CHAPTER V
DISCUSSION AND CONCLUSION

5.1 Discussion

Recent years have seen a lot of interest in how resveratrol influences the development of bovine embryos *in vitro*, which has prompted studies on its mechanisms of action and impact on embryo quality. This study showed that 0.5 μM resveratrol added to the IVC medium affects the different morphology of embryos at both the cleavage and blastocyst phases (Figure 1). The findings revealed that embryos at these stages exhibit different characteristics under different conditions. Consistent cleavage stage development in the control group embryos reflected normal early embryogenesis, as indicated by symmetric blastomeres with clear boundaries. Similar morphology of embryos treated with 0.5 μM resveratrol also suggested that resveratrol does not influence the early cleavage process. These findings were consistent with studies indicating that low resveratrol levels had little impact on early developmental stage cell division (Piras et al., 2020). Control group embryos showed suitable expansion and compaction at the blastocyst stage, suggesting normal blastocyst development. Embryos treated with 0.5 μM resveratrol showed increasing structural integrity, despite this, showing trophectoderm layers and well-formed inner cell masses. This result supports earlier research indicating that resveratrol may increase mitochondrial activity and lower oxidative stress, raising blastocyst quality (Piras et al., 2019). Resveratrol-treated embryos also showed no morphological abnormalities or developmental delays, hence verifying its probable role in improving embryonic viability (Gaviria et al., 2019). Consistent with its stated antioxidant qualities, the findings mostly suggested that resveratrol treatment at a dose of 0.5 μM had no impact on early cleavage and might enhance blastocyst quality. On the other hand, the results of resveratrol supplementation in a post-warming culture medium. The addition of 0.5 μM resveratrol to the post-warming culture medium at 24 and 48 h did not affect blastocyst survival rates or developmental competency.

Though after 48 h, the +R/-R group outperformed the -R/+R group in hatching rates, indicating a context-dependent effectiveness of resveratrol supplementation. These findings support recent studies indicating that the impact of resveratrol can vary depending on concentration, duration, and cellular environment, maybe because it modulates mitochondrial activity and oxidative stress (Hara et al., 2018). Surprisingly, resveratrol has been demonstrated to have both pro-oxidant and antioxidant actions, hence affecting the destinies of embryos through mitochondrial homeostasis and apoptosis. Higher hatching rates in the +R/-R group (Abe et al., 2017) suggest that resveratrol exposure before cryopreservation may have enhanced the balance of ROS, hence enhancing mitochondrial efficiency and developmental ability. Exposure to resveratrol after cryopreservation, such as in the -R/+R group, may, however, alter the redox balance in favor of pro-oxidative stress, hence reducing its antioxidant properties and showing the intricate function of resveratrol in embryonic systems (De La Lastra & Villegas, 2007). These findings help us to know more about the important link between mitochondrial function, antioxidant supplements, and embryonic survival. Future studies on the temporal modulation of resveratrol in culture systems should help to maximize its advantages and avoid any undesirable cytotoxic consequences. This will aid in strengthening techniques for improving embryo cryotolerance and developmental results in assisted reproductive technologies.

Moreover, the findings of this study revealed that including 0.5 μM resveratrol to bovine embryos, whether fresh or vitrified, has no noticeable impact on the total counts of ICM, TE. Not having any notable changes in cell counts suggested that resveratrol does not interfere with vital developmental pathways since ICM cells are involved in embryonic development and TE cells express interferon-tau (IFN-tau), which is required for implantation. Though the findings indicated that ICM or TE numbers were unaffected by 0.5 μM resveratrol, this does not negate potential molecular effects on embryonic physiology. Earlier research has suggested that, rather than directly affecting cell proliferation, resveratrol may work via pathways connected to mitochondrial bioenergetics, stress response modulation, and gene expression control (Yuan et al., 2015).

In this study, resveratrol-supplemented and vitrified bovine embryos showed notable variations in the expression of genes linked to antioxidant defense and cellular stress response. Interestingly, different expression patterns of *GPX4*, *HSP70*, *SOD1*, and *CAT*) were found. The group without resveratrol supplementation showed the highest expression of *HSP70* both before and after vitrification (-R/-R), indicating more cellular stress when antioxidant assistance is lacking. On the other hand, embryos supplemented with resveratrol consistently had low expression of *HSP70*, suggesting that the antioxidant qualities of resveratrol effectively reduce cellular stress (Arredondo et al., 2021) Every group that received resveratrol showed a notable rise in *SOD1*, a vital enzyme that shields embryos from oxidative stress by transforming superoxide radicals into less toxic forms (Zarbakhsh, 2021). Resveratrol supplementation can improve embryo survival under oxidative stress, as evidenced by the continued high expression of *SOD1* in vitrified embryos (Keane and Ealy, 2024). Additionally, embryos regularly treated with resveratrol showed much increased *CAT* gene expression, whereas all vitrification groups showed a dramatic drop in this gene expression. This implies that embryos are more susceptible to oxidative damage during vitrification. Such findings are consistent with earlier research showing that vitrification can increase oxidative stress to a level that may surpass the inherent antioxidant capability of embryos (Amin et al., 2014). An interesting pattern was shown by the significantly higher *GPX4* expression in vitrified groups, specifically -R/-R, +R/-R, and +R/+R, as compared to the control. This upregulated expression may be a compensatory reaction to elevated lipid peroxidation brought on by vitrification, which is in line with earlier findings in oxidative stress scenarios and highlights the critical function of *GPX4* in reducing the effects of lipid peroxidation during embryonic development (Saber et al., 2024). The results point to resveratrol as a useful additive for oxidative stress management in bovine embryo culture, especially during vitrification procedures. According to earlier studies, resveratrol effectively lowers the formation of ROS, raises intracellular glutathione levels, and has anti-apoptotic properties, all of which improve embryo survival after vitrification (Silva et al., 2021).

This study found that bovine embryos exposed to resveratrol supplementation and vitrification treatments showed notable changes in gene expression linked to stress resistance and mitochondrial function, namely the expression of *SIRT1* and *TFAM*. The main conclusions showed that whereas all vitrified groups showed a significant decrease in the expression of *SIRT1* and *TFAM*, embryos constantly supplied with resveratrol +R showed significantly greater expression levels of these genes. Resveratrol is a strong activator of *SIRT1*, which improves mitochondrial activity and may improve embryo developmental competence, as seen by the greater expression of *SIRT1* in the (+)R group when compared to the control. Prior research by Abe et al. (2017) showed that resveratrol-mediated activation of *SIRT1* promoted mitochondrial biogenesis and turnover through pathways involving phosphorylated AMP-activated protein kinase (AMPK), which in turn increased ATP generation and improved mitochondrial function. This is consistent with our research and implies that resveratrol supplementation may be a useful tactic for improving embryo viability, especially when mitochondrial quality is essential for later developmental success. On the other hand, *SIRT1* expression levels were markedly reduced following vitrification treatments, indicating mitochondrial malfunction and lower fetal viability post-warming culture. Vitrification-induced mitochondrial damage, a frequent side effect of cryopreservation techniques, may account for this phenomenon. According to earlier research, vitrification significantly impairs the integrity of the mitochondrial membrane, lowers ATP synthesis, and causes oxidative stress in embryos (Hara et al., 2018). *SIRT1* expression suppression in vitrified embryos may result from cryoinjury-induced mitochondrial malfunction and oxidative stress. Comparable patterns were noted for *TFAM*, a crucial modulator of transcription and replication of mitochondrial DNA. The positive impact of resveratrol in preserving the integrity and function of the mitochondrial genome is further supported by the notable increase in *TFAM* expression in the +R group supplemented with resveratrol. Especially, the +R/+R group showed the least *TFAM* expression after vitrification. This pattern could imply that cryopreservation does not provide benefits from extended or consecutive exposure to resveratrol before and after, and in some situations, may cause dysregulation of mitochondrial gene expression. One reasonable theory is that resveratrol supplementation during both IVC and post-warming culture media phases might excessively shift the redox balance, possibly cause mild cytotoxic effects or interfere with mitochondrial signaling pathways under post-thaw conditions (De la Lastra & Villegas, 2007; Hara et al., 2018). Particularly through altering mitochondrial dynamics and oxidative stress, resveratrol has been said to have both antioxidant and pro-oxidant effects depending on quantity, time, and cellular environment (Abe et al., 2017; Mesalam et al., 2018). This finding highlights the requirement of exact management of treatment duration and timing to prevent unintentional consequences on mitochondrial function and embryo viability, as well as the of the interaction between resveratrol and developmental physiology. These findings have significant ramifications for techniques involving IVP. A crucial part of embryo transfer programs in bovine breeding is that improved mitochondrial activity via resveratrol supplementation may significantly increase embryo survival rates following cryopreservation. According to Alarcón de la Lastra & Villegas (2007), resveratrol has strong antioxidant qualities that can potentially lessen cryo-induced mitochondrial damage by scavenging ROS and lowering oxidative stress. Since oxidative stress and mitochondrial dysfunction are the main factors limiting embryo quality after thawing, the judicious use of antioxidants like resveratrol could significantly enhance research and commercial results in IVP systems. However, there are certain restrictions on this study. First, actual protein activities or mitochondrial functional outcomes cannot be accurately represented by gene expression data alone. Consequently, assays for mitochondrial functional parameters such as ATP content, membrane potential, and mitochondrial turnover, as well as protein expression analysis, should be a part of future studies. According to Ren et al. (2019), additional research on the molecular signaling pathways that resveratrol activates, specifically SIRT1-mediated mechanisms involving PGC-1α and downstream transcription factors like NRFs and ERRs, may also shed light on mitochondrial biogenesis and function under stressful circumstances. Our findings highlight the potential benefits of resveratrol in enhancing mitochondrial activity and mitigating the negative effects of vitrification on bovine embryos. To fully utilize the positive effects of resveratrol on embryo quality and mitochondrial integrity, it is highly advised that future research refine resveratrol supplementation strategies, especially optimizing treatment timing about vitrification protocols, and conduct thorough functional studies.

The current study found that resveratrol supplementation had a significant impact on the expression of genes linked to apoptosis in bovine embryos, including *BAX* and *BCL2*, which have conflicting functions in controlling cell survival and death. The fresh control group, from -R group had the highest expression of the pro-apoptotic gene *BAX*, indicating that increased apoptotic signaling during *in vitro* embryo development may result from a lack of antioxidant support. On the other hand, resveratrol supplementation during *in vitro* culture markedly decreased *BAX* expression, which is in line with the preventive function of resveratrol against oxidative stress-induced apoptosis (Huang et al., 2007). The -R/+R group had the lowest *BAX* expression among the vitrified groups, suggesting that resveratrol supplementation in post-warming culture may be a more effective way to reduce cryoinjury-induced apoptosis than pre-warming treatment. This is consistent with research by Gaviria et al. (2019), who found that vitrification dramatically raises the expression of apoptotic genes and that antioxidant supplements, especially resveratrol, can reduce this effect, though the degree of protection may vary depending on the time of treatment and other factors. Conversely, the +R group had the highest and considerably higher expression of the anti-apoptotic gene *BCL2* compared to the other treatment groups. This finding supports the anti-apoptotic effects of resveratrol during culture, possibly through modulating mitochondrial integrity or activating *SIRT1* (Hayashi et al., 2018). Regardless of the degree of resveratrol treatment, *BCL2* expression was considerably lower in all vitrified groups. This implies that cellular stress caused by vitrification weakens anti-apoptotic defenses, which resveratrol by itself might not be able to fully repair. These trends suggest that whereas resveratrol effectively lowers the expression of apoptotic genes in non-cryopreserved circumstances, its protective effects are somewhat lessened in vitrified embryos. According to earlier research, vitrification-related injuries can overwhelm cellular defense systems, resulting in decreased anti-apoptotic responses, and the positive effects of antioxidants are more noticeable in fresh embryos (Lee et al., 2010; Gaviria et al., 2019). These findings significantly impact the improvement of embryo cryotolerance. To improve post-thaw embryo survival, *BAX* expression should be decreased and *BCL2* expression should be increased. The potential of resveratrol as a culture supplement is demonstrated by its capacity to change the *BAX/BCL2* ratio in fresh embryos toward a more survival-favorable condition. Its limited post-vitrification impact, however, highlights the necessity of combining strategies, perhaps incorporating synergistic antioxidant combinations or enhanced vitrification protocols to protect embryo quality better.

The expression of *DNMT1* and *DNMT3A* in +R group suggests better epigenetic programming, most likely due to the regulatory action of resveratrol on chromatin remodeling enzymes. According to Giroto et al. (2019), DNA methyltransferases like *DNMT1* preserve methylation patterns, while *DNMT3A* participates in de novo methylation throughout early embryogenesis. Both processes are critical for lineage commitment and genome stability. The decreased expression of these genes in every vitrified group supports research showing cryopreservation might affect epigenetic markers, resulting in problems with development (Yodruk et al., 2021). Importantly, it has been demonstrated that resveratrol activates *SIRT1* and promotes histone deacetylation and methylation, which indirectly supports DNMT activity and preserves the epigenetic integrity of the embryo (Adamkova et al., 2017). Similarly, the function of resveratrol in maintaining pluripotency is supported by the markedly increased *OCT4* expression in the +R group. One important indicator of inner cell mass and a major self-renewal regulator is *OCT4*. Cryopreservation lowers *OCT4* expression in bovine blastocysts, perhaps because of stress-induced cellular damage, according to Mori et al. (2015). Our findings build on existing findings by showing that resveratrol-pretreated embryos retain higher *OCT4* levels, suggesting improved resistance to stress brought on by vitrification. The findings of Sabre et al. (2024), who also documented an increase in *OCT4* expression after low-dose resveratrol intake, corroborate this observation. In bovine, a similar pattern was seen for *IFN-tau*, a cytokine generated from trophoblasts that is essential for the mother to recognize pregnancy. The expression of *IFN-tau* was lowest in the vitrified groups, particularly +R/+R, and highest in the +R group. This result illustrates the detrimental impact of vitrification on trophectoderm function as well as the restricted ability of resveratrol to reinstate *IFN-tau* expression when administered solely post-warming culture. Previous studies have also shown that vitrification impairs the expression of *IFN-tau*, which may lessen embryo-endometrial signaling (Mori et al., 2015).

5.2 Conclusions

The present study demonstrated that supplementing bovine embryo culture with 0.5 µM resveratrol during IVC or PWC modulates gene expression related to stress response, mitochondrial function, apoptosis regulation, epigenetic programming, and developmental competence in both fresh and vitrified embryos. In fresh embryos, resveratrol supplementation during IVC promoted favorable gene expression, including lower pro-apoptotic *BAX*, higher anti-apoptotic *BCL2*, enhanced antioxidant defense (*SOD1*, *CAT*), improved mitochondrial-related genes (*SIRT1*, *TFAM*), and increased expression of pluripotency (*OCT4*) and maternal recognition of pregnancy (*IFN-tau*) markers. These results indicate improved embryo quality and developmental potential under non-stressed conditions. For vitrified embryos, resveratrol treatment before vitrification (+R/-R) proved superior to supplementation in post-warming culture medium (-R/+R) in preserving embryo quality. The +R/-R group exhibited higher expression of mitochondrial function genes (*TFAM*), epigenetic regulator (*DNMT1*), and pluripotency marker (*OCT4*), along with improved antioxidant defense (*CAT*, *SOD1*, *GPX4*) and a reduced *BAX* level, contributing to better resistance to oxidative stress and higher hatching rates. In contrast, embryos supplemented only during post-warming culture (-R/+R) showed limited benefits, with persistent downregulation of *IFN-tau* expression and no significant improvement in other critical gene markers. Notably, embryos receiving continuous resveratrol treatment both before and after vitrification (+R/+R) displayed the lowest *IFN-tau* expression, suggesting that prolonged or excessive resveratrol exposure may impair embryo recovery or disrupt redox homeostasis following cryopreservation. Despite these positive effects, vitrification remained associated with downregulation of implantation (*IFN-tau*), epigenetic (*DNMT1*, *DNMT3A*), and mitochondrial (*TFAM*) genes across groups, highlighting the molecular stress induced by cryopreservation, which resveratrol alone could not fully overcome. These findings emphasize the importance of optimizing resveratrol treatment strategies, particularly in terms of timing and duration, to enhance cryotolerance and developmental potential in bovine embryos. Further research should explore protein-level changes, oxidative stress indicators (GSH, ROS), mitochondrial membrane potential, and apoptotic markers to refine supplementation protocols. Overall, this study supports the potential application of resveratrol as a culture supplement to improve embryo quality and developmental outcomes, particularly when administered prior to vitrification. (Figure 10)

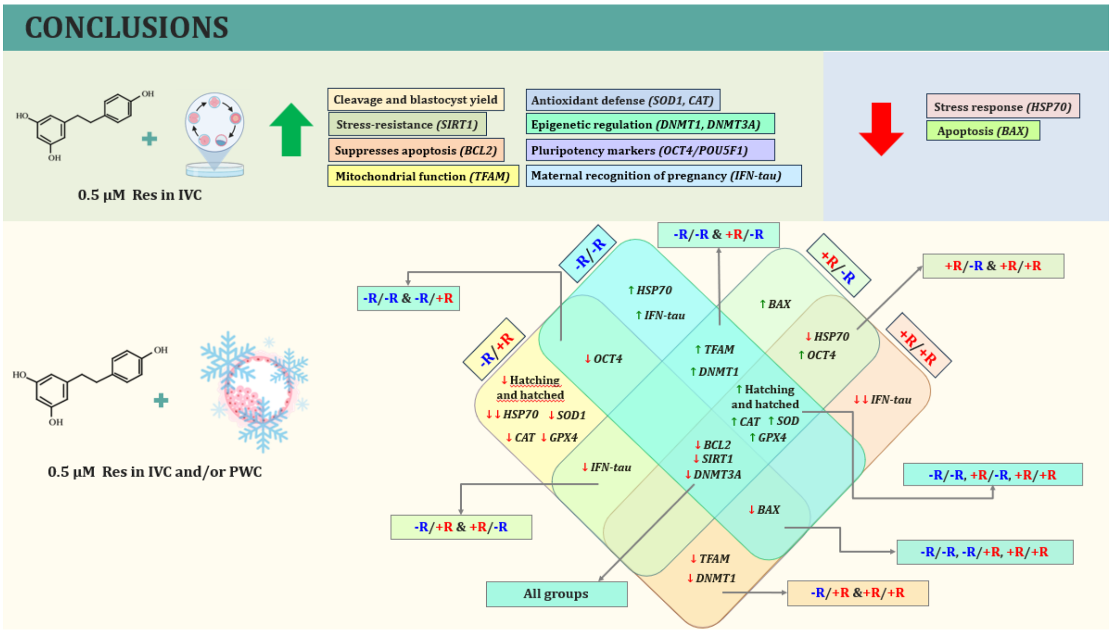

Figure 10 Graphical conclusions of resveratrol supplementation in IVC and post-warming culture media on bovine embryo development and gene expression.