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EFFECT OF ADDING NATURAL AGAINST SYNTHETIC ANTIOXIDANTS ON OVINE OOCYTE MATURATION, MALONDIALDEHYDE LEVELS, AND IN VITRO FERTILIZATION

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Abstract

A total of 608 ewe ovary samples were obtained from the abattoir and transported in a cool box containing normal saline. Oocytes (n=1368) were collected via aspiration and divided into eight treatment groups: T1 comprised Minimum Essential Media (MEM) + µmol 50 Capparis spinosa extract (CSE); T2 comprised MEM + 100 µmol Silymarin Extract (SE); T3 the MEM + 5 µmol Q10; C1 the MEM + control; T4 the Dulbecco's Modified Eagle Medium (DMEM) + 50 µmol CSE; T5 the DMEM + 100 µmol SE; T6 the DMEM + 5 µmol Q10; and C2 the MEM + Control. Increases were recorded for T1 and T5, the treatment with Silymarin extract (T2) and (T6) in all stages of embryonic development in vitro cleavage. There was a significant decrease (P<0.01) in MDA levels for the CSE and SE treatments in the same media as compared to the Q10 and control. In conclusion, treatment with CSE and SE improves the cleavage stage and development of morula and blastocysts though there was a decrease in malondialdehyde levels in the cultural media.

Keywords: Synthetic, Antioxidant, Reproduction, IVF, Sheep.

تأثير إضافة مضادات الأكسدة الطبيعية او الصناعية لوسط الإنضاج والإخصاب المختبري لبويضات النعاج ومستوى MDA

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الخلاصة

تم جمع 304 جهاز تناسلي أنثوي من المجزرة خلال الفترة من 1 كانون الثاني 2021 الى 1 تموز 2021، تم نقل العينات بحاوية مبردة تحتوي على محلول ملحي متعادل. حُصل منها على 1368 بويضة محاطة بالخلايا الركامية. قسمت الدراسة الى ثمانِ معاملات إذ كانت الاولى (T1، MEM + مستخلص الكُبر)؛ الثانية (T2، MEM + مستخلص الكلغان)؛ الثالثة (T3، Q10 + MEM)؛ الرابعة (C1، MEM + السيطرة)؛ الخامسة (T4، DMEM + مستخلص الكُبر) (T5، DMEM + مستخلص الكلغان) (T6، Q10 + DMEM) (C2، DMEM + السيطرة). أظهرت نتائج الدراسة الى وجود تحسن معنوي في معاملة مستخلص الكبر (T1) و(T4) ومعاملة مستخلص الكلغان في الوسطين (T2) و(T5) في جميع مراحل تكوين الأجنة مختبريًا مقارنة بباقي المعاملات. وأيضا لوحظ وجود انخفاض معنوي في مستوى (MDA) في معاملة CSE وSE في نوعي الوسط الزرعي مقارنة مع معاملة Q10 ومعاملة السيطرة. نستنتج من نتائج الدراسة أن المعاملة بـ CSE وSE حسنت من مراحل الانقسام وتكون التويتة والكيسة الأريمية، على الرغم من ذلك وأيضا انخفض مستوى MDA في الوسط الزرعي.

كلمات مفتاحية: مضادات صناعية، مضادات الاكسدة، الاخصاب المختبري، الاغنام.

Introduction

Improving reproductive performance is one of the targets for animal producers. Several methods, such as feed additives (22) and hormones (35), assisted reproductive technology (ART) (27 and 33), and in vitro fertilization (IVF), which is regarded as one of the biotechnologies, are used to increase reproductive efficiency in farm animals (13). Successful IVF includes oocyte maturation, in vitro fertilization, and in vitro culture (8 and 19). This technology treats reproductive issues by increasing the number of lambs with high genetic traits and minimizing generation intervals that accelerate genetic improvement programs (10). Oxidative stress is one of the hurdles impeding the progress of IVF (7), as it can upset the balance between the production of reactive oxygen species (ROS) and antioxidant defenses (3). This reaction produces free radicals that damage the cells (29) and the ability of the body to reduce damage from antioxidants (6). The antioxidant system in the body protects against damage resulting from free radicals in various biological molecules (25). Oocytes and sperm may be

subject to damage via oxidative factors caused by ROS that are present in the follicles and uterine tubes (12).

It has been shown that antioxidants introduced to the culture media prevents anomalies in embryonic development during pregnancy (1). Also, the addition of a natural or synthetic antioxidant to the maturation media improves embryonic development, increases intracytoplasmic oocyte glutathione concentrations, improves development rates, and enhances embryonic conditions during the cleavage division stage (4, 17 and 18). This study aimed to show the effect of adding natural or synthetic antioxidants on the maturation and in vitro fertilization media of Iraqi ewes oocytes, and to determine the level of malondialdehyde (MDA) in the cultural media.

Materials and Methods

The study was conducted on 608 ovaries of local Iraqi ewes. The samples were obtained from the abattoir of Fallujah/Al-Anbar province from January 3 to July 1, 2021 and transported in a cool box containing normal saline within one hour to the Reproductive Biotechnology Lab., College of Veterinary Medicine, University of Fallujah. The ovaries were separated from attached tissue, cleaned with normal saline and placed in a sterile glass container. Oocytes were recovered via aspiration using an 18-gauge needle with 5ml syringe filled with 3ml of DMEM (Euroclone®, Italy) or MEM (Gibco, USA) supplemented with penicillin-streptomycin (100 U/ml, 10µg/ml) and amphotericin B (25µg/ml). The aspirated oocytes were moved to a Petri dish containing 16 wells. Then, two oocytes were placed in each well in a sterile hood Cabinet (Nuve, Turkey). The oocytes were evaluated under an inverted microscope (Olympus, China) and graded according to (37) as good (A), fair (B), and poor (C) (Figures 1, 2 and 3, respectively) based on the presence of cumulus cells and uniform cytoplasms. They were calculated and incubated after the addition of the antioxidants.

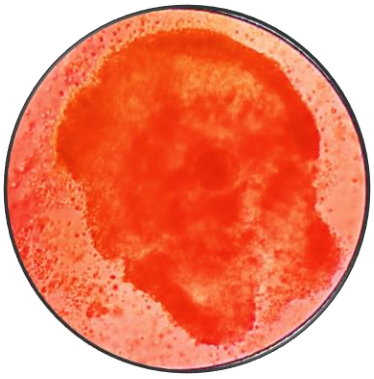

Figure 1: Oocyte grade A.

Figure 2: Oocyte grade B.

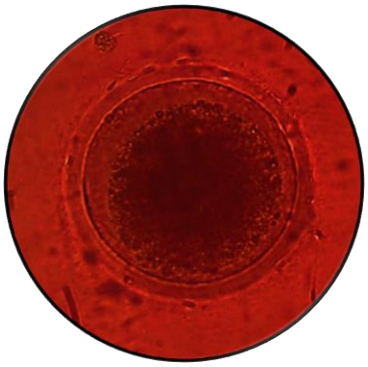

Figure 3: Oocyte grade C.

Preparation of Antioxidants: Silymarin extract (SE) (Bulk Supplements, USA) was prepared according to the method of (34) and 5 g were weighed and placed in 200 ml of dimethyl sulfoxide at a concentration of 100%. Coenzyme Q10 (Bulk Supplements, USA) was prepared according to (36) with 50 mg/ml in chloroform at a concentration of 40%. The capparis spinosa extract (CSE) was prepared according to (31), where the fruits of the capparis spinosa L were collected, dried completely, and ground into a fine powder. Following that, 200 gm 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 the mixture was filtered using filter paper on a glass container and sterilized using a Nalgene 0.45 µm filter and stored in test tubes at 5°C. The collected oocytes were divided into eight treatments after selection of grades A and B only. The maturation, fertilization and culture media and addition of antioxidant was as follows: DMEM and MEM for maturation, fertilization, and culture media. The treatments were divided into eight groups: the 1st group (T1) DMEM + 50 µmol CSE; the 2nd group (T2) DMEM + 100 µmol SE; the 3rd group (T3) DMEM + 5 µmol Q10; the 4th group (C1) DMEM + Control; the 5th group (T4) MEM + 50 µmol CSE; the 6th group (T5) MEM + 100 µmol SE; the 7th group (T6) MEM + 5 µmol Q10; and the 8th group (C2) MEM + Control. The Petri dish with 16 wells (Nunc ® IVF, Germany) were incubated at 38.5°C, 5% Co2 and 90% relative humidity for 24 hours. Oocytes maturation was indicated by the presence of a first polar body. The matured oocytes were calculated.

Preparation of Media: Complete media were prepared using culture media DMEM and MEM to which fetal bovine serum (10%), penicillin-streptomycin (100 IU/ml, 10µg/ml), and amphotericin B (25µg/ml) had been added. Then it was sterilized with a Nalgene 0.22 µm filter.

Semen Collection: Semen was collected from two fertile rams via electro ejaculator (ElectroJac 6, USA) and the pooled semen diluted 1:20 with MEM media. Capacitation of the sperm was done by adding 10 pg/ml heparin of diluted semen at a concentration of $40x10^6$.

In Vitro Fertilization: Diluted semen with heparin was added directly to the mature oocytes and incubated with oocytes 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 number of zygotes was calculated.

In Vitro Culture of Zygotes: The zygotes were cultured in different treated complete media and incubated at 38.5°C, 5% Co2, and 90% relative humidity. Half of the complete media were refreshed every 24 hours. The different stages of division were examined every 24 hours with an inverted microscope equipped with a camera, to observe cleavage stages, which included 2 cells after 24 hours, 4 cells after 48 hours, morula after 120 hours, blastocysts after 168 hours, and hatched blastocysts after 216 hours after fertilization.

Measurement of MDA: The MDA was measured to identify the level of oxidative stress resulting from lipid peroxidation. A total of 48 samples were collected in February, April, and June from different complete media treated with the antioxidants and control or during 216 in vitro culture. The samples were centrifuged at 3000 rpm for 15 minutes, and the supernatant fluid harvested and preserved at -20 °C till MDA analysis according to (5).

Statistical Analysis: Statistical analysis was done using the SAS statistical analysis system program (32). The data analyzed the effect of different factors for the traits under study according to treatment experiment (2 medium x 4 addition) based on the complete randomized design. The significant difference between means of MDA measurement and cleavage stage was evaluated using the Duncan multiple means range test (9). The mathematical model formulated was $Yijk = \mu + Ei + Aj + (EA)ij + eijk$.

Results and Discussion

The results in Table 1 show no difference (P>0.05) in the maturation percentages of the oocytes. The highest mean for oocyte maturation was in the first group at 78.3% followed by the second group at 74.5%. Figure 1 shows the mature oocyte with the first polar body. The fertilization rate was highest in T3 at 70.9% followed by C1 (control). Figure 2 shows a fertilized oocyte with the second polar body. Also, the results in the same table show that production at the morula stage was higher in T4, T5, T1, and T6. Figures 6, 7, 8, 9, 10, and 11 show the different stages of embryonic development. The presence of blastocysts in different treated groups showed higher percentages in T2, T1, and T3 compared with other treatments, and there were significant differences (P<0.05) between them. Figure 8 shows the blastocyst stage.

Table 1: Effect of the types of complete medium and the addition of natural or synthetic antioxidants in the formation stages of the laboratory embryos.

Median	Additive	Oocyte	Matured	Fertilized	Morula	Blastocyst
DMEM	**T1 CAPER**	171	(78.3%) 134	86 (64.1%)	(31.9%) 27	**17 (62.9%)**
	T2 Silymarin	173	(74.5%) 129	81 (62.7%)	(27.1%) 22	**15 (68.1%)**
	T3 Q 10	172	(70.9%) 122	80 (65.5%)	(28.7%) 23	**10 (43.7%)**
	C1	168	(68.4%) 115	79 (68.6%)	(22.7%) 18	**7 (38.8%)**
MEM	**T4 CAPER**	173	(72.8%) 126	80 (63.4%)	28 (35%)	**17 (60.7%)**
	T5 Silymarin	171	(72.5%) 124	75 (60.4%)	24 (32%)	**(45.8%) 11**
	T6 Q 10	170	(70.5%) 120	71 (59.1%)	(30.9%) 22	**(40.9%) 9**
	C2	170	(65.2%) 111	63 (56.7%)	(25.3%) 16	**(37.5%) 6**
Total		**1368**	**981**	**615**	**180**	**92**
Kay square value (x^2)		**NS 0.262**	**NS 0.307**	*** 0.825**	*** 4.062**	*** 4.955**

.(P≤0.05) *

(T) Treatment; (C) Control; (DMEM) Dulbecco's Modified Eagle Medium; (MEM) Minimum Essential Media

Table 2 shows the levels of MDA in different treatments over the three-month study period. Changes in the levels indicate the amount of lipid peroxidation resulting from the harmful effects of free radical attacks on the oxidation of unsaturated fatty acids. There was a decline in the level of MDA in the DMEM and MEM mediums at different periods in the first and second groups compared to the control group at the 5% significant difference level. The DMEM medium showed the best results in T1 compared to the others treatments. Also, there was a significant decrease in MDA levels in February and June between T4 and T5 compared to T3 and the control group.

Table 2: Effect of complete medium types and addition of natural or synthetic antioxidants and their interactions on MDA levels.

Medium	Additive	Period			Significance
		February	April	June	
DMEM	T1 CAPER	4.11± 15.11 AB ab	0.29± 11.02 B c	3.50± 23.22 A abc	**
	T2 Silymarin	1.92± 20.12 B ab	3.03± 12.48 C c	3.27± 27.30 A ab	**
	T3 Q 10	4.14± 35.41 A a	2.55± 33.58 A ab	1.26± 27.55 A ab	NS
	C1	5.89± 23.27 B ab	9.51± 36.81 A a	7.70± 30.04 AB ab	**
MEM	T4 CAPER	1.81± 10.79 B b	7.70± 19.25 A bc	3.50± 7.35 B c	**
	T5 Silymarin	6.70± 26.22 A ab	0.29± 11.96 B c	8.34± 17.32 B bc	**
	T6 Q 10	2.51± 29.46 A ab	2.10± 35.00 A ab	5.20± 35.40 A a	NS
	C2	5.29± 32.02 B ab	1.52± 42.00 A a	1.51± 25.67 B ab	**
Significance	--	*	**	*	---

Different small vertical letters and capital horizontal letters indicate significant differences among themselves

Small superscripts indicate significant difference (P<0-05) between different treatments

*(P≤0.05 , **(P≤0.01)

(T) Treatment; (C) Control; (DMEM) Dulbecco's Modified Eagle Medium; (MEM) Minimum Essential Media

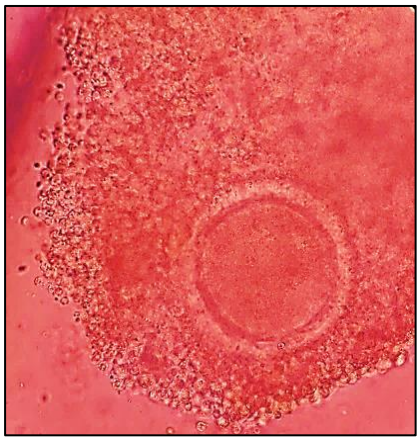

Figure 4: Mature Oocyte.

Figure 5: Fertilized Oocyte.

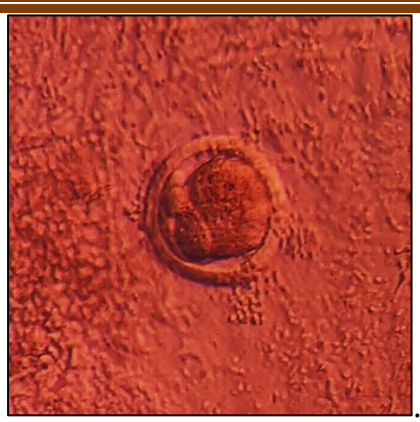

Figure 6: 2-Cell Stage.

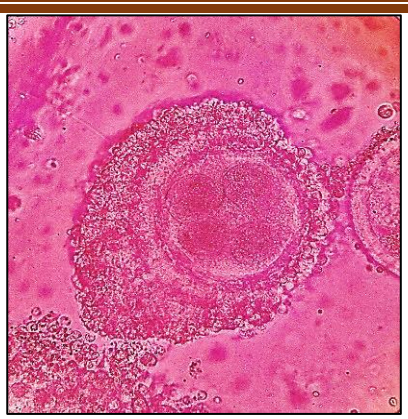

Figure 7: 4-Cell Stage.

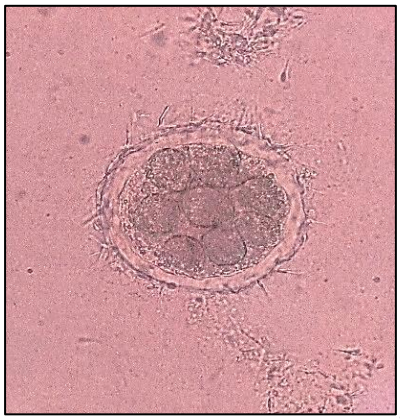

Figure 8: 8-Cell Stage.

Figure 9: 16-Cell Stage.

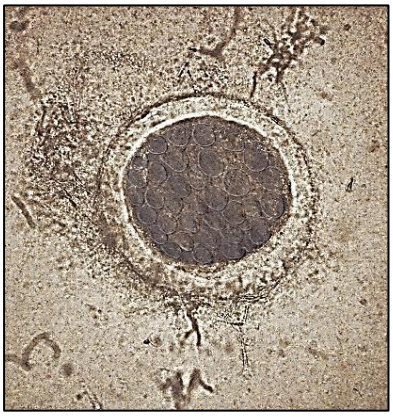

Figure 10: Morula Stage.

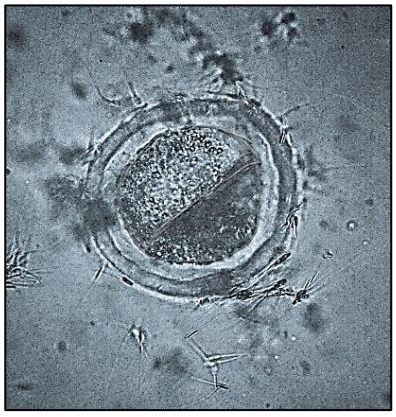

Figure 11: Blastocyst Stage.

The higher oocyte maturation rates for the CSE and SE treatments might be due to their flavonoid and phenol contents that act as an antioxidant and improved the media (39). Ghani (11) found that the CSE had a significant effect in treating seminal fluid due to its high flavonoids content. The superiority of the DMEM over the MEM media in terms of higher oocyte maturation rates is similar to that reported by (24 and 26). This study also agreed with (23) that treatment with SE reduces the number of programmatic cell deaths (apoptosis) due to its effect on oxidative factors. Rahimi Madiseh (30) observed that SE treatment may increase the number of motile sperm and decrease abnormal ones due to the action of antioxidants via inhibition, remove free radicals, and finally protect sperm from oxidation.

The superiority of treatments T3 and T4 in fertilization rates might be due to the positive effect of Q10 on the fertilization media. These results agree with (40) who reported that Q10 plays a role in protecting oocytes from oxidative stress that cause disturbances in mitochondrial functions. The results are also in agreement with (21) who observed that Q10 has no effect on cultural media during embryonic development till the formation of the blastocyst stage.

The levels of MDA increased from lipid peroxidation caused by the harmful effects of the free radical invasion leading to the oxidation of unsaturated fatty acids. This results in tissue damage and the failure of antioxidants to protect from oxidant factors (38). Oxidative factors act by invading the constituents of biological cells leading to harmful effects on apoptosis and damage to proteins and their functions (28).

Kalantari (15) explained that CSE contains high levels of phenols, quercetins, and other substances that are active as antioxidants. There is evidence of a significant decrease in MDA in mice treated with CSE due to its chemical composition that directly eliminates reactive oxygen species (ROS) by increasing the production of the cellular antioxidant glutathione (16). It has been observed that protection of the body from the harmful effects of increased levels of MDA are associated with the removal of free radicals via CSE through the high content of phenols which increases antioxidant enzymes and the glutathione content in cells (15). Jamalan (14) reported that treatment with rutin and quercetin which is one of the flavonoids present in high amounts in CSE significantly reduces MDA concentrations in humans. Similar observations have been made by (20) who noted that adding CSE to the cultural media as antioxidants markedly reduced MDA levels. These results of the SE antioxidant treatment are in line with (2) who reported that treating seminal fluids with SE leads to a significant decrease in MDA levels compared to the control groups.

Conclusions

The study showed that CSE, SE, and Q10 had beneficial effects for the maturation, fertilization, and cultural media in improving IVF outcomes and in reducing MDA levels.

Supplementary Materials:

No Supplementary Materials.

Author Contributions:

Author 1: writing original draft preparation; Author 2: methodology, laboratory analysis; preparing and checking figures, and approving the published version of the manuscript.

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Informed Consent Statement:

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Data Availability Statement:

No Data Availability Statement.

Conflicts of Interest:

The authors declare no conflict of interest.

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