

Original Article

Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

doi: 10.4103/apjr.apjr_136_23

Effect of coenzyme Q10 supplementation on post–vitrification mouse embryo development

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ABSTRACT

Objective: To investigate the effects of coenzyme Q10 (CoQ10) supplementation on post-vitrification embryo development and gross morphology.

Methods: Balb/c mouse embryos were cultured in potassium simplex optimised medium (KSOM) with varying CoQ10 concentrations [0 (control), 20, 40, and 60 µM]. The most effective CoQ10 concentration (40 µM) was selected for subsequent post-vitrification morphology study. Embryos were randomly divided into four groups: Group A (non-vitrified without CoQ10), Group B (non-vitrified with CoQ10), Group C (vitrified without CoQ10), and Group D (vitrified with CoQ10), followed by vitrification at the 8-cell stage. Survival rates and development until the blastocyst stage were evaluated through morphological examinations using ASEBIR's system, distinguishing normal and abnormal embryos.

Results: Supplementation of 40 µM CoQ10 significantly increased blastocyst formation (95%) compared to the control group (92%), 20 µM (62%), and 60 µM (56%) ($P<0.001$). Following vitrification, Group D exhibited a significant increase in blastocyst formation (92%) compared to Group C (82%) ($P<0.05$). Morphological assessments indicated superior embryo quality in Group B over Group D during the cleavage stage, morula, and blastocyst ($P<0.05$).

Conclusions: CoQ10 supplementation exhibits promising potential to enhance preimplantation embryo development, increase blastocyst formation rates, and improve embryo quality post-vitrification. This offers a promising approach to mitigate oxidative stress on embryos, potentially improving overall assisted reproductive technology outcomes.

KEYWORDS: Coenzyme Q10; Antioxidant; Embryo development; Vitrification; Morphology; Assisted reproductive technology

1. Introduction

Infertility is a condition characterized by the inability of a

couple to achieve pregnancy after consistent, unprotected intercourse for at least 12 months. This widespread issue affects millions of individuals of reproductive age worldwide, causing significant emotional and social distress[1]. Assisted reproductive technology (ART) encompasses a range of medical treatments and procedures involving the manipulation of human eggs, sperm, and embryos outside the body to facilitate pregnancy. Prominent forms of ART, such as *in-vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), gamete intrafallopian transfer, and pronuclear stage tubal transfer, have emerged as effective solutions. Nevertheless, despite advancements, only 35% of couples undergoing ART procedures achieve a live birth[2].

One promising advancement in the field of ART is the vitrification technique for cryopreserving mammalian embryos. Vitrification's

Significance

In recent years, assisted reproductive technology (ART) usage has surged due to global infertility. Despite advancements, success rates remain low. Factors such as oxidative stress from vitrification may contribute to this. Antioxidant supplementation has been shown to have beneficial effects on embryo development, but coenzyme Q10 research is limited. This study revealed that coenzyme Q10 supplementation positively impacted embryo development, notably increasing blastocyst formation and improving morphology. It potentially offers protective benefits against cryodamage and improves ART outcomes.

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How to cite this article: Anisa-Annur S, Wan-Hafizah WJ, Nor-Ashikin MNK, Muhammad-Zaki R. Effect of coenzyme Q10 supplementation on post-vitrification mouse embryo development. *Asian Pac J Reprod* 2024; 13(3): 126-132.

Article history: Received: 24 November 2023; Revision: 19 January 2024; Accepted: 18 February 2024; Available online: 31 May 2024

popularity has soared in ART laboratories globally, driven by its advantages over traditional slow-freezing methods in terms of cost and time efficiency. Vitrification involves rapid cooling and warming rates and highly concentrated cryoprotectant solutions in small volumes with high viscosity. These conditions collectively induce a transition into a glass-like physical state without forming ice crystals[3]. The method is known for its speed, cost-effectiveness, and successful application in the cryopreservation of embryos at various developmental stages across mammalian species[3].

Despite vitrification's increasing effectiveness, ongoing research is essential to investigate its potential impact on cell membranes and mitochondrial bioenergetic processes, which can be influenced by oxidative stress. Oxidative stress is recognized for its capacity to hinder enzyme activation, disrupt mitochondrial function, reduce adenosine triphosphate (ATP) levels, influence apoptotic pathways, and cause DNA fragmentation, all of which can contribute to developmental delays.

Recognizing that challenges in ART procedures persist, it is crucial to explore strategies for mitigating their adverse effects on embryo development. Previous studies have demonstrated that antioxidant supplementation such as glutathione[4], and vitamin C[5] improves gamete quality and strengthens the developing embryos.

Another antioxidant, coenzyme Q10 (CoQ10), is a fat-soluble component situated within the electron transport chain in the inner mitochondrial membrane. CoQ10 is pivotal role in ATP production and inhibitor of peroxidation and oxidation processes. The presence of CoQ10 in human follicular fluid has shown associations with embryo morphokinetics and pregnancy rates in animal reproduction. In mice, CoQ10 has been demonstrated to enhance the quality of both oocytes and cumulus cells by reversing mitochondrial dysfunction, including reducing oxidative phosphorylation and ATP levels. ATP levels have been linked to oocyte morphology and developmental potential[6].

However, compared to other antioxidants, CoQ10 has received relatively limited research attention, especially regarding *in vitro* supplementation in the culture media[7]. Therefore, the present study aims to address these research gaps by investigating the potential protective effects of CoQ10 on the preimplantation development of embryos following cryopreservation.

2. Materials and methods

2.1. Chemicals

The CoQ10 used in this study was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The following reagents and materials were also used: Potassium simplex optimised medium (KSOM) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotropin (hCG) were obtained from Prospect Bio, Israel.

2.2. Animals

A total of 24 female Balb/c mice 6 to 8 weeks of age, weighing 25 to 35 g, were used as embryos donors. A collective of 24 proven fertile male mice of the same strain, 6 to 8 weeks of age, weighing 25 to 40 g were used to set up natural mating. Animals were purchased from Laboratory Animal Facility and Management (LAFAM), Universiti Teknologi MARA (UiTM) and bred at Laboratory Animal Care (LACU), Institute for Molecular Biotechnology (IMMB), UiTM until the time of use. Mice were kept in an animal house under a 12:12 hour light: dark cycle at $(19.0 \pm 0.5)^\circ\text{C}$ and 50% to 60% humidity conditions. Females were kept in 290 mm $\times$ 220 mm $\times$ 140 mm dimension cages with a stainless-steel lid separating the water bottle and feed. Male mice were isolated in individual cages a week before being introduced to the female mice. This is to ensure optimal reproductive performance. The mice were fed with mouse pellets (Altromin, Malaysia) and provided reverse osmosis water *ad libitum*. Autoclaved sawdust was used as bedding material. The bedding was changed twice a week. Cages and water bottles were washed every week.

2.3. Superovulation of mice and collection of embryo

Female mice were superovulated by intraperitoneal (*i.p.*) injections of 5 IU of PMSG followed by 5 IU of hCG 48 h later by *i.p.* injection to induce ovulation. The female mice were subsequently mated with fertile males of the same strain in a 1:1 ratio. Copulation plug was inspected 24 h later to confirm mating. Plugged female mice had their oviduct excised, and embryos were flushed out using M2 medium.

2.4. Optimization of CoQ10 concentration

A preliminary study was conducted to determine the optimal concentration of CoQ10 for addition to the culture media of the treatment group. Two-cell stage embryos were cultured in 10 μL CoQ10 with varying concentrations (0, 20, 40, and 60 μM), combined with 40 μL of KSOM media in a humidified incubator[8]. The embryos were allowed to develop until they reached the blastocyst stage. The concentration of CoQ10 that resulted in the highest percentage of blastocyst formation was identified as the optimal amount to be added to the culture media.

2.5. Embryo culture

Embryos were divided into four groups, each consisting of a

different number of embryos: Group A consisted of 63 embryos (non-vitrified without CoQ10), Group B comprised 55 embryos (non-vitrified with CoQ10), Group C included 64 embryos (vitrified without CoQ10), and Group D comprised 64 embryos (vitrified with CoQ10). Group A received no CoQ10 and no vitrification, serving as the control group. Group B received CoQ10 but was not vitrified. Group C received no CoQ10 and vitrified. Group D was supplemented with CoQ10 and vitrified. Embryos in groups A and C were cultured in 50 μ L of KSOM, while embryos in groups B and D were cultured in 40 μ L of KSOM media + 10 μ L of CoQ10. Embryos in groups A and B were allowed to develop to the blastocyst stage without undergoing the vitrification process. The developmental viability of these embryos was evaluated every 24 h until the blastocyst stage. Embryos in groups C and D were cultured until 8-cell stage before being subjected to vitrification.

2.6. Vitrification and warming of embryos

Embryos were vitrified using Mochida's EFS20/40 vitrification method[9] with minor modifications[10]. Briefly, embryos were immersed in 20 μ L of EFS20 solution containing 20% ethylene glycol, 24% ficoll 70 and 0.4 M sucrose for 2 min. Then, they were transferred to 50 μ L of EFS40 solution containing 40% ethylene glycol, 18% Ficoll 70 and 0.3 M sucrose for 3 min. Subsequently, a 1-mL syringe fitted with a white pipette tip was used to load the EFS40 solution into the straw and immediately immersed into liquid nitrogen filled in a styrofoam box.

For warming, the straws were taken from the styrofoam box using forceps and then held in the air for 10 s before being placed in a 37°C water bath for another 10 s. The contents of the straws were expelled out into a culture dish containing a thawing solution composed of M2 medium with 0.5 M sucrose for 5 min. The culture dish was gently agitated to combine the dilution and cryoprotectant solutions thoroughly. The embryos were then transferred into M2 media, washed twice in fresh M2 media and prepared for culture.

2.7. Developmental study and morphological assessment

The post-vitrification survival rate of embryos was assessed by examining their morphology under an inverted microscope (Leica DM IRB, BZ:05) connected to the PC software (PixeLINK) at 20 $\times$ magnification followed by development *in vitro* until the blastocyst stage. The development of the embryos was recorded according to the ASEBIR's morphological scoring system[11]. The embryos were classified as normal or abnormal based on their morphology. Normal embryos had consistent size and shape among blastomeres, with fragmentation below 10%. Morula-stage embryos were compact with fully compacted blastomeres, while blastocysts had homogeneous trophectoderm (TE) cells, a larger blastocoel, and

a compacted inner cell mass (ICM). Abnormal embryos exhibited uneven blastomeres, fragmentation over 10%, partial compaction in morulae, non-homogeneous TE cells, smaller blastocoel, and non-compacted ICM in blastocysts.

2.8. Statistical analysis

The statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS) software version 29 from IBM Corp. The morphological embryonic outcomes and blastocyst formation rates were determined and reported as frequencies and percentages. The differences between groups were analyzed using the *Chi-square* test. A *P*-value of less than 0.05 was considered statistically significant.

2.9. Ethics statement

All animal care and use procedures were approved by the Animal Care and Use Committee (ACUC) under approval code UiTM Care: 387/2022.

3. Results

3.1. Optimization of CoQ10 concentration in culture media

The preliminary study's results demonstrated that the group with a CoQ10 concentration of 40 μ M exhibited the highest blastocyst formation (95%) compared to the control group (92%), the 20 μ M group (62%), and the 60 μ M group (56%) ($P<0.001$) (Figure 1). Therefore, this concentration (40 μ M) was selected for the treatment group.

3.2. Developmental study of non-vitrified and vitrified embryos

No significant difference in blastocyst formation was found between the control non-vitrified group (Group A) and the CoQ10-supplemented non-vitrified group (Group B) for embryos not subjected to vitrification. However, for vitrified embryos, there was a significantly higher blastocyst formation (92%) with the CoQ10 supplement (Group D) compared to the control vitrified group (Group C) (82%) ($P<0.05$) (Figure 2).

3.3. Cleavage embryos comparison between the treatment and control groups

As shown in Table 1, non-vitrified embryos in the control group (Group A) exhibited 80% normal morphology with even-sized blastomeres and low fragmentation, whereas the CoQ10-

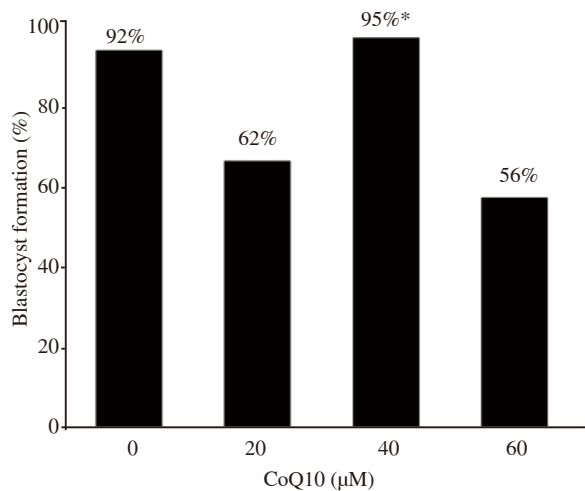


Figure 1. Comparison of blastocyst formation between different concentrations of CoQ10 in culture media. *40 μM *versus* 0, 20 and 60 μM ($P<0.001$).

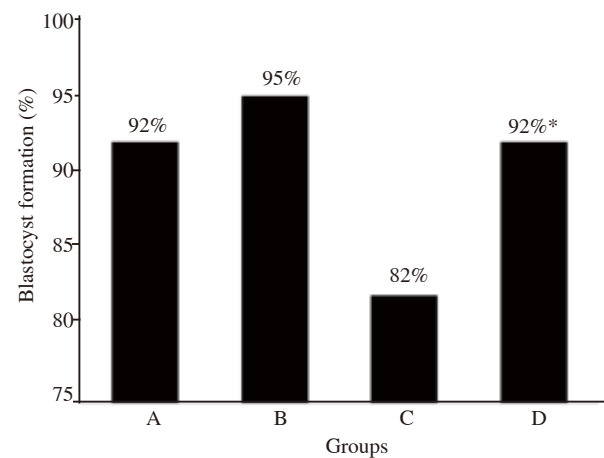


Figure 2. Comparison of blastocyst formation between the control and treated embryos in non-vitrified and vitrified groups. *Group C *versus* Group D embryos in vitrified group ($P<0.05$). Group A: non-vitrified without CoQ10; Group B: non-vitrified with CoQ10; Group C: vitrified without CoQ10; Group D: vitrified with CoQ10.

Table 1. The morphology of preimplantation embryos at the 8-cell stage, morula and blastocyst [n(%)].

Groups	8 cell		Morula		Blastocyst	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
A	40 (80)	10 (20)	39 (78)	11 (22)	37 (74)	13 (26)
B	44 (88)*	6 (11)*	42 (84)*	8 (16)*	43 (86)*	7 (14)*
C	29 (58)	21 (42)	30 (60)	20 (40)	32 (64)	17 (34)
D	33 (66)	17 (34)	33 (66)	17 (34)	34 (68)	16 (32)

*Group B *versus* Group D ($P<0.05$). *Chi-square* test is used. Group A: non-vitrified without CoQ10; Group B: non-vitrified with CoQ10; Group C: vitrified without CoQ10; Group D: vitrified with CoQ10.

supplemented embryos (Group B) demonstrated 88% (Table 1). For vitrified embryos, the control vitrified group (Group C) exhibited 58% normal morphology, while the CoQ10-supplemented vitrified embryos (Group D) showed 66%, both displaying even-sized blastomeres and low fragmentation (Table 1).

A significantly higher percentage of normal embryo morphology was observed in the non-vitrified treatment group (Group B) compared to the vitrified treatment group (Group D) (88% *versus* 66%) ($P<0.05$). The results showed no significant difference in the morphology of cleavage embryos between the control non-vitrified (Group A) and control vitrified (Group C) embryos.

3.4. Morula morphology comparison between the treatment and control groups

In addition, non-vitrified embryos showed 78% normal morphology for the control group (Group A) and 84% for CoQ10-supplemented embryos (Group B), characterized by compacted morula formation. Regarding vitrified embryos, the control vitrified group (Group C) displayed 60% normal morphology, while the CoQ10-supplemented vitrified embryos (Group D) exhibited 66%, indicating complete morula formation without distinguishable blastomeres.

Non-vitrified treatment group (Group B) exhibited significantly

higher normal morula morphology compared to the vitrified treatment group (Group D) (84% *versus* 66%) ($P<0.05$). The results showed no significant difference in morula morphology between the control non-vitrified (Group A) and control vitrified embryos (Group C) (Table 1).

3.5. Blastocyst morphology comparison between the treatment and control groups

Table 1 reveals that non-vitrified embryos showed 74% normal morphology of blastocyst for the control group (Group A) and 86% for CoQ10-supplemented embryos (Group B), characterized by homogenous trophectoderm cells, oval inner cell mass, and compacted structure. Regarding vitrified embryos, the control vitrified group (Group C) exhibited 64% normal morphology. In comparison, the CoQ10-supplemented vitrified embryos (Group D) showed 68%, displaying homogenous trophectoderm cells, oval inner cell mass, and compacted morphology (Table 1).

A significantly higher percentage of normal blastocyst morphology was observed in the non-vitrified treatment group (Group B), 86% compared to the vitrified control group (Group C). The results indicated no significant difference in blastocyst morphology between the control non-vitrified (Group A) and vitrified groups (Group C).

4. Discussion

The obtained result indicates that the most effective concentration for adding exogenous CoQ10 to the culture media is 40 μ M, as evidenced by the highest blastocyst formation. Conversely, the lowest blastocyst formation occurred at 60 μ M. In line with these observations, prior studies on the oocyte stage also reported positive outcomes for 40 μ M CoQ10, showing a significant reduction in oocyte death after 24 h of *in vitro* maturation (IVM) culture compared to 0, 20, or 60 μ M CoQ10. Furthermore, CoQ10 treatment at 40 μ M significantly increased ATP levels after 24 h of IVM culture compared to 0, 20, or 60 μ M. Furthermore, CoQ10 treatment at 40 μ M significantly increased ATP levels after 24 h of IVM culture compared to 0, 20, or 60 μ M[12]. The presence of 30 μ M CoQ10 was found to promote the hatching of expanded blastocysts, possibly through several mechanisms[13]. Firstly, CoQ10 and electron transport can automatically activate proton antiport, which is crucial for ion changes and fluid distribution in the blastocyst. Secondly, by enhancing cell proliferation and increasing ATP content, CoQ10 might speed up the formation of the blastocoel cavity, accelerating the hatching process. Lastly, CoQ10 could correct the ionic imbalance in embryos cultured in conventional media[13]. These previous studies suggest that the best CoQ10 concentration for achieving positive molecular and biological outcomes in the culture of bovine embryos falls within the midrange concentration[13].

CoQ10-supplemented vitrified embryos (Group D) were significantly higher than the control group of vitrified embryos (Group C) at eight-cell stage, morula, and blastocyst. This observation suggests that CoQ10 may have a beneficial effect on embryo development after exposure to the stress induced by the vitrification process. Indeed, individual antioxidants have been demonstrated to effectively alleviate oxidative stress in mammalian embryos during the cryopreservation process[14].

Furthermore, a previous study on oocytes found that supplementing them with 50 μ M CoQ10 during IVM is beneficial in preserving oocyte quality following vitrification. Specifically, it helps prevent premature cortical granule exocytosis. In other words, CoQ10 supplementation during IVM helps maintain the structural and functional quality of oocytes, preventing premature release of specific cellular components called cortical granules, which can be essential for successful fertilization and embryonic development[15]. The abnormal embryo development includes a higher grade of fragmentation and multinucleated blastomeres[16].

Notably, ascorbate was among the first antioxidants shown to enhance mouse embryo development and improve blastocyst's subsequent viability[17]. Additionally, including *N*-acetylcysteine in both vitrification and warming solutions had positive effects. This supplementation improved the distribution patterns of mitochondria and effectively eliminated reactive oxygen species (ROS), resulting in increased development of vitrified oocytes[18].

Moreover, studies conducted by Moawad *et al*[19] demonstrated successful outcomes from adding *L*-carnitine during the vitrification and IVM of germinal vesicle-stage mouse oocytes. This

supplementation significantly improved several crucial aspects of oocyte development, including blastocyst development, spindle assembly, chromosome alignment, mitochondrial distribution, and mitochondrial oxidative activity.

In all stages of development, the CoQ10-supplemented vitrified embryos consistently showed the highest percentage of normal morphology. Vitrified embryos without CoQ10 exhibited an increased occurrence of abnormal morphology. This suggests that vitrification may have influenced abnormal embryo morphology through cryodamage caused by the cryoprotectant, leading to increased oxidative stress and, consequently elevated toxicity in the embryo. The supplementation of CoQ10 in the culture medium enhanced cleavage and blastocyst formation rates. Furthermore, it contributed to the improvement of blastocyst quality by mitigating oxidative damage and reducing blastomere apoptosis[20].

During cytokinesis, various organelles, including mitochondria, distribute evenly into each new cell, referred to as a blastomere. Mitochondria play a crucial role in generating ATP, which is essential for supporting the energy demands during subsequent cleavage divisions or cell division processes. Unequal distribution of mitochondria can disrupt the cleavage process, leading to irregular blastomere sizes and the formation of embryos with 5, 6, or 7 cells, deviating from the typical even division[21].

Vitrification can cause alterations in mitochondrial distribution, reduced membrane potential in mouse pronuclear embryos[22], lowered ATP levels in human oocytes[23], and induce mitochondrial dysfunction in mouse oocytes and parthenogenetic embryos[24]. Natural antioxidants have shown significant positive effects by safeguarding mitochondrial function, regulating the synthesis and activity of antioxidant enzymes, and suppressing ROS production[25–27]. Embryos displaying uneven cleavage patterns often exhibit a higher incidence of aneuploidy (abnormal chromosome numbers) and multinucleation than embryos with uniformly sized blastomeres[28].

At the morula stage, it was observed that most CoQ10-supplemented vitrified embryos exhibited full blastomere compaction. Conversely, morula and blastocyst-stage embryos that underwent vitrification without CoQ10 supplementation displayed increased dichlorodihydrofluorescein fluorescence intensity, indicating heightened ROS production and a decrease in developmental rates[29]. This rise in ROS levels could be attributed to the conditions employed during the vitrification process, potentially inducing oxidative stress. This observation aligns with prior studies that reported the up-regulation of genes linked to oxidative stress mechanisms, such as Hsp70, MnSOD, and CuSOD, in vitrified mouse embryos[30].

Oxidative stress harms sperm function through various mechanisms, including lipid peroxidation, protein damage, and DNA strand breakage, significantly impacting an individual's fertility[31]. A utilizing hydrogen peroxide (H_2O_2) to induce oxidative stress in mouse spermatozoa[32], the results demonstrated that this stress caused damage to sperm, ultimately reducing the developmental potential of preimplantation-stage embryos and diminishing their

ability to implant. This emphasizes the critical need to mitigate oxidative stress to maintain optimal reproductive health.

The sustained higher blastocyst formation rate in CoQ10-supplemented vitrified embryos (Group D) compared to the control vitrified group (Group C) underscores the impact of vitrification on embryonic development. This suggests that CoQ10 plays a pivotal role in restoring embryo development post-vitrification. Additionally, it is noteworthy that blastocyst formation rates were notably lower in vitrified 2-cell stage embryos compared to those at the 4-cell or 8-cell stages. In mouse embryos, a common occurrence called 'cleavage arrest' often happens at the 2-cell stage, mainly attributing to the initiation of embryonic genome activation, commonly known as the 'two-cell block'[33].

The reduction in trophectoderm cell numbers observed in vitrified-warmed blastocysts could be attributed to delayed cleavage, likely resulting from some extent of cryoinjury experienced by the embryonic blastomeres during the vitrification process[34]. Supplementing with an antioxidant could enhance the survival rate. This statement strongly aligns with our findings, indicating that the CoQ10-receiving group exhibited the highest blastocyst formation percentage compared to the control group among the vitrified embryos.

The limitation of this study lies in its focus on preimplantation embryo development, suggesting the need for further research into fetal development effects. Additionally, exploring CoQ10's mechanisms, particularly its modulation of oxidative stress and gene expression, is essential for a comprehensive understanding.

In conclusion, the study revealed that supplementing the culture media with CoQ10 positively impacted embryo development. An optimal concentration of 40 μ M notably increased blastocyst formation. Moreover, CoQ10 supplementation improved cleavage, morula, and blastocyst morphology in both non-vitrified and vitrified embryos. This suggests that CoQ10 potentially offers protective benefits, especially advantageous for vitrified embryos, potentially reducing the impact of cryodamage and enhancing the overall quality of the embryos.

Conflict of interest statement

There was no conflict of interest.

Acknowledgement

The authors would like to thank the Ministry of Higher Education, Malaysia for providing financial support under Fundamental Research Grant Scheme (FRGS) [FRGS/1/2020/SKK06/UNIKL/02/1], and we wish to thank the Institute of Medical Molecular Biotechnology (IMMB), Faculty of Medicine, Universiti Teknologi MARA, Sungai Buloh Campus, Selangor Branch for providing technical supports for this study. We further thank the Laboratory Animal Care Unit (LACU) staff for providing animal care and maintenance.

Funding

This study was supported by the Fundamental Research Grant Scheme (FRGS) [FRGS/1/2020/SKK06/UNIKL/02/1], from the Ministry of Higher Education, Malaysia.

Authors' contributions

Funding for this study was provided by Wan-Hafizah WJ. Anisa-Annur S and Wan-Hafizah WJ contributed to the study's conception and design, conducted literature searches, and drafted the initial manuscript. Anisa-Annur S, Wan-Hafizah WJ, Nor-Ashikin MNK, and Muhammad-Zaki R were responsible for data analysis and interpretation, as well as contributing to manuscript drafting. Anisa-Annur S took the lead in writing the manuscript. Wan-Hafizah WJ, Nor-Ashikin MNK, and Muhammad-Zaki R participated in article screening and provided critical revisions to the manuscript. Wan-Hafizah WJ finalized and approved the manuscript. All authors reviewed and approved the final manuscript.

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