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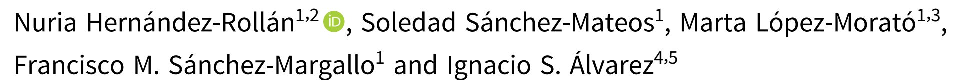
Supplementation of culture medium with quercetin improves mouse blastocyst quality and increases the expression of HIF-1α protein



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Summary

Regarding the low number of embryos that reach the blastocyst stage when cultured *in vitro*, this study aimed to evaluate the effects of quercetin on pre-implantation mouse (*Mus musculus*) embryos obtained using *in vitro* fertilization, especially during the passage from morula to blastocyst. Furthermore, we studied whether quercetin also affected the expression of hypoxia-inducible factor 1α (HIF-1α). The culture medium for the embryos was supplemented with quercetin, for long or short periods of time, and then the development potential, total cell number, apoptosis rates and expression of HIF-1α were studied to determine the effect of quercetin. Embryos failed to develop when cultured for long periods of time with quercetin, implying the possible toxic effects of this, alternatively antioxidant, compound. However, a short culture from morula to blastocyst significantly improved the development potential of *in vitro* produced embryos, increasing the final total cell number and reducing the apoptosis rate, observing similar results to those embryos cultured in low-oxygen concentrations or developed *in utero*. Furthermore, in embryos treated with quercetin for 2 or 4 h we found an increase in HIF-1α compared with untreated embryos. This work could imply a way to use quercetin in fertility clinics to improve the production of healthy blastocysts and, consequently, increase the success rates in assisted reproduction techniques.

Introduction

In vitro embryo culture is an important step to achieving success in reproductive technology and is challenging due to multiple factors. The development potential of embryos obtained using *in vitro* fertilization (IVF) is related to the composition of the culture medium (Paternot *et al*., 2010; Sameni *et al*., 2018). Even though many advances have been made in cell culture techniques, the proportion of embryos that reach the blastocyst stage remains variable (Sepúlveda *et al*., 2009; Sills and Palermo, 2010; Yu *et al*., 2014). It is well known that one of the factors that negatively affects embryonic development is oxidative stress and therefore the oxygen (O_2) concentration used in the incubation system emerges as a crucial factor to be considered. Some studies have reported that *in vivo* oxygen tension in the female reproductive tract of mammals ranges from 2% to 8% (Bishop, 1957; Mastroianni Jr and Jones, 1965; Morin, 2017) and that oxygen tension decreases around the time of blastocyst formation (Fischer and Bavister, 1993; Morin, 2017). Also, improved *in vitro* development of mammalian embryos under physiological oxygen tension has been reported (Whitten, 1969; Morin, 2017) and because the oxygen tension in the uterus is indeed lower than in the oviduct (Bishop, 1957; Steptoe *et al*., 1971; Morin 2017), a dynamic embryo culture system that adjusts oxygen concentration could represent the most physiological system.

Low O_2 tension triggers a wide range of cellular events in embryonic development, focused on the regulation of hypoxia-inducible factors (HIF) (Webster and Abela, 2007; Mantikou *et al*., 2013; Ma *et al*., 2017). HIFs are transcriptional regulators of hypoxic cellular responses and consist of two basic helix–loop–helix protein subunits, HIF-1α and HIF-1β. The α subunit is activated and stable only in cells under conditions of low O_2 content (Wang *et al*., 1995; Dunwoodie, 2009), producing the activation of a wide range of genes (Caramelo *et al*., 2006), although the expression and function of HIF-1α in embryogenesis is still unclear (Yoon *et al*., 2013). Conversely, it has been shown that the expression of HIF-1α protein can be induced using a variety of stimuli other than hypoxia, such as hormones, cytokines, growth factors (Carroll and Ashcroft, 2006; Pringle *et al*., 2010; Yoon *et al*., 2013), and flavonoids (Bogacz *et al*., 2021).

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In contrast, high levels of atmospheric O_2 favour a greater production of reactive oxygen species (ROS), which are highly harmful and can cause cell damage to oocytes and embryos (Guérin *et al.*, 2001; Martín-Romero *et al.*, 2008; Yu *et al.*, 2014; Sovernigo *et al.*, 2017) being an important cause of low efficiency in oocyte maturation and embryo development in several species (Luberda, 2005). The female reproductive system is rich in antioxidants, such as catalase and glutathione, among others (Carbone *et al.*, 2003) and it has been widely proposed that the use of substances with antioxidant properties could counteract the increase in ROS and improve embryo production (De Matos *et al.*, 2002; Kere *et al.*, 2013). Therefore, culture medium often contains a wide variety of antioxidants (Kere *et al.*, 2013; Mishra *et al.*, 2016; Rocha-Frigoni *et al.*, 2016) to reduce ROS that is produced by the medium during *in vitro* embryo culture (Martín-Romero *et al.*, 2008).

Flavonoids are phytophenolic compounds with a strong antioxidant effect that work as free radical scavengers. Within the flavonoid family, quercetin is the most potent scavenger of ROS (Cushnie and Lamb, 2005; Boots *et al.*, 2008) and nitrogen species (Heijnen *et al.*, 2002). These antioxidant properties could be attributed to its ability to chelate metals (Ferrali *et al.*, 1997; Aherne and O'Brien, 2000), for radical scavenging (Aherne and O'Brien, 2000), enzymatic inhibition (Nagao *et al.*, 1999), and/or induction of repair mechanisms (Myhrstad *et al.*, 2002). Quercetin is very abundant in fruits and vegetables such as apple, strawberry, onion, tea and broccoli (Kazuo *et al.*, 1977; Nakayama *et al.*, 1993; Mlcek *et al.*, 2016). The effect of this compound has been extensively studied by researchers over the past 35 years in hamster embryonic cells (Kazuo *et al.*, 1977), V79 Chinese hamster cells (Nakayama *et al.*, 1993), human sperm (Khanduja *et al.*, 2001), hepatocytes human HepG2 (Dong *et al.*, 2006), and porcine oocytes (Kang *et al.*, 2016). It has been reported to have antioxidant, antimutagenic, and anti-inflammatory activities, thereby reducing abnormalities in rat embryos, decreasing neural tube defects in diabetic mouse embryos, and reducing the damage to hair cells in zebrafish embryos (Karampour *et al.*, 2014; Lee *et al.*, 2015; Kang *et al.*, 2016).

In contrast, there have been several reports that indicated a possible toxic effect of quercetin in *in vitro* obtained mouse embryos through unknown mechanisms and, therefore, there is still controversy about its possible utilization in IVF (Pérez-Pastén *et al.*, 2010; Hashemzaei *et al.*, 2017). Consequently, the objective of this study was to gain insight into the effect of quercetin on pre-implantation mouse embryos obtained using IVF, especially during the passage from morula to blastocyst. Furthermore, we studied whether quercetin influenced embryonic development by stimulating the expression of HIF-1α.

Materials and methods

Animals

Female B6D2F1/J (C57BL/6JOlaHsd × DBA/2OlaHsd, Envigo RMS, The Netherlands) hybrid mice were used as oocyte and embryos donors, and male B6D2F1/J hybrid mice were used as semen donors. All mice were 8–12 weeks old. Animals were housed in the animalarium of the Jesús Usón minimally invasive surgery centre (certified by ES100370001499) under controlled conditions, 12 h light:12 h dark, 20–25°C temperature and 40–70% relative humidity, with food and water *ad libitum*. Males were housed individually in cages, whereas females were housed in groups of two or four per cage, and were controlled by the veterinarian throughout the entire realization of this work. Confounders were not controlled. The number of animals used for the experiments was divided as follows: 60 females were used to obtain embryos through IVF and treated with quercetin for long periods of time to study the development potential; 11 females were used to obtain embryos using IVF and treat with quercetin for short periods of time and to analyze the development potential, the cell number, the apoptosis rate, and the expression of HIF-1α; 11 females were used to obtain embryos *in utero* and treat them with quercetin for short periods of time and analyze the same parameters as before. This means, in total, 82 females were used in this work, along with, in total, 40 males. All experiments were approved by the Ethics Committee of the University of Extremadura according to RD 53/2013 1 February, and all procedures followed the guiding principles for research involving animals in accordance with the Institute for Laboratory Animal Research (ILAR) Guide for Care and Use of Laboratory Animals, and the Spanish Regulation (RD1201/2005).

Collection of *in utero* embryos and *in vitro* (IVF) obtained embryos

Embryos developed under physiological conditions

Mature female mice were stimulated with 8 IU equine chorionic gonadotropin (eCG; Syncrostim, CEVA Animal Health, S.A. Barcelona, Spain) and 48 h later, with 8 IU human chorionic gonadotropin (hCG; Veterin Corion, Divisa Farmavic, S.A. Barcelona, Spain). Females were mated with fertile males of the same age and strain to induce pregnancy (1♂:1♀) overnight. Pregnancy was confirmed by visualization of the vaginal plug on day 1 of embryo development. Females were sacrificed 72 h and 96 h post-hCG injection by cervical dislocation and developing embryos were recovered in potassium simplex optimized medium (KSOM; Merck-Millipore, Madrid, Spain) after flushing the uterus with M2 medium (Sigma-Aldrich, Barcelona, Spain).

Embryos obtained using IVF

Mature female mice were stimulated with 8 IU of eCG and 48 h later, with 8 IU of hCG. To perform IVF, male mice were euthanized by cervical dislocation and sperm was obtained from the caudal epididymides and then incubated in human tubal fluid (HTF) medium to allow capacitation. Mature oocytes were recovered from the ampulla of the oviduct of females sacrificed by cervical dislocation 12 h post-hCG injection and placed in HTF medium. Insemination was performed with capacitated spermatozoa (3×10^6) in 600 μl of HTF medium containing the oocytes. Fertilized oocytes were washed and cultured in KSOM medium at 37°C and atmospheric oxygen concentration (21%) until required for the experiments.

Quercetin treatment and low-oxygen concentration culture

Quercetin (Sigma-Aldrich, Barcelona, Spain) diluted in dimethyl sulphoxide (DMSO; 0.01%) (Sigma-Aldrich, Barcelona, Spain) at different concentrations (1 μM, 5 μM, 10 μM, or 50 μM), was added to the culture medium at different stages of development [pronucleus (PN) stage, 2-cell, 8–16-cell and morulae], and then embryos were cultured until the blastocyst stage, at 37°C. To study the effect of the vehicle DMSO, a set of embryos was cultured only with DMSO (0.01%), and all the results were compared with embryos cultured in KSOM medium throughout the entire development (Figure 1A).

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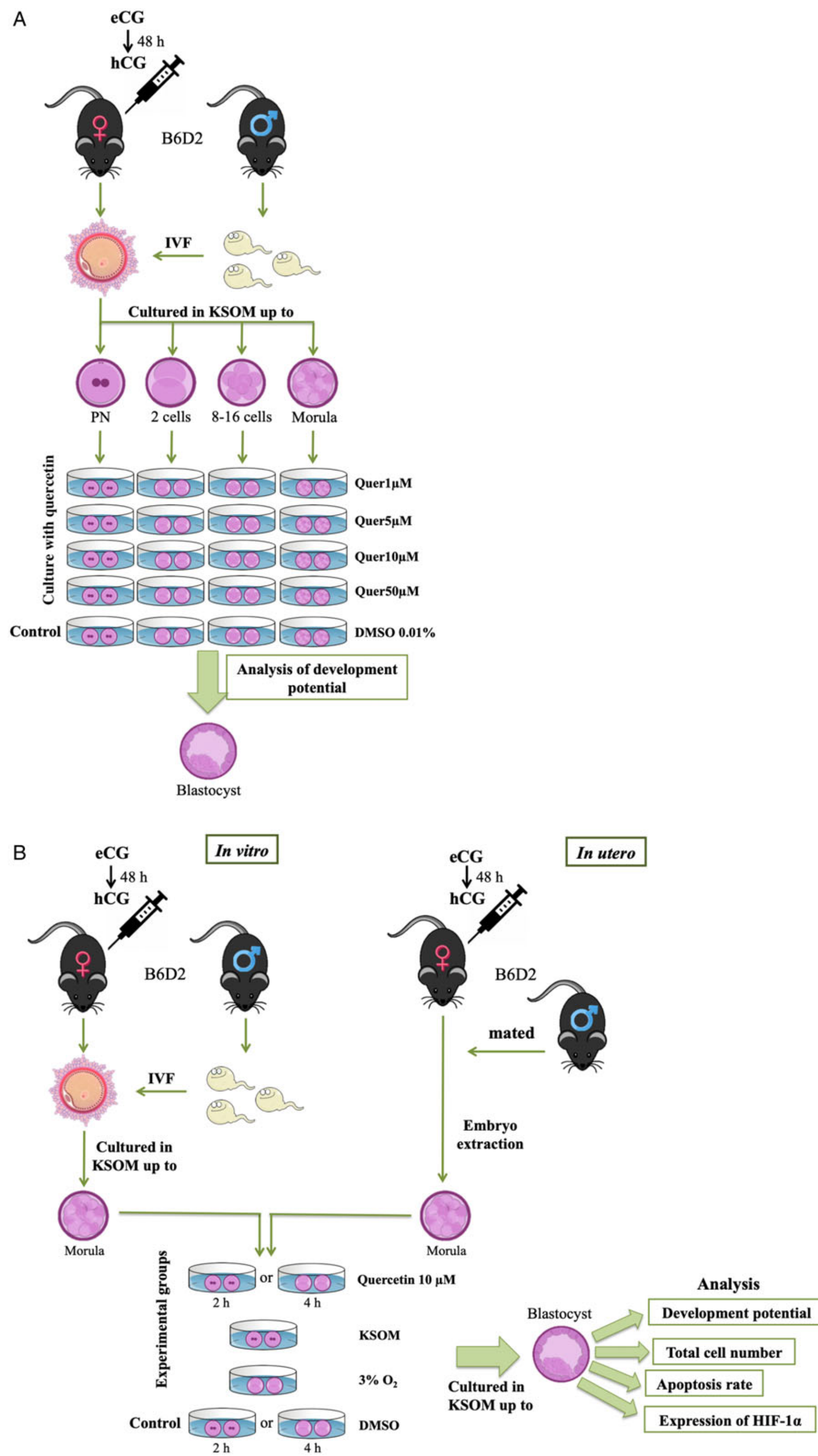

Figure 1. Diagram of the workflow of the treatment of the embryos with quercetin. (A) Treatment of the embryos during long periods of time. The top of the image shows how the embryos were obtained by *in vitro* fertilization and then cultured up to different stages of development. Embryos at different days of development were treated up to the blastocyst stage with different concentrations of quercetin, and then the development potential was studied (bottom part). (B) Treatment of the embryos for short periods of time. The top of the image shows obtaining the embryos for the experiments, by *in vitro* fertilization (left side) or by extraction from the uterus at the morula stage (right side). The bottom of the image explains how the embryos were treated with quercetin, or DMSO for the control, and what parameters were then analyzed.

Based on the results obtained, we performed new experiments exposing embryos to quercetin at 10 µM for short periods of time. We added this flavonoid to the culture medium of embryos at the morula stage for 2 h or 4 h. These embryos were both obtained using IVF or developed *in utero* (IU) (Figure 1B). Then, embryos were cultured again in conventional medium up to the blastocyst stage. For comparison, two other sets of embryos, both obtained using IVF and IU, were cultured either in KSOM medium or under 3% O_2. Finally, once the embryos reached day 4.5 of development, the percentage of embryonic development, total cell number, number of apoptotic cells, and expression of HIF-1α protein were evaluated and compared with the control groups consisting of

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Table 1. Development using *in vitro* fertilization of zygotes or embryos at different stages in the presence of quercetin, up to the blastocyst stage

Groups	% of zygotes/embryos that reached the blastocyst stage							
	Zygote stage	Blastocyst	Two-cell stage	Blastocyst	Six–eight-cell stage	Blastocyst	Morula stage	Blastocyst
Control (KSOM)	101	87 (86.14%)	100	88 (88%)	176	160 (90.90%)	155	145 (93.54%)
DMSO (0.01%)	102	42 (41.18%)	97	40 (41.23%)	156	85 (54.49%)	131	82 (62.59%)
Quer 1 µM	100	2 (2%)***	98	12 (12.24%)***	135	12 (8.88%)***	127	25 (19.68%)***
Quer 5 µM	96	2 (2.08%)***	97	2 (2.06%)***	118	8 (6.78%)***	125	20 (16%)***
Quer 10 µM	101	2 (1.98%)***	97	2 (2.06%)***	123	11 (8.94%)***	117	28 (23.93%)***
Quer 50 µM	103	9 (8.74%)***	97	1 (1.03%)***	120	7 (5.83%)***	116	24 (20.69%)***

Differences between the groups were calculated using the χ^2-test.
Quer, quercetin. ***$P < 0.001$ vs the control group.

embryos cultured with DMSO but no quercetin, for 2 h or 4 h and then cultured in KSOM medium up to blastocyst stage (Figure 1B).

Nuclear staining

To count the total cell number, 4% paraformaldehyde (PFA)-fixed blastocysts were stained with 2 µg/ml of Hoechst 33342 (Eugene, OR, USA) and the nuclei were examined using a confocal microscope (Nikon Eclipse TE2000-U). The cell number was analyzed using Fiji ImageJ software (1.45q, Wayne Rasband, NIH, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

Apoptotic cells in the embryos were detected using the *in situ* Cell Death Detection kit (TUNEL) from Roche (Mannheim, Germany) using the manufacturer's protocol and nuclei were counterstained with Hoechst 33342 as previously described. Fluorescence images were taken using the same confocal microscope.

Immunofluorescence of HIF-1α protein

The immunodetection of HIF-1α was performed by incubating fixed embryos with a 1:100 dilution of the HIF-1α monoclonal antibody (Novus Biologicals; NB100-105) and using fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse IgG (Novus Biologicals; NB720-F) at 1:400 dilution as the secondary antibody. Nuclei were counterstained again with Hoechst 33342 and embryos were observed under a confocal laser-scanning microscope (Nikon Eclipse TE2000-U). Control embryos were processed as described above, but the primary antibody was omitted. The expression of HIF-1α was analyzed in a qualitative manner, to detect possible changes in its expression.

Statistical analysis

Statistical analyses were performed using SPSS software (SPSS Onc., Chicago. IL, USA), and a P-value less than 0.05 was considered statistically significant. Analysis of variance (ANOVA) was used to compare embryo proliferation, whereas the Mann–Whitney–Wilcoxon non-parametric test was performed to compare development rate and results from the TUNEL assay. Data expressed as percentages were analyzed using the chi-squared (χ^2) test.

Results

Effect of quercetin on the development potential of mouse embryos

To investigate the effect of quercetin on embryo development, we first cultured *in vitro* obtained zygotes and embryos at different stages of development with different concentrations of quercetin (50 µM, 10 µM, 5 µM, and 1 µM) until they reached the blastocyst stage. Zygotes and embryos cultured in KSOM medium throughout the entire development were used as the control group. Our results showed that, regardless of the concentration of quercetin, the percentage of embryos that reached the blastocyst stage was significantly lower compared with the control (Table 1). Embryos cultured only with the solvent for quercetin, DMSO at a concentration of 0.01%, were also included in this experiment to analyze the potential effect of the dissolvent on the embryos and the development potential. Compared with control embryos, ~50% of the embryos reached the blastocyst stage, a number much higher than in the groups of embryos treated with quercetin. This first observation indicated a negative or toxic effect of quercetin on embryo development that could not be attributed to the dissolvent. Despite these results, the Quer 10 µM group presented the highest number of embryos that developed to the blastocyst stage (Table 1), so we decided to use this concentration for further studies described next to try to counteract the toxic effect of quercetin.

Based on the previous results that showed a detrimental effect of quercetin when used for long periods of time in the culture medium, the approach of this work was changed. Instead of studying the effect that quercetin produced on the development potential when used throughout the entire embryo culture, we decided to study its effects during short time intervals (2 h or 4 h) on embryos obtained directly from the uterus at the morula stage, a crucial stage in which embryos seemed to be less susceptible to the negative effects of quercetin/DMSO treatment (see data in Table 1). Interestingly, as can be seen in Figure 2, in this case, quercetin did not show a detrimental effect on embryo development potential but rather a beneficial effect, with a similar number of embryos reaching the blastocyst stage compared with control or low O_2 treated embryos.

Therefore, we further studied the effects of quercetin on embryos obtained using IVF, cultured up to the morula stage in KSOM medium, and then treated with quercetin for 2 or 4 h. Our results indicated that there were statistically significant differences in all the experimental groups (Figure 3) in favour of

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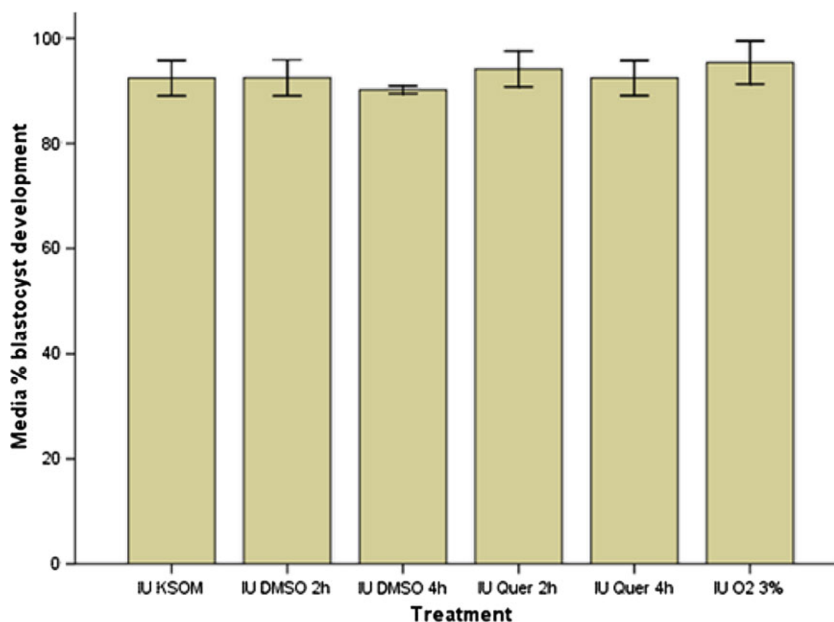

Figure 2. Results of the effect of quercetin culture for 2 h or 4 h in the pre-implantation development of embryos obtained *in utero* at the morula stage. IU KSOM group: embryos obtained from the uterus at the morula stage and developed to blastocyst in conventional culture medium, $n = 26$. IU DMSO 2 h group: embryos obtained from the uterus at the morula stage and cultured with DMSO for 2 h, $n = 24$. IU DMSO 4 h group: embryos obtained from the uterus at the morula stage and cultured with DMSO for 4 h, $n = 24$. IU Quer 2 h group: embryos obtained from the uterus at the morula stage and cultured with quercetin for 2 h, $n = 18$. IU Quer 4 h group: embryos obtained from the uterus at the morula stage and cultured with quercetin for 4 h, $n = 24$. IU O_2 3% group: embryos obtained from the uterus at the morula stage and cultured in low-oxygen concentrations until blastocyst stage, $n = 15$. The data are represented as the mean ± the standard deviation (SD). $P > 0.05$ for all statistical analyses. Mann–Whitney–Wilcoxon test.

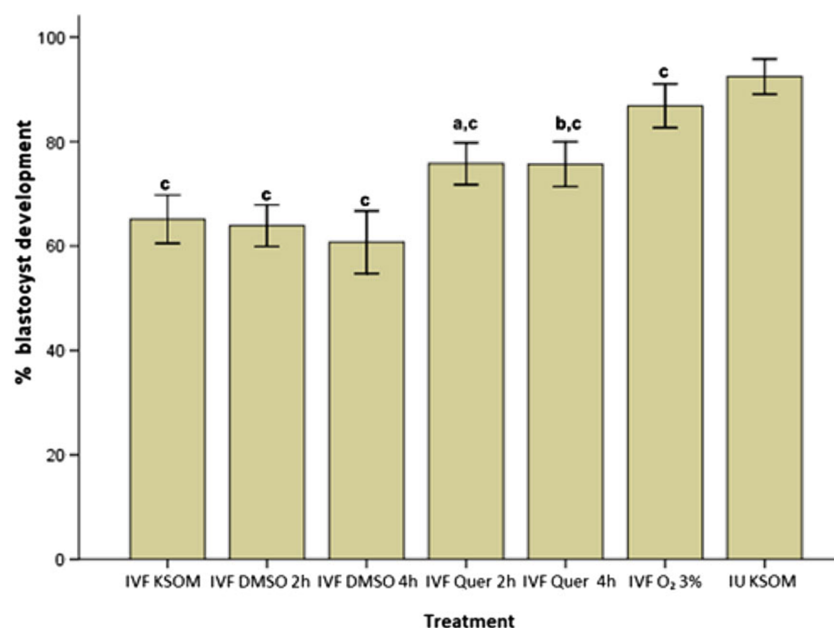

Figure 3. Results of the effect of quercetin culture for 2 h or 4 h in the pre-implantation development of embryos obtained by *in vitro* fertilization (IVF) and compared with *in utero* obtained embryos. IVF KSOM group: embryos obtained by IVF and developed to the blastocyst stage in conventional culture medium, $n = 18$. IVF DMSO 2 h group: embryos obtained by IVF and cultured with DMSO for 2 h at the morula stage, $n = 20$. IVF DMSO 4 h group: embryos obtained by IVF and cultured with DMSO for 4 h at the morula stage, $n = 16$. IVF Quer 2 h group: embryos obtained by IVF and cultured with quercetin for 2 h at the morula stage, $n = 16$. IVF Quer 4 h group: embryos obtained by IVF and cultured with quercetin for 4 h at the morula stage, $n = 20$. IVF O_2 3% group: embryos obtained by IVF and cultured with low concentrations of oxygen from morula to blastocyst stage, $n = 16$. IU KSOM group: embryos obtained from the uterus at the morula stage and developed to blastocyst in conventional culture medium, $n = 26$. The data are represented as the mean ± the standard deviation (SD). a, $P < 0.01$ vs IVF DMSO 2 h group; b, $P < 0.005$ vs IVF DMSO 4 h group; and c, $P < 0.001$ vs IU KSOM group. Mann–Whitney–Wilcoxon test.

embryos cultured with quercetin, both for 2 and 4 h. We also included for comparison embryos cultured at a low-oxygen concentration (IVF O_2 3% group), observing that they behave similarly to those developed to the morula stage in the uterus (IU KSOM group) and, in all the experimental cases, embryos had a higher development potential than those cultured in conventional KSOM medium (IVF KSOM group).

Effect of quercetin on the total cell number at the blastocyst stage

Once we confirmed the beneficial effects of quercetin on the development potential of embryos, we examined whether quercetin produced an increase in cell proliferation by analyzing the total cell number of the embryos that reached the blastocyst stage in our set of experiments. As can be seen in Figure 4, embryos that were developed *in utero* up to the morula stage and were treated with quercetin for either 2 or 4 h, presented a statistically significant higher average of cells at the blastocyst stage. For embryos treated for 4 h (IU Quer 4 h group), their total cell number was even higher than embryos cultured at low-oxygen concentrations (IU O_2 3% group) (Figure 4B). Cell count was also performed in embryos obtained using IVF and exposed to quercetin at the morula stage (Figure 5). Results showed again that embryos presented a higher total cell number in quercetin and in the low-oxygen groups, showing differences that were statistically significant (Figure 5B); the total cell numbers were similar to those found in embryos developed *in utero* up to the morula stage.

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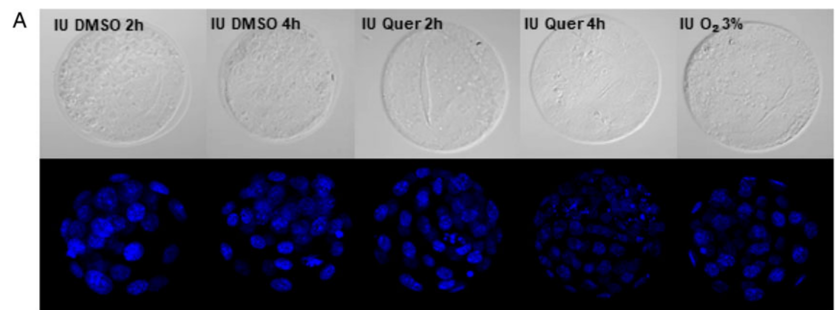

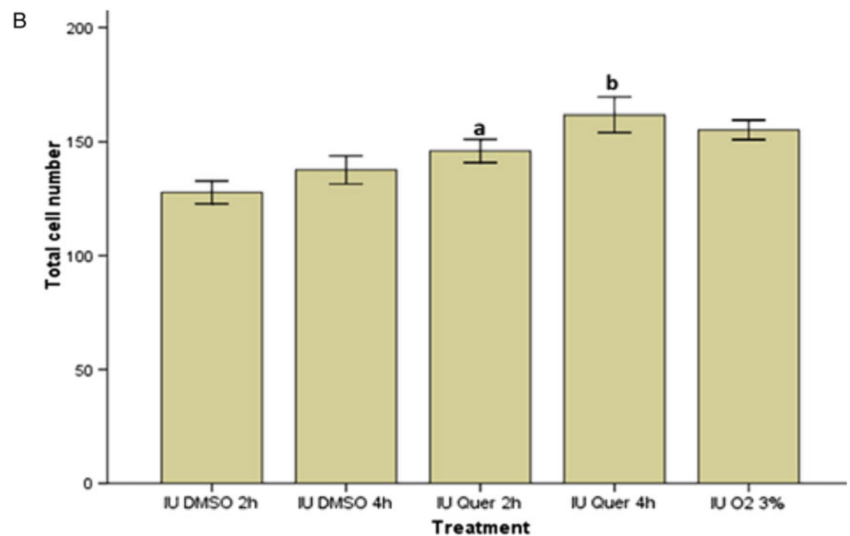

Figure 4. Results of the total cell number in blastocysts obtained *in utero* at the morula stage. IU DMSO 2 h group: embryos obtained directly from the uterus and cultured with DMSO for 2 h at the morula stage, $n = 30$. IU DMSO 4 h group: embryos obtained directly from the uterus and cultured with DMSO for 4 h at the morula stage, $n = 27$. IU Quer 2 h group: embryos obtained directly from the uterus and cultured with quercetin for 2 h at the morula stage, $n = 24$. IU Quer 4 h group: embryos obtained directly from the uterus and cultured with quercetin for 4 h at the morula stage, $n = 30$. IU O_2 3% group: embryos obtained directly from the uterus and cultured with low concentrations of oxygen from morula to blastocyst stage, $n = 24$. (A) Representative images of the total cell number, taken with a confocal microscope Nikon Eclipse TE2000-U, of embryos from each group. (B) Total cell number analysis. The data are represented as the mean ± the standard deviation (SD). a, $P < 0.001$ IU DMSO 2 h vs IU Quer 2 h; b, $P < 0.001$ IU DMSO 4 h vs IU Quer 4 h. Mann–Whitney–Wilcoxon test.

Effect of quercetin on the number of apoptotic cells in the blastocysts

We tested using TUNEL the number of apoptotic cells in *in utero* developed embryos up to the morula stage and then treated with quercetin for short periods of time. Results showed that treatment with quercetin for 4 h reduced the number of apoptotic cells found in embryos, even though differences were not statistically significant. Furthermore, morulae cultured in low-oxygen concentrations also presented a not statistically significant lower number of apoptotic cells (Figure 6; Table 2).

In addition, programmed cell death was also evaluated in embryos obtained using IVF, which usually presented more apoptosis. Results showed that embryos treated with quercetin for 4 h (IVF Quer 4 h group) presented lower apoptotic rates, and a higher number of embryos without apoptotic cells (Figure 7; Table 3), although differences were not statistically significant.

Expression of HIF-1α

Immunofluorescence was performed to study the expression of HIF-1α in embryos cultured under different conditions, as explained before. Results showed that in *in utero* obtained embryos, the expression of HIF-1α was similar in all groups despite the treatment used (Figure 8). Conversely, when we studied the protein expression in *in vitro* obtained embryos treated with quercetin, especially for 4 h, or in embryos cultured under low-oxygen conditions (IVF O_2 3% group), there was a significant increase in the expression of HIF-1α (Figure 9) compared with embryos cultured under conventional conditions, which clearly showed a reduction in the expression of HIF-1α. Noticeably, the fluorescence signal observed in embryos treated with quercetin was similar to that observed in embryos developed *in utero*. Finally, we must mention that a clear decrease in the expression of HIF-1α could also be observed in embryos cultured only with DMSO.

Discussion

The development potential of embryos obtained using IVF, as well as their quality and viability, depends among other things on the composition of the culture medium in which they are developed (Paternot *et al.*, 2010). A non-optimal culture can lead to a failure in the fertilization of the gametes and an inadequate development of the embryos, which do not reach the blastocyst stage or fail to implant in the uterus due to their poor quality. Therefore, optimal culture conditions are of utmost importance for the IVF of gametes, pre-implantation embryonic development, and implantation in all species. Therefore, increasing the *in vitro* development potential of early embryos by supplementation of the culture medium has been of great interest to many researchers and, consequently, there have been several works in which the culture medium has been enriched with many compounds that improved the quality of the embryos obtained using IVF.

In the present work, we analyzed the consequences of supplementation of the culture medium with quercetin, a flavonoid with antioxidant properties. Quercetin has been reported to have a protective effect on mouse embryos against H_2O_2, and a better rate of blastocyst formation was observed when embryos were cultured for 3 h with quercetin (Yu *et al.*, 2014). It has also been reported that more than 100 μM of quercetin in the culture medium caused some defects and abnormalities, whereas 3 μM of this molecule had

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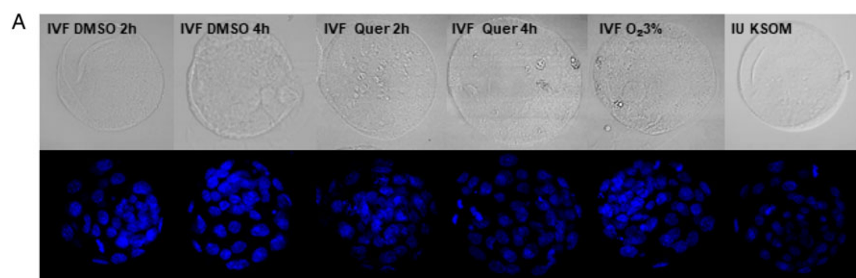

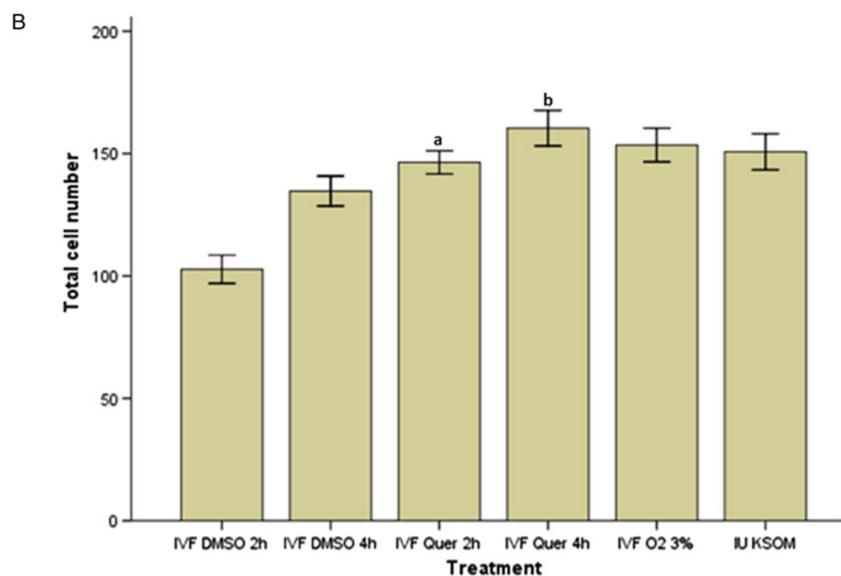

Figure 5. Results of the number of total cells in blastocysts obtained by *in vitro* fertilization (IVF). IVF DMSO 2 h group: embryos obtained by IVF and cultured with DMSO for 2 h at the morula stage, $n = 33$. IVF DMSO 4 h group: embryos obtained by IVF and cultured with DMSO for 4 h at the morula stage, $n = 27$. IVF Quer 2 h group: embryos obtained by IVF and cultured with quercetin for 2 h at the morula stage, $n = 35$. IVF Quer 4 h group: embryos obtained by IVF and cultured with quercetin for 4 h at the morula stage, $n = 29$. IVF O_2 3% group: embryos obtained by IVF and cultured in low concentrations of oxygen from morula to blastocyst stage, $n = 29$. IU KSOM group: embryos obtained from the uterus at the morula stage and developed to blastocyst in conventional culture medium, $n = 26$. (A) Representative images of the total cell number, taken with a confocal microscope Nikon Eclipse TE2000-U, of embryos from each group. (B) Total cell number analysis. The data are represented as the mean ± the standard deviation (SD). a, $P < 0.001$ IVF DMSO 2 h vs IVF Quer 2 h; b, $P < 0.001$ IVF DMSO 4 h vs IVF Quer group 4 h, ANOVA, post-hoc Tukey test.

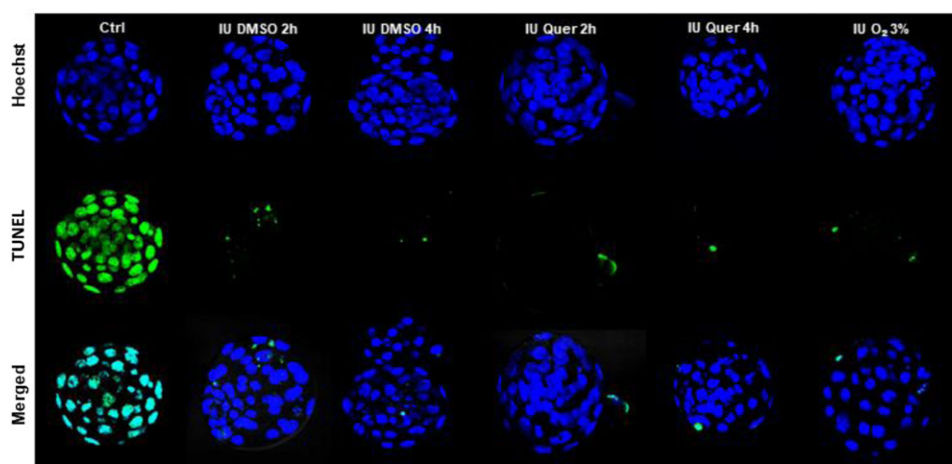

Figure 6. Representative images illustrating the incidence of cell death in mouse embryos obtained *in utero*. Nuclei were stained with Hoechst 33342 (blue). Fragmented DNA was labelled using the TUNEL assay (green). Merged images represent the proportion of positive TUNEL cells (apoptotic, middle row) with respect to the embryo total cell count (bottom row). IU DMSO 2 h group: embryos obtained *in utero* at the morula stage and cultured with DMSO for 2 h and grown to the blastocyst stage in conventional culture medium, $n = 25$. IU DMSO 4 h group: embryos obtained *in utero* at the morula stage and cultured with DMSO for 4 h and grown to the blastocyst stage in conventional culture medium, $n = 25$. IU Quer 2 h group: embryos obtained *in utero* at the morula stage and cultured with quercetin for 2 h and developed to the blastocyst stage in conventional culture medium, $n = 25$. IU Quer 4 h group: embryos obtained *in utero* at the morula stage and cultured with quercetin for 4 h and developed to the blastocyst stage in conventional culture medium, $n = 25$. IU O_2 3% group: embryos obtained *in utero* and cultured in low-oxygen concentrations from the morula to the blastocyst stage, $n = 25$. Ctrl: positive control, which consisted of *in utero* obtained embryos treated with DNase to induce apoptosis before incubation with TUNEL reagents.

a protective effect on mouse embryos against hydroxyurea (Pérez-Pastén *et al.*, 2010). Furthermore, a protective effect of quercetin was demonstrated in embryos against 0.005 μg/ml of actinomycin D (Sameni *et al.*, 2018). Our results suggested that quercetin produces a detrimental effect on embryonic development when cultured for long periods of time regardless of the stage of development. This could be caused by different things. First of all, after 24 h in the culture medium, quercetin stops being stable (Hu *et al.*, 2012) and loses its effectiveness as an antioxidant, producing toxic products for embryos. Another

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Table 2. Number of apoptotic cells in embryos obtained *in utero* from female mice and treated with quercetin or DMSO

Groups	No. embryos	TUNEL-positive nuclei (%)			
		0 apoptotic cells	1 apoptotic cell	2 apoptotic cells	>3 apoptotic cells
IU DMSO 2 h	25	20 (80%)	3 (12%)	2 (8%)	0 (0%)
IU DMSO 4 h	25	22 (88%)	1 (4%)	2 (8%)	0 (0%)
IU Quer 2 h	25	22 (88%)	3 (12%)	0 (0%)	0 (0%)
IU Quer 4 h	25	24 (96%)	1 (4%)	0 (0%)	0 (0%)
IU O_2 3%	25	23 (92%)	2 (8%)	0 (0%)	0 (0%)

Differences between the groups were calculated using the χ^2-test. Quer, quercetin.

Table 3. Number of apoptotic cells in embryos obtained by *in vitro* fertilization and treated with quercetin or DMSO

Groups	No. embryos	TUNEL-positive nuclei (%)			
		0 apoptotic cells	1 apoptotic cell	2 apoptotic cells	> 3 apoptotic cells
IVF DMSO 2 h	25	15 (60%)	6 (24%)	2 (8%)	2 (8%)
IVF DMSO 4 h	21	19 (90.47%)	1 (4.76%)	1 (4.76%)	0 (0%)
IVF Quer 2 h	25	20 (80%)	4 (16%)	0 (0%)	1 (4%)
IVF Quer 4 h	23	22 (95.26%)	1 (4.35%)	0 (0%)	0 (0%)
IVF O_2 3%	25	19 (76%)[a]	4 (16%)	1 (4%)	1 (4%)
IU KSOM	25	21 (84%)	3 (12%)	0 (0%)	1 (4%)

Differences between the groups were calculated using the χ^2-test. Quer, quercetin. [a]$P < 0.05$, IVF Quer 4 h vs IVF O2.

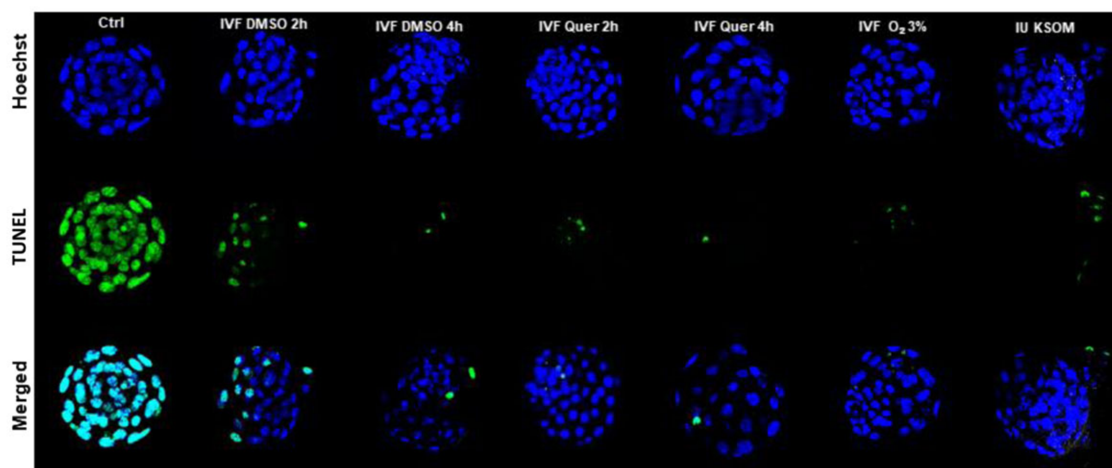

Figure 7. Representative images illustrating the incidence of cell death in mouse embryos obtained by *in vitro* fertilization. nuclei were stained with Hoechst 33342. Fragmented DNA was labelled using the TUNEL assay (green). Merged images represent the proportion of positive TUNEL cells (apoptotic, middle row) with respect to the embryo total cell count (bottom row). IVF DMSO 2 h group: embryos obtained by *in vitro* fertilization and cultured with DMSO for 2 h at the morula stage, $n = 25$. IVF DMSO 4 h group: embryos obtained by *in vitro* fertilization and cultured with DMSO for 4 h at the morula stage, $n = 21$. IVF Quer 2 h group: embryos obtained by *in vitro* fertilization and cultured with quercetin for 2 h at the morula stage, $n = 25$. IVF Quer 4 h group: embryos obtained by *in vitro* fertilization and cultured with quercetin for 4 h at the morula stage, $n = 23$. IVF O_2 3% group: embryos obtained by *in vitro* fertilization and cultured in low-oxygen concentrations from the morula to blastocyst stages, $n = 25$. IU KSOM group: embryos obtained *in utero* at the morula stage and developed to the blastocyst stage in conventional culture medium, $n = 25$. Ctrl: positive control, which consisted of *in vitro* obtained embryos treated with DNase to induce apoptosis before incubation with TUNEL reagents.

possibility is the fact that quercetin increases the expression of γ-glutamylcysteine synthetase, and therefore it promotes the synthesis of glutathione (Moskaug *et al.*, 2005). However, after long periods of time, quercetin sequesters cysteine from the culture medium, which is a precursor of glutathione, and it has been demonstrated that cleavage-stage embryos have a limited capacity to synthesize reduced glutathione, and appear to be negatively affected under conditions that deplete glutathione (Gardiner and Reed, 1995). It is also known that quercetin has been seen as an antiproliferative and proapoptotic compound in several lines of cancer cells and this property could explain its negative effects on embryos in culture because they cannot grow properly (Hashemzaei *et al.*, 2017). Additionally DMSO, which is the usual vehicle for dissolving quercetin, has been proved to have cytotoxic

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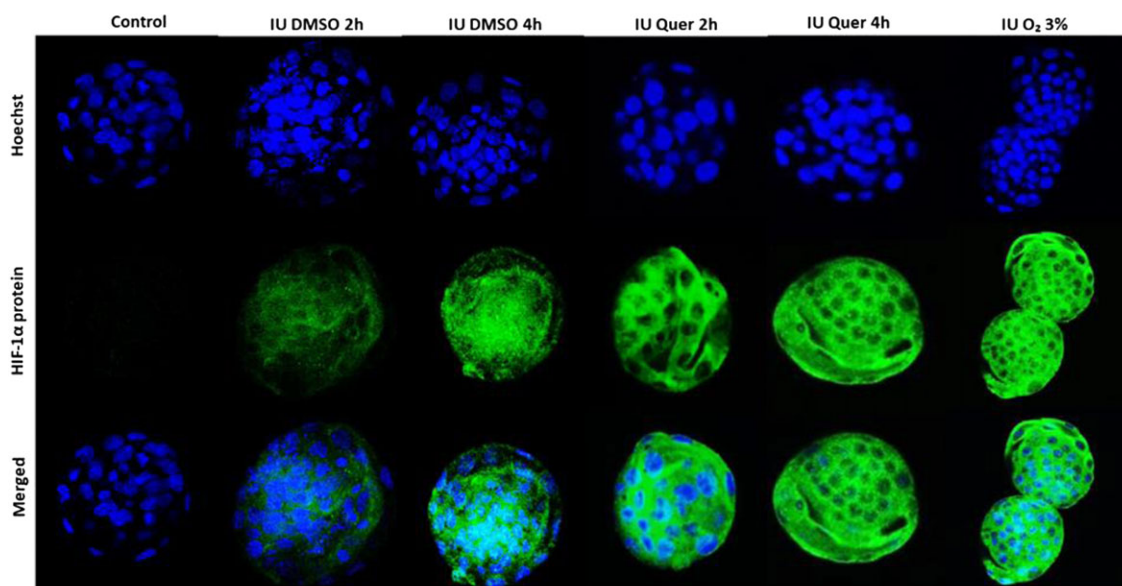

Figure 8. Immunofluorescence analysis of HIF-1α protein expression in mouse embryos obtained *in utero*. Nuclei were stained with Hoechst 33342 (blue). The expression of HIF-1α was marked using FITC (green). IU DMSO 2 h group: embryos developed *in utero* up to the morula stage and cultured with DMSO for 2 h and cultured until the blastocyst stage in conventional culture medium, $n = 25$. IU DMSO 4 h group: embryos developed *in utero* up to the morula stage and cultured with DMSO for 4 h, then cultured until the blastocyst stage in conventional culture medium, $n = 25$. IU Quer 2 h group: embryos developed *in utero* up to the morula stage and cultured with quercetin for 2 h, then developed until the blastocyst stage in conventional culture medium, $n = 25$. IU Quer 4 h group: embryos developed *in utero* up to the morula stage and cultured with quercetin for 4 h, then developed to the blastocyst stage in conventional culture medium, $n = 25$. IU O_2 3% group: embryos developed *in utero* up to the morula stage and cultured in low-oxygen concentrations to the blastocyst stage, $n = 25$. Ctrl: negative control, which consisted of embryos developed *in utero* from which the primary antibody was omitted.

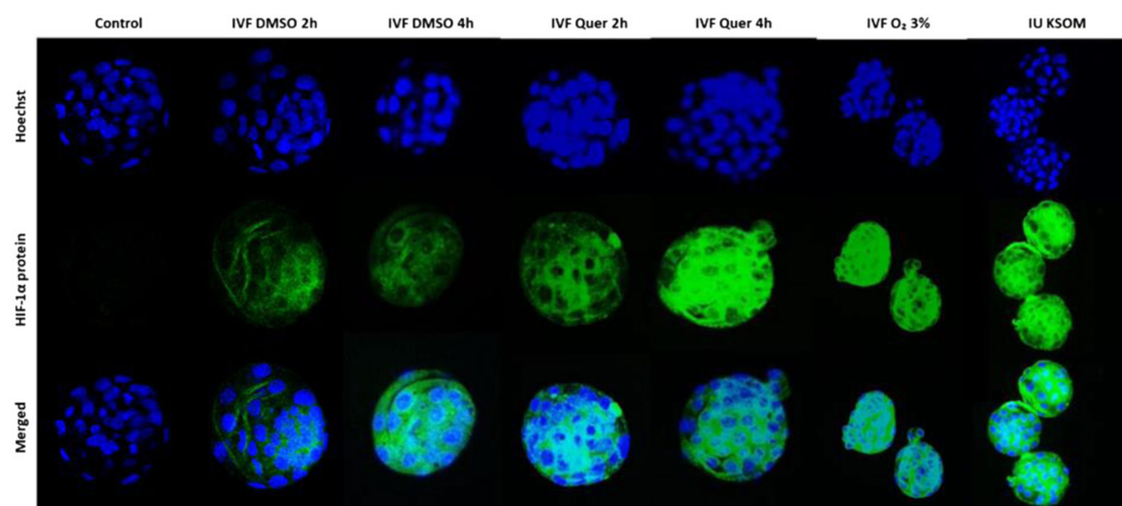

Figure 9. Immunofluorescence analysis of HIF-1α protein expression in mouse embryos obtained *in vitro*. Nuclei were stained with Hoechst 33342 (blue). The expression of HIF-1α was marked using FITC (green). IVF DMSO 2 h group: embryos obtained by IVF and cultured with DMSO for 2 h at the morula stage, $n = 25$. IVF DMSO 4 h group: embryos obtained by IVF and cultured with DMSO for 4 h at the morula stage, $n = 21$. IVF Quer 2 h group: embryos obtained by IVF and cultured with quercetin for 2 h at the morula stage, $n = 25$. IVF Quer 4 h group: embryos obtained by IVF and cultured with quercetin for 4 h at the morula stage, $n = 23$. IVF O_2 3% group: embryos obtained by IVF and cultured in low-oxygen concentrations from the morula to the blastocyst stage, $n = 25$. IU KSOM group: embryos obtained *in utero* at the morula stage and developed to the blastocyst stage in conventional culture medium, $n = 25$. Ctrl: negative control, which consisted of embryos obtained by IVF from which the primary antibody was omitted.

effects in mouse pre-implantation embryos (Kang *et al.*, 2017), which could explain some of the results observed here but not the drastic effects observed with quercetin. Another possibility is that this cytotoxic effect and the instability of quercetin after 24 h could be producing a synergic effect with DMSO and be responsible for the low rates of blastocyst development observed here.

Recently, Hernández *et al.* (2020) showed that an improvement in embryo quality occurs when $4OHE_2$ is added at the time of compaction (day 3 of pre-implantation development, morula stage), which indicates that embryos are more sensitive to certain environmental changes when they are at the morula stage. Nonetheless, in *in vitro* obtained embryos, our study corroborates that the addition of quercetin at the time of embryo compaction, for a period of 2 h or 4 h, results in a greater number of embryos that develop to the blastocyst stage, compared with embryos cultured in conventional medium, and results almost resemble

those observed in embryos cultured in low-oxygen concentrations. These results indicate that quercetin does not negatively affect embryos and, furthermore, appears to counteract the detrimental effects produced by the *in vitro* culture conditions.

Putting this information in the context of embryo development, it seems clear that exposing early cleavage embryos to certain compounds and for long periods of time impairs normal development and they fail to reach the blastocyst stage, possibly for some of the reasons explained above. This could apply to hormones and their metabolites, or antioxidants such as quercetin, which reduces the viability of embryos by the different means stated above. Nevertheless, the introduction of the same compounds in the morula to blastocyst transition for short periods of time can have a beneficial effect when embryos are cultured *in vitro*. Obviously, the possible implications of this situation on human embryology and the culture of human embryos must be manifested.

There are two conventional possibilities for *in vitro* human embryo culture for IVF techniques. One is sequential culture, which implies changing the culture medium on day 3 of development to a more appropriate medium for extended culture and the other is the unique or one-step culture, in which the same culture medium is used during the entire embryo development (Tao *et al.*, 2022). Our results would be in favour of the first type of culture, because the needs and susceptibility of the embryos are very different during the early steps from zygote to morula, compared with later on when the blastocyst forms. Our data could gain insight into the possible modification of the culture medium at the morula stage to achieve better results in IVF. This information could also help some laboratories that do not have access to low-oxygen incubators, because the addition of quercetin at the morula stage resembles the success that these systems provide in the IVF clinics.

In addition to improving the development potential, our results showed that there was an increase in the total cell number when we use quercetin, data in concordance with other works that also showed this increase after using quercetin (Yu *et al.*, 2014; Sameni *et al.*, 2018). It is important to mention that the increase was not uncontrolled and the maximum number of total cells observed remained at ~160. This observation is interesting because a higher number of cells do not relate to a higher implantation potential, and it can be indicative of bad quality (Wigger *et al.*, 2017).

Regarding apoptosis rates, several studies have demonstrated that there was a reduction in the number of apoptotic cells in blastocysts when embryos were treated with quercetin after treatment with stressful substances or in aged mice (Yu *et al.*, 2014; Sameni *et al.*, 2018; Cao *et al.*, 2020). Our results show that, indeed, culturing embryos obtained using IVF with quercetin produced a reduction in the number of apoptotic cells per embryo, finding fewer embryos with apoptotic cells when they were cultured for 4 h with quercetin. However, when embryos were obtained from the uterus at the morula stage and cultured with quercetin, a reduction in the number of embryos that had apoptotic cells was not observed, and many of the embryos presented only a single apoptotic cell, matching results observed in the control. Again, these results showing the effects of quercetin in *in vitro* obtained embryos, but not in embryos developed *in utero* up to the morula stage, suggested that quercetin does not affect the embryos per se, but it appears to counteract the negative effects that the components and conditions of the *in vitro* culture medium produce in the embryos fertilized *in vitro*. The effects produced by quercetin could be attributed to its antioxidant properties (De Matos *et al.*, 2002; Kere *et al.*, 2013; Rocha-Frigoni *et al.*, 2016).

Oxygen tension is known to influence embryo development and cell number in various species and one of its major negative consequences is the production of ROS. Conversely, oxygen levels have a significant influence on gene expression patterns that are mediated by the heterodimeric transcription factor HIF-1α (Myhrstad *et al.*, 2002; Rocha-Frigoni *et al.*, 2016). Harvey (Mastroianni Jr and Jones 1965; Khanduja *et al.*, 2001) suggested that HIF may be involved in the molecular mechanisms that respond to changes in oxygen status during embryo development in mouse and bovine blastocysts. Some studies have shown that HIF-1α could be activated by the flavonoid quercetin to heal ulcers (Jeon *et al.*, 2007) and produced an increase of HIF-1α in processes related to inflammation and vascular diseases in human placental cells (Bogacz *et al.*, 2021). Our study demonstrated that the localization of HIF-1α was predominantly in the cytoplasm of cells, which is consistent with the previous study by Thompson *et al.* (2004) in which HIF-1α was detected in the cytoplasm of mouse blastocysts when cultured in low-oxygen concentrations (2%) during compaction and blastulation. Furthermore, embryos obtained using IVF and cultured with quercetin for 4 h had a higher expression of the HIF-1α protein. This increase was similar to that observed in embryos obtained using IVF cultured with 3% O_2, and in embryos obtained directly from the uterus. In contrast, embryos cultured in conventional medium hardly expressed HIF-1α protein. Putting these results together, we can determine that quercetin, when added to the culture medium of embryos for short periods of time, produces an increase in the expression of HIF-1α, which could produce an increase in the activation of the genes necessary for the reduction of ROS levels and repair mechanisms.

In conclusion, this study illustrates how changing the experimental conditions can turn a molecule from being toxic for the embryos when left in the culture medium for long periods of time, to be beneficial for them when the exposure time is reduced to a short period. The results provided here illustrate a potential strategy for obtaining better quality embryos and improving the success rates of assisted reproductive technologies, especially for those who do not have access to incubators with low-oxygen concentrations. Nonetheless, the translation of the use of quercetin to the clinic needs much more data about its effects and mechanisms of action, as an excess of polyphenols can result in negative effects in early embryos (Yang *et al.,* 2008).

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Conflicts of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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