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Reducing Oxidative Stress in *in Vitro* Rat Embryo Culture through Antioxidant Supplementation

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تقليل الإجهاد التأكسدي في زراعة أجنة الفئران في المختبر *(in vitro)* من خلال التكميل بمضادات الأكسدة

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Abstract:
Oxidative stress is considered one of the major limitations during mammalian embryo culture due to an imbalance between the generation of ROS and the ability of the developing embryo to resist this oxidative stress. This study aimed to determine whether antioxidants supplementation by ascorbic acid and resveratrol enhances the redox state and developmental capability of rat embryos when cultured in laboratory settings in Libya. Rat embryos were collected after superovulation of Wistar Albino rats and cultured using KSOM-R medium. The obtained embryos were randomly assigned into control group (without antioxidants), ascorbic acid (50 µM and 100 µM), and resveratrol (0.25 µM and 0.50 µM). Embryos' development was studied for 96 hours of culture, besides measuring oxidative stress factors such as ROS. Morphological images were taken for selected stages of development. Antioxidants supplementation significantly increased cleavage rate and beyond 2-cell stage and decreased degeneration rate of embryos. The greatest protection from the three concentrations of resveratrol was achieved by 0.50 µM resveratrol, with the highest percentage of blastocyst production and least ROS level. A negative relationship existed between ROS levels and developmental ability for all groups. This study concludes that supplementation with antioxidants, especially resveratrol, helps to reduce oxidative stress and improve embryonic development in the lab environment in Libya, hence optimizing the redox state of culture medium for mammalian embryos' study.

Keywords: oxidative stress; embryo culture; antioxidants; resveratrol; ascorbic acid; rat embryos.

الملخص:
يُعدّ الإجهاد التأكسدي أحد أهمّ المعوقات التي تواجه زراعة أجنة الثدييات، وذلك نتيجةً لاختلال التوازن بين إنتاج أنواع الأكسجين التفاعلية وقدرة الجنين النامي على مقاومة هذا الإجهاد. هدفت هذه الدراسة إلى تحديد ما إذا كان تناول مضادات الأكسدة، مثل حمض الأسكوربيك والريسفيراترول، يُحسّن حالة الأكسدة والاختزال وقدرة نمو أجنة الفئران عند زراعتها في المختبر في ليبيا. جُمعت أجنة الفئران بعد تحفيز الإباضة لدى فئران ويستار البيضاء، وزُرعت باستخدام وسط KSOM-R. وُزّعت الأجنة المُستحصلة عشوائيًا على ثلاث مجموعات: مجموعة ضابطة (بدون مضادات أكسدة)، ومجموعة حمض الأسكوربيك (بتركيز 50 و100 ميكرومولار)، ومجموعة الريسفيراترول (بتركيز 0.25 و0.50 ميكرومولار). دُرست مراحل نمو الأجنة لمدة 96 ساعة من الزراعة، بالإضافة إلى قياس عوامل الإجهاد التأكسدي، مثل أنواع الأكسجين التفاعلية. والتقطت صور مورفولوجية لمراحل مُختارة من النمو. أدى تناول مضادات الأكسدة كمكمل غذائي إلى زيادة ملحوظة في معدل انقسام الأجنة وتجاوز مرحلة الخليتين، وانخفاض معدل تدهورها. وقد حقق تركيز

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0.50 ميكرومول من الريسفيراترول أعلى مستوى من الحماية من بين التراكيز الثلاثة، حيث سجل أعلى نسبة إنتاج للكيسة الأريمية وأقل مستوى من أنواع الأكسجين التفاعلية. ولوحظ وجود علاقة عكسية بين مستويات أنواع الأكسجين التفاعلية وقدرة النمو في جميع المجموعات. وخلصت هذه الدراسة إلى أن تناول مضادات الأكسدة، وخاصة الريسفيراترول، كمكمل غذائي يساعد على تقليل الإجهاد التأكسدي وتحسين نمو الأجنة في بيئة المختبر في ليبيا، مما يُسهم في تحسين حالة الأكسدة والاختزال لوسط الاستنبات لدراسة أجنة الثدييات.

الكلمات المفتاحية: الإجهاد التأكسدي؛ زراعة الأجنة؛ مضادات الأكسدة؛ ريسفيراترول؛ حمض الأسكوربيك؛ أجنة الفئران.

1-Introduction

In vitro embryo culture is a key technique in reproductive and developmental biology, toxicology, and assisted reproduction research. It allows for the study of cellular and molecular mechanisms involved in the process of embryogenesis and the creation of an experimental system for reproductive technologies [1]. On the other hand, in vitro culture of embryos creates an artificial environment differing from physiological conditions in terms of composition of the culture medium, gaseous mixture, temperature, and illumination [2].

An important problem in the process of in vitro embryo culture is the occurrence of oxidative stress. Reactive oxygen species (ROS) produced mainly as a result of cellular metabolism and mitochondrial respiration have physiological importance as signaling molecules. An excessive level of ROS generation might surpass the natural antioxidant potential of the cell resulting in damage to lipids, proteins, and DNA, dysfunction of mitochondria and stimulation of apoptosis [3,4]. As a result, high oxidative stress leads to lower rate of cleavage and development, as well as failure in blastocyst formation in mammalian embryos [1,5]. Redox balance is thus critical for embryo culture success.

One possible approach to modulate the amount of ROS within the system and provide a protection for the developing embryos from their adverse effects could be supplementation of the culture media with exogenous antioxidants [2,6]. Vitamin C, or ascorbic acid, which is one of the soluble antioxidants with free radical scavenging activity, showed its positive effects on embryo development in animal studies [7,8]. Resveratrol, a natural compound of the polyphenols group, with antioxidant and cytoprotective effects, has been studied for oocyte and embryo culture [9]. However, the antioxidant effects depend on the dose and are species-specific [5,10].

The rat (*Rattus norvegicus*) is a widely accepted model organism for reproductive studies and research owing to its known reproductive physiology, fecundity and ability to conduct embryo culture experiments [11]. The rat embryos have endogenous antioxidant mechanisms that include superoxide dismutase, catalase and glutathione peroxidase [3]. The rising usage of laboratory animals such as Wistar Albino rats as models for reproductive studies in Libya makes the development of reproducible approaches possible in reproductive research [12]. However, there is a lack of literature on the use of antioxidants to optimize in vitro culturing of rat embryos in Libyan laboratories [13].

Thus, the aim of this study was to test the influence of ascorbic acid and resveratrol supplements on oxidative stress and embryo development competence during in vitro culture. Our hypothesis is that supplementation with proper antioxidants will decrease oxidative stress and increase the results of embryonic development in experimental conditions in Libya.

2. Materials and Methods

2.1 Study Location and Laboratory Setting

This experimental study was carried out in the Department of Biology, Faculty of Education, Bani Waleed University, Libya. All experiments were carried out in the research laboratory of the university from January to June 2025. Laboratory equipment for conducting the experiments consisted of standard equipment for embryo culture such as CO_2 incubator, stereomicroscope, and laminar flow hood for aseptic work.

2.2 Ethical Clearance and Care of Animals

Guidelines for the care and use of laboratory animals were strictly followed in maintaining animals. Rats were kept in polypropylene cages under controlled environmental conditions such as temperature 22 ± 2°C, relative humidity 50 ± 10%, and 12 hours light and dark cycles (07:00-19:00). Animals were allowed free access to a standard pelleted rat diet and drinking water.

2.3 Experimental Animals

The experiment was carried out on a total of 20 female and 10 male Wistar Albino rats that were 10-12 weeks old and weighed between 180-220 g (see Figure 1). The female rats were nulliparous and had shown at least two consecutive estrous cycles prior to superovulation through vaginal smear test each day. Males were sexually mature and proven fertile.

Figure 1: Adult Wistar albino rats used in the experimental study

2.4 Superovulation and Mating

The female rats underwent superovulation by intraperitoneal injection of 20 IU pregnant mare serum gonadotropin (PMSG; Folligon, Intervet) followed by injection of 30 IU human chorionic gonadotropin (hCG; Chorulon, Intervet) after 48 hours, according to the procedure already established [12]. Mating immediately followed the administration of hCG, whereby individual female rats were put with fertile males at a 1:1 ratio. Proof of successful mating was evident the next morning by the detection of vaginal plugs and spermatozoa in vaginal smears. The day was considered Day 0 of development.

2.5 Embryo Recovery

Female rat was killed through cervical dislocation on day 1 (18-20 h after hCG administration). Oviducts were removed and rinsed with HEPES-buffered KSOM solution using a 30-gauge needle. Zygotes at one-cell stage were retrieved, washed three times in freshly prepared HEPES-KSOM solution, and evaluated for morphological integrity under a stereomicroscope. Morphologically normal one-cell zygotes with prominent pronuclei, intact zona pellucida, and homogenous cytoplasm were cultured (Figure 2).

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Figure 2. One-cell zygotes with visible sperm tails (indicated by arrows) collected 18-19 hours after hCG injection

2.6 Experimental Design

A completely randomized experimental design was employed with six treatment groups:

Group	Treatment	Antioxidant Concentration
Control	KSOM-R only	None
AA50	KSOM-R + Ascorbic acid	50 µM
AA100	KSOM-R + Ascorbic acid	100 µM
RSV0.25	KSOM-R + Resveratrol	0.25 µM
RSV0.50	KSOM-R + Resveratrol	0.50 µM

Stock solutions of ascorbic acid (Sigma-Aldrich, A5960) and resveratrol (Sigma-Aldrich, R5010) were made in sterile phosphate-buffered saline and diluted directly into KSOM-R medium at the time of use. The treatments included at least 60 embryos per group, which were divided into three biological replicates (20 embryos per replicate). The concentrations were used according to previously published reports using rodents embryo culture systems [8,9].

2.7 Embryo Culture

The embryos were incubated in 50 µL droplets of KSOM-R medium (Millipore, MR-106-D) overlaid by mineral oil (Sigma-Aldrich, M8410) in 35 mm culture dishes. Each droplet contained 15-20 embryos. Culture was performed at 37°C in a humidified atmosphere of 5% CO_2 in air (approximately 20% O_2) for 96 hours. Embryo development was assessed at 24-hour intervals under an inverted microscope (40× magnification) and recorded at the following stages: cleavage (2-cell), 4-cell, 8-cell, morula, and blastocyst. Embryos that failed

to cleave or showed signs of degeneration (fragmentation, cytoplasmic abnormalities) were classified as degenerated/arrested. Representative images were captured at 43-44 hours and 94-96 hours post-ovulation to document developmental progression.

At 43-44 hours post-ovulation, embryos were evaluated for early cleavage events. Two-cell embryos were identified by the presence of two distinct blastomeres of equal size. Degenerate embryos were characterized by dark, granular cytoplasm and absence of cleavage. Fragmented embryos showed irregular cytoplasmic fragments of varying sizes (Figure 3).

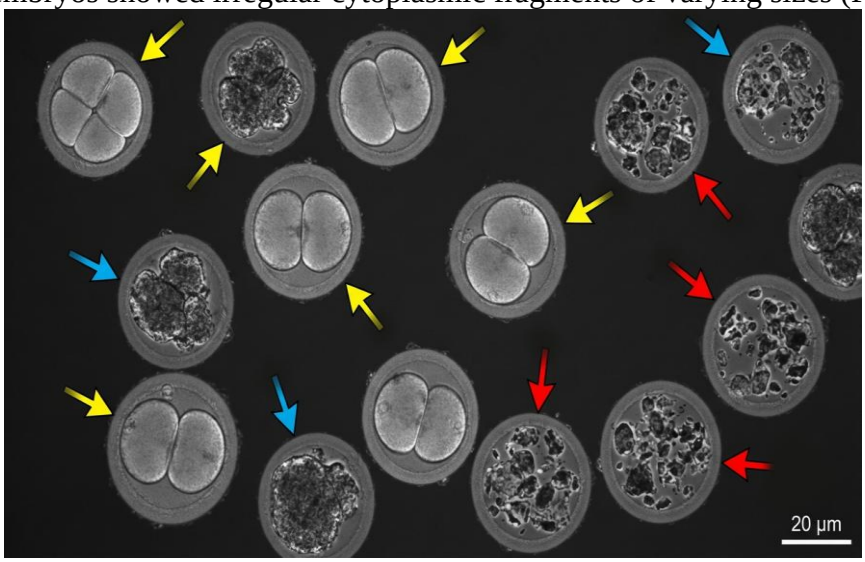

Figure 3. Embryos at 43-44 hours post-ovulation

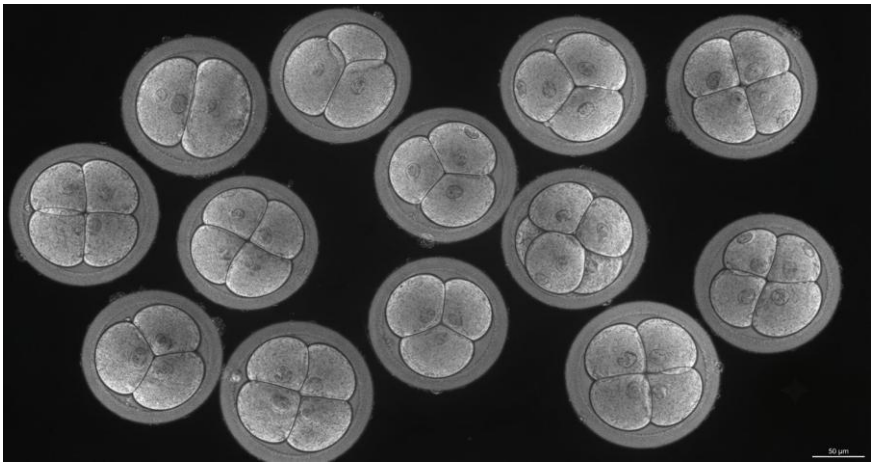

Figure 4. Embryos that progressed beyond the two-cell stage (three to eight blastomeres) at 94-96 hours post-ovulation

At 94-96 hours post-ovulation, embryos that had progressed beyond the two-cell stage were evaluated. Embryos with three to eight blastomeres were classified as having progressed beyond the two-cell stage, representing successful embryonic genome activation and early cleavage divisions (Figure 4).

2.8 Oxidative Stress Assessment

Intracellular ROS levels were measured on Day 4 (96 hours of culture) using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, D6883). Briefly, embryos were washed twice in phosphate-buffered saline (PBS) and incubated in 10 µM DCFH-DA in PBS at 37°C for 30 minutes in the dark. After three washes in PBS, fluorescence intensity was measured using a fluorescence microscope with an excitation

wavelength of 488 nm and emission at 530 nm. Images were captured under identical exposure settings, and fluorescence intensity was quantified using ImageJ software. ROS levels were expressed as relative fluorescence units (RFU) per embryo. Moreover, the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) was determined using an assay kit (Abcam, ab138881) following the protocol of the manufacturer.

2.9 Statistical Analysis

All statistical tests were conducted using SPSS 26.0 (IBM Corp., Armonk, NY, USA). All values are expressed as mean ± SD. Comparisons of the developmental data were done using the Chi-square test with Bonferroni correction, whereas ROS and GSH/GSSG were tested by one-way ANOVA with Tukey's post-hoc test. Pearson correlation was used to determine the relationship between ROS and developmental indicators. $P<0.05$ was regarded as statistically significant. Each experiment was replicated three times biologically, and the number of embryos used for the analysis is mentioned in the Results section.

3. Results and Discussion

3.1 Experimental Procedure

An overview of the experimental procedure is provided in Figure 5 below.

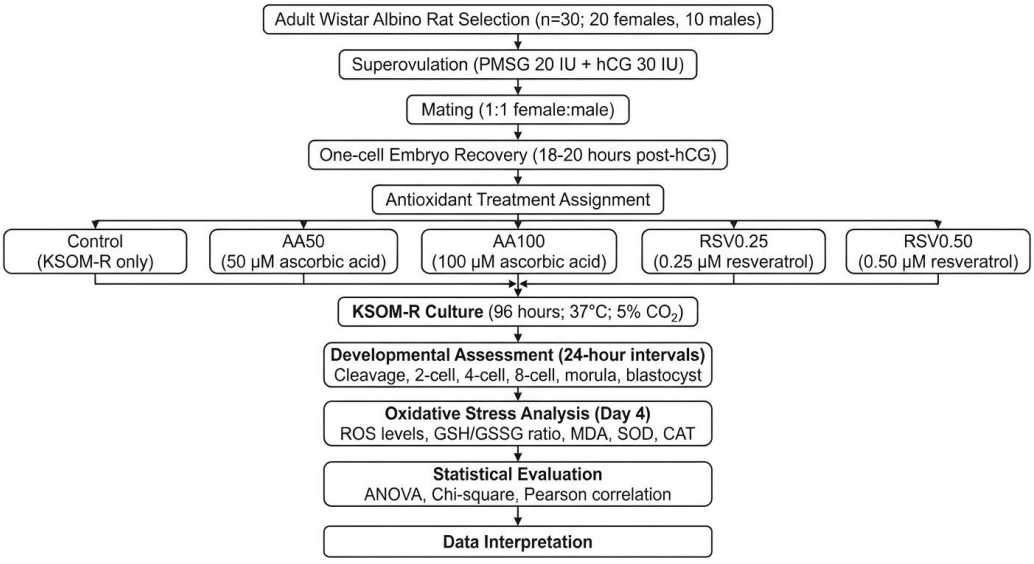

Figure 5. Experimental workflow of the Libyan rat embryo culture experiment

3.2 Effect of Antioxidant Supplementation on Embryo Development

The number of one-cell embryos used was 360 (60 embryos for each treatment group), and the culture period was 96 hours. The morphology of the embryos during early and late stages of culture is shown in Figures 3 and 4, while the results of development are demonstrated in Table 1.

Table 1. Influence of antioxidant supplementation on the developmental competence of rat embryos

Treatment	Embryos (n)	Cleavage (%)	≥2-cell (%)	Morula (%)	Blastocyst (%)	Degeneration (%)
Control	60	58.3 ± 3.2^{a}	51.7 ± 4.1^{a}	31.7 ± 3.8^{a}	18.3 ± 2.9^{a}	41.7 ± 3.2^{a}
AA50	60	71.7 ± 3.8^{b}	65.0 ± 3.5^{b}	48.3 ± 4.2^{b}	30.0 ± 3.6^{b}	28.3 ± 3.8^{b}
AA100	60	73.3 ± 4.3^{b}	66.7 ± 4.7^{b}	51.7 ± 3.9^{bc}	33.3 ± 4.1^{b}	26.7 ± 4.3^{b}
RSV0.25	60	76.7 ± 3.4^{bc}	70.0 ± 3.8^{bc}	56.7 ± 4.1^{bc}	38.3 ± 3.4^{c}	23.3 ± 3.4^{bc}
RSV0.50	60	83.3 ± 4.1^{c}	78.3 ± 3.2^{c}	65.0 ± 3.9^{c}	46.7 ± 3.8^{d}	16.7 ± 4.1^{c}

Values are expressed as means ± SD from three biological replicates. Superscripts with different letters in the same column are significantly different ($P < 0.05$, Chi-square test with Bonferroni correction).

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The cleavage rate was significantly higher in all antioxidant supplemented groups than control ($P < 0.05$). The highest cleavage rate was achieved in the group treated with RSV0.50 (83.3%), followed by RSV0.25 (76.7%), AA100 (73.3%) and AA50 (71.7%). Likewise, the developmental rate to morula and blastocyst stages was significantly increased by antioxidants. The rate of blastocyst formation in groups ranged between 18.3% in control to 46.7% in the group RSV0.50 which indicated 2.5-fold increase. On the other hand, the degeneration rate was significantly lower in all antioxidant-supplemented groups. The lowest rate was achieved in the group RSV0.50 (16.7% versus 41.7% in control). The results demonstrated that both ascorbic acid and resveratrol enhanced embryo developmental capability, with the last one at 0.50 µM being the best treatment.

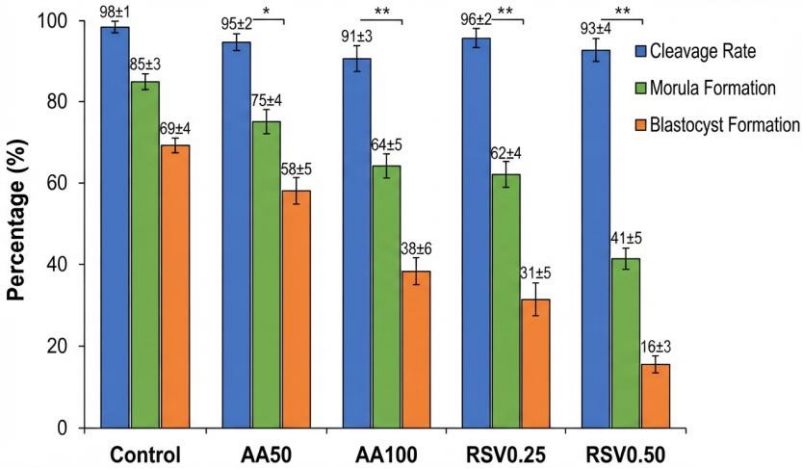

Figure 6. Effect of antioxidant supplementation on embryo developmental progression [Error bars representing SD. *P < 0.05, **P < 0.01 compared to control*]

3.3 Effect of Antioxidant Supplementation on Oxidative Stress Parameters

ROS content and GSH/GSSG ratios were determined for the redox state of the embryos in the various experimental groups (Table 2).

Table 2. Oxidative stress parameters in the rat embryos treated with antioxidants

Treatment	ROS Levels (RFU/embryo)	GSH/GSSG Ratio	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
Control	142.3 ± 8.7^{a}	3.2 ± 0.4^{a}	4.87 ± 0.42^{a}	12.4 ± 1.2^{a}	8.6 ± 0.8^{a}
AA50	108.6 ± 7.2^{b}	5.1 ± 0.5^{b}	3.56 ± 0.38^{b}	16.8 ± 1.5^{b}	11.2 ± 1.1^{b}
AA100	102.4 ± 6.8^{b}	5.4 ± 0.6^{b}	3.21 ± 0.35^{b}	17.5 ± 1.6^{b}	12.1 ± 1.2^{b}
RSV0.25	89.7 ± 5.9^{c}	6.3 ± 0.7^{c}	2.68 ± 0.29^{c}	20.3 ± 1.8^{c}	14.5 ± 1.4^{c}
RSV0.50	76.5 ± 5.3^{d}	7.8 ± 0.8^{d}	2.14 ± 0.25^{d}	23.7 ± 2.1^{d}	16.8 ± 1.6^{d}

ROS levels were significantly lowered in all the antioxidant treated groups in comparison to control groups ($P < 0.001$). The lowest ROS level was observed in the RSV0.50 group (76.5 RFU/embryo) and that is a 46% decrease in comparison to the control groups (142.3 RFU/embryo). The use of both ascorbic acids also significantly lowered ROS levels and while AA100 showed a slight advantage over AA50 (102.4 vs. 108.6 RFU/embryo), there was no significant difference between them ($P > 0.05$).

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GSH/GSSG ratio, which is an indication of the cellular redox balance, was significantly higher in all antioxidant treated groups in comparison to control groups. The highest GSH/GSSG ratio was observed in the RSV0.50 group (7.8), then RSV0.25 (6.3), AA1.

The amount of lipid peroxidation was significantly lower in antioxidant-supplemented groups and measured using the level of malondialdehyde (MDA). The RSV0.50 group exhibited the lowest MDA content (2.14 nmol/mg protein) in comparison with controls (4.87 nmol/mg protein).

The activity of endogenous antioxidant enzymes (SOD and CAT) was significantly higher in antioxidant-supplemented groups in comparison with controls. The RSV0.50 group had the highest level of SOD (23.7 U/mg protein) and CAT (16.8 U/mg protein) activity. It can be assumed that the antioxidant supplement can stimulate endogenous antioxidant protection mechanisms.

These findings prove that antioxidant supplementation effectively improves redox status in in vitro cultured embryos, resveratrol being the most effective antioxidant.

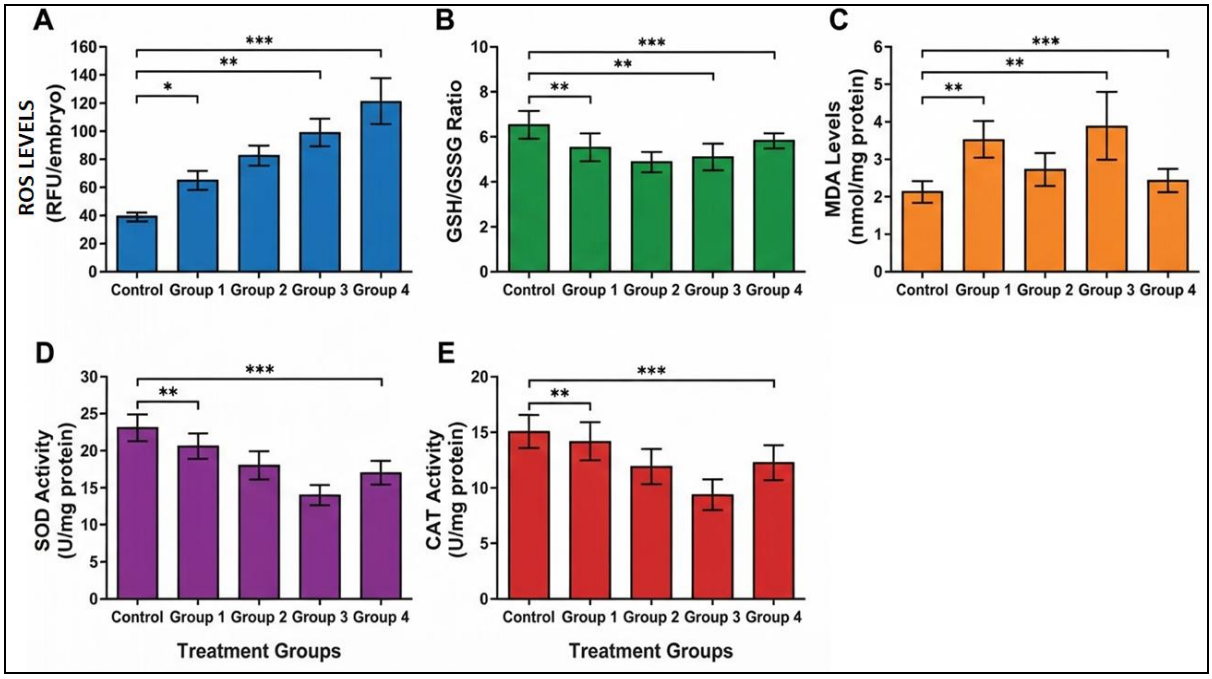

Figure 7. Effect of antioxidant supplementation on oxidative stress parameters

Figure 7 showing ROS levels (A), GSH/GSSG ratio (B), MDA levels (C), SOD activity (D), and CAT activity (E) across treatment groups. Bars represent mean ± SD. *P < 0.05, **P < 0.01, **P < 0.001 compared to control.*

3.4 Association Between Oxidative Stress and Developmental Competence

Results of Pearson correlation analysis showed that there was a significant negative correlation between the ROS level and the rate of blastocyst formation among all the treatment groups (r = -0.86, P < 0.001; n = 360 embryos). Embryos having a low level of ROS showed high blastocyst formation rates. Likewise, a significant positive correlation was found between the GSH/GSSG ratio and blastocyst formation (r = 0.83, P < 0.001). Moreover, high correlations were found between the SOD activity and blastocyst formation (r = 0.79, P < 0.001) and CAT activity and blastocyst formation (r = 0.76, P < 0.001).

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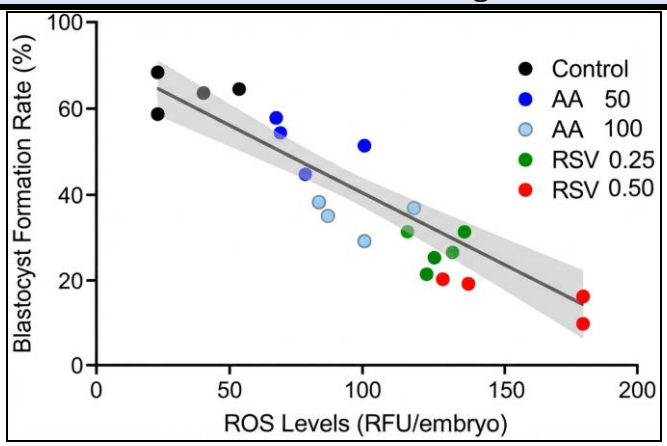

Figure 8. The relationship between oxidative stress and embryo development capability

Scatter plot (Figure 8) illustrating negative correlation between the ROS level (RFU/embryo) and the blastocyst formation percentage (%). Each point indicates an individual experiment.

The current study confirms the potential of ascorbic acid and resveratrol supplementation for reducing oxidative stress and enhancing developmental competence of in vitro cultured rat embryos in a Libyan laboratory setting. In particular, resveratrol was the most effective compound, especially at the concentration of 0.50 µM, implying that antioxidant activity is concentration- and compound-dependent.

The decreases in ROS and MDA, as well as the increases in GSH/GSSG, SOD, and CAT, imply that redox balance is improved through the antioxidant treatment. While ascorbic acid is a free radical scavenger, resveratrol might also regulate endogenous antioxidant system [6,9]. The high level of ROS due to presence of atmospheric oxygen (20% O_2) in the culture medium might be responsible for oxidative stress in untreated embryos [2,3]. Reduced MDA further suggests protection against lipid peroxidation and cellular membrane damage.

Improved cleavage and progression beyond the two-cell stage may reflect protection from ROS-induced damage to DNA, proteins, lipids, and mitochondria [3,4]. This is particularly relevant during early embryonic development and genome activation. The lower degeneration and fragmentation observed in treated embryos further support the protective effect of antioxidant supplementation. These findings are consistent with previous studies reporting beneficial effects of ascorbic acid and resveratrol in mammalian embryo culture [5,7–10].

The dose-dependent response emphasizes the importance of optimizing antioxidant concentrations because excessive supplementation may exert undesirable effects [5]. The significant inverse association between ROS and developmental competence further supports a relationship between redox status and embryo development.

Importantly, this study provides experimental evidence from a Libyan laboratory setting and demonstrates the feasibility of establishing reproducible rat embryo-culture protocols locally. The findings may support further reproductive and developmental research in Libya. Future studies should evaluate antioxidant combinations and molecular mechanisms, including gene-expression and signaling pathways, to further optimize embryo-culture conditions.

5. Conclusion

This study demonstrates that ascorbic acid and resveratrol supplementation significantly reduced oxidative stress and improved the developmental competence of *in vitro* cultured rat embryos. Resveratrol at 0.50 µM showed the strongest protective effect, with the highest blastocyst formation (46.7%) and lowest ROS level (76.5 RFU/embryo). The inverse relationship between ROS and developmental competence supports the role of oxidative stress in limiting embryo development. Changes in ROS, GSH/GSSG, MDA, SOD, and CAT further confirmed improved redox balance following antioxidant treatment. These findings provide

experimental evidence from a Libyan laboratory and support antioxidant supplementation as a strategy for optimizing rat embryo culture. The established model provides a basis for future reproductive research in Libya, including molecular investigations and evaluation of other mammalian embryo systems.

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