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RELATIONSHIP BETWEEN REACTIVE OXYGEN SPECIES, VITAMIN E, CATALASE AND *CHLAMYDIA TRACHOMATIS* IN SERUM OF INFERTILE FEMALE

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ABSTRACT

Background: The failure in female reproductive system could be cause infertility according to different factors such as sperm isoantobody, zona pellucid antibody, ovarian antibody, *Chlamydia trachomatis* infection, hormonal imbalance and even between oxygen radical and antioxygen radical. These factors might deteriorate female infertility by different mechanisms. **Material and Methods:** retrospective case-control study was performed on 105 infertile Jordanian female including 75(71.4%) with primary infertility, and 30(28.6%) with secondary infertility. Thirty healthy women were used as a control. Enzyme labeled and Biotin labeled antibody kits were used in the serum and cervical secretion for determination. Polymerase Chain Reaction (PCR) assay was used to assess the *Chlamydia trachomatis* in the cervical secretion. Finally, the mean level of Reactive Oxygen Species (ROS) and anti ROS including Vitamin E, and Catalase (CAT) enzyme were estimated in the cervical secretion **Results:** Serum samples obtained from fertile and infertile females were analyzed for the presence of *C. trachomatis* specific IgG. 5 (4.76%) of tubal damage infertile females showed the presence of Chlamydial antibody with no detectable level in the serum of fertile females. To confirm the presence of *C. trachomatis*, PCR was used to identify Chlamydia DNA in the cervical secretion. Only positive serum samples revealed clear, sharp bands of amplified Chlamydial DNA with 241bp. The presence of reactive oxygen species (ROS) within the cervical secretion cancontribute to female infertility if dose not controlled by anti-oxidants. The results revealed no statistical difference in the mean level of ROS, and anti ROS among infertility cases and control. However, the mean antioxidant capacity (vitamin E and catalase) among infertile females with combine infertility problems. **Conclusion:** We concluded that isoimmunity to sperm and autoimmunity to ova antigens accompanied by elevation in the reactive oxygen species play an ambiguous role in fertility impairment.

KEYWORDS: *Chlamydia trachomatis*, ROS, Catalase, Vitamin E.

INTRODUCTION

Infertility is an inability to obtain a clinical pregnancy after one year or more of regular unprotected coition (Hochschild *et al*., 2009). On other words, infertility is the failure of a sexually active, non-contraception couple to achieve pregnancy in one year. Infertility can be due to the woman or the man, or both. The male or female partner could be estimated fertility or subfertility by using sets of clinical interferences, and also from laboratory chemical tests of semen (Menkveld, 2010). Demographers usually use a longer period of five to seven years in their definition of infertility. Normally, a distinction is made between primary infertility, when a woman has never conceived or given birth to a live child, and secondary infertility, when a woman has given birth at least once, and subsequently becomes infertile (WHO, 1975).

An increasing number of studies reported that, for several reasons, infertility is a serious problem in the developing world. First, it has been found that infertility has many serious psychological and especially social

consequences for people in developing countries. It appears that men, and especially women, with fertility problems are often reject and denounce in their culture, and pressurized by their family to solve their fertility problems (Gerrits *et al*., 1997; Inhorn, 1994).

In general infertility in the medical literature identifies various reasons for male infertility such as problems of the sperm (e.g. not enough sperms, low sperm motility), problems on the seminal fluid (e.g. containing pus) or blocked ejaculatory seminal duct (Wood, 1994; McConell, 1993). These problems can be either congenital or occurs by environmental factors, like infections. Points out those psychosexual dysfunctions, like premature ejaculation or impotence can also contribute to fertility problems (Barden-O'Fallon, 2005).

Also oxidative stress has been shown to be a major player in many gynecological diseases, such as fibroids, endometriosis, and postoperative adhesions. Han *et al*. (2009) found that bacterial viginitis was able to stimulate the production of proinflammatory cytokine and nitric oxide (NO) and the expression of NOS (Han *et al*., 2009). Ray and Chakrabarti (1999) found that the lipid peroxidation and antioxidant potential was altered in human uterine tumors (Ray and Chakrabarti, 1999). However, the role of antioxidants in the vaginal discharge on vaginitis development has not been reported.

The non-hormonal medical treatment has a relevant role in female and male infertility since it may solve the cause of infertility. The scientific evidence of the effectiveness of such a treatment has been scanty. Anti-inflammatory, fibrinolytic, and antioxidant compounds and vitamin supplementation are used. Antioxidant treatment for subfertile couples has been also prescribed to the female and male partner of couples undergoing assisted reproduction techniques (ART). Further studies are required to clarify the role of these drugs (Showell et.al.2014).

As well as under physiological conditions, catalase plays a role in the nitric oxide-induced sperm capacitation (Walczak-Jedrzejowska et. al. 2013). Its activity has been associated with low sperm quality. In fact, H_2O_2, which increases in case of catalase deficiency or reduced activity, plays a substantial role on sperm motility due to the decrease in membrane fluidity that is important for sperm-oocyte fusion (Makker et.al. 2013).

MATERIALS AND METHODS

1. Subjects

A total of 135 women (105 infertile and 30 fertile) with ages ranged between 21-44 years old which were investigated. Infertile cases were divided into four age groups. The blood samples were collected from Jordan University Hospital, Department of in vitro fertilization (IVF).

2. Sample collection

Serum (5ml) and Cervical mucus samples (0.5ml) were collected from 105 infertile females who visiting the Specializing Medical Center in Amman with at least (2 or 3) years duration of infertility. The control group was consisted of thirty apparently healthy fertile women.

3. Cervical secretion collection

The samples were collected and transported directly to the laboratory. Samples of cervical secretions were collected from spontaneously ovulating infertile and fertile women. A sterile speculum will inserted into the vagina and a 5–10 mL syringe used to collect cervical secretions from the endocervical canal. About 0.5 ml of cervical secretion was taken by syringe from high cervix using Cusco speculum, placed in sterile tubes and stored at –21 ºC until analyzed.

The mucus must be liquefied by mucolytic agent of N-acetyl L cysteine at concentration 0.2 mg/ml which prepared by weight 0.2 mg of N-acetyl L- cysteine and complete to one milliliter with distilled water (White in 2007).

4. Diagnosis

At the detection of Chlamydia Chlamydia trachomatis of IgG was measured by ELISA from NovaTec Immundiagnostica GmbH Technologie & Waldpark (Germany).

Human Reactive Oxygen Species (ROS) were quantified using Mybiosource ELISA kits (Mybiosource, USA). Plate was precoated with human ROS monoantibodies and the detecting antibodies were Biotin labeled polyclonal ROS. Human Catalase (HCAT) and Vitamin E was measured in diluted samples using ELISA kit (Sinnow Medical, China) coated with anti-CAT antibody. Aliquot of 50µl of each sample and standard (0, 5, 10, 20, 40, 80U/ml) were dispensed into each well in duplicate along with 100µl of enzyme HRP-conjugate and then incubated for 60 minutes at 37 °C. **Statistical analysis:** All ELISA assay results were analyzed using (SPSS, V20). Results were presented as the mean ± SD (standard deviation of the mean) by using ANOVA multiple range tests.

RESULTS

A total of one hundred and thirty five Jordanian females were enrolled in this study, including 105 (77.8%) infertile females and 30 (22.2%) fertile control. All the females were attended the Infertility Department at Jordan University Hospital during the period Feb -Sept 2018. The mean age of infertile females was 31.27± 0.558 years old, and the mean duration of infertility was (4.53±2.875). According to the age, infertile females were divided into four groups including; group I (20-25 years), group II (26-30 years), group III (31-35 years), group IV (36-42 years). No statistically significant differences in the mean age of the infertile and fertile females used as a control (25.1±5.90) was reported ($p >$

0.05).

According to infertility, cases were divided into primary infertility which include 75 (71.4%) of the cases and secondary which include 30 (28.6%) of the infertility cases. Furthermore, females were grouped with respect to the causes of infertility to nine groups (Hamad et al., 2018).

Chlamydial DNA with 241bp (Figure 1) was analyzed by PCR. The prevalence of *C. trachomatis* among infertile females was significantly correlated to the presence of ASA, and AZP antibodies in the serum and cervical secretion ($p < 0.05$).

Table 1: Distribution of females with primary and secondary infertility, according to the causes of infertility (Cited by Hamad et.al. 2018).

Causes of Infertili			Infertility		
			Primary infertility	Secondary infertility	Total
I	Unexplained	Count	23	6	29
		% within infertility group	79.3%	20.7%	100.0%
		% within status	30.7%	20.0%	21.5%
II	Polycystic ovary	Count	20	12	32
		% within infertility group	62.5%	37.5%	100.0%
		% within status	26.7%	40.0%	23.7%
III	Endometriosis	Count	6	0	6
		% within infertility group	100.0%	0.0%	100.0%
		% within status	8.0%	0.0%	4.4%
IV	Tubal damage	Count	15	7	22
		% within infertility group	68.2%	31.8%	100.0%
		% within status	20.0%	23.3%	16.3%
V	Hormonal imbalance	Count	5	4	9
		% within infertility group	55.6%	44.4%	100.0%
		% within status	6.7%	13.3%	6.7%
VI	Polycystic ovary + Endometriosis	Count	1	0	1
		% within infertility group	100.0%	0.0%	100.0%
		% within status	1.3%	0.0%	0.7%
VII	Polycystic ovary + Tubal damage	Count	2	0	2
		% within infertility group	100.0%	0.0%	100.0%
		% within status	2.7%	0.0%	1.5%
VIII	Polycystic + Hormonal tubal	Count	2	1	3
		% within infertility group	66.7%	33.3%	100.0%
		% within status	2.7%	3.3%	2.2%
IX	Multiple causes	Count	1	0	1
		% within infertility group	100.0%	0.0%	100.0%
		% within status	1.3%	0.0%	0.7%
Total		Count	75	30	105
		% within infertility group	55.6%	22.2%	100.0%
		% within status	100.0%	100.0%	100.0%

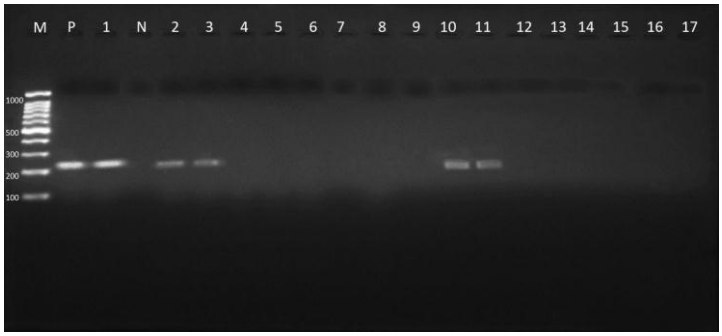

Figure 1: Detection of *C. trachomatis* in the serum of infertile females by PCR. Lane M; DNA markers; Lane 1 to 17 cervical specimens; Lane 1, 2, 3, 10, and 11 are positive for *C. trachomatis*; N is a negative control; P is a positive control. (Cited by Hamad et.al. 2018).

Table 2: Incidence of *C. trachomatis* in the primary and secondary infertility cases as detected by ELISA and PCR. (Cited by Hamad et.al. 2018).

			C. trachomatis		Total
			Negative	Positive	
Status	Primary infertility	Count	70	5	75
		% within status	93.3%	6.7%	100.0%
		% of Total	66.7%	4.8%	71.4%
	Secondary infertility	Count	30	0	30
		% within status	100.0%	0.0%	100.0%
		% of Total	28.6%	0.0%	28.6%
Total		Count	100	5	105
		% within status	95.2%	4.8%	100.0%
		% of Total	95.2%	4.8%	100.0%

Detection of ROS, Vitamin E and Catalase enzyme in the cervical secretion

The concentration of oxygen radical (ROS), and antioxygen radical (vitamin E and Catalase enzyme) was determined in the cervical secretion of infertile and fertile females and analyzed using ELISA. The highest level of ROS was detected in infertile females with multiple causes of infertility (63.10U/ml) followed by (44.31U/ml) in females with unexplained infertility, and (43.70U/ml) in female with polycystic overy and tubal damage (Table 3). No statistically significant relationship was found between ROS and infertility groups (p= 0.733)

Table 3: The mean concentration of ROS, vitamin E and Catalase enzyme in the cervical secretion of infertile and fertile females.

Infertility groups	ROS(U/ml)		Vitamin E (Umo1/L)		Catalase (U/ml)	
	Mean± SD	Range	Mean±SD	Range	Mean ± SD	Range
Group I	44.31±26.47	5.5 – 87.3	55.73±23.98	8.7 -255.5	147.9±145.45	50.5 – 708.6
Group II	38.18±27.44	5.6 – 90.3	59.09±58.95	8.8 – 300.0	130.7±80.24	47.60-1006.7
Group III	34.32±29.62	4.70 -66.10	142.8±86.54	44.50 – 123.9	34.32±29.62	51.4 – 277.1
Group IV	37.78±25.47	6.0 -78.5	52.56±25.87	8.70 – 96.80	320.1±25.47	50.5 – 1281.0
Group V	34.38±33.23	5.60 – 85.40	49.55±17.67	9.4- 188.4	66.54±15.54	51.4 – 100.0
Group VI	22.80±0.00	22.8 -22.8	10.30±0.00	22.8 -22.8	63.80±0.00	22.8 -22.8
Group VII	43.70±16.66	10.7- 76.7	9.35±0.63	8.9- 9.80	64.30±14.14	54.30-74.3
Group VIII	11.36±2.64	8.50- 13.70	45.63±1.79	44.5-47.70	116.8±108.3	53.3- 241.9
Group IX	63.10±0.00	63.10-63.10	60.0±0.00	60.0-60.0	50.50±0.00	50.0-50.0
Fertile females	26.79±24.0	0.60-91.80	35.51±15.8	0.6-342.6	383.6±174.8	119.0-677.10
P- value	NS		NS		NS	

Detection of *C.trachomatis* by Immunological and Molecular methods

Serum samples obtained from fertile and infertile females were analyzed for the presence of *C. trachomatis* specific IgG using ELISA method. Five Out of 105 serum samples obtained from infertile females screened, 5 (4.76%) showed the presence of anti *C. trachomatis* IgG antibodies. No detectable level of IgG specific for *C. trachomatis* was recorded in the serum samples obtained from fertile females.

Females with anti-Chlamydia antibodies showed no apparent causes for infertility, thus belong to unexplained infertility groups (Group I). In order to reconfirm the presence of *C. trachomatis* among the infertile females, polymerase chain reaction was used to identify Chlamydia DNA bands in the serum of female with infertility. Positive serum samples for Chlamydial specific antibodies detected by ELISA revealed clear, sharp bands of amplified Chlamydial DNA with 241bp (Figure 1) analyzed by PCR. The prevalence of *C. trachomatis* among infertile females was significantly correlated to the presence of ASA, and AZP antibodies in the serum and cervical secretion (p< 0.05).

Table 4: Incidence of *C. trachomatis* in the primary and secondary infertility cases as detected by ELISA and PCR.

			C. trachomatis		Total
			Negative	Positive	
Status	Primary infertility	Count	70	5	75
		% within status	93.3%	6.7%	100.0%
		% of Total	66.7%	4.8%	71.4%
	Secondary infertility	Count	30	0	30
		% within status	100.0%	0.0%	100.0%
		% of Total	28.6%	0.0%	28.6%
Total		Count	100	5	105
		% within status	95.2%	4.8%	100.0%
		% of Total	95.2%	4.8%	100.0%

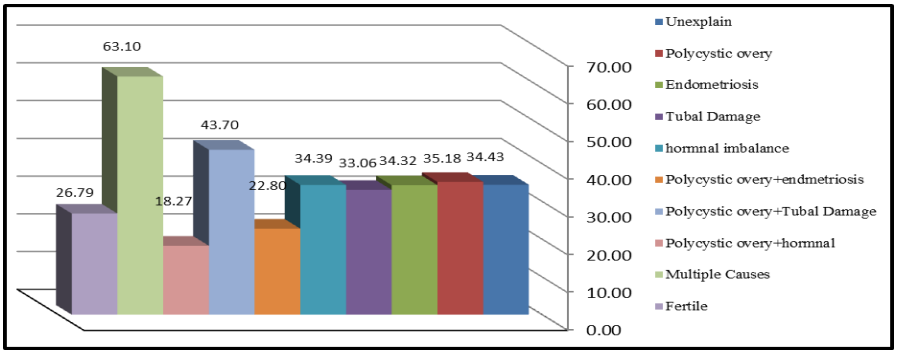

Figure 2: Variation in ROS levels (U/ml) in the cervical secretion of fertile and infertile groups.

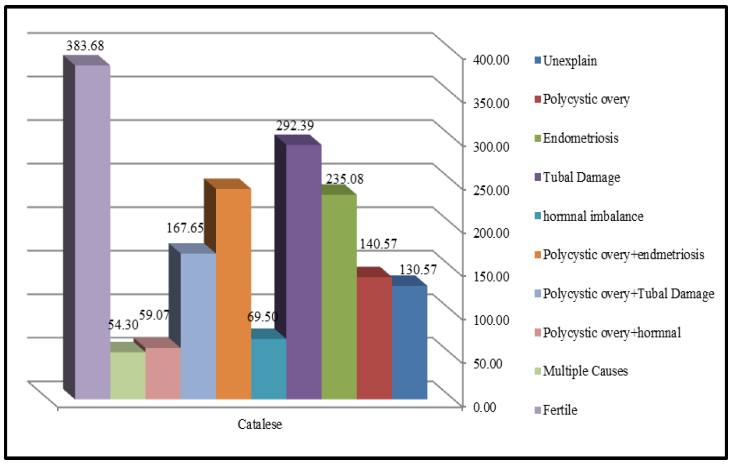

Figure 3: Variation in catalase levels (U/ml) in the cervical secretion of fertile and infertile groups.

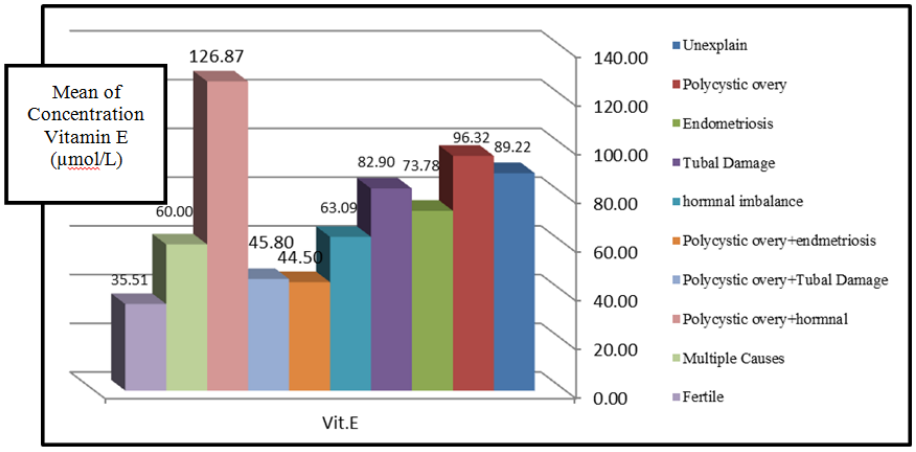

Figure 4: Variation in vitamin E levels (µmol/L) in the cervical secretion of fertile and infertile groups.

Additionally, the mean antioxidant capacity (vitamin E and catalase) was detected in the cervical secretion and the results revealed that infertile females with endometriosis showed the highest mean level of vitamin E group III (142.8Umol/L), while infertile females with combine infertility problems (polycystic ovary and tubal damage) showed the lowest mean level group VII (9.35 Umol/L). In turn, the means catalase concentration also varies in infertile females with the highest mean level in tubal damage related infertility group IV (320.1 U/ml). In all cases, the variation in ROS, Vitamin E, and catalase dose doesn't reach a statistical significant value (Fig. 3 and 4).

Endometriosis shows the highest value in variation of ROS levels by 63.1 U/ml compared with polycystic ovary and hormonal changes has the least value of 18.27 U/ml (Fig. 2), polycystic ovary and tubal damage has in between value 43.7 U/ml, there is slightly differentiation among others**.** Fertile patients have huge variation in catalase level by 383.68 U/ml, there is fluctuations between groups in the catalase levels, the least value is about 54.3 U/ml in multiple causes group and the second high value after fertile group is in tubal damage 292.39 U/ml**.** (Table 5).

Table 5: The mean concentrations of ROS, vitamin E, and catalase enzyme in the cervical secretion of infertile and fertile females.

Infertility groups	ROS(U/ml)		Vitamin E (Umo1/L)		Catalase (U/ml)	
	Mean± SD	Range	Mean±SD	Range	Mean ± SD	Range
Group I	44.31±26.47	5.5 – 87.3	55.73±23.98	8.7 -255.5	147.9±145.45	50.5 – 708.6
Group II	38.18±27.44	5.6 – 90.3	59.09±58.95	8.8 – 300.0	130.7±80.24	47.60-1006.7
Group III	34.32±29.62	4.70 -66.10	142.8±86.54	44.50 – 123.9	34.32±29.62	51.4 – 277.1
Group IV	37.78±25.47	6.0 -78.5	52.56±25.87	8.70 – 96.80	320.1±25.47	50.5 – 1281.0
Group V	34.38±33.23	5.60 – 85.40	49.55±17.67	9.4- 188.4	66.54±15.54	51.4 – 100.0
Group VI	22.80±0.00	22.8 -22.8	10.30±0.00	22.8 -22.8	63.80±0.00	22.8 -22.8
Group VII	43.70±16.66	10.7- 76.7	9.35±0.63	8.9- 9.80	64.30±14.14	54.30-74.3
Group VIII	11.36±2.64	8.50- 13.70	45.63±1.79	44.5-47.70	116.8±108.3	53.3- 241.9
Group IX	63.10±0.00	63.10-63.10	60.0±0.00	60.0-60.0	50.50±0.00	50.0-50.0
Fertile females	26.79±24.0	0.60-91.80	35.51±15.8	0.6-342.6	383.6±174.8	119.0-677.10
P- value	NS		NS		NS	

DISCUSSION

Reproductive failure is a major medical issue adversely affecting human health in the 21st century. It is widely accepted that during the last two decades, that the average age of having children has increased and this is a key factor for infertility. Throughout the progress of the world, the age of delivery is increased, the reproductive capacity is decreased, the ovary becomes low active, the frequency of sexual intercourse is decreased and the possibility of chromosomal abnormalities and miscarriage is increased (Roupa *et al*., 2009).

Other female infertility causes such as endometriosis, unexplained infertility, and tubal damage are also associated with anti-ovarian autoimmunity. These finding was confirmed by others (Pires *et al*., 2007; Luborsky *et. al.,* 2002). It has been established that anti-ovarian antibodies (AOA) could be a contributing factor towards female infertility. Although a non-significant relation was appeared between AOA and other auto and isoantibodies, our findings suggest that AOA are useful marker for infertility investigation mainly cases with POF.

One of the most common sexually transmitted diseases is *Chlamydia trachomatis* infection that could impair fertility through different mechanisms (Siam and Hefzy, 2011). Chlamydia infection, often simply known as Chlamydia, is a common sexually transmitted disease associated with the bacterium *C. trachomatis*, which can damage a woman's reproductive tissues, thus, affecting fertility. The overall prevalence of IgG specific to Chlamydia in infertile women was 4.76% which seems to be lower than in other studies (Ahmadi *et al*., 2016). All cases positive to *Chlamydia trachomatis* IgG were associated with primary infertility. IgG antibodies in serum were the best predictor of a current or potential past Chlamydia infection. Host immune response plays important roles in the pathogenesis of long-term complications after Chlamydia infections (Bas *et al*., 2008; Bohring *et al.,* 2003). Positive serum samples for Chlamydia specific IgG antibodies revealed bands of amplified Chlamydial DNA by PCR.

The three major types of ROS are: superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\bullet}$). The hydroxyl ion can modify purines and pyrimidine's and cause strand breaks resulting in DNA damage (Mello *et al*., 1984). Vitamin E is a major chain breaking antioxidant in membranes, located mainly in the ovary especially in follicular fluid. Vitamin E directly neutralizes superoxide anion, hydrogen peroxide, and hydroxyl radical; so increase these types of free radical may lead to depletion of vitamin E. The concentration of oxygen radical (ROS), and antioxygen radical (vitamin E and Catalase enzyme) was determined in the cervical secretion of infertile and fertile females and analyzed using ELISA. The highest level of ROS was detected in infertile females with multiple causes of infertility

(63.10U/ml) followed by (44.31U/ml) in females with unexplained infertility, and (43.70U/ml) in female with polycystic ovary and tubal damage. Reactive oxygen species such as superoxide anion, hydrogen peroxide, and the hydroxyl radical are types of oxidants that can attack biomolecules such as lipids, proteins, and nucleic acids. Antioxidants, on the other hand, can help prevent that damage (Sikka, 2004). Antioxidants stabilize ROS by donating electrons to oxygen-based free radicals (Gupta *et al.*, 2006). In the follicular fluid of healthy patients, antioxidants protect oocytes from ROS damage. In endometriosis, ROS production increases due to stimulated peritoneal fluid mononuclear cells and macrophages (Zeller *et al.*, 1987).

In this study, the contribution of reproductive biomarkers of oxidative stress in female to the development of Chlamydia encourage infertility was examined in Chlamydia positive women (Nsonwu-Anyanwu et al. 2015). The Mechanisms of damage is related to DNA oxidation and reduced antioxidant capacity which might induce infertility related to tubal damage (Nsonwu-Anyanwu *et al*., 2015). The mean level of antioxidant also have been shown to be significantly related to iso antisperm antibodies among the infertile females in comparison to fertile one (Isong *et al.*,(2016). In the current study the results shows that no statistical significant difference in the mean level of ROS, and anti ROS among infertility cases and control. Nevertheless, a significant correlation was appeared between AZP- Ab, ASA, and ROS in the cervical secretion. The mean antioxidant capacity (vitamin E and catalase) among infertile females with combine infertility problems (polycystic ovary and tubal damage) showed the lowest level (9.35 Umol/L) in comparison to other infertility etiology. This finding matches with the results cited by others that showed a significant elevation in vitamin E levels in fertile women as compared to infertile (Mehendale, *et al*., 2009; Nedim Cicek et.al. 2012).

CONCLUSION

Reproductive failure is a major medical issue adversely affecting human health in the 21st century. Female fertility can be affected by infectious diseases, dysfunctions of reproductive tract, neuroendocrine system, increase ROS, and immune system. We concluded that isoimmunity to sperm and autoimmunity to ova antigens accompanied by elevation in the reactive oxygen species play an ambiguous role in fertility impairment.

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