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Archives of Veterinary Science
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META-ANALYSIS OF THE ACTION OF FORSKOLIN DURING *IN VITRO* CULTURE OF BOVINE EMBRYOS AND ITS IMPLICATION ON CRYOPRESERVATION

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Submitted: *28/03/2023*
Accepted: *03/09/2023*

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Abstract: Increased rates of embryonic re-expansion and implantation have been reported following the use of forskolin during embryo culture, primarily attributed to the reduction of intraplasmic lipids, which improves cryopreservation. The aim of this meta-analysis was to compare the occurrence of embryonic re-expansion among different studies that utilized forskolin in vitro for embryo production. Five articles, out of 159, assessing forskolin at concentrations of 2.5, 5.0, and/or 10µM in embryo culture were considered from 1980 to 2022, comparing them to the control group (in vitro culture with forskolin). The Restricted Maximum Likelihood Method (REML) was employed to compare the results of the articles. The Q test was used to identify heterogeneity among the studies, and the I^2 analysis was used to quantify the heterogeneity between the studies and to quantify the heterogeneity between the studies. Based on the statistical analysis, it is inferred that embryos cultivated with forskolin at a concentration of 10µM are 71% more likely to re-expand, compared to the control group, with a 95% confidence interval, ranging from 27 to 132%. There was no statistically significant difference in the likelihood of embryonic re-expansion when comparing embryos treated with a forskolin at 5µM, and the control group, suggesting that a concentration of 10µM would enhance the quality of cryopreserved bovine embryos. Further experiments are required to define the correct concentration of forskolin in vitro for bovine embryos.
Keywords: Delipidation; Cryotolerance; in vitro fertilization.

1. Introduction

In vitro embryo production is a widely used technology in bovine reproduction. According to the International Embryo Technology Society (VIANA et al., 2019), the majority of bovine embryos are produced through *in vitro* fertilization worldwide. To optimize these techniques, the use of delipidation chemical products during embryo culture has been the focus of studies, since it has been suggested that IVF embryos are not as resistant to cryopreservation methods as *in vivo-produced* embryos, due to the higher amount of lipids associated with the culture conditions (ABE et al., 2015).

Lipids are the main molecules of intracellular energy storage (CARRO, M. et al., 2013). During their metabolism, oxidative stress is generated, via oxidative phosphorylation, leading to lipid peroxidation, and the proliferation of oxygen reactive species (ROS), apoptosis, and mitochondrial dysfunction (YIN et al., 2015). Cryopreservation further exacerbates this damage, causing structural alterations, such as cytoskeleton fractures, and functional alterations, including changes in mitochondrial activity patterns and delayed protein synthesis (DALCIN et al., 2013; AKSU et al., 2012). Additionally, at physiological temperatures, lipid droplets are generally in a fluid state, providing flexibility to membranes and intracellular structures. However, after the cooling process, the diffusion rate of hydrophobic molecules and the permeability of membranes decrease, resulting in reduced flexibility (SUDANO et al., 2011).

Therefore, the use of compounds that reduce the excessive amount of lipids is an alternative approach. Forskolin (7-acetoxy-8,13-epoxy-1,6,9-trihydroxy-labd-14-en-11-one, $C_{22}H_{34}O_7$), a derivative product from the diterpenes family, can be added to induce an increase of cAMP levels. This, in turn, activates endogenous lipases through the cAMP/kinase protein pathway, leading to triacylglycerol lipolysis, and the release of fatty acids and glycerol release (CUELLO et al., 2013). The objective of this meta-analysis was to determine the most effective concentration of forskolin (2.5, 5.0 or 10.0 µM) for *in vitro* embryo culture, specifically focusing on the concentration that yields the best blastocyst re-expansion after cryopreservation of *in vitro*-produced embryos.

2. Method

The meta-analysis was conducted using peer-reviewed full articles that aimed to evaluate the effects of forskolin concentrations on bovine embryo culture and their correlation with re-expansion rates. Studies that focused on forskolin's effects on oocyte maturation and embryo fertilization were excluded from the analysis.

The research strategy involved online searches in databases such as Capes Periódicos, PubMed, BVS, and Wiley Online Base. There were no language restrictions, and the search covered articles published from 1980 (as there were no previous publications on this topic) until 2022. The search keywords used were "embryo", "bovine", and "forskolin" in both English and Portuguese. No Boolean operators or filters were applied to determine the material type.

The primary method of identification involved reading the titles and abstracts of the studies, with a focus on the use of forskolin *in vitro* for bovine embryo culture assessing cryopreservation. Only studies directly relevant to answering the research question were included, while those that did not fit within this scope were excluded during the screening process. Additionally, non-peer-reviewed articles were eliminated from the analysis. Duplicate records in the database were not automatically excluded; rather, human judgment was employed to remove duplicates. By following these rigorous inclusion and exclusion criteria, the meta-analysis aimed to provide a comprehensive and reliable assessment of the effects of forskolin concentrations on bovine embryo culture and their impact on cryopreservation outcomes.

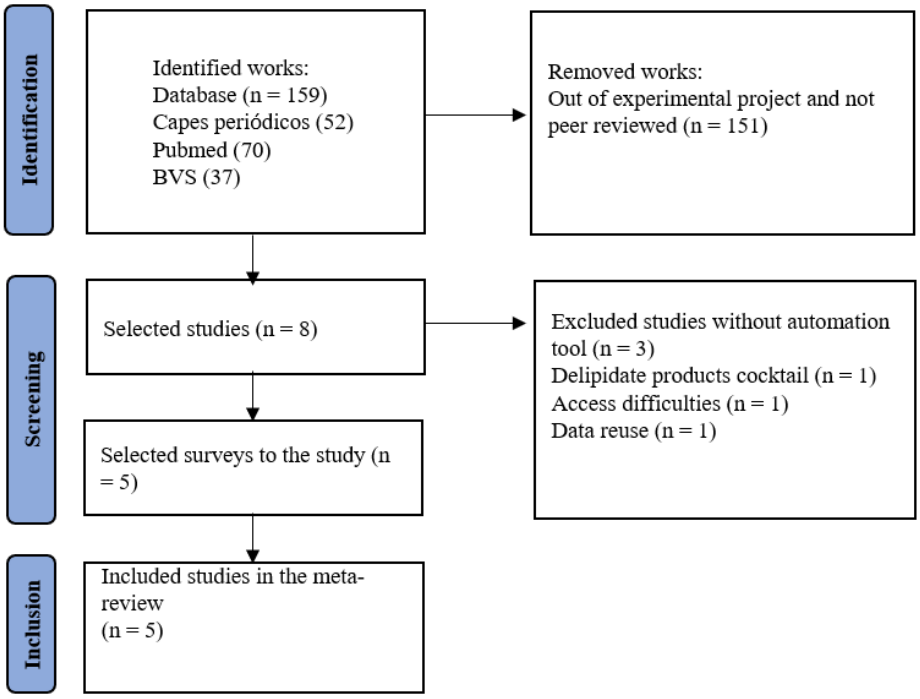

Figure 1 – Flowchart of identification of the studies via database and registers.

Despite the specific experimental criteria set for this study, three out of the eight selected articles could not be used. Oliveira et al. (2021) used a cocktail of delipidation products, making it impossible to isolate the effect of forskolin. Paschoal et al. (2014,2017) published two articles that were compatible with the study's criteria, but one had to be excluded because both studies were part of the same experiment. The second study analyzed the addition of a distinct treatment, which would result in duplicate data and potential errors in the evaluation. Therefore, in order to maintain data integrity, the decision was to exclude the study with additional treatments. Unfortunately, the full text of Barceló-Fimbres et al. (2010) study could not be accessed.

Although the focus of this study is on bovines, the meta-analysis included the study by Panyaboriban et al. (2018), which involved experiments in both buffalos and bovines. The presented results were separated by species, allowing the extraction of relevant data to bovine embryos. Five articles published between 2013 and 2018 were selected for the meta-analysis. A researcher extracted relevant data using a standardized sheet, which included information about the authors, year of publication, and study design.

To evaluate the significance of heterogeneity among the studies, the Q test was employed. This test examines the null hypothesis that all studies are assessing the same effect. Heterogeneity was quantified using I^2 value ranging from 0.0 to 100%, where values near 0% indicate no heterogeneity, values near 25% indicate low heterogeneity, values near 50% indicate moderate heterogeneity, and values near and above 75% indicate high heterogeneity among the studies (HIGGINS et al., 2003).

Fixed or random models were used to estimate the odds ratio of forskolin compared to the control group. The fixed model was employed when low heterogeneity was observed, assuming that the effect of interest is the same in every study and that differences were due to sampling errors. The random model was used when significant heterogeneity was detected among the studies, assuming a probability distribution, typically considered normal, connecting the studies (BORENSTEIN et al., 2009).

The evaluation of publication bias could not be performed due to the limited number of studies. Visual assessment of funnel plots and statistical tests for the hypothesis is not recommended when there are fewer than 10 studies, as they have low power to detect possible publication bias (HIGGINS et al., 2011). Publication bias refers to the tendency of published results to systematically differ from reality. This study attempted to minimize this bias by conducting extensive and sensitive literature searches without language restrictions.

3. Results

Figures 2 and 3 display the *forest plot* for forskolin concentrations of 10µM and 5µM, respectively. The *forest plot* is a visual representation of estimated measurements and their confidence intervals. Each study is represented by a box indicating the estimation, along with a horizontal line representing the confidence interval. The size of the box corresponds to the sample size of the study, while shorter horizontal lines indicate higher precision of the results. A study with a larger box and shorter line has a greater impact on the effect size estimated by the meta-analysis.

Figure 2 demonstrates that forskolin, at a concentration of 10µM, is associated with a 71% higher likelihood of re-expansion compared to the control group, with a 95% confidence interval ranging from 27 to 132%. The studies included in this analysis exhibited low heterogeneity (I^2= 20%), indicating similarities in their findings. Therefore, the fixed effects model was employed to calculate the *odds ratio* (OR), which represents the ratio between the likelihood of an effect occurring in the exposed group and the occurrence of the same event in the control group.

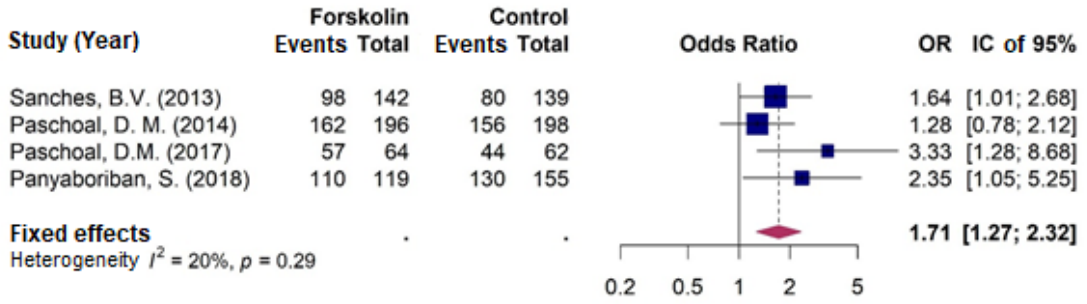

Figure 2 – Forest plot of the results obtained in the experiments that used 10 µM of Forskolin in the experimental groups.

Figure 3 illustrates that there is no statistically significant difference in the chance of re-expansion between the forskolin at 5µM concentration and the control group. However, it is important to note that high heterogeneity among the studies was observed (I^2 = 77%), therefore, a random-effect model was employed to calculate the *odds ratio* (OR).

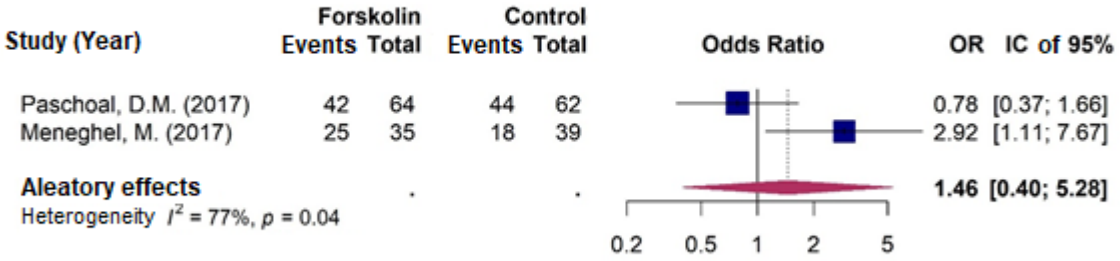

Figure 3 – Forest plot of the results obtained in the experiments that used 5 µM of Forskolin in the experimental groups.

4. Discussion

After conducting a throughout literature review, five articles were selected for inclusion in this meta-analysis. These studies investigated the effects of forskolin on the lipid parameters of bovine embryos and also examined its role in re-expansion after cryopreservation. However, the results obtained from these studies presented divergent conclusions. Among the five studies included, two did not directly attribute the efficacy of forskolin at 10µM concentration to re-expansion post-cryopreservation. Paschoal et al. (2017) observed no significant differences in blastocyst productivity following a 24-hour treatment with forskolin at 10µM. However, they did report an effective lipolytic action when treating bovine embryos with lower concentrations of forskolin,

leading to a greater number of cells per embryo. Similarly, Meneghel et al. (2017) found that embryo production was lower in the group treated with forskolin at 10µM compared to 2.5 and 5.0µM groups. Both of these studies diverged from the findings of this meta-analysis.

In addition to the previously mentioned studies, Sanches et al. (2013) conducted research in a laboratory setting and found no significant differences in blastocyst production rates following a 48-hour treatment with forskolin 10µM when compared to non-treated embryos. However, they obtained more favorable results in a livestock scenario. Cows that hosted treated embryos presented significantly higher pregnancy rates compared to those that received control embryos. Paschoal et al. (2014) and Panyaboriban et al. (2018) also reported improved outcomes with the use of forskolin at the 10µM concentration. All of these studies agree that forskolin enhances cryopreservation outcomes.

The statistical analysis revealed that the 10µM concentration offers a 71% higher chance of re-expansion compared to the control group, with a 95% confidence interval. However, Meneghel, et al. (2017) also obtained results indicating a reduction in cytoplasmic lipid content and improvement in re-expansion with the use of the 5µM dose. Given this scenario, it was decided to investigate whether there were significant differences between the use of forskolin at 5µM concentration and the control group, in terms of the chance of re-expansion. However, no significant difference was found. There was high heterogeneity observed among the studies conducted by Paschoal et al. (2017) and Meneghel et al. (2017) studies ($I^2 = 77\%$), indicating the need for further research to enhance the reliability of this analysis, especially regarding forskolin at the 2.5 e 5µM concentrations.

Furthermore, it is important to note that while cyclic AMP is regulating various physiological functions, its specific sites of action on embryonic cells are still poorly elucidated (SANCHES et al. 2013). Other aspects that warrant investigation include the duration of embryo exposure to forskolin and the developmental stage at which it is most beneficial to add the compound. Currently, there is no consensus and insufficient studies to formulate a comprehensive review of these aspects. Assessing pregnancy rates in recipient cows, as studied by Sanches et al. (2013), could provide valuable insights, as laboratory results may not fully reflect the embryo's actual potential.

5. Conclusion

The meta-analytical results obtained from this study confirm that the utilization of forskolin as a lipolytic agent at a concentration of 10µM during embryo culture is a valuable strategy for improving *in-vitro*-produced (IVP) bovine embryos. This approach allows for the development of blastocysts with higher survival rates after cryopreservation. However, it is important to note that there is a lack of research concerning the other concentrations investigated in this study. By conducting additional research, a more comprehensive understanding of the effects of forskolin at varying doses can be obtained, enabling the development of optimized protocols for bovine embryo culture and cryopreservation.

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