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Original Article

Effect of Culture Medium on *in vitro* Fertilization in Local Iraqi Ewes

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Abstract

in vitro fertilization (IVF) is considered to be the most important reproductive biotechnological method having great potential to accelerate genetic improvement in ruminants as well as for research on embryonic development. The present study aimed to investigate the effect of culture medium and the addition of natural and synthetic antioxidants on *in vitro* maturation (IVM), fertilization (IVF), and culture (IVC) in local Iraqi ewes. A total of 304 reproductive systems of local ewes were collected from a slaughterhouse in Fallujah, Anbar Province, Iraq from 3, January to 1, July 2021. The study was conducted in the Reproductive Biotechnology Laboratory, Department of Surgery and Theriogenology, College of Veterinary Medicine, University of Fallujah, Iraq. A total of 1368 oocytes were recovered from 608 ovaries surrounded by cumulus cells. The method of collection was aspiration and oocytes were divided into eight treatments. The first (T1), the second (T2), the third (T3), the fourth (T4), the fifth (T5), the sixth (T6), the seventh (T7), and the eighth (T8) treatments were MEM + Capparis spinosa extract 50µmol, MEM + Silymarin extract 100µmol, MEM + Coenzyme Q10 5 µmol, MEM only serves as a control, DMEM + Capparis spinosa extract 50 µmol, DMEM + Silymarin extract 100µmol, DMEM + Coenzyme Q10 5µmol and DMEM only serves as a control, respectively. The results indicated a significant difference ($P \leq 0.05$) between T5 (DMEM + Capparis spinosa extract) and other controls or treatments. Cultural medium DMEM with Capparis spinosa extract (as an antioxidant) presents the best results in the morula and blastocyst stage.

Keywords: Antioxidant, Culture Medium, DMEM, IVF, Local Iraqi Ewes, MEM

1. Introduction

in vitro fertilization (IVF) is considered to be the most important reproductive biotechnological approach which has a high potential to accelerate genetic improvement in ruminants and also for the research of embryonic development (1-3). Several factors can usually interfere with the fusion between sperm and ovum. Therefore, using *in vitro* embryo production (IVEP) would be desirable to transfer large numbers of embryos (4). The application of IVEP allows the study of embryonic development in mammals under a controlled environment. The recovered oocytes which are cultured *in vitro* are affected by several environmental factors and chemicals which lead to a significant decrease in the oocyte competence. The decrease in oocyte competence is a serious problem in the effectiveness of IVF which may be due to its sensitivity to the composition of the culture medium and the lack of natural antioxidants which only exist in the body of living animals (5, 6). The production of reactive oxygen species (ROS), OH, and H_2O_2 inside the follicle or Agarwal, Gupta (7) may lead to inducing

oxidative stress which reduces the quality of oocytes and consequently, resulting in a significant reduction of competence and ability of oocytes to develop *in vitro* (8, 9). ROS damages cell membrane lipids and nucleic acid and accelerates the apoptosis of cells (10). The culture medium plays an essential role in the success of IVF (11); therefore, adding an antioxidant to the medium may have a beneficial effect on IVEP. Therefore, the present study aimed to investigate the effect of culture medium and antioxidant addition on IVEP.

2. Materials and Methods

The study was conducted on 304 female reproductive systems recovered from local Iraqi ewes, collected from a slaughterhouse in Fallujah, Anbar Province, Iraq from 3, January to 1, July 2021. All recovered ovaries were transferred to the Reproductive Biotechnology Laboratory, Department of Surgery and Theriogenology, Faculty of Veterinary Medicine, the University of Fallujah within an hour with a cool box containing normal saline. The ovaries were cleaned and isolated and then placed in a sterile beaker. Oocytes were collected by aspiration using a 5 ml syringe containing a 3 ml oocyte washing medium with an 18-gauge needle. The recovered oocytes were transferred to a sterile Petri Dish with multiple wells (16 wells) containing MEM and DMEM culture media, then examined under an inverted microscope.

The collected oocytes were graded as good (grade A), fair (grade B), and poor (grade C) according to the presence of cumulus cells and uniform cytoplasm using. For more details about the different stages of oocyte and embryo development please see the supplementary figure.

2.1. Preparation of Antioxidant

Capparis spinosa extract (CSE) was prepared according to the method of Rios, Recio (12). Silymarin extract (SE) and coenzyme Q10 were also prepared according to the methods of Shiau, Shih (13), and Talevi, Barbato (14), respectively.

2.2. *in vitro* Maturation of Oocytes

The oocytes were examined according to Wani, Wani (15). Only grade A and B oocytes were taken and washed with MEM and DMEM media. The oocytes were cultured in the multi-well Petri Dish (16×wells) containing MEM medium. The antioxidant has been added to eight different treatments. The first (T1), the second (T2), the third (T3), the fourth (T4), the fifth (T5), the sixth (T6), the seventh (T7), and the eighth (T8) treatments were MEM + Capparis spinosa extract 50µmol, MEM + Silymarin extract 100µmol, MEM + Coenzyme Q10 5 µmol, MEM only serves as a control, DMEM + Capparis spinosa extract 50 µmol, DMEM + Silymarin extract 100µmol, DMEM + Coenzyme Q10 5µmol and DMEM only serves as a control, respectively. The Petri Dish was incubated at 38.5°C, 5% CO_2, and 90% relative humidity for 24 h. Then, the Petri Dish was examined under an inverted microscope. The presence of the first polar body was a good indicator of oocyte maturation (16).

2.3. Semen Collection

Semen was collected from two fertile rams with Electro ejaculation (ElectroJac 6, USA) pooled semen was diluted at a ratio of l:20 with MEM medium. Sperm capacitation was determined by adding heparin 10 pg/ml.

2.4. *in vitro* Fertilization

Semen contained heparin directly added to mature oocytes and incubated at 38.5ºC, 5% CO_2 at 90% relative humidity for 24 h. Then, the Petri Dish was examined under an inverted microscope.

The presence of the second polar body was a good indicator of successful fertilization. The number of zygotes was calculated.

2.5. *in vitro* Culture of Zygotes

Fertilized oocytes were cultured in different media with different antioxidant treatments and incubated at 38.5 °C, 5% CO_2, and 90% relative humidity. Embryonic development was observed every 24 h by refreshing 50% of the medium with a new one. Number of cleavedzygotes was observed after 48, 72, 120, 168, and 216 h for 2-cell, 4-cell, morula, blastocyst, and expanded blastocyst stages, respectively.

2.6. Statistical Analysis

Data analysis was performed using SAS software (version 9) (13).Randomized Design (CRD) was used

to show the effects of different factors of studied traits according to experimental factors (2×4). A significant difference was observed in the comparison of means using Duncan's Multiple Range test (17). Chi-squared test was used to compare the significant difference between percentages.

3. Results and Discussion

The recorded data revealed no significant difference between different treatments (antioxidants). However, a significant increase in the success of IVM, IVF, and IVC was observed in the treated group (DMEM with Capparis spinosa extract) compared with the control group and other treatments (Figures 1, 2). The superiority of natural antioxidantshas also been observed compared to synthetic antioxidants which may be due to increased adaptation of natural antioxidants with the living cells, while synthetic antioxidants may have a toxic effect which may lead to the reduction in the oocyte and early embryo competence. Previously published studies have shown that synthetic antioxidants have side effects such as cancer which limit their application (18). These results are consistent with Kharche, Goel (19) who claim thatantioxidants in different types in the maturation medium may improve the embryonic development of oocytes. The findings are also in line with Torres-Osorio, Urrego (20) who reported that the use of natural substances with antioxidant activity may improve the environment of *in vitro* maturation.

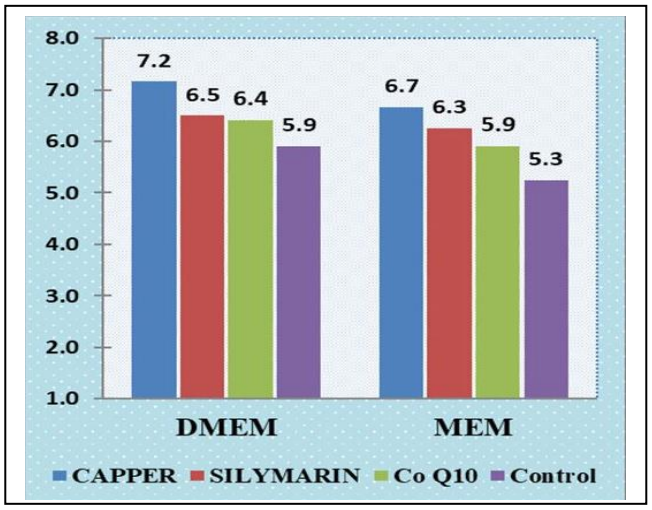

Figure 1. *in vitro* maturation rate

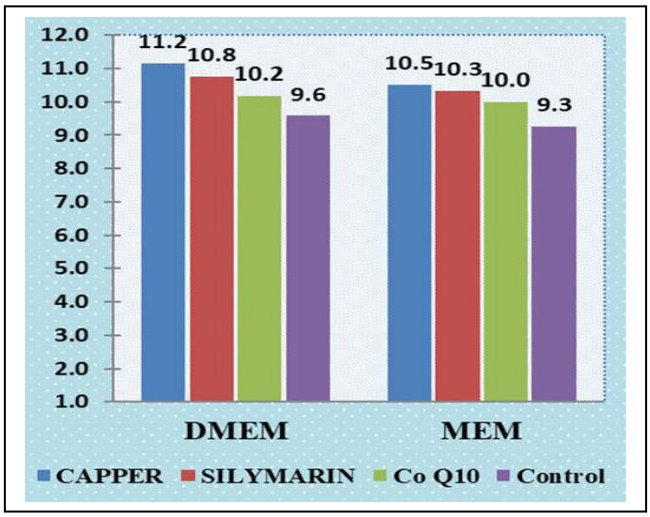

Figure 2. *in vitro* fertilization rate

Figures 3, 4, and 5 indicate no significant difference between treatment and its intervention in 2-cell, 4-cell, and 8-cell stages of embryonic development. Significant superiorityhas been observed in Capparis spinosa extract treatment in DMEM compared to control and other treatments using two types of culture media for all embryonic stages. The oxidative stress may harm the metabolic process of the cells and ultimately decrease the mean of embryo development *in vitro*; therefore, the culture medium should be strengthened with antioxidants (21). Several studies have shown that the presence of balanced antioxidants with ROS in a culture medium can be beneficial for embryonic development (22). The active ingredient in the used natural substance has been shown to contain phenolate, flavins, and tannins exist in the aqueous extract of Capparis spinosa and silymarin by improving the natural levels of enzymes that activate the key enzymatic function (23) which acts as a strong antioxidant with an active effect on signaling pathways in apoptosis and prevents morphological changes that increase apoptosis which stops the biological activity (24-26).

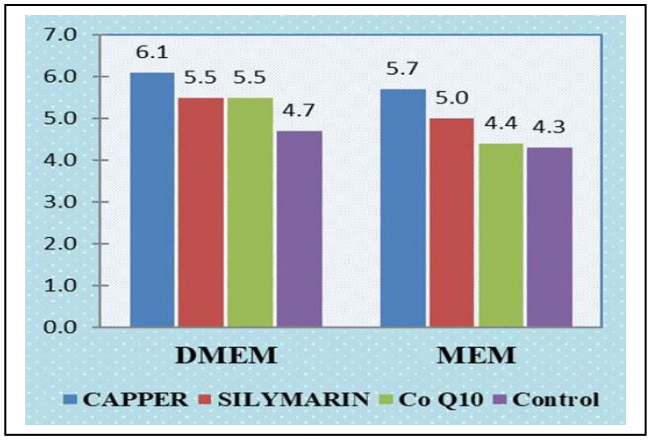

Figure 3. Percentages of 2-cell stage embryo

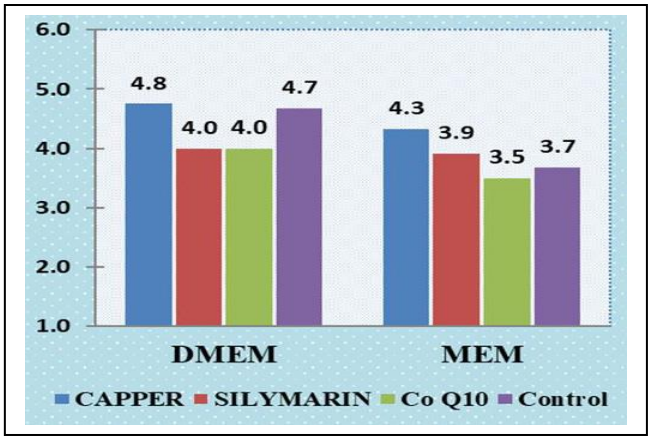

Figure 4. Percentages of 4-cell stage embryo

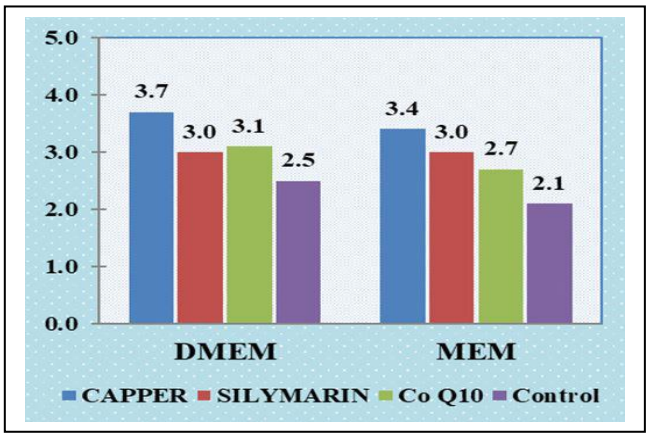

Figure 5. Percentages of 8-cell stage embryo

Tables 1 and 2 represent significant differences between the aqueous extract of Capparis spinosa compared to the control treatment in morula and blastocyst stages, while no significant difference was observed between Capparis spinosa, silymarin, and the Coenzyme (Q10) in both culture media. Also, the results showed the superiority of treatment between the natural and synthetic antioxidants. Cell damage may be due to the accumulation of a different type of ROS which reduces cell activity and destructs their membranes which ultimately interferes with growth and development *in vitro* (27, 28). These results are consistent with those of de Oliveira Santos, Borges (21) who proved that natural antioxidants are resistant to the types of ROS compared to synthetic ones. Natural antioxidants are also low-cost alternatives with high activity. Also, the results are in line with those of Kharche, Goel (19) who observed that adding antioxidants of different types to the maturation medium may improve the embryonic development of oocytes. These results may be similar to those of Gonçalves, Barretto (29) which reveal that access to a culture medium with synthetic antioxidants can weaken spermatozoa and normal nucleus growth and the formation of the blastocyst stage. These results may agree with those explained by Maside, Martinez (30) who stated that adding Q10 at different concentrations fails to affect oocyte development and percentage of fertilization, and the formation of morula and blastocyst. Also, these results are consistent with those of Natarajan, Bhawani (28) who observed that adding L-ascorbic as an antioxidant to the medium improved blastocyst formation rate.

Table 1. Effect of the type of culture medium and addition of natural and artificial antioxidants in morula stages

Addition	Media		Mean
	MEM	DMEM	
CAPER	0.52 ± 2.41^{a}	0.51 ± 2.33^{ab}	0.36 ± 2.37^{a}
Silymarin	0.45 ± 2.08^{ab}	0.52 ± 2.08^{ab}	0.34 ± 2.08^{ab}
Q10	0.45 ± 1.83^{ab}	0.46 ± 2.08^{ab}	0.32 ± 1.95^{ab}
Control	0.39 ± 1.41^{b}	0.39 ± 1.58^{ab}	0.27 ± 1.50^{b}
Mean	0.23 ± 1.93^{a}	0.23 ± 2.02^{a}	---

The means with different letters are significantly different from each other.
*($P \leq 0.05$)

Table 2. Effect of the type of culture medium and addition of natural and artificial antioxidants in blastocyst stages

Addition	Media		Mean
	DMEM	MEM	
CAPER	0.31 ± 1.42^{a}	0.31 ± 1.42^{a}	0.21 ± 1.42^{a}
Silymarin	0.32 ± 1.25^{ab}	0.25 ± 0.91^{ab}	0.20 ± 1.0^{ab}
Q10	0.23 ± 0.83^{ab}	0.2 ± 0.75^{ab}	0.18 ± 0.79^{b}
Control	0.23 ± 0.58^{b}	0.19 ± 0.50^{b}	0.14 ± 0.54^{b}
Mean	0.14 ± 1.02^{a}	0.13 ± 0.89^{a}	---

The means with different letters are significantly different from each other.
*($P \leq 0.05$)

Authors' Contribution

Study concept and design: Z. W. K. K.
Acquisition of data: Z. W. K. K. and Z. I. I.
Analysis and interpretation of data: F. A. A.
Drafting of the manuscript: Z. I. I.
Critical revision of the manuscript for important intellectual content: Z. I. I.
Statistical analysis: Z. W. K. K.
Administrative, technical, and material support: Z. W. K. K.

Ethics

The human study was approved by the University of Basrah, Basrah, Iraq ethics committee.

Conflict of Interest

The authors declare that they have no conflict of interest.

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