https://doi.org/10.17221/61/2026-CJAS

Improved cryotolerance of bovine blastocysts by 1α,25-dihydroxyvitamin D_3 supplementation during *in vitro* culture and post-thaw recovery

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Citation: Lee H.K., Kim D.H., Kwon D.J. (2026): Improved cryotolerance of bovine blastocysts by 1α,25-dihydroxyvitamin D_3 supplementation during *in vitro* culture and post-thaw recovery. Czech J. Anim. Sci., 71: 319–329.

Abstract: *In vitro* embryo production (IVP) is a valuable technique for advancing cattle breeding and reproductive biotechnology. Nevertheless, embryos produced *in vitro* often display reduced developmental competence and cryotolerance compared with their *in vivo* counterparts. Vitamin D is known to regulate many cellular functions, including growth and antioxidant defence. This study evaluated the impact of vitamin D supplementation during *in vitro* culture on bovine embryo development and post-thaw recovery. Oocytes were matured and fertilised *in vitro* and presumptive zygotes were cultured with 0 (control), 25, or 50 ng/ml of 1α,25-dihydroxyvitamin D_3. Supplementation of 25 ng/ml vitamin D did not significantly alter cleavage or blastocyst rates; however, it increased the expression of *IGF2R* and *SOD2*. In contrast, 50 ng/ml impaired embryonic development. the VD/VD group showed higher blastocyst hatching rates, lower mitochondrial reactive oxygen species (ROS) levels, increased IGF2R expression and reduced *BAX* expression and *BAX*/*BCL2L1* ratio ($P < 0.05$). Overall, these findings suggest that the low-dose vitamin D supplementation may support embryonic recovery from cryo-induced stress, potentially through the modulation of the oxidative balance and growth-related pathways, rather than by enhancing the pre-freezing developmental competence.

Keywords: apoptosis; cattle; cryopreservation; embryo quality

In vitro embryo production (IVP) in cattle has become indispensable in modern livestock breeding and biotechnology, offering valuable applications in genetic improvement, conservation of endangered breeds and commercial embryo transfer programmes (Sirard 2018; Ferre et al. 2020). Despite

Carried out with the support of the "Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01620003)" Rural Development Administration, Republic of Korea, and supported by research funds of Jeonbuk National University in 2025.

https://doi.org/10.17221/61/2026-CJAS

these advances, the efficiency of IVP remains lower than that achieved *in vivo*, with suboptimal rates of fertilisation, cleavage, and blastocyst formation (Banliat et al. 2022; Krisher and Herrick 2024). Recent studies have confirmed that *in vitro* produced bovine embryos show the poorer survival after freeze-thawing and larger differences in developmental competence compared with *in vivo*-derived embryos (Janati Idrissi et al. 2021; Banliat et al. 2022). These limitations are primarily attributed to the artificial environment in which gametes and embryos are maintained, which lacks many physiological cues, signalling molecules and growth factors naturally provided by the maternal reproductive tract (Wetscher et al. 2005). Therefore, considerable attention has been directed toward improving *in vitro* culture systems by supplementing media with bioactive molecules that mimic the oviductal and uterine milieu (Lopera-Vasquez et al. 2017).

Among the factors of interest is vitamin D, namely, a secosteroid hormone classically known for its regulation of calcium and phosphorus metabolism but increasingly recognised for its pleiotropic effects on cell growth, immune function, and differentiation (Christakos et al. 2016). The discovery of vitamin D receptors (VDRs) and vitamin D-metabolising enzymes in reproductive tissues, including bovine oocytes, granulosa cells and in the uterus, indicated that vitamin D may play a physiological role in reproduction and early embryogenesis (Xu et al. 2021). Vitamin D metabolites have been detected in the bovine follicular fluid, suggesting their direct influence on oocyte competence and follicular development (de Souza et al. 2022). Moreover, VDR expression has been reported in the reproductive tissues of various mammalian species, including bovine reproductive organs, implying that vitamin D signalling can modulate meiotic progression and cytoplasmic maturation processes critical for subsequent embryonic development (Xu et al. 2021). These findings are consistent with those of studies in humans and other mammals, showing that vitamin D deficiency can impair fertility and reproductive outcomes (Grzechocinska et al. 2013).

In vitro culture conditions can influence not only embryo development but also the expression of genes related to growth regulation, oxidative stress and embryo quality. Previous studies have shown that suboptimal culture environments may alter embryonic gene expression without markedly affecting morphological development (Arias et al. 2012; Cagnone and Sirard 2016). In particular, the genes involved in growth regulation and oxidative stress responses, such as *IGF2R* and *SOD2*, are highly sensitive to *in vitro* conditions and redox balance (Ludwig et al. 1996; Young et al. 2001; Arias et al. 2012). Despite continuous improvements in *in vitro* culture systems, the optimal culture environment required to support appropriate embryonic gene expression remains incompletely defined (Cagnone and Sirard 2016; Krisher and Herrick 2024). Further studies are required to determine the optimal culture conditions that support the appropriate expression of key embryonic development-related genes.

Embryos produced by *in vitro* culture are also susceptible to cryopreservation-induced stress. Previous studies have shown that cryopreservation can impair the mitochondrial function, resulting in elevated oxidative stress and reduced developmental competence after thawing (Marsico et al. 2019; Cao et al. 2022). The role of vitamin D may extend beyond oocyte maturation and may also influence pre-implantation embryonic development. VDR expression has been detected in mammalian oocytes and early embryos of several species, including mice and humans, suggesting potential vitamin D responsiveness during early embryogenesis (Xu et al. 2018; Li et al. 2024). However, the specific effects of vitamin D on embryonic quality and developmental competence are unclear. *In vitro* studies have shown that supplementation of active vitamin D can influence blastocyst formation and quality-related gene expression, although the available data remain limited and suggest species-specific and dose-dependent responses (de Souza et al. 2022; Bognar et al. 2023).

In this study, we investigated the effects of vitamin D supplementation during bovine *in vitro* culture (IVC) and post-warming recovery on embryo development and cryotolerance. We evaluated developmental competence, post-warming survival and hatching, mitochondrial oxidative stress, and the expression of genes associated with embryo quality. We hypothesised that vitamin D supplementation would improve the post-warming developmental competence of vitrified bovine blastocysts by reducing oxidative stress and modulating the expression of genes related to embryo quality.

https://doi.org/10.17221/61/2026-CJAS

MATERIAL AND METHODS

***In vitro* embryo production.** Ovaries were collected from slaughtered cows and immediately placed in physiological saline maintained at 30–35 °C for transportation to the laboratory. Follicles of 2–7 mm in diameter were aspirated and cumulus-oocyte complexes (COCs) displaying homogeneous cytoplasm and compact cumulus layers were selected. The COCs were matured for 20–22 h at 38.5 °C under 5% CO_2 in TCM-199 medium supplemented with 10% foetal bovine serum (FBS; Gibco-BRL, NY, USA), 0.2 mM sodium pyruvate, 50 µg/ml gentamicin (Sigma, St. Louis, MO, USA), 0.02 IU/ml follicle-stimulating hormone (FSH), 5 µg/ml luteinising hormone (LH) and 1 µg/ml oestradiol (Sigma).

For fertilisation, frozen semen was thawed at 37 °C for 45 s and purified by Percoll gradient centrifugation at 1 500 × *g* for 10 minutes. The sperm (1×10^6/ml) were added to fertilisation droplets containing matured oocytes (approximately $5–10 \times 10^3$ sperm per oocyte). Insemination was carried out for 8 h in IVF100 medium (Research Institute for Functional Peptides, Yamagata, Japan). After insemination, cumulus cells were removed with 300 IU hyaluronidase and presumptive zygotes were collected for further culture.

Presumptive zygotes were cultured for 168 h post fertilisation (hpf) at 38.5 °C under 5% CO_2 and 5% O_2 in the following groups: (*i*) control, modified synthetic oviductal fluid with amino acids (mSOFaa medium); (*ii*) 25VD, mSOFaa medium with 25 ng/ml of 1α,25-dihydroxyvitamin D_3 (vitamin D; Sigma); (*iii*) 50VD, mSOFaa medium with 50 ng/ml of vitamin D. A vitamin D stock solution (50 µg/ml) was prepared by dissolving the compound in dimethyl sulfoxide (DMSO) and storing the resulting solution below –20 °C. The final DMSO concentration in the culture medium was maintained at 0.1% for all groups, including the control group. The control and 25VD groups were generated from nine independent IVP replicates, whereas the 50VD group was generated from five replicates because this treatment yielded very few blastocysts across the batches. The additional blastocysts required for vitrification were obtained only from the control and 25VD groups. Embryonic development was assessed at 48, 120, and 168 hpf by determining the cleavage, 8-cell and morula, and blastocyst rates, respectively (Bo and Mapletoft 2013).

Vitrification and warming of blastocysts. Embryos were vitrified using the Cryotop method (Liu et al. 2008). Blastocysts (168 hpf) were washed in phosphate-buffered saline (PBS) containing 20% FBS and equilibrated with 7.5% dimethyl sulfoxide (DMSO) and 7.5% ethylene glycol (EG) for 3 min at room temperature. They were subsequently transferred to 15% DMSO, 15% EG, and 0.5 M sucrose for 1 min, and four blastocysts were loaded onto a Cryotop (Kitazato Supply Co., Fujinomiya, Japan), and plunged into liquid nitrogen. For warming, Cryotops were placed into a 0.5 M sucrose solution containing 20% FBS, followed by stepwise dilution in 0.5, 0.3 and 0.2 M sucrose for 5 min each. Blastocysts were washed in PBS containing 20% FBS for 5 min and cultured for 48 h in the IVC medium.

Four experimental groups were used to assess the effects of vitamin D supplementation after vitrification. For clarity, the treatment combinations after vitrification-warming were designated as follows: C/C = blastocysts derived from the control group (0 ng/ml vitamin D) and cultured in control medium during post-warming culture; C/VD = blastocysts derived from the control group and cultured with vitamin D (25 ng/ml) during post-warming culture; VD/C = blastocysts derived from the 25VD group (25 ng/ml vitamin D) and cultured in control medium during post-warming culture; and VD/VD = blastocysts derived from the 25VD group and cultured with vitamin D (25 ng/ml) during post-warming culture. Following vitrification and warming, the survival rate was defined as the percentage of warmed blastocysts that re-expanded with an intact blastocoel within 24 h and the proportion of hatched blastocysts was calculated based on the number of surviving embryos after 48 h of culture. All the groups were replicated four times, except for the VD/C group, which was replicated three times.

Apoptosis analysis and detection of reactive oxygen species (ROS). Apoptotic cells within blastocysts were identified using the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay (Kwon et al. 2023). Randomly selected blastocysts from each treatment group were fixed in 4% (w/v) paraformaldehyde for 30 min and permeabilised with 0.5% Triton X-100 (Sigma) for 30 min at room temperature. The fixed embryos were labelled with an *In-Situ* Cell Death Detection Kit (TMR Red; Roche, Germany) for 1 h at 38.5 °C in the dark. The stained embryos were then mount-

https://doi.org/10.17221/61/2026-CJAS

ed on slides with ProLong Gold Antifade Mountant containing DAPI (Thermo Fisher Scientific, OR, USA) and covered with glass coverslips before fluorescence imaging. Images were captured under an inverted epifluorescence microscope under identical microscope settings. The total cell numbers were counted manually using the Cell Counter tool in ImageJ software (v1.54d; NIH, USA). Because the images were acquired as single-plane 2D projections, nuclei were identified based on the DAPI signal shape, boundary contrast and intensity differences. To minimise subjectivity, partially overlapping nuclei were carefully distinguished by examining the differences in contour and signal intensity, ensuring that all identifiable nuclei within each blastocyst were consistently counted. The apoptotic index was calculated as the ratio of TUNEL-positive to total DAPI-stained nuclei per blastocyst.

For ROS detection, blastocysts were incubated with 1 µM MitoSOX (Thermo Fisher Scientific, OR, USA) at 48 hpf after warming for 30 min at 38.5 °C under 5% CO_2. After staining, embryos were mounted as described above and fluorescence images were taken at 200× magnification using an inverted microscope under identical microscope settings. The semi-quantitative MitoSOX intensity was estimated using the ImageJ software. For each embryo, the entire embryonic area was manually outlined using a freehand selection tool while excluding the *zona pellucida* and background regions. Mean intensity and embryo area were measured using a freehand selection tool (Kageyama et al. 2021).

Gene expression analysis. Total RNA extraction and complementary DNA (cDNA) synthesis were performed using the manufacturer's protocols (Qiagen). RNA from individual blastocysts (five blastocysts from each treatment group) in each group was converted to cDNA with the FastLane Cell cDNA Kit (Qiagen, Valencia, CA, USA). Quantitative RT-PCR was conducted using PowerUp™ SYBR™ Green Master Mix (Invitrogen) on a Real-Time PCR System (Life Technologies). The amplification programme included an initial step at 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 minute. Data were processed with QuantStudio™ Design & Analysis Software (v1.5.2). The genes analysed in this study are key regulators of embryo quality: insulin-like growth factor 2 (*IGF2R*), superoxide dismutase (*SOD2*), chloride intracellular channel proteins (*CLIC1*), BCL-2-associated X protein (*BAX*) and BCL2 Like 1 (*BCL2L1*). The *BAX*/*BCL2L1* ratio was calculated using relative quantification (RQ) values by dividing the normalised expression value of *BAX* by that of *BCL2L1*. All experiments were performed in triplicate. Primer sequences used for target genes are listed in Table 1.

Statistical analysis. All analyses were performed using the SPSS software v23.0 (IBM, USA). Developmental rates, gene expression, apoptotic index and MitoSOX fluorescence intensity were analysed using one-way ANOVA followed by Tukey's HSD post-hoc tests. Differences in cell numbers were analysed using an independent sample *t*-test. Statistical significance was accepted at $P < 0.05$.

Table 1. Primer sequences used for quantitative RT-PCR analysis

Gene		Primer sequences (5' to 3')	Product size (bps)	Accession No.
BAX	F-	GCAGAGGATGATCGCAGCTG	197	U92569
	R-	CCAATGTCCAGCCCATGATG		
BCL2L1	F-	CGTGGAAAGCGTAGACAAGGAG	133	AB238936
	R-	GTAGAGTTCCACAAAAGTGTC		
SOD2	F-	GTGAACAACCTCAACGTCGC	165	NM_201527.2
	R-	GGGTTCTCCACCACCGTTAG		
CLIC1	F-	GGCTCCTGAAAGCCCTGAAA	132	NM_001015608.1
	R-	CCAGAGTGAGCTCATTGCCA		
IGF2R	F-	GCTGCGGTGTGCCAAGTGAAAAAG	201	NM_174352.2
	R-	AGCCCCTCTGCCATTGTTACCT		
GAPDH	F-	GCCATCAATGACCCCTTCAT	70	NM_001034034.2
	R-	TGCCGTGGGTGGAATCA		

https://doi.org/10.17221/61/2026-CJAS

RESULTS

***In vitro* development of fresh embryos after IVF.** Cleavage, 8-cell, morula and blastocyst formation rates are summarised in Table 2. Cleavage and development to the 8-cell stage did not differ significantly between groups. However, progression to the morula and blastocyst stages was significantly reduced in the 50VD group (23.2 ± 9.5% and 16.8 ± 9.2%, respectively) compared with that in the control (43.0 ± 12.3% and 34.9 ± 13.3%, respectively) and 25VD groups (36.2 ± 10.5% and 33.3 ± 10.2%, respectively) ($P < 0.05$). No differences were observed between the control and 25VD groups, indicating that high concentrations of vitamin D impaired late-stage embryonic development, whereas low concentrations did not adversely affect developmental competence.

Total cell number, apoptosis and gene expression in fresh blastocysts. The total cell number and apoptotic cell index of the blastocysts did not differ between the groups (Figure 1A,B). The relative gene expression analysis revealed that *IGF2R* and *SOD2* were significantly upregulated

Table 2. Effects of vitamin D supplementation on the *in vitro* development of bovine embryos

Group	Cultured embryos	No. (%) of embryos developed to:			
		cleaved	8-cell	morula	blastocysts
Control	272	191 (70.2 ± 14.5)	146 (53.7 ± 13.6)	117 (43.0 ± 12.3)[a]	95 (34.9 ± 13.3)[a]
25VD	246	162 (65.8 ± 7.8)	131 (53.3 ± 10.8)	89 (36.2 ± 10.5)[a]	82 (33.3 ± 10.2)[a]
50VD	125	70 (56.0 ± 21.0)	49 (39.2 ± 18.1)	29 (23.2 ± 9.53)[b]	21 (16.8 ± 9.2)[b]

[a,b]Different superscripts within the same column indicate significant differences ($P < 0.05$); Values are presented as mean ± SEM
Cleavage, 8-cell, morula, and blastocyst rates were evaluated at 48, 120, and 168 hpf of culture in the control, 25VD (25 ng/ml vitamin D), and 50VD (50 ng/ml vitamin D) groups, respectively

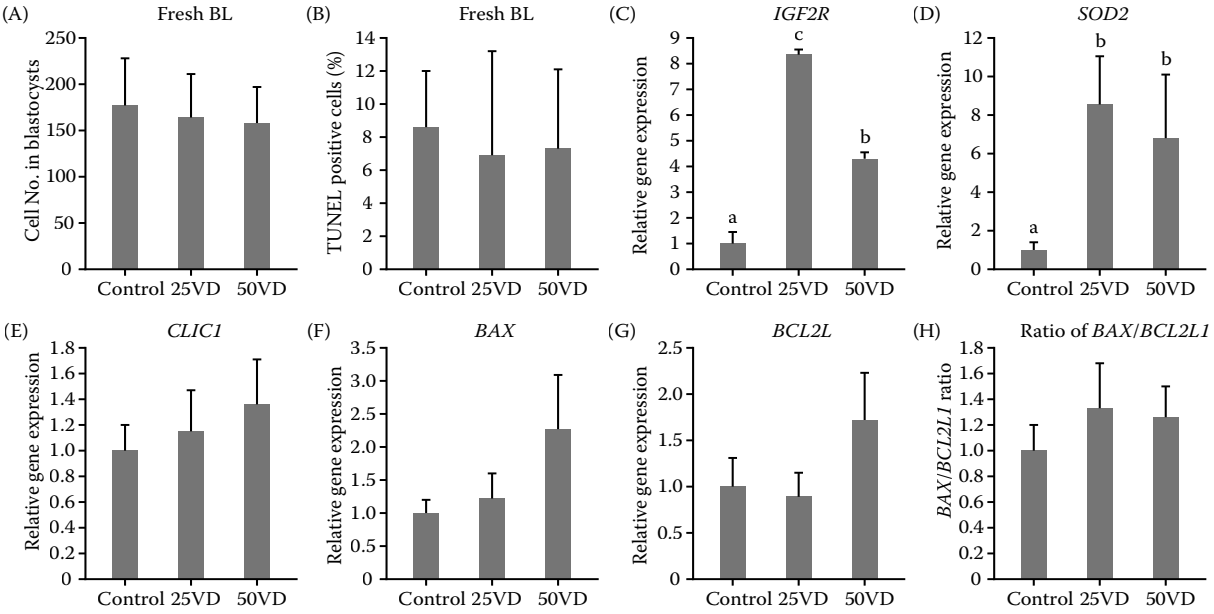

Figure 1. Assessment of blastocyst (BL) quality in the control and vitamin D groups
Total cell numbers (A) and apoptotic index (B) were analysed in the control ($n = 15$), 25VD (25 ng/ml vitamin D, $n = 15$) and 50VD (50 ng/ml vitamin D, $n = 13$) groups. Apoptotic index = TUNEL-positive cells/total DAPI-stained cells per blastocyst. Gene expression was determined by quantitative RT-PCR, normalised to GAPDH and expressed as relative fold changes with the control group set to 1 (C–H). RNA was extracted from individual blastocysts (five from each treatment group at 168 hpf of culture). The *BAX*/*BCL2L1* ratio was calculated by dividing the normalised expression value of *BAX* by that of *BCL2L1*
[a–c]Different superscripts denote significant differences between the groups ($P < 0.05$); Bars represent mean ± SEM

https://doi.org/10.17221/61/2026-CJAS

in the 25VD and 50VD groups compared with those in the control group (Figure 1C,D; $P < 0.05$). Expression of *CLIC1* did not differ between the groups (Figure 1E). Although *BAX* and *BCL2L1* levels were slightly increased in the 50VD group, these differences were not found to be statistically significant (Figure 1F,G). Consequently, the *BAX*/*BCL2L1* ratio did not differ between the treatments (Figure 1H). These results show that vitamin D does not affect cellular morphology or apoptosis in fresh blastocysts but it enhances the expression of growth-related genes and genes encoding antioxidant enzymes.

Survival and hatching of frozen-thawed blastocysts. As shown in Table 3, the survival rate of frozen-thawed blastocysts was lowest in the C/C

Table 3. Survival and hatching rates of frozen-thawed blastocysts from the control and 25VD IVC groups following vitamin D treatment

Treatment (replicate)	No. of blastocysts thawed	No. (%) of blastocysts survived	No. (%) of hatched blastocysts
C/C	41	28 (68.3 ± 10.0)	8 (28.6 ± 11.4)[a]
C/VD	46	38 (82.6 ± 12.6)	27 (71.1 ± 12.8)[b]
VD/C	32	25 (78.1 ± 11.8)	10 (40.0 ± 11.4)[a]
VD/VD	42	37 (88.1 ± 12.1)	32 (86.5 ± 14.5)[b]

[a,b]Different superscripts within the same column indicate significant differences ($P < 0.05$); Values are presented as mean ± SEM
Post-warmed blastocysts from C and 25VD groups were cultured either without vitamin D (C/C, VD/C) or with 25 ng/ml vitamin D (C/VD, VD/VD). The survival rate was assessed at 24 h and the hatching rate was assessed at 48 h after warming and calculated based on the number of surviving embryos

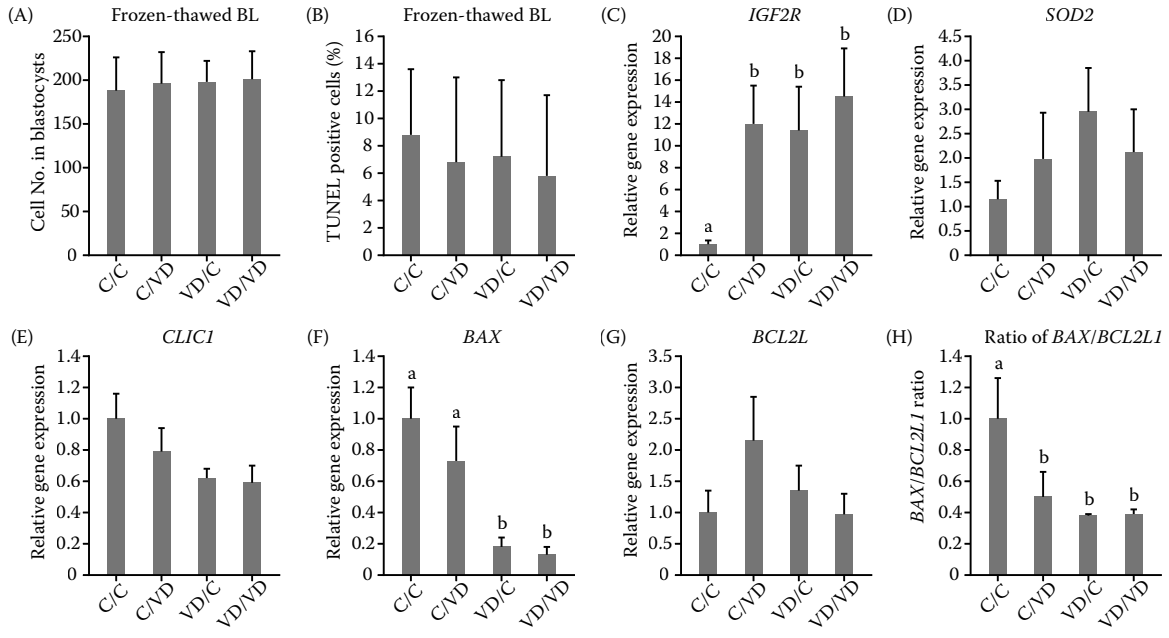

Figure 2. Quality evaluation of frozen-thawed blastocysts (BL) from the control and vitamin D groups
Post-warmed blastocysts from C and 25VD groups were cultured either without vitamin D (C/C, VD/C) or with 25 ng/ml vitamin D (C/VD, VD/VD). Total cell numbers (A) and apoptotic index (B) were analysed in the C/C (n = 14), C/VD (n = 15), VD/C (n = 14), and VD/VD (n = 14) groups. Apoptotic index = TUNEL-positive cells/total DAPI-stained cells per blastocyst. Gene expression was determined by quantitative RT-PCR, normalised to GAPDH and expressed as relative fold changes with the C/C group set to 1 (C–H). RNA was extracted from individual blastocysts (five from each treatment group at 48 hpf of culture). The *BAX*/*BCL2L1* ratio was calculated by dividing the normalised expression value of *BAX* by that of *BCL2L1*
[a,b]Different superscripts denote significant differences between the groups ($P < 0.05$); Bars represent mean ± SEM

https://doi.org/10.17221/61/2026-CJAS

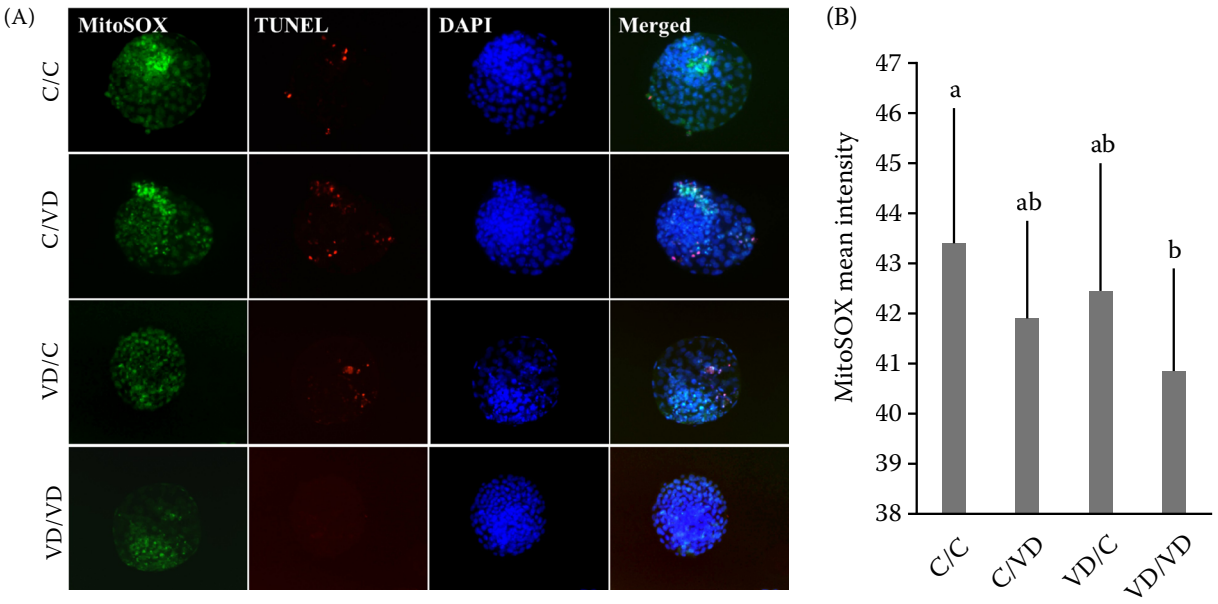

Figure 3. MitoSOX fluorescence intensity in frozen-thawed bovine blastocysts following vitamin D treatment
Post-warmed blastocysts from C and 25VD groups were cultured either without vitamin D (C/C, VD/C) or with 25 ng/ml vitamin D (C/VD, VD/VD). (A) Representative fluorescence images of the blastocysts from the C/C (n = 16), C/VD (n = 12), VD/C (n = 13) and VD/VD (n = 16) groups. Blastocysts were stained with MitoSOX (green) to detect mitochondrial ROS, TUNEL (red) for apoptotic nuclei and DAPI (blue) for total nuclei, and images were merged. (B) Quantification of MitoSOX fluorescence intensity. Scale bars indicate 100 μm
[a,b]Different superscripts denote significant differences between the groups (P < 0.05); Bars represent mean ± SEM

group (68.3% ± 10.0%) and it increased in the C/VD (82.6% ± 12.6%), VD/C (78.1% ± 11.8%), and VD/VD (88.1% ± 12.1%) groups, although these differences were not statistically significant. In contrast, hatching rates differed markedly between the treatments. The C/C group showed a low hatching rate (28.6 ± 11.4%), whereas post-warming vitamin D supplementation significantly increased hatching in the C/VD (71.1 ± 12.8%) and VD/VD (86.5 ± 14.5%) groups (P < 0.05). These findings indicate that vitamin D may enhance the post-thaw development by exerting beneficial effects during the post-warming culture period of vitrified blastocysts.

Cellular quality, gene expression, and oxidative stress in frozen-thawed blastocysts. Total cell numbers and apoptotic index did not differ between the vitrified-warmed blastocysts in the C/C, C/VD, VD/C and VD/VD groups (Figure 2A,B). In contrast, the gene expression analysis revealed clear effects of vitamin D. *IGF2R* expression was significantly higher in all vitamin D-treated groups compared with C/C (P < 0.05; Figure 2C). Although *SOD2* expression showed an increasing tendency in vitamin D groups, the differences were not significant (Figure 2D). *CLIC1* expression did not differ (Figure 2E). The pro-apoptotic gene *BAX* was significantly reduced in the VD/C and VD/VD groups (P < 0.05; Figure 2F). Although *BCL2L1* expression showed no significant difference (Figure 2G), the *BAX*/*BCL2L1* ratio was markedly lower in the C/VD, VD/C and VD/VD groups than in the C/C group (P < 0.05; Figure 2H).

As shown in Figure 3A,B, MitoSOX intensity, representing mitochondrial ROS, was significantly reduced in VD/VD (40.87 ± 2.04) compared with that in C/C (43.39 ± 2.72; P < 0.05). Intermediate values were observed in C/VD (41.92 ± 1.94) and VD/C (42.47 ± 2.53). These results indicate that VD supplementation significantly enhanced antioxidant defence, reduced oxidative stress and promoted the expression of survival-related genes after vitrification.

DISCUSSION

Vitamin D is a secosteroid hormone known to regulate calcium homeostasis, immunity, and cellular growth (Christakos et al. 2016). Several studies have reported its role in reproductive pro-

https://doi.org/10.17221/61/2026-CJAS

cesses, such as ovarian function, implantation and early embryonic development (Banker et al. 2017; Keane et al. 2017; de Souza et al. 2022; Bognar et al. 2023). However, most previous studies have focused on maternal physiology or early cleavage-stage embryos, whereas the potential influence of vitamin D on embryo quality and survival after vitrification has not been investigated.

In the present study, vitamin D supplementation showed concentration- and stage-dependent associations with bovine embryo outcomes, with effects mainly observed during the post-warming period rather than before freezing. High-dose vitamin D (50 ng/ml) impaired morula and blastocyst formation, whereas low-dose vitamin D supplementation (25 ng/ml) was associated with improved post-warming blastocyst hatching and quality, while pre-freezing developmental rates remained unchanged. In this study, vitamin D supplementation did not significantly improve the pre-freezing developmental rates, as cleavage and blastocyst formation were comparable between the control and groups with 25 ng/ml of vitamin D. In contrast, de Souza et al. (2022) reported that vitamin D (50 ng/ml) supplementation in cattle increases oocyte maturation and blastocyst formation rates, which may reflect differences in IVC conditions (Peixoto de Souza et al. 2022). Nevertheless, although the developmental rates did not differ, 25 ng/ml vitamin D influenced embryo quality, as indicated by the upregulation of *IGF2R*, a growth-related gene, and *SOD2*, an antioxidant/stress-related gene. Vitamin D has been reported to exert cell state-dependent effects, including the ability to inhibit proliferation and induce the VDR-dependent cell-cycle arrest in TMK1 cells (Consiglio et al. 2015; Li et al. 2017). These observations suggest that vitamin D signalling may differentially influence cellular metabolism depending on the physiological state of the embryo. Therefore, the reduced development observed at 50 ng/ml may be associated with metabolic or mitochondrial stress exceeding the optimal signalling range for the embryos rather than a direct apoptotic response. Thus, under the present IVC conditions, vitamin D may modulate the molecular features linked to embryo quality without increasing the overall developmental capacity.

Although vitamin D did not enhance the pre-freezing developmental rates, its potential benefits were evident after vitrification. Notably, the total cell numbers did not differ significantly between the groups, indicating that vitamin D did not promote proliferation. Instead, vitamin D supplementation may be associated with changes in the mitochondrial oxidative status. Cryopreservation induces substantial mitochondrial dysfunction, including loss of membrane potential, increased ROS generation, calcium overload, opening of the mitochondrial permeability transition pore and disruption of the cristae structure (Gualtieri et al. 2021). These alterations may impair ATP production and cellular homeostasis and they are likely to contribute to the reduced developmental competence of embryos after thawing. Meanwhile, vitamin D plays a regulatory role in maintaining the mitochondrial structure and function. Vitamin D supplementation has been shown to restore disrupted cristae morphology, normalise fusion-fission dynamics, increase ATP production and reduce ROS accumulation in mitochondria-damaged cells (Ren et al. 2020). These findings suggest that vitamin D promotes mitochondrial stability under stressful conditions. In the present study, the relatively low hatching rate observed in the C/C group may be related to an increased sensitivity to vitrification-induced stress, as evidenced by elevated mitochondrial ROS levels and an increased *BAX*/*BCL2L1* ratio; this result is consistent with previous reports showing that vitrification and warming induce mitochondrial dysfunction and oxidative stress in embryos (Marsico et al. 2019; Cao et al. 2022). In contrast, the C/VD and VD/VD groups showed high hatching rates, low mitochondrial ROS levels and reduced *BAX*/*BCL2L1* ratios. The VD/C group showed a slight increase in hatching rates and this improvement was limited when compared to the other vitamin D-supplemented groups. These results suggest that the beneficial effects of vitamin D depend on its availability during the post-warming recovery phase rather than during IVC alone. The limited improvement observed in the VD/C group may be related to the insufficient protection against cryo-induced stress during IVC, and the absence of vitamin D during the recovery period, which may have disturbed mitochondrial restoration after warming.

The VD/VD group showed the highest hatching rate, accompanied by lower mitochondrial ROS levels, as indicated by the reduced MitoSOX intensity. Simultaneously, *CLIC1* – recognised as an ROS-responsive marker in cellular and embryo culture systems (Averaimo et al. 2010; Cajas et al. 2020) –

https://doi.org/10.17221/61/2026-CJAS

was downregulated in the vitamin D-treated groups, supporting an association between vitamin D supplementation and reduced intracellular ROS accumulation after warming. Moreover, *SOD2* – a mitochondrial superoxide dismutase that directly detoxifies superoxide and serves as a key antioxidant defence in preimplantation embryos (Arias et al. 2012; Cagnone and Sirard 2016) – was upregulated following vitamin D supplementation. Although these gene expression changes were modest, small shifts in the antioxidant- and stress-related pathways may influence the embryo competence, given that the functional thresholds during early embryonic development are not fully defined. Taken together, these aforementioned results suggest that vitamin D supplementation may support the mitochondrial redox balance after warming rather than promoting the cell proliferation.

In this study, the expression of *IGF2R* was increased in vitamin D-treated blastocysts. *IGF2R* is a maternally expressed, imprinted gene that regulates *IGF2* signalling by internalising and degrading excess *IGF2* ligands. The proper expression of *IGF2R* limits *IGF2* activity and is critical for maintaining normal growth and development. Loss of the *IGF2R* function in animal models leads to unchecked *IGF2* signalling, overgrowth and abnormal development, highlighting its essential role in regulating cell proliferation, differentiation and placental formation (Ludwig et al. 1996; Tveden-Nyborg et al. 2008; Plasschaert and Bartolomei 2015). Dysregulation of *IGF2R* expression or methylation in bovine IVP embryos has been frequently reported and is associated with impaired blastocyst development, implantation failure and abnormal foetal growth, including large offspring syndrome (LOS) (Young et al. 2001; Chen et al. 2013; Smith et al. 2015). In this context, the increased *IGF2R* expression observed following the vitamin D supplementation may contribute to a more balanced regulation of growth-related signalling pathways, although its functional implications require further investigation.

CONCLUSION

In summary, low-dose vitamin D supplementation (25 ng/ml) did not improve pre-freezing embryonic development under the *in vitro* culture conditions of the present study, as developmental rates before freezing were not affected. The potential effects of vitamin D were mainly observed after vitrification and warming, where low-dose supplementation (25 ng/ml) was associated with modest improvements in blastocyst hatching and embryo quality. Vitamin D supplementation during IVC may contribute to a limited increase in the resistance to cryo-induced stress, whereas supplementation after warming appears to be more important for supporting mitochondrial recovery. Consistently, the hatching outcomes were more favourable when vitamin D was present during the post-warming phase (C/VD and VD/VD), whereas supplementation restricted to the pre-freezing period (VD/C) resulted in only a limited improvement. These post-warming effects were not related to increased cell proliferation, but they may be associated with reduced mitochondrial oxidative stress and changes in stress- and growth-related gene expression. Overall, the present results suggest that vitamin D may support post-warming recovery from cryogenic stress rather than directly improving the intrinsic embryo quality.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Arias ME, Sanchez R, Felmer R. Evaluation of different culture systems with low oxygen tension on the development, quality and oxidative stress-related genes of bovine embryos produced in vitro. Zygote. 2012 Aug;20(3):209-17.

Averaimo S, Milton RH, Duchen MR, Mazzanti M. Chloride intracellular channel 1 (CLIC1): Sensor and effector during oxidative stress. FEBS Lett. 2010 May 17;584(10):2076-84.

Banker M, Sorathiya D, Shah S. Vitamin D deficiency does not influence reproductive outcomes of IVF-ICSI: A study of oocyte donors and recipients. J Hum Reprod Sci. 2017 Apr-Jun;10(2):79-85.

Banliat C, Mahe C, Lavigne R, Com E, Pineau C, Labas V, Guyonnet B, Mermillod P, Saint-Dizier M. The proteomic analysis of bovine embryos developed in vivo or in vitro reveals the contribution of the maternal environment to early embryo. BMC Genomics. 2022 Dec 19;23(1):839.

Bo GA, Mapletoft RJ. Evaluation and classification of bovine embryos. Anim Reprod. 2013;10(3):344-8.

Bognar Z, Csabai-Tanics TJ, Gorgey E, Miko E, Horvath-Szalai Z, Szekeres-Bartho J. The effect of calcitriol on the

https://doi.org/10.17221/61/2026-CJAS

development and implantation capacity of embryos from hyper-stimulated mice. Front Immunol. 2023 Jul 21;14: 1200704.

Cagnone G, Sirard MA. The embryonic stress response to in vitro culture: Insight from genomic analysis. Reproduction. 2016 Dec;152(6):R247-61.

Cajas YN, Canon-Beltran K, Ladron de Guevara M, Millan de la Blanca MG, Ramos-Ibeas P, Gutierrez-Adan A, Rizos D, Gonzalez EM. Antioxidant nobiletin enhances oocyte maturation and subsequent embryo development and quality. Int J Mol Sci. 2020 Jul 27;21(15):5340.

Cao B, Qin J, Pan B, Qazi IH, Ye J, Fang Y, Zhou G. Oxidative stress and oocyte cryopreservation: Recent advances in mitigation strategies involving antioxidants. Cells. 2022 Nov 11;11(22):3573.

Chen Z, Robbins KM, Wells KD, Rivera RM. Large offspring syndrome: A bovine model for the human loss-of-imprinting overgrowth syndrome Beckwith–Wiedemann. Epigenetics. 2013 Jun;8(6):591-601.

Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, molecular mechanism of action, and pleiotropic effects. Physiol Rev. 2016 Jan;96(1):365-408.

Consiglio M, Viano M, Casarin S, Castagnoli C, Pescarmona G, Silvagno F. Mitochondrial and lipogenic effects of vitamin D on differentiating and proliferating human keratinocytes. Exp Dermatol. 2015 Oct;24(10):748-53.

de Souza VP, Jensen J, Whitler W, Estill CT, Bishop CV. Increasing vitamin D levels to improve fertilization rates in cattle. J Anim Sci. 2022 Jul 1;100(7):skac168.

Ferre LB, Kjelland ME, Strobech LB, Hyttel P, Mermillod P, Ross PJ. Review: Recent advances in bovine in vitro embryo production: Reproductive biotechnology history and methods. Animal. 2020 May;14(5):991-1004.

Grzechocinska B, Dabrowski FA, Cyganek A, Wielgos M. The role of vitamin D in impaired fertility treatment. Neuro Endocrinol Lett. 2013;34(8):756-62.

Gualtieri R, Kalthur G, Barbato V, Di Nardo M, Adiga SK, Talevi R. Mitochondrial dysfunction and oxidative stress caused by cryopreservation in reproductive cells. Antioxidants (Basel). 2021 Feb 24;10(3):337.

Janati Idrissi S, Le Bourhis D, Lefevre A, Emond P, Le Berre L, Desnoes O, Joly T, Buff S, Maillard V, Schibler L, Salvetti P, Elis S. Lipid profile of bovine grade-1 blastocysts produced either in vivo or in vitro before and after slow freezing process. Sci Rep. 2021 Jun 2;11(1):11618.

Kageyama M, Ito J, Shirasuna K, Kuwayama T, Iwata H. Mitochondrial reactive oxygen species regulate mitochondrial biogenesis in porcine embryos. J Reprod Dev. 2021 Apr 21;67(2):141-7.

Keane KN, Cruzat VF, Calton EK, Hart PH, Soares MJ, Newsholme P, Yovich JL. Molecular actions of vitamin D in reproductive cell biology. Reproduction. 2017 Jan 1; 153(1):R29-R42.

Krisher RL, Herrick JR. Bovine embryo production in vitro: Evolution of culture media and commercial perspectives. Anim Reprod. 2024 Sep 23;21(3):e20240051.

Kwon DJ, Park KD, Lee H, Oh JD. Lipid-rich bovine serum albumin is beneficial for the early development of bovine embryos, but induces lipid droplet formation in the blastocyst. Czech J Anim Sci. 2023 Dec 1;68(11):443-50.

Li M, Hu S, Sun J, Zhang Y. The role of vitamin D3 in follicle development. J Ovarian Res. 2024 Jul 17;17(1):148.

Li M, Li L, Zhang L, Hu W, Shen J, Xiao Z, Wu X, Chan FL, Cho CH. 1,25-Dihydroxyvitamin D3 suppresses gastric cancer cell growth through VDR- and mutant p53-mediated induction of p21. Life Sci. 2017 Jun 15;179:88-97.

Liu Y, Du Y, Lin L, Li J, Kragh PM, Kuwayama M, Bolund L, Yang H, Vajta G. Comparison of efficiency of open pulled straw (OPS) and Cryotop vitrification for cryopreservation of in vitro matured pig oocytes. CryoLetters. 2008 Jul-Aug;29(4):315-20.

Lopera-Vasquez R, Hamdi M, Maillo V, Gutierrez-Adan A, Bermejo-Alvarez P, Ramirez MA, Yanez-Mo M, Rizos D. Effect of bovine oviductal extracellular vesicles on embryo development and quality in vitro. Reproduction. 2017 Apr;153(4):461-70.

Ludwig T, Eggenschwiler J, Fisher P, D'Ercole AJ, Davenport ML, Efstratiadis A. Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. Dev Biol. 1996 Aug 1;177(2):517-35.

Marsico TV, de Camargo J, Valente RS, Sudano MJ. Embryo competence and cryosurvival: Molecular and cellular features. Anim Reprod. 2019 Oct 23;16(3):423-39.

Plasschaert RN, Bartolomei MS. Tissue-specific regulation and function of Grb10 during growth and neuronal commitment. Proc Natl Acad Sci U S A. 2015 Jun 2;112(22): 6841-7.

Ren L, Xuan L, Han F, Zhang J, Gong L, Lv Y, Zhang W, Yang S, Xu B, Yan Y, Guo L, Liu H, Wan Z, Liu L. Vitamin D supplementation rescues simvastatin induced myopathy in mice via improving mitochondrial cristae shape. Toxicol Appl Pharmacol. 2020 Aug 15;401:115076.

Sirard MA. 40 years of bovine IVF in the new genomic selection context. Reproduction. 2018 Jul;156(1):R1-7.

Smith LC, Therrien J, Filion F, Bressan F, Meirelles FV. Epigenetic consequences of artificial reproductive technologies to the bovine imprinted genes SNRPN, H19/IGF2, and IGF2R. Front Genet. 2015 Feb 25;6:58.

Tveden-Nyborg PY, Alexopoulos NI, Cooney MA, French AJ, Tecirlioglu RT, Holland MK, D'Cruz NT. Analysis of the expression of putatively imprinted genes in bovine

https://doi.org/10.17221/61/2026-CJAS

peri-implantation embryos. Theriogenology. 2008 Oct 15;70(7):1119-28.

Wetscher F, Havlicek V, Huber T, Gilles M, Tesfaye D, Griese J, Wimmers K, Schellander K, Muller M, Brem G, Besenfelder U. Intrafallopian transfer of gametes and early stage embryos for in vivo culture in cattle. Theriogenology. 2005 Jul 1;64(1):30-40.

Xu F, Wolf S, Green O, Xu J. Vitamin D in follicular development and oocyte maturation. Reproduction. 2021 May 5; 161(6):R129-37.

Xu J, Lawson MS, Xu F, Du Y, Tkachenko OY, Bishop CV, Pejovic-Nezhat L, Seifer DB, Hennebold JD. Vitamin D3 regulates follicular development and intrafollicular vitamin D biosynthesis and signaling in the primate ovary. Front Physiol. 2018 Nov 14;9:1600.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet. 2001 Feb;27(2):153-4.

Received: May 14, 2026
Accepted: June 18, 2026
Published online: July 24, 2026