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EFFECTS OF OXIDATIVE STRESS ON MALE REPRODUCTIVE TRACT OF MICE INFECTED WITH BRUCELLA MELITENSIS

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INTRODUCTION

Reproductive is important public health problem (Ruder etal., 2008). Ménézo etal.,(2012) recorded that about one out six couples were suffering from infertility in human and 40 t0 50% of the cases return to females and 30% was return for male factor of infertility without clear causes (Agarwal etal.,2014) however, oxidative stress were considered one of these etiology of infertility in females and male worldwide (Agarwal etal.,2014 ; WHO,2010).

Free radical mean reactive unstable molecules or atoms that have at least single unpaired electron in their outer orbital and they try to become stable through taken electron from other molecules such as lipid, protein, carbohydrate ,, sugar and nucleic acid (Agarwal etal.,2012)

ROS in normal concentration play role in certain physiological process of male reproductive system including capacitation, hyper activation, acrosome reaction and sperm-oocyte fusion (Agarwal etal.,2010), low concentration of ROS are required to function and maturation of the spermatozoa in addition to sperm motility, oolemma, ROS release from immature spermatozoa and leukocytes in the semen fluid (Saalu, 2010)

however, overproduction of ROS can be harmful for both male and female reproductive system, the Free radical in the semen come from leukocytes, immature sperms and varicocele, (Agarwal etal., 2014) in addition to radiation, toxin and smoking which were considered exogenous source of free radicals in the semen fluid, (Agarwal etal.,2014).

Defect in the spermatogesesis and fertility are the major problem of pathological changes in the male reproductive organs (Desai etal., 2009). oxidative stress were considered important cause of male infertility due to their effects on the sperms (Saalu , 2010), and it cause decrease count ,disturbed sperm motility alter sperm capacity to fertility ,all these defects were considered main cause of men infertility (Saalu , 2010),also ROS induced decrease in concentration as well as decrease fusion of sperm with oocyte (Ruder etal.,2008).

Brucella strain can generate ROS endogenously due to their aerobic respiratory metabolism (Pizarro-Cerda etal.,1998), also the important virulence factor of these pathogen are antioxidant enzyme such as superoxide dismutase that consumed the host antioxidant such as glutathione peroxidase (GSH-Px) at beginning of infection to detoxify the ROS that may be release from inflammatory responses (Erdogan et al.,2010)

Few studies about the influence of *B.melitensis* on male mice reproductive tract (Abdullah etal.,2013) but in Iraq, there is no researches about the influence of oxidative stress associated with *B. melitensis* on male reproductive tract ,therefore the aims of the present study were to: To determine the influence of oxidative stress induced by *B.melitensis* infection on male reproductive tract and to investigate the effects of both chitosan and Rev I vaccine on *B.melitensis* infection producing oxidative stress.

MATERIALS AND METHODS

Bacterial isolate: The virulent *Brucella melitensis* isolate was obtained from Al-Nahdha Veterinary Laboratories /Baghdad, the growth and biochemical tests were done to these isolates to confirm diagnosis according to Quinn etal., (2004), It was used different culture media including.

Tryptic Soya Agar, Tryptic Soya Broth (TSB), Blood Agar and These media were prepared according to manufactured instruction, the strain was confirmed diagnosis by gram stain and biochemical test according to Quinn etal.,(2004)

Preparation of chitosan diet: The commercial assorted pellets were grinded by food grinder and weighed , then 1gm of chitosan (fatsorb®) was added to each kilogram of grinded pellets mixed well and converted into paste which passed through meat grinder to mould the paste in to the original pellets form , then left exposed to dry in room temperature (Shakir. 2012).

The challenge dose was 1x10-8 CFU /ml.

Experimental design: One sixty white male mice, average age 8to 10 weeks were divided randomly into five group equally in addition to 12 female mice and treatment as following

1st group was considered control positive group(G1)

2nd group was immunized with *B.melitensis* Rev 1 vaccine S\C, two dose, two weeks interval.(G2)

3rd group was vaccinated as 2nd group and fed diet supplement with 1g\kg diet of chitosan for the end of the experiment.(G3)

4th group was fed diet supplement with 1g\kg diet of chitosan for the end of the experiment.(G4)

5th group was administrated orally with PBS and served as control negative group.(G5)

30days post immunization, animals of 1st ,2nd ,3rd ,and 4th groups were divided into two sub group A and B equally , subgroup A were infected I\P with o,3ml of bacterial suspension containing 1X10^8 CFU of viable virulence *B.melitensis* and subgroup B was persisting as control for subgroup A, at 30 days post infection, 6 infected males of subgroup A were mated with 12 normal females in ratio1:2 for 10 days , then both sexes will be separated and determine pregnancy percentage, after 60 day post infection, all animals were sacrificed, blood samples were taken for hormonal and determined the levels of oxidative stress.

Collection of blood

Blood was collected immediately after anesthesia, directly from the heart by using insulin syringe 1 ml ,blood samples directly transferred in to a Eppendrof tubes ,after that kept in refrigerator for period of time in stand position then centrifuged at 1500 rpm for 3 minutes, the serum was stored in the freeze at -20 Ċ until hormonal analysis for testosterone kit.

Measurement of total antioxidant status (TAS)

Serum TAS was determined using an automated measurement method developed by (Erel, 2004).. This method utilizes the hydroxyl radical, the most potent biological radical. In the assay, a ferrous ion solution, which is present in reagent 1, is mixed with hydrogen peroxide, which is present in reagent 2. Other potent radicals are produced, such as brown dianisidinyl radical cation, which is produced by the hydroxyl radicals. This method measures the antioxidant effects of the sample against the potent free radical reactions initiated by the

hydroxyl radical. The assay has excellent precision values of < 3%. The results are expressed as millimoles of Trolox equivalent per liter (mmol Trolox equiv./l).

Serum malondialdehyde (MDA)concentration was done according to (Yagi , 1976.)

Semen collection: The testes were removed along with the epididymis. the caudal epididymis were separated from the testis, blotted with filter papers and lacerated to collect the semen.

Sperm morphology: A part of sperm suspension was used for preparing smears to evaluate the sperm shape abnormalities according to(Wyrobek etal.,1974,1983),and sperm morphology were determined according to (Narayana etal., 2002),Sperm viability was determined according to (Kodama etal., 1997).

RESULT

Serum testosterone hormone: The results showed increased mean of serum levels of testosterone hormone in immunized with feed supplement of chitosan mice(1.749 ± 0.03) as compared vaccinated group (0.645 ± 0.04) ,chitosan group(0.896 ± 0.02) and control negative group (0.632) , post infection, these values were decline in G1(0.362 ± 0.02), G2(0.848 ± 0.02)but the levels of testosterone in control positive were significantly decline as compared to other groups 0.186 **table(1).**

Table (1): Effect of Immunized, immunized+ chitosan, chitosan on Testosterone hormones of males mice at 60 days post infection

Traits	immunized Mean ± SE	immunized+ chitosan Mean ± SE	Chitosan Mean ± SE	C-	C+
(non-infection) **	0.645 ± 0.04 C	1.749 ± 0.03A	0.896 ± 0.02B	0.632 ± 0.03 A	
(post-infection)**	0.462 ± 0.02 C	0.848 ± 0.02 A	0.562 ± 0.01 B	0.632 ± 0.03 A	0.186 ± 0.04A

Sperm morphology

The result showed that high % of sperm deformity in infected animals that appeared in head (20.00%, neck,(23.5%), tail(21.5%) ,with low percentage of normal sperm (35%) and viability (28%) as compared with control negative group, (94.8%,7.4%, 10.2%,70.1% and 87% respectively) ,with immunized chitosan infected group (4.8%,7.4%,10.2%,79.6% and 80% respectively),with immunized infected animals(12%,10.3%,12.5%,60.2% and 66%

respectively) and with chitosan infected group (7.3%, 8.0%,10.5%,74.2% and 72% respectively) (Table:2) ,also the result revealed varies sperm deformity including amorphous with inverted head(Fig:1), coiled tail (fig:2), dabble head (Fig:3).

Table (2): index of sperm deformity.

Sperm deformity					
Group	**Head deformity (%)**	**Neck deformity (%)**	**Tail deformity (%)**	**Normal**	**Sperms viability**
GI	12	10.3	12.5	60.2	66%
GII	4.8	7.4	10.2	79.6	80%
GIII	7.3	8.0	10.5	74.2	72%
GIV	20.0	23.5	21.5	35	28%
GV	4.8	7.4	10.2	70.1	87%

G1= infected immunized GII= infected immunized +chitosan. GIII= infected +chitosan .GIV=control +. GV=control -

also the result revealed various sperm deformity including amorphous with inverted head(Fig:4), coiled tail (fig:5), dabble head (Fig:6).

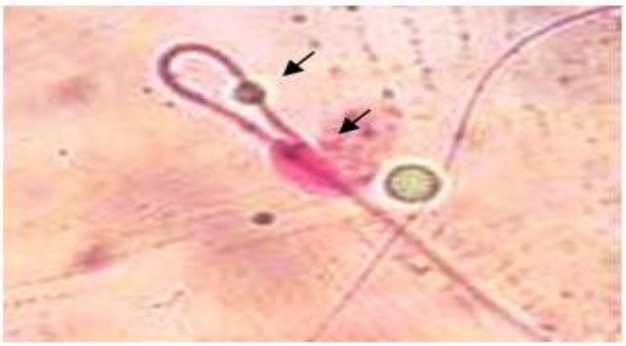

Fig (1): shows abnormal epididymal sperm in infected mice with *B.melitensis* after 30 days of infection, the arrow show amorphous with inverted head.

Fig (2): shows abnormal epididymal sperm in infected mice with *B.melitensis* after 60 days of infection, the arrow refers to coiled tail.

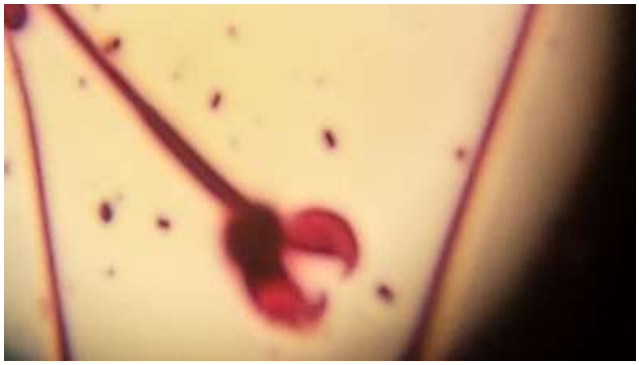

Fig. (3): shows deformity in Epididymal sperm (dabble head) of infected mice with *B.melitensis* at 30 days post infection. (stain

Pregnancy percentage of normal females mated with infected males: The results of pregnancy index of normal females that mated with infected males show high parentage (100% , 100% , 100%) in control negative group and infected groups of males that fed on diet supplement of chitosan with and without immunization (GII), (GIII) and control negative respectively, comparing with infected immunized groups only (GI) (66.66%) while control+ groups show the lowest percentage (16.66%), table (3).

Table (3): Pregnancy index of normal females mated with infected males 60 days post infection.

Group	Total number	No. of pregnant	percentage
GI	6	4	66.66%
GII	6	6	100%
GIII	6	6	100%
C+	6	1	16.66%
C-	6	6	100%

Biochemical analysis

4.3.1. Measerment of total antioxidant concentration level in serum: The result show elevated level of antioxidant concentration in groups that fed on diet supplement of chitosan with or without infection comparing with those of immunized groups with or without infection(table,4) , also Level of MDA show elevation in infected groups comparing with non-infected groups ,as well as significantly decline in the serum levels of MDA in infected animals feed diet supplement with chitosan only followed by immune –chitosan infected group and immunized infected animals as compared with control infected group.

Table (4): mean of serum levels of Total antioxidant status (TAC) in non-infected and infected immunized male animals at 60 days post infection

Males					
trait	**G1**	**GII**	**GIII**	**C-**	**C+**
Non- Infection**	3. 514± 0.001D	4.941 ± 0.04 B	5.599 ± 0.15 A	3.232± 0.01 A	
Post- Infection**	3.001 ± 0.003D	4.400 ± 0.07 B	4.293 ± 0.06 B	3.232± 0.01A	2.174± 0.01 B

Table (5): mean of serum levels of MAD in non-infected and infected immunized male animals at 60 days post infection

Males					
trait	**G1**	**GII**	**GIII**	**C-**	**C+**
Non- Infection**	357.086 ±4.48B	60.521 ±2.88D	33.077 ±0.24 F	83.67± 41.67A	
Post- Infection**	520.874 ±1.28B	106.150 ±4.69E	104.333 ±8.68C	83.67± 41.67A	728.30 ± 0.01 A

DISCUSSION

Abnormal structure of the sperms in the infected animals in present study may indicated that *Brucella* infection associated with generation of ROS that induced abnormal shape of sperm with decrease number of sperm , these idea was agreement with (Agarwal and Said, 2005), and Colagar etal.,(2007) who reported that ROS cause male infertility due to the cause impairment function of the sperm with poor their quality and quantity.

According to above result it was suggested that infection by *B.melitensis* was important etiology of infertility in male due to production of the free radical which considered important cause of morphological abnormality of sperm due to lipid peroxidation of sperm membrane ,these idea was in consistent with observation of, Agarwal and Sekhon,(2011) who found that morphological and function changes of the sperm were considered important etiology of male infertility in addition to sperm concentration .

The present finding demonstrated that sperm viability was low in infected non treatment animals but these percentage was high in immunized chitosan group and chitosan group as compared with immunized group only post infection, these observation may indicated that brucellosis cause oxidative stress that cause damage of the sperm and chitosan can act as antioxidants and prevent damage of the sperms ,these observation was supported idea of Lanzafame etal,(2009) who suggested that one of important cause of male infertility is oxidative stress and they found that 30 t0 40% of men suffering from infertility expressed

high levels of ROS in seminal plasma ,the first cell responsible for ROS is spermatozoa because these cells were unable to regenerate oxidative damage as a result these cells were lack cytoplasmic enzymes that play role in the repair (Saleh and Agarwal,2002) but its cell membrane was rich with polyunsaturated fatty acids which highly susceptible for ROS damage by lipid peroxidation that associated with axonal damage, depression sperm viability and defect in sperm morphology and decrease sperm motility (Bansal and Bilaspuri, 2010; Gharagozloo and Aitken, 2011).

The deformity of the sperms in the current result may be due to oxidative damage as a result its cell membrane was rich with polyunsaturated fatty acid to facilitated fusion with oocyte (Aitken and De Iuliis, 2010)

The present study showed decrease in the levels of testosterone , and abnormal shape of sperm in animals infection by *B. melitensis*, these result may be due to oxidative stress induced by these bacteria that influence on sperms, these idea was in consistent with Mendelson *et*al., (2003) who found that rat treatment with nicotine which generated ROS can cause diminish in sperms concentration, abnormal motility of sperms with abnormal morphological structure.

The current study showed the values of serum testosterone in animals administrated with chitosan were higher than those values in control animals and immunized animals these results may indicated that the chitosan improve the fertility of the males due to testosterone play essential role in the production of the sperm, these idea was agreement with He et al.,(2008) who recorded that chitosan can improve fertility in human.

It was reported ,in the present study, that infected animals with *B.melitensis* showed highly significant decline in values of testosterone as compared with non-infected group, these result may indicated that the bacterial infection effect on the fertility of the animals through directed damage testicular tissue or due to generate of free radical ,these idea was supported by present finding which revealed that infected animals have diet supplementing with chitosan expressed less decline in levels of serum values as compared with other groups, these idea was agreement with Agarwal et al.,(2014), who found that oxidative stress cause suppression of testosterone production.

Also the present study showed the levels of serum testosterone in immunized animals were less than those values in immunized chitosan treated animals after infection these results

may indicated that chitosan act as antioxidant that remove the ROS also the result explained that immunized chitosan group revealed high levels of serum testosterone post infection as compared in immunized animals only ,these result may indicated the vaccinated animals fed diet supplement with chitosan expressed protective immune response against Brucella infected and subsequently decrease free radicals that effect on testosterone production as well as chitosan may act as antioxidant ,these observation was agreement with Mariam et al.,(2014) who demonstrated that chitosan act as antioxidant against toxicity of the endocrine system ,sperm abnormalities and Oxidative stress cause depress male fertility.

These results may indicated that chitosan can improve testosterone levels ,these idea was agreed with He et al.,(2008)who recorded that chitosan improve fertility ,also it was found that chitosan decrease level of MDA due to it is protective membrane integrity from lipid peroxidation since it act against oxidative stress as antioxidant action (Osman etal.,2010;Mariam et al.,2014).

The low percentage of pregnancy in normal females which were mated by infected males, in the present study, may be related to ability of sperms to fertilize ova , these observation may supported by lower of testosterone levels in infected males that using in the mating of normal females , these result may indicated that the free radical influence on GnRH which regulated action of LH on Leydig cells to produce testosterone through the expression of 17-B hydroxysteroid dehydrogenase to converted gonad producing androstenedione to testosterone and the ability of sperm to fertilize egg was influence by sperm concentration, motility and normal morphology(Sanocka and Kurpisz, 2004), also the present result may be due to ROS cause damage of DNA of sperm and deletion (Zribi etal.,2011; Wright etal.,2014; Tahmasbpour etal.,2014) with apoptosis of germ cells that associated with decrease sperm count and poor fertility of the male (Singh etal,.2003).

The sperm deformity in the current study may considered the essential factor of decreasing percentage of pregnancy of normal females that were mated by infected males, , The criteria using to determine potential sperm fertilization are sperm concentration, motility and normal morphology of the sperms from ejaculate also breakdown of DNA strands was essential factors influence on sperm viability to fertilization due to chromatin of The DNA are more sensitive to ROS(Zribi etal.,2011).

The high percentage of pregnancy of normal females mated by males fed chitosan with or without vaccinated , post infection may indicated the vaccine provided good immune response that augment by chitosan which protective the animals against Brucella infection and subsequence no oxidative stress occur which induced chain chemical reaction in the sperm membrane called LPO (Makker etal.,2009) and these reaction lead to remove about 60% of lipid in the sperm membrane which associated with increase its permeability ,losses ions, inactivated membrane receptors and enzymes and abnormal morphological changes that lead to impair sperm activity (Makker etal.,2009).

In non-infected animals, the current result showed that high levels of serum total antioxidant in animals fed diet supplement with chitosan as comparing with other groups while immunized animals expressed ,low levels of serum total antioxidant as compared with other groups ,these result may indicated that chitosan act as antioxidant agent since the host commonly exposure to endogenous ROS in which chitosan may neutralized them , the low levels of TAC in immunized animals in the present study may indicated that the immune responses may associated with oxidant production since the levels of TAC was high in immunized chitosan group as compared with immunized and control negative group ,these indicated also chitosan act as antioxidant The present finding revealed high levels of serum TAC in chitosan group followed by immunized chitosan group as compared with other groups of non-infected animals, these result may supported idea that chitosan act as antioxidant better than immunization alone it was found no changes in the serum antioxidant levels before and after immunization, these result was recorded by (Schneeberger etal., 2013) after injected bats with LPS.

The current result may confirmed idea that Brucella infection induced generated free radicals , since, lowest levels of TAS were seen in non-immunized infected animals but these values were higher in immunized chitosan and chitosan only group post infection ,these evidence was in consistent with Karaagac etal.,(.2010) , who found diminished in the levels of TAS in the serum of human suffering from brucellosis, also the result of MAD examination may supported idea that *B.melitensis* was associated with oxidative stress.

Kivanc et al.,(2009) found that significantly increase in the levels of Plasma total peroxide and malondialdehyde in patients with brucellosis as compared with healthy controls and they suggested that Oxidative stress was increased in patients with brucellosis .the present finding may indicated that generating of ROS due brucellosis cause lipid peroxidation that

lead to MAD production ,these idea was agreement with Ragab etal., (2013) and Sarhan etal.,(2012), who suggested that high levels of MDA indicated lipid peroxidation, essential marker of oxidative damage and insufficiently antioxidant mechanism to prevent oxidative stress. Decrease of MDA in animals feed chitosan post infection, in present study may be indicated that chitosan scavenger of free radicals due to its antioxidant features or inhibited lipid peroxidation through antilipidemic activity (Anraku etal.,2010), also El-Fattah etal.,(2013) found that chitosan have highly activity against certain highly toxic environmental pollutants such as TCDD also chitosan can protected against radiotherapy (Mohamed, 2011).

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