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Effect of Melatonin and Vitamin C as Antioxidants in Vitro Embryo Production in Local Iraqi Ewes

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ABSTRACT

Melatonin and vitamin C were tested on 155 reproductive tracts (310 ovaries) of local Iraqi sheep in aimed to investigate their effects on blastocyst generation. Oocytes were counted and cultured for 24 hours in a CO2 incubator with melatonin and vitamin C as antioxidants. Fresh semen was collected from two fertile rams by electroejaculator with the addition of heparin for capacitation at a rate of 10pg/ml. Matured oocytes grade good and fair were incubated in a CO2 incubator for 24 hours. Fertilised oocytes were cultivated in TCM-199 media with melatonin and vit c. Daily media replacement was 50%. The divided zygote developed every 24 hours until it formed a blastocyst after 168 hours and hatched after 216 hours after fertilisation. Addition of melatonin increased maturation rate to 60% (27/45) from 37.7% (20/53) with vitamin C and 22.2% (4/18) without antioxidants. Melatonin significantly differed ($p<0.05$) from vitamin C or control groups in the maturation group. The melatonin group had 59.2% (16/27) fertilisation, while the vitamin C 65% (13/20). There was no significant difference between groups. Maturing and fertilisation rates differed between the treated and control groups 22.2% (4/18) and 25% (1/4), respectively ($p<0.5$). With melatonin, blastocyst production was 62.5% (10/16). It was 53.8% (7/13) in vitamin C. Blastocysts were absent in the control group. Blastocyst production differed significantly ($p<0.5$) between the melatonin, vitamin C, and control groups. It was concluded from this study that the addition of an antioxidant (melatonin or vitamin C) could improve IVM, IVF, and IVEP in sheep.

Introduction

In vitro fertilization is an important technology in animal breeding. Recently has been regarded as another alternative for sheep genetic enhancement. Maturation of oocyte is required for the success of IVF procedure (1). So, These technologies are designed with the objective of augmenting the quantity of births with superior genetic characteristics, while simultaneously reducing the generation interval in order to expedite genetic enhancement (2).

Moreover, oxidative stress plays a role in the overall yield of viable embryos. The defence mechanism of antioxidant present in male and female genital system acts against reactive oxygen species (ROS) and maintain the healthy balance between production of ROS and antioxidant, which protect gametes from oxidative damage for high reproductive activity (3). Oxidative stress may be resulted from exposure to light, gametes manipulation, high O_2 concentration, uncommon metabolic activity and substances concentration during the procedure of IVF (4). Due to adequate natural defence mechanism in the body of antioxidant and the presence of ROS

inducing sources (5).

Vitamin C, also known as ascorbic acid, has potent antioxidant properties by functioning as a reducing agent, thereby safeguarding biological systems against reactive oxygen species (ROS). This substance functions as both an antioxidant and a cofactor for enzymes (6). One example of an antioxidant is vitamin C, also known as ascorbic acid. This compound is classified as water-soluble and can be found in several reproductive organs, such as the ovaries, corpus luteum, and follicular fluid. Additionally, it plays a role in the reduction of follicular apoptosis. (7).

Melatonin (5-methoxy-N-acety tryptamine) synthesized via pineal gland from tryptophan an essential amino acid. Also, melatonin plays a role as a biological clock synchronizer and a regulator of seasonality in animal reproduction (8). It has an effect on reproductive system through a decrease of oxidative stress, changing the ovarian steroidogenesis capacity of the C.L. and follicles; the shape of follicles. (9).

The aim of the study was designed to show the effect of melatonin and vitamin C on IVF in local Iraqi sheep.

Materials and Methods

Ethics approval

We approve that the experimental animals were treated according to the standards of the ethical committee of the Veterinary College/University of Fallujah.

Study area, animals and samples

The study was performed on 155 Reproductive tracts (310 ovaries) of local Iraqi sheep. Collected samples from AL-Fallujah slaughter house / AL-Anbar province during July to december,1,2022. Samples were transported via a cool box contained normal saline within one to two hours to the laboratory of reproductive biotechnology, college of Veterinary medicine, university of Fallujah. ovaries were isolated from attached tissue and cleaned with normal saline and put in a sterile glass container. Oocytes were aspirated with 18_gauge sterile needle with 5ml disposable syringe containing 3ml of TCM_199. Aspirated oocytes were transferred to petri dish having 16 wells in a cabinet sterilized with UV light. The oocytes that were retrieved were observed using an inverted microscope in order to assess their quality. These oocytes were then categorised into three grades: good (A), fair (B), and bad (C), based on established criteria (10). The classification was determined by evaluating the existence of cumulus cells and the uniformity of the cytoplasm. Oocytes were counted and incubated after the addition of melatonin and vit C as antioxidants. Vit c (Ascorbic acid) was added at a rate of 50 µg /ml while melatonin was added at a rate of 100 µg /ml.

IN Vitro Maturation of oocytes:

Grade A and B oocytes were selected (10) and washed with TCM-199 media. Oocytes were cultured in petridish having 16 wells. The antioxidant has been adding to Three different treatment. The first treatment (T1) includes TCM-199 plus 100 µg /ml melatonin the second treatment (T2) includes TCM-199 media plus vit C (Ascorbic acid 50 µg /ml). the third treatment (T3) includes TCM-199 media without addition of antioxidants. Petridish with 16 wells were incubated at 38.5C, 5%CO2 and relative humidity 90% for 24 hrs. Oocytes maturation was indicated by the presence of 1st polar body. The maturation oocytes were calculated.

Semen collection:

Semen was collected via electro ejaculator (Electro Jac 6, USA) from two fertile rams presented at the farm of college veterinary medicine., university of Fallujah. Pooled semen were diluted at a ratio of 1:20 with TCM-199 media according to (11). Heparin was added as a capacitating factor at a rate of 10pg/ml.

In vitro fertilization:

Heparin (10pg/m) was added to semen and the mature oocytes and incubation of semen plus oocytes at 38.5C, 5%CO2, and relative humidity 90% for 24 hours. presence of 2nd polar body was indicated to fertilization. The numbers of fertilized oocytes (zygote) were calculated.

In vitro culture of fertilized oocytes (zygote):

Fertilized oocytes were cultured in culture media (TCM-199) with addition of melatonin and ascorbic acid (vit c) and incubated at 38.5C, 5% CO2, and relative humidity 90%. Half of the media was replaced every 24 hours. Development of the different stages of division were observed every 24 hours till formation of blastocyst stage after 168 hours and hatched blastocyst after 216 hours after fertilization.

Statistical analysis

Chi-square test and Duncan multiple range test were used.

Results

Table-1 showed the effect of antioxidants (melatonin and vitamin c) on maturation, fertilization rate and blastocyst production in TCM-199 media as compared with control. The results showed a higher percentage of maturation rate 60% (27/45) treated with melatonin as antioxidant while maturation rate was 37.7% (20/53) treated with vitamin c. as a maturation rate of 22.2%(4/18) without antioxidant. There was a statistical difference ($p<0.05$) between melatonin treated group as compared with vit c or control group, in maturation rate.

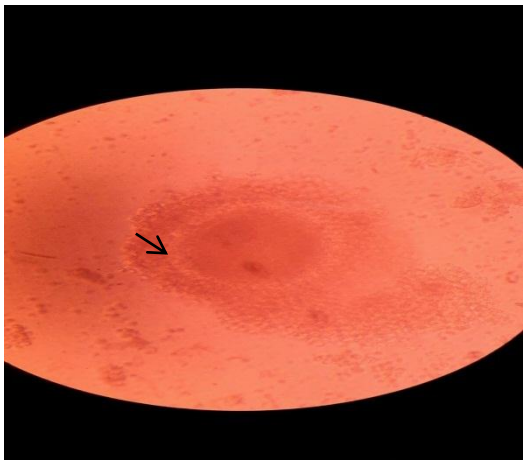

Figure 1: Matured oocyte. Arrow showed first polar body.

Table.1. Effect of antioxidant on maturation rate, fertilization rate and blastocyst production in TCM_199 medium.

Antioxidant	No.	Maturation % (n/n)	Fertilization % (n/n)	Blastocyst production % (n/n)
Melatonin	45	60% **a** (27/45)	59.2% (16/27) **a**	62.5% (10/16) **a**
Vitamin C	53	37.7% **b** (20/53)	65% (13/20) **a**	53.8% (7/13) **a**
Control	18	22.2% **c** (4/18)	25% (1/4) **b**	0% (0/1) **b**
Total	**116**	**43.9% (51/116)**	**58.8% (30/51)**	**56.6% (17/30)**

The values with different litters showed significant difference ($p \leq 0.05$)

The fertilisation rate showed 59.2% (16/27) in the melatonin-treated group, while it was 65% (13/20) in the vitamin C-treated group. There was no statistical difference between the two groups. There was a statistical difference ($p<0.5$) in maturation rate and fertilisation rate between the treated and control groups (22.2% (4/18) and 25% (1/4), respectively.

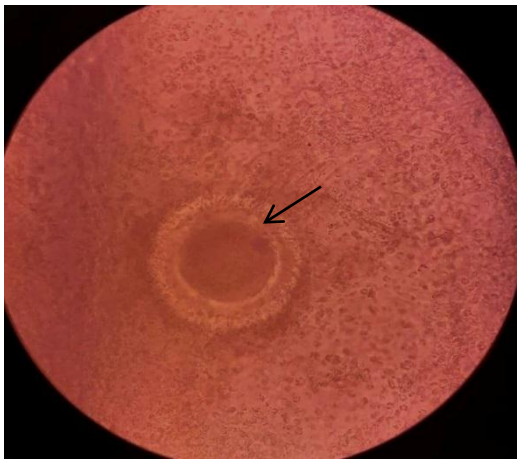

Figure 2: Fertilized oocyte. Arrow showed second polar body.

Also, Table 1 showed that the blastocyst production rate, or percent, was 62.5% (10/16) in the melatonin-treated group. While it was 53.8% (7/13) in the vitamin C-treated group. The control group showed no development of blastocysts. There was a statistical difference in blastocyst production ($p<0.5$) between the melatonin, vitamin C-treated group and the control group.

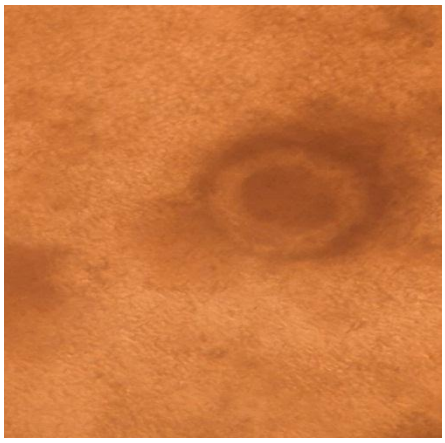

Figure 3: 2-cells embryo.

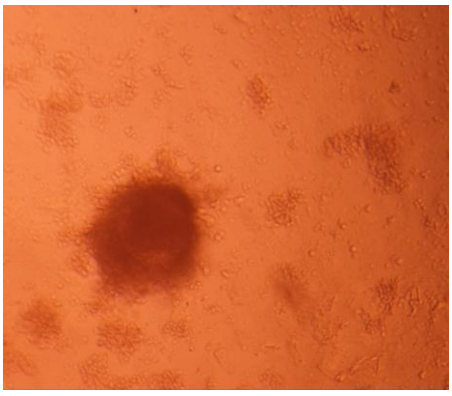

Figure 4: 4-cells embryo.

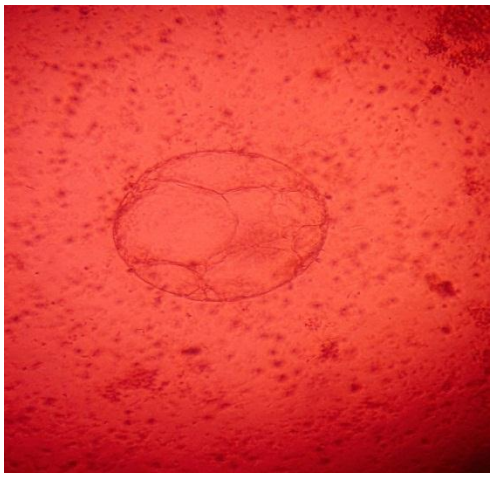

Figure 5: 8-cells embryo.

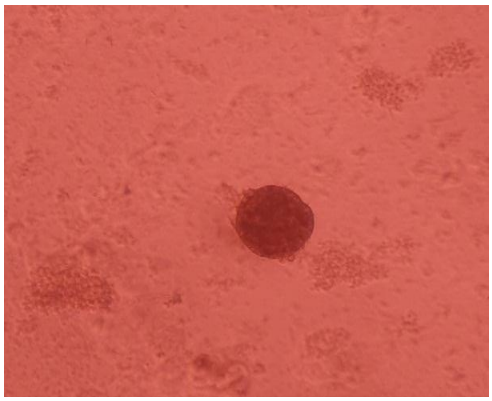

Figure 6: 16-cells embryo.

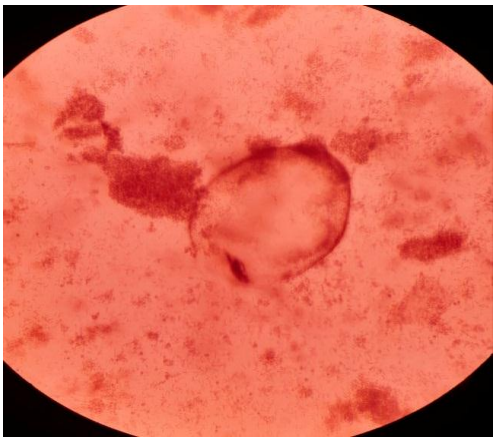

Figure 7: Blastocyst formation.

Discussion

The results are similar observation have been made by (12,13). Higher maturation percentage observed in melatonin treated group might be due to that melatonin supplemented to maturation, fertilization and culture medium for IVF acts as potent antioxidant and scavenger against free radicals and reactive oxygen species and apoptosis of embryo during their development. It is also improving the development of follicles (14,15).

Melatonin plays an important role in physiological functions such as regulation of seasonality, as well as sleep anti-aging and antioxidant actions in addition to their influence on the gene expression of antioxidant enzymes (16).

Several studies have presented evidence indicating that melatonin exerts a direct influence on the quality and development of oocytes. The inclusion of melatonin in the IVM medium has been found to enhance the maturation of juvenile oocytes in goats, resulting in an increased generation of high-quality blastocysts. The findings were elucidated by the decrease in reactive oxygen species (ROS) and the antioxidative properties of melatonin. (17). According to reports, the utilisation of ascorbic acid (Vitamin C) has been found to enhance the in vitro maturation and developmental efficacy of buffalo oocytes (18).

The results of fertilisation rate Similar observations have been made in IVF in sheep (19). The observed outcomes might perhaps be attributed to the presence of ascorbic acid (50 µg) throughout the process of in vitro maturation (IVM), which resulted in a reduction of oxidative stress by decreasing the amounts of reactive oxygen species (ROS) associated with oxidative stress in oocytes. Consequently, this improvement in the quality and quantity of embryos was seen (13).

The blastocyst production rate findings align with the data reported by Al-Hafedh and Cedden (19) as well as Tripothi et al. (13). The potential reason for this outcome could be attributed to the inclusion of antioxidants, such as melatonin and vitamin C, in the culture medium. The potential benefits of antioxidants in relation to oocyte maturation and embryo development lie in their ability to enhance mitochondrial function and regulate reactive oxygen species (ROS) levels (20). It was concluded from this study that the addition of an antioxidant (melatonin or vitamin C) could improve IVM, IVF, and IVEP in sheep.

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