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Effect of Natural and Artificial Antioxidants Different Culture Media in the Maturation and Fertilization of Ewe Ova Fertilized Using Epididymis Sperm

H A Dakhel^{1*}, A A Munther², M S Al-Kubaisi³, A F Majeed⁴, A A Omar³, E M Kadim⁵

¹University Headquarter, University of Anbar, Ramadi, Iraq

²Monitoring of Trade and Finance, Ministry of Trade, Baghdad, Iraq

³College of Veterinary Medicine, University of Fallujah, Fallujah, Iraq

⁴Al Maarif University, Ramadi, Iraq

³College of Veterinary Medicine, University of Fallujah, Fallujah, Iraq

⁵Sunni Endowment Diwan, Baghdad, Iraq

Corresponding author's e-mail: hmeed.a.dakhil@uoanbar.edu.iq.

Abstract. The current study was based on Ewes slaughtered in the slaughterhouse of Fallujah /Anbar Governorate as a source for obtaining ovaries for the purpose of conducting the research. 370 female ovaries were collected and approximately 710 oocytes surrounded by different cumulus were obtained during the period from March 10, 2021, to July 1, 2021, where research was conducted in the Reproduction Techno. Labo. at the Veterinary Medicine/ University of Fallujah. This study was divided into eight treated groups; The First (T1) (Minimum Essential Media (MEM) + μmol 50 Capparis spinosa extract)(CSE); Second (T2) (MEM + 100 μmol Silymarin Extract)(SE); Third (T3) (MEM + 5 μmol Q10); Fourth (C1) (MEM + control); Fifth (T4) (Dulbecco's Modified Eagle Medium (DMEM) + 50 μmol CSE); Sixth (T5) (DMEM + 100 μmol SE); Seventh (T6) (DMEM + 5 μmol Q10); eighth (C2) (MEM + Control). The study results showed that no significant between the culture medium DMEM and the medium MEM in the medium of ripening, fertilization, and subsequent embryonic development, as the difference in the types of culture media had no significant role. Also, the results showed that there were no significant with statistical significance between the study treatments in the cleavage stage, fertilization, and the stages of mitosis of sheep oocytes.

1. Introduction

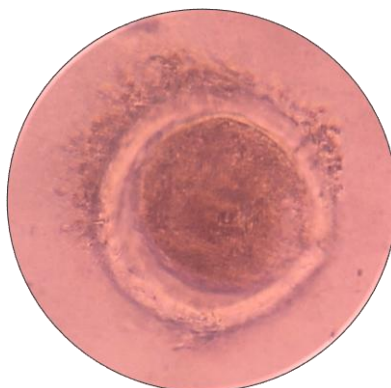
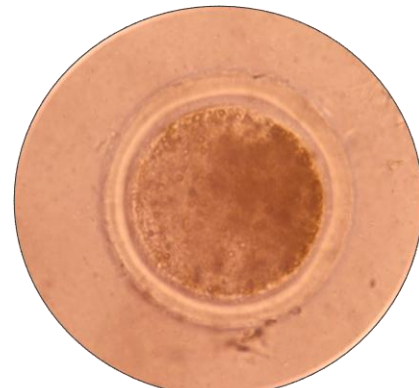
In vitro fertilization (IVF) is considered to be the most important reproductive biotechnological method having great potential to accelerate genetic improvement in ruminants as well as for research on embryonic development [1]. Low reproductive efficiency is a major concern for economic loss, as high reproductive efficiency is essential to maintain herd profitability [2]. Unfortunately, there are many factors influences that prevent the union of the ova with the sperm, these include the survival of sperm in the uterus, the migration of sperm within the female reproductive system, the morphological and physiological changes to sperm, and also the interaction of sperm with ova in the oviduct [3]. Therefore, the goal that farm animal breeders seek to improve reproductive efficiency. Nutrient supply [4], and other additive like, antioxidant [5] and exogenous hormones [6], also many reproductive



techniques, including embryo transfer and artificial insemination are methods have been used by many researchers to improve reproductive efficiency. In vitro fertilization (IVF) defined as steps that required for fertilizing oocyte, which involved growth, maturation, and fertilization the ova in vitro, also sperm capacitation is one of IVF steps [7]. This technology faces many difficulties that hinder researchers from advancing this technology, the most prominent of which are oxidizing agents, which are defined as a chemical reaction that transfer electrons from the oxidizing substance to the oxidizing agent. This allows this reaction to produce free radicals that may cause damage or cell apoptosis [8]. Cells in vivo have the ability to reduce oxidative damaged through many antioxidants' factors Cells in vivo have the ability to reduce oxidative damaged through many antioxidants' factors, such as the glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT), to protect themselves from ROS-induced cellular damage [9]. In vitro the antioxidant system that exists naturally within the body loses which made a challenge for embryo development due to the accumulation of oxidative agent [10]. Therefore, the media should be enriched with antioxidants factors that prevent defects in the development of embryos during incubation in vitro [11]. Several research have proven that addition of antioxidants to the medium in various natural and artificial types during incubation and fertilization media may improve the embryonic development [12]. In addition, it significantly improves blastocyst development, and this indicates that oxidizing agents have a restrictive effect during embryo implantation [13]. The research aimed to study effect of addition of natural and artificial antioxidant and the culture medium to maturation media in vitro fertilization on the embryonic development.

2. Materials and Methods

The research was conducted on 370 ovaries of Local Iraqi ewes. The samples were collected from the Slaughterhouse of Fallujah / Province of Anbar during the period from March 10, 2021, to July 1, 2021. Within one hour, and in cooled normal saline ovaries were transported by cool box to the Reproductive Biotech. Lab./ Veterinary Medicine/ University of Fallujah. The ovaries have been isolated from the attached tissue, washed by normal saline and placed in container. Oocytes were recovered via aspiration method using a needle 18-gauge with syringe filled 5 ml with 3 ml of complete medium without fetal calf serum. The aspirated Oocytes were transported to a Petri dish containing 16 wells, 2 oocytes in each well in a sterile hood Cabinet (Nuve, Turkey). The Oocytes were examined under inverted microscope (Olympus, China) to evaluate the Oocytes and graded as good ova (A), Fair ova (B), and Poor ova (C) as seen in Figures (1,2 and 3) respectively, according to [14] which were graded according to the presence of cumulus cells and uniform cytoplasm, only grades selected A and B. The Oocytes were calculated and incubated in CO₂ incubator (Mettler, Germany).

**Fig 1.** Oocytes grade (A)**Fig 2.** Oocyte grade (B)**Fig 3.** Oocyte grade (C)

2.1 Preparation of complete Media

Complete media were prepared for one week using culture medium Dulbecco's Modified Eagle's, Euroclone®, Italy (DMEM) or Minimum Essential Medium, Gibco, USA (MEM), (100IU/ml, 10µg/ml) Penicillin-Streptomycin, (25µg/ml) Amphotericin B and (10%) Fetal calf Serum, then it has been sterilized with Nalgene filter 0.22µm.

2.2 Preparation of Antioxidant

Silymarin Extract (SE) (Bulk Supplements, USA) has been prepared according to the method of [15], Where 5 g of Silymarin was weighed and placed in 200 ml of Dimethyl sulfoxide at a concentration of 100%. Coenzyme Q10 (Bulk Supplements, USA) has been prepared according to the method of [16], 50 mg/ml in (Chloroform) at a concentration of 40%. While Capparis Spinosa Extract (CSE) prepare according to the method of [17], where the fruits of Capparis spinosa L. were collected manually and then dried completely and then ground into a fine powder. 200 gram in 1 Liter of distilled water and a hotplate stirrer was used to mix the mixture well with water at a temperature of 30°C, then all the mixture was filtered using filter paper on a glass container and then sterilized using (Nalgene filter 0.45 µm) after that. Store in test tubes at 5°C. Collected Oocytes were divided into eight treatments after selection of grades A and B only. Maturation, fertilization and culture media and addition of antioxidant as follows; (C₁ and C₂) serve as control group, DMEM, MEM respectively. (T₁) DMEM+50µmol CSE, (T₂) DMEM+100µmol SE, (T₃) DMEM+5µmol Q10, (T₄) MEM+50µmol CSE, (T₅) MEM+100µmol SE, (T₆) MEM+5µmol Q10. The Petri dish with 16 wells (Nunc ® IVF, Germany) were incubated at incubated at 5% Co₂, 38.5°C and 90% relative humidity for 2 days. The indicated of maturation Oocytes by the presence of a first polar body. The matured Oocytes were calculated number.

2.3 Collecting sperm from the tail of epididymis

Semen was obtained from tail epididymis from slaughtered rams within one hour, Sperm prepared and matured according to [18]. Capacitation of the sperm was done by the addition of 10 pg/ml heparin of diluted semen at concentration (40x10⁶).

2.4 *In vitro* fertilization

Diluted and capacitated semen was added to the mature Oocytes directly and incubated at 38.5°C, 5% CO₂, and 90% relative humidity for 24 hours. The fertilized Oocytes (Zygote) were indicated by the presence of the 2nd polar body. The numbers of Zygote were calculated.

2.5 *In vitro* Culture of Zygote

Zygote was cultured in different treated culture medium and incubated at 5% CO₂, 38.5°C and 90% relative humidity. Half of medium were refreshed every 24 hours. Investigation of the stages different of division every 24 hours with inverted microscope equipped with a camera, to observe cleavage stages which include; 2 Cells after 1 day; 4 cells after 2 day; Morula after 5 days; blastocyst after 7 days, and hatched blastocyst after 9 days after fertilization [19].

2.6 Statistical Analysis

SAS-Statistical analysis system programs were used [20]. Data of this study analyzed the effect of difference factors for the traits under study according to treatment experiment (2 medium x 4 addition) according to complete Randomized Design. The significant difference between means of MDA measurement and cleavage stage. using Duncan multiple means range test [21]. The mathematical model: $Y_{ijk} = \mu + E_i + A_j + (EA)_{ij} + e_{ijk}$.

3. Results and Discussion

Several research have shown that the balanced of antioxidants and reactive oxygen species (ROS) in the culture media may be beneficial for the stage of embryonic development [22]. Studies have shown that active substances in the natural materials used, such as phenols, flavonoids, and tannins present in Capparis spinosa extract and Silymarin extract, lead to improving the natural levels of enzymes and activating their regulatory switches [23], which act as a powerful antioxidant that has an effective effect on apoptosis, cell death signaling pathways and prevent changes of morphological promoted by apoptosis that disrupt their vital functions [24].

Our results listed in the table below shown that were no significant between DMEM and MEM in terms of maturation, fertilization, and subsequent embryonic development, as the difference in the types of culture media had no role in process of maturation and fertilization of the ewe's Ova.

Also, the results showed no significant differences between treatments and control groups in maturation rate, fertilization rate and division stages of ewe's Ova. The non-significant between treatments and control groups confirmed the important role of natural antioxidants in contrast of its availability & cheapest price as compared with artificial antioxidants. The results are agreed with Torres-Osorio [25] who's reported that the use of natural antioxidant materials improves Ova maturation in vitro. Mishra [26] reported that adding some antioxidants in different concentrations to the medium didn't affect the maturation rate, however it had an important role in improving the division of Ova and reaching Morula stage.

The results agreed with our previous study [4], we found that adding various types of antioxidants to maturation media may improvement the oocytes embryonic development without significant differences. Oliveira Santos [27] also showed that natural antioxidants are highly efficient against ROS as compared to synthetic ones as well as being a low-cost alternative with high effectiveness. Kharche and Ozturkler [12, 28] shown that adding antioxidants didn't affect the stages of maturation and cell division (2 cells, 4 cells) of sheep Ova as compared with control treatment. The results also showed agreement with Jang et al., [29], as their study aimed to evaluate the effect of

Silymarin in preventing or alleviating oxidative damage caused by nitric oxide through its effect on the apoptosis and development of bovine Ova. The results of the study showed no significant difference, but an improvement in Ova maturation. The development of Morula is believed to be a result from the secretion of fetal nutritional factors and the suppression of free radicals resulting from oxidative stress.

Regarding the artificial addition, the results agreed with Maside [30], as a compound for adding CoQ10 at different concentrations influenced the stages of Ova development and the fertilization rate but did not affect Morula stage.

Table 1. Effect of the type of culture media, natural and synthetic antioxidants materials in the embryo cleavage

Treatment		Trait							
		Oocyte	Matured	Fertilized	2Cell	4Cell	8Cell	Morula	Balastocyst
Media	M1	14.1	10.2	6.29	5.45	4.16	3.25	2.00	1.08
	M2	14.2	10.0	5.95	4.79	3.83	3.04	2.20	1.12
Significant		N. S	N. S	N. S	N. S	N. S	N. S	N. S	N. S
Additives	T1	14.2	10.75	7.00	6.00	4.91	3.83	2.58	1.500
	T2	14.4	10.33	6.41	5.50	4.33	3.41	2.33	1.333
	T3	14.1	10.16	5.91	4.91	3.75	2.91	2.00	0.916
	C	14.0	9.41	5.16	4.08	3.00	2.41	1.50	0.666
Significant		N. S	N. S	N. S	N. S	N. S	N. S	N. S	N. S
Interaction	T1	14.00	11.00	7.16	6.33	5.16	4.00	2.33	1.333
	T2	14.50	10.66	6.50	5.83	4.33	3.50	2.33	1.500
	T3	14.16	10.16	6.16	5.50	4.00	3.00	1.83	0.833
	C1	14.00	9.33	5.33	4.16	3.16	2.50	1.50	0.666
	T4	14.50	10.50	6.83	5.66	4.66	3.66	2.83	1.666
	T5	14.33	10.00	6.33	5.16	4.33	3.33	2.33	1.166
	T6	14.16	10.16	5.66	4.33	3.50	2.83	2.16	1.000
	C2	14.00	9.50	5.00	4.00	2.83	2.33	1.50	0.666
Significant		N. S	N. S	N. S	N. S	N. S	N. S	N. S	N. S
Mean Trait		14.2	10.1	6.12	5.12	4.00	3.14	2.10	1.10
SEM		0.992	0.847	0.763	0.651	0.525	0.418	0.240	0.143

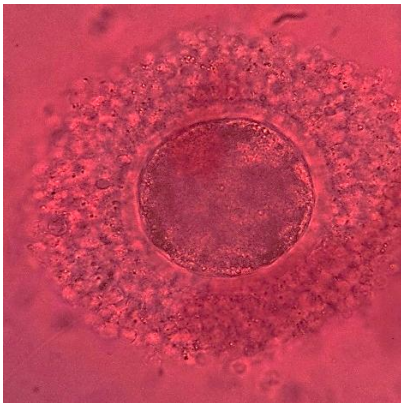


Fig 4. Mature Oocyte

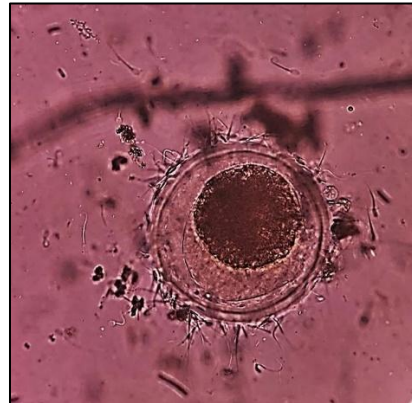


Fig 5. Fertilized Oocyte

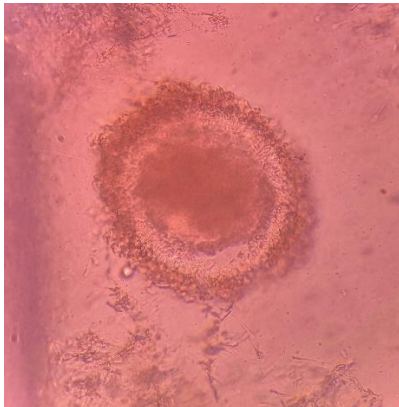


Fig 6. 2-Cell Stage

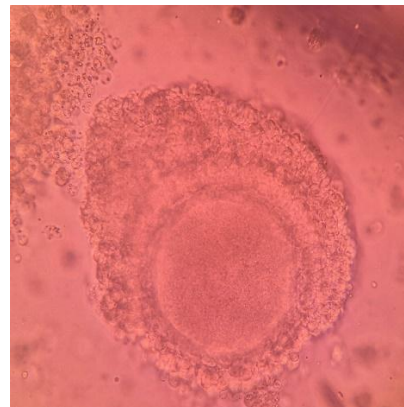


Fig 7. 4 - Cell Stage

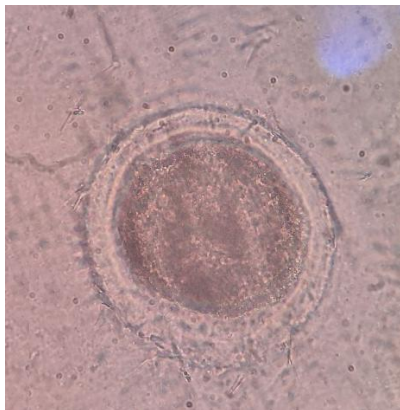


Fig 8. 8-Cell Stage

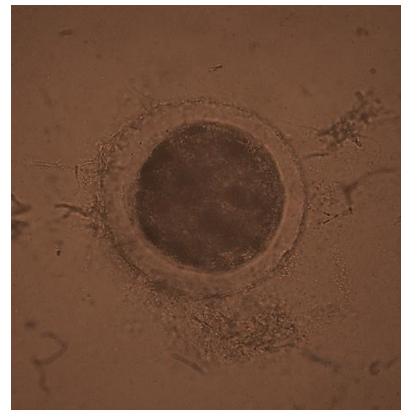
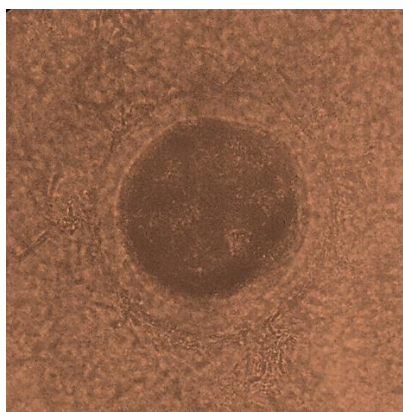
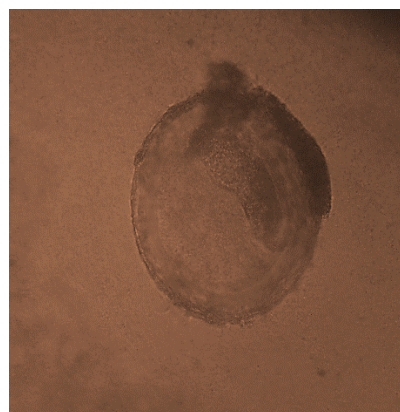


Fig 9. 16 - Cell Stage

**Fig 10.** Morula Stage**Fig 11.** Blastocyst Stage

4. Conclusion

It was concluded from the results of the study that there is no effect of DMEM and MEM culture medium on maturation rate, fertilization rate, and embryonic development. The results also showed that there was no effect of the additives on the division, fertilization, and division stages.

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