CHAPTER IV

RESULTS

4.1 Experiment 1: Effects of supplemented resveratrol in IVC medium on embryo development.

Embryo development was evaluated based on cleavage and blastocyst rates in embryos cultured with 0.5 μM resveratrol (Table 4.1). Cleavage rate was significantly higher in the resveratrol group (81.70% ± 3.20) vs control (75.13% ± 4.04), ($P < 0.05$). Similarly, the blastocyst development rate in the resveratrol-treated group (37.75% ± 2.31) was also significantly higher ($P < 0.05$) than that in the control group (29.82% ± 1.95). The present results show that supplementation with 0.5 μM resveratrol during IVC improved cleavage and blastocyst rates, with enhanced early embryo development achieved.

Table 2. Effects of resveratrol during *in vitro* culture (IVC) of bovine embryos on cleavage rates and blastocyst formation rates.

Groups	No. Oocytes	No. (%) Cleavage (Mean ± SEM)	No. (%) Blastocyst (Mean ± SEM)
Control	546	404 (75.13 % ± 4.04)[a]	153 (29.82 % ± 1.95)[a]
0.5 μM Resveratrol	546	441 (81.70 % ± 3.20)[b]	196 (37.75 % ± 2.31)[b]

10 replicate; %: Mean ± SEM. [a b] Values with different superscripts are significantly different at $P < 0.05$ (ANOVA)

4.2 Experiment 2: Effects of supplemented resveratrol in IVC and post-warming culture (PWC) media on the developmental potential of vitrified blastocysts, ICM, TE, and total cell numbers of fresh and gene expression.

4.2.1 Effects of supplemented resveratrol in IVC and post-warming culture (PWC) media on the developmental potential of vitrified blastocysts

The effect of resveratrol addition in post-warming culture medium on embryonic developmental competency following post-warming is shown in Table 3. The post-warming culture medium supplementation of 0.5 μM resveratrol could not significantly improve the survival rate of blastocysts or developmental competency. The survival rates at 24 h post-warming were not significantly different among all groups, ranging from 88.96 ± 3.55% to 93.85 ± 2.88% ($P > 0.05$). Likewise, there were no significant differences in the re-expanded blastocyst percentage between treatments, ranging from 51.01 ± 7.82% to 64.02 ± 5.97% ($P > 0.05$). At 48 h following post-warming culture, all groups had similar survival rates (81.80 ± 4.56% to 90.67 ± 2.87%, $P > 0.05$). In contrast to the −R/+R group (45.03 ± 5.48%, $P < 0.05$), the group that was cultured with resveratrol during IVC but not in post-warming culture (+R/−R) exhibited the highest hatching and hatched rate at 71.50 ± 5.35%. There was no appreciable variance in the fully expanded blastocyst rates, which ranged from 19.18 ± 3.41% to 36.77 ± 5.42% ($P > 0.05$). These findings suggest that whereas resveratrol supplementation had no discernible effect on developmental competency or overall survival, the use of resveratrol during IVC combined with its absence in the post-warming culture medium may enhance blastocyst hatching and hatched competency.

Table 3. Effect of resveratrol supplementation in post-warming culture on developmental competency of embryos after post-warming.

Resveratrol in IVC	Resveratrol in post-warming culture medium	No. Blastocyst	Post-warming culture					
			24 h.			48 h.		
			No. (%) Survival	No. (%) Re-expanded blastocysts	No. (%) Hatching and hatched blastocysts	No. (%) Survival	No. (%) Fully expanded blastocysts	No. (%) Hatching and hatched blastocysts
-	-	91	82	57	25	77	24	53
			(90.16 ± 3.72)	(64.02 ± 5.97)	(26.14 ± 5.39)	(83.22 ± 4.71)	(27.22 ± 4.13)	(56.00 ± 4.82)^{ab}
-	+	91	81	58	22	74	34	40
			(88.96 ± 3.55)	(63.73 ± 4.22)	(22.73 ± 4.48)	(81.80 ± 4.56)	(36.77 ± 5.42)	(45.03 ± 5.48)^{b}
+	-	95	89	54	34	86	19	67
			(93.85 ± 2.88)	(54.81 ± 6.64)	(36.58 ± 8.02)	(90.67 ± 2.87)	(19.18 ± 3.41)	(71.50 ± 5.35)^{a}
+	+	95	87	51	35	81	22	59
			(90.83 ± 3.54)	(51.01 ± 7.82)	(37.32 ± 6.50)	(84.06 ± 5.05)	(22.67 ± 5.98)	(61.39 ± 6.45)^{ab}

10 replicates; %: Mean ± SEM. $^{a\,b}$ Values with different superscripts are significantly different at $P < 0.05$ (ANOVA)

4.2.2 Effect of resveratrol supplemented in IVC and post-warming culture media on ICM, TE, and total cell numbers of fresh and vitrified bovine embryo

Figure 3 presents the cell counts of both fresh and vitrified blastocysts following post-warming culture. The ICM, TE, and total cell counts were not significantly different between the vitrified and fresh control groups, regardless of resveratrol treatment. Fresh blastocysts with and without resveratrol had higher ICM, TE, and total cell counts than vitrified groups, but these differences were not statistically significant. These results imply that resveratrol supplementation does not affect the development of cells in both fresh and cultured bovine embryos.

Table 4. Effect of resveratrol supplemented in IVC and post-warming culture on ICM, TE, and total cell numbers of fresh and vitrified bovine embryos

Groups	No. blastocyst	No. ICM Mean ± SEM	No. TE Mean ± SEM	Total cell numbers Mean ± SEM
-R	10	54.50 ± 3.24	155.7 ± 5.34	210.2 ± 7.242
+R	10	53.70 ± 1.606	156.7 ± 4.95	210.4 ± 5.82
-R/-R	10	51.50 ± 4.11	144.7 ± 9.66	196.2 ± 12.65
-R/+R	10	50.90 ± 1.87	152.2 ± 8.30	203.1 ± 9.01
+R/-R	10	54.00 ± 0.83	150.9 ± 13.07	204.9 ± 13.39
+R/+R	10	53.80 ±1 .988	150.1 ± 13.50	203.9 ± 13.72

A total of 10 embryos per group were analyzed. Data are presented as mean ± SEM. No significant difference at $P > 0.05$ (ANOVA).

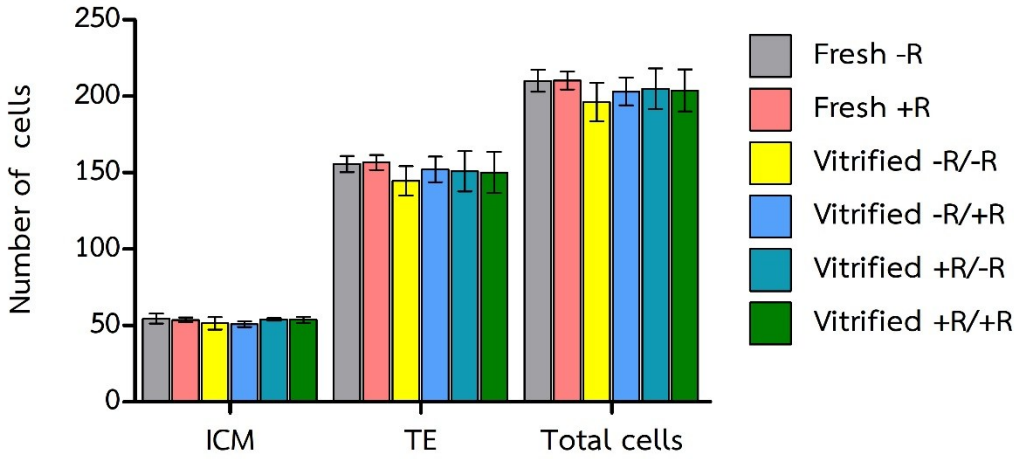

Figure 3. Analysis of the TE, ICM, and total cell numbers of fresh and vitrified embryos cultured in IVC with or without 0.5 µM resveratrol. A total of 10 embryos per group were analyzed. Data are presented as mean ± SEM. No significant difference at P > 0.05 (ANOVA).

4.2.3 Effect of resveratrol supplementation on fresh and vitrified bovine blastocyst gene expression in IVC and/or post-warming culture media

Gene expression related to stress response and antioxidant defense

HSP70, a heat shock protein involved in the cellular stress response, displayed a unique expression pattern with the largest increase in the -R/-R group ($P < 0.05$), whereas the +R groups displayed similar low expressions. *HSP70* expression differed between the control and the -R/-R group ($P < 0.05$) in the other vitrification groups (-R/+R, +R/-R, and +R/+R). All resveratrol-treated groups showed a significant increase in the antioxidant enzyme *SOD1* ($P < 0.05$) when compared to the control. The +R and -R/-R groups had the highest *SOD1* expression (Figure 4), whereas the vitrification groups, including resveratrol, maintained high expression throughout certain treatment stages. *CAT* expression was much higher in the +R group than in any other group ($P < 0.05$). The vitrification treatments resulted in significantly decreased *CAT* expression ($P < 0.05$) than both the -R and +R groups. *GPX4* showed a distinct expression pattern with significant overexpression in the -R/-R, +R/-R, and +R/+R groups compared to the -R and +R groups ($P < 0.05$). Unless resveratrol is added only

post-warming culture (-R/+R), this suggests that vitrification may result in overexpression of *GPX4* (Figure 5).

Gene expression related to stress-resistance and mitochondrial function

SIRT1 exhibited significantly higher expression in the +R group compared to the control ($P < 0.05$). Conversely, all vitrified groups had markedly reduced *SIRT1* levels, with no statistically significant variations among them ($P > 0.05$). Similarly, *TFAM* expressions significantly increased in the +R group compared to the other groups ($P < 0.05$). The +R/+R group had the lowest *TFAM* expression ($P < 0.05$) following vitrification treatments (Figure 6).

Gene expression related to apoptosis

The fresh control group without resveratrol supplementation had the highest relative expression of the pro-apoptotic gene *BAX* ($p < 0.05$). When compared to the -R group, resveratrol supplementation during IVC significantly decreased *BAX* expression ($p < 0.05$). The *BAX* expression levels in vitrified embryos were lowest in the -R/+R group and significantly lower than in the -R/-R and +R/-R groups ($p < 0.05$). On the other hand, the +R group had the highest expression of the anti-apoptotic gene *BCL2*, which was significantly higher than that of any other group ($p < 0.05$). Regardless of when resveratrol was administered, all vitrification groups showed a significant decrease in *BCL2* expression ($p < 0.05$), and there were no significant differences among the vitrified groups ($P > 0.05$) (Figure 7).

Gene expression related to cell-programming, pluripotency and recognition of pregnancy

The +R group had significantly higher *DNMT1* expression than any other group ($p < 0.05$). The -R/-R and +R/-R groups showed intermediate levels of expression, which were significantly higher than both the -R/+R and +R/+R groups ($p < 0.05$) but significantly lower than the +R group. The -R group had the lowest *DNMT1* expression ($p < 0.05$). The +R group once again showed the highest expression of *DNMT3A* ($p < 0.05$), whereas all vitrified groups (-R/-R, -R/+R, +R/-R, and +R/+R) showed significantly lower expression levels that did not differ significantly from one another ($p > 0.05$). The expression level of the -R group was intermediate; it was lower than that of the

+R group but significantly higher than all vitrified groups ($p < 0.05$) (Figure 8). The +R group showed significantly higher levels of *OCT4/POU5F1* expression than all other groups, according to the analysis ($p < 0.05$). Although the expression of the -R group was significantly lower than that of the +R group, it was higher than that of other vitrified groups ($p < 0.05$). The -R/+R group had the lowest expression ($p < 0.05$) among vitrified embryos, while the -R/-R, +R/-R, and +R/+R groups all had comparably low levels with no significant differences ($p > 0.05$). The +R group had the highest expression of *IFN-tau*, higher than all other groups by a significant amount ($p < 0.05$). While the -R/+R and +R/-R groups had moderate expression levels that were not significantly different from the other groups ($p > 0.05$), they were significantly lower than the +R group ($p < 0.05$). The -R/-R group also showed higher expression than the -R group ($p < 0.05$). Comparable to the -R control, the +R/+R group had the lowest expression of all vitrified groups ($p > 0.05$) (Figure 9).

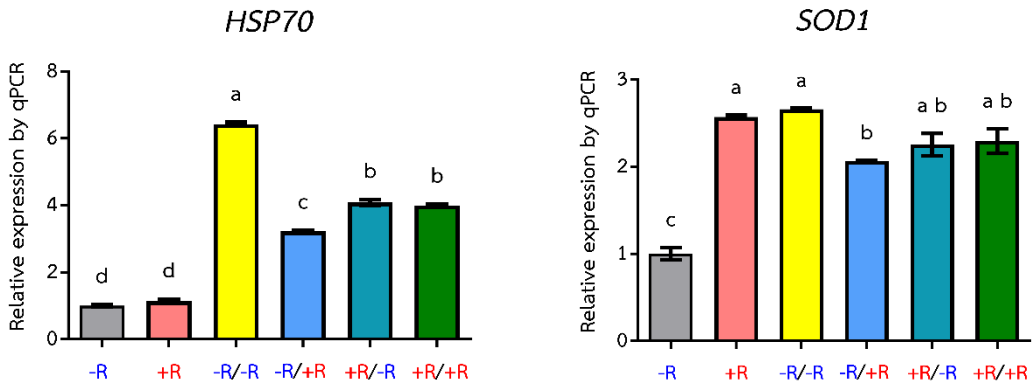

Figure 4 Expression levels of *HSP70* and *SOD1* genes in bovine embryos produced *in vitro* cultured under different conditions. A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at $P < 0.05$ (ANOVA).

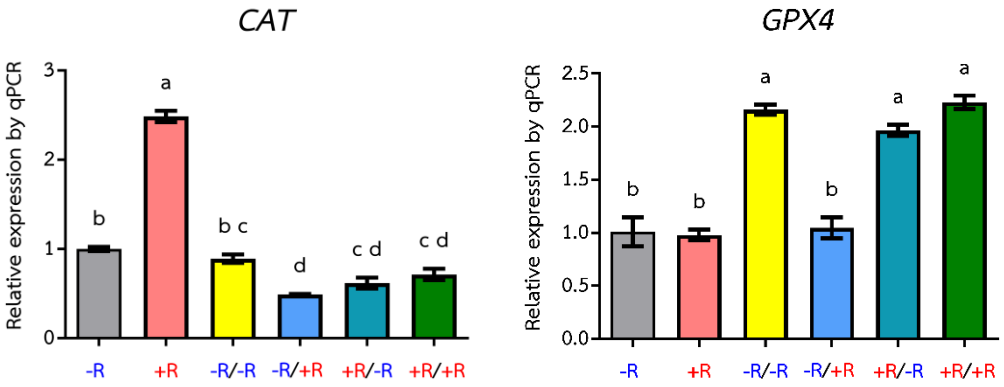

Figure 5 Expression levels of *CAT* and *GPX4* genes in bovine embryos produced *in vitro* cultured under different conditions. A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at P < 0.05 (ANOVA).

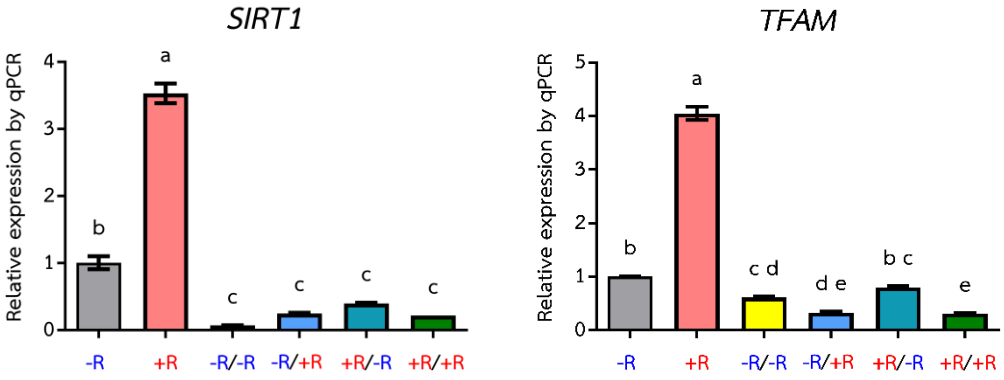

Figure 6 Expression levels of *SIRT1* and *TFAM* genes in bovine embryos produced *in vitro* cultured under different conditions. A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at P < 0.05 (ANOVA).

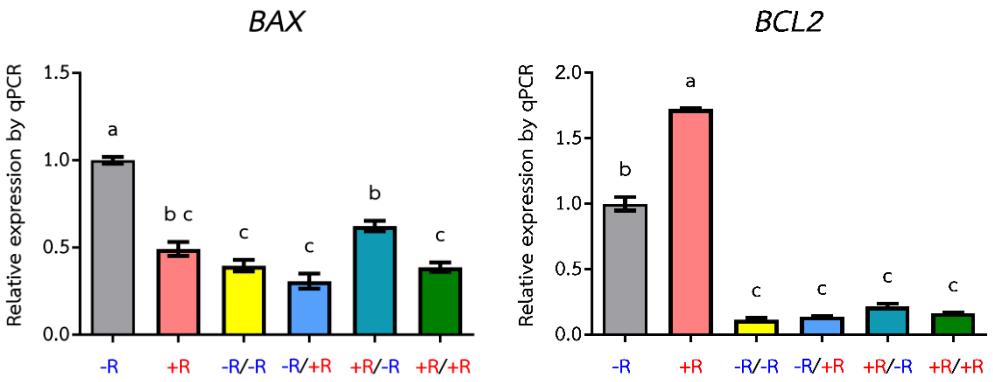

Figure 7 Expression levels of *BAX* and *BCL2* genes in bovine embryos produced *in vitro* cultured under different conditions. A total of 20blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at P < 0.05 (ANOVA test).

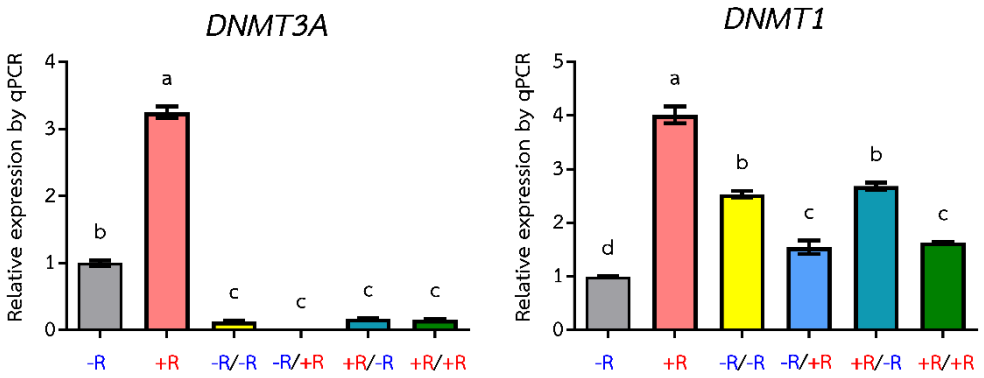

Figure 8 Expression levels of *DNMT1* and *DNMT3A genes* in bovine embryos produced *in vitro* cultured under different conditions. A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at P < 0.05 (ANOVA).

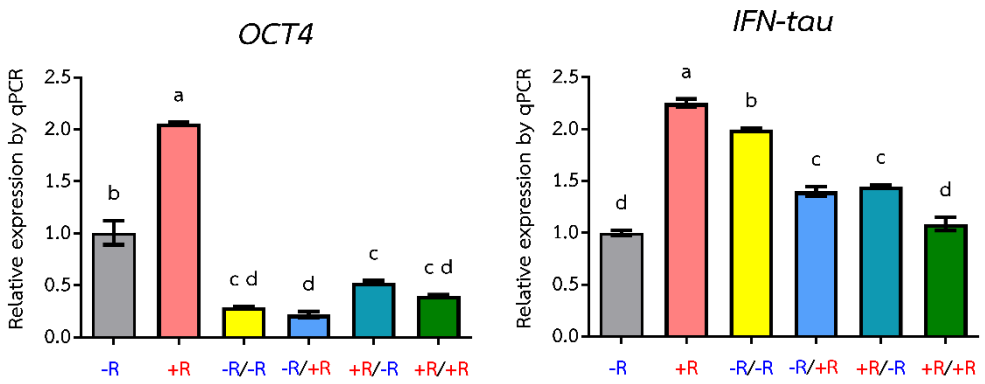

Figure 9 Expression levels of *OCT4* and *IFN-tau genes* in bovine embryos produced *in vitro* cultured under different conditions. A total of 20 blastocysts per group were analyzed. Data are presented as mean ± SEM. [a b] Values with different superscripts are significantly different at $P < 0.05$ (ANOVA).