CHAPTER V
DISCUSSION AND CONCLUSION

5.1 Discussion

In the present study, the blastocyst formation rate of IVC- fresh embryos was 32.40%, whereas it increased to 35.20% in IVC+ fresh embryos. However, there was no statistical difference between both groups. These results indicated no positive effects of resveratrol on bovine embryo development. Similarly, several reports focused on resveratrol supplementation have shown a neutral benefit to bovine embryo development. For example, Salzano et al. (2014) investigated the effect of supplementing the IVC medium with varied concentrations of resveratrol at 0, 0.25, 0.5, and 1 μM. They found that resveratrol did not significantly increase blastocyst yields, which were 57.1%, 57.7%, 59.2%, and 46.6%, respectively. Afterward, Gaviria et al. (2019) reported that supplementing the culture medium with 0.5 μM resveratrol resulted in no significant difference in blastocyst rates compared to the control group. It is generally accepted that resveratrol might contribute to fostering embryo development, potentially due to its antioxidant properties and its ability to influence cellular processes (Hayashi et al., 2018). Resveratrol can also reduce mitochondrial ROS production. Anti-superoxide radicals inhibit fat oxidation and modulate the expression of cofactors and antioxidant enzymes (Abbasi et al., 2021; Abe et al., 2017; Agarwal and Majzoub, 2017). Additionally, it has been reported that supplementing the culture medium with 0.5 μM resveratrol can enhance the hatching rate of blastocysts (Iwata, 2021). Therefore, a potential strategy to alleviate oxidative stress-induced damage involves enriching culture media with antioxidant molecules, such as resveratrol. However, in the present study, a slight improvement of embryo development was noticed in IVC+ fresh group. This phenomenon may be a consequence of different culture environments. Since supplementation of resveratrol in IVC medium was not necessary in the study, our IVC condition may be optimal for the embryos by producing less ROS.

Post-warmed vitrified blastocysts in IVC-/VT- showed the significantly lowest survival rate (87.04%), compared to other groups. Despite no significant difference observed, the

addition of resveratrol yielded higher survival rate of blastocysts in the ranges of 90.74 – 94.34%. In accordance with Gaviria et al. (2019), resveratrol added into the IVC medium caused an increased survival rates after 4 hours of post-warming culture. Moreover, in this study, vitrified blastocysts derived from IVC+/VT+ exhibited the highest developmental rates (81.25%), indicated by the highest expanded and hatching blastocyst stages after culturing in warming solution. However, no significant difference was found in the developmental rate after 24 hours of culture. These results were in contrast with the findings performed by Salzano et al. (2014), who reported an increase in developmental and hatching rates of post-warmed embryos with 0.5 μM resveratrol supplement in IVC medium. Other studies have also shown that resveratrol supplementation during embryo culture or as a pretreatment before cryopreservation could improve viability and hatching rates after thawing (Abe et al., 2017; Hayashi et al., 2019). Despite most of the research indicating that low concentrations of resveratrol positively affect embryo competence, the results of this study did not show a substantial improvement. This suggests that resveratrol effects may not be sufficiently robust to create statistically significant differences in this context. These results were consistent with the report by Gaviria et al. (2019), who found no difference in the hatching rate after 24 hours of culture with resveratrol added to both the IVC medium and the vitrification solution. It is possible that resveratrol alters some metabolic characteristics of the embryo, potentially generating detrimental effects following vitrification e.g. increase in the proportion of cell death (Gadacha et al., 2009; Gueguen et al., 2015). This may be related to induction of cell cycle arrest by resveratrol through the upregulation of p53 expression leading to efflux of cytochrome c into cytosol and the activation of the caspase apoptotic pathway (Brill et al., 1999; Yuan et al., 2015). Some features, such as concentration, effective cell uptake, species, cell type, cellular redox state, and stressor factor might interact and form a negative effect by the added supplement (Lastra and Villegas, 2007; Gadacha et al., 2009)). In addition, similar survival rate and developmental competency of post-warmed embryos between the control and resveratrol treated groups may be associated with gene expression as observed in the present study. Apoptotic inducing *BAX* gene was increased simultaneously with a decrease in anti-apoptotic *BCL2* gene in post-warmed embryos despite resveratrol addition. This may be explained why no significant enhancement of survival in post-warmed embryos in resveratrol treated solution. The present study indicated that resveratrol supplemented had no beneficial effects on stress resistance and

survival of bovine embryos following vitrification. Comparatively, IVC+ fresh and IVC- fresh embryos showed significantly higher developmental rates to expanded and hatching blastocyst stages than all vitrified groups. This outcome underscores the inherent advantage of fresh embryos over vitrified ones, likely due to the absence of stressors associated with the freezing and thawing processes. This finding suggested that while resveratrol supplementation in IVC media can improve certain developmental outcomes for bovine embryos, particularly in fresh conditions, its benefits for vitrified embryos are limited. These insights highlight the complexity of optimizing embryo culture and cryopreservation protocols and the need for further research to enhance the efficacy of these techniques.

The findings presented in the impact of resveratrol supplementation on the cell numbers of vitrified blastocysts following thawing and culturing in warming solution. Specifically, the inner cell mass (ICM), trophectoderm (TE), and total cell numbers did not show significant differences between vitrified blastocysts supplemented with resveratrol (VT+) and those without resveratrol (VT-). Moreover, IVC+ fresh blastocysts exhibited significantly higher ICM, TE, and total cell numbers compared to vitrified-warmed blastocysts incubated in warming solution without resveratrol (VT-). The results of Salzano et al. (2014) were consistent with the present finding. They reported that the adding 0.5 μM resveratrol to the culture media did not affect the cell allocation between the ICM and TE or the total cell number within the embryos. Similarly, Abe et al. (2017) found no difference in total cell number between the control group and the 0.5 μM resveratrol group (65.5 and 65.4, respectively). This observation underscores the potential vulnerability of blastocysts to cryopreservation-induced cellular damage in the absence of resveratrol (Nashtaei et al., 2018). Interestingly, no significant differences were noted between the control group and the group subjected to vitrification with resveratrol supplementation regarding the counts of ICM cells, TE cells, or total cell numbers. These findings suggested that the absence of resveratrol supplemented during both culture and post-warming stages resulted in a significant reduction in cell numbers in vitrified blastocysts. However, when comparing these blastocysts to those from groups with resveratrol supplementation, the difference in cell numbers is less pronounced. Further investigations focused on the mechanisms underlying the protective effect and the optimal supplementation protocols are warranted to improve the success rates of cryopreservation in assisted reproductive technologies.

Vitrification/warming technique not only ensures high cryo-survival rates but also maintains biological functions, leading to higher developmental competency after warming. Thus, we evaluated the potential impact of resveratrol supplementation in conjunction with the vitrification process on the expression of apoptosis-related and transcription factor genes. Apoptosis is a regulated program that initiates cell death, with members of the *BCL2* gene family playing a crucial role in its regulation. *BCL2* is anti-apoptotic and promotes cell survival, whereas *BAX* is pro-apoptotic and induces cell death (Yang and Rajamahendran, 2002). In this study, the aim was to evaluate whether resveratrol addition during IVC or post-warming caused change in the relative expression of genes related to mitochondrial function, antioxidant defense and embryo quality. The results of this study indicated a significant impact of vitrification on the relative expression of *BAX* and *BCL2* genes in bovine blastocysts. As expected, vitrified embryos exhibited significantly higher *BAX* and lower *BCL2* expression, compared to both IVC- and IVC+ fresh embryos. Similar results have been reported by Torres et al., 2018 and Gaviria et al., 2018. This suggests that the vitrification process alters the expression of pro-apoptotic genes, potentially affecting the viability and quality of the embryos. Despite the known antioxidant properties of resveratrol, the supplementation in the IVC medium and/or warming solution did not confer any beneficial effect on *BAX* expression in vitrified embryos. Interestingly, the IVC+/VT+ embryos showed slightly lower *BAX* expression compared to other vitrified groups. This marginal reduction, while not indicative of a substantial protective effect, suggests a minor influence of resveratrol on *BAX* regulation under vitrification stress conditions. In contrast, vitrification had a pronounced detrimental effect on *BCL2* expression, a gene associated with anti-apoptotic functions. The relative *BCL2* expression in all vitrified embryos (IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+) were significantly lower than those observed in IVC- and IVC+ fresh embryos. The ratio of *BAX* and *BCL2* can be used to evaluate the vulnerability of cells to a mortality signal (Gross et al., 1999). Fresh embryos represented low *BAX/BCL2* ratio while IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ vitrified embryos presented a higher ratio, indicating a major tendency to apoptosis. Obviously, in the present study, the supplementation of the IVC medium with resveratrol (IVC+) significantly increased *BCL2* expression levels in fresh embryos, highlighting the potential of resveratrol to enhance embryo resilience under non-vitrified conditions (Gross et al., 1999). However, this beneficial effect was not observed in IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ vitrified embryos, indicating that

the protective mechanisms of resveratrol may be insufficient to counteract the severe stress induced by the vitrification process.

The findings of this study highlight the differential impact of resveratrol supplementation on gene expression in vitrified bovine blastocysts. *SIRT1* is known for its role in promoting cell survival and longevity by deacetylating key transcription factors and enzymes involved in stress resistance, DNA repair, and metabolism (Brooks and Gu, 2009). There was no significant difference of the expression of *SIRT1* gene between fresh and vitrified groups in this study. Similarly, Gaviria et al. (2019) reported that culturing bovine blastocysts in the IVC medium and/or the vitrification/warming process did not alter *SIRT1* mRNA expression. On the contrary, recent studies have reported that the addition of 0.5 or 1 μM resveratrol into the IVC medium increased the expression of *SIRT1* protein (Abe et al., 2017; Hayashi et al., 2018, 2019). However, Torres et al. (2018) found that bovine blastocysts cultured with 1 μM resveratrol in complex with cyclodextrin (RV-CD) showed a significant reduction in *SIRT1* mRNA expression compared to the control.

This study also found that the addition of resveratrol to IVC+ fresh embryos resulted in a slight increase in *FOXO3A* and *PNPLA2* gene expressions. *FOXO3A*, a transcription factor regulated by *SIRT1*, is critical for the expression of genes involved in apoptosis, oxidative stress resistance, and metabolism (Salminen et al., 2013). The upregulation of *FOXO3A* suggests enhanced cellular defense mechanisms and stress response pathways in resveratrol-treated groups (Qin et al., 2021; Yun et al., 2012). *PNPLA2*, an enzyme involved in lipid metabolism and energy homeostasis through triglyceride hydrolysis resulting to the formation of diglyceride and fatty acid (Taxiarchis et al., 2019; Zhang et al., 2019). The slight increase in *FOXO3A* and *PNPLA2* expression in the present study may imply partial maintaining cellular energy balance and membrane integrity of resveratrol during and after vitrification. Resveratrol supplemented in the IVC medium or warm solution may help restore the initial quieter, or lower metabolic state of bovine blastocysts (Baumann et al., 2007; Gaviria et al., 2019; Krisher and Prather, 2012). Furthermore, the supplementation of resveratrol in the IVC medium and/or warming solution (IVC-/VT+, IVC+/VT- and IVC+/VT+) in this study did not change the expression of *FOXO3A* and *PNPLA2* in post-warmed embryos compared to IVC- and IVC+ fresh embryos, but vitrification significantly increased both gene expression. Similar result was reported by Gaviria et al. (2019). They observed no alteration in *FOXO3A* and *PNPLA2* gene expression

following resveratrol administration, but vitrification-waring cycle was the main cause of the increased relative abundance of *FOXO3A* and *PNPLA2* genes. A previous study demonstrated that the vitrification-warming cycle increases active mitochondria *in vitro* produced bovine blastocysts, leading to an increase in mitochondrial O_2^- production. The significant increase in expression of *FOXO3A* and *PNPLA2* reflects the elevated embryo metabolism in response to cryopreservation (Gaviria et al., 2019). When embryos are exposed to stressors, they activate energy-consuming repair mechanisms, such as apoptosis, which increases their ATP consumption (Baumann et al., 2007; Marsico et al., 2019). In this context, the increased expression of *PNPLA2* may indicate the hydrolysis of triacyl glycerides stored in cytosolic lipid droplets and subsequent production of fatty acid to support energy demand of IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ embryos in the present study (Gruber et al., 2010; Schweiger et al., 2008).

5.2 Conclusion

Our study reveals the potential benefits of resveratrol supplementation in the development and cryopreservation of bovine embryos. Supplementing the culture medium with resveratrol slightly increased blastocyst formation rates, suggesting a positive impact on embryo development, although this increase was not statistically significant. Resveratrol had no desired effects on cryotolerance improvement of vitrified embryos evidenced by similar survival rate and development of blastocysts in post-warming solution. Fresh embryos cultured with resveratrol had the highest ICM, TE and total cell mass, while similar ICM, TE and total cell mass were produced in IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ embryos. However, these numbers were not statistically significant. In addition, resveratrol did not play a role in any changes of expression of genes related to apoptosis and stress response indicated by no significant difference of the gene expression in the IVC-/VT-, IVC-/VT+, IVC+/VT- and IVC+/VT+ embryos. However, vitrification played an important role in the alteration of targeted genes. Vitrification caused significant increases in *BAX*, *PNPLA2* and *FOXO3A* expression while significant decrease in *BCL2* expression. No changes were observed in the expression of *SIRT1* gene following vitrification and/or resveratrol supplementation.

Further research is necessary to optimize the concentration and timing of resveratrol supplementation to maximize its benefits. Understanding the precise molecular mechanisms through which resveratrol exerts its effects will be crucial in refining

cryopreservation techniques and improving embryo quality. By advancing our knowledge and application of resveratrol in embryo culture, we can enhance reproductive success rates and the overall efficiency of breeding programs.