendran et al (1984). 100µL of sperm suspension was mixed with 1mL of hypo osmotic solution (equal parts of 150mOsmol fructose and 150mOsmol sodium citrate solution), and incubated at 37°C for 30 minutes. After incubation, one drop was observed under the light microscope. A minimum of 200 spermatozoa were examined per slide and the percentage of spermatozoa that showed typical tail abnormalities indicative of swelling were calculated.

4.2.5. Chromatin condensation (DNA integrity) (Chromomycine test CMA_3)

Chromomycin CMA3 was used to predict the percentage of mature sperm cells. CMA_3 was performed as previously described by Bianchi et al (1996). Briefly, a semen aliquot was washed in Dulbecco's Ca-Mg free phosphate buffer saline (PBS) and centrifuged at 250 xg for 10 minutes. The spermatozoa were washed again and then fixed in methanol/glacial

acetic acid (3:1) at 4°C for 5 minutes and spread on clean slides.

The CMA_3 (Sigma St. Louis, MO, USA) was dissolved in Mc Ilvaine's buffer (pH, 7.0) supplemented with 10 mmol/L $MgCl_2$ (17 mL of 0.1 mol/L citric acid mixed with 83mL 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 minutes with 100µl of CMA_3 solution at 25°C in the dark. Slides were then washed in PBS, rinsed in PBS buffer and mounted with buffered-glycerol. The fluorochrome was examined using a Zeiss photomicroscope III, via a combination of exciter dichroic barrier filters of BP 436/10: FT 580: LP 470. A total of 200 spermatozoa were analysed for each sample, at least 200 cells were counted: cells positive for CMA3 displayed bright yellow–green fluorescence (presumably defective chromatin packaging), while those negative for CMA3 showed dull yellow staining (normal chromatin packaging).

4.2.6. DNA Fragmentation (TUNEL-Test)

(Terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL).

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

Briefly, ejaculated sperm samples were washed from seminal plasma by slow speed centrifugation (250xg; 10 minutes), smeared on microscope slides, air dried, fixed with 4% paraformaldehyde in phosphate–buffered pH 7.4 saline at 4°C for 25 minutes, and permeabilized with 0.1% triton X-100 in 0.1% sodium citrate.

The TdT-labeled nucleotide mixture was added to each slide and incubated in a humidified chamber at 37°C for 60 minutes in the dark. Later, slides were rinsed twice in PBS and counterstained with 10 mg/mL 4', 6 diamidoino-2-phenylindole (DAPI). Negative controls without TdT enzyme were run in each replicate. A total of 500 sperm per individual were evaluated using fluorescence microscopy by the same examiner. The number of sperm per field stained with DAPI (blue) was first counted; the number of cells with green fluorescence (TUNEL positive) was expressed as a percentage of the total sample.

4.2.7. DNA denaturation (Acridine orange Test)

The Acridine orange assay (AO) was used to evaluate the susceptibility of in situ DNA denaturation. The AO assay was performed following the protocol of Tejada et al (1984). The fluorochrome AO intercalates into double-stranded DNA as a monomer and binds to

single–stranded DNA as an aggregate. The monomeric AO which bound to native DNA fluoresces green, whereas the aggregated AO on denatured DNA fluoresces red.

10 µl of each semen sample was spread onto a pre-cleaned slide that was allowed to dry at room temperature. Slides were then fixed overnight in freshly prepared Carnoys solution (3 parts methanol and 1 part glacial acetic acid) and allowed to air dry for a few minutes before being stained with AO (CI 46005; sigma Chemical Company, St Louis, Mo).

A stock solution of AO was prepared (1g/L in distilled water) and stored in the dark at 4°C. The staining solution consisted of 10mL of stock solution, 40 mL of 0.1 M citric acid, and 2.5 mL of 0.3 M $Na_2 HPO_4 . 7H_2O$. the final pH of the solution was adjusted to 2.5. Slides were stained in AO solution for 5 minutes at ambient temperature in the dark in an aluminium foil covered 500 mL glass container, gently rinsed with deionised water, and mounted with phenylenediamine mounting medium. The AO slides were kept in the dark and analysed by means of a fluorescence microscope (Leitz, Oberkochen, Germany) equipped with a 490-nm excitation filter and 530-nm barrier. At least 200 spermatozoa were assessed per slide. Spermatozoa with normal DNA content revealed a distinct green fluorescent colour, whereas sperm heads displaying a fluorescent spectrum varying from yellow-green to red were considered as being denatured (Abnormal DNA; Tejada et al., 1984).

4.2.8. Reactive oxygen species measurement

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

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

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

4.2.9. Total antioxidants measurements

Semen antioxidant capacity was determined by a method described by Miller et al., (1993), and Rice-Evance and Miller (1994), developed for the evaluation of antioxidant capacity in

blood plasma. This is a Colorimetric assay using ABTS® (2, 2'-Azino-di-[3ethylbenzthiazoline sulphonate], and a commercially available kit, TAS (Randox Laboratory, Ltd Krefeld, Germany).

Assay Principle: The method is based on the antioxidants inhibition of the absorbance of the radical cation 2, 2`-azinobis (3-ethylbenzothiazoline-6-sulphonate ($ABTS^{\cdot +}$) formed by the interaction between ABTS and ferrylmyoglobin radical species, generated by activation of metmyoglobin with H_2O_2. This has a relatively stable blue-green colour, which is measured at 600nm. Antioxidants added to the sample cause suppression of this colour production to a degree which is proportional to their concentration. Range 1.30-1.77mmol/L plasma (Miller et al. 1993)

4.2.10. Cotinine measurement: Bio Quant (Cotinine Direct ELISA)

Summary and Explanation: The BQ Cotinine Direct Elisa Kit is a specific and sensitive in – vitro test to detect the presence of Cotinine in serum and urine. Exposure to tobacco smoke can be detected by nicotine and its metabolites. Nicotine has a short half life and is not used as a marker for tobacco smoke exposure. Cotinine due to its longer half life has been used to research as a reliable marker for smoking status and smoking cessation studies.

Principles of the test: This continine Direct ELISA Kit is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture. A 10 µl aliquot of a diluted unknown specimen is incubated with a 100 µl dilution of enzyme (Horseradish peroxidase) labeled Cotinine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed IS inversely proportional to the concentration of drug in the sample. The technique is sensitive to 1 ng/ml.

ASSAY PROCEDURE: All reagents must be brought to room temperature (18-26 °C) before use. The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor. Use urine calibrators for urine and serum calibrators for serum. Depending on the cut offs a sample dilution may be required for urine applications.

1. Add 10 µl of calibrators and standards to each well in duplicate.
2. Add 10 µl of the specimens in duplicate (recommended) to each well.
3. Add 100 µl of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.

4. Incubate for 60 minutes at room temperature (18-26 °C) preferably in the dark, after addition of enzyme conjugate to the last well.
5. Wash the wells 6 times with 300 µl distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells.
6. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
7. Add 100 µl of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
8. Incubate for 30 minutes at room temperature, preferably in the dark.
9. Add 100 µl of Stop Solution to each well, to change the blue color to yellow.
10. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
11. Wells should be read within 15 minutes of yellow color development.
The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run. A dose response curve or a cutoff calibrator should be run with every plate.
Sensitivity: Assay sensitivity based on the minimum Cotinine concentration required to produce a three standard deviation from assay A0 is 1 ng/ml.

4.2.11. Selenium and zinc measurement

Determination of the concentration of selenium and zinc was carried out by means of instrumental neutron activation analysis at the Hahn-Meitner –Institute, Department of Trace Element in Health and Nutrition, Free University, Berlin, Germany. The method has been described in detail elsewhere (Behne and Jürgensen, 1978; Behne et al., 1988). Briefly, samples were centrifuged for 20 minute at 10.000Xg. Aliquots of 200 µl of seminal plasma were transferred into ampoules made of highly pure silica and lyophilized. The sealed ampoules, together with standards, were irradiated for 10 days at a thermal neutron flux density of $7x10^{13}$ cm^{-2} sec^{-1}. The gamma ray spectra of the radionuclide thus produced were measured by means of a Ge (Li) detector, after a decay time of 3 months, which was necessary to reduce sufficiently the interfering phosphorus-^{32} activity. The element contents were determined by comparing the intensities of the gamma rays of the selenium-75, and zinc-66 in sample and standards.

4.2.12. Female patient characteristics.

The female patients underwent either IVF (n= 57) or ICSI (n=37) therapy. The female patients underwent ovarian hyperstimulation as previously described (Hammadeh et al., 2002). 478 oocytes were retrieved (2798 from IVF and 1666 from ICSI patients). 29 pregnancies were achieved (30.9%). ROS, TAS, Cotinine concentration were measured in the seminal plasma and the results were correlated with sperm parameters and IVF/ICSI outcome. The following data were collected from female patients: ovarian stimulation protocol, number of retrieved, mature, injected oocytes and the fertilization, cleavage and pregnancy rates.

4.2.13. Statistical analysis.

Data analysis was performed using the personal computer of the local area computer network of the Institute of Medical Biometrics and Medical Information, University of Saarland, Homburg/Saar, Germany, using the SPSS 11 for Windows Software Package (SPSS Inc., Chicago, IL, USA). Data were expressed as mean±SD and range. The relationship between ROS, TAS, and DNA integrity, motility and morphology and their effect on fertilization rate after IVF and ICSI treatment was analysed. Furthermore, semen characteristics, ROS and TAS and cotinine levels were analysed in seminal plasma in Smokers and non-smokers and their effect on ART results were analysed using parametric and nonparametric methods. Differences in cotinine distribution and characteristics of sperm quality were evaluated between smokers and nonsmokers. Statistical significance was defined as $p<0.05$. The Mann-Whitney (U-test) was used for non–paired data. The results were presented by the mean ± SD values. Correlations were analysed by means of chi-square test or exact Fisher-test and by Spearman's test. They were considered statistically significant when $P<0.05$.

5-Results

Semen and sperm characteristics are illustrated in table 2. A total of 94 semen speciemens of men underwent either IVF (n=57) or ICSI therapy (n=37) were analysed for count, motility morphology, DNA integrity, DNA fragmentation (TUNEL-Test, Acridine Orange-Test), ROS, TAS, Zink and Selenium concentration in seminal plasma.

Sperm concentration, the proportion of motile spermatozoa, sperm viability and the percentage of TUNEL positive spermatozoa are shown in table 2.

Mean values for the sperm concentration in the native semen samples were 65.32±37.16 mill/ml and decreased after semen preparation with pure sperm gradients centrifugation to 31.91±29.33mill /ml.

Sperm motility increased after semen preparation in comparison to the value in the native semen samples (32.69±18.291% versus 62.23±30.68%). Sperm preparation by gradients centrifugation increased the percentage of sperm motility compared with the original semen sample.

Besides, 8.02 ±6.57% and 5.83±5.46% of spermatozoa with normal head displayed DNA fragmentation after staining with Tunel and Acridine Orange respectively .

Moreover, the percentage of sperm parameters such as concentrations, motility, morphology, chromatin condensation, CMA3, DNA Fragmentation (Tunel) and ROS, TAS, mean number of retrieved, cleaved, transferred and pregnancy were compared between IVF/ICSI patient groups and the results were reported in table (3) and figures (4-15). The correlations were illustrated in table (4 a,b) and figures (16-21).

Statistical analysis demonstrated significantly lower semen quality of ICSI in comparison to IVF patients. Sperm concentration, motility and vitality were significantly higher in IVF patients group in comparison to ICSI group(Fig. 4, 5, 6, 7). The mean percentage of morphologically normal spermatozoa was higher, but not significant of patients underwent IVF (14.05±15. 87%) in comparison to those of ICSI therapy (10.39±10.34%,) (Fig. 8)..

Chromatin integrity (sperm stained by CMA_3) of ICSI patients group was significantly (p= 0.001) lower in comparison to IVF group (30.97±20.50% vs. 17.81±9.64%) (Fig. 9). Whereras, the DNA fragmentation (TUNEL) in the spermatozoa of ICSI patients group was higher (8.14±6.58% vs. 7.95±6.618%; p=0.892) than that in the IVF group (Fig.10). ROS concentration in seminal plasma of IVF patients was, non-significant, higher than that of ICSI patients (73.7±74.3 versus 50.8±34.63, p= 0.130 respecticvely) (Fig. 11). Whereas, the

concentration of TAS in seminal plasma was similar in both groups (1.36±0.52 versus 1.42±0.510; p= 0.564) (Fig.12).

Pregnancy rate was higher in patients underwent IVF in comparison to those who underwent ICSI therapy (31.5% vs. 29.7%; p=0.392).

In IVF patients group 18 (18/57) pregnancies were achieved. Whereas only 11 women become pregnant (11/37) after ICSI treatment. However, only 40 women became transfer in IVF and 18 in ICSI groups, the other patients ended up with total fertilization or cleavage failure (Tab. 3; Figure. 15).

In addition, the semen and sperm quality as well as comparisons between Smokers and non smoker's patient groups were illustrated in table (5-6) and figures (22-34).

The correlations between semen parameters, ROS, TAS, Cotinine concentration and IVF/ ICSI outcome of smokers and non-smokers group were presented in table 7a, b and diagrams (35-42).

A significant difference in sperm concentration, motility and morphology was been shown between smokers and non-smokers patient group. Other semen parameters were higher in non smoker group in comparison to smokers. However, these values failed to achieve statistical significance. The data is summarized in table 6. Eleven pregnancies out of 32 patients became transfer in the non-smokers groups (34% pregnancy rate). However, in the smoker's patients group only 3 pregnancies were achieved out of 11 patients who became transfer (27.3%). The pregnancy rate was significantly higher of non-smokers group in comparison to smoker group (p=0.001) (Table 6; Figure 34).

Tab. 2: Semen qualitative characteristics of patients undergoing assisted reproduction treatments (n= 94)

	M±SD
Sperm concentration in the native semen sample(mill/ml)	65.32±37.16
Sperm concentration after semen preparation	31.91±29.33
Motility (%) in the native semen sample	32.69±18.291
Motility (%) after semen preparation	62.23±30.688
Vitality (stained with Eosin test) (%)	63.66±22.647
Membrane Integrity (%) HOS-test in the native semen samples	43.66±20.709
Morphologically normal spermatozoa in the native semen samples	12.63±14.049
Chromatin condensation in the native semen samples positive	22.99±16.142
DNA Fragmentation TUNEL-test in the native semen samples	8.02±6.570
DNA Denaturation (AO) in the native semen samples	5.83±5.467
ROS (µmol/l) level in seminal plasma	64.75±62.62
TAS (mmol/l) level in seminal plasma	1.38±0.516
Selenium (µgSe/kg dry mass) in seminal plasma	3.504E-02±1.355E-02
Zinc in seminal plasma	129.31±69.38
Mean number of collected oocytes	9.67±6.084
Mean number of fertilized oocyte	5.41±5.015
Fertilization rate (%)	53.9±28.1
Mean number of transferred embryos	1.91±0.86
Pregnancy	0.93±0.83

Tab. 3: Semen qualitative characteristics of patients undergoing assisted reproduction treatments

	IVF M±SD(n=57)	ICSI M±SD (n=37)	P-Value
Sperm concentration (mill/ml) in the native semen samples	75.51±31.946	49.63±39.51	0.002
Sperm concentration (mill/ml)	35.87±28.39	25.82±30.07	0.010
Motility (%)in the native Semen	39.30±18.16	22.22±12.95	0.001
Motility (%) after semen preparation	73.68±23.95	44.59±31.82	0.001
Vitality (%) Eosin test	58.77±21.82	71.19±22.09	0.003
Membrane integrity (HOS-Test)	41.84±20.49	46.53±21.00	0.277
Morphologically normal spermatozoa	14,05±15,87	10.39±10.34	0.259
Chromatin condensation (CMA3) native	17.81±9.64	30.97±20.50	0.001
DNA Fragmentation TUNEL-test	7.95±6.618	8.14±6.58	0.892
DNA Denaturation AO test	5.33±4.903	6.63±6.25	0.470
ROS concentration	73.7±74.3	50.8±34.63	0.130
TAS concentration	1.36±0.52	1.42±0.510	0.564
Selenium (µgSe/kg dry mass) in seminal plasma	131.0±77.47	127.31±60.160	0.992
Zinc concentration in seminal plasma	0.034±0.016	0.036±0.00	0.619
Retrieved oocytes	10.39±6.85 2798	8.57±4.53 1666	0.480
Fertilized oocyte	5.52±5.66 2578,00	5.0±3.70 1793.00	0.670
Fertilization rate	50.56±28.89	59.23±26.47	0.124
Cleaved and transferred Oocytes	1.77±0.86	2.14±0.82	0.036
Pregnancy	1.55±0.50 18/57 (31.5%)	1.39±0.50 11/37 (29.7%)	0.392

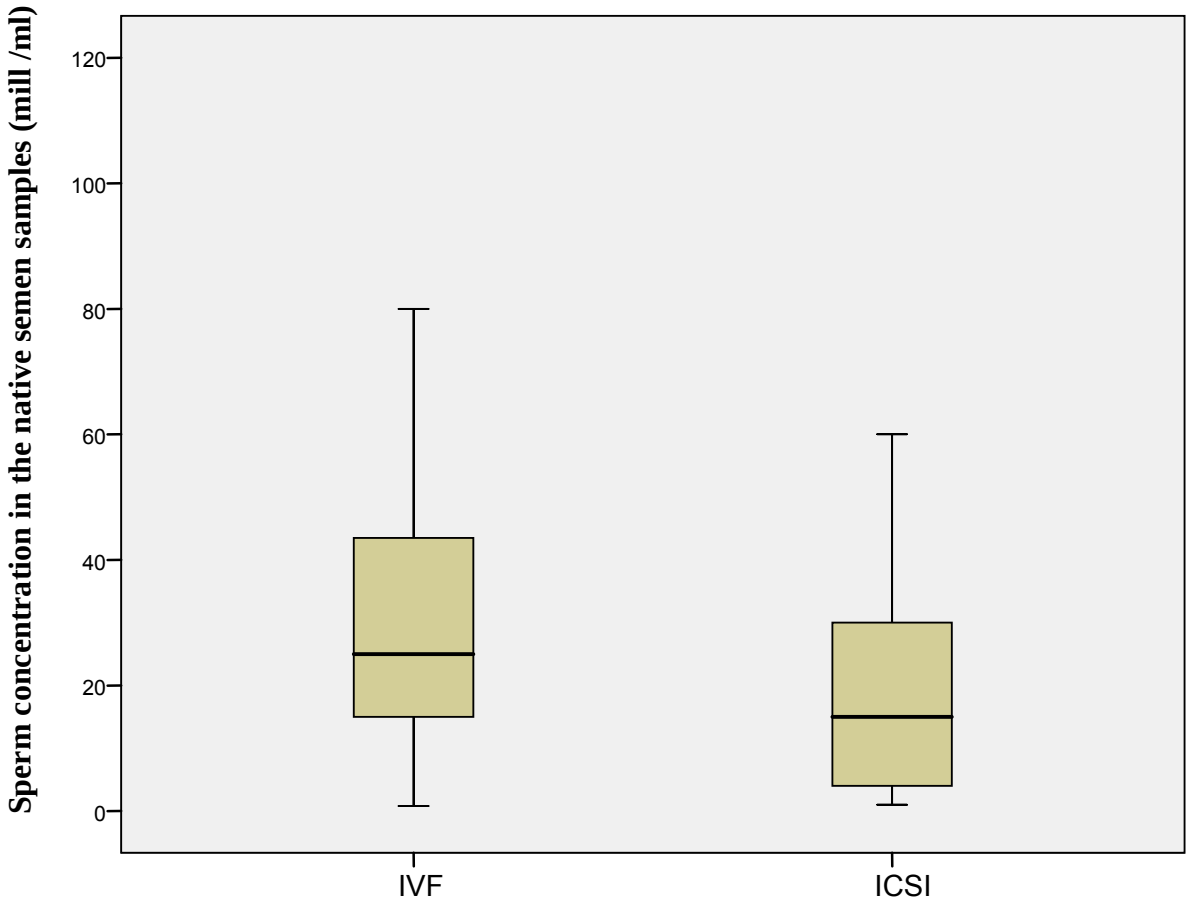

Figure 4: Box-plots showing the mean, median, and range of sperm concentration (mill /ml) after semen preparation of IVF/ICSI patients. Sperm concentration, was significantly higher in IVF patients group in comparison to ICSI group in comparison to those of ICSI therapy not only in the native semen sample (75.51±31.946±49.63±39.51mill/ml P=0.002) but also after semen processing (35.87±28.39±25.82±30.07mill /ml; p= 0.010).

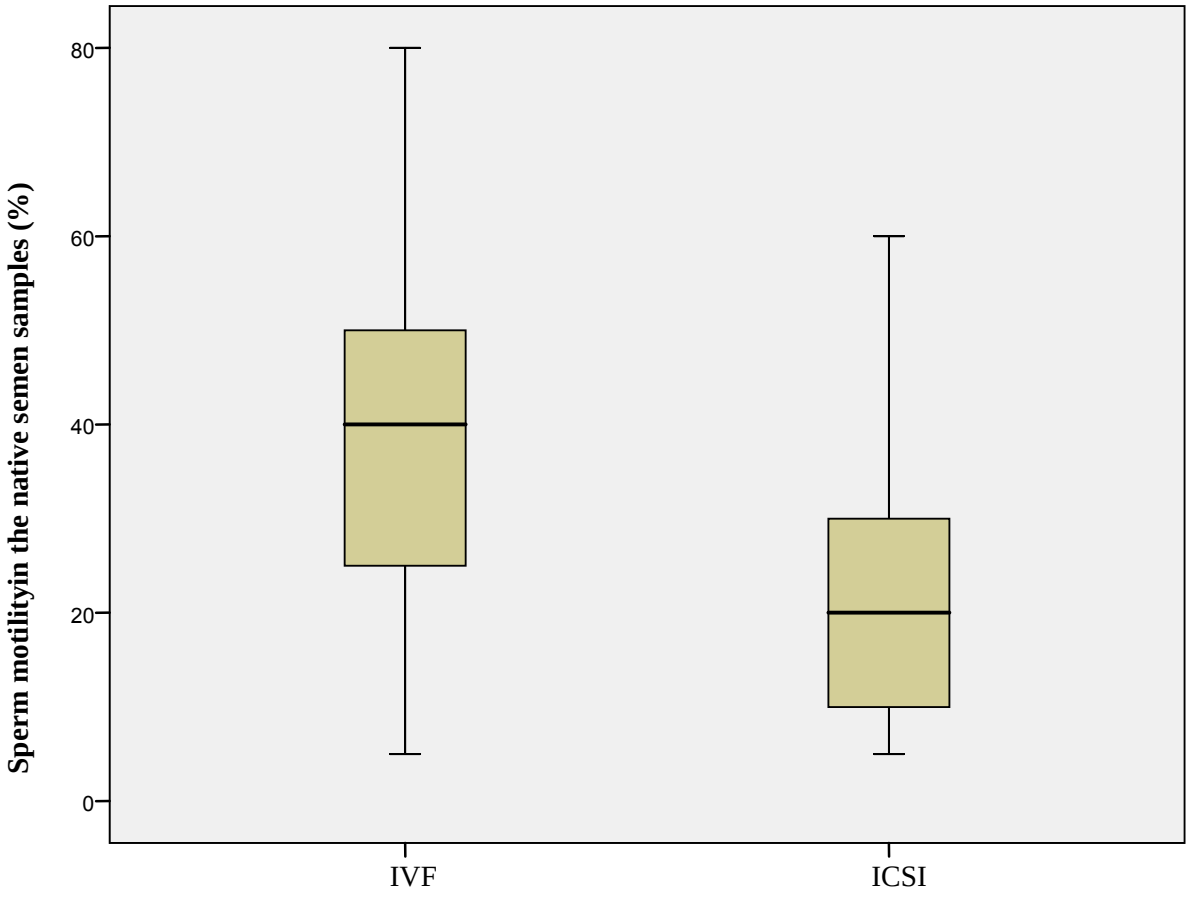

Figure 5: Box-plots showing the mean, median, and range of sperm motility (%) in the native semen samples of IVF/ICSI patients. Sperm motility were significantly higher in IVF patients group in comparison to ICSI group. The mean percentage of motile spermatozoa in the native semen samples was significantly higher (p=0.001) of patients underwent IVF (39.30±18.16) in comparison to those of ICSI therapy (22.22±12.95).

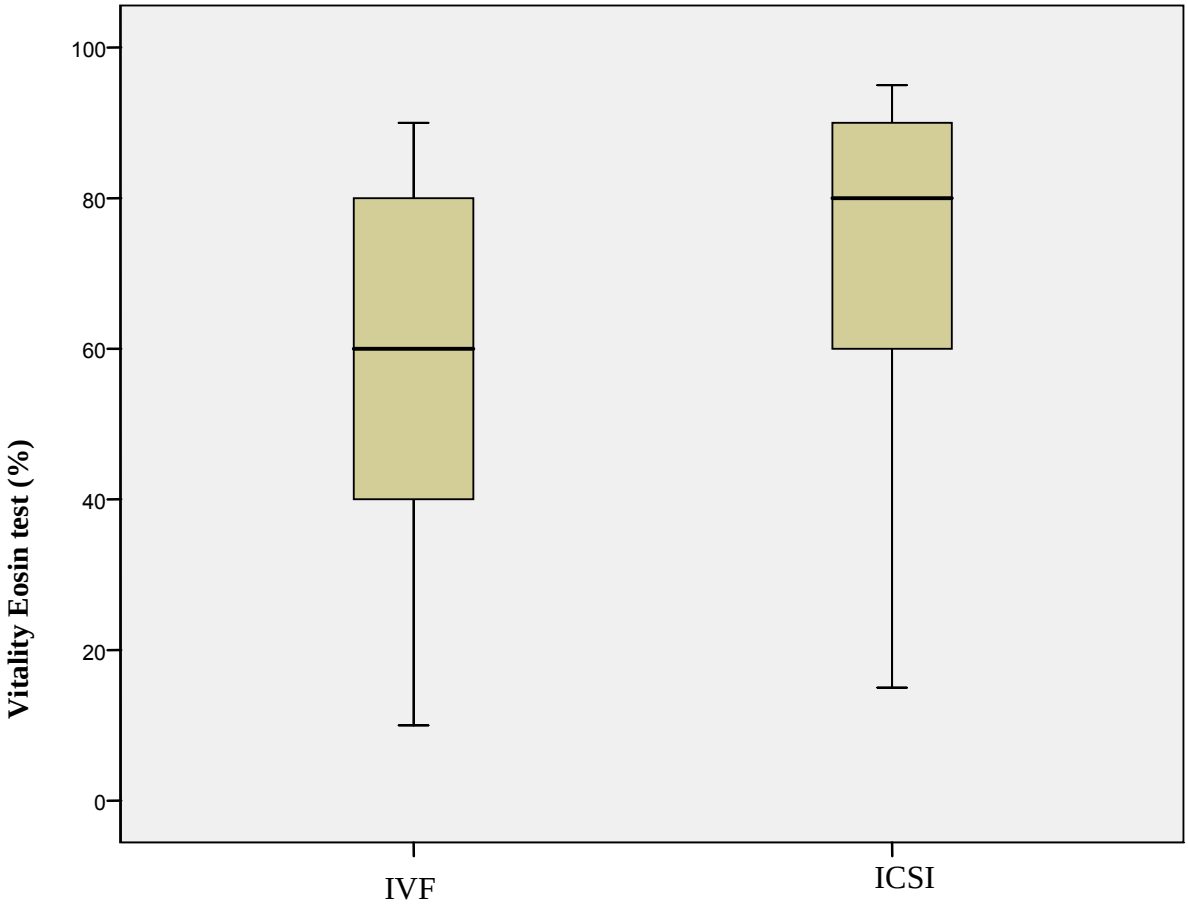

Figure 6: Box-plots showing the mean, median and range of sperm vitality (%) assessed by Eosin test of IVF/ICSI patients. The mean percentage of vital spermatozoa in the native semen samples was significantly higher in IVF patients group in comparison to ICSI group (58.77±21.82 versus 71.19±22.09; p=0.003).

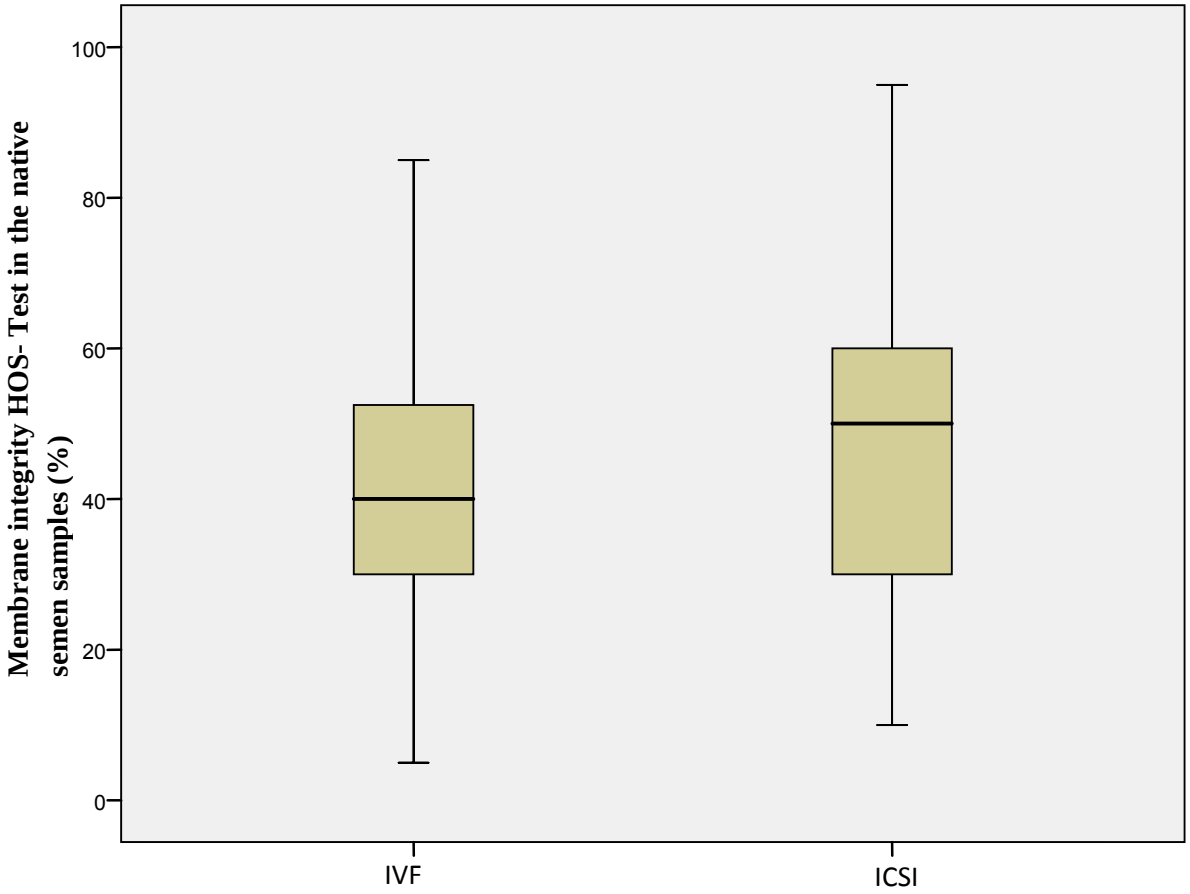

Figure 7: Box-plots showing the mean, median and range of sperm membrane integrity (Hypo-osmotic swell- Test; HOS-Test) of IVF/ICSI patients. The mean percentage of membrane integrity was similar in both groups (41.84±20.49 versus 46.53±21.00; p=0.277).

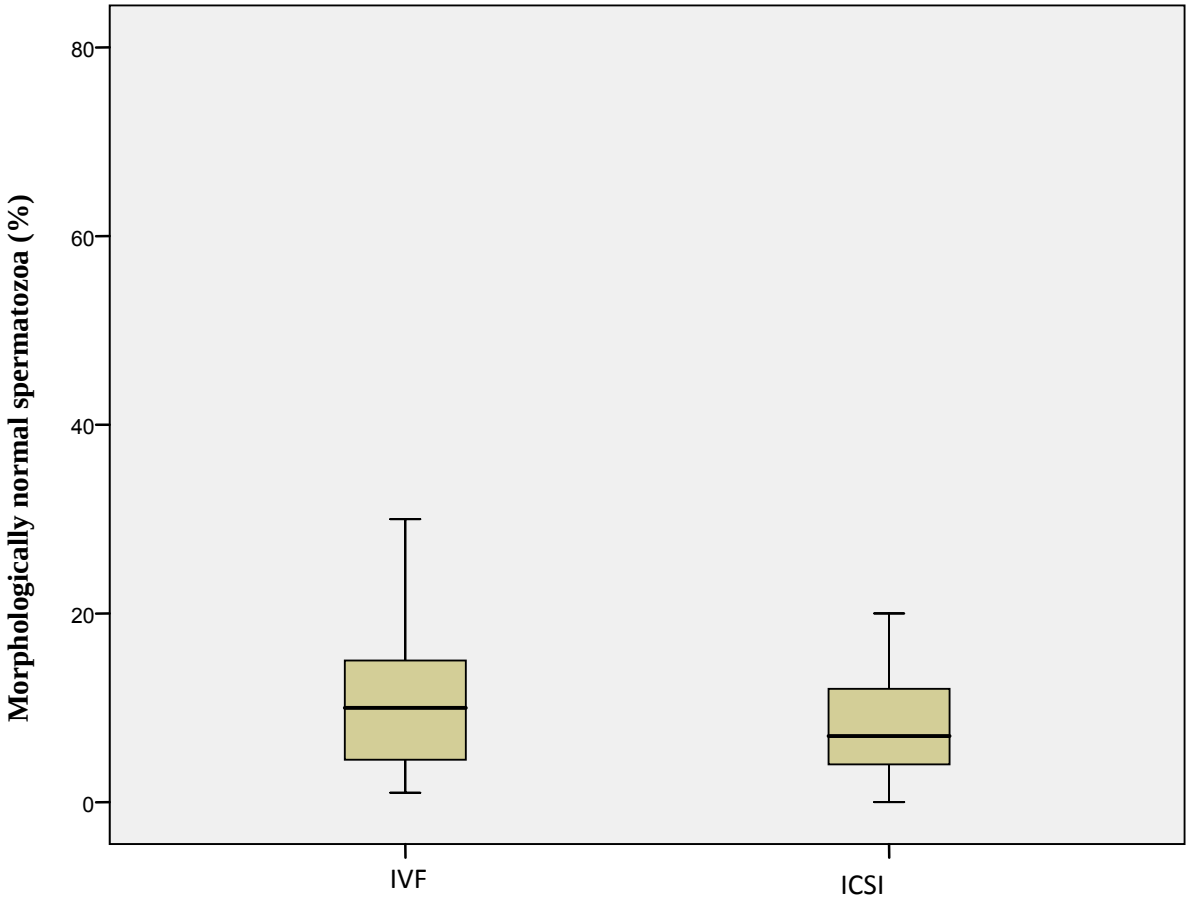

Figure 8: Box-plots showing the mean, median and range of morphologically normal spermatozoa (%) of IVF/ICSI patients. The mean percentage of morphologically normal spermatozoa was higher, but not significant, of patients underwent IVF in comparison to those of ICSI therapy (14.05±15. 87% versus 10.39±10.34%; p=0.259).

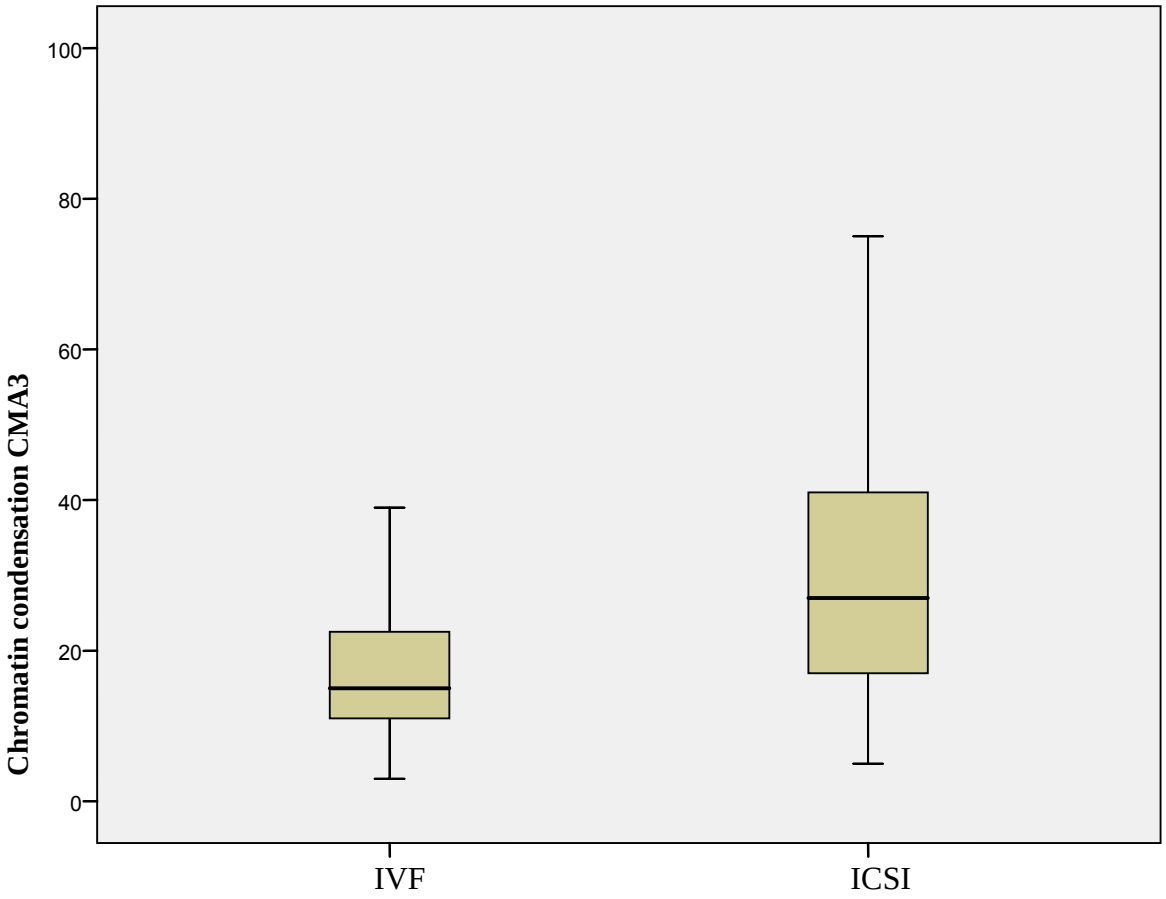

Figure 9: Box-plots showing the mean, median and range of chromatin condensation (non-condensed chromatin stained with CMA3) of spermatozoa after assessment with chromomycin (CMA3) of IVF/ICSI patients. The mean percentage of stained spermatozoa with chromomycin CMA3 in spermatozoa of male partner of patients underwent IVF was significantly (p=0.001) lower (17.81±9.64%) than those in spermatozoa of patients underwent ICSI program (30.97±20.50%; p=0.001).

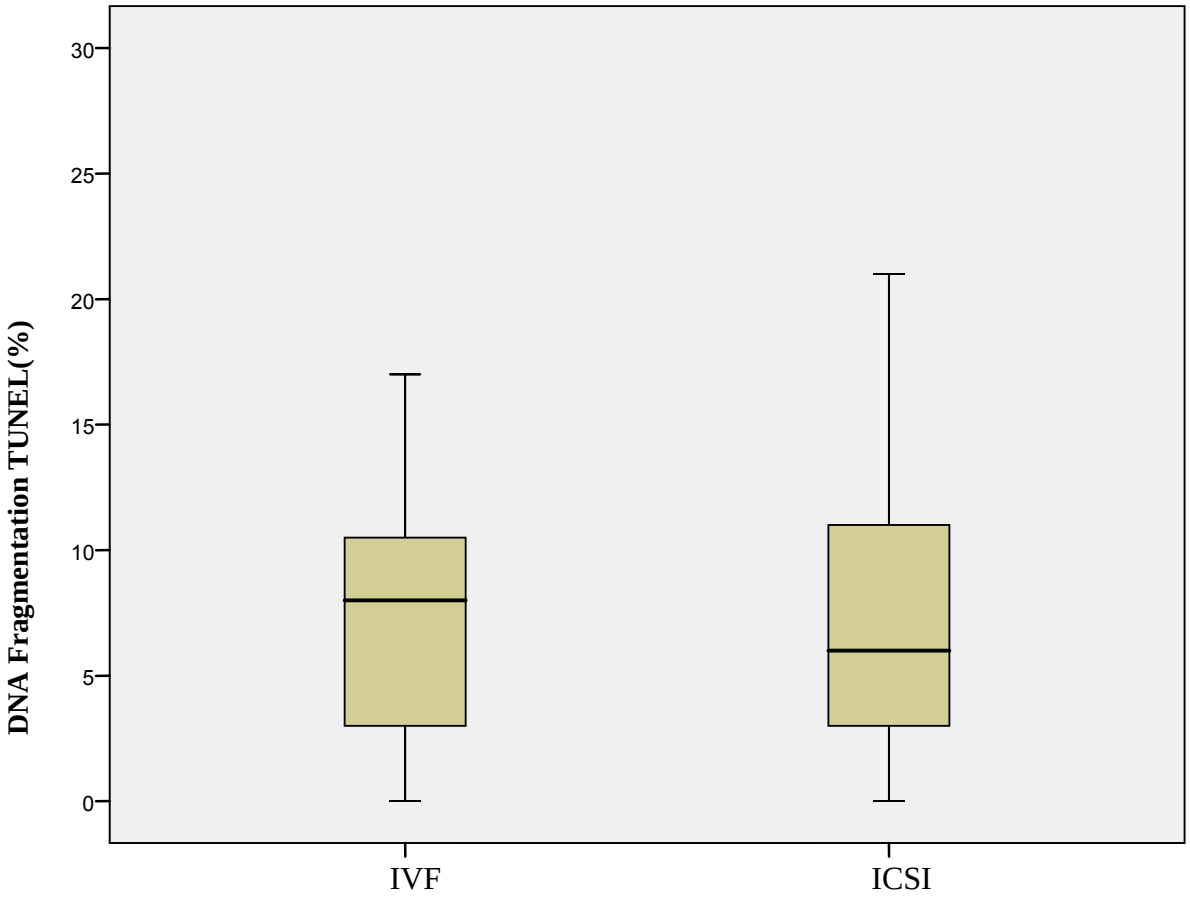

Figure 10: Box-plots showing the mean, median and range of DNA Fragmentation TUNEL in spermatozoa of IVF/ICSI patients. DNA fragmentation of IVF patients was 7.95±6.618% and similar value was found of ICSI patients (7.95±6.618; p= 0.892).

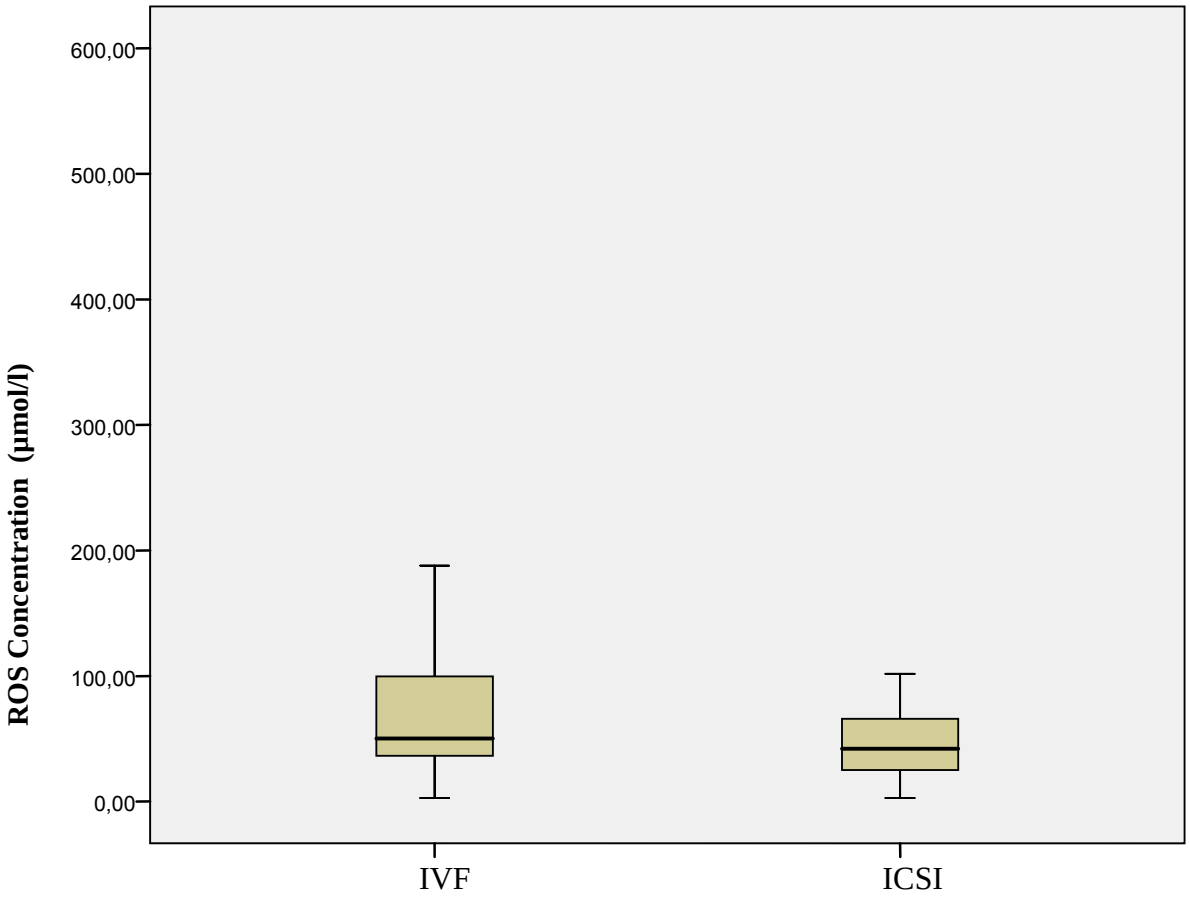

Figure 11: Box-plots showing the mean, median and range of ROS concentration (µmol/l) in seminal plasma of IVF/ICSI patients. ROS concentration was higher, but non significant, in IVF group in comparison to ICSI group (73.7±74.3 versus 50.8±34.63; p= 0.130).

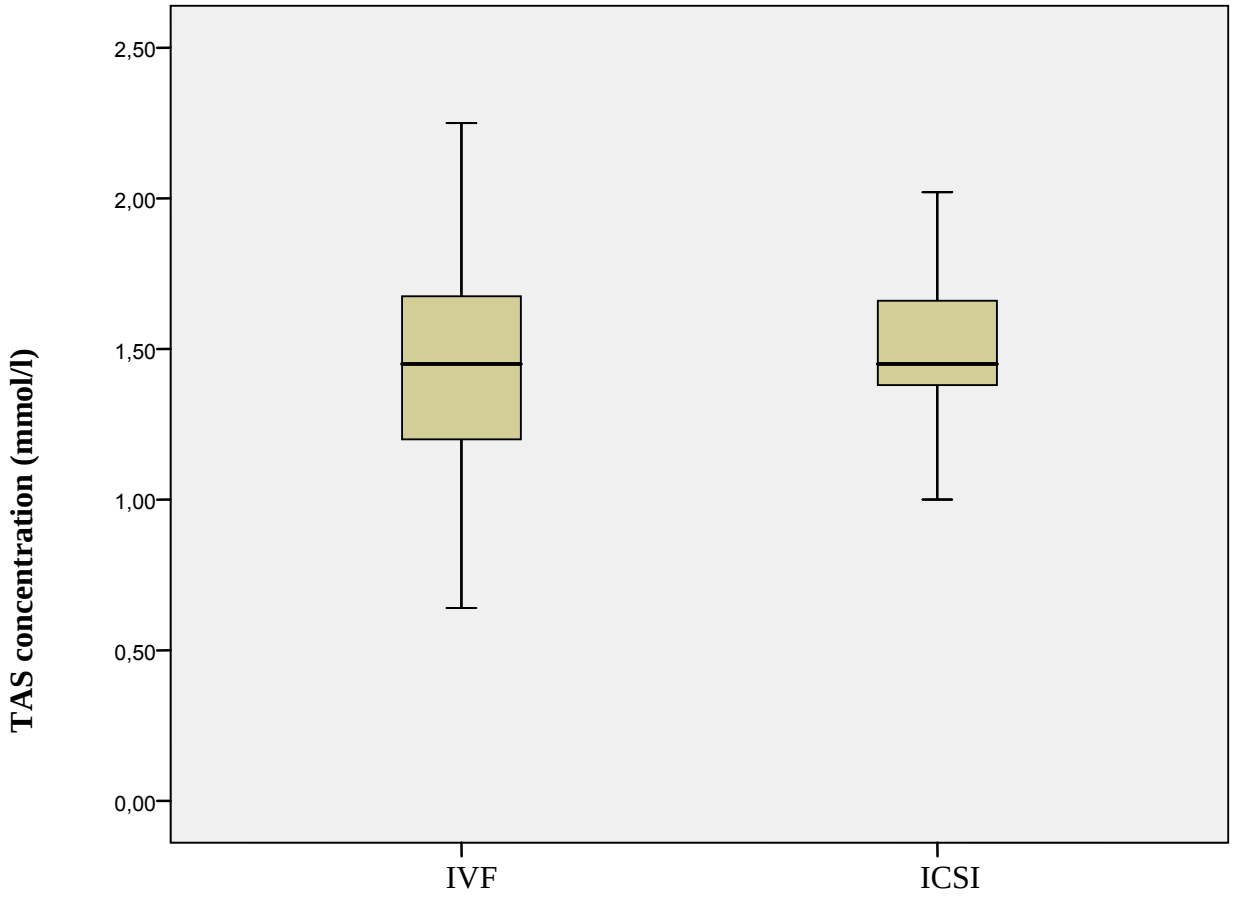

Figure 12: Box-plots showing the mean, median and range of TAS concentration in seminal plasma (mmol/l) of IVF/ICSI patients. TAS concentration was slightly higher in IVF vs. ICSI patient group (1.36±0.52 versus 1.42±0.510; p= 0.564).

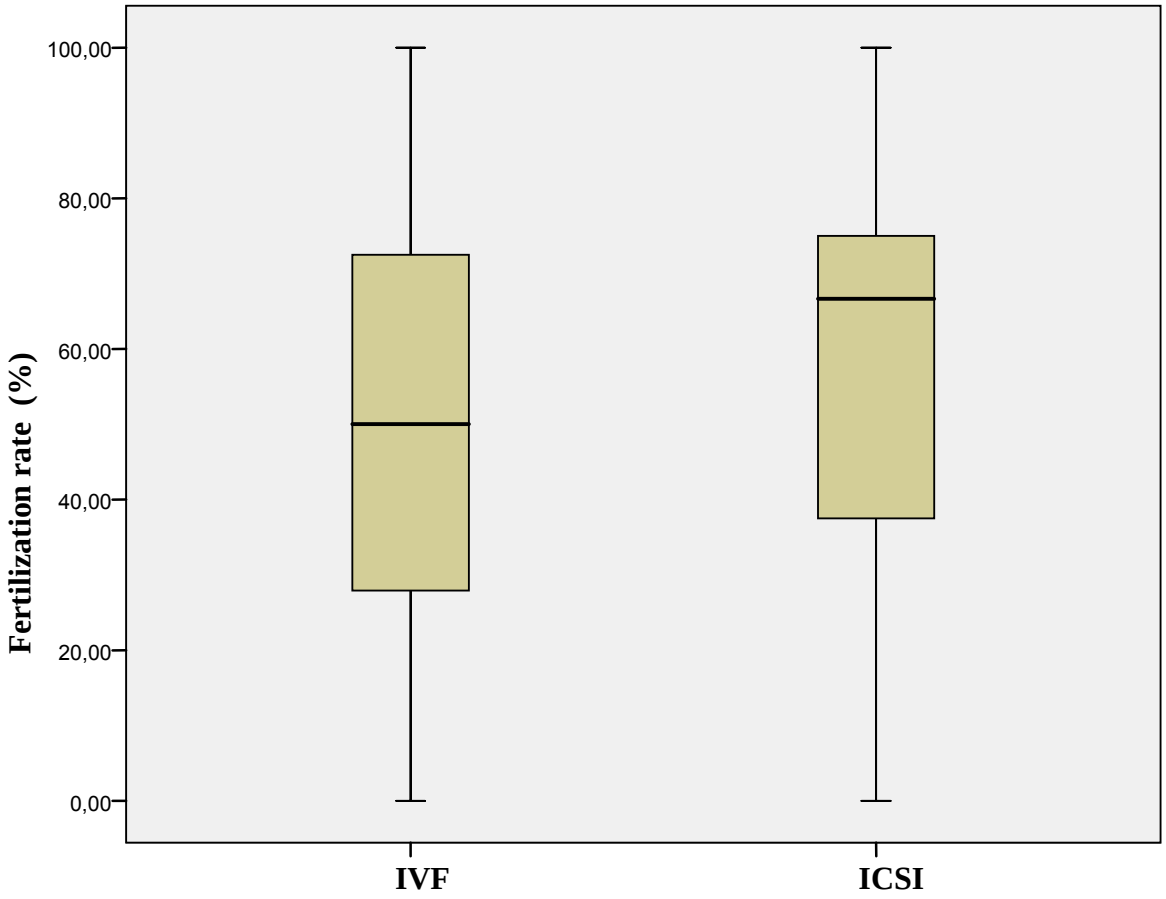

Figure: 13: Box-plots showing the mean, median and range of fertilization rate (%) of patients underwent IVF/ICSI therapy. The fertilization rate of oocytes in IVF was 50.56±28.89% and the corresponding value of ICSI was 59.23±26.47%. no significant difference was found bwtween both group (p=0.124)

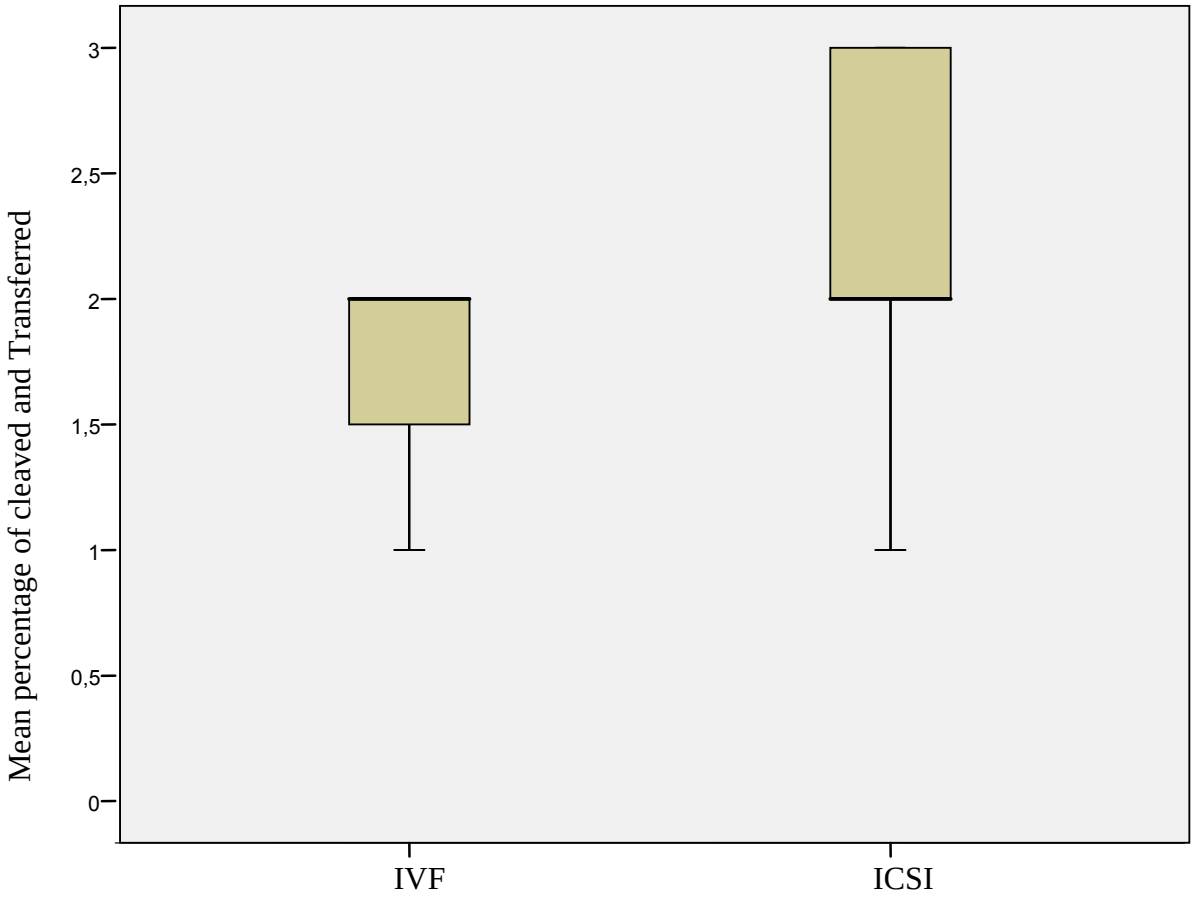

Figure 14: Box-plots showing the mean, median and range of the number of cleaved and transferred oocytes of IVF/ICSI patients. The mean number of cleaved and transferred oocytes in IVF was 1.77±0.86% and of ICSI was 2.14±0.82%. A significant difference was found between the groups (p=0.036). It should here be mentioned that according to German embryo protection law only three embryos can be produced and transferred.

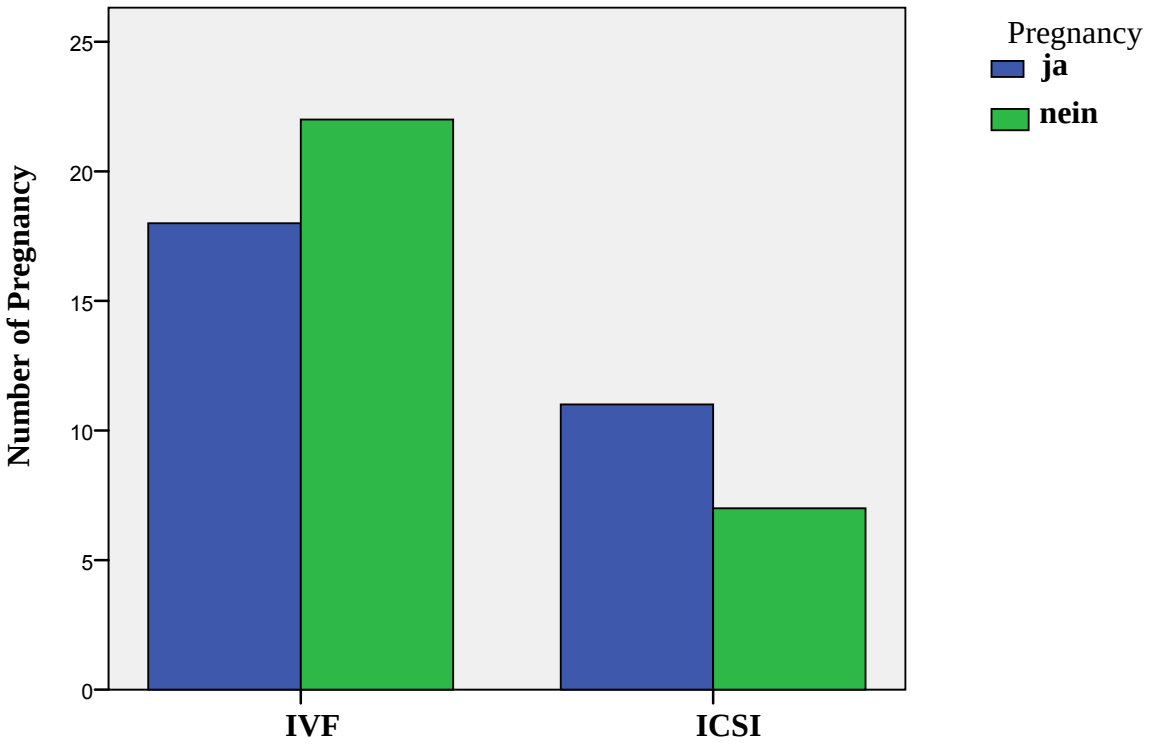

Figure 15: Mean number of clinical pregnancy rate of IVF/ICSI patients. Out of the 57 patients enrolled in IVF program only (n=40) women become transfer and of patient underwent ICSI therapy (n=37) only 18 patient became embryo transfer. The other patients ended up with total fertilization or cleavage failure. In IVF patients group 18 (18/ 57) pregnancies were achieved. Whereas only (11 women become pregnant (11/37) in ICSI patients group.

A significant positive correlation between semen concentration and motility before (r=0.427; p=0.003) and after semen processing (r= 0.326; p=0.027) has been demonstrated. Whereas, a negative correlation between DNA integrity (CMA_3) (r=-0.418; p=0.004) and DNA fragmentation (Tunel) (r=-0.292; p=0.049) was shown. Besides, a linear regression analysis of the data in this study indicated that there was a significantly positive relationship between Chromatin integrity (CMA_3) and sperm vitality (Eosin) (r=0.0332; p=0.029).(Tab. $4^{a.b}$)

The mean percentage of morphological normal spermatozoa correlated positively with sperm motility (r=0.382; p=0.010), vitality (r=0.318; p=0.033) and negatively with DNA integrity (CMA_3) (r= -0.162; p=0.288) (fig; 17), DNA fragmentation (TUNEL) (r=-0.123; p=0.419, ROS concentration in seminal plasma (r=-0.347; p=0.20) (Fig.18), fertilization rate (r=-0.021; p= 0.889) and cleavage rate (r=-0.096; p=0.530). However, the percentage of morphologically normal sperm in the specimens did correlate negatively significant with ROS concentration (r=-0.347; p=0.020) Fig. 18). However, TAS concentration in seminal plasma correlated positively significant with cleavage rate (r=0.313; p=0.034) (Fig. 19).

A positive correlation was found between chromatin condensation (CMA_3) of spermatozoa and sperm concentration (r=-0.418; p=0.004), motility (r=-0236; p=0.118), vitality (r=0.322; p=0.029) and morphology (r=-0.162; p= 0.288) (Fig. 17). An inverse correlation (but not significant) was shown between spermatozoa DNA fragmentation (Tunel) and motility (r= -0.107; p=0.484), morphology (r=-0.123; P=0.419), fertilization rate (r= -0.175; p=0.245), and pregnancy (r=-0.048; p= 0.750) (Fig. 20). ROS correlated negatively with sperm vitality (r=-0.111, p=0.453), membrane integrity (r=-0009; p=0.953) and morphology (r=-0.347; p=0.020)(Fig. 18). Besides, there was an inverse correlation (r=-0.275; p=0.064) between ROS concentration in seminal plasma and fertilization rate in IVF/ICSI patients.
Other inverse correlation, but not significant, was shown between Acridine orange staining and sperm vitality (r=-0.048; p=0.765); ROS concentration in seminal plasma (r=-0.072; p=0.652); TAS (r=-0.148; p=0.349); fertilization rate (r=-0.211; p=0.180); cleavage rate (r=-0. 214; p= 0.174) and pregnancy rate (r= -0. 033; p= 0.834) (Fig.21)..

Tab 4a: Correlation coefficient of semen parameters and sperm quality of IVF/ICSI patients.

		Sperm concent. native	semen preparation	Motility	Vitality Eosintest	integrity HOS-Test	Morphology	Chromatin condensation CMA3	Fragmentation (TUNEL)
Sperm concentration (mill/ml) in the native semen	r	1.000	0.314(*)	0.427(**)	-0.264	-0.107	0.074	-0.418(**)	-0.292(*)
	p	.	0.034	0.003	0.076	0.485	0.629	0.004	0.049
Motility in the native semen samples (%)	r	0.427(**)	0.269	1.000	-0.594(**)	-0.397(**)	-0.143	-0.236	-0.107
	p	0.003	0.074	.	0.000	0.008	0.348	0.118	0.484
Motility after semen preparation (%)	r	0.326(*)	-0.282	0.233	-0.156	-0.004	0.382(**)	-0.256	-0.031
	p	0.027	0.058	0.124	0.301	0.978	0.010	0.086	0.839
Vitality (%) Eosin test	r	-0.264	-0.110	-0.594(**)	1.000	0.238	0.318(*)	0.322(*)	0.100
	p	0.076	0.467	0.000	.	0.116	0.033	0.029	0.510
Membrane integrity HOS-test (%)	r	-0.107	-0.201	-0.397(**)	0.238	1.000	0.251	-0.251	0.008
	p	0.485	0.185	0.008	0.116	.	0.100	0.096	0.956
Morphologically normal sperm (%)	r	0.074	-0.360(*)	-0.143	0.318(*)	0.251	1.000	-0.162	-0.123
	p	0.629	0.015	0.348	0.033	0.100	.	0.288	0.419
Chromatin condensation CMA3 (%)	r	-0.418(**)	-0.042	-0.236	0.322(*)	-0.251	-0.162	1.000	0.287
	p	0.004	0.780	0.118	0.029	0.096	0.288	.	0.053
DNA Fragmentation – TUNEL (%)	r	-0.292(*)	-0.122	-0.107	0.100	0.008	-0.123	0.287	1.000
	p	0.049	0.419	0.484	0.510	0.956	0.419	0.053	.
DNA denatiuration (AO) (%)	r	-0.191	0.096	0.034	-0.048	0.090	0.032	0.077	0.219
	p	0.227	0.547	0.834	0.765	0.576	0.845	0.628	0.163
ROS (µmol/l)	r	0.192	-0.051	0.203	-0.163	-0.009	-0.347(*)	-0.127	-0.112
	p	0.201	0.738	0.181	0.280	0.953	0.020	0.401	0.460
TAS (mmol/l)	r	0.035	-0.154	-0.086	-0.130	0.307(*)	0.045	-0.143	-0.094
	p	0.820	0.306	0.573	0.390	0.040	0.768	0.344	0.532
Fertilization rate	r	0.188	-0.028	0.022	0.084	0.023	-0.021	-0.204	-0.175
	p	0.210	0.853	0.887	0.579	0.883	0.889	0.175	0.245
Cleaved and transferred oocytes	r	-0.074	0.095	0.000	0.076	0.235	-0.096	-0.088	-0.215
	p	0.627	0.528	0.999	0.616	0.120	0.530	0.559	0.152
Pregnancy	r	-0.066	0.059	0.070	-0.028	0.012	0.019	-0.072	-0.048
	p	0.663	0.695	0.648	0.853	0.935	0.900	0.635	0.750

Tab:4b: Correlation coefficient of semen parameters and sperm quality of IVF/ICSI patients.

		DNA fragmentation (AO)	ROS	TAS-	Fertilization rate	Cleaved and Transfer	Pregnancy
Sperm concentration (mill/ml) in the native semen	r	-0.191	0.192	0.035	0.188	-0.074	-0.066
	p	0.227	0.201	0.820	0.210	0.627	0.663
Motility (%) in the native semen	r	0.034	0.203	-0.086	0.022	0.000	0.070
	p	0.834	0.181	0.573	0.887	0.999	0.648
Vitality (%) Eosin test	r	-0.048	-0.163	-0.130	0.084	0.076	-0.028
	p	0.765	0.280	0.390	0.579	0.616	0.853
Membrane integrity (HOS) test (%)	r	0.090	-0.009	0.307(*)	0.023	0.235	0.012
	p	0.576	0.953	0.040	0.883	0.120	0.935
Morphologically normal spermatozoa (%)	r	0.032	-0.347(*)	0.045	-0.021	-0.096	0.019
	p	0.845	0.020	0.768	0.889	0.530	0.900
Chromatin integrity (CMA_3)	r	0.077	-0.127	-0.143	-0.204	-0.088	-0.072
	p	0.628	0.401	0.344	0.175	0.559	0.635
DNA fragmentation (TUNEL) (%)	r	0.219	-0.112	-0.094	-0.215	-0.048	0.115
	p	0.163	0.460	0.532	0.152	0.750	0.447
DNA Denaturation (AO) (%)	r	1.000	-0.072	-0.148	-0.214	-0.033	0.124
	p	.	0.652	0.349	0.174	0.834	0.434
ROS (µmol/l)	r	-0.072	1.000	0.033	0.275	0.059	-0.114
	p	0.652	.	0.828	0.064	0.696	0.451
TAS (mmol/l)	r	-0.148	0.033	1.000	0.071	0.313(*)	0.086
	p	0.349	0.828	.	0.638	0.034	0.571
Fertilzation rate	r	-0.211	0.275	0.071	1.000	0.192	-0.271
	p	0.180	0.064	0.638	.	0.200	0.069
Cleaved and transferred oocytes	r	-0.214	0.059	0.313(*)	0.192	1.000	0.019
	p	0.174	0.696	0.034	0.200	.	0.900
Pregnancy	r	-0.033	-0.114	0.086	-0.271	0.019	1.000
	p	0.834	0.451	0.571	0.069	0.900	.

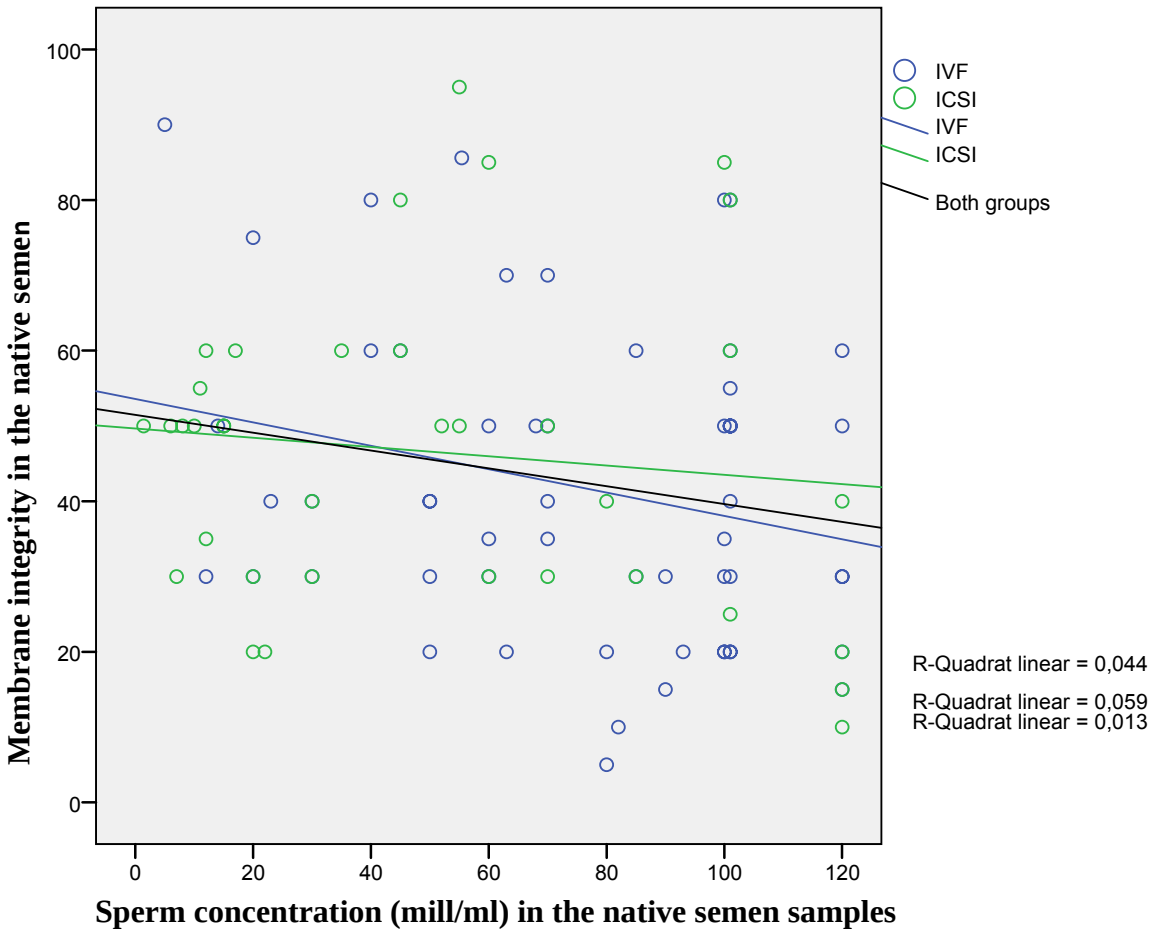

Figure 16: Scatter plot of correlation between sperm concentration (mill/ml) in the native semen samples and sperm membrane integrity (HOS-Test) of patients underwent IVF/ICSI therapy. A negative correlation, but non-significant, was found between sperm concentration in seminal plasma and membrane integrity in the of spermatozoa in the native semen samples (r=-0.201; p= 0.185).

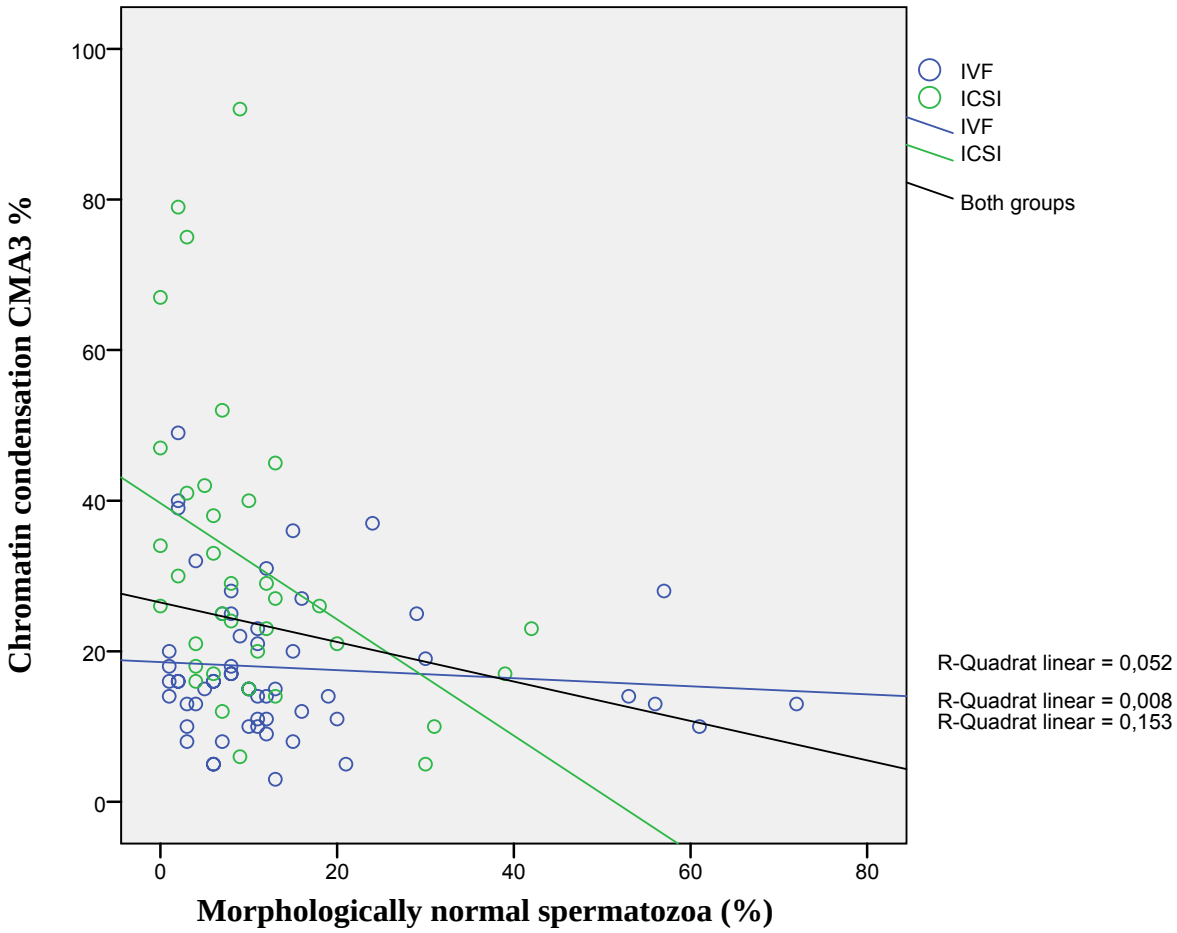

Figure 17: Scatter plot of correlation between morphologically normal spermatozoa and chromatin condensation (non condensed sperm after chromomycin staining (CMA3) of patients underwent IVF/ICSI therapy. A negative non statistically significant correlation was found between the mean percentage of the morphologically normal spermatozoa in the ejaculate and the mean percentage of condensed chromatin (r= -0.162; p=0.288)

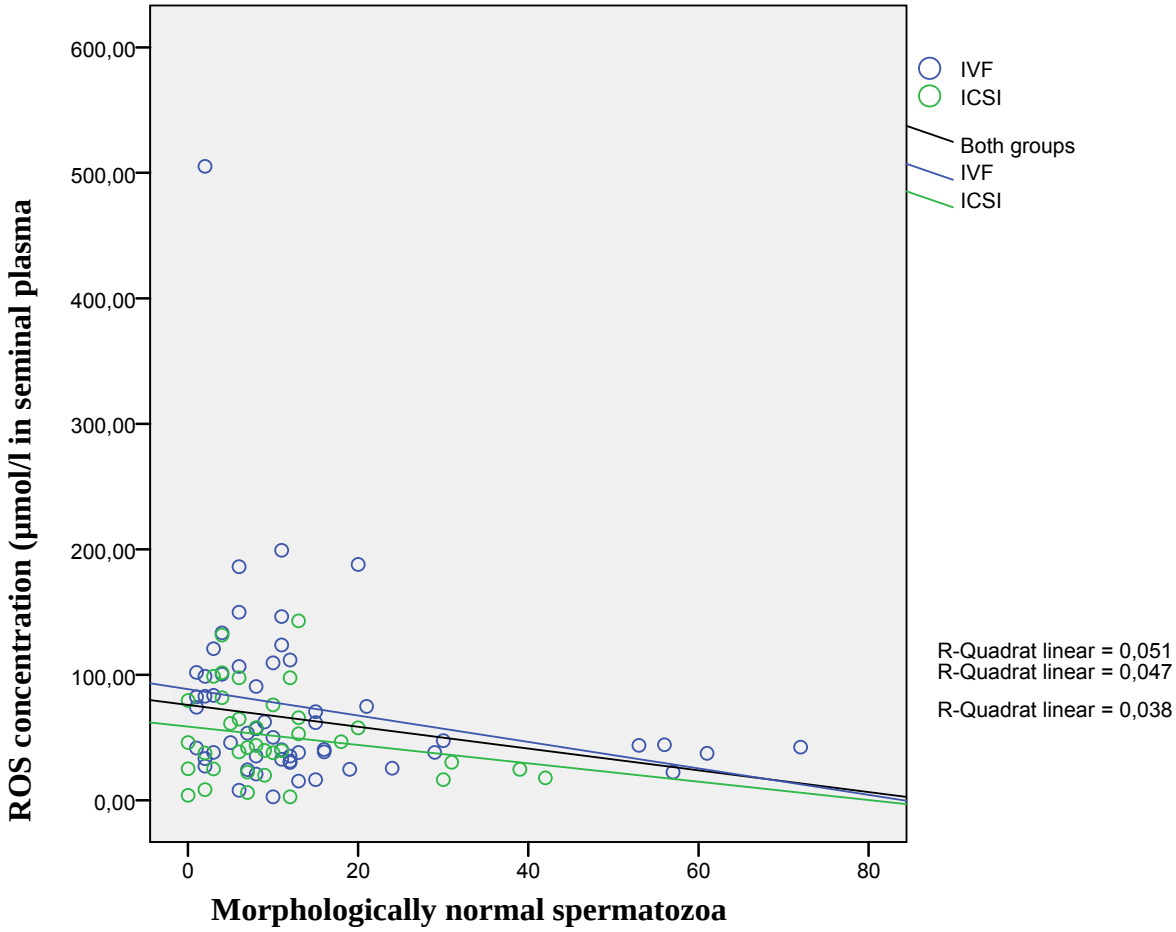

Figure 18: Scatter plot of correlation between morphologically normal spermatozoa and reactive oxygen species concentration (µmol/l) in seminal plasma of patients underwent IVF/ICSI therapy. No negatively significant correlation was found between the mean concentration of ROS in seminal plasma and the mean percentage of morphologically normal spermatozoa (r=-0.347; p=0.020).

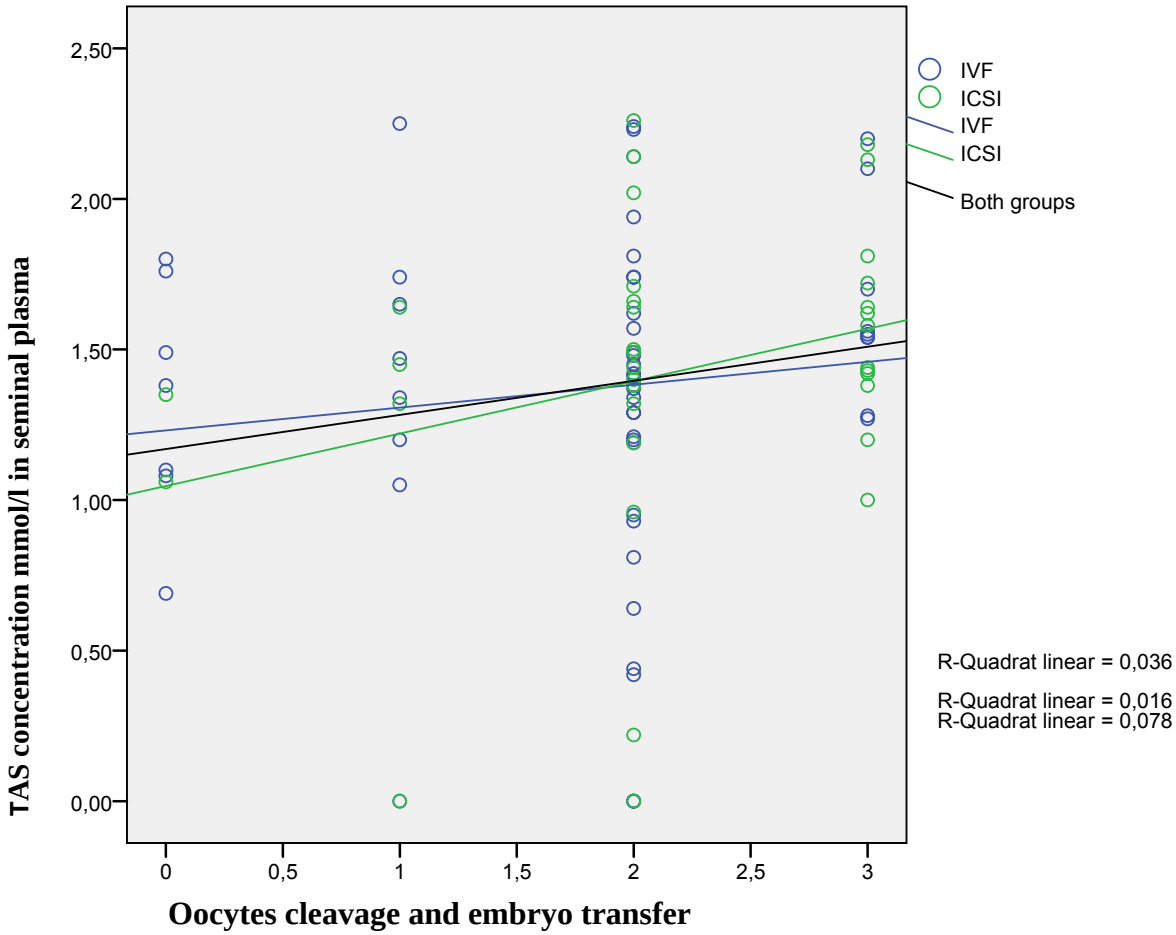

Figure 19: Scatter plot of correlation between the mean number of cleaved Oocytes (Embryo-transferred) and total anti oxidant (mmol/l) concentration in seminal plasma of patients underwent IVF/ICSI therapy. A statistically significant positive correlation was found between TAS concentration in seminal plasma and the mean number of cleaved (embryo transferred) oocytes (r=0.313; p=0.034).

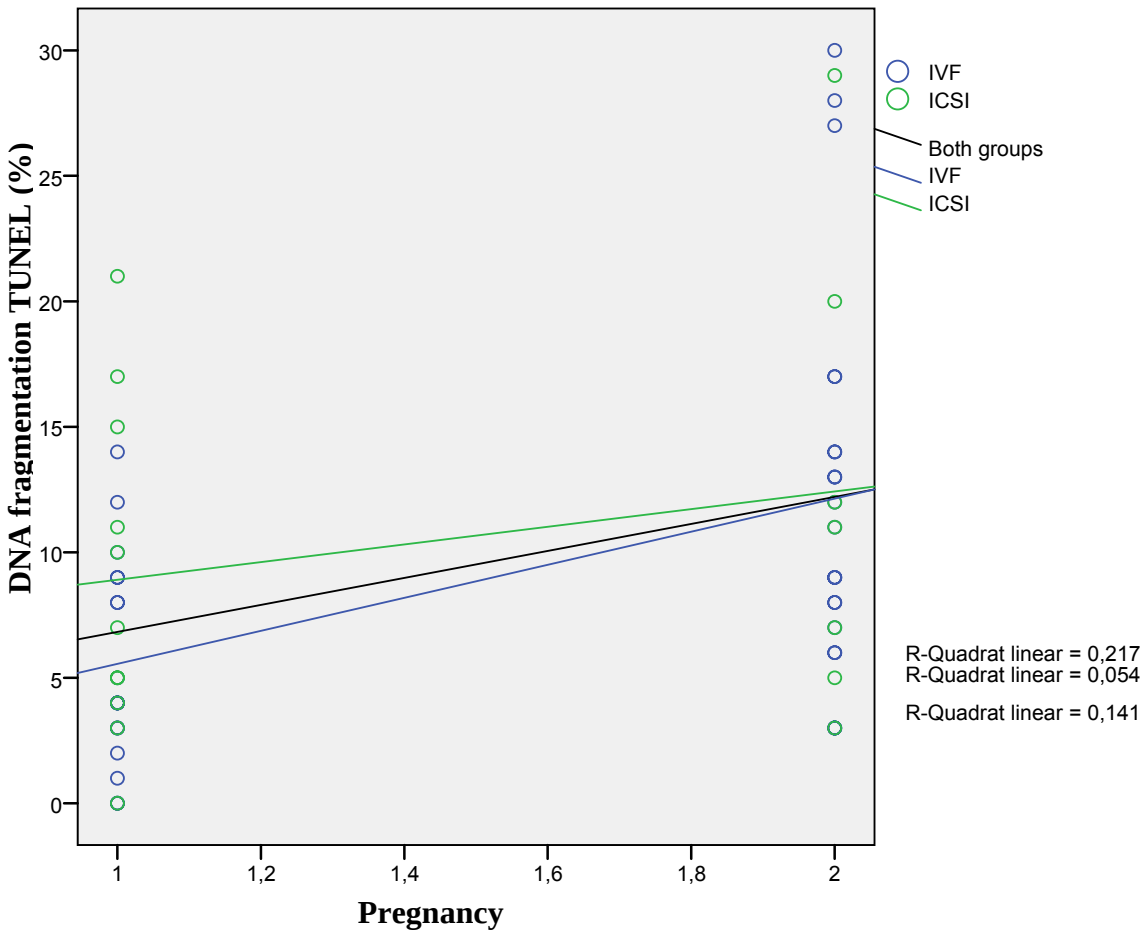

Figure 20: Scatter plot of ccorrelation between DNA fragmentation (TUNEL) of spermatozoa and pregnancy of patients underwent IVF/ICSI therapy. The percentage of DNA fragmentation of spermatozoa correlated negatively, but statistically not significant; with pregnancy (r=-0.04; p=0.750).

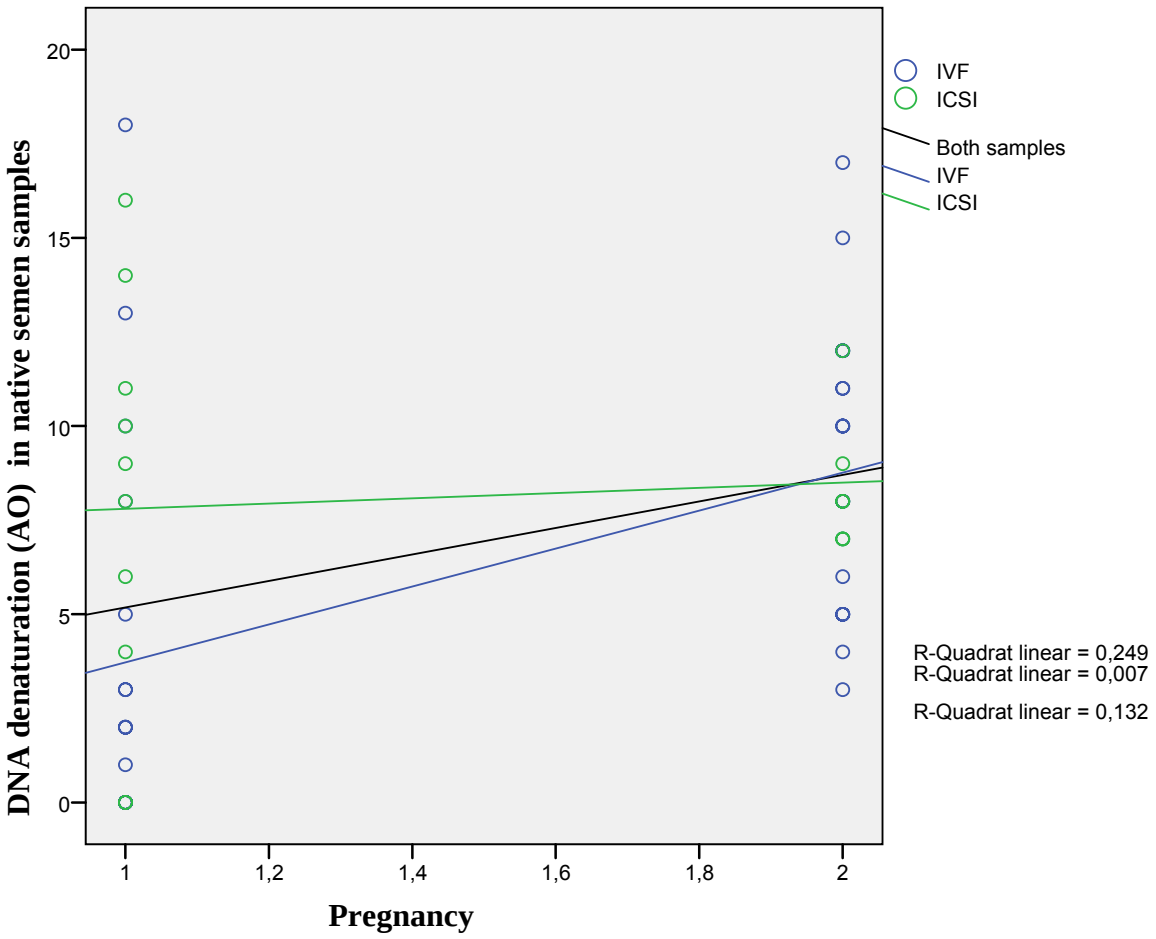

Figure 21: Scatter plot of correlation between DNA denaturation (AO) of spermatozoa and pregnancy of patients underwent IVF/ICSI therapy. No statistically significant correlation was observed between DNA denaturation (AO) in native semen samples and pregnancy achieved (r= -0.033; p=0.834).

A total of 46 semen samples of smokers (n=11) and non-smokers (n=35) were analysed before and after processing by Pure Sperm gradient centrifugation techniques. The results are shown in table 5.

The mean semen parameters were as follows: volume 3.5±1.3, concentration 70.0±33.42mill/ml, motility 33.22±17.09% motility after semen processing 64.24±31.52%, morphology 16.9±18.4%, DNA integrity (CMA_3) 16.7±8.23%, DNA fragmentation TUNEL 11.15±7.04% and membrane integrity 48.89±23.0%. The mean concentration of ROS and TAS in seminal plasma was (52.58±27.7µmol/l and 1.29±0.68(mmol/l).(Tab.5).

The mean number of retrieved oocytes was (5.22±4.52%) and the fertilizationrate and the clinical pregnancy rates were (55.40±24.67% and 32.6% respectively) (Tab.5).

Tab 5: Semen characteristic and sperm quality of smoker and non smoker patients underwent assisted reproduction technology program.

	N	M±SD	Percentile		
			75.	25.	50
Volume (ml)	46	3.478±1.27	2.875	3.500	4.500
pH-value	46	8.659±0.32	8.500	8.700	9.000
Sperm concentration (mill/ml)	46	70.00±33.42	50.00	75.00	101.00
Sperm concentration after semen preparation	46	39.55±31.786	14.25	31.00	56.25
Motility (%)	45	33.22±17.095	20.00	30.00	47.50
Motility after semen preparation (%)	46	64.24±31.517	38.75	80.00	90.00
Vitality (Eosin-test)	46	67.83±18.02	57.50	70.00	80.00
Membrane integrity (HOST-test)	45	48.89±23.00	30.00	50.00	65.00
Morphologically normal spermatozoa (%)	45	16.89±18.38	4.00	10.00	20.50
Chromatin condensation (CMA_3)	46	16.74±8.231	11.50	15.00	20.00
DNA Fragmentation TUNEL-Test	46	11.15±7.036	6.75	9.50	14.00
DNA Denaturation (Acridine Orange-test) (AO)	42	8.36±3.92	5.00	8.00	11.00
ROS (µmol/l) concentration in seminal plasma	46	52.58±27.77	33.08	44.04	74.347
TAS-concentration (mmol/l) in seminal plasma	46	1.29±0.681	0.953	1.38	1.757
Cotinine (µg/dl) concentration in seminal plasma	46	49.19±72.20	10.93	14.93	31.43
Fertilized Oocytes	45	5.22±4.52	2.00	3.00	8.00
Fertilization rate (%)	46	55.40±24.67	33.33	57.52	69.67
Transfer	46	1.89±0.795	1.75	2.00	2.00
Pregnancy (pregnancy rate)	43	14 (32. 6%)			

In this study, the ejaculat volume (ml) was similar in both groups (3.66±1.2 vs. 3.04±1.4; p=0.365). Vitality of the spermatozoa, membrane integrity (HOS-test), chromatin condensation (CMA3), DNA fragmentation TUNEL and DNA denaturation (Acridine orange) in the native semen of smokers were (64.6±19.16%, 47.73±16.03%; 15.73±7.18; 12.82±7.9 and 8.90±3.5) and the corresponding values in nonsmokers were (68.9±17.79; 49.26±25.04; 17.06±8.61; 10.6±6.8 and 8.2±4.1 respectively). A non significant decline in these parameters in somker in comparison to non-smokers was observed. Therefor, a significant differences in standard sperm parameters (mean percentage of sperm, motility, percentage of sperm vitality, membrane integrity, chromatin condensation, DNA stand breaks and DNA naturation) between infertile smokers and infertile nonsmokers could not found. Differences in the ROS and TAS values among the infertile smokers (50.94±28.20 and 1.09±0.73) and nonsmoker (53.1±28.03 and 1.35±0.66) groups were not statistically significant (Tab. 6).

On there other side, sperm concentration in the ejaculate of smokers was significantly lower than non-smokers (50.7±39.2 vs. 76.16±29.5, p=0.006). Whereas seminal plasma cotinine levels were found to be significantly higher (p=0.001) in smokers versus non smokers (144.29±82.19 vs. 19.30±32.69µg/ml, p=0.001)). Likewise the mean percentage of of motile spermatozoa after semen preparation from smokers (61.36±33.09%) was significantly lower in comparison to non-smokers (65.14±31.44%, p=0.021). The mean percentage of cleaved and transferred embryos and the pregnancy rate was significantly lower in smokers group (1.91±0.70% and 27.3%) in comparison to non smokers (1.89±0.83% and 34%, p=0.001).

Tab. 6: Comparison between semen characteristics and semen quality of smoker and non smoker patients underwent assisted reproduction treatments.

Semen characteristics	Non-smoker 35	Smokers 11	P-value
Volume (ml)	3.66±1.2	3.04±1.4	0.365
Sperm concentration (mill/ml) of the native semen samples	76.16±29.5	50.7±39.2	0.006
Sperm concentration (mill/ml) after Semen preparation.	41.32±29.89	33.91±38.23	0.412
Motility in the native semen samples (%)	31.57±15.98	39.0±20.38	0.061
Motility after semen preparation (%)	65.14±31.44	61.36±33.09	0.021
Vitality in the native semen samples (%)	68.9±17.79	64.6±19.16	0.107
Membrane integrity (HOS) in native semen (%)	49.26±25.04	47.73±16.03	0.140
Morphologically normal spermatozoa (%)	16.06±18.53	19.80±18.52	0.018
Chromatin condensation in the native semen sample (CMA3)	17.06±8.61	15.73±7.18	0.210
DNA fragmentation (TUNEL) (%)	10.6±6.8	12.82±7.9	0.210
DNA denaturation (Acridine orange) (%)	8.2±4.1	8.90±3.5	0.714
ROS concentration[µmol/l]	53.1±28.03	50.94±28.20	0.197
TAS concentration[mmol/l]	1.35±0.66	1.09±0.73	0.589
Mean number of collected Oocytes	8.9±5.7 (312)	10.82±7.8 (119)	0.018
Mean number of fertilized Oocytes	5.12±4.2 (174)	5.55±5.7 (61)	0.018
Fertilization rate (%)	58.5±24.6	45.6±23.5	0.591
Cleaved and transferred Oocytes	1.89±0.83 (66)	1.91±0.70 (21)	0.001
Pregnancy (Pregnancy rate)	11 /32 (34%)	3/11 (27.3%)	0.001
Cotinine (µg/dl)	19.30±32.69	144.29±82.19	0.001

Statistically significant ($p<0.05$)

Figures (22-34) demonstrate the standard semen parameters in both smoker and non-smoker groups.

Before semen processing the mean number of spermatozoa of non-smokers was significantly higher than for smokers (76.16±29.5 versus 50.7±39.2mill/ml)(Fig. 24).

In addition, the mean number of motile spermatozoa in the native semen samples of non-smokers and smokers group was similar (31.57 ±15.98% versus 39.0 ±20.30%; p=0.061) (Fig. 25). However, the mean percentage of motile spermatozoa of non-smoker group was increased significantly (p=0.021) after semen processing in comparison to the smokers (65.14±31.44% versus 61.36±33.09%). When the morphological distribution of spermatozoa was analysed, smokers spermatozoa had a significantly higher mean percentage (19.80±18.52%) of abnormal forms in comparison to non-smoker samples (16.06±18.53%; p= 0.018) (Fig.27). However, the percentage of DNA integrity (Chromomycin CMA_3) was not different between smokers and non smokers specimens (15.73±7.18% versus 17.06± 8.61% ; p=0.210)(Fig. 28). No difference in other parameters were seen between smokers and non-smokers group. Also, There was no statistically significant difference in the mean number of sperm concentration after semen processing and the motility, vitality (Fig. 23), and ROS (Fig.30), TAS concentration (Fig.31) in the native semen samples between smoker and non–smokers group. The differences in sperm DNA damage, as assessed by Acridin orange and Tunel, between the infertile smokers and non smokers did not reach a statistically significance (Fig. 29).

Cotinine is the main metabolit of nicotine was significantly higher (144.29± 82.19µg/dl) in the smoking than non smoking group (19.30±32.69 p= 0.001) (Fig.33).

Besides, statistically significant difference were found in the mean number of fertilized (Fig. 32), cleaved oocytes and pregnancy rates. The ART success rate in 46 patients in term of clinical pregnancies in women with smoking male partners was 27.3%; the rate was 34% with non-smoking patients group (Tab. 6 &; Fig. 34).

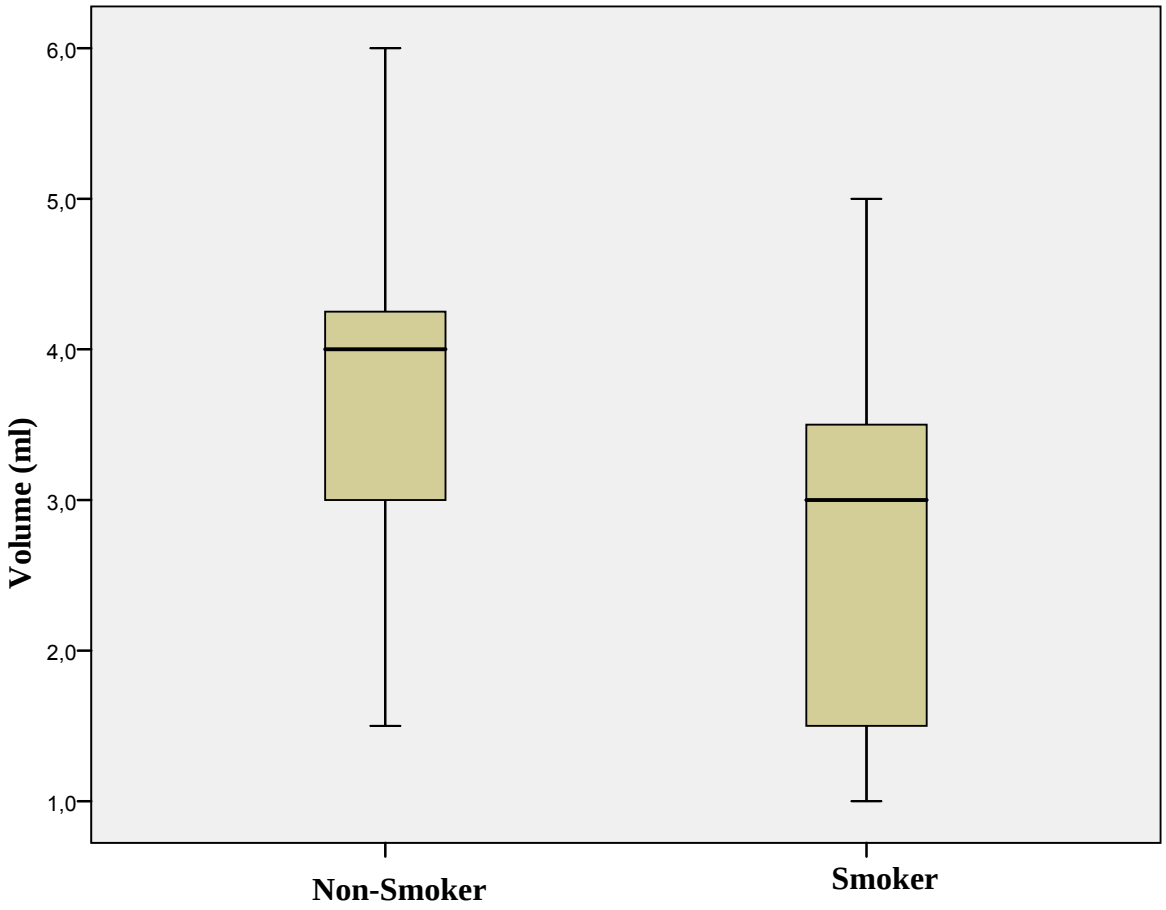

Figure 22: Box-and whisker-plots showing the mean, median, lower and upper quartiles and range of semen volume (ml) of smoking and non-smoking men underwent IVF/ICSI therapy. The mean value of ejaculate volume was not different between smoker (3.04±1.4) and non-smoker group (3.66±1.2ml; p= 0.365) .

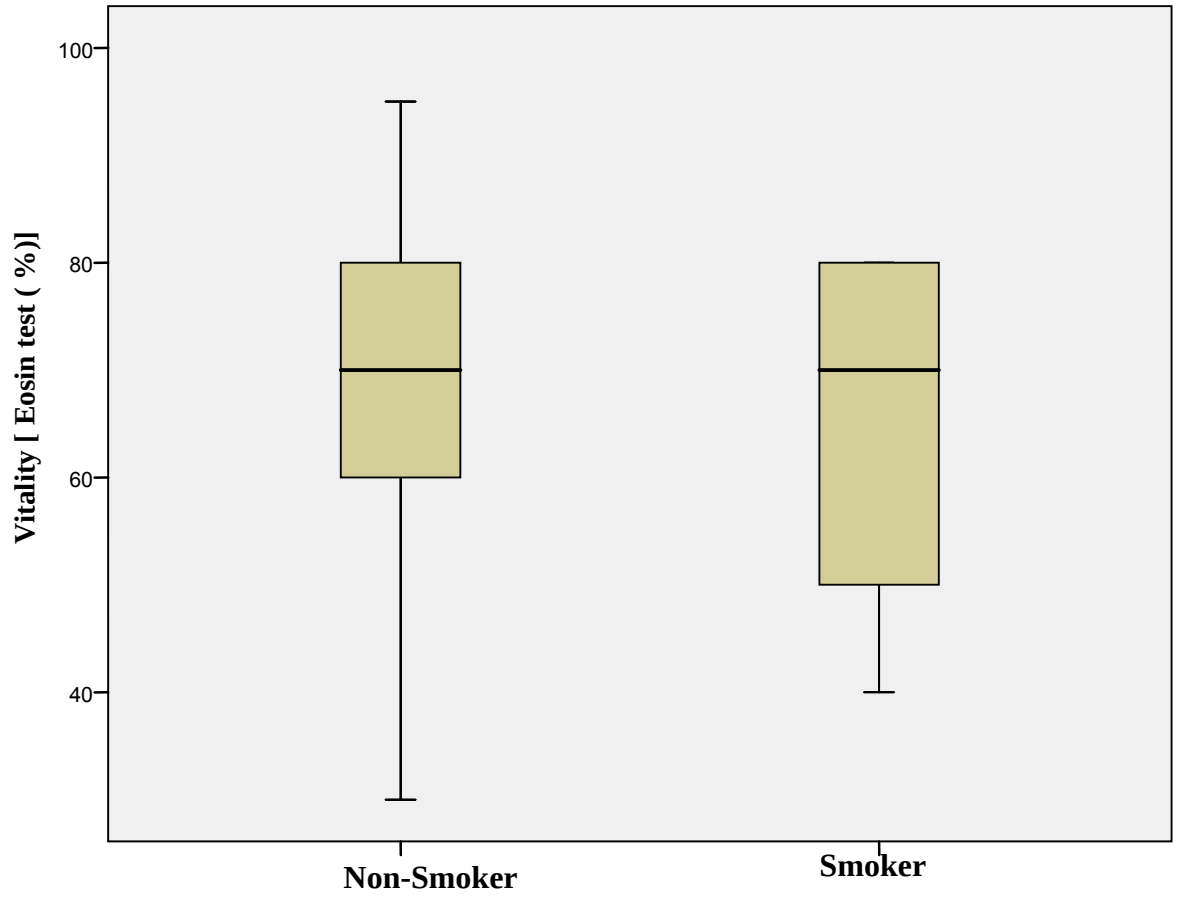

Figure 23: Box-and whisker-plots showing the mean, median and range of sperm vitality of the native semen samples of smokers and non smoker's underwent IVF/ICSI therapy. No significant difference was found between the groups (64.6±19.16% versus 68.9±17.79%; p=0.107).

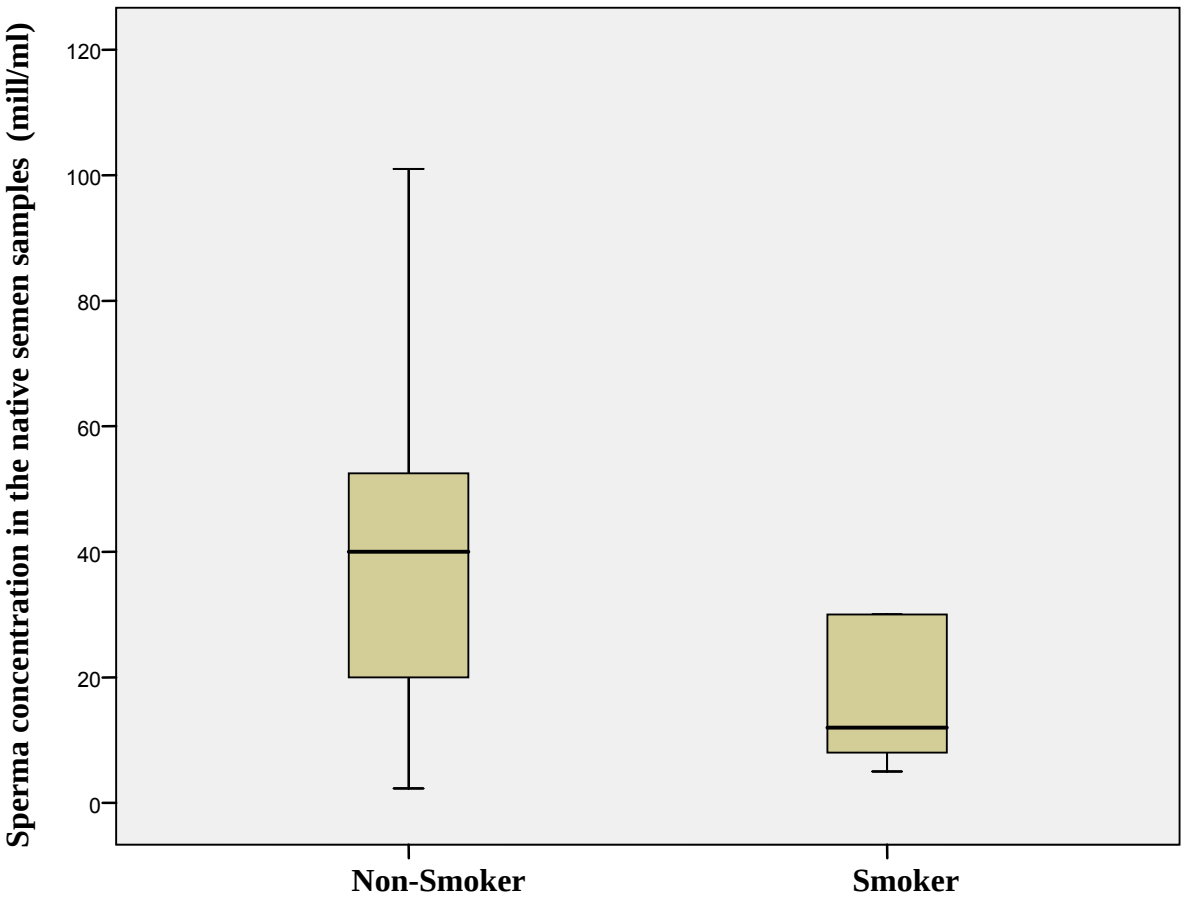

Figure 24: Box-and whisker-plots showing the mean, median, and range of sperm concentration in the native semen samples of smokers and non smokers underwent IVF/ICSI therapy. A statistically significant higher (p=0.006) sperm concentration could be found in non- smokers (76.16±29.5) in comparison to smokers (50.7±39.2).

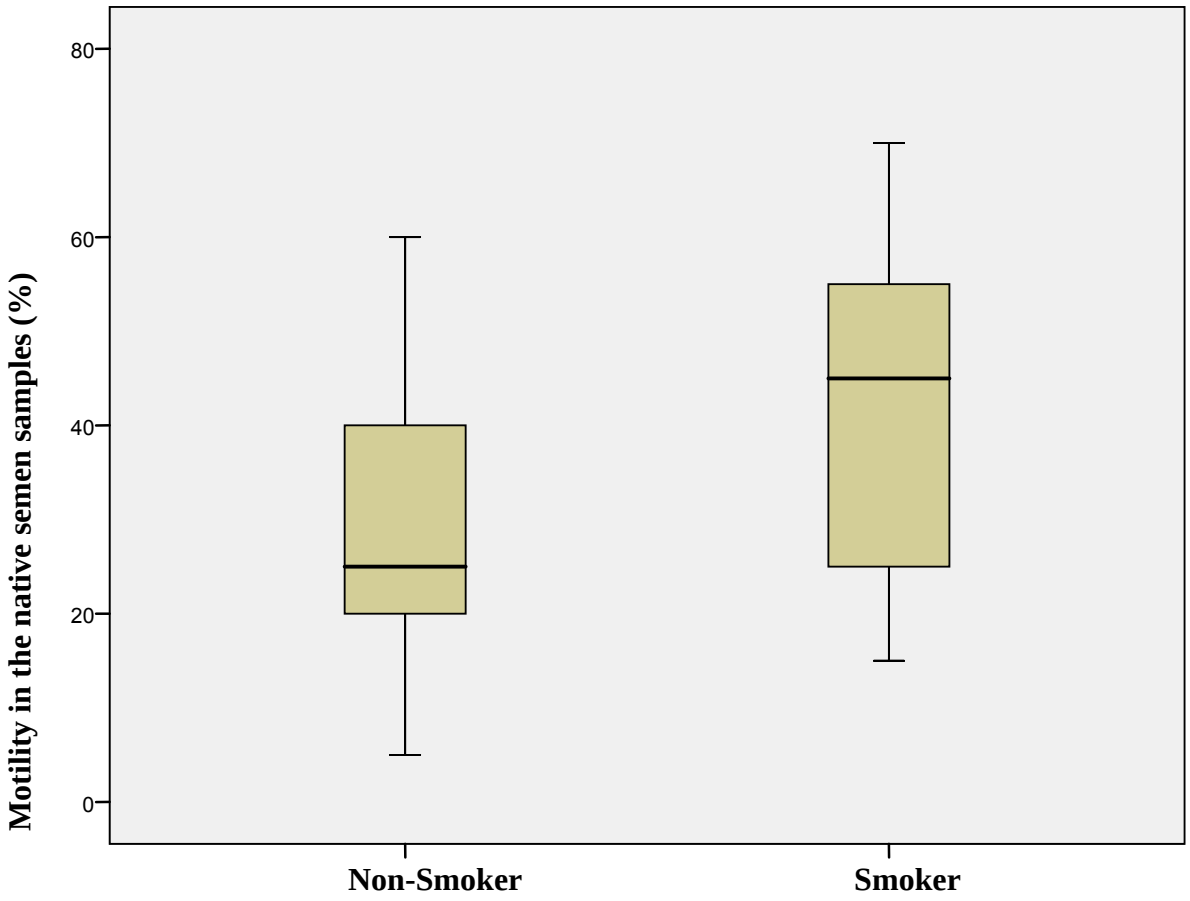

Figure 25: Box-and whisker-plots showing the mean, median and range of sperm motility in the native semen samples of smokers (39.0±20.38) and non smokers (31.57±15.98) men underwent IVF/ICSI therapy. There was no signifant difference (p= 0.061) between the two investigated groups.

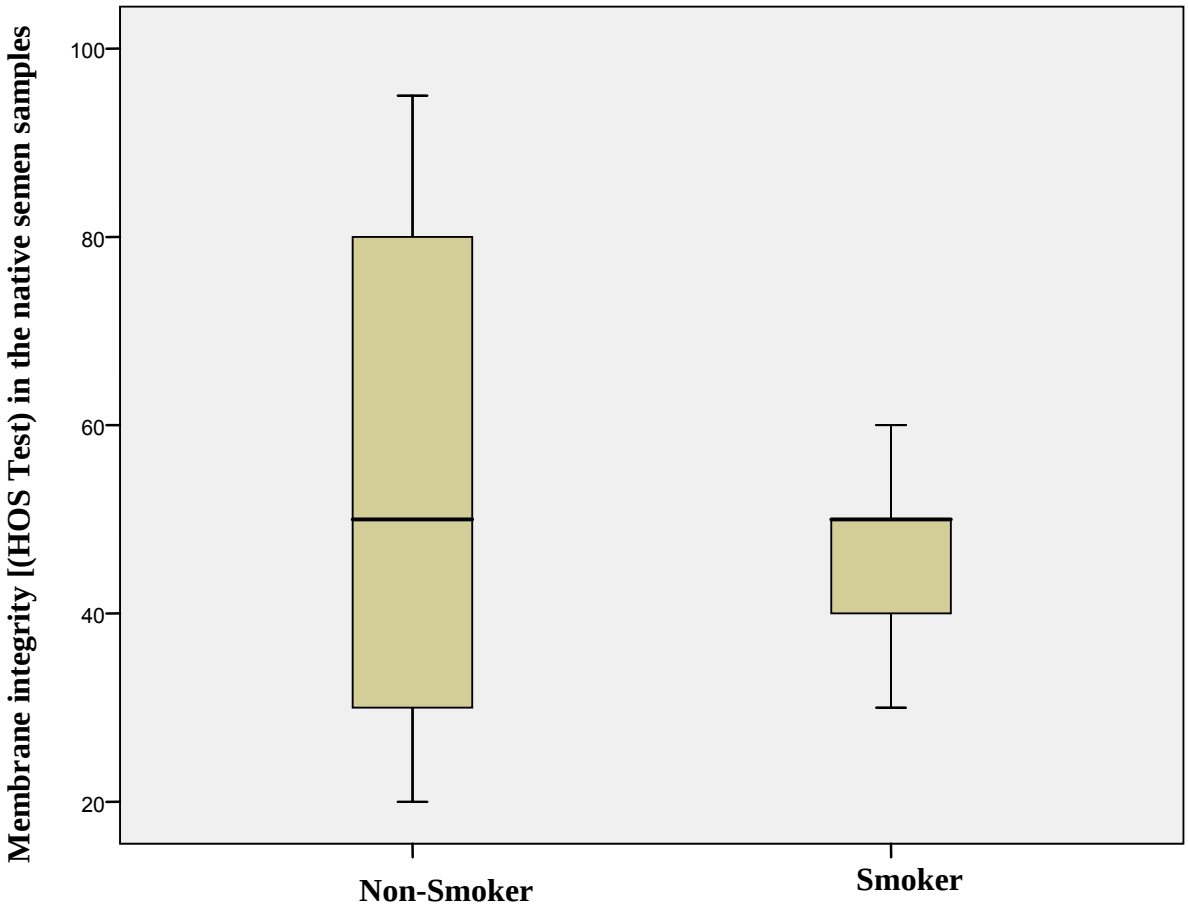

Figure 26: Box-and whisker-plots showing the mean, median and range of semen of membrane integrity (HOS-test) of spermatozoa in the native semen samples of smokers (47.73±16.03) and non smokers (49.26±25.04) underwent IVF/ICSI therapy. No statistical significance was observed between non-smoker and smoker group (p= 0.140).

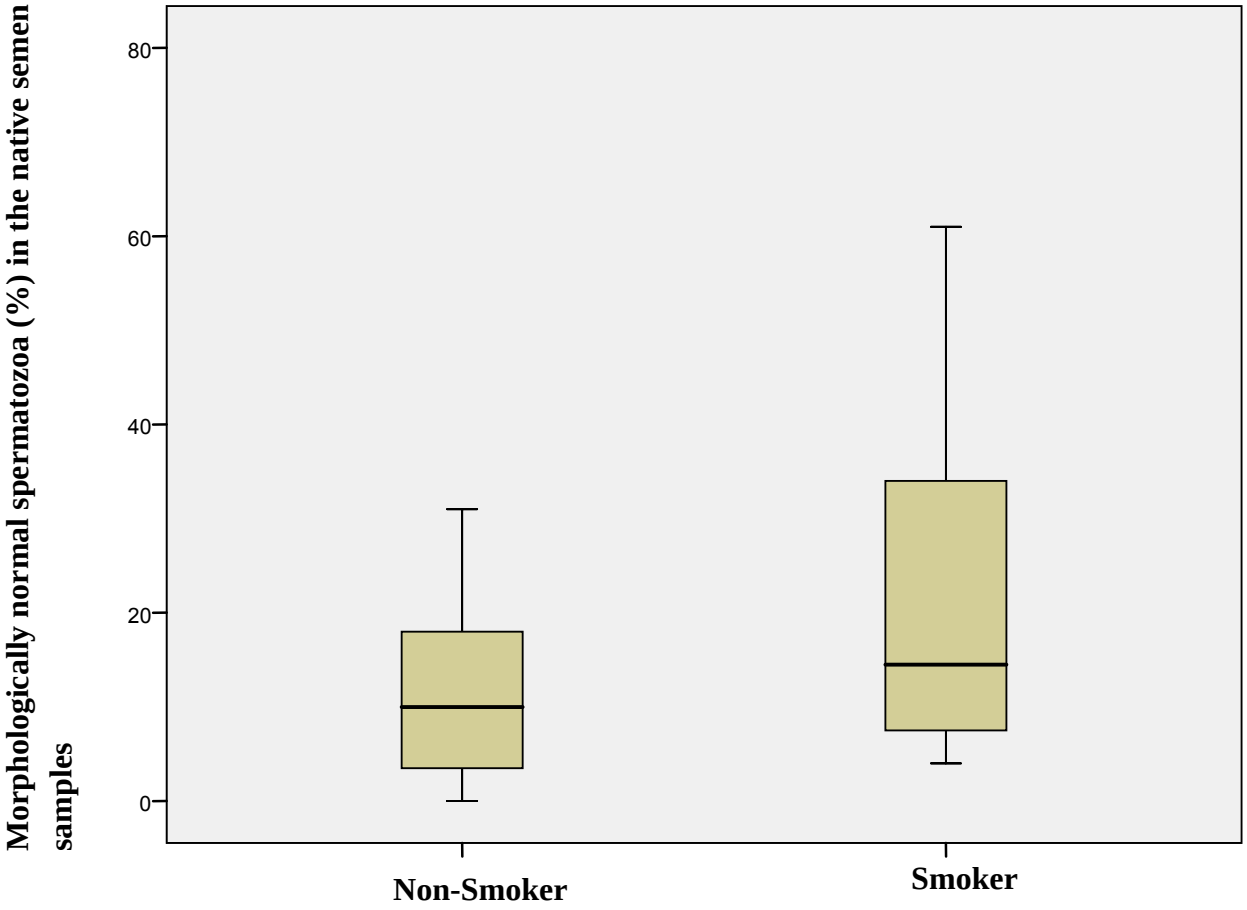

Figure 27: Box-and whisker-plots showing the mean, median and range of morphologically normal spermatozoa in the native semen samples of smokers and non-smoker's underwent IVF/ICSI therapy. The men percentage of morphologically normal spermatozoa was significantly higher (p=0.018) of spermatozoa of non-smoker (16.06±18.53%) in comparison to smoker patients group (16.06±18.53%)

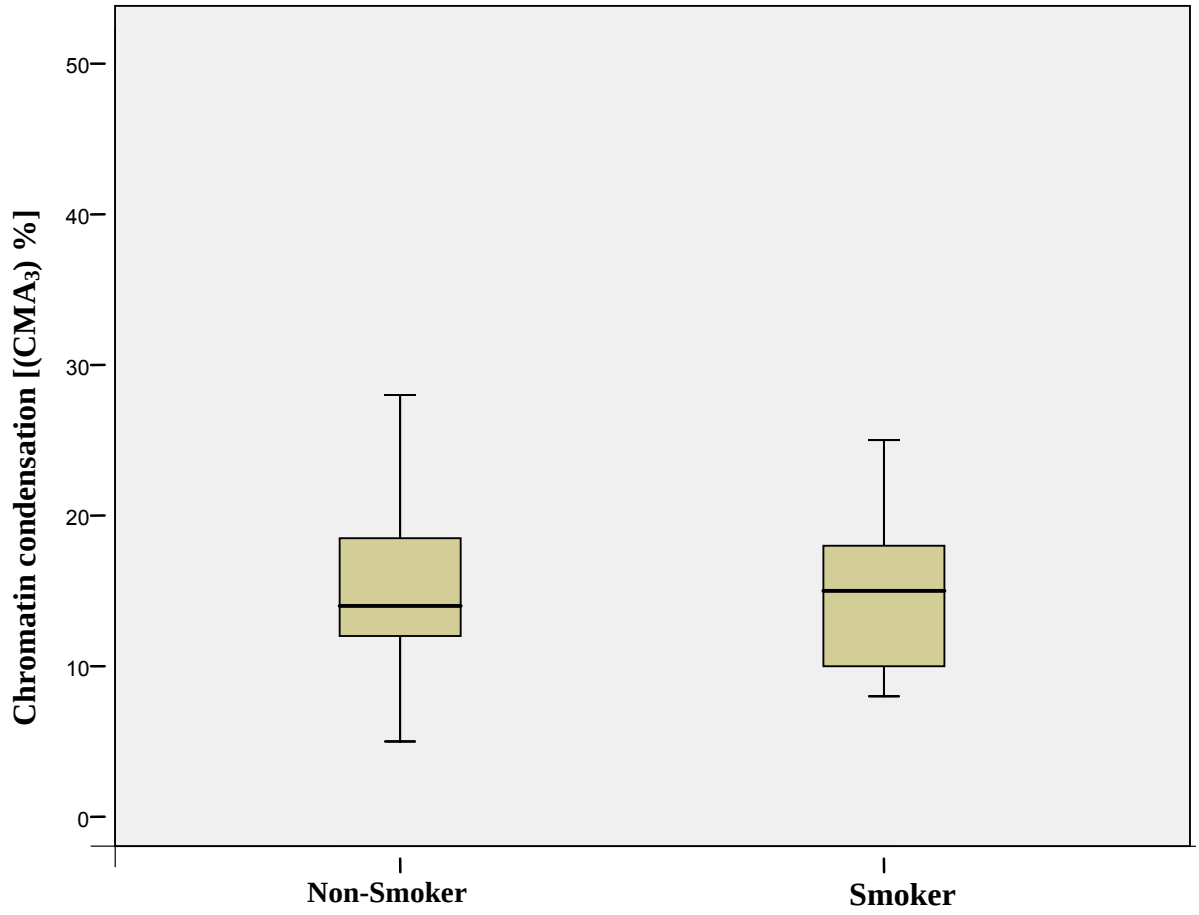

Figure 28: Box-and whisker-plots showing the mean, median and range of sperm chromatin condensation (CMA_3) in the native semen sample of smokers and non smokers underwent ART therapy. The proportion of DNA denaturation (AO-test) was similar between smoker and non smoker group (8.2±4.1 VS; 8.90±3.5; p=0.714).

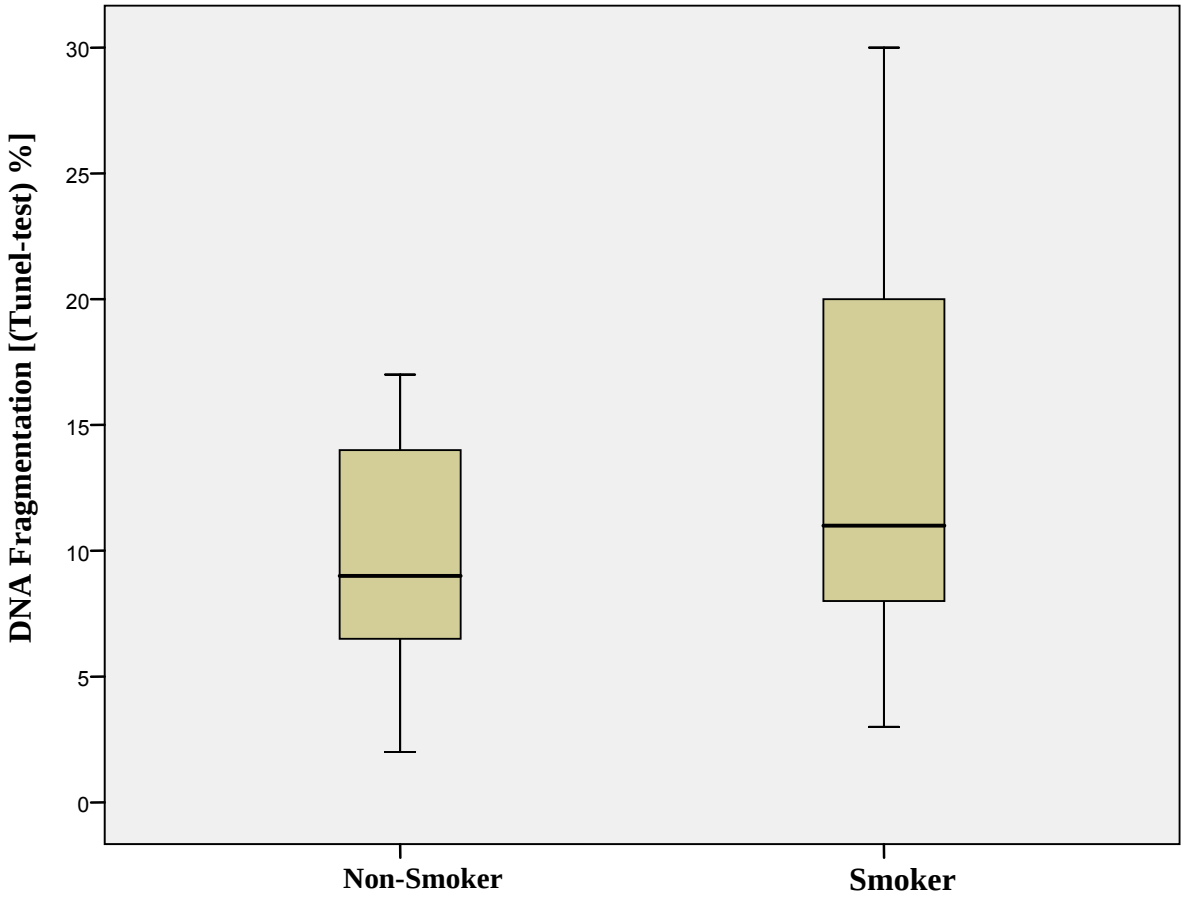

Figure 29: Box-and whisker-plots showing the mean, median and range of sperm DNA fragmentation (TUNEL) in the native semen sample of smokers and non-smokers underwent ART therapy, the proportion of DNA fragmentation (TUNEL-test) was not different between smoker and non smoker group (10.6±6.8 versus 12.82±7.9; p=0.210) .

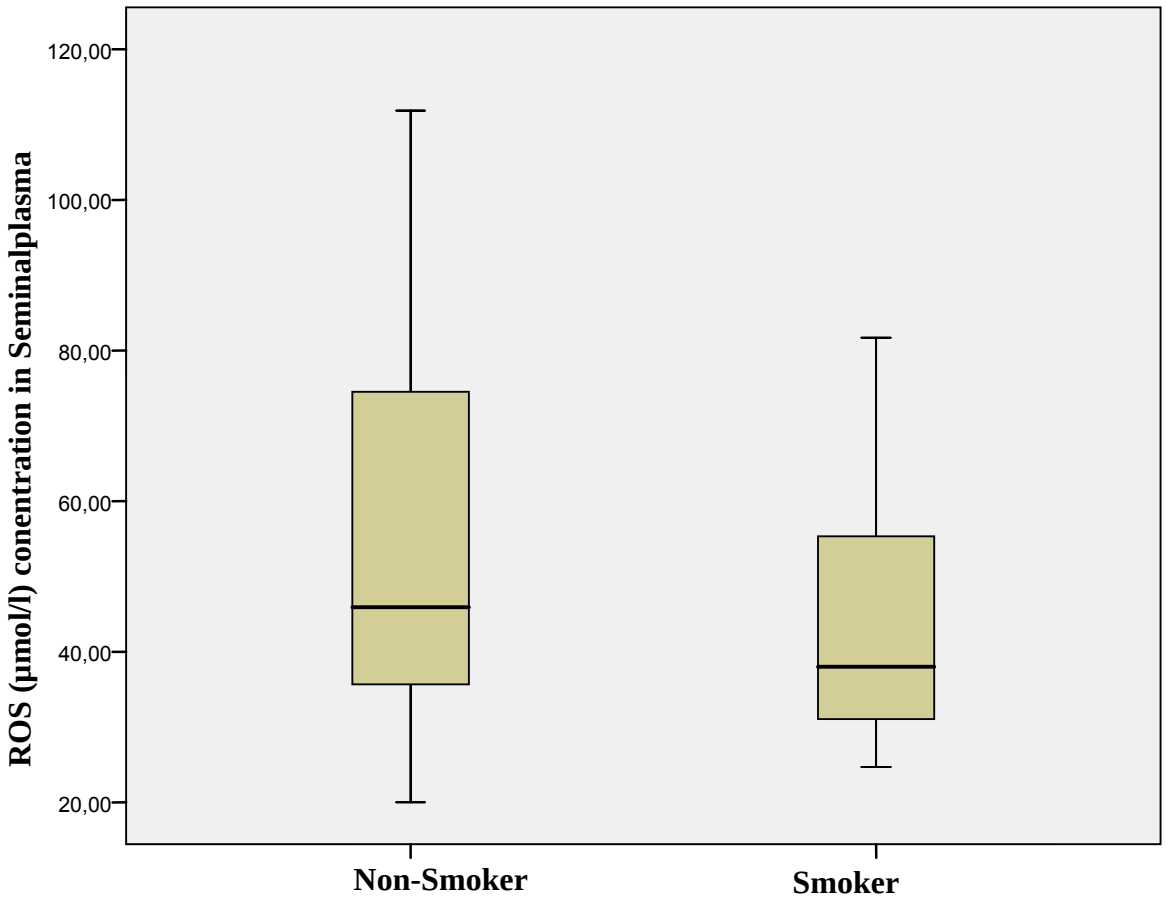

Figure 30: Box-and whisker-plots showing the mean, median and range of semen ROS concentration (µmol/l) of smoking and non-smoking patients underwent IVF/ICSI therapy. No statistical significance (p= 0.197) was observed between non-smoker (53.1±28.03) and smoker group (50.94±28.20).

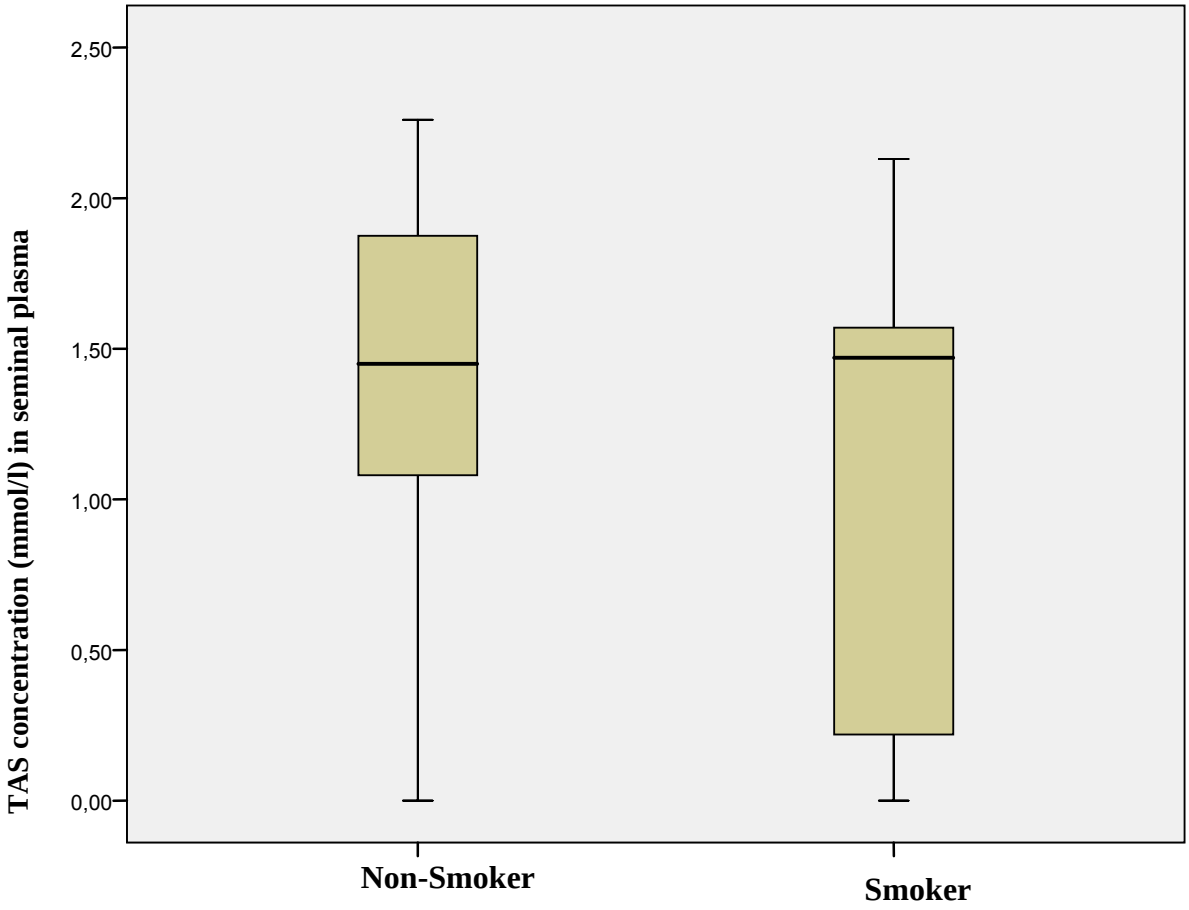

Figure 31: Box-and whisker-plots showing the mean, median and range of semen TAS concentration (mmol/l) of smoking and non-smoking patients underwent IVF/ICSI therapy. The mean concentration of TAS in seminal plasma differed non-significantly (p= 0.589) between non-smokers (1.35±0.66 mmol/l) and smokers group (1.09±0.73 mmol/l)

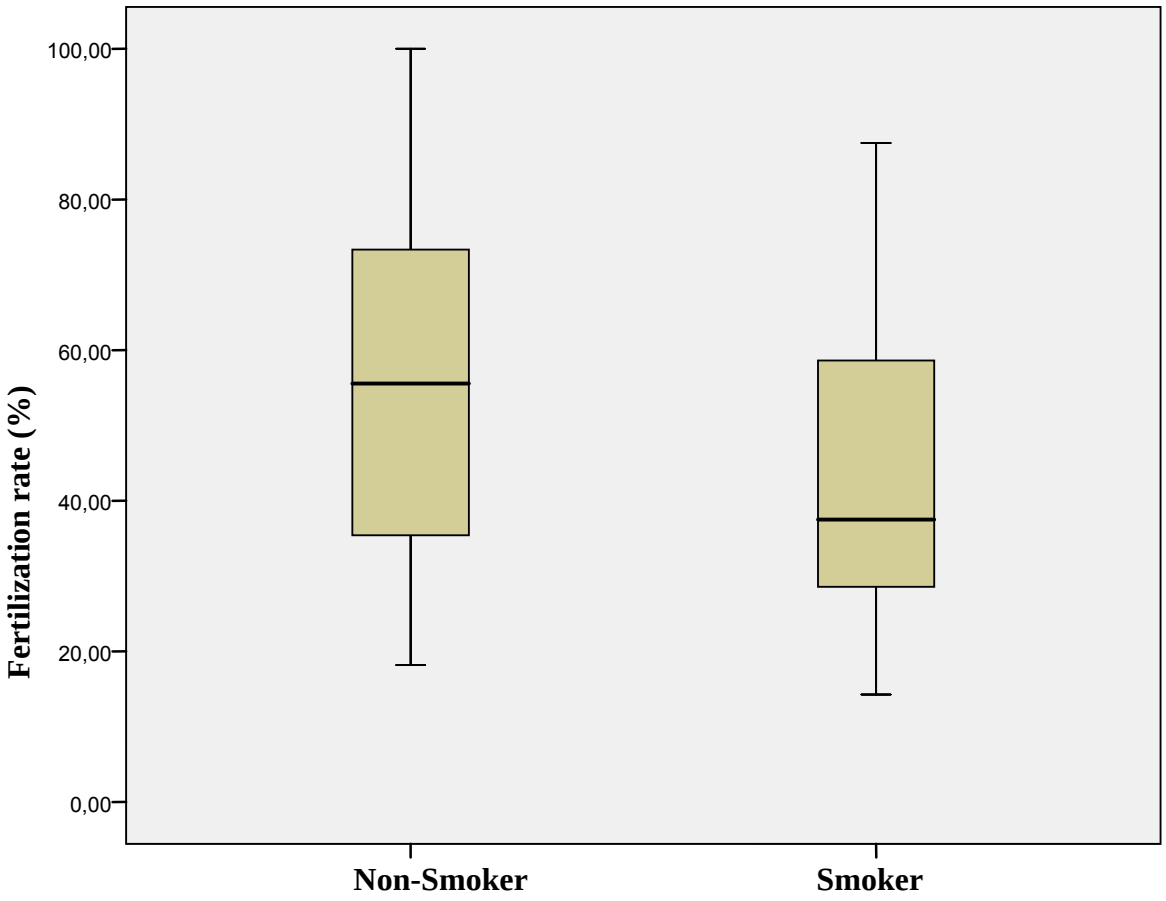

Figure 32: Box-and whisker-plots demonstate the mean, median, lower and upper quartiles and range of fertilization rate of smokers and non smokers group. The pregnancy rate was (p=34%) for non smokers and 27% for smokers, resulting in a significant difference between non-smokers and smokers (p=0.001).

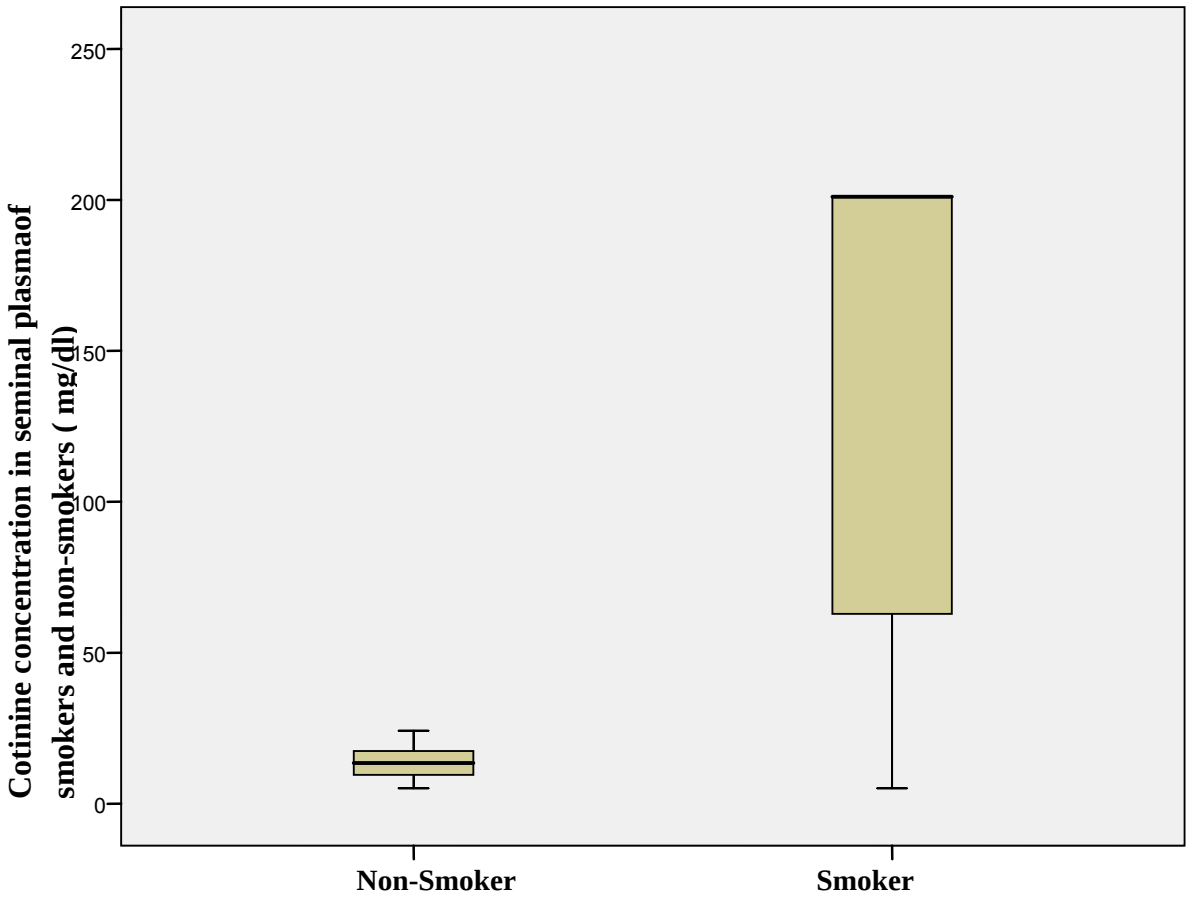

Figure 33: Box–and-whisker plots indicate mean, median, lower and upper quartiles and range of semen cotinine concentration of smoking and non-smoking patients underwent IVF/ICSI therapy. In this study, the mean semen cotinine levels of smokers and non smokers were (144.29±82.19 vs. 19.30±32.69 mg/dl; p=0.001 mg/dl, respectively).

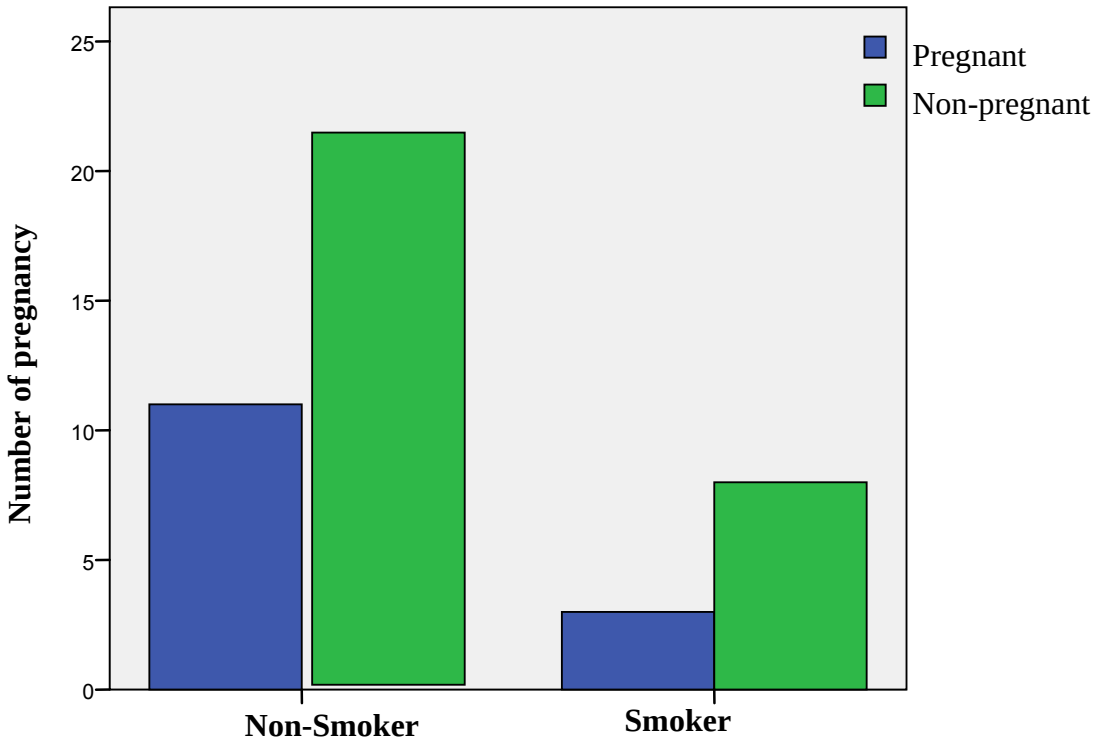

Figure 34: Pregnancy outcome of smokers and non-smokers patients underwent IVF/ICSI therapy. Eleven pregnancies out of 32 patients became transfer in the non smoker groups (34% pregnancy rate). However, in the smoker's patient group only 3 pregnancies were achieved out of 11 patients who became transfer (27.3%). Also the pregnancy rate was significantly higher in non-smokers group in comparison to smoker group (p=0.0001).

Correlation of semen parameters of smoker patients are demonstrated in Table 7a, b. The vitality (Eosin test) showed a non-significant negative correlation with sperm motility (r=-0.571; p=0.085), membrane integrity (r=-0.015; p=0.965), DNA fragmentation (r=-0.172; p=0.613), TAS concentration (r=-0.492, p=0.124), cleaved and transferred embryos (r=-0.319; p=0.864) and pregnancy (r=-0.342; p=0.303). A more non significant negative linear trend could be shown in sperm motility and membrane integrity (r=0.252, p= 0.483) (Fig. 36), DNA integrity (CMA_3) (r=-319; p=0.369) and Cotinine concentration (r=0.120; p=0.741).

In addition, sperm membrane integrity (HOS-test) correlated negatively, but non-significant with sperm concentration (r=-0.117; p=0.731), DNA integrity (r=0.044; p= 0.899), ROS concentration (r=-550; p= 0.079), TAS concentration in seminal plasma (r=-0.270; p=421), fertilization rate (r= -0.265; p=0.430) (Fig. 41), cleaved rate (r=-0.059; p=0.864) (Fig.42) and pregnancy rate (R= -0.342; p=0.303).

The mean percentage of morphologically normal spermatozoa demonstrated a negatively significant correlation with ROS concentration (r= -0.869; p=0.014), TAS concentration in seminal plasma (r=-0.742; p=0.014) and negatively non significant correlation with sperm motility (r=-0.104; p=0.776), DNA fragmentation (r=-0.401; p=0.250) and fertilization rate (r=-394; p= 0.784) (Fig. 37)..A more non significant negative linear trend could be seen in sperm motility and membrane integrity (r=-0.252, p= 0.483) (Fig. 36), DNA integrity (CMA_3) (r=-319; p=0.369) and Cotinine concentration (r=0.120; p=0.741).

Moreover, a statistical significant negative linear trend in cotinine concentration in seminal plasma and sperm DNA fragmentation (TUNEL) was observed (r=-0.791; p=0.004). Besides, there were a statistically, non-significant, negative correlations between cotinine concentration in seminal plasma and sperm motility (r=-0.120; 0,741), DNA integrity (CMA_3) (r=-0.058; p=0.865), total anti oxidant concentration (TAS) (r=-0.042; p=0.902), fertilization (r=-0.074; p=0.829) and pregnancy rate (r=-0.075; p=0.827) (Tab.7[a,b]).

DNA fragmentation (TUNEL) correlate negatively with the following variable: Sperm vitality (r=-0.172; p= 0.613), morphologicaaly normal spermatozoa (r=-0.401; p=0.250), fertilization rate (r=-0.046; p= 0.894). Seminal plsam cotinine concentration demonstrated a significantly negative correlation (r=-0.791; p=0.004) with sperm DNA fragmentation (TUNEL) and a negative non significant correlation with sperm motility (r= -0.120; p=0.741); chromatin condensation (r=-0.058; p= 0.865), TAS concentration (r=-0.042; p=0.902), fertilization rat (r=-0.074; p=0.829), cleavage rate (r=-0.372; p=0.259) and pregnancy rate (r=-0.075; 0.827) (Tab. 7a, b).

Seminal plasma ROS of smokers demonstrates significant negative correlation with sperm morphology (r=-0.869; P=0.001) and TAS concentration (r= 0.753; p=0.007). Non significant correlations were elicited with semen concentration (r=-0.055; p=0.871), motility in the native semen sample (r= 0.358; p= 0.310) vitality (r=-0.306; p= 0.360) , membrane integrity (r=-0.550; 0.079), Chromatin condensation CMA3 (r=0.137; p=0.687), DNA fragmentation (TUNEL) (r= 0.313; p=0.349) and cotinine concentration (r= -0.338; p= 0.310), fertilization and pregnancy rate (r=0.320; p= 0.338 und r=-0.291; p=0.385 respectively)

Table 7a: Correlation of semen parameters of smoker patients (n=11).

	Volume	Concentration in native semen	Motility in the native semen	Vitality Eosin test	Membrane integrity (HOS)	Morphology	CMA_3	DNA-TUNEL-
Sperm concention (ml) in native semen	-0.481	1.000	0.429	0.443	-0.117	0.315	-0.132	0.016
	0.134	.	0.217	0.172	0.731	0.376	0.699	0.962
Motility in native semen (%)	0.071	0.429	1.000	-0.571	-0.252	-0.104	-0.319	0.092
	0.845	0.217	.	0.085	0.483	0.776	0.369	0.801
Vitality Eosin (%)	-0.557	0.443	-0.571	1.000	-0.015	0.420	0.224	-0.172
	0.075	0.172	0.085	.	0.965	0.227	0.508	0.613
Membrane integrity (HOS) (%)	-0.552	-0.117	-0.252	-0.015	1.000	0.437	-0.044	0.208
	0.078	0.731	0.483	0.965	.	0.206	0.899	0.540
Morphologically normal sperm (%)	-0.554	0.315	-0.104	0.420	0.437	1.000	0.201	-0.401
	0.097	0.376	0.776	0.227	0.206	.	0.577	0.250
Chromatin Condensation. (MA3)	-0.234	-0.132	-0.319	0.224	-0.044	0.201	1.000	0.156
	.489	0.699	0.369	0.508	0.899	0.577	.	0.648
DNA fragmentation Tunel -test	-0.392	0.016	0.092	-0.172	0.208	-0.401	0.156	1.000
	0.232	0.962	0.801	0.613	0.540	0.250	0.648	.
ROS (µmol/l)	0.438	-0.055	0.358	-0.306	-0.550	-0.869(**)	0.137	0.313
	0.178	0.871	0.310	0.360	0.079	0.001	0.687	0.349
TAS (mmol/l)	1.000	-0.139	0.174	-0.492	-0.270	-0.742(*)	0.101	0.205
	.	0.684	0.630	0.124	0.421	0.014	0.768	0.544
Cotinine (mg/ml)	0.168	0.208	-0.120	0.196	0.045	0.451	-0.058	-0.791(**)
	0.621	0.539	0.741	0.564	0.896	0.191	0.865	0.004
Fertilization rate (%)	0.129	0.178	-0.305	0.532	-0.265	-0.394	-0.094	-0.046
	0.706	0.601	0.392	0.092	0.430	0.260	0.784	0.894
Cleaved and transferred oocytes	0.321	-0.176	0.321	-0.319	-0.059	-0.461	-0.174	0.227
	0.366	0.605	0.366	0.338	0.864	0.180	0.609	0.503
Pregnancy	-0.132	-0.065	0.268	-0.200	-0.342	0.342	0.032	-0.226
	0.698	0.848	0.455	0.555	0.303	0.334	0.925	0.503

Table. 7b: Correlation of semen parameters of smoking patients (n=11).

	DNA - TUNEL	DNA - AO	ROS	TAS	Cotinine	Fertilization rate	Transfer	Pregnancy
Sperm concentration in native semen (mill/ml)	0.016	-0.593	-0.055	-0.139	0.208	0.178	-0.176	-0.065
	0.962	0.071	0.871	0.684	0.539	0.601	0.605	0.848
Motility in the native semen(%)	0.092	0.416	0.358	0.174	-0.120	-0.305	0.321	0.268
	0.801	0.265	0.310	0.630	0.741	0.392	0.366	0.455
Vitality (%) Eosin	-0.172	-0.720(*)	-0.306	-0.492	0.196	0.532	-0.319	-0.200
	0.613	0.019	0.360	0.124	0.564	0.092	0.338	0.555
Membrane integrity (HOS) (%)	0.208	0.114	-0.550	-0.270	0.045	-0.265	-0.059	-0.342
	0.540	0.753	0.079	0.421	0.896	0.430	0.864	0.303
Morphologically normal sperm (%)	-0.401	-0.050	-0.869(**)	-0.742(*)	0.451	-0.394	-0.461	0.342
	0.250	0.898	0.001	0.014	0.191	0.260	0.180	0.334
Chromatin condensation (CMA_3)	0.156	0.160	0.137	0.101	-0.058	-0.094	-0.174	0.032
	0.648	0.660	0.687	0.768	0.865	0.784	0.609	0.925
DNA Fragmentation TUNEL-test (%)	1.000	-0.055	0.313	0.205	-0.791(**)	-0.046	0.227	-0.226
	.	0.880	0.349	0.544	0.004	0.894	0.503	0.503
ROS (µmol/l)	0.313	0.239	1.000	0.753(**)	-0.338	0.320	0.491	-0.291
	0.349	0.507	.	0.007	0.310	0.338	0.125	0.385
TAS (mmol/l)	0.205	0.260	0.753(**)	1.000	-0.042	0.237	0.441	-0.323
	0.544	0.468	0.007	.	0.902	0.482	0.175	0.332
Cotinine (mg/dl)	-0.791(**)	-0.240	-0.338	-0.042	1.000	-0.074	-0.372	-0.075
	0.004	0.504	0.310	0.902	.	0.829	0.259	0.827
Fertilized oocytes	-0.199	-0.379	-0.065	0.044	0.139	0.896(**)	0.183	-0.327
	0.558	0.280	0.850	0.898	0.684	0.000	0.589	0.326
Fertilization rate (%)	-0.046	-0.165	0.320	0.237	-0.074	1.000	0.438	-0.453
	0.894	0.648	0.338	0.482	0.829	.	0.178	0.162
Cleaved and transferred oocytes	0.227	0.523	0.491	0.441	-0.372	0.438	1.000	-0.392
	0.503	0.121	0.125	0.175	0.259	0.178	.	0.232
Pregnancy	-0.226	0.191	-0.291	-0.323	-0.075	-0.453	-0.392	1.000
	0.503	0.597	0.385	0.332	0.827	0.162	0.232	.

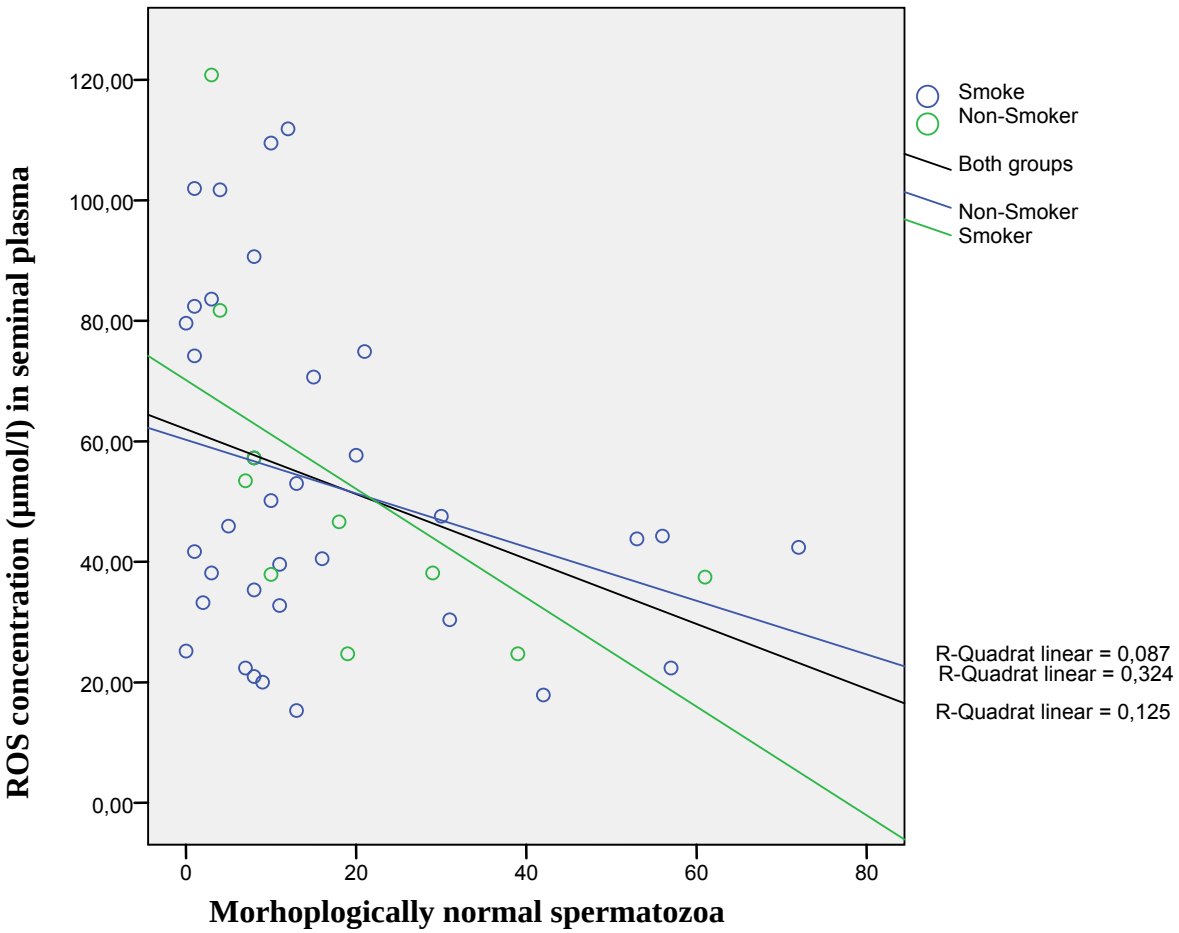

Figure 35: Scatter plot demonstrate the correlation between ROS concentration in seminal plasma and the percentage of morphologically normal spermatozoa in both groups (r=-0.347; p=0.020), in smokers (r=-0.869; p= 0.001)) and non smokers group (r=-0.228; p=0.188) of patients underwent IVF/ICSI therapy.

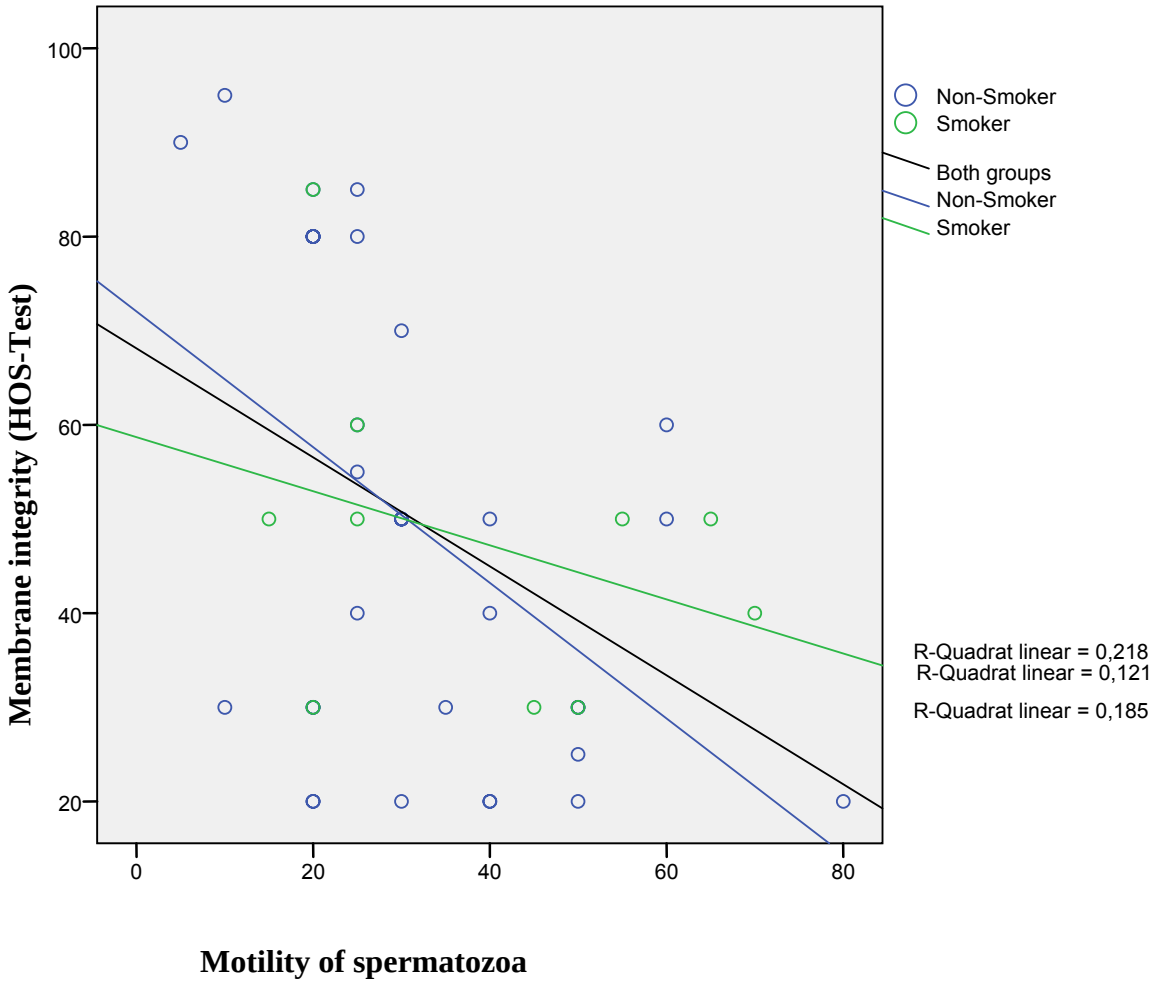

Figure 36: Scatter plot of correlation between sperm membrane integrity of smokers, non smokers and motility of patients underwent IVF/ICSI therapy. There was an inverse correlation between sperm motility and sperm membrane integrity not only by smoker groups (r=- 0.252; p= 0.483) but also by non-smokers too (r= -0.450; p=0.007).

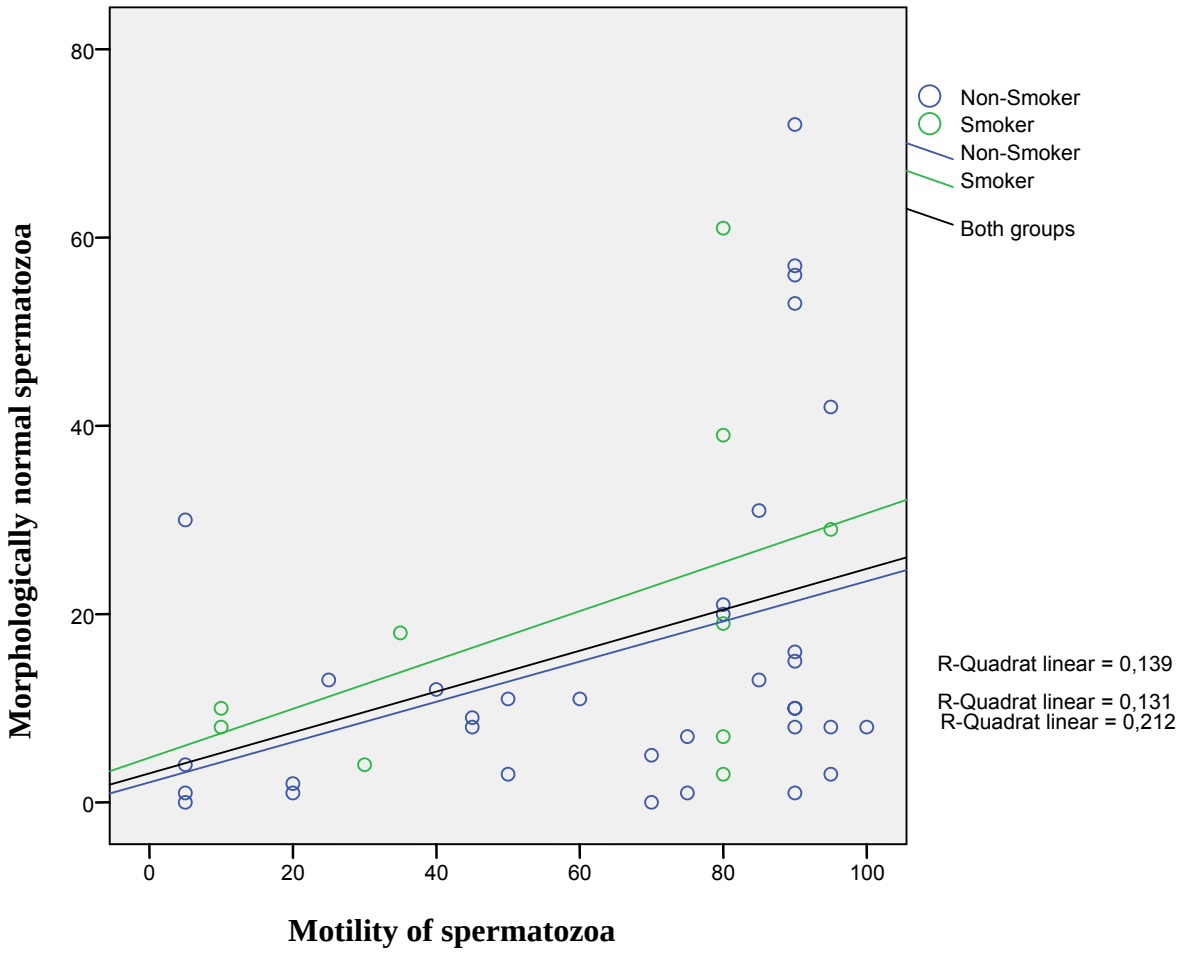

Figure 37: Scatter plot of correlation between morphologically normal spermatozoa of smokers, non smokers and motility of patients underwent IVF/ICSI therapy. A negative non significant correlation was observed between the morphologically normal spermatozoa and the mean percentage of motile spermatozoa in the native semen samples of smokers (r= -0.104; p=0.776) and non –smokers group (r=-0.158; p=0.365).

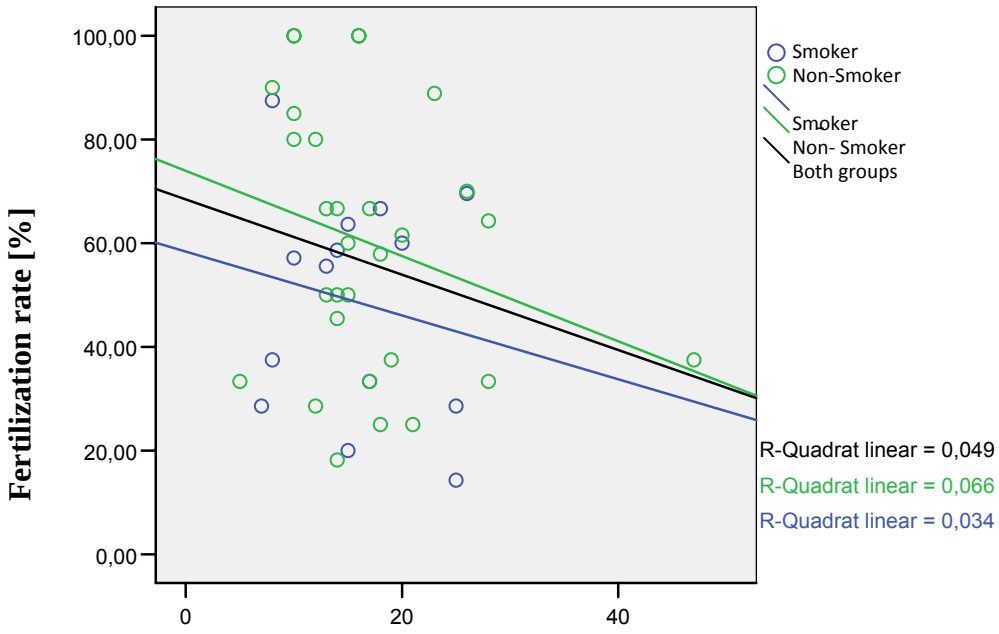

Figure 38: Scatter plot demonstrate the correlation between DNA integrity (CMA3) of spermatozoa and fertilization rate in both groups (r=-0.204; p=0.175), in smokers (r=-0.094; p= 0.784)) and non smokres group (r=-0.263; p=0.126) of patients underwent IVF/ICSI therapy.

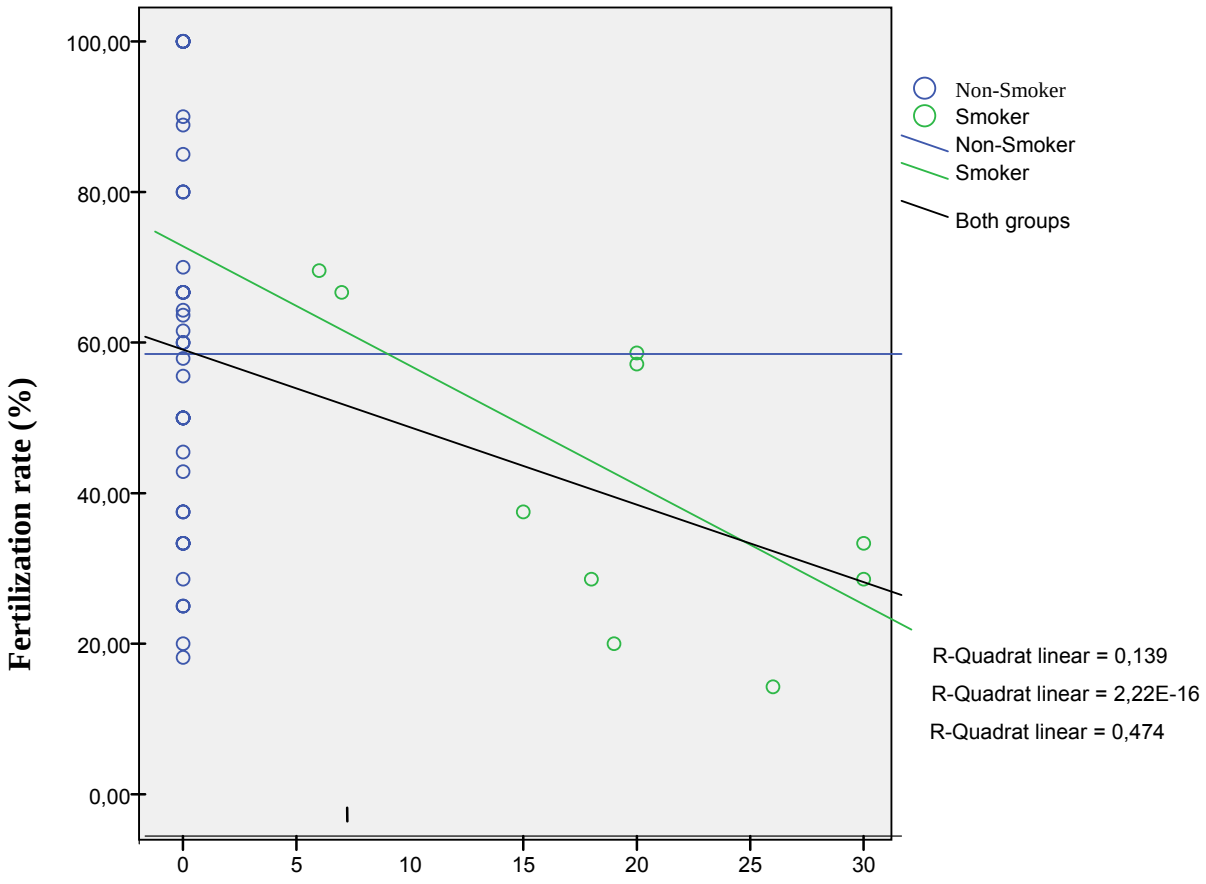

Figure 39: Scatter plot demonstrate the correlation between cotinine concentration in seminal plasma and fertilization rate in both groups (r=-0.107; p=480), smoker (r= -0.074; p=0.829), non smokers (n=-0.048; 0.786) of patients underwent IVF/ICSI therapy.

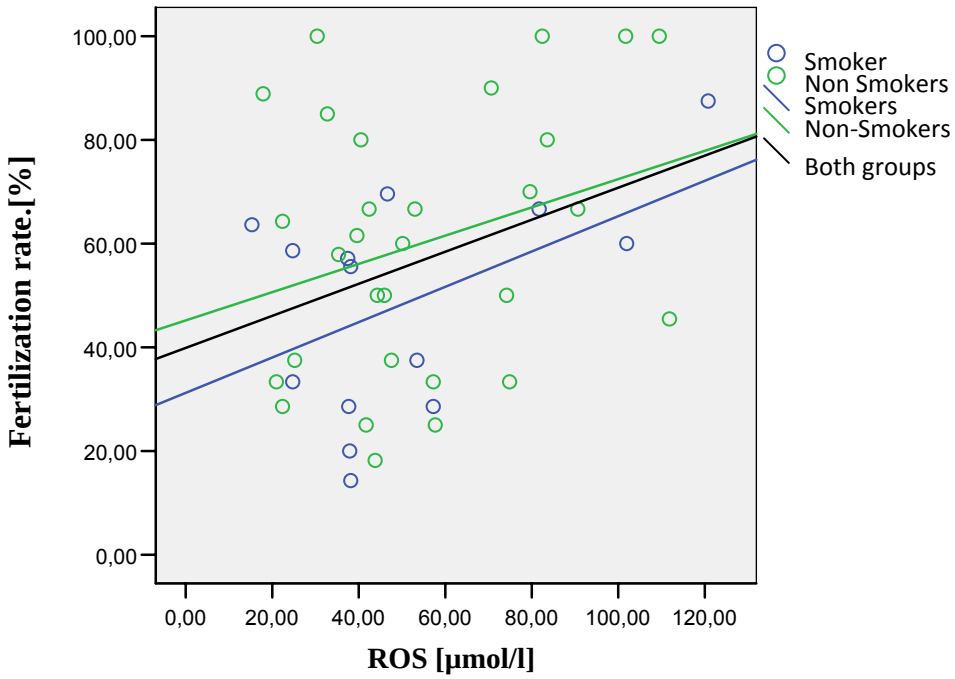

Figure 40: Scatter plot of Correlation between ROS concentration in seminal plasma of male partners of women underwent IVF/ICSI therapy and the fertilization rate of the oocytes. Statistically non significant positive correlation was observed between the ROS concentration and fertilization rate (r=0.320; p=0.338). Whereas, a negative correlation was shown between ROS concentration in seminal plasma and the mean number of fertilized oocytes (r=-0.065; p= 0.850).

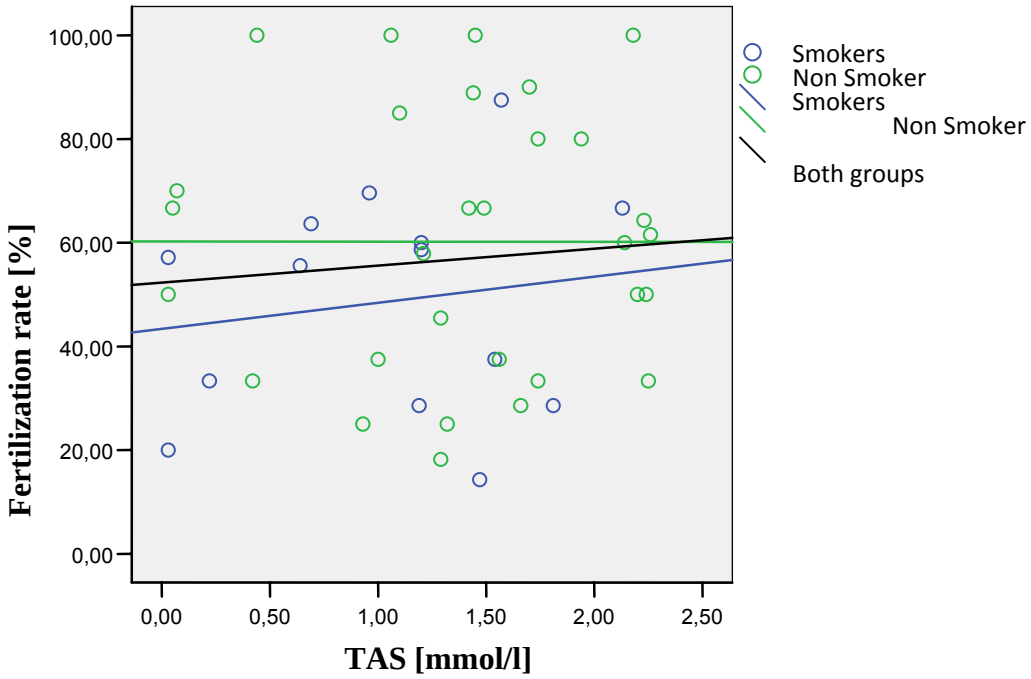

Figure 41: Scatter plot of correlation between TAS concentrations in seminal plasma of male partners of women underwent IVF/ICSI therapy and the fertilization rate of the Oocytes. A significant positive correlation between TAS concentration in seminal plasma of smokers (r=0.237; p= 0.482) and fertilization rate has been shown.

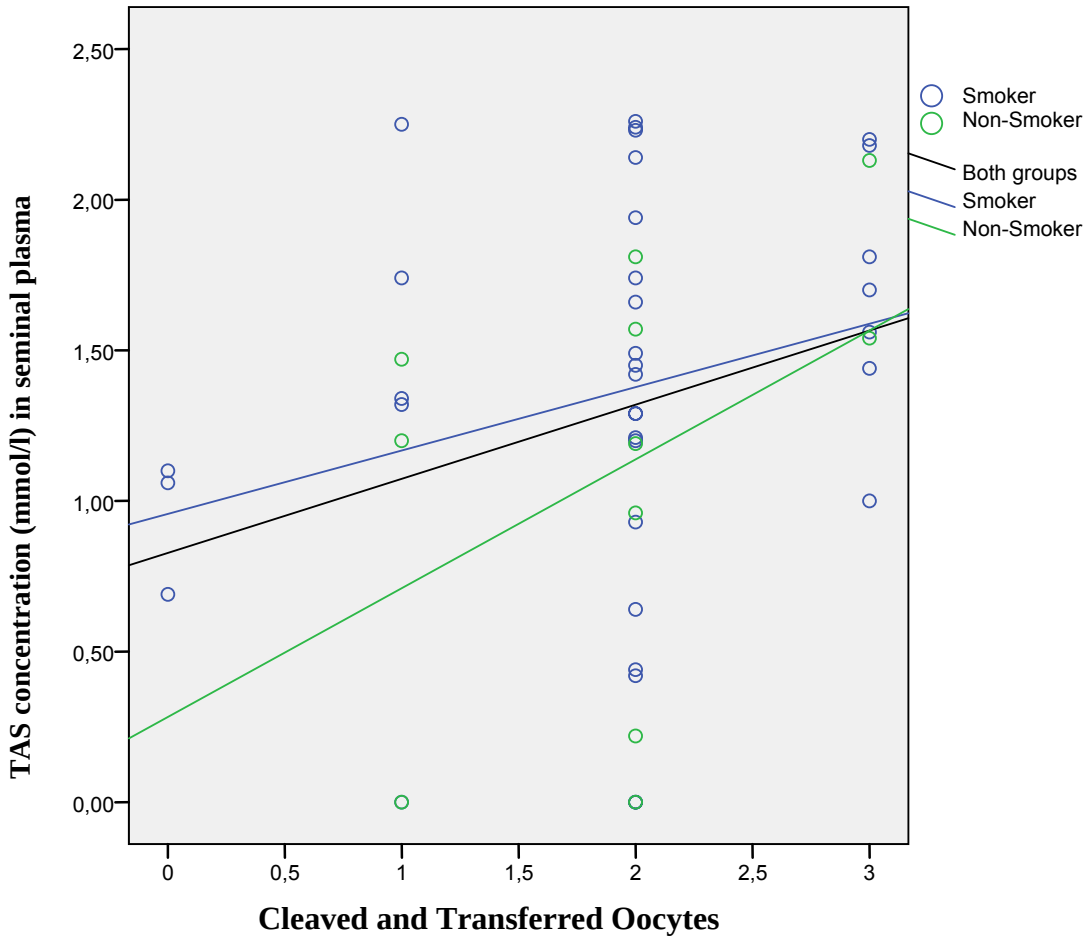

Figure 42: Scatter plot of correlation between TAS concentrations in seminal plasma of male partners of women underwent IVF/ICSI therapy and the cleaved Oocytes (Transferred Embryos). There was a positive, non significant, correlation between TAS concentration in seminal plasma and the mean number of cleaved oocytes in smoker (r=-0.441; p=0.175) and non smoker (r=0.269; p=0. 119).

6- Discussion

Normal spermatozoa are considered those that successfully undergo a number of steps necessary for fertilization of the oocyte. When the spermatozoa are unable to lead to natural fertilization of an oocyte and nucleolus formation, then we talk about impaired sperm function. Oxidative damage to spermatozoa, induced by excessive production of free oxidants or impairment of the native antioxidant mechanisms, has been identified as a parameter of male subfertility (Sikka et al., 1995). Nevertheless, reactive oxygen species have been associated with the pathology of numerous diseases. Small controlled amounts of ROS are vital for sperm maturation and development into normal spermatozoa capable of fertilization (Gagnon et al., 1991; Aitken et al., 1997; Aitken., 1999). In addition, de Lamirande et al. and Zini et al. reported that H_2O_2 and O_2- promote sperm capacitation and acrosome reaction, as well as that H_2O_2 promotes hyperactivation and oocyte fusion (Zini et al., 1996; de Lamirande and Gagnon1993a). Therefore, hydrogen peroxide and superoxide anion are of great importance to spermatozoa, necessary for controlling the tyrosine phosphorylation events associated with sperm capacitation (Aitken et al., 1991; Aitken et al., 1998a). Additionally, ROS are produced by leukocytes, which are present in the male reproductive system and in the ejaculate, as a result of their role in the immunological defense against pathogenic germs (Henkel., 2005; Tomlinson et al., 1992). Only in case of excessive production of ROS or malfunction of the native antioxidant-production mechanisms, do the free radicals cause problems, by putting the several tissues under oxidative stress (OS) (Aitken ., 2005; Balercia et al., 2003; Agarwal et al., 2003). The function of controlling excessive production of ROS is carried out by antioxidants (Young and Woodside 2001) present in the seminal plasma (Henkel., 2005; Armstronng et al., 1998; Sies., 1993). A number of reports have discussed the

possible origin of antioxidants in semen, but our knowledge on that is still limited. Some of the studies supported the testicular origin of semen antioxidants, while others have presented evidence that the source of antioxidant activity is post-testicular. Therefore, eexcessive ROS production that exceeds critical level can overwhelm all antioxidant defence systems of spermatozoa and seminal plasma causing oxidative stress (de Lamirande et al., 1997; Sikka, 2001). Also, oxidative stress when oxidants outnumber antioxidant (Sies, 1993). It is an imbalance between levels of ROS production and antioxidants protection in semen. Therefore, assessment of oxidative stress requires the measurement of total antioxidant capacity (TAC) and ROS production. Infertile men with male factor or idiopathic diagnosis had significantly lower ROS –TAC scores than controls (Sharma et al., 1999).

6.1. The relationship between ROS level in seminal fluid and sperm quality and IVF/ICSI outcome.

ROS are related to poor sperm parameters such as progressive motility, vitality and morphology; they are supposed to be produced by morphologically abnormal spermatozoa (Iwasaki and Gagnon, 1992). According to Aitken et al (1998b) reactive oxygen metabolites are known to disrupt sperm DNA integrity. According to their study, as the level of oxidative stress increased, the spermatozoa exhibited significantly elevated levels of DNA damage ($p<0.001$). Fragmentation of DNA caused by free radical attack induces activation of the poly (ADP ribose) synthetase enzyme. This splits NAD+ to aid the repair of DNA. However, if the damage is extensive, NAD^+ levels may become depleted to the extent that the cell may no longer be able to function and will die.The relative proportion of ROS–producing immature sperm was directly correlated with nuclear DNA damage value in mature sperm and inversely correlated with recovery of motile, mature sperm. This led to the hypothesis that oxidative damage of mature sperm by ROS-producing immature sperm during their co-migration from seminiferous tubules to the epididymis may be an important cause of male infertility (Agarwal, 2004).

Increased generation of ROS resulted in a marked decline in sperm motility; the peroxidation of polyunsaturated fatty acids (PUFAS) in membrane lipids is one frequently cited mechanism for the decline in sperm motility after exposure to oxidative stress (Aitken et al., 1989, 1998; Armstrong et al., (1999). De Lamirande and Gagnon (1992) suggested that the inhibition of sperm motility after incubation with ROS was caused by a depletion of ATP

In the present study, sperm concentration (75.5±31.9 vs. 49.6±39.5 mill/ml $p<0.013$), motility (39.3±18.16 vs.22.2±12.9% $p<0.0012$), morphology (14.0±15.8 vs. 10.4±10.3%) vitality (58.8±21.8 vs. 71.2±22.1%), and the mean percentage of non condensed chromatin (17.8±9.6 vs. 30.9±20.5%) were significantly better in IVF group in comparison to ICSI group (Tab.3). Remarkably, the concentration of ROS in seminal plasma was higher in IVF than ICSI patients group (73.78± 74.37 vs. 50.84±34.63) (Tab. 3; Fig 11). Though these differences were not statistically significant ($p= 0.130$). Mazilli et al., (1994) found significantly high levels of superoxide anion in 87% and 55% of infertile and fertile normospermic patients respectively.

ROS (in both groups, IVF/ICSI) were negatively correlated; but not significant, with vitality of the spermatozoa ($r=-0.163$, $p=0.280$), membrane integrity ($r=-009$, $p=0.953$), morphology ($r=-0.347$, $p=0.020$), Tunel ($r=-0112$, $p=0.460$) and pregnancy ($r=-0.114$, $p=0.451$) (Tab. 4a,b). However, In IVF groups a correlation (but not significant) was found between ROS concentration in the seminal plasma and sperm concentration ($r=-0.105$, $p=0.435$, motility ($r=-0.047$; $p= 0.726$), morphology ($r=-308$; $p=0.020$), chromatin condensation ($r=-0.072$;

p=0.595), fertilization rate (r=0.047, p=0.733) and pregnancy (r=-0.009; p= 0.719). (Tab.8a b Appendix). this is in agreement with our previous studies (Hammadeh et al., 2006).
Similar correlation was registered in ICSI group. Also, ROS concentration in seminal fluid was negatively significant correlated with sperm concentration (r=-0.357, p=<0.030) and none significantly with motility (r=-0.116; P=0.499) (Tab. 9 Appendix). These results are in accordance with those of (Zorn et al., 2003) who found that ROS concentration in seminal fluid was negatively correlated with progressive motility, normal morphology and positively with abnormal sperm head morphology. ROS can directly damage spermatozoa by inducing peroxidation of the lipid–containing sperm plasma membrane and may also affect sperm motility by damaging the axonemal structure (Saleh et al., 2002).

In addition, during sperm preparation in assisted reproductive techniques, ROS generation by defective spermatozoa can be increased by the removal of seminal plasma which has endogenous antioxidant mechanisms. Centrifugation of sperm during preparation also plays a role in rapid production of ROS (Gagnon et al, 1991) and may enhance the generation of ROS 2-5 fold above baseline, within 5 minutes, which can damage sperm membrane and DNA. Oxidative stress has also been correlated with high frequencies of single and double DNA strand breaks (Twigg et al., 1998a: b; Aitken and Krausz, 2001). Sperm are particularly susceptible to oxidative stress due to the high content of unsaturated fatty acids in their membranes, as well as their limited stores of antioxidant enzymes (Baker and Aitken, 2005). Infertile men with poor sperm motility and morphology have increased DNA fragmentation compared with individuals with normal semen parameters (Lopes et al, 1998b; Irvine et al., 2000; Zini et al, 2001a, b).

In many studies, the packaging quality of sperm chromatin in connection with fertility status has been assessed by staining the sperm with CMA_3 fluorochrome. A correlation has been reported to exist between abnormal sperm chromatin packaging as evaluated by CMA_3 staining, and the presence of DNA strand breaks, decreased sperm penetration, the absence of sperm decondensation within the oocyte and IVF and ICSI failure (Sailer et al., 1995; Nasr-Esfahani et al., 2001; Esterhuizen et al., 2002; Razavi et al., 2003).

In addition, Gopalkrishnan et al (1999) observed greater than 50% green fluorescence in samples from fertile donors and used this as a normal cut-off value for Acridine orange staining test (AOT). Hoshi et al (1996) also reported that in vitro fertilization (IVF) was successful when sperm exhibited more than or equal 50% green Acridine orange fluorescence and no pregnancies were obtained when green-fluorescing sperm were less than 50% even though an average 26% of Oocytes were able to be fertilized using intracytoplasmic sperm injection (ICSI). Therefore, Sperm DNA fragmentation is being

recognized as an important cause of infertility. In vitro fertilization of metaphase II Oocytes with spermatozoa that have damaged DNA could potentially lead to failed fertilization, defective embryo development, implantation failure, or early abortion (Genesca et al., 1992; Parinaud et al., 1993; Evenson et al., 1999).

Besides, many clinical studies indicated that DNA fragmentation levels above 30% measured by the Sperm Chromatin Structure Assay (SCSA) are not compatible with the initiation and maintenance of a term pregnancy (Larson et al., 2000c). Other demonstrated that, production of ROS positively correlates with the sperm deformity index, calculated by dividing the total number of deformities observed by the number of sperm evaluated (Aziz et al., 2004). Moreover, Said et al. (2005) suggest that Sperm deformity index (SDI) may be a useful tool to detect the prevalence of sperm DNA damage and to identify potential infertile men. Infertile patients with semen sample containing high proportion of morphologically abnormal spermatozoa and specifically those with cytoplasm droplets may be more susceptible to develop ROS–mediated sperm DNA damage (Said et al., 2005). In the current study, the mean percentage of non condensed chromatin (CMA_3) significantly higher of spermatozoa of ICSI patients than those of IVF patients (30.97±20.50 vs. 17.81±. 9 64%; p=0.001). In addition, DNA denaturation (AO) and DNA fragmentations were higher in ICSI than in IVF (Tab. 5). And correlate negatively with fertilization and pregnancy rate (Tab. 3; Fig. 9; 10).

Besides, the main percentage of stained spermatozoa with Chromomycin (CMA_3) of IVF patients correlate significantly negative with sperm concentration (r=-0.424; p=0.001), and fertilization rate (r=-0.324; p=0.014) (Tab.8a,b Appendix). In ICSI patient groups a negative significant correlations were found between CMA_3 and sperm motility (r=-0.0333; p=0.048), morphologically normal spermatozoa (r=-0.472; mp=0.004) and pregnancy rate (r=-0.550; p= 0.018). Other authors did not observe any difference between IVF and ICSI in case of spermatozoa DNA fragmentation (Ahmadi and Ng, 1999, Host et al., 2000, Zitzmann et al., 2003) (Tab. 9a,b).

Spermatozoa with DNA fragmentation can fulfill the fertilization of the Oocytes, but the following steps leading to pregnancy may be disturbed. This explains the negative correlations of the Tunel with fertilization rate(r= -0.175; p=0.0245) (Tab.4a,b) and with the number of pregnancy of both IVF/ICSI program (r=-0.048; p=0.750). Therefore, our data showed no correlation between sperm DNA integrity and percentage of morphologically normal spermatozoa(r=-0.0162; p=0.288) (Fig. 17) and sperm concentration (r=0.074; p=0.629) and confirm previously reported results (Muratori et al., 2000; Tomlinson et al., 2001). Moreover, these results confirm the previous results of Pasqualotto et al. (2000) who found that male partners of couples who achieved pregnancy did not have significantly different ROS-TAS scores than controls (Pasqualotto et al., 2000).

6.2. TAS concentration in seminal plasma and sperm quality and IVF/ICSI outcome.

Mammals have evolved several mechanisms to suppress oxidative stress and minimize damage by ROS (Halliwell and Gutteridge, 1998). The enzyme system scavenges harmful ROS before they have an opportunity to react with other important molecules and terminates the subsequent chain reaction. The concentration of vitamin E in sperm membranes was lower in sample with poor motility and was inversely correlated with the concentration of leukocytes in the semen (Therond et al., 1996).

The other system (low molecular weight compounds such as vitamins is a reduction – oxidation (redox) system that not only functions to detoxify harmful oxidations but reductively repairs oxidized molecules as well. However, both systems are involved in cross-talk and are not clearly separated (Tanaka et al., 2000). The enzyme scavengers and low–molecular weight antioxidant make-up the total antioxidant capacity of seminal plasma (Smith et al., 1996) Therefore, seminal plasma contain a high concentration of some organic substances and enzymes such as fructose, citric acid, glycerylphosphocholine, acid phosphates, 5-nucleotidase and ascorbic acid. Human seminal plasma contains about 10mgdl^{-1} ascorbic acid, which is considerably more than that in the human blood plasma (range: 0.6–2.5mg/dl) (Dawson et al., 1992).

Previous research has shown that seminal superoxide dismutase, catalase, glutathione peroxidase and sulfhydryl group levels are significantly lower in infertile patients than those in controls, thus, clearly indicating their direct implication in male fertility (Nakamura et al., 2002). In other studies (Zalata et al., 1995; Smithet al., 1996; Potts et al., 1999) showed that fertile males have higher antioxidant capacity than infertile controls, suggesting that antioxidant capacity impairment may play a key role in infertility. However, previously published data on the effect of antioxidant on sperm concentration, motility, and morphology are contradictory (Review in Agarwal, 2004).

In the present study, no significant difference could be found between IVF and ICSI patients group with regard to the concentrations in seminal plasma of total antioxidant level TAS (1.36.52 vs. 1.42±0.51 mmol/l, p=0.153), zinc (131.0±77.47 vs.127.3±60.16, p=0.992) selenium (3.412E-2±1.681E-02 vs3.614E-02±8.481E-03) (Tab. 3). These results are in accordance with those of Greco et al., (2005). Who found no difference in basic semen parameters between groups of male treated with antioxidant and those of placebo group controlled before and after treatment. However, the percentage of DNA–fragmented spermatozoa was significantly reduced ($p<0.001$) in the antioxidant treatment group after the treatment. An improvement of basic sperm parameters by oral treatment with antioxidants

has been reported in a number of studies (reviewed in Agarwal, 2004). However, DNA damage has been investigated in a few studies and a direct assessment for the detection of DNA strand breaks was not used (Geva et al., 1996; Suleiman et al., 1996; Comhaire et al., 2000). In the present study, an adverse negative correlation was observed between total antioxidants concentration, motility (r=-0.086; p=0.573) and the main percentage of fragmented DNA assessed by TUNEL (r=-112; p=0. 460) or Acridine orange (r=-0.148; p=0.349) (Tab. 4a,b). Besides, TAS concentration in seminal plasma correlate significantly positive with sperm membrane integrity (r= 0.307; p= 0.04), fertilization rate (Tab.4a,b) oocyte cleavage rate (transfer rate) (r=313; p= 0.034).

On the other hand, Zinc levels in seminal plasma have been positively associated with sperm concentration and motility in some studies (Fuse et al., 1999; Chia et al., 2000), but not others (Lewis-Jones et al., 1996). Twigg et al. (1998a, b) examined the use of specific antioxidants in order to reduce the oxidative stress of spermatozoa, they showed that albumin was effective in reducing NADPH induced oxidative stress, it failed to protect against the resultant DNA damage but was beneficial in reducing DNA fragmentation. Furthermore, in vitro studies have demonstrated a protective effect of several antioxidants on sperm DNA integrity (Twigg et al., 1998a, b; Donnelly et al., 1999; Dobrzynska et al., 2004). Fraga et al (1991) found that seminal plasma levels of vitamin C were directly associated with the level of oxidative damage in human sperm DNA. Tikkiwal et al. (1987) showed an improvement in sperm count and the number of progressive motile and normal sperm after oral intake of 89 mg zinc sulphate for four months (n=14; idiopathic oligospermia males). Besides, three pregnancies occurred during the study. When taking all together as a composite score, antioxidant intake was positively related to sperm concentration, motility, and progressive motility (Eskenazi et al., 2005). However, zinc intake was somewhat negatively associated with volume (Eskenazi et al., 2005).

In addition, serum selenium is reported to be lower in men with oligospermia and azoospermia than in controls (Krsnjavi et al., 1992). Many authors have shown an association between high seminal plasma selenium with impaired sperm motility in human **(**Bleau et al., 1984; Takasaki et al., 1987; Hansen and Deguchi, 1996). A significant inverse correlation was observed between7-hydro-8-oxy2-Deoxyguanosin [8-OHdG] (a Marker of DNA fragmentation) and selenium concentration (r=-0.40, p<0.01). This result indicates that selenium could protect against oxidative DNA damage in human sperm cells (Xu, et al., 2001). In another study Vezina et al. (1996) demonstrated that the combination of vitamin E and selenium significantly increases sperm motility and the overall percentage of morphologically normal spermatozoa. Also, the sperm quality was significantly better in IVF group in comparison to ICSI group, however, the fertilization rate was higher in ICSI than in IVF group (50.56±28.89% vs. 59.23±26.47%; p= 0.124). These values failed to achieve

statistically significance. The cleavage rate and the mean number of embryos transferred in ICSI group was significantly higher (p=0.036) than those in IVF group (2.41±0.82 vs. 1.77±0.86 respectively). The pregnancy rate was similar in both groups. In the IVF group 18 patients became pregnant (31.5%) in comparison to 11 pregnancies in ICSI group (29.7%) The results of the present investigation also revealed that low quality spermatozoa used for intracytoplasmic sperm injection could achieve similar fertilization rate like good quality spermatozoa used for IVF.

6.3. Smoking and male infertility

Cigarette smoking is a serious health problem of most societies. Consumption of tobacco exerts widely adverse effects on different aspects of health (O'Dowd, 2006). Studies about the relation between smoking and semen quality reveal conflicting results. The production and function of healthy normal spermatozoa was affected by the number of cigarettes smoked per day, the years of smoking and the level of nicotine by-products present in the body fluids, which correlate negatively with semen and sperm quantity and quality (Zavos, 1989; Chi et al., 1994a; b). More specifically, a number of studies have shown a positive correlation between male smoking and decreased concentration and impaired motility and morphology (Evans et al., 1981, Handelsman et al., 1984, Mak et al., 2000, Sofikitis et al., 1995). Taszarek *et al.* (2005) and Agarwal et al. (2005) demonstrated that cigarette smoking alters semen quality, which could worsen the fertilizing capability in infertile men. On the contrary, other studies have not found any significant association between smoking and male infertility (Vogt et al., 1986; Dikshit et al., 1987, Vine et al., 1996; Trummer et al., 2002). The average values of conventional sperm parameters (sperm density, motility, normal morphology, viability) were normal (Sepaniak et al., 2006). Moreover, there was not even a trend to an alternation of these conventional parameters in smokers, as has been suggested by other (Zavos et al., 1998; Wong et al., 2000).

6.3.1.1. EFFECTS OF SMOKING ON SEMEN VOLUME

The first variable assessed in a common sperm analysis is the semen volume, and therefore several subsequent studies have included that in their assessment of tobacco influence on male fertility.

In 1987, Saaranen et al carried out a study on the effect of smoking on conventional semen parameters and especially sperm motility, by assessing men of reproductive age. They found that there was an effect of smoking on semen volume and specifically, sperm volume of heavy smokers was significantly lower than that of non-smokers (Saaranen et al., 1987). However, the same year, Dikshit et al studied tobacco chewers, tobacco smokers and controls and found only a small, statistically insignificant effect of tobacco on semen volume

(Dikshit et al., 1987). Four years later, Holzki et al, after assessing 90 healthy patients (50 non- and 40 smokers), showed that smokers had significantly smaller semen volumes compared to non-smokers (Holzki et al., 1991), a result which was in agreement with that of Saaranen in 1987.

Following Holzki et al in 1992, Osser et al performed a dose-dependent study on the effect of smoking on sperm parameters. Although they did not find any negative effect of tobacco on most conventional semen parameters, they noticed, however, a dose dependent deleterious effect of tobacco on semen volume and sperm counts (Osser et al., 1992). Two years later, Chia et al studied the effects of cadmium concentrations in blood and semen and smoking on semen quality and found that cadmium concentration in seminal plasma was negatively correlated with years of smoking and semen volume. Given that cadmium is a component of cigarettes, they suggested that smoking might be partially responsible for decreased semen density and volume (Chia et al., 1994b). Their results supported by second study they carried out in the same year (Chia et al., 1994a).

Four years later, Chia et al conducted another study on Singapore males to assess again the effect of smoking on sperm parameters; most male subjects had normal sperm parameters, according to WHO guidelines, namely, normal values for volume, concentration, motility and viability. Their results showed that cigarette smoking was significantly negatively associated with sperm volume, because smoking males presented lower semen volumes than those who did not smoke or had no history of smoking (Chia et al., 1998). These results of Chia et al were supported by another study in 2000 by Zhang et al, who performed a study on infertile males in Shandong Province, China. They concluded that cigarette smoking had a detrimental effect on semen volume of smoking infertile males, compared to non-smoking infertile and control fertile ones (Zhang et al., 2000).

Pasqualotto et al carried out a study in 2005, assessing the effect of smoking on the semen variables and concluded that male smoking does not significantly impair the usual sperm parameters, namely, concentration, motility and morphology. However, they found that sperm volume if affected by smoking in a dose-depended manner. More precisely, sperm volume declined significantly between non-, mild, moderate and heavy smokers, with a mean 2.8ml, 2,4ml, 2,3ml and 2.1ml respectively (Pasqualotto et al., 2005).

Additionally, Sobreiro et al., in 2005 evaluated the effect of smoking, among other parameters, on semen characteristics of 500 fertile men, who attended a clinic for vasectomy for sterilization purposes. These men were asked to provide samples before the vasectomy and the groups results showed that smoking has no influence on semen parameters whatsoever (Sobreiro et al., 2005). On the contrary, Ramlau-Hansen et al presented an overall study one year later, which supported that there is an inverse dose-response relation between smoking and semen volume. This study incorporated seven separate occupational

or environmental semen studies performed from 1987 to 2004, resulting in a total of 2562 men evaluated (Ramlau-Hansen et al., 2006).
In the present investigation, no significant difference (p=0.365) in the ejaculate volume between smokers (3.04±1.39ml) and non smoker groups (3.66±1.2ml) (Tab. 6; Fig 22).

6.3.1.2. EFFECT OF SMOKING ON SPERM CONCENTRATION.

Decreased sperm concentration due to smoking has also been reported (Chia et al., 1994a; b.; Handelsman et al., 1984) but there are also contradictory results in this regard (Dikshit et al., 1987).

Osser et al., (1992) have also found no statistically significant effects of cigarette smoking on sperm density. However, the difference was disclosed between heavy smokers and non-smoking men (Goverde et al 1995).
Other several investigators have evaluated the effect of cigarette smoking on sperm concentration (Sofikitis et al., 2000, Wong et al., 2000, Zhang et al., 2000). In 2001, Wang et al studied the semen parameters of men that worked at petrochemical plants, smokers and non smokers and compared them with non-smoking/not exposed controls. Their findings showed that males that were not exposed and smoked regularly had significantly decreased sperm density compared to control subjects who did not smoke and were also not exposed to any petrochemicals (Wang et al., 2001).

An association between cigarette smoking and sperm density (10^6/ml) was found to exist according to the investigation carried out by Kunzle et al (2003). The men they included in their study had attended the infertility clinic with their couple, seeking for causative factors concerning their infertility issues and the overall result was a significant decrease in sperm density of smoking males, compared to non-smoking controls (Kunzle et al., 2003). One year later, several published studies have conquered and supported the fact that tobacco smoking significantly affects semen quality (Chen et al., 2004, Marinelli et al., 2004, Martini et al., 2004, Pasqualotto et al., 2005, Stutz et al., 2004).
In more detail; Martini et al showed that tobacco smoking does not significantly alter semen parameter. However, smoking in combination with alcohol consumption was found to alter semen parameters in general and decreased sperm concentration in particular (Martini et al., 2004). Stuzt et al also supported the detrimental effect of tobacco smoking on semen parameters, although their study concluded that alcohol and tobacco nonsignificantly reduced sperm concentration (Stutz et al., 2004). A limited effect of smoking on conventional sperm parameters was also reported by Marinelli et al., (2004) and a non-statistically significant relationship supported by Chen et al, despite the fact that smoking men in the latter's study generally appeared to have lower sperm concentration (Chen et al., 2004).

In 2005, Pasqualotto et al evaluated a group of men who attended the clinic for vasectomy for sterilization purposes, by dividing them in non-smokers, mild smokers, moderate smokers and heavy smokers. All conventional semen parameters were evaluated and they found no significant differences in sperm concentration among the four groups (Pasqualotto et al., 2005). Similarly, Sobreiro et al found no differences among the evaluated smoker and non-smoker groups of men, attending the clinic also for sterilization purposes (Sobreiro et al., 2005). Additionally, Hassa et el., also found no significant negative correlation between tobacco smoking and sperm concentration (Hassa et al., 2006). On the other hand, Ramlau-Hansen et al, in a study carried out in 2006, found a 19% lower sperm concentration in smoking men, compared to non-smokers (Ramlau-Hansen et al., 2006). Using Meta analysis Vine et al., (1996) reported that sperm concentration of smokers was 13-17% lower than that of non-smoke.

In this study, the mean percentage of spermatozoa count of non-smokers patients group (76.06±29.48mill/ml) was significantly higher (p=0.006) than those of smokers (50.73±30.17mill/ml). This is in agreement with the findings of (Vini et al., 1996, Wong et al, 2000; Kunzle; 2003; Ramlau-Hansen et al., 2006) (Tab. 6, Fig. 24).
In addition, Jensen et al., have proposed that prenatal exposure to maternal tobacco smoking is a stronger predictor of poor semen quality than current smoking and that the association between current smoking and decreased semen quality may be confounded by the prenatal exposure. Among 1770 young men from five European countries, current smoking had no independent effect on semen quality, whereas prenatally exposed men had 20% lower sperm concentration and a 25% lower total sperm count compared with prenatally unexposed men (Jensen et al., 2004). Prenatally exposed men have also been reported to have decreased semen quality compared with unexposed men in two other studies (Storgaard et al., 2003; Jensen et al., 2005).

6.3.1.3. EFFECT OF SMOKING ON SPERM VITALITY

In the category of environmental factors nicotine was shown to be a potential oxidant agent, which affects plasma membrane and DNA integrity. In addition there was a strong negative correlation between lipid peroxidation (LPO) and percentage of viable sperm cells. Sperm viability of smokers patients group (64.60±19.16%) was significantly lower (p=0.107) than those of non smokers (68.90±17.79%). (Tab. 6; Fig. 23). Many studies have suggested a detrimental direct effect of Cotinine on sperm membrane permeability and sperm membrane function. Thus, the spermatozoa may not have optimal ability to undergo capacitation and hyperactivation within the female reproductive tract (Sofikitis et al., 2000).

6.3.1.4. EFFECT OF SMOKING ON SPERM MOTILITY

The concentration of cotinine and hydroxycotinine in the seminal plasma is significantly correlated with total sperm motility of spermatozoa (Pacifici et al., 1993).
Heavy smokers seem to have an enhancing effect on progressively rapid motile sperm compared to light-smoking infertile men (Ozgur et al., 2003). This finding in agreement of that of (Dikhitel et al., 1987; Goverde et al., 1995; Vine et al., 1996). The possible explanations for this enhancement of rapidly progressive motility due to heavy smoking in comparison to light smoking is a definite number of cigarettes per day may constitute a threshold for the enhancement of rapid progressive motility in smokers. Some researchers have found a significant correlation between smoking and decreased sperm motility or morphology and lower sperm density (Evans et al., 2004; Shararwy & Mahmoud 1982; Vine et al., 1994; Merino et al., 1998; Zenzes, 2000).

Besides, smoking has been found to affect the function of accessory glands (e.g. prostate and seminal vesicles (Pakrashi and Chatterjeg, 1995). Reduced the secretion may diminish the content of chromatin zinc and, thereby, the stability of the sperm chromatin; this contributes to reproductive failure or has consequences in fetal development (Oldereid et al., 1994). Both Zinc and Copper are linked to structure /activity center of superoxide dismutase (SOD), which is closely related to sperm motility. Decrease in the Zn and Cu level leads to lowering of the SOD activity. In addition, smoking increases the production of free radicals (ROS) that synthesis and/or augment the consumption of SOD (Zhang et al., 1999).The sperm mitochondrial membrane is a major target of ROS and therefore the sperm midpiece is prone to damage due to oxidative stress. The mitochondrial membranes are directly exposed to the superoxide anions produced during cellular respiration. Hydrogen peroxide is known to directly affect mitochondrial ATP generation (Sikka, 2001). Disruption of sperm mitochondrial function directly affects sperm motility by decreasing the intra-cellular ATP level and also subsequently affects sperm viability.

Our results demonstrated that the mean percentage of sperm motility in the native semen samples was significantly (p=0.061) higher in smokers than non smokers groups (39.0±20.38 vs. 31.57±15.98 respectively) (Tab. 6; Fig 25). However, after semen preparation with Pure sperm gradient centrifugation the mean percentage of motile sperm was higher of non smoker patients groups in comparison to smokers (65.14±31.44 vs. 61.36±33.09; p= 0.021) (Tab. 6). The present study showed that initial sperm motility type was higher in specimens of smokers, but more spermatozoa with abnormal forms and sperm with cytoplasm retention may be presented in the ejaculate. These results are supported by the finding of Jedrzejczak et al. (2004) who reported that that smokers had significantly fewer spermatozoa with motility

grades A, B and C, and more abnormal forms in the ejaculate especially with head defects and cytoplasm droplets. Besides, the results presented here demonstrated that sperm motility in the native semen samples correlate negatively with cotinine concentration in seminal plasma (r= -0.120; p=0.741) (Tab. 7a,b)

6.3.1.5. EFFECT OF SMOKING ON SPERM MORPHOLOGY

The effects of cigarette smoking on sperm morphology were also evaluated in several studies. Namely, the one of (Goverde et al., 1995; Rantala & Koskinies 1987) about smoking and sperm morphology and (Chia et al ., 1994a: b ; Osser et al.,1992), who found lower percentage of normal sperm morphology. Also, increased head piece abnormalities (Chia et al., 1994a; b) have also been reported. A review of the epidemiological studies indicates that cigarette smoking is associated with modest reduction in semen quality, including sperm concentration, motility and morphology. It also reveals that the association between smoking and sperm concentration and motility is stronger among studies of healthy men (e.g. Volunteers and sperm donor) than among men attending infertility clinics (Vine. 1996). Others have found that cigarette smoking could significantly reduced the mean percentage of normal sperm morphology, ejaculate volume and sperm vitality (Kunzle et al., 2003). Ozgur et al. (2003) demonstrated that the morphological evaluation results were better for the non-smokers than the heavy smokers in terms of tail anomalies and the percent of coild tails. Mak et al. (2000) indicated that cigarette smoking is associated with retention of sperm cytoplasmic droplets in infertile men, a morphologic characteristic associated with impaired sperm function. In the present study the mean percentage of morphologically normal spermatozoa was significantly higher (p=0.018) of smokers group (19.8±18.5%) than non smokers (16.06±18.53%) (Fig. 27).. In this regard, it is noteworthy to mention here that the morphology assessment of spermatozoa was carried out according to strict criteria described by Krüger et al, (1986), whereas others evaluated sperm morphology according to WHO criteria. Therefore, a direct comparison with other study is difficult.

6.3.1.6. EFFECT OF SMOKING ON SPERM CHROMATIN INTEGRITY (DNA)

There are currently various techniques for the evaluation of sperm DNA fragmentation. These techniques have been recently reviewed (Evenson and Wixon, 2006 a: b). In the present study, Chromomycin (CMA3), Acridine Orange and TUNEL- technique have been used to evaluate the DNA integrity, denaturation and fragmentation respectively. Furthermore, it has been shown that DNA strand-breaks occur during the process of

apoptosis and that the nicks in the DNA molecule can be detected via the Tunel assay (Berg et al., 1998).

The etiology of sperm DNA fragmentation is still poorly understood, but relationship between cigarette smoking and increased of DNA damage has been shown in infertile smokers compared of non-smokers (Saleh et al., 2003; Zenzes, 2000). Oldereid et al. (1994) pointed out that smoking affects the function and may diminish the zinc secretory from accessory glands and consequently the stability of the sperm chromatin. Therefore, paternal smoking has been associated with a significant increase in the percentage of spermatozoa with DNA damage and higher risk of birth defect and childhood cancers in the offspring (Sorahan et al., 1997; Saleh et al., 2002). However other studies found no correlation between cigarette smoking and semen quality, sperm function or sperm nuclear DNA damage (Vogt et al 1986; Sergerie et al 2000). In the present study, sperm from smoker showed increased DNA fragmentation compared with sperm from non smoker under CMA_3 (15.73±7.2% vs. 17.1±8.6%; p=0.761), and TUNEL test (12.8±7.9% vs. 10.6±6.8%; p=0.430). However, No difference was observed for overall DNA fragmentation using AO test between smoker men and non smoking in the present study (Tab. 6; Fig. 29). These results are in close agreement with other reports in which it revealed that semen samples from smokers have a significantly higher ratio of single-strand-to double-strand DNA spermatozoa (Sofikitis et al., 1995). Studies by Zenses (1999) and (2000) have proposed that smoking induces DNA damage in spermatozoa and in generated preimplantation embryos. The percentage of sperm with DNA fragmentation is higher in smokers and the repair capacity is significantly decreased in ejaculated spermatozoa. Genetic damage is passed on to the embryo and further development, in women undergoing both IVF and ICSI, as clinical pregnancy rates are drastically reduced in couples with smoking males. This seems to be the case especially in older women who undergo ICSI (Zenzes, 2000). Nicotine could induce double-strand DNA breaks (11% in 0.75 mM concentration) in the sperm nuclei. Moreover smoking can be linked to significantly increased levels of seminal Reactive Oxygen Species (ROS) (Saleh et al., 2002). It is hypothesized that spermatozoa with some type of DNA damage defects because of the male exposure to cigarette smoke may not have impaired capacity (via ICSI) to induce fertilization, participate syngamy and trigger embryonic development, but they may be responsible for the observed lower implantation rate of the development embryos.

6. 4. ASSOCIATION BETWEEN SMOKING AND OXIDATIVE STRESS

Sperm DNA damage is significantly increased in men with idiopathic and male factor infertility and in men who failed to initiate a pregnancy after assisted reproduction techniques. Such an increase may be related to high levels of seminal oxidative stress (Saleh et al., 2003).

Smoking was associated with a 107% increase in reactive oxygen species (ROS) levels and a 10-point decrease in ROS-total antioxidant capacity (TAC) scores (Saleh et al., 2002). DNA damage is commonly observed in spermatozoa of infertile patients and may be mediated by high ROS (Kodama et al., 1997). The main ROS which create this problem are superoxide anion ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{-}) (Sukcharoen et al., 1996; Agarwal et al., 2003). A recent study has reported that sperm DNA damage values greater than 27% are highly predictive of pregnancy failure after conventional IVF or intracytoplasmic sperm injection (Larson et al., 2000). A significant reduction of ROS-TAC scores associated with smoking can be attributed to a significant increase in seminal ROS levels, which is related to the significant increase in leukocyte concentration in the semen of infertile smokers (Close et al., 1990).
It should be emphasized that cigarette smoke has been associated with increased frequency of aneuploidy in sperm (Twigg et al., 1998a,b), lower seminal plasma antioxidant levels and increased oxidative damage to DNA (Fraga et al., 1995; Shen et al., 1997).

The intracellular antioxidant enzymes can not protect the plasma membrane that surround the acrosome and the tail, forcing spermatozoa to supplement their limited intrinsic antioxidant defenses by depending on the protection afforded by the seminal plasma, which paths these cells (Iwasaki, 1992). Besides, the seminal plasma protects spermatozoa from excessive ROS by means of small molecular weight free radical scavengers, such as ascorbate and tocopherol, uric acid and ROS-metabolizing enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Alvarez and Storey, 1989). However, it was demonstrated that heavy smoking in males is associated with a 20–40% decrease in serum ascorbic acid levels associated with increased sperm abnormalities (Kul'Krauchas et al., 1985). Dawson et al. (1992) suggested supplementation of ascorbic acid to heavy smokers could improve sperm quality. Mehran, (2005) and Arabi and Moshtaghi, (2005) showed that exposure of spermatozoa from smokers to the seminal plasma from nonsmokers resulted in an improvement of sperm function.

The results from this work showed that ROS and TAS concentration in seminal plasma of

smokers are similar to those of non-smokers groups (53.1±28.03 µmol/l and 1.35± 0.66mmol/l vs. 50.94±28.20µmol/l and 1.09±0.73mmol/l respectively) (Tab. 6; Fig. 30-31). Besides, in both groups, ROS concentration in seminal plasma correlate negatively with sperm motility after semen preparation (-0.172; p= 0.253), with vitality (-0.163; p= 280) membrane integrity (-0.009; p=0.953) morphology (-0.347; p=0.02) chromatin condensation (CMA3).(r=-0.127; p=0.401) DNA fragmentation (TUNEL) (r=-0.112; p=0.460) and DNA denaturation AO (r=-0..072; p=0.652) and positively with cotinine concentration (r=0.017, p= 0.910) (Tab. 10a,b Appendix). TAS concentration in seminal plasma correlate significant positively with membrane integrity of spermatozoa (r=0.307; p= 0.040) (Tab. 10a,p Appendix). Similarly (Armstrong et al., 1998) demonstrated that the high levels of seminal oxidative stress have been correlated with sperm dysfunction through different mechanisms that include lipid peroxidation of sperm plasma membrane and impairment of sperm metabolism, motility, and fertilizing capacity (Armstrong et al., 1998).

6.5. COTININE LEVEL IN SEMINAL PLASMA.

Exposure to cigarette smoking appears to be an important risk factor for male infertility. Significant smoker-non-smoker differences were found in semen cotinine concentration and sperm quality. Cotinine may decrease male fertility by inhibiting density, reducing total progressively motile sperm count, and increasing the percentage of sperm with abnormal morphology. Cotinine is the major metabolite of nicotine, which is the major psychoactive substance found in cigarette smoke (Hulka 1991 ; Zenzes et al. 1996). Because it is easily detectable in human body fluids, such as urine, saliva (Zenzes et al. 1996 ; Wall et al. 1988), and seminal plasma (Pacifici et al. 1993), cotinine has been used as a specific biomarker of cigarette smoking (Hulka 1991). Besides, cotinine with a half-life in sperm of 5–7days is a better indicator of long-term exposure to cigarette smoke than urinary metabolites index (Jarvis et al. 1988).

There is a small but significant correlation between cotinine concentration in seminal plasma and abnormal sperm morphology, but not for other semen variables (Wong et al., 2000). Cotinine concentration of 400-800ng/ml impairs sperm motility, membrane function, and their ability to undergo capacitation (Sofikitis et al., 1995). In assisted reproduction program, smokers with high seminal plasma cotinine concentration may be treated with intracytoplasmic sperm injection, rather than conventional in vitro fertilization (Sofikitis et al., 2000).

Box-plots in Fig. 33 and Table 6 show the difference in semen cotinine levels between smokers and non-smokers patient groups. Seminal plasma cotinine level was found to be significantly higher in smokers versus nonsmokers. The results were (144.29±82.19µg/l) in

the smokers group and (19.30±32.69µg/l) in the non smokers group (p<*0.001)*, (Tab. 6). These results demonstrate cigarette smoking is the main contributor to semen cotinine.

Seminal plasma cotinine of either smokers or nonsmokers demonstrated non significant negative correlation with semen volume (*r*=-0.254; p=0.089) and sperm concentration (*r*=-0.144, p=0.341), sperm morphology (r=-0.027; p=0.861), besides, the motility of sperm (r= 0.103; P= 0.503) and ROS concentration (r= 0.017; p= 0.910) in seminal plasma correlate positive with cotinine level. However, non significant negative correlation with sperm DNA fragmentation (TUNEl- test) (r=-0.137; p=0.363), DNA denaturation (AO) (r=-0.026, p=0.869), TAS concentration (r=-0.215, P=.151) could be found (Tab. 7a,b).

The relation of semen cotinine characteristics to sperm quality in our study is consistent with past reports. Pacifici et al. (1993) who found that total motility of spermatozoa was significantly negatively correlated with concentrations of cotinine and hydroxycotinine.

Moreover, these results in accordance with Yang's results (Yang et al., 2000) showing that urinary cotinine levels of non-smokers are related to second-hand smoke exposure. The results of the present investigation also revealed that nicotine from cigarettes is actively transferred from the arterial testicularis via the Sertoli cell into the seminiferous tubules and seminal plasma and supported earlier finding. A previous research found nicotine and cotinine in the seminal plasma of smokers and suggested that other harmful components of tobacco smoke would pass through the blood–testis barrier (Vine et al. 1993*).*

6. 6. The effect of smoking on IVF/ICSI outcome.

6.6. 1. EFFECT OF SPERM OF SMOKERS AND NON SMOKERS ON FERTILIZATION, EMBRYONAL CLEAVAGE (TRANSFER).

It is hypothesized that smoking has an adverse effect on all biological endpoints of IVF and GIFT including sperm parameters, number of oocyte retrieved, fertilization rates, number of embryos transferred and pregnancy rate, resulting in lower success rate in live birth delivery rates. Moreover, the thesis that exposure to cigarette smoke influences the sperm's capacity or epigenetic factor does not affect sperm capacity to fertilize oocyte (via ICSI) or to trigger early embryonic development, but it causes an impaired capacity for implantation of the development blastocyst, is very strongly supported by (Ubaldi et al., 1999). They showed that some testicular defects have negative impact on the reproductive performance of testicular spermatozoa, resulting in a decreased embryonic potential for implantation, without any apparent effect on fertilization and early embryonic development. Recent studies also show that smoking may alter uterine-fallopian tube function (Knoll & Talbot, 1998; Saraiya et al., 1998). Zenzes et al., determined that smoking alters the meiotic spindle of gametes, leading to chromosome errors (Zenzes, 2000).

6.6.2. EFFECT OF SPERMATOZOA OF SMOKERS AND NON SMOKERS ON PREGNANCY.

Furthermore, cotinine is present in minimal vesicular secretion in rats exposed to cigarette smoke. In addition, rat models, show very vividly that male cigarette smoke exposure does not alter the potential of spermatozoa, to decondense within the ooplasm, transform into male pronucleus and trigger embryonic development up to the blastocyst stage, after ICSI techniques. In contrast, other results show that Fertilization Rate (FR), Cleavage Rate (CR) and Blastocyst Development Rate (BDR) after ICSI trials are lower when spermatozoa are recovered from animals exposed to cigarette smoke, compared with those of the control group. It may be of clinical significance that ICSI techniques, in contrast to IVF techniques using spermatozoa derived from animal exposed to cigarette smoke don't generate significantly less number of blastocyst compare with control animal (Klonoff-Cohen, 2001). In the contrary, (Kapawa, et al., 2004) reported that in rats exposed to cigarette smoke both ICSI/IVF trials lead to a significant decrease in the number of alive offspring's per transferred embryo, compared to the control group (Kapawa et al., 2004). In the resent study, the mean number of fertilized oocyte in smokers and non smokers patients groups (5.55±5.7 vs. 5.15±4.18; p= 0.591) and the fertilization rate (45.6±23.50 vs. 58.48±24.60; p=0.151) were similar. However, the Cleavage rate (the mean number of transferred embryo) was significantly higher in smokers than in smokers (1.89±0.83 vs. 1.9±0.70; p=0.001). Moreover, the pregnancy rate in non smoking patients group (34%) was significantly higher (27%) than those of smokers (Tab. 6).

Semen cotinine concentration was clearly correlated negative with sperm quality (Table 7a,b). Semen cotinine concentration was highly negatively correlated with total number of motile sperm (r =−0.120; p=0.741), with percentage of morphologically normal spermatozoa (r =-0.451; p=0.191). Besides, non significant correlation were elicited with fertilization rate (r= -0.074; P= 0.829) and transfer rate (r= -0.372, p=0.259) (Tab.7a,b).

There is a lack of correlation between smoking and DNA damage in sperm cells of healthy men. As sperm DNA damage may affect the final events of fertilization, this may results in early embryonic death (Sakkas et al., 1999).

Paternal transmission of altered genetic or epigenetic factors may comprise the embryonic capacity for implantation, resulting in failed implantation or early pregnancy loss. In other studies, damaged sperm DNA could diminish the chance of successful pregnancy by

increasing the spontaneous abortion rate (Ahmedi and Ng, 1999; Host et al., 2000; Zitzmann et al., 2003). Although the effects of smoking of male fertility seem to be contradictory between researchers, the majority conclude that it has detrimental effects on sperm parameters and male partners should refrain from that. In general, male partners are advised to quit smoking, among others (e.g. alcohol) and pay attention to their weight, in order to present better sperm parameters and have higher chances of achieving fertilization (Larkin, 1998).

Furthermore, epidemiological studies in women of reproductive age have shown that cigarette smoking has a dose related effect that can delay time of conception by two months, advance the start of menopause by two years and that it causes a small but clinically significant increase in the risk of spontaneous abortion, reducing fecundity among those undergoing assisted conception (Howe et al., 1985; Windham et al., 1992.; Pasqualotto et al., 2005). Normal fecundity is re-established then women stop smoking, providing compiling evidence of the relationship between smoking and reduced fertility in women.

In conclusions, the findings of the present study suggest that excessive reactive oxygen species production reduces the quality of the spermatozoa and hence its fertilization capacity, which in turn may lead to fail fertilization or decrease the embryos implantation ability and consequently IVF/ICSI outcome. Besides, the results of the present study suggest a negative biological effect of smoking on spermatozoa quality. Therefore, cigarette smoking exposure appears to be an important risk factor for male infertility. Significant smoker-non-smoker differences were found in semen cotinine concentration and sperm quality. Cotinine may decrease male fertility by inhibiting density, reducing total progressively motile sperm count, increasing the percentage of sperm with abnormal morphology and increase the production of reactive oxygen species.

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Anhang

Tab. 8a: Correlation coefficient of semen parameters, sperm quality and IVF outcome.

		Volume	Sperm Concentration	concentration after semen prep.aration	Motility Native	semen praparaaion	Vitality	HOST test	Morphology
Sperm concentration (mill/ml) in the native semen	r	-0.190	1.000	0.259	0.117	0.283(*)	-0.266(*)	-0.175	0.120
	p	0.158	.	0.052	0.384	0.033	0.046	0.193	0.375
Motility (%) in the native semen sample	r	0.052	0.117	0.260	1.000	0.026	-0.485(**)	-0.314(*)	0.070
	p	0.702	0.384	0.050	.	0.849	0.000	0.017	0.606
Vitality (%)	r	-0.052	-0.266(*)	-0.275(*)	-0.485(**)	-0.078	1.000	0.342(**)	0.087
	p	0.701	0.046	0.039	0.000	0.566	.	0.009	0.520
Membrane integrity (HOS)	r	0.253	-0.175	-0.198	-0.314(*)	0.145	0.342(**)	1.000	0.140
	p	0.058	0.193	0.141	0.017	0.283	0.009	.	0.300
Morphologicaly normal Sperm (%)	r	0.087	0.120	-0.109	0.070	0.206	0.087	0.140	1.000
	p	0.518	0.375	0.422	0.606	0.125	0.520	0.300	.
Chromatin condensation. (CMA3)	r	0.033	-0.424(**)	-0.118	-0.273(*)	-0.087	0.142	0.084	-0.135
	p	0.805	0.001	0.382	0.040	0.520	0.293	0.535	0.317
DNA fragmentation (TUNEL)	r	0.250	0.025	0.277(*)	-0.025	0.080	0.225	0.093	0.085
	p	0.061	0.852	0.037	0.851	0.555	0.093	0.490	0.531
DNA denaturation (AO)	r	-0.102	-0.088	0.205	-0.077	0.016	0.217	0.046	0.024
	p	0.457	0.525	0.134	0.578	0.910	0.112	0.737	0.860
ROS (µmol/l)	r	0.007	0.297(*)	-0.105	0.024	-0.047	0.160	0.177	-0.308(*)
	p	0.959	0.025	0.435	0.859	0.726	0.235	0.188	0.020
TAS (mmol/l)	r	0.180	0.039	-0.418(**)	-0.228	0.186	-0.105	0.182	-0.119
	p	0.181	0.775	0.001	0.088	0.165	0.439	0.176	0.377
Fertilization rat (%)	r	0.282(*)	0.093	0.032	0.050	0.125	0.143	0.016	0.030
	p	0.034	0.490	0.815	0.714	0.356	0.290	0.908	0.825
Cleaved and transferred oocytes	r	0.018	0.030	0.011	0.053	-0.043	-0.082	0.172	0.007
	p	0.896	0.822	0.934	0.696	0.749	0.542	0.202	0.960
Pregnancy	r	-0.095	-0.101	0.124	-0.138	0.144	0.189	0.110	-0.096
	p	0.561	0.534	0.444	0.395	0.375	0.243	0.499	0.556

Tab. 8b: Correlation coefficient of semen parameters, sperm quality and IVF outcome.

		ion CMA3	DNA fragmentation	denaturation (AO)	ROS	TAS	Fertilization rate	(Transferred)	Pregnancy
Sperm cononcentration(mill/ml) in native semen	r	-0.424(**)	0.025	-0.088	0.297(*)	0.039	0.093	0.030	-0.101
	p	0.001	0.852	0.525	0.025	0.775	0.490	0.822	0.534
Motility in the native semen (%)	r	-0.273(*)	-0.025	-0.077	0.024	-0.228	0.050	0.053	-0.138
	p	0.040	0.851	0.578	0.859	0.088	0.714	0.696	0.395
Vitality (%)	r	0.142	0.225	0.217	0.160	-0.105	0.143	-0.082	0.189
	p	0.293	0.093	0.112	0.235	0.439	0.290	0.542	0.243
Membrane integrity (HOS)	r	0.084	0.093	0.046	0.177	0.182	0.016	0.172	0.110
	p	0.535	0.490	0.737	0.188	0.176	0.908	0.202	0.499
Morphologically normal sperm (%)	r	-0.135	0.085	0.024	-0.308(*)	-0.119	0.030	0.007	-0.096
	p	0.317	0.531	0.860	0.020	0.377	0.825	0.960	0.556
Chromatin Condensation (CMA_3)	r	1.000	0.055	-0.153	-0.072	0.013	-0.324(*)	0.064	0.055
	p	.	0.685	0.264	0.595	0.926	0.014	0.635	0.738
DNA Fragmentation (TUNEL)	r	0.055	1.000	0.361(**)	-0.100	-0.133	0.096	0.043	0.470(**)
	p	0.685	.	0.007	0.458	0.323	0.478	0.750	0.002
DNA Denaturation (AO)	r	-0.153	0.361(**)	1.000	-0.249	-0.085	-0.041	-0.174	0.561(**)
	p	0.264	0.007	.	0.067	0.535	0.764	0.204	0.000
ROS (µmol/l)	r	-0.072	-0.100	-0.249	1.000	0.118	-0.046	0.156	-0.009
	p	0.595	0.458	0.067	.	0.382	0.733	0.245	0.957
TAS (mmol/l)	r	0.013	-0.133	-0.085	0.118	1.000	-0.119	0.155	-0.059
	p	0.926	0.323	0.535	0.382	.	0.377	0.248	0.719
Fertilization rat (%)	r	-0.324(*)	0.096	-0.041	-0.046	-0.119	1.000	0.060	-0.131
	p	0.014	0.478	0.764	0.733	0.377	.	0.658	0.421
Cleaved and transferred oocytes	r	0.064	0.043	-0.174	0.156	0.155	0.060	1.000	-0.392(*)
	p	0.635	0.750	0.204	0.245	0.248	0.658	.	0.012
Pregnancy	r	0.055	0.470(**)	0.561(**)	-0.009	-0.059	-0.131	-0.392(*)	1.000
	p	0.738	0.002	0.000	0.957	0.719	0.421	0.012	.

Tab.9a: Correlation coefficient of semen parameters, sperm quality and ICSI outcome.

		Volume	native semen	after.preparation	motility in native semen	Motility after semen preparation	Vitality (%)	(HOS-test)	Morphology
Sperm concentration (mill/ml) of native semen	r	-0.094	1.000	0.587(**)	0.343(*)	0.385(*)	-0.288	-0.166	0.050
	p	0.578	.	0.000	0.040	0.019	0.084	0.333	0.773
Motility of sperm in the native semen (%)	r	0.186	0.343(*)	0.429(**)	1.000	0.514(**)	-0.705(**)	-0.279	0.039
	p	0.277	0.040	0.009	.	0.001	0.000	0.104	0.823
Vitality (%)	r	-0.189	-0.288	-0.210	-0.705(**)	-0.387(*)	1.000	0.217	-0.025
	p	0.263	0.084	0.211	0.000	0.018	.	0.203	0.886
Membrane integrity HOS- test	r	-0.162	-0.166	-0.215	-0.279	-0.197	0.217	1.000	0.178
	p	0.345	0.333	0.208	0.104	0.249	0.203	.	0.306
Morphological mormal sperm (%)	r	0.221	0.050	0.040	0.039	0.408(*)	-0.025	0.178	1.000
	p	0.195	0.773	0.816	0.823	0.014	0.886	0.306	.
Chromatin condensation (CMA3)	r	-0.049	-0.264	-0.121	-0.333(*)	-0.440(**)	0.293	-0.296	-0.472(**)
	p	0.772	0.114	0.476	0.048	0.006	0.079	0.080	0.004
DNA Fragmentation (TUNEL)	r	-0.134	-0.053	-0.182	-0.007	-0.035	-0.064	0.319	0.116
	p	0.429	0.756	0.281	0.968	0.836	0.709	0.058	0.500
ROS (µmol/ml)	r	0.242	-0.357(*)	-0.167	-0.116	-0.313	0.064	0.034	-0.101
	p	0.149	0.030	0.322	0.499	0.059	0.708	0.844	0.556
TAS (mmol/l)	r	0.141	0.118	0.218	0.147	0.108	-0.050	0.041	-0.106
	p	0.405	0.488	0.194	0.393	0.525	0.770	0.811	0.537
Fertilization rate (%)	r	-0.037	0.014	-0.060	0.097	0.061	-0.032	-0.053	0.029
	p								
Cleaved and transferred oocytes	r	0.009	-0.112	0.069	-0.237	-0.053	0.226	0.253	0.051
	p	0.956	0.510	0.683	0.164	0.755	0.179	0.136	0.768
Pregnancy	r	0.033	-0.275	0.066	-0.013	0.286	0.100	0.355	0.453
	p	0.896	0.269	0.795	0.961	0.250	0.694	0.162	0.068

Tab. 9b: Correlation coefficient of semen parameters, sperm quality and ICSI outcome.

		n. CMA3	TUNEL	Denaturation (AO)	ROS	TAS	Fertilized rate	Transferred oocytes	Pregnancy
Sperm concent.in native semen samples (mill/ml)	r	-0.264	-0.053	-0.112	-0.357(*)	0.118	0.014	-0.112	-0.275
	p	0.114	0.756	0.522	0.030	0.488	0.934	0.510	0.269
Sperm motility (%) in the native semen	r	-0.333(*)	-0.007	-0.047	-0.116	0.147	0.097	-0.237	-0.013
	p	0.048	0.968	0.790	0.499	0.393	0.575	0.164	0.961
Vitality (Eosin test)(%)	r	0.293	-0.064	0.037	0.064	-0.050	-0.032	0.226	0.100
	p	0.079	0.709	0.834	0.708	0.770	0.851	0.179	0.694
Membrane integrity (HOS) test (%)	r	-0.296	0.319	0.051	0.034	0.041	-0.053	0.253	0.355
	p	0.080	0.058	0.775	0.844	0.811	0.757	0.136	0.162
Morphologicaly normal spermatozoa (%)	r	-0.472(**)	0.116	0.000	-0.101	-0.106	0.029	0.051	0.453
	p	0.004	0.500	0.999	0.556	0.537	0.866	0.768	0.068
Chromatin condensation (CMA_3) (%)	r	1.000	-0.252	-0.095	0.001	-0.109	-0.087	0.083	-0.550(*)
	p	.	0.132	0.588	0.997	0.521	0.610	0.625	0.018
DNA Fragmentation (TUNEL) (%)	r	-0.252	1.000	0.012	0.049	-0.226	-0.185	0.140	0.198
	p	0.132	.	0.944	0.775	0.179	0.272	0.410	0.430
ROS (µmol/l)	r	0.001	0.049	0.000	1.000	-0.037	0.154	0.003	-0.494(*)
	p	0.997	0.775	0.999	.	0.826	0.361	0.984	0.037
TAS (mmol/l)	r	-0.109	-0.226	0.044	-0.037	1.000	0.279	0.297	-0.011
	p	0.521	0.179	0.800	0.826	.	0.094	0.074	0.965
Fertilized rate	r	-0.087	-0.185	0.033	0.154	0.279	1.000	0.209	-0.077
	p								
Cleaved and transferred oocytes	r	0.083	0.140	-0.133	0.003	0.297	0.209	1.000	0.099
	p	0.625	0.410	0.446	0.984	0.074	0.215	.	0.697
Pregnancy	r	-0.550(*)	0.198	0.014	-0.494(*)	-0.011	-0.077	0.099	1.000
	p	0.018	0.430	0.959	0.037	0.965	0.761	0.697	.

Tab 10a: Correlation of semen parameters of smoker and non smoker patients (n=46).

	Volume	Sperm concnt. In the native semen	Motility in the native semen	Vitality-Eosin-test	Membrane integrity HOS-test	Morphology	CMA_3
Volume (mll)	1.000	-0.044	0.161	-0.139	-0.093	0.038	0.012
	.	0.774	0.292	0.359	0.545	0.802	0.938
Sperm concentration native semen (mill /ml)	-0.044	1.000	0.427(**)	-0.264	-0.107	0.074	-0.418(**)
	0.774	.	0.003	0.076	0.485	0.629	0.004
Motility (%) of native semen samples	0.161	0.427(**)	1.000	-0.594(**)	-0.397(**)	-0.143	-0.236
	0.292	0.003	.	0.000	0.008	0.348	0.118
Motility(%) after semen preparation	0.176	0.326(*)	0.233	-0.156	-0.004	0.382(**)	-0.256
	0.242	0.027	0.124	0.301	0.978	0.010	0.086
Vitality (%)	-0.139	-0.264	-0.594(**)	1.000	0.238	0.318(*)	0.322(*)
	0.359	0.076	0.000	.	0.116	0.033	0.029
Membrane integrity (HOS)	-0.093	-0.107	-0.397(**)	0.238	1.000	0.251	-0.251
	0.545	0.485	0.008	0.116	.	0.100	0.096
Morphological normal sperm	0.038	0.074	-0.143	0.318(*)	0.251	1.000	-0.162
	0.802	0.629	0.348	0.033	0.100	.	0.288
Chromatin Condensation CMA_3	0.012	-0.418(**)	-0.236	0.322(*)	-0.251	-0.162	1.000
	0.938	0.004	0.118	0.029	0.096	0.288	.
DNA Fragmentation TUNEL	-0.115	-0.292(*)	-0.107	0.100	0.008	-0.123	0.287
	0.448	0.049	0.484	0.510	0.956	0.419	0.053
ROS (µmol/l)	0.060	0.192	0.203	-0.163	-0.009	-0.347(*)	-0.127
	0.694	0.201	0.181	0.280	0.953	0.020	0.401
TAS (mmol/l)	0.223	0.035	-0.086	-0.130	0.307(*)	0.045	-0.143
	0.137	0.820	0.573	0.390	0.040	0.768	0.344
Cotinine (mg/dl)	-0.254	-0.144	0.103	0.009	-0.061	-0.027	0.067
	0.089	0.341	0.503	0.951	0.691	0.861	0.657
Fertilization rate (%)	0.130	0.188	0.022	0.084	0.023	-0.021	-0.204
	0.390	0.210	0.887	0.579	0.883	0.889	0.175
Cleaved and transferred oocytes	-0.032	-0.074	0.000	0.076	0.235	-0.096	-0.088
	0.834	0.627	0.999	-0.102	-0.235	0.075	-0.080
Pregnancy	-0.020	-0.147	0.078	-0.103	-0.084	0.015	-0.128
	0.897	0.348	0.624	0.511	0.595	0.927	0.412

Tab. 10b: Correlation of semen parameters of smoker and non smoker patients (n=46).

	DNA - TUNEL-test	DNA AO	ROS	TAS-	Cotinine	Fertilized Oocytes	Fertilization rate (%)	Transfer	pregnancy
Volume (ml)	-0.115	0.085	0.060	0.223	-0.254	0.113	0.130	-0.032	-.020
	0.448	0.592	0.694	0.137	0.089	0.461	0.390	0.834	0.897
Sperm concentration (mill/ml) in native semen	-0.292(*)	-0.191	0.192	0.035	-0.144	0.144	0.188	-0.074	0.147
	0.049	0.227	0.201	0.820	0.341	0.345	0.210	0.627	0.348
Motility (%) native	-0.107	0.034	0.203	-0.086	0.103	0.141	0.022	0.000	0.078
	0.484	0.834	0.181	0.573	0.503	0.363	0.887	0.999	0.624
Vitality (HOS) in native semen samples	0.100	-0.048	-0.163	-0.130	0.009	-0.102	0.084	0.076	-0.084
	0.510	0.765	0.280	0.390	0.951	0.505	0.579	0.616	0.103
Membrane	0.008	0.090	-0.009	0.307(*)		-0.235	0.023	0.235	-0.511
integrity (HOS)	0.956	0.576	0.953	0.040	0.691	0.125	0.883	0.120	0.595
Morphological normal sperm (%)	-0.123	0.032	-0.347(*)	0.045	-0.027	0.075	-0.021	-0.096	0.015
	0.419	0.845	0.020	0.768	0.861	0.629	0.889	0.530	0.927
Chromatin condensation (CMA3)	0.287	0.077	-0.127	-0.143	0.067	-0.080	-0.204	-0.088	-128
	0.053	0.628	0.401	0.344	0.657	0.604	0.175	0.559	0.412
DNAFragmentation	1.000	0.219	-0.112	-0.094	-0.137	-0.168	-0.215	-0.048	-0.164
TUNEL-test	.	0.163	0.460	0.532	0.363	0.271	0.152	0.750	0.292
ROS (µmol/l)	-0.112	-0.072	1.000	0.033	0.017	-0.025	0.275	0.059	-0.214
	0.460	0.652	.	0.828	0.910	0.871	0.064	0.696	0.168
TAS (mmol/l)	-0.094	-0.148	0.033	1.000	-0.215	-0.022	0.071	0.313(*)	-0.026
	0.532	0.349	0.828	.	0.151	0.886	0.638	0.034	0.868
Cotinine (mg/dl)	-0.137	-0.026	0.017	-0.215	1.000	0.119	-0.107	-0.077	0.008
	0.363	0.869	0.910	0.151	.	0.436	0.480	0.613	0.959
Fertilization rate (%)	-0.215	-0.214	0.275	0.071	-0.107	0.658(**)	1.000	0.192	0.154
	0.152	0.174	0.064	0.638	0.480	0.000	.	0.200	0.323
Cleaved and transferred oocytes	-0.048	-0.033	0.059	0.313(*)	-0.077	0.280	0.192	1.000	0.291
	-0.168	-0.211	-0.025	-0.022	0.119	0.063	0.200	.	0.059
Pregnancy	0.164	0.146	-0.214	-0.026	0.008	-0.077	-0.154	-0.291	1.000
	0.292	0.368	0.168	0.868	0.959	0.625	0.323	0.059	0.

Tab. 11a: Correlation of semen parameters of non smoker patients (n=35).

	Sperm concentration native semen	Motility in the native semen	Vitality- Eosin test	Membrane integriy HOST-test	Morphology	CMA_3
Sperma concentration (mill/ml) in native semen	1.000	0.411(*)	-0.505(**)	-0.116	0.072	-0.512(**)
	.	0.014	0.002	0.515	0.682	0.002
Motility (%) in the native semen samples	0.411(*)	1.000	-0.615(**)	-0.452(**)	-0.158	-0.191
	0.014	.	0.000	0.007	0.365	0.272
Vitality (%) (Eosin-test)	-0.505(**)	-0.615(**)	1.000	0.316	0.259	0.401(*)
	0.002	0.000	.	0.069	0.133	0.017
Membrane integrity (%) (HOS) Test.	-0.116	-0.452(**)	0.316	1.000	0.213	-0.302
	0.515	0.007	0.069	.	0.227	0.083
Morphology normal spermatozoa (%)	0.072	-0.158	0.259	0.213	1.000	-0.277
	0.682	0.365	0.133	0.227	.	0.107
Chromatin condensation (CMA_3)	-0.512(**)	-0.191	0.401(*)	-0.302	-0.277	1.000
	0.002	0.272	0.017	0.083	0.107	.
DNA fragmentation-TUNEL-test (%)	-0.343(*)	-0.153	0.236	-0.015	-0.078	0.343(*)
	0.044	0.382	0.173	0.931	0.655	0.044
ROS (µmol/l)	0.232	0.144	-0.131	0.098	-0.228	-0.146
	0.179	0.408	0.454	0.582	0.188	0.401
TAS (mmol/L)	0.019	-0.228	-0.009	0.426(*)	0.285	-0.254
	0.914	0.188	0.958	0.012	0.097	0.141
Cotinine (mg/dl)	-0.100	0.161	-0.036	-0.180	-0.220	0.044
	0.566	0.355	0.838	0.307	0.204	0.803
Fertilization rate (%)	0.165	0.147	-0.079	0.075	0.086	-0.263
	0.344	0.399	0.652	0.672	0.625	0.126
Cleaved and transferred oocytes	-0.013	-0.090	0.199	0.298	0.002	-0.044
	0.940	0.605	0.252	0.087	0.991	0.800
Pregnancy	-0.157	0.011	-0.054	-0.011	-0.064	-0.189
	0.391	0.953	0.769	0.951	0.727	0.300

Tab. 11b: Correlation of semen parameters of non smoker patients (n=35).

	DNA -Tunel	DNA -AO	ROS	TAS	Cotinine	Fertilized oocytes	Fertilization rate	Transfer	Pregnancy
Sperm concentration in native semen samples (mill/ml)	-0.343(*)	-0.079	0.232	0.019	-0.100	0.180	0.165	-0.013	-0.157
	0.044	0.667	0.179	0.914	0.566	0.308	0.344	0.940	0.391
Motility (%) of native semen samples	-0.153	-0.098	0.144	-0.228	0.161	0.374(*)	0.147	-0.090	0.011
	0.382	0.592	0.408	0.188	0.355	0.030	0.399	0.605	0.953
Vitality (%)(Eosin)	0.236	0.159	-0.131	-0.009	-0.036	-0.377(*)	-0.079	0.199	-0.054
	0.173	0.384	0.454	0.958	0.838	0.028	0.652	0.252	0.769
Membrane integrity (%)(HOS)-test	-0.015	0.113	0.098	0.426(*)	-0.180	-0.273	0.075	0.298	-0.011
	0.931	0.543	0.582	0.012	0.307	0.125	0.672	0.087	0.951
Morphologically Normal sperm	-0.078	0.058	-0.228	0.285	-0.220	0.072	0.086	0.002	-0.064
	0.655	0.751	0.188	0.097	0.204	0.686	0.625	0.991	0.727
Chromatin condensation (%) CMA_3	0.343(*)	0.056	-0.146	-0.254	0.044	-0.106	-0.263	-0.044	-0.189
	0.044	0.763	0.401	0.141	0.803	0.552	0.126	0.800	0.300
DNA Fragmentation (%)-TUNEL-test	1.000	0.282	-0.199	-0.179	-0.115	-0.143	-0.240	-0.127	0.279
	.	0.118	0.252	0.304	0.510	0.419	0.164	0.467	0.122
DNA denaturation (AO)	0.282	1.000	-0.123	-0.240	-0.124	-0.122	-0.167	-0.184	0.103
	0.118	.	0.503	0.185	0.500	0.507	0.361	0.312	0.590
ROS (µmol/l)	-0.199	-0.123	1.000	-0.157	0.080	-0.077	0.211	-0.088	-0.125
	0.252	0.503	.	0.367	0.648	0.665	0.224	0.615	0.496
TAS (mmol/l)	-0.179	-0.240	-0.157	1.000	-0.226	-0.004	-0.004	0.269	0.064
	0.304	0.185	0.367	.	0.192	0.983	0.982	0.119	0.727
Cotinine (mg/dl)	-0.115	-0.124	0.080	-0.226	1.000	0.141	-0.048	0.015	0.025
	0.510	0.500	0.648	0.192	.	0.427	0.786	0.931	0.892
Fertilization rate (%)	-0.240	-0.167	0.211	-0.004	-0.048	0.599(**)	1.000	0.122	-0.050
	0.164	0.361	0.224	0.982	0.786	0.000	.	0.486	0.786
Cleaved and Transferred oocytes	-0.127	-0.184	-0.088	0.269	0.015	0.318	0.122	1.000	-0.252
	0.467	0.312	0.615	0.119	0.931	0.067	0.486	.	0.164
Pregnancy	0.279	0.103	-0.125	0.064	0.025	-0.007	-0.050	-0.252	1.000
	0.122	0.590	0.496	0.727	0.892	0.969	0.786	0.164	.

Tab 12: Semen characteristics of patient undergoing (IVF) treatments

	N	Minim	Maxim	M±SD
Volume (ml)	57	0.5	6.5	3.59±1.32
Sperm concentration (mill/ml) in native semen samples	57	5.0	120	75.51±31.95
Sperm concentration (mill/ml)	57	1.0	101	35.87±28.39
Motility (%)in the native semen samples	57	5.0	80	39.30±18.16
Motility (%) after semen preparation	57	5.0	100	73.68±23.95
Vitality (%) Eosin test (Stained sperm)	57	10	90	58.77±21.82
Membrane integrity (HOS-Test)	57	5.0	90	41.84±20.49
Morphologically normal spermatozoa (%) in native semen	57	1.0	72	14.05±15.88
Chromatinkondensation (%) CMA3. (Stained samples)	57	3.0	49	17.81±9.646
DNA Fragmentation (%) TUNEL-test	57	0.0	30	7.95±6.62
DNA Denaturation (%) Acridine orange test AO test	55	0.0	18	5.33±4.90
ROS (µmmol/l) concentration in seminal plasma	57	2.84	505.17	73.78±74.37
TAS (mmol/l) concentration in seminal plasma	57	0.00	2.25	1.36± 0.52
Selenium (µgSe/kg dry mass) in seminal plasma	26	15	317 E-2	3.412E-2±1.681E-02
Zinc concentration in seminal plasma	26	0.01	0.08	131.0±77.47
Retrieved Oocytes	57	2.0	30	10.39±6.850 (2798.50)
Fertilized oocyte	56	0	24	5.2±5.66 (2578.00)
Fertilization rate (%)	57	0.00	100.00	50.56±28.89
Transferred Oocytes	57	0	3	1.77±0.866(2461.50)
Pregnancy	40	1.0	2.0	1.55±0.504 (18/22)(45%)

Tab 13: Semen characteristics of patient undergoing (ICSI) treatments

	N	Minim	Maxim	M±SD
Volume (ml)	37	1.0	8.0	3.33±1.50
Sperm concentration (mill/ml) in the native semen samples	37	1.0	120	49.63±39.52
Sperm count in the native semen (mill/ml)	37	1.0	101	25.82±30.07
Motility (%)in the native semen	36	5.0	60	22.22±12.95
Motility (%) after semen preparation	37	5.0	95	44.59±31.83
Vitality (%) Eosin test	37	10	95	71.19±22.09
Membrane integrity (HOS-Test)	36	10	95	46.53±2.00
Morphologically normal spermatozoa	36	0.0	42	10.39±10.34
Chromatin condensation-native	37	5.0	92	30.97±20.50
DNA Fragmentation TUNEL-test	37	0.0	29	8.14±6.58
DNA Denaturation AO test	35	0.0	23	6.63±6.25
ROS concentration	37	2.84	143.04	50.84±34.63
TAS concentration	37	0.00	2.26	3.614-02
Selenium (µgSe/kg dry mass) in seminal plasma	22	36	268	3.614E-02±8.481E-03 3.500E-02
Zinc concentration in seminal plasma	22	0.02	0.06	127.31±60.16
Retrieved Oocytes	37	2.0	23	8.57±4.537 1666.50
Fertilized oocyte	37	0.0	16	5.03±3.700 1793.00
Fertilization rate (%)	37	0.00	100.00	59.23±26.47231
Transferred Oocytes	37	0.0	3	2.14±0.822 2003
Pregnancy	18	1.0	2.0	1.39±o.50 11/7 (61.1%)

Tab 14: Semen characteristic and sperm quality of non smoker patients underwent assisted reproduction technology program.

	N	Mini	Maxi	Sum	M±SD
Volume (ml)	35	1.5	6.0	128.0	3.65±1.22
pH-Wert	35	7.9	9.5	302.5	8.64±0.33
Sperm concentration (mill/ml) the native semen sample	35	5	101	2662	76.06±29.49
Sperm concentration (mill/ml) after semen preparation	35	2	101	1446	41.32±29.89
Motility (%) of native semen samples	35	5	80	1105	31.57±15.99
Motility after semen preparation (%)	35	5	100	2280	65.14±31.45
Vitality (%) Eosin-test	35	30	95	2410	68.86±17.78
Membrane integrity (HOS) test	34	20	95	1675	49.26±25.05
Morphologically normal sperm	35	0	72	562	16.06±18.53
Chromatin condensation (CMA3)	35	5	47	597	17.06±8.60
DNA–Fragmentation (TUNEL-test)	35	2	29	372	10.63±6.77
DNA-Denaturation Acridine Orange-test (AO)	32	1	18	262	8.19±4.09
ROS (µmol/ml) level in seminal plasma	35	15.30	111.84	1858.47	53.09±28.03
TAS (mmol/ml) level in seminal plasma	35	0.00	2.26	47.38	1.35±0.66
Cotinine (µg/dl) concentration in seminal plasma	35	5	201	676	19.30±32.69
Oocytes	35	2	22	312	8.91±5.66
Fertilized Oocytes	34	1	17	174	5.12±4.17
Fertilization rate (%)	35	18.18	100.00	2046.86	58.48±24.55
Cleaved (Transferred) embryos	35	0	3	66	1.89±0.83

Tab 15: Semen characteristic and sperm quality of smoker patients underwent assisted reproduction technology program.

	N	Mini	Maxi	Sum	M±SD
Volume (ml)	11	1.0	5.0	32.0	2.909±1.33
pH-Wert	11	8.3	9.0	95.8	8.709±0.31
Sperm concentration (mill/ml) in the native semen sample	11	6	101	558	50.73±39.17
Sperm concentration (mill/ml) after semen preparation	11	2	101	373	33.91±38.23
Motility (%) of native semen samples	10	15	70	390	39.00±20.38
Motility after semen preparation (%)	11	10	95	675	61.36±33.09
Vitality (%) Eosin-test	11	40	90	710	64.55±19.16
Membrane integrity (HOS)-test	11	30	85	525	47.73±16.03
Morphologically normal sperm (%)	10	3	61	198	19.80±18.52
Chromatin condensation (CMA3)	11	7	26	173	15.73±7.185
DNA–Fragmentation TUNEL-test	11	3	30	141	12.82±7.93
DNA-Denaturation Acridine Orange-test (AO)	10	3	14	89	8.90±3.51
ROS (µmol/ml) level in seminal plasma	11	24,72	120,79	560,38	50.94±28.20
TAS (mmol/ml) level in seminal plasma	11	0,00	2,13	12,09	1.09±0.73
Cotinine (µg/dl)	11	5	201	1587	144.29±82.19
Oocytes	11	5	29	119	10.82±7.82
Fertilized Oocytes	11	1	17	61	5.55±5.68
Fertilization rate (%)	11	14.29	87.50	501.76	45.61±23.47
Cleaved (Transferred)embryos	11	1	3	21	1.91±0.70