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Assessment Of Lipid Peroxidation And Glutathione Levels In Serum And Seminal Plasma For Unexplained Infertile Men In Thi-Qar Governorate

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Abstract

Infertility is the inability of a sexually active, non-contracepting couple to achieve spontaneous pregnancy in one year. unexplained male infertility (UMI) is reserved for infertile of unknown origin and accounts for 6-27% of infertile men with normal semen analyses on two or more occasions with no demonstrable physical or endocrine abnormalities and female factor infertility has been ruled out. Oxidative stress (OS) is a consequence of an imbalance between the production of reactive oxygen species(ROS) and the body's antioxidant defense mechanisms. However oxidative stress(OS) is a common pathology seen in approximately half of all infertile men. It has been shown that reactive oxygen species (ROS) can lead to deleterious effects on a range of sperm parameters. The monitoring of MDA levels in different biological systems can be used as an important indicator of lipid peroxidation .Humans have developed a highly ,organized and complex antioxidant defense system to protection cells and organ systems of the body against reactive oxygen species(ROS) such as glutathione is capable of reducing ROS levels and lipid peroxidation..

Serum and seminal plasma MDA was significantly increased in unexplained infertile(smoker and nonsmoker)groups when compared to fertile (smoker and nonsmoker)groups. The serum and seminal plasma glutathione were significantly decreased in unexplained infertile(smoker and nonsmoker)groups when compared to fertile (smoker and nonsmoker)groups. The decrease in antioxidant levels appears to be mainly a consequence of increased oxidative stress.

Keywords: unexplained infertile men, Oxidative stress, Lipid peroxidation, Antioxidants, MDA, Glutathione

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تقييم مستوى الأكسدة الفوقية للدهون والكلوتاثيون في المصل والبلازما المنوية لحالة عقم الرجال الغير المفسر في محافظة ذي قار

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الخلاصة

العقم هو عدم قدرة الزوجين الناشطين جنسياً وبدون استخدام موانع الحمل على حدوث الحمل التلقائي في سنة واحدة. العقم الذكوري غير المفسر (UMI) يشير لعقم غير معروف السبب, وبمعدل ٦-٢٧٪ من الرجال الذين يعانون من العقم مع تحاليل السائل المنوي الطبيعي في مناسبتين أو أكثر بدون أي تشوهات جسدية أو هرمونية ظاهرية واستبعاد عامل العقم عند النساء. الإجهاد التأكسدي (OS) هو نتيجة لعدم التوازن بين إنتاج أنواع الأكسجين التفاعلية (ROS) وآليات الدفاع المضادة للأكسدة في الجسم. ومع ذلك ، فإن الإجهاد التأكسدي (OS) هو حالة مرضية شائعة تظهر تقريبا في نصف الرجال المصابين بالعقم. لقد ثبت أن أنواع الأكسجين التفاعلية (ROS) يمكن أن تؤدي إلى تأثيرات ضارة على مجموعة من معايير الحيوانات المنوية. يمكن استخدام مراقبة مستويات MDA في أنظمة بيولوجية مختلفة كمؤشر مهم على بيروكسيد الدهون. لقد طور البشر نظام دفاع مضاد للأكسدة عالي التنظيم ومنظم لحماية خلايا الجسم وأنظمة الجسم ضد أنواع الأكسجين التفاعلية (ROS) مثل الجلوتاثيون Glutathione قادر على الحد من مستويات ROS و بيروكسيد الدهون .

كان الهدف من الدراسة هو تحديد مستوى بيروكسيد الشحوم كما هو محدد من قبل MDA(malondialdehyde) و الجلوتاثيون Glutathione في المصل والبلازما المنوية للرجال ذات العقم غير المفسر مقارنة مع الرجال الخصبين.

وجد زيادة MDA المصل والبلازما المنوية بشكل كبير في مجموعات العقيم غير المفسر (المدخن وغير المدخنين) بالمقارنة مع المجموعات الخصبة (المدخنة وغير المدخنين). انخفض الجلوتاثيونGlutathione (المصل والسائل المنوي) بشكل ملحوظ في مجموعات العقم (المدخن وغير المدخنين) غير المبررة بالمقارنة مع المجموعات الخصبة (المدخنة وغير المدخنين). ويبدو أن الانخفاض في مستويات مضادات الأكسدة هو أساسا نتيجة لزيادة الإجهاد التأكسدي.

Introduction

Infertility remains both prevalent and problematic affecting 13-15% of the couples worldwide medically and psychosocially . It is clinically defined as failure the inability to achieve a successful pregnancy after 12 months of unprotected intercourse or therapeutic donor insemination *(Hamada et al., 2012).* According to World Health Organization (WHO) , "Infertility is the inability of a sexually active, non-contracepting couple to achieve spontaneous pregnancy in one year" *(WHO, 2000).* It classified to(primary infertility) refers to woman with no prior pregnancies (when no pregnancy has ever occurred) *(Obeisat et al., 2012).* Or Infertility is considered secondary if it occurs after one or more pregnancies ,approximately 15% of couples attempting their first conception meet with a failure *(Geidam et al., 2008).* Thus, those who repeatedly spontaneously miscarry or whose pregnancy results in a stillbirth *(Gurunath et al., 2011).*

Infertility of unknown origin (no identifiable cause) accounts for 37–58% is divided into two groups unexplained male infertility and idiopathic male infertility; it is a condition in which fertility impairment occurs spontaneously or due to an obscure or unknown cause *(Hamada et al., 2011).*

The category 'unexplained male infertility' (UMI) is reserved for infertile of unknown origin and accounts for 6-27% of infertile men with normal semen analyses on two or more occasions with no demonstrable physical or endocrine abnormalities and female factor infertility has been ruled out *(Alaa et al., 2011).*

oxidative stress (OS) is a consequence of an imbalance between the production of reactive oxygen species(ROS) and the body's antioxidant defense mechanisms *(Sies, 1993)* , this ratio can be altered by increased levels of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS), or a decrease in antioxidant defence mechanisms *(Agarwal et al., 2012).*

Oxidative stress (OS) has been identified as one factor that affects fertility by causing sperm dysfunction status and thus, has been extensively studied in recent years *(Sies,1993).* Oxidative stress(OS) is a common pathology seen in approximately half of all infertile men, it damages sperm function and DNA integrity *(Asbagh et al., 2010).* The mechanism of loss of sperm function by ROS appears to be multifold . ROS may affect the quality and number of spermatozoa reaching the ovum in the female reproductive tract. In addition, ROS impair the fertilization process by preventing the initiation of sperm-oocyte fusion events . Finally, ROS can impair embryo development and affect the health of offspring by damaging sperm DNA *(Agarwal et al., 2004).*

Malondialdehyde (MDA) is the organic compound with the nominal formula $CH_2(CHO)_2$,it occurs naturally as an end product of lipid peroxidation (LPO) which is a process where reactive

oxygen species degrade polyunsaturated lipids. The monitoring of MDA levels in different biological systems can be used as an important indicator of lipid peroxidation both in-vitro and in vivo for various health disorders. Human sperm cells in contrast with other cells are particularly susceptible to oxidation of their plasma membranes due to the existence of a high concentration of polyunsaturated fatty acids(PUFA) in the membrane . PUFA play an important role in ion transport and sperm membrane fluidity, therefore oxidation of sperm membrane PUFA by oxidants (ROS) cause to deficiency in membrane function and sperm death.Thus, MDA is used in biochemical arrays to monitor the degree of peroxidative damage to spermatozoa *(Agarwal et al., 2014).*

Humans have developed a highly ,organized and complex antioxidant defense system to protection the body's cells and organ systems of the body against reactive oxygen species(ROS). The system involves a synergy between various endogenous and exogenous components , that function interactively and synergistically to neutralize free radicals *(Sheweita et al., 2005; Adewoyin et al., 2017)*.. Nevertheless, the pathologic levels of ROS detected in the semen of infertile men are more likely caused by increased ROS production than by reduced antioxidant capacity of the seminal plasma . Unfortunately, spermatozoa are unable to repair the damage induced by oxidative stress, because they lack the cytoplasmic enzyme systems required to accomplish the repair *(*Zini *et al., 1993).*

Glutathione (GSH), It is a tri- peptide composed of the amino acids glutamic acid , cysteine and glycine, present in most cells of the body, and much smaller concentrations in blood.

L-glutamate +L-cysteine +ATP $\xleftrightarrow{\text{γ-glutamyl -cysteine synthetase}}$ γ- L-glutamyl-L-cysteine +ADP+Pi

γ-L-glutamyl-L-cysteine + glycine +ATP $\xleftrightarrow{\text{GSH synthetase}}$ GSH+ADP+Pi

Glutathione (GSH) is an important antioxidant in plants, animals, fungi, and some bacteria. It is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides, and heavy metals *(Pompella et al., 2003).*In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered indicative of oxidative stress *(Pompella et al., 2003)*,it has been suggested that deficiency in the consumption of these is an important factor in the development of many cancers *(Jones et al., 1992).* As glutathione is part of an essential defence mechanism to protect germ cells from mutagens and permatozoa from oxidative injury *(Ochsendorf et al., 1998)*, also is essential to the formation of

phospholipid hydroperoxide glutathione peroxidase an enzyme present in spermatids which becomes a structural protein in the midpiece of mature spermatozoa *(Sheweita et al., 2005).*

The aim of this study determination the level of lipid peroxidation as indicated by malondialdehyde (MDA) and glutathione in serum and seminal plasma of unexplained male infertility men compare with fertile men.

Material and Methods

This study was conducted in infertility unit at Al-Hussein teaching hospital Thi-Qar governorate during the period collect from 1 \8\ 2016 to 1 \5\ 2017. A total number of cases ,one hundred men with infertility of the ages (19– 49) years and compare the results with fifty fertile men of the ages (20 – 52)years. Thus, the present study including (130) men, which were divided in to two groups:

1. Control group (Fertile men): included (50) healthy fertile men (32 Non smoker and 18 Smoker) .
2. Case group (infertile men): included (80) infertile men who unexplained infertility were diagnosed with normal semen analyses on two or more occasions with no demonstrable physical (55Non smoker and 25 Smoker) .

All the infertile subjects had a history of infertility for at least 2 years with no indication of hormonal, medical, or surgical causes for their infertility. The wives of the infertile subjects included had no obvious causes for infertility like tubal blockage or ovulation disorders.

Exclusion criteria included(Oligoasthenoteratozoospermics, Azoospermia, a history of epididymo-orchitis, prostatitis, genital surgery; genital disease or varicocele, presence of any endocrinopathy; of taking alcohol Patients were also excluded from analysis known to be associated with decreased fertility;hepatobiliary disease; significant renal insufficiency ,diabetes mallites and patients taking drugs like vitamin E, vitamin C supplementation within the past three months were also excluded from the study.

Serum Samples: (6) ml of blood samples was collected by veinpuncture using a sterile disposable syringe from patients and control group. The blood sample was dispensed in a gel tube, and left for 15 minutes at room temperature to clot. Then, it was centrifuged at 3000 rpm for 10 minutes to collect serum and kept in the freezer (-20ºC) until use unless used immediately to analyze biochemical parameters

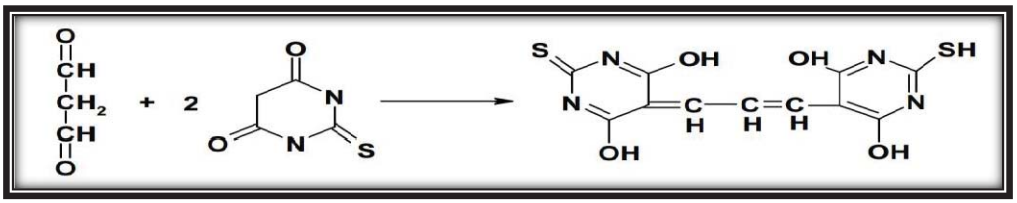
Semen samples were collected by masturbation into a sterile, wide mouthed container, after at least 72 hours (3- 4days) of sexual abstinence. Samples were allowed to liquefy at temperature (37ºc) for

at least 30 minutes .Seminal plasma was separated from spermatozoa by centrifugation at 3500 rpm for 15 minutes and stored at –(45-65)°C until analysis .

1- Measurement of Serum and seminal plasma MDA levels by TBA method : Thiobarbituric acid assay-Lipid peroxidation is a complex process leading to the formation of various aldehydes including malonaldehyde (MDA) *(Fong et al., 1973; Rao et al., 1989).*

► ***Principle:***

In this method, MDA formed from the oxidation of polyunsaturated fatty acids was identified as the product of Lipid peroxidation (LPO) that react with thiobarbituric acid (TBA), in coexisting trichloroacetic acid (TCA).



► ***Reagents :***

- 17.5% trichloroacetic acid(TCA): (17.5 gram of trichloroacetic acid make up to100ml with distilled water).
- 66% thiobarbituric acid(TBA): (66 gram of thiobarbituric acid make up to 100ml with distilled water).
- 70% trichloroacetic acid(TCA): (70 gram of trichloroacetic acid make up to 100ml with distilled water).
- 0.67% thiobarbituric acid(TBA): (0.67 gram of thiobarbituric acid make up to 100ml with distilled water).
- 0.5 g NaOH and 100 ml glacial acetic acid .

► ***Procedure***

A- Serum

The level of serum MDA was determined spectrophotometerically with a thiobarbituric acid(TBA) solution:

- 150 µl serum sample was added to 1 ml of 17.5% trichloroacetic acid(TCA) and 1ml of 66% thiobarbituric acid(TBA) mixed well by vortex, incubated in a boiling water bath for 15 minutes and then allowed to cool.

- 1 ml of (70% trichloroacetic acid) was added and mixed well ,stand at room temperature for (20 min) .
- each tube was centrifuged for 15 min at 2,000 rpm and the supernatant absorbance of these was read on a spectrophotometer at 532 nm.

B- seminal plasma

Seminal MDA levels were analyzed according to *(Rao et al. 1989)* .

- 100 µl of seminal plasma was added in 900 µl of distilled water into glass tube.
- To each tube, 500 µl of thiobarbituric acid reagent(0.67%) (0.67 g of 2-thiobarbituric acid dissolved in 100 ml of distilled water with 0.5 g NaOH and 100 ml glacial acetic acid added) was added .
- Heated for 1 h in a boiling water bath. After cooling temperature.
- each tube was centrifuged for 10 min at 4,000g and the supernatant absorbance of these was read on a spectrophotometer at 534 nm.

$$\text{Concentration of Malondialdehyde (MDA)} = \frac{\text{Abs at 532or 534nm}}{L \times \varepsilon} \times D \times 10^6$$

L: Light path(1cm) D: Dilution factor=21

Ɛ: Molar Extinction coefficient 1.56×10^5 mol^{-1} Lcm^{-1}

2- Serum reduced glutathione (GSH) was measured by thiol concentration according to Ellman's method *(Ellman,1959)* and seminal plasma glutathione was estimated by methods based on principles of methods of Moron et al *(Moron et al., 1979; Shete et al., 2012).*

► ***Principle:***

Thiol concentration was measured according to Ellman's method*(Ellman,1959)*. This method depend on the reaction of 5, 5'-Dithio bis(2-nitrobenzioc acid) (DTNB)with aliphatic thiol compounds at pH8.0 to produce one mole of p-nitrothiol phenol anion permolethiol. Since this anion highly colored (ε=13600 at 412 nm), it can be used to measure the thiol concentration.

► ***Reagents:***

A- Serum

1- Phosphate buffer pH=8:

- 0.1M Na_2HPO_4 was prepared by dissolving 3.54 gm in 250 mL distilled water.
- 0.1 M NaH_2PO_4 was prepared by dissolving 12 gm in 250 mL distilled water.

Then mixed 94.7 ml from 0.1M $Na_2HPO_4$with 5.3 mL of 0.1M NaH_2PO_4.

2- Phosphate buffer PH=7:

- 61 mL of 0.1 M Na_2HPO_4 was mixed with 39 mL of 0.1M NaH_2PO_4.

3- DTNB solution:

- This solution was prepared by dissolving 39.6mg of DTNB in 10mL phosphate buffer PH=7.

► *Procedure:*

1. Blank: 20 µl of plasma was added to 9mL distilled water, then added 1mL of phosphate buffer pH8, mixed well.

2. Test: 20 µl of DTNB was added to 3mL blank solution, mixed well, read absorbance at 420 nm after waiting for one hour.

$GSH\ (\mu mol/L) = A \times \frac{H}{t} \times 1000$

Where: $A = A_T - A_B$

A_T : absorbance of test A_B : absorbance of blank D: Diluting factor

E = Molar extension coefficient equal to 13600 $mol^{-1}L\ cm^{-1}$ $\frac{H}{t} = 36.8$

B-Seminal plasma

Reduced glutathione (GSH) is estimated by methods based on principles of methods of *(Moron et al., 1979; Shete et al., 2012).*

- 0.5 ml of seminal plasma was taken in a test tube and 2ml of distilled water was added.
- mixed well. Then centrifuged for 5 min at 5000 rpm.
- 0.5 ml of supernatant was taken, to which 0.5ml of TCA (5%) was added and then centrifuged for 10 min at 10,000 rpm.
- 0.5 ml of supernatant was taken to which 2.5ml of phosphate buffer (pH 8) was added.
- 1ml DTNB was added. This solution was inverted for 3 times to mix.
- The absorbance was read on spectrophotometer at 412nm within 4 min.

Results and Discussion

Table 1 : Comparison between different parameters in the studied groups

Groups	Fertile(Control) (n=50)		Unexplained infertile (n=80)	
	Smoker (n=18)	Non smoker (n=32)	Smoker (n=25)	Non smoker (n=55)

Serum	Malondialdehyde (nmol\ml)	Mean± SD	4.32±0.98	2.95±.81	5.35±0.64	4.28±0.65
		P-value	<0.05[a]		<0.05[b]	
			<0.05[c]	>0.05[d]	<0.005[e]	<0.005[f]
	Glutathione (µmol\L)	Mean± SD	9.01±0.85	10.48±1.51	7.08±0.94	8.15±1.34
		P-value	<0.05[a]		<0.05[b]	
			<0.05[c]	<0.05[d]	<0.005[e]	<0.05[f]
Seminal plasma	Malondialdehyde (nmol\ml)	Mean± SD	0.83±0.24	0.70±0.17	1.55±0.45	1.24±0.42
		P-value	<0.05[a]		<0.05[b]	
			<0.005[c]	<0.05[d]	<0.005[e]	<0.005[f]
	Glutathione (µmol\L)	Mean± SD	3.86±0.48	4.92±0.72	2.48±0.58	3.43±0.59
		P-value	<0.05[a]		<0.05[b]	
			<0.05[c]	<0.05[d]	<0.005[e]	<0.05[f]

a: compared between Fertile (Smoker) and (Non smoker)

b: compared between unexplained Infertile (Smoker) and (Non smoker)

c: compared between Fertile (Smoker) and unexplained Infertile (Smoker)

d: compared between Fertile (Smoker) and unexplained Infertile (Non smoker)

e: compared between Fertile (Non smoker) and unexplained Infertile (Smoker)

f: compared between Fertile (Non smoker) and unexplained Infertile (Non smoker)

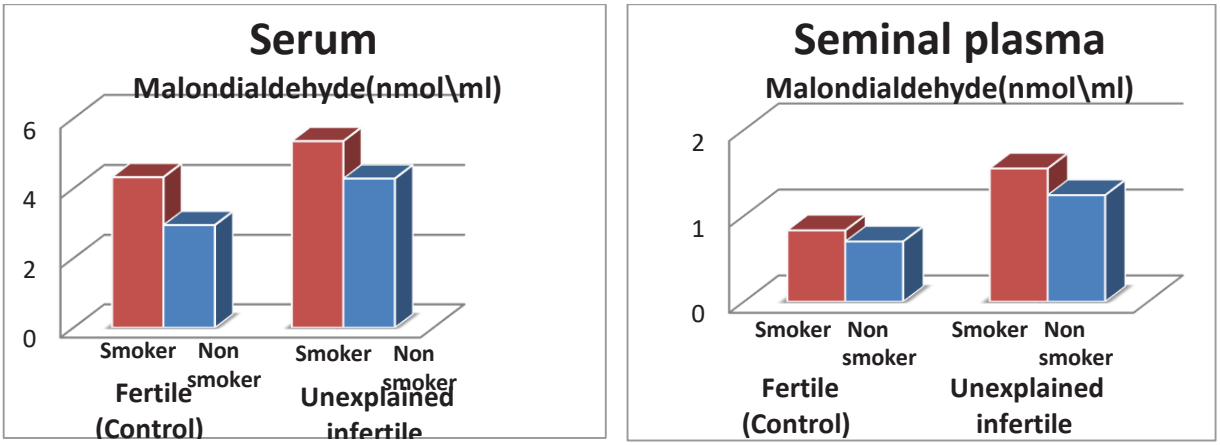


Figure (1-a) Serum and Seminal Plasma Malondialdehide concentrations in fertile(Control) and unexplained infertile

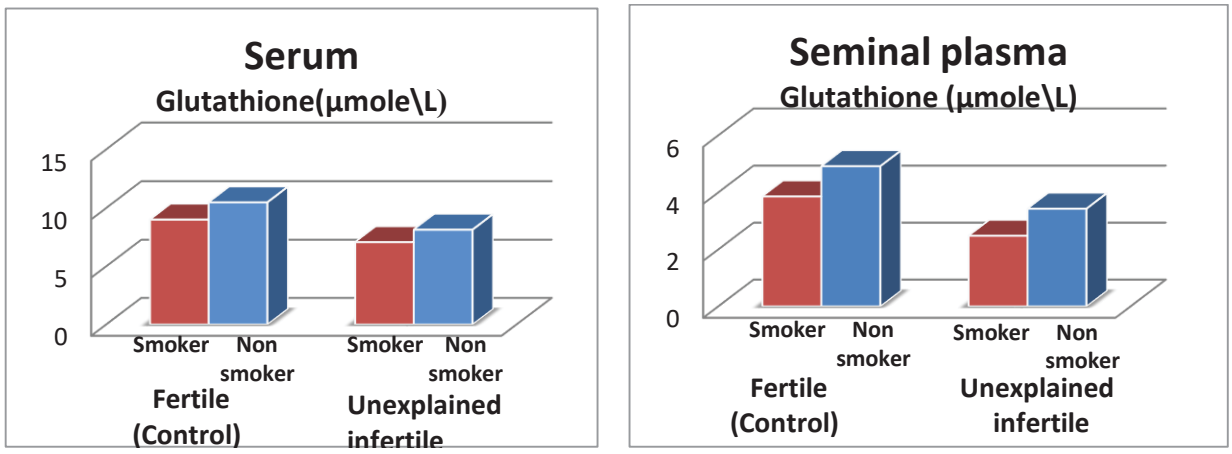


Figure (1-b) Serum and Seminal Plasma Glutathione concentrations in fertile (Control) and unexplained infertile

Through the table(1) and figure (1-a) the result showed a significant increase in serum malondialdehyde (MDA) , in unexplained Infertile (Smoker and Non smoker) groups compared fertile(Control) groups, the results revealed a significant change of all groups ($p<0.05$). Increased MDA concentration in unexplained Infertile is due to increased production of reactive oxygen species and hence more lipoxidation products . Increased levels of MDA in the peroxidation products which have been associated with a variety of acute and chronic pathophysiological processes in human beings . Increased free radical generation occurs in states of tissue hypoxia, hyperoxygenation, various infective and metabolic disorders *(Haue et al., 2012).* Cigarette smoking has been reported to contain many free radicals and other highly reactive molecules, it is suggested that the observed increase in the levels of MDA in the serum is linked to the increase in the levels of exogenous free radicals generated from cigarette smoke that ultimately leads to lipid peroxidation *(Bello et al., 2017).*

The result of study was , the mean value of seminal plasma MDA is higher significant in unexplained Infertile group compared with fertile (Control) group. Which mean the level of MDA significant different between groups.

Studies suggested that detection of MDA concentrations in seminal plasma has an indicative value on the diagnosis of male infertility induced by overproduction of reactive oxygen species in male reproductive system *(Asbagh et al., 2010).*

it has been suggested that cigarette smoking negatively affects every system involved in the reproductive process. Spermatozoa from smokers have reduced fertilising capacity, and embryos display lower implantation rates .Associated smoking with a 48% increase in seminal leukocytes,an increase in ROS levels, higher level of MDA and a 10-point decrease in ROS-TAC scores *(Mostafa et al., 2010).*

ROS are usually associated with poor semen quality and male infertility. It has been shown that 40%–88% of non selected infertile patients have high levels of seminal ROS . However, recent reports found that normospermic (normal semen analysis) infertile men have higher ROS levels and reduced total antioxidant capacity (TAC) levels than the normospermic fertile counterparts . The exact prevalence of oxidative stress (OS) in normospermic infertile men is unknown. Nevertheless, a controlled study on limited number of patients detected that 11% of normospermic infertile men have oxidative stress (OS) *(Alaa et al., 2011).*

table(1)and figure(1-b) show that mean level of glutathione was significantly lower in serum unexplained Infertile (Smoker and Non smoker)groups comparison with fertile (Smoker and Nonsmoker) groups, the results revealed a significant change of all groups ($p<0.05$). Evident that the increase in free radicals generation, and lipid peroxidation for many causes which sure decreases the levels of antioxidants in the serum as a results of their constant destruction during the neutralization of these free radicals , which indicates that the antioxidant defense system was impaired in infertile subjects. Therefore; ill-health related to smoking may be linked to increased production of ROS and impaired antioxidant defense system in infertile subjects .Many studies have reported a negative impact of smoking on semen analysis parameters and male infertility . Cigarette or hookah smoking induces almost the same causes of decreased levels of glutathione . Also significant decreased in GSH concentration may be return to the decreased in NDPH Co–enzyme which consider as reduction substance and essential in build Glutathione or as cataleptic substance for Glutathione Reductase enzyme which work to return Glutathione from GSSH form to GSH *(Lee et al., 2010).*

In this study glutathione level ,in seminal plasma is significantly decrease ($p < 0.05$) unexplained infertile groups (Smoker and Non smoker) when compared with fertile(control) groups .

In human reproductive tissues all components of the glutathione cycle have been demonstrated in different concentrations and localizations, including seminal plasma and spermatozoa.The glutathione content of mammalian epididymis reached about 10% of the testes . The glutathione content decreased about 75% during sperm maturation from caput epididymis spermatozoa to ejaculated spermatozoa . The reduction in the glutathione concentration during spermatogenesis especially rendered late spermatids susceptible to mutagenesis by free radicals or other compounds *(Ochsendorf et al.,1998).*

Thus the findings of the present study shows an association between low antioxidant level(Glutathione) and infertility, the obvious of the present study is confirm the low level of

antioxidant itself lead to inflicted damage on the spermatozoa (such as peroxidation of membrane lipids, DNA modification or apoptosis).

Smoking is also associated with decreased plasma glutathione levels (GSH), quitting smoking raises plasma GSH levels *(Lane et al.,1998).*

Given the large number of men world-wide who smoke, and the fact that cigarette smoke is aknown somatic cell mutagen and carcinogen, there is a major concern that smoking may adversely affect male reproductive health . A number of studies have shown that smoking has a detrimental effect on antioxidant level and sperm quality, most significantly sperm concentration, motility, and morphology *(Hamad ,2014).*

Conclusion

Present study demonstrates there is increased oxidative stress in Serum and seminal plasma unexplained infertile male compared to fertile male .This study also demonstrates the decreased antioxidant level glutathione (GSH) in unexplained infertile male(smokers) compared to fertile(non-smokers) . thus ,malondialdehyde can serve as an important marker and its value may indicate the prognosis or severity of infertility.

This study found a significant negative correlation between GSH and MDA levels of seminal plasma. Therefore, GSH might have some fertility enhancing role by reducing lipid per-oxidation. It could therefore be proposed that the concentration of reduced glutathione, a non-enzymatic antioxidant of seminal plasma, could be used as a chemical parameter to assess male fertility, whereas high levels of MDA can stand for poor semen quality. The beneficial role of GSH to minimize oxidative damage to the sperms can make it a suitable candidate for therapeutic usage in the treatment of male infertility. A series of clinical trial is needed to explore the possibility.

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