STUDY OF EFFECT OF REACTIVE OXYGEN SPECIES (ROS) ON HUMAN SPERM MORPHOLOGY

By

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ABSTRACT:

Introduction: Infertility affects 10%–15% of couples worldwide and male infertility accounts for approximately 50% of these problems. Infertility becomes a public health problem in recent decades. Physicians and embryologist dealing with practical aspects of male infertility are driven toward identifying why spermatozoa from a particular male have not achieved fertilization and whether spermatozoa from a particular male have the capacity to fertilize an oocyte. ROS can be either harmful or beneficial to the body. **Aim of work:** Aiming to improve ICSI outcome using additional criteria to enhance the sperm selection. In this study we made an attempt to establish ROS measurement as an important tool in the diagnosis of seminal oxidative stress (OS) in infertile Egyptian men. **Patients and Methods:** A prospective study included a population of 200 semen samples were obtained from patients. Semen will be examined followed by division of the semen sample in 3 tubes. The sample in **1st** tube will be examined by Halo sperm G2 kit for evaluation of DNA fragmentation based on the Sperm Chromatin Dispersion (SCD) Test. The sample in **2nd** tube will be examined by oxi-sperm kit for determination of Reactive Oxygen Species (ROS) level in the sperm sample. The sample in **3rd** tube will be examined by electron microscope after preparation to evaluate the sperm morphology. **Result(s):** The motility of spermatozoa in control group was significantly higher ($P < 0.05$) compared to motility of spermatozoa in different groups. The abnormal morphology of spermatozoa in infertility groups was significantly higher ($P < 0.05$) compared to abnormal morphology of spermatozoa in control group. The head, middle piece and

tail abnormality of spermatozoa in infertility groups was significantly higher ($P < 0.05$) compared to head, middle piece and tail abnormal morphology of spermatozoa in control group. **Conclusion(s):** ROS measurement should be established as an important tool in the diagnosis of seminal OS in infertile Egyptian men. From our study, the diagnosis of reactive oxygen species can help us to explain and resolve most of the problems of 1ry infertility, 2ry infertility and repeated ICSI failure.

Keywords: ROS- male infertility-Sperm morphology.

INTRODUCTION

Infertility affects 10%–15% of couples worldwide and has become a public health problem in recent decades **(Bashamboo et al., 2010).** Male infertility accounts for approximately half of these problems, and decreased semen quality has been widely reported in recent decades **(Bolan et al., 2014).** A free radical is defined as "any atom or molecule that possesses one or more unpaired electrons" **(Rivlin et al., 2004)**. Like all cells living under aerobic conditions, spermatozoa produce reactive oxygen species (ROS), mostly originating from normal metabolic activity. ROS are highly reactive oxidizing agents belonging to the class of free radicals. They are highly unstable oxidants that react with many biochemical substances like lipids, amino acids, carbohydrates, protein, and DNA. Therefore ROS are considered as a causative factor for a variety of diseases **(Venkatesh et al., 2009).**

It is evident from researches that oxidative stress(OS), sperm DNA damage and apoptosis are the possible independent or interlinked molecular events, in infertile males, that are associated with various clinical and laboratory manifestations. Sperm DNA contributes one half of the genomic material to offspring and the integrity of sperm DNA is of crucial importance for balanced transmission of genetic information to future generations **.**The incidence of DNA-fragmented sperm in human ejaculate is documented, particularly in men with poor semen quality **(Irvine et al., 2000).**

Poor chromatin packaging has been shown to correlate with numerous reproductive outcomes: decreased fertility of couples after intercourse **(Spano et al.,**

2000), poor fertilization after IVF and ICSI and a higher incidence of pregnancy loss **(Esterhuizen et al., 2000).** Conflicting reports on the pre-fertility necessity of sperm nuclear DNA screening have emerged over the last decade. Normal and fertile sperm donors were found to have lower levels of nuclear DNA defects when compared to men undergoing fertility work-ups **(Irvine et al., 2000).**

ROS at low level facilitate hyper activation, capacitation, acrosome reaction, motility, fertilization and oocyte adhesion of spermatozoa **(de Lamirande & O'Flaherty, 2008),** but higher ROS damages a variety of biomolecules such as lipids, amino acids, carbohydrates, protein and DNA and adversely affect the sperm function **(Rivlin et al., 2004).** Some studies have focused the impact of free radicals on male fertility as they are believed to cause sperm dysfunction either by damaging sperm plasma membrane or DNA **(Venkatesh et al., 2009)**.

ROS levels are elevated in semen of infertile men. The oxidative phosphorylation system of mitochondria is suspected to be both the production and target site of ROS, as it is closely associated with the inner mitochondrial membrane **(Agarwal et al., 2006, 2008)**. Moreover increased free radicals and accumulation of mitochondrial DNA (mtDNA) mutation have also been associated with increase in age. So it is vital to detect the seminal oxidative stress (OS) at the earliest in the male reproductive evaluation to prevent/treat OS-associated male infertility **(Beckman & Ames, 1998).** There are no reports that document the availability of ROS measurement as a routine diagnostic method in the infertility clinic in Egypt. Therefore in this study we made an attempt to establish ROS measurement as an important tool in the diagnosis of seminal OS in infertile Egyptian men.

PATIENTS AND METHODS:

A prospective study was conducted at the Assisted Reproduction Unit, International Islamic Center for Population Studies and Researches, Al-Azhar University (IICPSR); during the period between July 2015 and July 2016. The study was approved by Ethics Committee of Faculty of Medicine and (IICPSR), Al-Azhar University. All participating patients had to sign informed written consent after

thorough explanation of the purpose and procedure of the study. The study included 200 male ages ranged between 25 and 40 years and cases of 1ry infertility or cases of 2ry infertility or cases with history of repeated ICSI failure. While the excluded couples were excluded due to negative DNA fragmentation index (DFI) or negative Reactive Oxygen Species (ROS) or female factor. Subjects were divided into 4 groups (50 subjects from each group).

1. **Control group**: individuals who had never smoked, no varicocele after clinical testicular examination, no drugs uptake (hashish and tramadol) and no urinary tract infection.
2. **1ry infertility group**: refers to couples who have not become pregnant at least1year having sex without using birth control methods.
3. **2ry infertility group**: refers to couples who have been able to get pregnant at least once, but now are unable.
4. **Repeated ICSI failure group**: concerned with couples with previous failed ICSI trial due to low grade embryo transfer.

   Semen will be examined followed by division of the semen sample.

Halosperm study: The sample in 1st tube will be examined by Halo sperm G2 kit which used for evaluation of DNA fragmentation based on the Sperm Chromatin Dispersion (SCD) Test.

ROS study: The sample in 2nd tube will be examined by oxi-sperm kit which used for determination of Reactive Oxygen Species (ROS) level in the sperm sample.

Electron microscopic study: The sample in 3rd tube will be examined by electron microscope after preparation to evaluate the sperm morphology. A fresh semen sample was processed in each of the patients for Electron microscopy (EM) within one hour of collection. At least 5 longitudinal and 5 cross-sections per sample were photographed.

STATISTICAL ANALYSIS:

The data were analyzed using statistical analysis software package **(SAS, 2002).** Chi-square test (x^2) was used as appropriate test for the studied variables treated in categories and *t* test for the studied continuous variables. The P values less than 0.05 were considered significant in all statistical analyses.

RESULTS:

Table (1): Clinical data among studied group:

Patient group	No. of patients	Age of husband	Age of wife	No. of years married
Control	50	35.5±5.42	31±5.5	6.5±4.17
1ry infertility	50	33.5±4.5	29.05±4.21	5.66±3.99
2ry infertility	50	37.62±8.14	30.5±3.5	5.09±3
Repeated ICSI failure	50	37.5±4.5	32.6±5.5	8.9±5.6

Table (2): Characteristics of semen specimens among studied group:

Patient group	Volume (mL)	Count ($x10^6$)	Motility (%)	Abnormal morphology (%)
Control	2.13±0.33	46.5±20.15	56.7±15	87.7±8.8
1ry infertility	1.9±0.67	25.3±25.88	23.9±21.8	96.3±8.8
2ry infertility	2.02±0.41	44.5±22.4	46.6±17.3	92.04±5.08
Repeated ICSI failure	1.9±0.6	31.24±25.3	25.12±18.9	94.6±6

Table (3): Relation between motility of spermatozoa in the studied group:

Groups	Motility	Significance		
		1ry infertility & Control	2ry infertility & Control	Repeated ICSI failure & Control
Control	56.7±15	P < 0.05*	P < 0.05*	P < 0.05*
1ry infertility	23.9±21.8			
2ry infertility	46.6±17.3			
Repeated ICSI failure	25.12±18.9			

The motility of spermatozoa was (56.7± 15%) in control group that was significantly higher (P < 0.05) compared to motility of spermatozoa in different groups as follow (23.9± 21.8%) in 1ry infertility group, (46.6± 17.3) in 2ry infertility group and (25.12± 18.9) in repeated ICSI failure group.

Table (4): abnormal morphology of spermatozoa among studied group:

Groups	Abnormal morphology %	Significance		
		1ry infertility & Control	2ry infertility & Control	Repeated ICSI failure &Control
Control	87.7±8.8	P < 0.05*	P < 0.05*	P < 0.05*
1ry infertility	96.3±8.8			
2ry infertility	92.04±5.08			
Repeated ICSI failure	94.6±6			

The abnormal morphology of spermatozoa was (96.3±8.8%) in 1ry infertility group, (92.04±5.08%) in 2ry infertility group and (94.6±6%) in repeated ICSI failure group which was significantly higher (P < 0.05) compared to abnormal morphology of spermatozoa (87.7±8.8%) in control group.

Table (5): Head abnormality of spermatozoa among studied group:

Groups	Head abnormality %	Significance		
		1ry infertility & Control	2ry infertility & Control	Repeated ICSI failure &Control
Control	34.34±14.1	P < 0.05*	P < 0.05*	P < 0.05*
1ry infertility	62.5±21.5			
2ry infertility	51.9±23.66			
Repeated ICSI failure	52.5±24.5			

The head abnormality of spermatozoa was (62.5±21.5%) in 1ry infertility group, (51.9±23.66%) in 2ry infertility group and (52.5±24.5%) in repeated ICSI failure group which was significantly higher (P < 0.05) compared to head abnormal morphology of spermatozoa (34.34±14.1%) in control group.

Table (6): middle piece abnormalities of spermatozoa among studied group:

Groups	Middle piece abnormality %	Significance		
		1ry infertility & Control	2ry infertility & Control	Repeated ICSI failure & Control
Control	25.8±7.4	P < 0.05*	P < 0.05*	P < 0.05*
1ry infertility	17.12±10.5			
2ry infertility	22.8±13.04			
Repeated ICSI failure	19.7±10.40			

The middle piece abnormality of spermatozoa was (17.12±10.5%) in 1ry infertility group, (22.8±13.04%) in 2ry infertility group and (19.7±10.40%) in repeated ICSI failure group which was significantly higher (P < 0.05) compared to middle piece abnormal morphology of spermatozoa (25.8±7.4%) in control group.

Table (7): Relation between tail abnormalities of spermatozoa among studied group:

Groups	Tail abnormality %	Significance		
		1ry infertility & Control	2ry infertility & Control	Repeated ICSI failure &Control
Control	27.8±8.2	P < 0.05*	P < 0.05*	P < 0.05*
1ry infertility	16.02±9.7			
2ry infertility	17.2±8.8			
Repeated ICSI failure	22.4±11.6			

The tail abnormality of spermatozoa was (16.02±9.7%) in 1ry infertility group, (17.2±8.8%) in 2ry infertility group and (22.4±11.6%) in repeated ICSI failure group which was significantly higher (P < 0.05) compared to tail abnormal morphology of spermatozoa (27.8±8.2%) in control group.

Result of human spermatozoa stained with halosperm G2 kit:

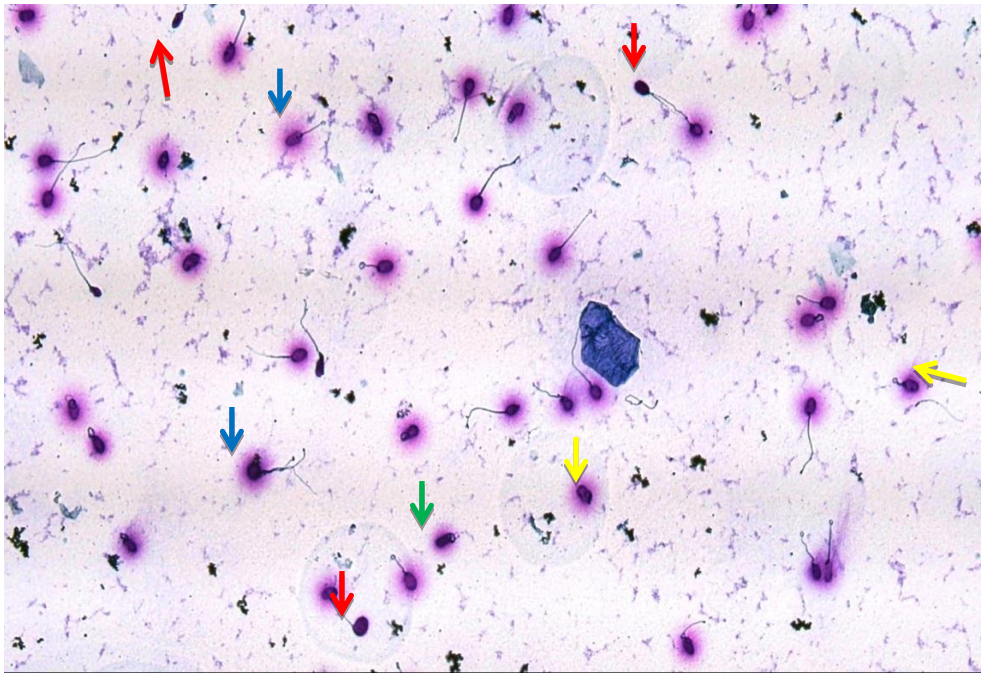

Fig. (1): The light micrograph shows sperms with fragmented DNA: Sperm with small halo (green arrow); Sperm without halo (red arrow) and sperms without fragmented DNA: Sperm with big halo (blue arrow); Sperm with medium-sized halo (yellow arrow). (Magnification x 50) stained with halosperm G2 kit (Halotech DNA).

Result of human spermatozoa stained with oxisperm kit:

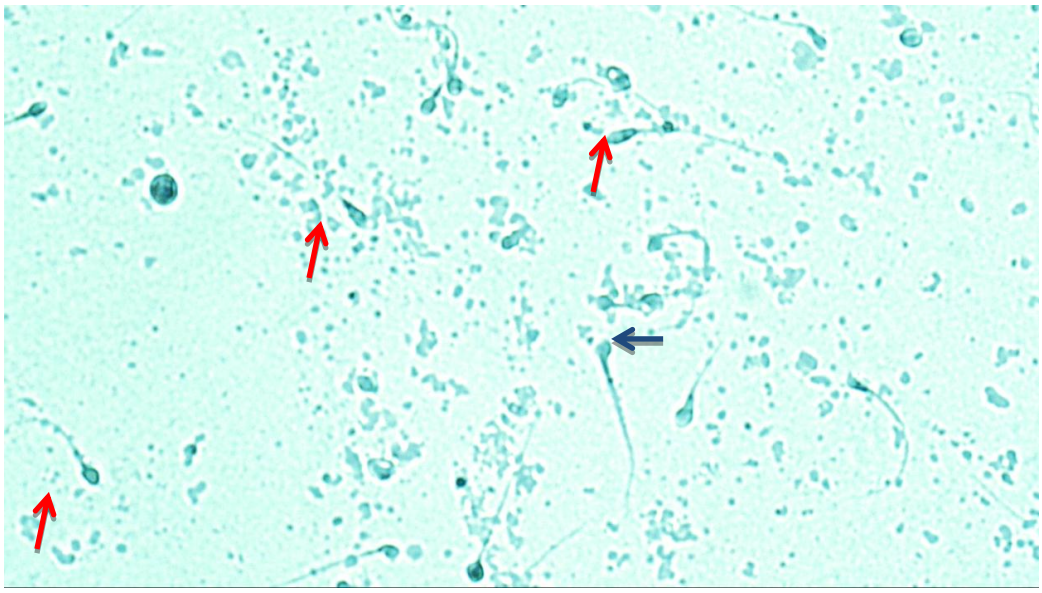

Fig. (2) : The light micrograph shows spermatozoa that were affected by oxidative reaction (red arrow); and other where the effect is null (blue arrow). As the number of molecules deposited on the sperm surface increases the color intensity of the reaction increases. (Magnification x 50) stained with oxisperm kit (Halotech DNA).

Result of human spermatozoa by transmission electron microscope (TEM):

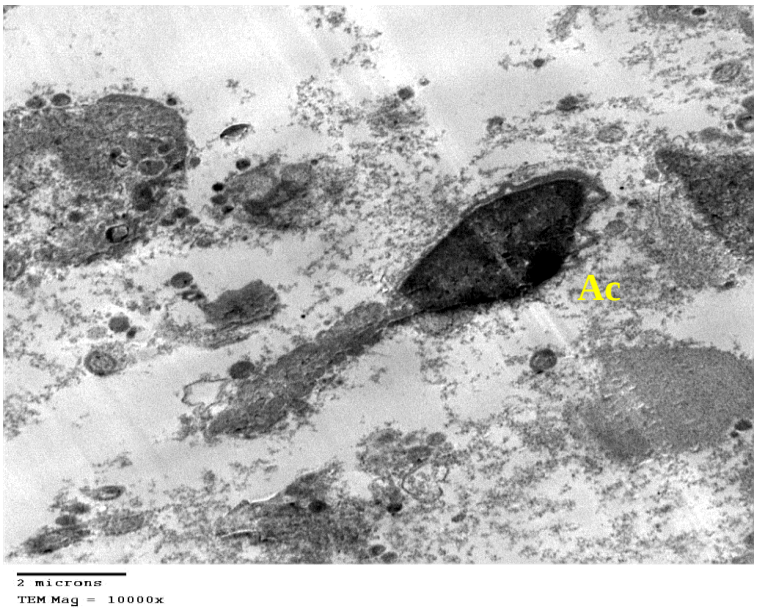

Fig. (3): Electron micrograph of sperm head showing; distorted shape of the acrosome, granular chromatin; Ac: acrosome; (TEM = 10000x).

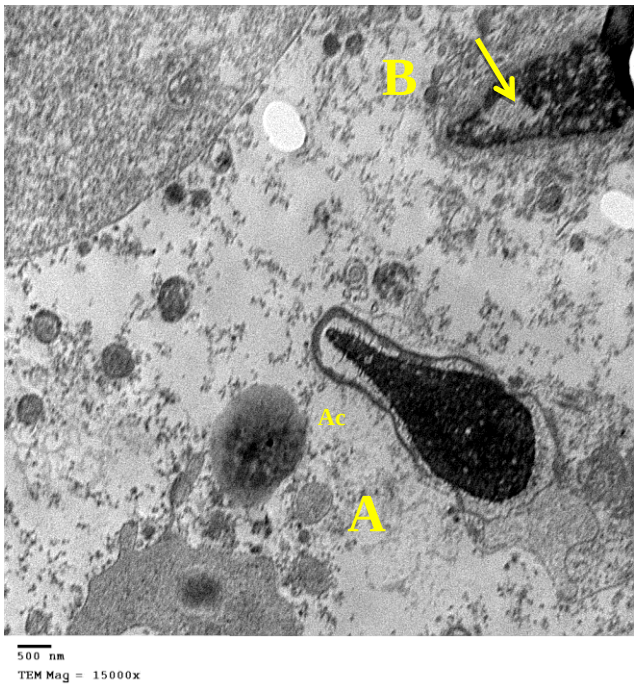

Fig. (4): Electron micrograph of sperm head showing: **[A]** Altered shape of the acrosome, abnormal taper head, granular chromatin and swollen middle piece. [**B]** Vacuolar defect of the chromatin, granular chromatin. Ac: acrosome; scale bars = 500 nm. (TEM Mag = 15000x).

DISCUSSION:

Reactive oxygen species (ROS) is a collective term used for oxygen derived free radicals (superoxide, hydroxyl radical, nitric oxide) and non-radical oxygen derivatives of high reactivity (singlet oxygen, hydrogen peroxide, peroxynitrite, hypochlorite). ROS can be either harmful or beneficial to the body. An imbalance between formation and removal of free radicals can lead to a pathological condition called as oxidative stress **(Saalu et al., 2008)**. Oxidative stress is a state in which an oxidant-generating system overcomes an antioxidant defense system, a process that is involved in many diseases including male factor infertility and/or subfertility. ROS are products of normal cellular metabolism and are formed during the normal enzymatic reactions of intercellular and intracellular signaling **(Agarwal et al., 2006).** So this study was designed to demonstrate the effect of reactive oxygen species (ROS) on human spermatozoa morphology by electron microscope in infertile men as an attempt to establish ROS measurement as an important tool in the diagnosis of seminal OS in infertile Egyptian men.

Our findings in 50 control subjects and 150 subjects with male factor infertility (1ry infertility, 2ry infertility and repeated ICSI failure) were monitored. In our study, human spermatozoa stained with halo sperm G2 kit for evaluation of DNA fragmentation showed that sperm DNA fragmentation exists in abundance in subjects with male factor infertility.

The oxidative stress is correlated with DNA fragmentation has been demonstrated in many studies. Firstly, the DNA in the ejaculates of infertile men is commonly associated with oxidative damage as reflected by measurements of 8-hydroxydeoxyguanosine (8-OHdG) **(Shen and Ong, 2000).** Secondly, correlations have been observed between oxygen radical generation and DNA damage in

ejaculated spermatozoa **(Barroso et al., 2000).**Also, this finding was in accordance with **Makker et al., 2009** who said that the plasma membrane contains high levels of lipids in the form of poly-unsaturated fatty acid (PUFAs). These lipids contain unconjugated double bonds separated by methylene groups. **sikka, (2001)** reported that many pathological effects of Lipid Peroxidation (LPO) on sperm function. Overall, LPO damages DNA and proteins through oxidation from lipid peroxyl or alkoxyl radicals. DNA damage by LPO can occur via base modifications, strand breaks or cross-linking.

Our results suggest that ROS level is conversely proportional to the sperm motility grade. Our result agree with **Mohammad et al., (2007)** who said that a significant negative correlation between sperm morphology and ROS production (p=0.05).This finding is in accordance with **Aziz et al., (2004)** study, in which a significant negative correlation was observed between sperm ROS production and the proportion of sperm with normal morphology.

Our findings moreover support the results of **Khorrowbeygi & Zarghami, (2007)** that levels of Total Antioxidant Capacity (TAC) have a positive correlation with sperm motility and morphology. Also, the same results have been reported by **Anju et al., (2013).** Also, **Singh et al., (2015)** concluded that high level of ROS may be responsible for the morphological deformities of the sperm cells. In our study we looked for reasons that could lead to ROS and we found that the most important of these reasons are first, smoking second, varicocele third, infection and finally drugs (tramadol and hashish) in Egyptian infertile men. In turn, activated leukocytes can generate high levels of ROS in semen, which may overwhelm the antioxidant strategies, resulting in OS. This finding explains our result. The link between cigarette smoking and increased levels of seminal ROS may be, at least in part, related to the significant increase in leukocyte concentrations in the semen of infertile smokers. An earlier study also reported an association between cigarette smoking in infertile men and increased leukocyte infiltration into semen **(Ramadan et al., 2002).**

The finding of increased levels of seminal OS in association with cigarette smoking is of significance and may have important implications in the fertilizing potential of infertile men. Spermatozoa are particularly susceptible to damage induced by excessive ROS because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes **(Sikka, 2001).**

In addition to the above explanations tobacco smoking is also an abundant source of nitric oxide and other reactive nitrogen species (RNS) which contribute to oxidation reactions and modify macromolecules. Nitrosative/ oxidative insult to proteins results in protein nitrosylation, moreover, it was found that RNS are capable of reducing endogenous antioxidants **(Van der et al., 1994).**We found that the result we have reached that smoking is one of the causes of the increase in reactive oxygen species is consistent with the findings of its **Ewa Ignatowicz et al., (2013)**. **Palanisamy Pasupathi et al., (2009)** reported that cigarette smoking is one of the most important exogenous factors, which cause 3- fold higher incidence of oxidative stress in smokers. A number of papers related to male smoking have suggested that severe DNA damage could be a cause of infertility. But we observe in our study that the varicocele is an important factor for the occurrence of ROS. This outcome confirmed by study carried out by **G.G. Akunna et al., (2013)** who reported that varicocele caused a significant ($P<0.005$) decrease in both testicular weights and testicular volumes in the animal models that were used.

The last cause in our study for generation of ROS is drugs (tramadol and hashish) and we can explain the mechanism by which drugs produce ROS is their oxidative metabolism, which generates ROS and reactive metabolites. This metabolism occurs mainly in the liver, but the metabolites may reach other tissues through the circulatory system. This interpretation is consistent with the (**Cunha-Oliveira et al., 2013).** Another explanation by (**Cunha-Oliveira et al., 2008)** mitochondrial dysfunction may be another source of oxidative stress and many drugs of abuse have been shown to affect mitochondrial functions. Most of the related reports and researches showed that a decrease in male fertility and increase in

abnormal semen parameters has occurred over recent years. One of the main reasons for the increase in impaired semen parameters is believed to be increasing exposure to toxicants in the environment. The causative agents may be chemical materials, stress, ionizing radiation, as well as substance abuse **(Petrelli & Mantovani, 2002; Claman, 2004).** Also, the same result observed in other studies which was found that morphine increased the lipid peroxidation in tissues whereas heroin led to oxidative DNA damage, protein oxidation and lipid peroxidation in the brain of mice **(Ozmen et al., 2007; Qiusheng et al., 2005).** Opioid drugs also exerts effect on the activity of antioxidant systems, as observed by decrease in the total antioxidant capacity in the blood of human heroin addict when compared to the control groups **(Pereska et al., 2007).** In other studies, **Pan et al., (2005)** noted a reduction in total antioxidant capacity in the serum and in the antioxidant enzymes, such as SOD, catalase, Gx and elevated markers of oxidative damage of DNA, proteins and lipids in heroin induced mice **(Xu et al., 2006).**

CONCLUSION:

We conclude that most of the infertility group's patients in our study had poor semen characteristics as regard (motility and morphology) compared to semen characteristics of control group and this is due to increase of ROS. So ROS measurement should be established as an important tool in the diagnosis of seminal OS in infertile Egyptian men. During our study, we found that the most important reasons that lead to an increase generation of ROS or decrease Total Antioxidant Capacity (TAC) are smoking, varicocele, male genital tract infection and drugs. We believe that the diagnosis of reactive oxygen species can explain and resolve most of the problems of 1ry infertility, 2ry infertility and repeated ICSI failure.

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دراسة تأثير أنواع الأكسجين التفاعلية على شكل الحيوانات المنوية البشرية

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** وحدة الإخصاب الطبى المساعد - المركز الدولى الإسلامى للدراسات والبحوث السكانية – جامعة الأزهر - القاهرة

مقدمة البحث:

العقم مشكلة طبية كبيرة، تؤثر على المجتمع طبيا واجتماعيا ونفسيا.

تشير البيانات المتراكمة على مدى العقود القليلة الماضية إلى أن العقم يتراوح من ١٠- ١٥% بين المتزوجين على مستوى العالم ، ويعزى السبب في العقم إلى الذكور فيما يقرب من ٥٠٪ من الأزواج الذين يعانون من العقم.

في بعض الحالات تشير الفحوصات القياسية لزوجين عقيمين إلى أنه لايوجد سبب طبي واضح للعقم، وبالتالي يتم تشخيص الزوجين بالعقم الأولي غير المفسر أو مجهول السبب كما أن فحص السائل المنوي القياسي ليس كاف للحكم على سلامة الحيوانات المنوية.

الهدف من الدراسة :

تم تصميم هذه الدراسة لبيان تأثير أنواع الأكسجين التفاعلية (ROS) على شكل الحيوانات المنوية البشرية عن طريق المجهر الإلكتروني في الرجال الذين يعانون من العقم كمحاولة لتوطيد قياس أنواع الأكسجين التفاعلية باعتباره أداة هامة في تشخيص أكسدة السائل المنوي لدى الرجال المصريين الذين يعانون من العقم .

طريقة البحث:

أجريت هذه الدراسة في وحدة الإخصاب الطبي المساعد في المركز الدولي الإسلامي للدراسات والبحوث السكانية، بجامعة الأزهر في الفترة من يوليو ٢٠١٥م إلى يوليو ٢٠١٦م.

توافق ٢٠٠ زوج مع خواص الدراسة، وطبقا للتاريخ المرضي وفحص المرضى تم تقسيم المرضى إلى ٤ مجموعات : المجموعة الضابطة ، مجموعة العقم الأولي ، مجموعة العقم الثانوي ، ومجموعة فشل الحقن المجهري المتكرر.

تم جمع عينات السائل المنوي عن طريق الاستمناء بعد فترة من الامتناع عن ممارسة الجنس من ٢- ٥ أيام ثم تم عمل تحليل كامل للسائل المنوي.

تم معالجة عينات السائل المنوي بعد الفحص المجهري وتم تقسيم عينة السائل المنوي في ٣ أنابيب.العينة التي في الأنبوبة (١) تم اختبارها بالهالوسبيرم جي ٢ (Halosperm G2 kit) لتقييم تجزئة الحمض النـــــووي، بينما العينة في الأنبوبة (٢) تم اختبارها بالأكسي سبيـــرم (oxisperm kit) لتقييم مستوى أنواع الأكسجينات التفاعلية (ROS) والعينة في الأنبوبة (٣) تم فحصها بالمجهر الإلكتروني بعد إعدادها لتقييم شكل وتركيب الحيوانات المنوية.

أهم النتائج:

لاحظنا أن نتائج معظم العينات التي تم أخذها من مرضى العقم في دراستنا كان لديهم خصائص السائل المنوي الفقيرة بخصوص (الحركة والشكل) بالمقارنة مع خصائص السائل المنوي في المجموعة الضابطة وهذا يرجع إلى زيادة أنواع الأكسجين التفاعلية ROS.

وخلال دراستنا، وجدنا أن أهم الأسباب التي تؤدي إلى زيادة توليد أنواع الأكسجين التفاعلية ROS أو نقصان إجمالي القدرة المضادة للأكسدة TAC هي التدخين، دوالي الخصية، عدوى الجهاز التناسلي للذكور و المخدرات.

ولسنا بحاجة لقول أن العديد من التغيرات قد تحدث في شكل الحيوانات المنوية حيث أن رأس الحيوانات المنوية تأثرت في جميع المجموعات الخاضعة للبحث (ويعزى ذلك إلى التأثير الضار لأنواع الأكسجين التفاعلية على شكل رأس الحيوانات المنوية وكذلك محتوى الحمض النووي بها). بينما حصل تشوه في منطقة الوسط للحيوانات المنوية كتغير إضافي في مجموعة العقم الثانوية، بينما كانت التغيرات الشكلية أسوأ في مجموعة فشل الحقن المجهري المتكرر لأنها لم تؤثر فقط على رأس الحيوان المنوي بل أثرت أيضا على ذيله.

الخلاصة والتوصيات:

ينبغي وضع قياس أنواع الأكسجين التفاعلية ROS بوصفها أداة هامة في تشخيص أكسدة السائل المنوي لدى الرجال المصريين الذين يعانون من العقم .

ويتضح من خلال دراستنا أن تشخيص أنواع الاكسجين التفاعلية يمكن أن يساعد في تفسير وحل معظم مشاكل العقم الأولي والعقم الثانوي وفشل الحقن المجهري المتكرر.