ABSTRACT

Background: Ova Max® is a new nutrient supplements containing vitamins, minerals, enzymes, amino acids and hormones. All may sustain the oocyte quality and ovarian function, at the same time protect oocyte from free radicals damage. Objective: The goal of the thesis is to examine the effect of Ova Max® on oocyte maturation.

Materials and Methods: Forty mature female mice were involved in this study that conducted at the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies through the period from September 2014 to April 2015. The animals were divided into three groups. The first group, the mice ages was (16) weeks old and The second group, the mice ages was (32) weeks old: independently the mice of each group were divided into three subgroups according to Ova Max® doses (0.2, 0.4, 0.8 mg / kg). The third group was the control group divided into two main groups, Five mice (16 weeks old) and Five (32 weeks old). Similar sub grouping and parameters assessment of treated mice was done for the control group. All the female mice were stimulated with super ovulation induction program. Ova Max® was treated by oral administration for 30 day. Results: The results indicated that the treatment with Ova Max® showed a significance (p< 0.05) improvement in oocytes maturation status in treated group of dose 0.2 and 0.4 mg/kg OM in age (32) week compared with control group at the same age. Conclusions: It was concluded that the treatment by Ova Max® has a great improvement in oocyte maturation with low dose for aged mice. This data can be used for human and other mammals to sustain the fertilization capacity and normal embryonic development.
KEYWORDS: Ova Max®, oocytes quality aged mice.

INTRODUCTION

Infertility is defined by International Committee for Monitoring Assisted Reproductive Technology and the World Health Organization (WHO) as a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse.\[1\] There are two major types of infertility and these includes, primary infertility and secondary infertility.\[2\]

The causes of infertility are either resulted from the male and/or the female. Male fertility can be reduced as a result of numerous causes such as

1 - congenital or acquired urogenital abnormalities. 2 - malignancies. 3- urogenital tract infections. 4-increased scrotal temperature (e.g. as a consequence of varicocele]. 5- endocrine disturbances.6-genetic abnormalities.7-immunological factors and others.\[3\]

Whereas the factors of female infertility are identified as risk and causative factors for female infertility including, 1-aging.\[4\] 2- blockage or damage of the fallopian tubes.\[2\] 3- endometriosis.\[6\] 4- ovarian insufficiency.\[7\] 5- pelvic adhesions.\[8\] 6-thyroid problems.\[9\] and so many other cancer\[10\], chemotherapy\[11\], caesarian sections\[12\], delayed puberty\[13\], obesity\[14\], amenorrhea\[15\], tobacco and alcohol use\[16\], genetic abnormalities\[17\], vitamin D deficiency\[18\], and unidentified factors.\[19\] Treatment of infertility depends on the cause, how long they have been infertile, age and partner's age, and many personal preferences.\[20\]

Maternal age is a significant factor in infertility treatment. Ovarian function and Oocytes quality decrease with age, whereas the frequency of chromosomal abnormalities increases.

Accordingly, fertilization, development, and pregnancy rates decrease, whereas the spontaneous abortion rate increases.\[21\]

Oocytes quality markedly decreases in women aged > 35 years and chromosomal aneuploidy increases: correspondingly, the implantation rate following embryo transfer diminishes.\[22,23\]

Different nutrient supplements were produced world wild, one of these supplements is the Ova Max®. This new medicine containing vitamins, minerals, enzymes, amino acids and hormones all may sustain the ova quality and ovarian function. At the same time Protect oocyte from free radicals damage (ROS).\[24\]
In addition, Ova Max contains Coenzyme Q10 (CoQ10), which is also a powerful antioxidant. But, CoQ10 also plays an important role in energy production within oocyte, once conception occurs, the amount of cell division that occurs to allow the oocyte to grown into an embryo and successfully implant into the wall of the uterus requires a tremendous amount of energy. CoQ10 works within the mitochondria of the oocyte to help ensure that energy production is optimal. Finally, OvaMax contains three additional antioxidant ingredients, Vitamin E, grape seed extract and alpha lipoic acid, to help ensure your oocytes are adequately protected from the damaging effects of free radicals.\[25\]

However, in our knowledge there are a lack in the studies regarding the effect of this supplement on oocyte quality and embryonic development therefore the study was designed to search on these effects.

**MATERIALS AND METHODS**

1. **Housing and management of experimental animals.**

Thirty mature male and Two hundred female Albino – Swiss mice their ages were 16 and 32 weeks old and 28-32 gm body weight were obtained from the Animal House at High Institute of Infertility Diagnosis and Assisted Reproductive Technologies /AL-Nahrain University during the period from October 2014 to March 2015. They were chosen as the fertile mice and kept in an air conditioned room (25°C) with a photoperiod of 13+2 hours. The mice were housed in box cage of plastic measuring (29×15×12) cm. Its floor covered with wooden shave. Each cage was containing four animals. The tap water and diet were freely available for the animals. The isolated females were kept in separate cages to make sure there was no meeting between them and the males happened and no pregnancy taking place by natural mating. The mice examined clearly every week, abnormal and sick mice were excluded from the experiment. The cages were cleaned and sterilized with 70% ethyl alcohol once a week regularly.

2. **Preparation of Culture Media and Ova Max®**

2.1- **Preparation of Phosphate Buffer Saline (PBS):** (Ca\(^{++}\), Mg\(^{++}\)free)

One liter of PBS was prepared by dissolving the component (8gm of NaCl,0.1444gm of KH\(_2\)PO\(_4\) (BDH) and 0.795gm of Na2HPO\(_4\)) in 990 ml deH\(_2\)O then adjust pH to 7.4 after that the volume was corrected to 1000 ml of deH\(_2\)O. This solution was filtered by using 0.45 μm Millipore filter, and it was sterilized by autoclaving at 121 °C and 15 pound/inch for 15 minutes. Finally, it was stored at 4 °C temperatures in sterile bottle.\[26\]
2.3- Preparation of Ova Max®
In the beginning, the powder inside the Ova Max® capsule was weighted using Electric balance the weight of powder was (600) mg \ capsule then the mice were weight. The powder was dissolved in 10 ml of distilled water and proceed to mitigation to reach the concentrations required three doses(0.2,0.4,0.8)mg After the adoption of the following equations.

Human weight / animal weight = human dose / animal dose.
HED (mg/kg) = Animal Dose (mg/kg) x [Animal Km / Human Km].[28]

3- Detection of female estrus cycle
Stages of estrus cycle of female mice were detected and reported using vaginal smears. The smear performed daily between 8:00 am and 10:00 am.

4- Ovarian Stimulation (super ovulation induction)
Super ovulation was induced hormonally and induction of multiple ovulations was accomplished by injection intra peritoneal (IP) of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG).

(PMSG) has FSH-like effect, while HCG has LH-like effect acting on the somatic cells of the mammalian ovarian follicle to stimulate ovulation.[29]

Super ovulation was performed by IP injection of 7.5 I.U. of PMSG, and then followed by IP injection of 7.5 I.U. of HCG, 48 hours later. Oocytes were recovered 13 hours post-HCG as shown in Figure (3-6).

4.1. Oocytes Collection
Under sterile condition in the laminar air flow hood, the Oocytes were collected. After scarified female mouse by cervical dislocation after 13-14 hours from HCG injection. The Fallopian tube was flushed using one ml (insulin) syringe and a blunt needle gauge -30. Which contain 0.5 ml of Ham’s F-12 medium. The injection was done through any side of Fallopian tube by slow pushing the medium through the duct, and then the oocytes were collected.
4.2. Slicing of Fallopian Tube
This procedure was accomplished using microsurgical set; it involved small slicing of the Fallopian tube under dissecting microscope as shown in (Figure 3.9), to grade the oocytes surrounded with cumulus. Then the collected oocytes were cultured in Hams –F12 medium in the 5%CO₂ incubator.

5. Identification of Immature Oocytes
The super ovulated oocytes were obtained by flushing the Fallopian tube. In order to determine whether the oocytes were mature or not, a special observation techniques was employed as follows:

5.1. Sliding
During cumulus – oocytes complex (COC) sliding, it was possible to observe clearly whether or not oocytes cytoplasm contains a germinal vesicle (GV) or if the oocytes has extruded a first polar body (1PB) into the perivitelline space (PVS). If neither GV was seen in the Oocytes cytoplasm nor 1PB found in PVS, the oocytes was defined as germinal vesicle breakdown (GVBD) or metaphase-I stages (M-I).[31]

When the corona radiate was compacted but still distinct from the cumulus, it was usually associated with a metaphase-I (M-I) oocytes with absence of the first polar body; this oocytes was classified as "immature " or "intermediate". A sunburst-like corona radiate and a well-expanded cumulus is generally associated with a metaphase II (MII) oocytes, and was classified as "mature" oocytes.[32]

Statistical analysis: All statistical analysis was performed by using version 20 IBM SPSS and also Microsoft Excel Work Sheet 2007. Chi square test was used to compare values of the treated and control groups oocyte maturation states .The differences between the values were considered statistically significant at (P<0.05).[33]

RESULTS
Comparison of maturation status and total number of oocytes between female mice aged (16) and (32) weeks old.
The differences in the rate of oocytes maturation following the treatment with Ova Max® for 30 days using three doses between mice ages 16 weeks and 32 weeks old (0.2mg/Kg=78.76% vs. 72.09% ,0.4mg/Kg= 75.45% vs. 71.57% and 0.8mg/Kg=74.76 vs.
64.83% shown no statistical significant (0.2 mg/Kg $P=0.523$, 0.4mg/Kg $P=0.783$ and 0.8mg/Kg $P=0.244$, respectively). There was no significant ($P>0.05$) differences in total number of collected oocytes following the treatment with three doses of Ova Max® between the ages 16 and 32 mice groups, as shown in tables (1).

**Tables (1): Comparison of maturation status of mice Oocytes Between (16) and (32) week age.**

<table>
<thead>
<tr>
<th>Mice group</th>
<th>maturation status</th>
<th>Mice ages</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16weeks</td>
<td>32 weeks</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NO.</td>
<td>%</td>
<td>NO.</td>
</tr>
<tr>
<td>Control group</td>
<td>mature Oocytes</td>
<td>68</td>
<td>66.7%</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>immature Oocytes</td>
<td>29</td>
<td>28.4%</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Atretic Oocytes</td>
<td>5</td>
<td>4.9%</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>102</td>
<td>100.0%</td>
<td>74</td>
</tr>
<tr>
<td>Treated group with 0.2 mg/kg Ova-max</td>
<td>mature Oocytes</td>
<td>89</td>
<td>78.76%</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>immature Oocytes</td>
<td>18</td>
<td>15.92%</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Atretic Oocytes</td>
<td>6</td>
<td>5.30%</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>113</td>
<td>100.0%</td>
<td>86</td>
</tr>
<tr>
<td>Treated group with 0.4 mg/kg Ova-max</td>
<td>mature Oocytes</td>
<td>83</td>
<td>75.45%</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>immature Oocytes</td>
<td>20</td>
<td>18.18%</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Atretic Oocytes</td>
<td>7</td>
<td>6.36%</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>110</td>
<td>100.0%</td>
<td>95</td>
</tr>
<tr>
<td>Treated group with 0.8 mg/kg Ova-max</td>
<td>mature Oocytes</td>
<td>81</td>
<td>74.76%</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>immature Oocytes</td>
<td>19</td>
<td>17.75%</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Atretic Oocytes</td>
<td>7</td>
<td>7.47%</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>107</td>
<td>100.0%</td>
<td>91</td>
</tr>
</tbody>
</table>

Chi–square test at 0.05 level of significance

**DISCUSSION**

The present study demonstrated that treatment with Ova Max® have a positive effect on maturation of the oocytes in the two mice groups treated after a 30 day of treatment.

Successful oocyte maturation that cause capability of the oocyte to undergo normal fertilization, embryonic development and pregnancy rates in the present study depends on the components of Ova Max® which contains folic acid, vitamin E, CoQ10, myoinositol, L-arginine, grape seed extract, melatonin, lipoic acid and chasteberry. Animal studies have
shown that supplementation with antioxidants reduces age-related ovarian senescence and prevents decline in oocyte quality and numbers in mouse.\[^{34}\] It has been found that vitamin E treatment have similar positive effect on oocyte maturation rates.\[^{35,36}\] Vitamin E is a vital antioxidant for reproduction and fertility. This powerful antioxidant can play a critical role in oocyte maturation because the follicular fluid (FF) found in oocyte is rich with vitamin E, the environment of the FF is thought to play a critical role in oocyte maturation and the eventual development of an embryo.\[^{37}\] Moreover, the FF is known to be metabolically active and contains steroid hormones, growth factors, cytokines, and granulose cells and leukocytes.\[^{38,39}\]

The results of current study may enhanced by the role of Myo-inositol (MI) action that is a part of Ova Max\(^{\circ}\) composition. It has been found that MI plays a crucial role in cell morphogenesis and cytokinesis, it is involved in cell membrane formation, lipid synthesis and cell growth.\[^{40}\] The other important component added to the Ova Max\(^{\circ}\) is the melatonin. In addition to the role of melatonin to stimulate antioxidative enzymes.\[^{41}\] It has been demonstrated that there is a direct correlation between melatonin concentrations in FF and oocyte quality.\[^{42}\] Therefore, the larger preovulatory follicles had higher concentrations of FF melatonin than smaller immature follicles.\[^{43}\]

The positive results in oocyte maturation of aged mice in this study may by due to the Ova Max\(^{\circ}\) supplement containing Coenzyme Q10. The Coenzyme Q10 (CoQ10), is a powerful antioxidant. At the same time, CoQ10 plays an important role in energy production within egg cells. Once conception occurs, the amount of cell division that occurs to allow the egg to grown into an embryo and successfully implant into the wall of the uterus requires a tremendous amount of energy. CoQ10 works within the mitochondria (the energy powerhouse of the cell) of the egg cell to help ensure that energy production is optimal.\[^{24}\]

The decreased production of matured ova in aged mice may resulted from energy production and ovarian reserve. Decreased energy production in aged oocytes may be related to a deficiency of CoQ10.\[^{44}\] Several trials in mice have found that administration of CoQ10 resulted in a significant increase in ovulated oocytes.\[^{45,46}\] CoQ10 concentrations decline with age, leading to increased ROS production, lipid oxidation, and cell necrosis\[^{47}\], it has been demonstrated that administration of CoQ10 for aged mice contribute to increasing the number of ovulated oocytes via improvement of oocyte mitochondrial function.\[^{48}\] This study has been postulated that the pool of respiring mitochondria was decreased in aged oocytes and increased by CoQ10 treatment to the level similar to that of young controls.\[^{49}\]
The supplement composed of folic acid too. Folate, water-soluble vitamin B, is necessary for energy production and healthy cell division, and it is also important for the formation of the red blood cells.\[50\] The current study suspected that addition of folate through Ova Max® is important for oocyte quality and maturation, implantation, placenta-ion, fetal growth and organ development.\[51\] It is considered to be important for oocyte quality and maturation as well as for implantation and normal continuation of pregnancy.\[52\] It has been emphasized that successful infertility treatment is associated with high folic acid supplement intake.\[53\]

The other important composition of Ova Max® is the lipoic acid(LA) which sustain the results of oocyte maturation in this work. Several studies showed LA effect on oocyte. It has been recommended that LA beside its main role as a coenzyme of various enzymes like pyruvate dehydrogenase complex that catalyses the utilization of pyruvate in mice oocytes and follicles.\[54,55\]

REFERENCES
32. Chain RC, Buckett WM and Tulandi T. Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women with polycystic ovarian syndrome. Hum Reprod. 2000; (15).
33. Cha KY, Han SY and Chung HM. Pregnancies and Deliveries after In Vitro maturation culture followed by in vitro fertilization and embryo transfer without stimulation in women with polycystic ovary syndrome. Fertil Steril. 2000; (73): 978-983.145.


