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Antioxidant and developmental capacity of retinol on the *in vitro* culture of rabbit embryos

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Summary

Oxidative stress is a major cause of defective embryo development during *in vitro* culture. Retinoids are recognized as non-enzymatic antioxidants and may have an important role in the regulation of cell differentiation and vertebrate development. However, there are not enough reports discussing the antioxidant and developmental capacity of retinoids, including retinol (RT), on the *in vitro* development of embryos recovered from livestock animals, particularly in rabbit species. Therefore, morula embryos obtained from nulliparous Red Baladi rabbit does were cultured for 48 h in TCM199 medium in the absence of RT (control group) or in the presence of RT at concentrations of 10, 100 and 1000 nM. The developmental capacity to the hatched blastocyst stage, the antioxidant biomarker assay and the expression of several selected genes were analyzed in each RT group. The data show that RT significantly ($P < 0.001$) promoted the embryo hatchability rate at the concentration of 1000 nM to 69.44% versus 29.71% for the control. The activity of malondialdehyde (MDA) level was significantly ($P < 0.05$) lower in the RT groups than in the control group, while the total antioxidant capacity (TAC), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were significantly ($P < 0.05$) higher following treatment with RT. Furthermore, RT treatment considerably upregulated the relative expression of gap junction protein alpha 1 (*GJA1*), POU class 5 homeobox 1 (*POU5F1*) and superoxide dismutase 1 (*SOD1*) genes compared with the control group. The current study highlights the potential effects of RT as antioxidant in the culture medium on the *in vitro* development of rabbit embryos.

Introduction

The *in vitro* production of embryos has become a traditional tool to increase the reproductive efficiency, to augment the genetic gain and to reduce generation intervals in livestock animals (Keefer *et al.*, 2001; Peippo *et al.*, 2001; Conceição *et al.*, 2016). However, retardation and low development rate of embryos have been induced due to their exposure to free radicals and reactive oxygen species (ROS) that arise during *in vitro* culture conditions (Agarwal *et al.*, 2003; Pasqualotto *et al.*, 2004; Mehaisen & Saeed, 2015). Excessive ROS causes detrimental injuries to embryos, including membrane's lipid peroxidation, metabolism disruption, intracellular milieu impairment, cell death and apoptosis (Maity *et al.*, 2009; Succu *et al.*, 2014; Poprac *et al.*, 2017; Sharma *et al.*, 2017; Zidane, 2017). In addition, the expression of several genes responsible for embryo compaction and development, such as *GJA1* and *POU5F1* were found to be suppressed as consequences of exposure to oxidative stress during *in vitro* culture (Gomez *et al.*, 2009; Kawasumi *et al.*, 2009).

The natural metabolites of retinol (RT) such as all-*trans* retinoic acid (t-RA) and 9-*cis* RA are collectively known as retinoids and are considered as non-enzymatic antioxidants (Guerin *et al.*, 2001). Recent protocols have used these compounds in the culture medium of embryos taking into account their deep effect upon cellular survival, proliferation, differentiation and embryonic morphogenesis (Noy, 2010; Rajesh *et al.*, 2010; Rhinn & Dollé, 2012; Conceição *et al.*, 2015; Conceição *et al.*, 2016). It was found that the addition of retinoids to the culture medium enhances the subsequent preimplantation embryonic development in bovine embryos (Lima *et al.*, 2004; Ahmed *et al.*, 2016), and in goat embryos (Duque *et al.*, 2002a,b; Lima *et al.*, 2006; Chiamenti *et al.*, 2010, 2012; Conceição *et al.*, 2015). The positive effect of retinoids on embryo development could be induced directly by modulating growth factor gene expression or indirectly by improving the quality of mRNAs (Livingston *et al.*, 2004). Furthermore, it was found that retinoids can affect the cell antioxidant defence mechanisms by reducing the content of ROS and lipid peroxidation (Pu *et al.*, 2014), and by increasing the

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levels of antioxidant enzymes such as superoxide dismutase and catalase (Livingston, 2003; Rajesh *et al.*, 2010).

To the best of our knowledge, there are not enough reports discussing the effect of retinoids on the *in vitro* development of embryos recovered from livestock animals, particularly in rabbit species. Therefore, the present study aims at evaluating the effect of retinol (RT) on the *in vitro* development of preimplantation rabbit embryos, focusing on the morphological aspects of embryo development. The antioxidant biomarkers in developed embryos were also assayed via evaluating the levels of malondialdehyde (MDA) and total antioxidant capacity (TAC) as oxidative substrates, and the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) as antioxidant enzymes. In addition, the effect of RT on the expression of two developmental genes namely gap junction protein alpha 1 (*GJA1*) and POU class 5 homeobox 1 (*POU5F1*), and an antioxidant gene of superoxide dismutase 1 (*SOD1*) were analyzed in the developed embryos.

Materials and methods

Chemicals and reagents

All the chemicals and reagents in this experiment were purchased from Sigma-Aldrich (S.A., Egypt) unless otherwise stated.

Source of embryos

In total, 60 nulliparous 5–6-month-old Red Baladi breed (Khalil, 2002) rabbit does (average 2.5 kg wt) were used in the current study. During the study period, all experimental animals were housed in a semi-closed housing system (Agricultural Experiment Station, Faculty of Agriculture, Cairo University) and kept in individual cages, supplied with feeding hoppers made of galvanized steel sheets and nipples for automatic drinker. They were raised under the same standard environmental conditions, fed with the same commercial diet and had free access to water.

The nulliparous females were synchronized for the receptivity by an intramuscular (im) injection with 20 IU eCG (Folligon, Intervet, The Netherlands) 60 h before insemination. The females were artificially inseminated with a semen pool from adult males of the same breed and immediately received an im injection of 2 μg buserelin acetate (Receptal, Intervet Co., Egypt). The method of semen recovery, evaluation and subsequent insemination was described earlier (Lavara *et al.*, 2000). The embryos were collected after sacrificing the does, 72 h post-insemination, by uterine flushing at room temperature (20–25°C). The flushing medium consisted of 1 L Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.132 g calcium chloride, 0.2% bovine serum albumin (BSA) and 0.1% antibiotics (10,000 units penicillin-G and 10 mg streptomycin per ml; penicillin–streptomycin solution 100 ×, BioShop, Canada Inc.).

Treatments and embryo culture

Retinol solution was added to the embryo culture medium (TCM199 + 20% fetal bovine serum + 1% antibiotics) in four different levels (0, 10, 100 or 1000 nM). Only the normal recovered embryos (compact morulae with intact mucin coat and zona pellucida) from each donor doe were randomly distributed to the four retinol treatment groups in a 4-well embryo culture dish (Nunc A/S, Thermo Fisher Scientific, Roskilde Site, Denmark). In total, 574 embryos were cultured; 141 as controls, 147 in 10 nM RT, 144 in 100 nM RT, and 142 in 1000 nM RT. The embryos were cultured for 48 h at 38.5°C, 5% CO_2 and saturated humidity. The numbers of total blastocysts, expanded and hatched blastocysts were recorded at the end of culture period for each treatment group.

Antioxidant biomarkers assay

Three samples per treatment group (each sample consisted of 1 ml culture medium with at least 10 developed blastocysts) were centrifuged at 1030 *g* for 10 min at 4°C. The precipitates were washed three times with phosphate-buffered saline (PBS) and collected by centrifugation. The embryo pellets were re-suspended in 1 ml of deionized water, and then were snap-frozen and stored at −80°C until further biochemical analysis. The samples were thawed, shook up, and centrifuged at 1030 *g* for 15 min at 4°C. The supernatant was then collected to evaluate the levels of MDA and TAC as oxidative substrates, and the activities of CAT, SOD and GPx as antioxidant enzymes, using colorimetric assay kits as mentioned below. The data were obtained for all analyses by using an automatic scanning spectrophotometer (CE1010, Cecil Instruments Limited, Cambridge, United Kingdom) and the calculations were adjusted per mg protein for each assay.

First, the total protein content was determined in the supernatant using the protein-Biuret colorimetric method (Gornall *et al.*, 1949). According to the kit's protocol (TP-2020, Bio-Diagnostic, Inc., Egypt), 25 μl of the sample or the standard solution were mixed with 1 ml Biuret reagent and incubated at 37°C for 10 min. The absorbance of the sample (A_{sample}) and standard ($A_{standard}$) against blank reagent was read at 550 nm after colour stability for 1 h and linearity up to 10 g/dL. The protein concentration was calculated using the equation: $A_{sample}/A_{standard} \times 5$.

The level of MDA was determined using colorimetric assay kits (MDA-2529, BioDiagnostic, Inc., Egypt) according to the methods described by Kei (1978). In summary, 200 μl of the sample or standard was mixed with 1.0 ml of chromogen, heated in boiling water bath for 30 min and then cooled. The absorbance of sample (A_{sample}) against blank and standard ($A_{standard}$) against distilled water was measured at 534 nm with colour stability for 6 h and linearity up to 100 nM/ml. The MDA in the sample = $A_{sample}/A_{standard} \times 10$.

The TAC was also determined using colorimetric kits (TAC--2513, BioDiagnostic, Inc., Egypt) according to previously described methods (Koracevic *et al.*, 2001). Briefly, 20 μl of each sample was added to 500 μl of substrate (H_2O_2) then incubated at 37°C for 10 min. After that, 500 μl of working reagent (1.0 ml chromogen + 1.0 ml enzyme buffer) was added to the reaction mixture and incubated for 5 min at 37°C. The absorbance of blank (AB) and sample (AS) against distilled water was read immediately at 505 nm and linearity up to 2.0 mM/l. The TAC concentration was calculated as (AB − AS × 3.3).

CAT activity was assayed using colorimetric assay kits (CAT-2517, BioDiagnostic, Inc., Egypt) according to previous methods (Aebi, 1984). In brief, 50 μl of each sample or standard solution was mixed with 0.5 ml phosphate buffer (pH 7.0) and 100 μl of diluted H_2O_2 then incubated for 1 min at 25°C. After that, 200 μl of chromogen-inhibitor and 0.5 ml of peroxidase 4-aminoantipyrine enzyme were added to the mixture and incubated for 10 min at 37°C. The same steps were repeated for the sample and standard blanks (without addition of H_2O_2). The absorbance of the sample (A_{sample}) against sample blank

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and the standard ($A_{standard}$) against standard blank was read at 510 nm after colour stable for 1 h. The CAT activity was calculated as ($A_{standard}$ − $A_{sample}/A_{standard}$ × 1000).

The SOD activity was measured using colorimetric assay kits (SOD-2521, BioDiagnostic, Inc., Egypt) according to the methods previously described (Nishikimi *et al.*, 1972). Briefly, 100 µl of the sample or control (distilled water) was mixed well with 1.0 ml of working reagent [10 ml phosphate buffer pH 8.5 + 1.0 ml nitroblue tetrazolium (NBT) + 1.0 ml NADH], and then added to 100 µl of phenazine methosulphate (PMS) to initiate the reaction. The increase in absorbance at 560 nm over 5 min following the addition of PMS was measured for the control ($\Delta A_{control}$) and for the sample (ΔA_{sample}) at 25°C. SOD activity = % inhibition × 3.75 (as % inhibition = $\Delta A_{control} - \Delta A_{sample}/\Delta A_{control}$ × 100).

GPx activity was determined according to the methods described by Paglia and Valentine (1967). According to the kits' protocol (GPx-2524; BioDiagnostic, Inc., Egypt), 10 µl of each sample was mixed well with the reaction mixture contained 1.0 ml of assay buffer (phosphate buffer and Triton X-100, pH 7.0), 100 µl of nicotinamide adenine dinucleotide phosphate (NADPH) reagent (glutathione, glutathione reductase and NADPH), and 100 µl of H_2O_2 (previously diluted 100 times). The decrease in absorbance at 340 nm per min (ΔA_{340}) was recorded over a period of 3 min against deionized water. A convenient sample dilution was used to control the start of A_{340} at 1.5 and ΔA_{340} at 0.05 per min. The following equation was used to calculate the GPx activity: $\Delta A_{340}/0.00622$ × dilution factor.

mRNA expression analysis

At the end of embryo culture, 30 blastocysts from each group (three biological replicates containing pool of 10 hatched embryos each) were kept in cryogenic vials (Corning Incorporated, Corning, NY, USA) and directly plunged into liquid nitrogen (LN_2) for later analysis. Total RNA isolation was performed using the Arcturs1 PicoPure1 RNA isolation kit (Applied Biosystems, Carlsbad, USA) per manufacturer's instruction. Genomic DNA contamination was removed by performing column DNA digestion using RNase-free DNase (Qiagen GmbH, Hilden, Germany).

RNA was eluted in 11 µl of elution buffer. The RNA from each replicate was reverse transcribed using 1 mM oligo(dT) primers and the Rever-AidcDNA synthesis kit (Thermo Fisher Scientific, Heidelberg, Germany) per manufacturer's recommendations. Sequence-specific primers (Table 1) for the real-time PCR were designed using the Primerblast web interface (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) and each pair of primers was tested to achieve efficiencies close to 1. qRT-PCR for expression analysis of two developmental GJA1 and POU5F1 genes, an antioxidant *SOD1* gene, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the housekeeping gene was performed in a 20 µl reaction volume containing Maxima SYBR Green qPCR Master Mixes with ROX (Thermo Fisher Scientific, Heidelberg, Germany), the cDNA samples and the specific forward and reverse primers in Mx3000P™ real-time PCR system (Stratagene). The thermal cycling parameter was set to 95°C for 3 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. After the end of the last cycle, melting curve was generated by starting the fluorescence acquisition at 60°C and taking measurements every 7 s intervals until the temperature reached 95°C. According to the comparative cycle threshold (C_T) method, fold changes in the relative gene expression of the target were determined using the equation $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

Statistical analysis

The experimental design was completely randomized with four groups of embryos treated with different levels of retinol (0, 10, 100 and 1000 nM). The variances in the data were homogeneous, as indicated by the HOVTEST option of the GLM procedure (SAS, 2004). Non-normally distributed data (parameters of *in vitro* development rates) were analyzed by using the CATMOD procedure (SAS, 2004). One-way analysis of variance (ANOVA) was used for statistical analysis of antioxidant enzymatic activity and gene expression parameters using GLM procedure. Tukey's test was performed to determine differences among embryo groups for all traits. Results presented in the tables are least square means. The mathematical model included the main effect as follows:

$$Y_{ij} = \mu + T_j + e_{ij}$$

where: Y_{ij} = An observation of individual 'i' of the treatment 'j'; μ = Overall population mean; T_j = Treatment effect; and e_{ij} = Experimental error. The significance level was set at 5%.

Results

In vitro development rates

The overall data and *in vitro* developmental rates of embryos cultured with retinol are shown in Table 2. There was no significant difference in the total blastocyst rate between retinol groups and the control group. The expanded blastocyst rate was significantly ($P < 0.001$) lower at the concentration of 1000 nM RT (30.56%) compared with the other groups (70.29, 71.80 and 60.65% for 0, 10 and 100 nM RT groups, respectively). In contrast,

Table 1. Details of primers used for real-time PCR quantitative analysis

Gene symbol	Gene full name	GenBank accession number	Primer sequences (5' → 3')	Annealing temperature (°C)	Product size (bp)
GJA1	Gap junction protein alpha 1	NM_001198948	F:atgagcagtctgcctttcgt R:cgttgacaccatcagtttgg	55	228
POU5F1	POU class 5 homeobox 1	NM_001099957	F:gagatttgcaaagcggagac R:cggttacagaaccacacacg	55	188
SOD1	Superoxide dismutase 1	NM_001082627	F:cacttcgagcagaagggaac R:cgtgcctctcttcatccttc	54	184
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001082253	F:aggtcatccacgaccacttc R:gtgagtttcccgttcagctc	57	202

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Table 2. *In vitro* development rates of rabbit embryos cultured with different levels of retinol

	Retinol (nM)				
Parameters	0	10	100	1000	*P*-value
Cultured embryos (*n*)	141	147	144	142	
Total blastocysts (%)[1]	$89.33^{a,b}$	82.67^{b}	98.5^{a}	84.39^{b}	0.0019
Expanded embryos (%)[2]	70.29^{a}	71.80^{a}	60.65^{a}	30.56^{b}	0.0001
Hatched embryos (%)[2]	29.71^{b}	28.20^{b}	39.35^{b}	69.44^{a}	0.0001

[1]Calculated as a percentage of cultured embryos.
[2]Calculated as a percentage of developed blastocysts.
[a,b]Least squares mean (LSM) with different superscripts, within the same row, are significantly different ($P < 0.05$).

the hatchability rate was significantly ($P < 0.001$) higher in the 1000 RT group (69.44%) than in the other groups (29.71, 28.20 and 39.35% for 0, 10 and 100 RT groups, respectively).

Antioxidant biomarkers

The results of antioxidant biomarkers in embryos treated or not treated with retinol are presented in Table 3. Retinol presence significantly ($P < 0.05$) resulted in lower MDA levels when compared with the control group. The TAC levels were significantly ($P < 0.05$) higher in the RT groups of 100 and 1000 nM than that in the 0 and 10 nM RT groups. Retinol at 100 and 1000 nM also induced a significant ($P < 0.05$) increase in the activity of SOD and GPx antioxidant enzymes when compared with the control group. No significant differences were found in the CAT enzyme among RT groups.

mRNA expression

Results of quantitative real-time PCR for the examined developmental-related genes *GJA1* and *POU5F1* and antioxidant-related gene *SOD1* in embryos treated or not treated with retinol are illustrated in Fig. 1. The concentrations of 100 and 1000 nM significantly ($P < 0.05$) enhanced the expression of *GJA1* gene by 4.37-fold and 6.92-fold, respectively, compared with the control group. Supplementing the culture medium with 10, 100 and 1000 RT significantly ($P < 0.05$) upregulated the expression of *POU5F1* by 2.76, 4.17 and 2.40 fold, respectively, compared with the controls. Furthermore, the expression of *SOD1* gene was upregulated by retinol treatments, however this increase was significant ($P < 0.05$) only at concentrations of 10 nM and 100 nM by 2.60- and 5.19-fold, respectively, compared with the control group.

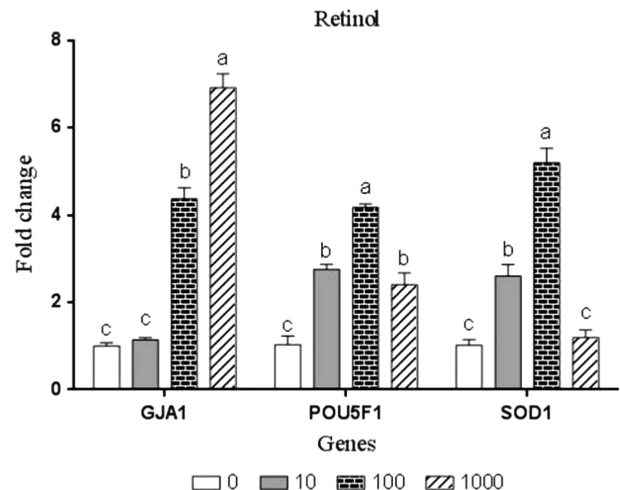

Figure 1. Gene expression analysis in rabbit embryos cultured with different levels of retinol (nM). Bars express the least squared means (LSM) ± standard error (SE) (n = 3). [a,b,c]Bars with different superscripts represent significant difference ($P < 0.05$).

Discussion

Retinoid compounds are found in the female reproductive tract and they were suggested to be able to improve the *in vitro* development of preimplantation embryos in several species (Duque *et al.*, 2002a,b; Lima *et al.*, 2004; Chiamenti *et al.*, 2010, 2012). Retinoids are considered as a biological antioxidant network and an important regulators of redox signalling pathways (Ikeda *et al.*, 2005; Alminana *et al.*, 2008). In the present study, over 574 rabbit embryos at morulae stages were used to evaluate the effect of retinol supplementation on the embryonic development to the blastocyst stage. The results show that the supplementation of retinol at the concentration of 1000 nM significantly increased the hatchability rate by 39.73% compared with the control group. In agreement with this finding, Vahedi *et al.* (2009) found that all-*trans*-RA supplementation at a concentration of 1 μM to the *in vitro* maturation (IVM) medium significantly increased bovine oocyte maturation. It was also reported (Kim *et al.*, 2011) that RA could enhance the developmental competence of mouse oocytes through the suppression of apoptosis and the enhancement of cells differentiation during IVM. In addition, it was concluded that RA increased the number of oocytes attained the two-cell stage and also improved IVM quality (Abouzaripour *et al.*, 2018).

Retinoids protect against oxidative damage by maintaining adequate endogenous competency and levels of antioxidants that are essential for oocyte maturation and embryonic development (Guerin *et al.*, 2001; Alminana *et al.*, 2008). Therefore, the current work investigated further roles of retinol in the activity of many antioxidant biomarkers in all experimental groups. The low MDA levels and high TAC levels in the RT-treated groups, particularly at concentrations of 100 and 1000 nM, indicated that retinol addition to the embryo culture medium reduces the degree of lipid peroxidation and oxidative stress damages in cultured embryos (Pasqualotto *et al.*, 2004; Agarwal *et al.*, 2008). In the present study, the activity of SOD and GPx antioxidant enzymes in the cultured embryos were significantly higher due to the high levels of retinol, while RT treatment did not affect CAT activity. It is well documented (Suzuki *et al.*, 1999; Guerin *et al.*, 2001; Zelko *et al.*, 2002; Lasota *et al.*, 2011; Wang, 2012) that SOD enzyme has an important role in protecting cells against ROS reactions that may otherwise be toxic to the embryos. Also, GPx enzymes are essential for the glutathione redox cycle as a major source of protection against low levels of oxidant stress (Guerin *et al.*, 2001), whereas CAT enzymes become more significant in protecting against severe oxidant stress (Yan & Harding, 1997; Mehaisen *et al.*, 2015). These observations could explain the higher competence of embryos cultured with retinol, showing higher hatchability rates for these embryos compared with controls.

Our results demonstrated that the embryo groups cultured with 10 and 100 nM RT expressed a higher mRNA for the *SOD1* gene compared with the control group. The high expression in antioxidant- and oxidative stress-related genes was previously

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Table 3. Antioxidant biomarkers of rabbit embryos cultured with different levels of retinol

	Retinol (nM)			
Parameters[1]	0	10	100	1000
MDA (nM/mg)	0.172 ± 0.0031[a]	0.102 ± 0.0042[b]	0.105 ± 0.0018[b]	0.092 ± 0.0031[b]
TAC (nM/mg)	4.68 ± 0.229[c]	5.13 ± 0.373[c]	7.48 ± 0.440[b]	9.15 ± 0.147[a]
CAT (U/mg)	0.045 ± 0.0014	0.047 ± 0.0037	0.054 ± 0.0064	0.063 ± 0.0040
SOD (U/mg)	1.06 ± 0.058[c]	1.43 ± 0.151[b,c]	2.25 ± 0.158[a,b]	2.36 ± 0.286[a]
GPx (mU/mg)	1.77 ± 0.088[b]	2.51 ± 0.234[a,b]	2.87 ± 0.109[a]	3.11 ± 0.241[a]

[1]All parameters are presented as per mg of total protein. CAT, catalase; GPx, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase; TAC, total antioxidant capacity.
[a,b,c]Least squared means (LSM) ± standard error (SE) ($n = 3$) with different superscripts, within the same row, are significantly different ($P < 0.05$).

reported (Mehaisen *et al.*, 2015) in embryos cultured with other antioxidant materials like melatonin. In the present study, mRNA expressions of *GJA1* and *POU5F1* as important developmental-related genes were analyzed in all experimental groups. The results show a higher expression in these genes when embryos were cultured with 100 and 1000 nM RT compared with the control embryo group. GJA1 (also named Cx43) is one of intercellular coupling connexins that allows the embryonic cells to share low molecular weight metabolites and second messengers, thus facilitating homeostatic and developmental processes in the cultured embryos (Houghton *et al.*, 2002). Other reports (De Sousa *et al.*, 1993; Mehaisen *et al.*, 2015) diminish the role of GJA1 in embryo development because the possible presence of any of the other connexins, such as Cx30, Cx31, Cx40, and Cx45, is enough to start the developmental regulation process. Conversely, POU5F1 is essential for the regulation and differentiation of the preimplantation embryonic cells toward the inner cell mass (ICM) (Ovitt & Schöler, 1998; Kirchhof *et al.*, 2000; Kazemi *et al.*, 2016). It was reported that POU5F1 is the early expressed factor, which has a critical role in cell differentiation and potential development of preimplantation embryos (Panda *et al.*, 2017). Furthermore, recent studies suggested that the expression of POU5F1 was reduced by the oxidative stress induced during the *in vitro* culture and vitrification of rabbit embryos (Mehaisen *et al.*, 2015). Similarly to the positive effects of retinol on rabbit embryos, in the present study, these authors found a positive effect of melatonin, as an antioxidant reagent, on the development and hatchability of embryos accompanied with high expression in the POU5F1 gene (Mehaisen *et al.*, 2015).

In conclusion, the current study shows that the addition of retinol at 100 or 1000 nM levels enhances the developmental capacity of rabbit embryos at the morula stage. The high expression of embryo-developmentally key genes, such as *GJA1* and *POU5F1* genes, appears to be one of the main mechanisms by which retinol improves the embryonic development. Furthermore, retinol treatment provides the cultured embryos with a high antioxidant capacity as presented by the high activity of SOD and GPx antioxidant enzymes. The antioxidant capacity of retinol seems to be another important pathway that can protect embryos from oxidative substrates produced during *in vitro* culture and, consequently, improves their potential development under such conditions.

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Conflict of interest. There are no conflicts of interest.

Ethical standards. The method of sacrificing the rabbit does is slitting the neck for cutting the carotid arteries, jugular veins, oesophagus and trachea, without severing the spinal cord or the head, while the animal is alive. The authors assert that all procedures contributing to this work comply with the ethical standards of Cairo University Ethics Committee for the Care and Use of Experimental Animals in education and Scientific Research (CU-IACUC).

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