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L-carnitine reduces the adverse effects of ROS and up-regulates the expression of implantation related genes in *in vitro* developed mouse embryos

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ABSTRACT

In vitro developed embryos are inevitably exposed to various reactive oxygen species (ROS) which may decrease the embryo's competence in assisted reproductive technology (ART) procedures. Optimization of embryo culture media using antioxidant agents could help to improve embryo quality and could overcome failures in current ART. The aim of this study was to evaluate the effects of L-carnitine (LC), an enhancer of mitochondrial activity and free radical scavenger, in culture media on early embryo competence and expression of ErbB1 and ErbB4 implantation related genes. Two-cell mouse embryos were cultured in the following four conditions: 1. LC group in media containing LC; 2.H $_2O_2$ group exposed to H_2O_2 for 30 min and then transferred into a simple media; 3.H_2O_2+LC group exposed to H_2O_2 for 30 min and then transferred into a simple media containing LC; 4.the control group kept throughout in simple media. All groups were allowed to develop until the blastocyst stage. ErbB1 and ErbB4 expression were evaluated by Real-time PCR and immunocytochemistry. The expression of Sirt3 gene was also evaluated. Intracellular ROS levels were examined by DCFH-DA fluorescence intensity. In order to assess the morphological quality of the embryos, ICM and OCM number blastocyst cells were evaluated by using Hoechst and propidium iodide (PI) staining. ErbB1, ErbB4, ROS levels and cell number were compared across all *in vitro* groups. Our data reveal that LC significantly increases ErbB1 and ErbB4 gene and protein expression with intracellular ROS levels and Sirt3 gene expression significantly decreased after LC treatment. It is worth noting that an elevated cell number was observed in the LC-treated group compared with the other groups suggesting increased viability and/or proliferation. Our findings suggest that the use of LC could be helpful to improve preimplantation embryo culture media through its effects in decreasing ROS levels and the increase of implantation-related genes.

1. Introduction

Infertility is a significant worldwide problem [1]. It is defined by "the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (WHO-ICMART glossary). According to the World Health Organization, up to one in six couples has an infertility problem [2]. The universal infertility rate is reported as varying from 3.5% to 22% and in Iran, it is estimated to be at 10.6% [3]. Studies indicate that Infertility can be due to many factors and causes, including physical and psychological and also show that most infertility issues are related to females [4]. Therapeutic strategies include psychotherapy and counseling, pharmacological and surgical interventions and finally assisted reproductive technology (ART) [5]. Recently, various methods of ART treatment such as *in vitro* fertilization (IVF), *in vitro* maturation (IVM) *in vitro* culture (IVC). have become more successful and more frequently used with nearly 3.9% of all new births now the result of these methods [1].

However, even though advances in the field of *in vitro* production and maturation of embryos have grown in the last few years,

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pregnancy success rates remain low. Unavoidable environmental sources of reactive oxygen species (ROS) such as laboratory light, excess temperature, pH fluctuation of culture media are recognized to impair embryo competency and resulted in reducing optimal pregnancy rates [6,7]. The natural origin of ROS within the cell is the mitochondria that are a major source of ROS and a determinant of the developmental competence of preimplantation embryos [8]. Sirt3 (a member of the sirtuin family) is a known mitochondrial deacetylase that is involved in the regulation of mitochondrial electron transport and is also capable of protecting *in vitro* fertilized mouse preimplantation embryos against oxidative stress [9,10]. Oxidative stress occurs when the generation of reactive oxidant molecules exceeds the antioxidant defense capacity of the cell. Embryo development incompetency and embryo growth retardation follow oxidative stress through induced cell-membrane damage, DNA damage, and apoptosis.

Hence, oxidative stress must be regulated by the addition of antioxidants to the culture media in ART laboratories [11,12]. Superoxide anion radical (O_2–•), the hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2) are the most common types of ROS. Hydrogen peroxide (H_2O_2) is one of the strongest oxidants and is an important ROS [12]. Therefore, antioxidants likely take a critical role during ART techniques and also in diminishing consequent loss of or damage to the embryo [13]. L-Carnitine (LC), an essential energy-providing compound to the cell, has a pivotal role in β-oxidation by delivering fatty acids to the mitochondrial matrix for ATP production. This can improve embryo development and blastocyst formation rates. On the other hand, it is known as an important antioxidant that neutralizes the toxic effects of H_2O_2 on the embryo and protects them against oxidative damage-induced apoptosis [14,15]. Although embryo competence is satisfactory, sadly the rate of successful pregnancies following *in vitro* development remains only 13% following embryo transfer. Since successful implantation depends on the expression of ErbB receptors including ErbB1, ErbB2, ErbB3, and ErbB4 on the expanded blastocysts and their interaction with uterine ligands so, both uterine investigations and molecular studies are needed to fully understand the problem [16]. The earliest expressed genes on preimplantation embryos are ErbB1 and ErbB4 [17]. Thus, following the successful introduction of optimal *in vitro* conditions for embryo culture involving neutralizing excess ROS, attention has now turned to improve the rate of preimplantation embryo development and successful implantation.

Limited data is available for gene expression in manipulated embryos and to our knowledge, no study has reported the effect of LC on the earlier pre-implantation gene expression for ErbB1 and ErbB4, and on the mitochondrial impairment by analyzing the rate of sirt3 gene expression.

The aim of this study was therefore to investigate the effects of LC against environmental stress factors on pre-implantation embryo competence and, thereby, to clarify whether LC exerts its effects on expression of the primary implantation receptors, ErbB1 and ErbB4 and/or on mitochondrial functions by sirt3 gene expression.

2. Materials and methods

2.1. Animal preparation and ethical considerations

All experiments were carried out according to the Iranian Council's guidelines for the Use and Care of Animals and confirmed by ethical committee of Kashan University of Medical Sciences (IR.KAUMS.MEDNT.REC.1396.45). A total of 90 NMRI mice with a mean weight of 30 ± 5 *gr* were purchased from the Research Center of Kashan University of Medical Sciences (Kashan, Isfahan, Iran). Six to 8-week old females and 10-week old males were housed individually under controlled conditions of temperature (25 ± 2 °C), 12 h light and 12 h dark cycle (7 a.m.: 7 p.m.) and with free access to food and water [18].

2.2. Embryos Collecting and grouping

Female mice were superovulated by an intraperitoneal (IP) injection of 8 IU pregnant mare serum gonadotropin (PMSG, Sigma Aldrich, China). After 48 h an injection of 8 IU human chorionic gonadotropin (HCG, pregnylOrganon, Germany) was also given to the females. These treated females were paired with proven fertile males overnight. Mice with vaginal plugs were taken as pregnant mothers. At 42–48 h after the HCG injection, under a stereomicroscope (Olympus SZX9, Japan), 2-cell embryos were flushed into Ham's F10 media supplemented with 10% human serum albumin (N6908, Sigma, USA) [17]. After washing three times, morphologically normal embryos i.e. those with blastomeres of equal size and with no cytoplasmic fragmentation were collected into a few drops of Global simple medium (LGGT-030, Life Global, USA). Collected 2-cell embryos were randomly divided into four *in vitro* groups including control, H_2O_2, LC, LC + H_2O_2 groups (60 embryos in each group) (diagram 1). After 72–96 h, the expanded blastocysts were used for further experiments. Four *in vitro* embryos groups were given the following treatments:

1. Control group; Embryos were kept throughout in the same Global simple medium.
2. H_2O_2 group; Embryos exposed to 50 μM H_2O_2 for 30 min. Then, embryos were washed with fresh Ham's F10 medium and transferred into the Global medium.
3. LC group; Embryos transferred into Global medium containing 0.3 mg/ml LC [14].
4. LC + H_2O_2 group; Embryos exposed to50 μM H_2O_2 in the Global medium for 30 min [19], washed with fresh Ham's F10 medium and transferred into a Global medium containing 0.3 mg/ml LC.

2.3. Quantitative RT-PCR assay

Expression of *ErB1, ErbB4, and Sirt3* genes in expanded blastocysts were assessed by using quantitative RT-PCR. RNeasy Micro kit (74004, Qiagen, USA) was used to isolate total RNA from 20 expanded blastocysts of each group according to manufacturer's protocol [20,21]. The extracted RNA was used for complementary DNA (cDNA) synthesis using the Prime Script™ RT reagent kit (Takara Bio Inc, Shiga, Japan). Reverse transcription was carried out using 500 ng of total RNA following the manufacturer's protocol. Real-Time qPCR was performed with 2 μl of the synthetized cDNA using SYBR green master mix (Biofact, Corea) in a total reaction volume of 10 μl using the IQ5 Real-time PCR system (Biorad, Germany). The sequences of specific primers used are shown in Table 1. The changes in long non-coding RNAs expression were normalized by GAPDH mRNA expression and these data were analyzed using $2^{-\Delta\Delta c}$.

2.4. Immunocytochemistry of mouse blastocysts

Three to five expanded blastocysts from each group were randomly selected for ICC using each antibody. Blastocysts were washed in PBS and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After washing three times with PBS they were permeabilized with PBS containing 0.5% Triton X-100 for 1–2 min, incubated in blocking solution (10% goat serum, Sigma, USA) for 10 min, before incubation with one of the primary

Table 1
List of specific primers used in real-time polymerase chain reaction assay.

Gene name	Accession no.	Designed oligonucleotide (5'→3')	Product size (bp)
ErbB1	NM_207655	F: GGCACAAGTAACAGGCTCAC R: AGTTGGACAGGATGGCTAAGG	277
ErbB4	L47241	F: GAGGAGAACTGGATGAAGAAGG R: CGAAGGTGTTGAGGTATAGAGG	213
Sirt3	NM_022433	F: TTTCATGTTGGCCAAGGAGC R: GCTGTTACAAAGGTCCCGTG	186
GAPDH	MH759770	F: AACTTTGGCATTGTGGAAGG R: ACACATTGGGGGTAGGAACA	233

antibodies, anti-EGFR antibody (ErbB1) (1:50 dilution, Abcam, ab52894, USA) and anti-ErbB4 antibody (1:50 dilution, Abcam, ab32375, USA) at 4 °C overnight. After washing in PBS, they were further incubated with secondary antibody conjugated to fluorescein isothiocyanate (FITC, 1:1000 dilution, Abcam, USA) for 20 min at room temperature in the dark. After that, the stained blastocysts were washed in PBS then for staining nuclei; the embryos were incubated in 25 μg/ml bisbenzimide (Hoechst 33342, Sigma, Germany) in absolute alcohol for 20 min in dark. Finally, they mounted in 90% glycerol in PBS and then visualized by fluorescence microscopy using a Nikon Eclipse (Ti, Japan) microscope. The brightness of each blastocyst was analyzed using the Image Quant software (TotalLab Quant, UK) [22,23].

2.5. Measurement of ROS content in embryos

To measure the relative levels of ROS, fluorescent DCF dye (DCFH-DA; 2 μM, Sigma, USA) was used. Five to eight expanded blastocysts from each group were placed in Ham'sF10 containing 2 , 7 -dichlorofluorescein diacetate (DCFH-DA; 2 μM, Sigma, D6883, USA) at 37 °C in the dark for 20 min. Samples were washed with Ham'sF10 medium plus 10%HSA and mounted on glass slides to observe each blastocyst using fluorescence microscopy with 450–490 nm (excitation) & 520 nm (emission) filters on a Nikon Eclipse (Ti, Japan) microscope. Finally, the brightness of each blastocyst was analyzed using the Image Quant software (TotalLab Quant, UK) [20,24].

2.6. Differential staining of blastocysts

Differential staining was conducted to assess the quality of expanded blastocysts as described by with minor modifications [25]. Briefly, 5–8 expanded blastocysts from each group (control, H_2O_2, LC, and LC + H_2O_2) were randomly selected and placed in Ham's F10 medium supplemented with 1%Triton X-100 plus 10 μg/ml propidium iodide (PI) (P4170, Sigma, Germany) at 37 °C for approximately 10 s. When blastomeres were visibly changed to a light pink color, the embryos were incubated in 25 μg/ml bisbenzimide (Hoechst 33342, Sigma, Germany) in absolute alcohol for 20 min in a dark and humid chamber. Fluorescence microscopy utilized a Nikon Eclipse (Ti, Japan) microscope equipped with a UV filter. For detecting the ICM an excitation wavelength of 355 nm was used in combination with a barrier filter having a cutoff at 465 nm. A second filter set used an excitation wave length of 530 nm and a barrier filter with a cutoff at 615 nm. Total numbers of nuclei stained were manually counted from photomicrographs. Under above mentioned conditions, ICM and OCM appeared in red and blue respectively [26].

2.7. Statistical analysis

At least three replicates were performed for each experiment and the data were expressed as the mean ± standard deviation. To evaluate the statistical significance between different groups, statistical analysis was performed using GraphPad Prism 8, San Diego, CA, USA and one-way analysis of variance (ANOVA) followed by Tukey's and Tamhane's post hoc tests with SPSS ver 16. The *P*-values less than 0.05 ($P < 0.05$) were considered statistically significant.

3. Results

3.1. Effect of LC on the genes expression of ErbB1, ErbB4, and Sirt3

The results of qRT-PCR showed that blastocysts treated with 0.3 mg/ml LC up-regulate ErbB1 and ErbB4 genes and down-regulate Sirt3 gene compared with control and the other treatment groups ($P < 0.05$). Statistical analysis revealed that culture media supplemented with 50 μM H_2O_2 can significantly ($P < 0.05$) decrease the expression of ErbB1 and ErbB4 and increase expression of Sirt3 in comparison to the other treatments (Figs. 1 and 2). Fig. 1a and 1b show that in the LC + H_2O_2 group, the LC had a protective effect over the damaging effects of H_2O_2 and significantly ($P < 0.05$) enhanced the expression of ErbB1 and ErbB4 genes. On the other hand, LC decreased the expression of Sirt3 gene in LC + H_2O_2 group in comparison with H_2O_2 group (Fig. 2).

3.2. Effect of LC on the proteins expression of ErbB1 and ErbB4

To evaluate the changes of protein expression in expanded blastocysts, the immunocytochemistry method was used and the quantitation of ErbB1 and ErbB4 were performed using the Image Quant software (TotalLab Quant, UK). The significant over-expression of ErbB1 and ErbB4 proteins were detected in all expanded blastocysts in LC group in comparison with other groups ($P < 0.05$). As the data show, the lowest ErbB1 and ErbB4 proteins expression are related to H_2O_2 group but treatment with L-carnitine in LC + H_2O_2 group significantly ($P < 0.05$) increased the expression of ErbB1 and ErbB4 proteins among those embryos damaged following H_2O_2 exposure (Figs. 3 and 4).

3.3. Effect of LC and H_2O_2 on the intracellular ROS level

To investigate whether the embryonic developmental arrest is caused by oxidative stress, we explored the intracellular ROS level at the expanded blastocyst stage. The quantification analysis showed that supplementing culture media with LC can significantly decrease the mean intracellular ROS concentration in the expanded blastocysts in comparison with control and the other groups ($P < 0.05$). The results showed that ROS levels were highest in supplemented culture media with H_2O_2 (H_2O_2 group) but that treatment with LC in this group (LC + H_2O_2 group) can significantly decrease ($P < 0.05$) the level of ROS (Fig. 5).

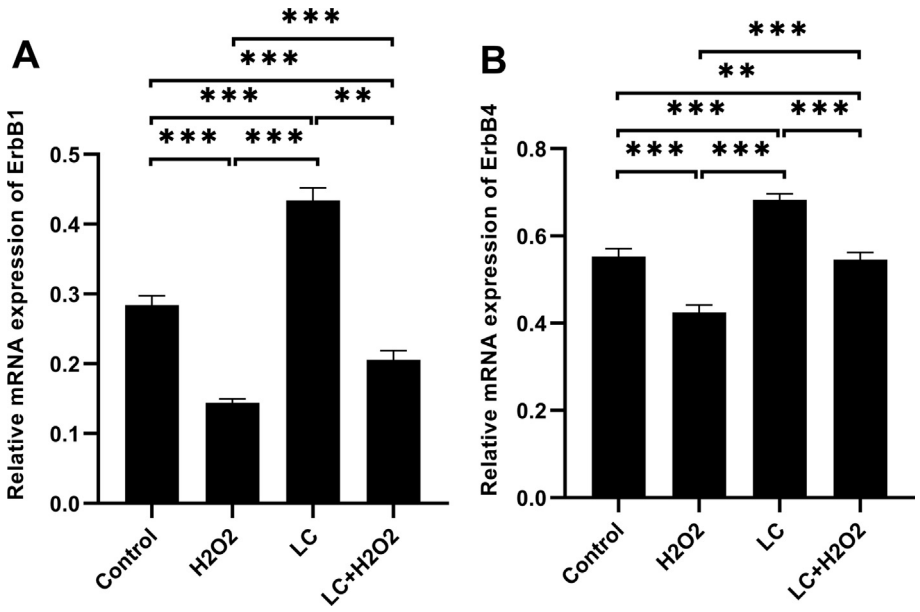


Fig. 1. Up-regulation of ErbB1 and ErbB4 genes expression in embryos with LC treatment. A) Quantitative real-time PCR (qRT-PCR) analysis of ErbB1 mRNA.B) Quantitative real-time PCR (qRT-PCR) analysis of ErbB4 mRNA. Data are presented as the mean ± SD from each group. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

3.4. Effect of LC on the total blastomere numbers and the number of cells in ICM

Differential blastocyst staining was done to determine the exact number of cells present in the inner cell mass (ICM), the outer cell mass (OCM) and the total cell numbers. Under fluorescence microscopy, ICM and OCM nuclei appeared blue and red, respectively.

As depicted in Fig. 6a and b treating media with LC can significantly ($P < 0.05$) enhance the ICM nuclei and total blastomere numbers in LC group in comparison to the other groups.

Interestingly, when the number of cells were compared in H_2O_2 and LC + H_2O_2 groups, LC decreased the negative effects of H_2O_2 exposure and increased the number of blastomeres in LC + H_2O_2 group in compare to H_2O_2 group.

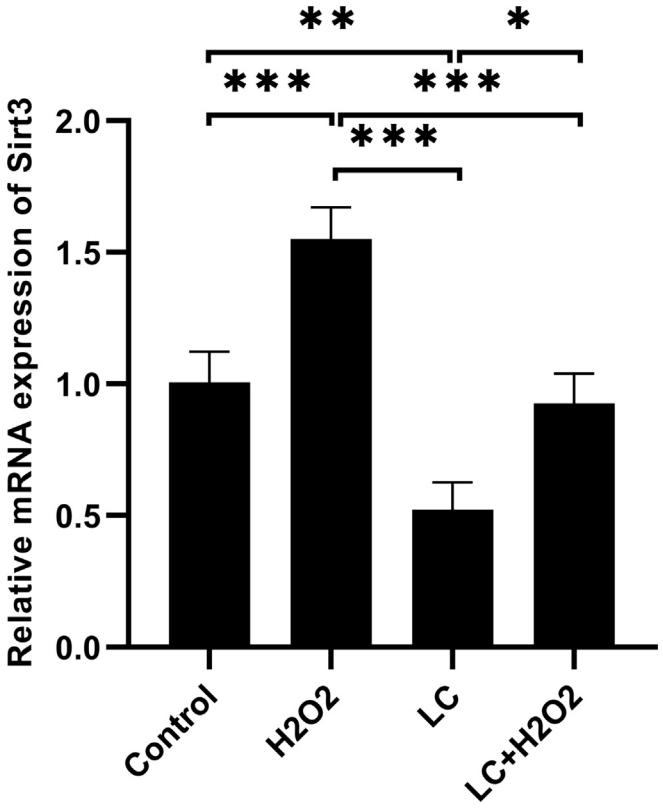


Fig. 2. Down-regulation of Sirt3 gene expression in embryos with LC treatment. Quantitative real-time PCR (qRT-PCR) analysis of Sirt3 mRNA. Data are presented as mean ± SD. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

4. Discussion

Even though there have been many advances in ART, the rate of successful implantation in laboratory-produced embryos remains disappointingly low [27,28]. indicating that critical factors have yet to be identified and addressed. Successful implantation requires interactions between the embryo and the mother's endometrium [29]. Both partners, the mother as well as the embryo, take equal roles in maternal-embryonic dialogue, the embryonic part being the main target in this study [30]. Published reports suggest that the *in vitro* produced embryos are weaker and more sensitive than embryos produced through natural, *in vivo* conception, therefore, show a lower pregnancy rate [31]. *In vitro* produced embryos are reported to show slower growth rate [31] and a higher incidence of cytogenetical abnormalities than normal embryos [32]. Addressing this issue, researchers have suggested the effects of environmental excess of ROS as a major factor in the reduction in the quality of embryos and suggested the use of antioxidants to neutralize ROS in embryonic culture media [33]. However, controversy continues in discussions on whether adding antioxidants to the embryonic culture media can lead to successful implantation of these embryos or not [28,33]. Thus, using different types of antioxidants are a hot topic in this area of ART.

In the present study, we investigated the effect of using LC on embryo competency. Two-cell embryos were collected and randomly divided into 4 groups including; 1. Simple culture media supplemented with H_2O_2; 2. Supplemented with LC; 3. Supplemented with both H_2O_2 and LC (LC + H_2O_2); and 4. Simple media without adding supplements (control). In each group, the

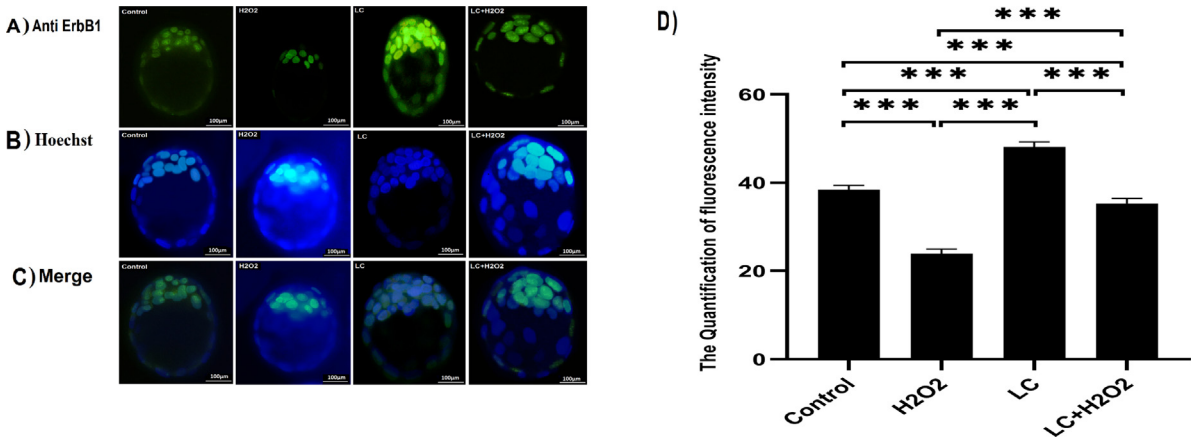


Fig. 3. Increased protein expression of ErbB1 following treatment with LC. Images show embryos stained with anti-ErbB1 fluorescence (A; in green), Hoechst dye (B; nuclei in blue) and merged (C) in control, H_2O_2, LC and LC + H_2O_2 groups. Anti-ErbB1 staining was quantified using Image Quant software (TotalLab Quant). D) Data are presented as mean ± SD. $^{***}P \leq 0.001$.

development of the embryos was studied morphologically and molecularly from the two cells to the blastocyst stage, and then compared to the other groups. The results of this study confirmed our hypothesis that adding LC to embryonic culture media could increase the total cell blastomere numbers compared with embryos grown in the other group conditions. Moreover, when Inner Cell Mass was counted, increased numbers of blastomere were found in the LC group. In contrast, the lowest blastomere number of ICM and the total cell blastomeres were counted in H2O2 group. The most remarkable finding was that LC addition in the LC + H2O2 group could cancel the destructive effect of H2O2 to exposed embryos and also increase the blastomere numbers of the ICM. LC thus improved embryonic competency and may imply the possibility of significantly improved pregnancy rates using this in ART procedures [34,35]. The Present results are in agreement with other studies defining the effective role of LC in blastocyst expansion, zona pellucida thinness and finally, successful hatching which are required for implantation [14,36–39].

According to the above results LC may improve embryo competency and thus give higher pregnancy rates. However, high morphological quality, as we have found, cannot guarantee equivalent molecular quality needed for actual successful implantation. We, therefore, investigated some of the important molecular markers associated with competent implantation receptors on the blastocyst, including ErbB1 and ErbB4. Our results confirm that the morphological assessment of LC supplement is also reflected in a significantly increased the level of ErbB1 and ErbB4 mRNAs and proteins. By contrast, the results showed that exposure of embryos to 50 μM H_2O_2 for 30 min could significantly reduce ErbB1 and ErbB4 mRNA expressions, but that treatment with LC improved mRNA expression levels of the ErbB1 and ErbB4 even in the presence of H_2O_2 indicating that LC can improve both the quality of *in vitro* embryos as well as the likelihood of successful implantation through improved expression of ErbB1 and ErbB4.

Based on the broad search on the web, there were no data that declared the effect of LC on the expression rate of the primary implantation receptors, ErbB1, and ErbB4, in *in vitro* cultured embryos and our findings may be the first.

According to our data, morphological and molecular changes inevitably appear in *in vitro* produced embryos but the use of appropriate antioxidants can reduce the intensity of detrimental environmental effects. In this study, we investigated the role of ROS in impairing implantation receptors on laboratory produced embryos. Our data showed that embryo exposure to H_2O_2 increased the intracellular ROS, and treatment with LC scavenged this intracellular ROS and protected the embryos. Indeed, the amount of intracellular ROS in the LC + H_2O_2 group was similar to that in the control group with no significant difference between then,

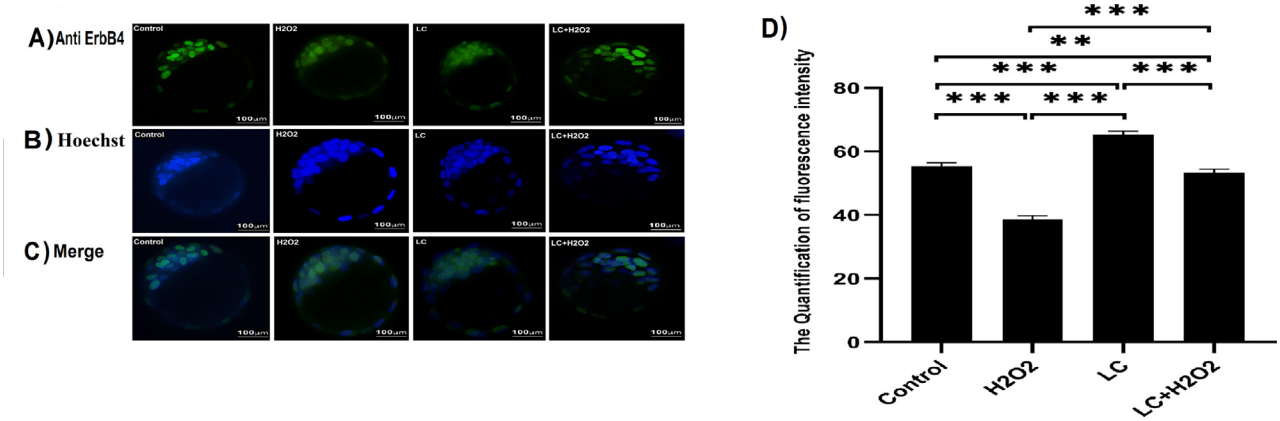


Fig. 4. Increased protein expression of ErbB4 following treatment with LC. Images show embryos stained with anti-ErbB1 fluorescence (A; in green), Hoechst dye (B; nuclei in blue) and merged (C) in control, H_2O_2, LC and LC + H_2O_2 groups. Anti-ErbB1 staining was quantified using Image Quant software (TotalLab Quant). D) Data are presented as mean ± SD. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

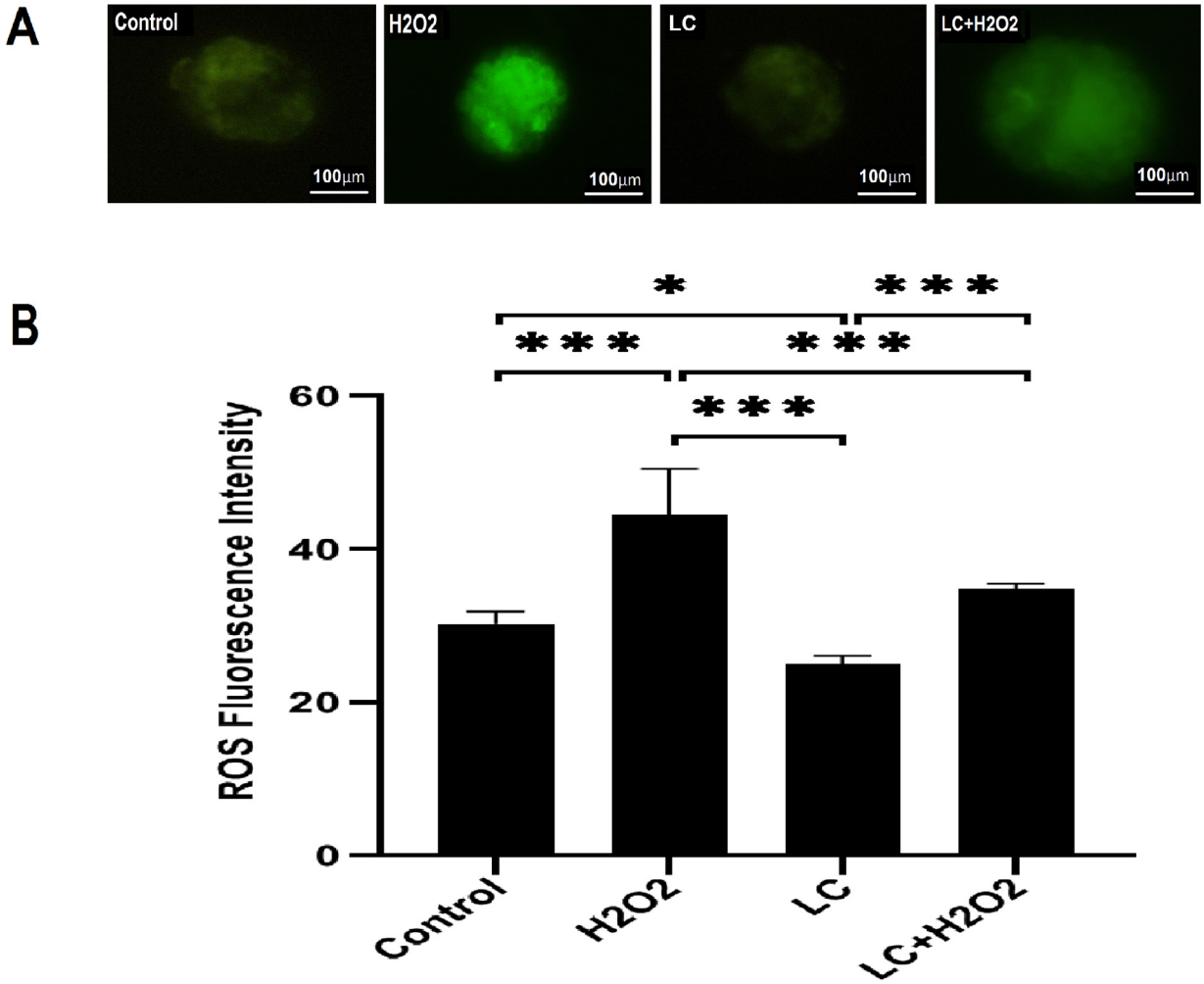


Fig. 5. LC decreases intracellular ROS levels in mouse expanded blastocysts. A) Intracellular ROS measured by the DCFH-DA fluorescence (in green) in all control, H_2O_2, LC, and LC + H_2O_2 studied groups. B) Measures of intensity of staining are presented as mean ± SD. $^{*}P \leq 0.05$; $^{***}P \leq 0.001$.

demonstrating full protection against ROS with the LC treatment with no deleterious effects observable in embryos in the LC + H_2O_2 group. As mentioned above, the mRNA expression of ErbB1 and ErbB4 were amplified in embryos treated with LC thus, intracellular ROS can be directly responsible for decreased expression of the early implantation receptors, ErbB1 and ErbB4. Consistent with our results, previous studies have also reported beneficial effects of LC on the expression of three important genes, GDF-9, MAPK1, and CDK1, involved in meiosis, oogenesis, maturation, and fertilization of mouse oocytes [40].

Published reports show that excess intracellular ROS results in cellular damage, DNA damage, mitochondrial dysfunction, embryo apoptosis and consequential implantation failure [13,41]. Mitochondrial damage, including mitochondrial DNA deficiency and poor mitochondrial genes expression, have been found to lead to the reduction of ATP concentrations in mouse embryos and result in low-quality embryos and implantation failure [9,42]. Therefore, maintaining the balance between the production and scavenging of ROS plays a vital role in successful implantation. In physiological condition, SIRT3, which is located in mitochondria, reduces ROS activity by activating antioxidant enzymes and thereby protect cells against oxidative damage [43,44]. Increased levels of intracellular ROS and resulting mitochondrial damage occur in SIRT3 knockout mice in Mouse Embryonic Fibroblasts (MEFs) [45] According to previous studies, the mRNA level of SIRT3 expression is an indicator of mitochondrial function [46] so in the current study we evaluated mitochondrial competency through SIRT3 expression in embryos exposed to H_2O_2 and the same embryos treated with LC. We found the highest and lowest levels of SIRT3 expression in the H_2O_2 and LC groups respectively. Although SIRT3 increased as expected in response to the ROS, this was associated with mitochondrial incompetency and poor quality embryos in the presence of H_2O_2 suggesting the response could not cancel the effects of the ROS by itself. However, when the same exposed embryos were treated with LC the expression of SIRT3 reduced, indicating that the ROS effect was canceled by the antioxidant. Thus, using appropriate type and concentration of antioxidants, such as LC, can neutralize the negative and destructive effects of intracellular ROS maintain mitochondrial function and, thereby, improve embryo quality in *in vitro* cultures. According to several studies, mitochondrial dysfunction is directly related to decreased ATP production and, as genes expression is ATP-dependent [47] it can be concluded that following mitochondrial impairment, the amount of ATP production was decreased and the reduced expression of ErbB1 and ErbB4 and implantation failure could be expected, and was rescued with LC addition to the embryo culture media.

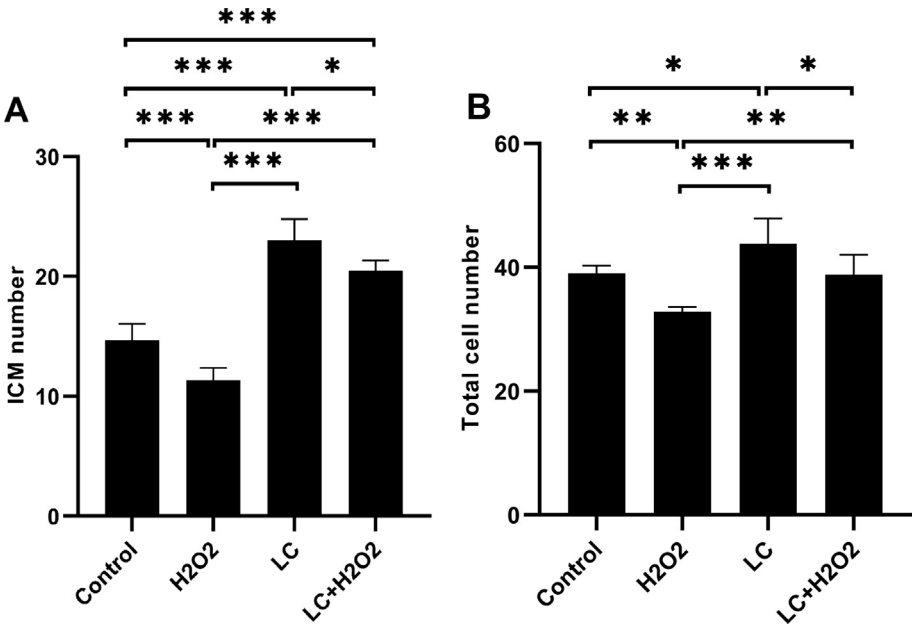
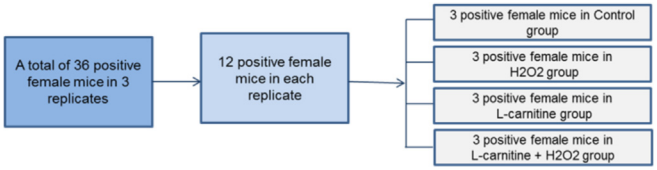


Fig. 6. LC increases the number of cells in the ICM and the total blastomere numbers in the expanded blastocysts. A) Data related to ICM. B) Data related to total blastomere numbers. Bars show the mean of blastomere numbers ±SD in all studied groups. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.



Groups	Total two-cell embryos number	Total expanded blastocyst number
Control	60	47-49
H_2O_2	60	38-42
LC	60	55-58
LC + H_2O_2	60	46-48

Diagram 1. Mapping of the Embryos Collecting and Grouping.
As Scheme depicts, 36 positive female mice were used for three replications. From each experiment, 12 positive female mice were chosen and divided into 4 groups (Control, H2O2, LC, H2O2 + LC(. Generally, 60 two-cell embryos were randomly chosen from each group and data were recorded to expand blastocyst stage.

Author contributions

GM, HN and JM designed and supervised the project. GS, MA, and JAM performed the experimental tests. GM, GS, and MA performed the data analysis. GS and MA contributed to preparing the drafted manuscript. All of the authors reviewed and finalized the paper.

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