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EFFECT OF SULFORAPHANE ON *IN VITRO* PRODUCTION OF BOVINE EMBRYOS

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ABSTRACT

The aim of this study was to evaluate the antioxidant effects of sulforaphane (SFN) on bovine embryo production *in vitro*. Ovaries from slaughterhouses were collected to obtain viable oocytes, which were selected and matured for 24 h in an incubator under a controlled atmosphere. Mature oocytes were fertilized and incubated for 9 h. After this period, 5 µM SFN was added in the final third of the *in vitro* fertilization step (final 3 h). Then, the probable zygotes were cultured, and embryonic development was evaluated at the cleavage, morula, blastocyst, and hatching stages. The data obtained were analyzed using the Wilcoxon test at a 5% significance level. No significant difference was observed in the cleavage rates (79.07% vs. 78.06%) and morulae (61.36% vs. 54.66%) between the Control and SFN groups. In contrast, the blastocyst rate (34.53% vs. 27.24%) and hatching rate (22.0% vs. 15.36%) were significantly higher in the Control group than in the SFN group. The addition of SFN in the final stage of *in vitro* fertilization did not significantly improve embryo production rates for bovine species. Further studies are needed to elucidate the effects of this antioxidant in *in vitro* culture systems of embryonic cells.

Keywords: Antioxidant; Biotechnology; Oxidative stress.

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INTRODUCTION

Reproductive biotechnologies applied to cattle raising have significantly contributed to the advancement of the livestock sector via an increase in reproductive parameters and the genetic improvement of herds. Among reproductive biotechnologies, the *in vitro* production of bovine embryos plays a prominent role, as it allows for the best use of females with quality genetics, increases the number of embryos produced by selected individuals, enables the use of young animals, and reduces the interval between generations (GONÇALVES *et al*., 2008).

The production process involves three main steps: *in vitro* maturation, *in vitro* fertilization, and *in vitro* cultivation. During this process, oocytes can suffer from oxygen tension, exposure to light, and excessive handling, which contribute to the increased production of reactive oxygen species (ROS), especially superoxide (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl (OH), resulting from the cellular metabolic processes of cells (Silva et al., 2010). Excessive exposure to these substances causes oxidative stress, which has deleterious effects on embryonic development (ZHANG *et al*., 2015).

Antioxidant addition to *in vitro* embryo production is an interesting alternative to reduce the damage caused by oxidative stress (ZULLO *et al*., 2016). Generally, antioxidants are substances capable of minimizing the oxidation of molecules and protecting them from free radicals. The main enzymatic antioxidants produced by living organisms are superoxide dismutase, catalase, and glutathione, the latter of which is found in higher concentrations (BIANCHI; ANTUNES, 1999).

As the enzymatic system becomes ineffective at certain times at preventing oxidative stress, non-enzymatic antioxidants have been highlighted as potential components of *in vitro* media aiming to balance the concentration of ROS. However, this type of supplementation has unstable results, making it necessary to continue the search for substances capable of improving the efficiency of current culture systems for the *in vitro* production of bovine embryos.

Sulforaphane ($C_6H_{11}NOS_2$), an isothiocyanate with a molar mass of 177.29 g/mol, synthesized in cruciferous vegetables of the genus *Brassica*, such as cauliflower, broccoli, kale, mustard, and watercress (FAHEY *et al*., 2001), has been the subject of many studies because of its excellent antioxidant properties, which increase the activity of enzymes acting against oxidative stress (LANGSTON-COX *et al*. 2020; SOHEL *et al*., 2018; QIN *et al.,* 2021). An example of this is the increased synthesis of glutathione in mouse cells, which is an endogenous antioxidant enzyme, promoting cellular protection against cytotoxic effects (PRIYA *et al*., 2011). However, there are no reports of this component in embryo cultures. Thus, the aim of this study was to evaluate the effect of adding sulforaphane as an antioxidant in the *in vitro* production of bovine embryos.

MATERIAL AND METHODS

Ethical considerations

The study was approved by the Council on Ethics in the Use of Animals of the Federal University of Acre (Protocol 31/2019 - CEUA-UFAC), following the guidelines of the National Council for Animal Control and Experimentation (CONCEA, Law No. 11794, October 8th, 2008) and other subsequent normative resolutions.

Place of execution

The experiments were conducted at the Animal Reproduction Laboratory of the Center for Biological and Nature Sciences at the Federal University of Acre (UFAC), Rio Branco Campus.

Oocyte recovery and selection

For oocyte recovery and selection, ovaries were collected from abattoirs in Rio Branco, Brazil. Ovaries were collected immediately after slaughter, placed in a thermostat, and transported to the laboratory for approximately 30 min. In the laboratory, the ovaries were washed with a NaCl solution (0.9%) and acclimated in a water bath at 36 °C.

To collect oocytes, antral follicles (3–8 mm in diameter) present in the ovaries were aspirated using a needle (18 G) and syringe (10 mL). The follicular fluid containing the oocytes was transferred to a 15-mL sterile tube and kept in a water bath at 36 °C for oocyte sedimentation. Subsequently, the contents of the tubes were filtered and transferred to a 100-mm petri dish

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containing Dulbecco's modified phosphate-buffered saline and 1% fetal bovine serum. Then, the structures were evaluated under a stereoscopic microscope, and oocytes (n = 1658) with homogeneous cytoplasm, without degeneration, hyperpigmentation, or extremely dark. Other selection criteria were oocytes with brown coloration, with two or more complete layers of cumulus cells.

In vitro maturation

At this stage, the selected oocytes were bathed in drops (50 µL) of washing medium and then in maturation medium. After bathing, the oocytes were transferred to a Petri dish containing microdrops (150 µL) of maturation medium and covered with mineral oil. There were approximately 25 oocytes per drop, which were kept remained for 24 hours in the incubator with a controlled atmosphere (38.5°C, maximum humidity and an atmosphere with 5% CO2).

In vitro fertilization

Semen from bulls with proven fertility and similar production characteristics was used for *in vitro* fertilization. Semen straws were thawed using an automatic defroster at 36 °C for 30 s. Before processing, a 30-µL sample of semen was evaluated under a microscope to determine post-thaw motility.

Semen was deposited in a microtube containing a Percoll gradient (45% and 90%) and centrifuged at 9.5 ×*g* for 5 min to obtain mobile sperm and, consequently, remove the extender and seminal plasma. After centrifugation, 100 µL of the formed pellet was removed and transferred to a microtube containing 1,000 µL of fertilization medium, which was centrifuged at 9.5 ×*g* for 3 min. Then, 50 µL of the newly formed pellet was removed and diluted in 50 µL of fertilization medium. After processing, the insemination dose was determined based on sperm concentration using a Neubauer camera, following the methodology described by Gonçalves *et al*. (2008).

Mature oocytes were bathed in TL semen drops and then in fertilization medium. Immediately after bathing, the cells were transferred to a new Petri dish containing microdrops (100 µL) of fertilization medium, covered with mineral oil, and stabilized in the incubator for 2 h. The inseminating dose (5 µL) was deposited in drops containing mature oocytes. The plate was incubated in a 5% CO_2 atmosphere at 38.5 °C for 12 h.

Addition of sulforaphane

The supplementation methodology was designed based on previous studies aimed at determining a dose-dependent response. In this previous study, structures were supplemented with 0 µM, 5 µM, 10 µM, and 20 µM, with the concentration that yielded the most satisfactory results being 5 µM, with behavior similar to the control group. The higher concentrations promoted cytotoxicity, with hyperpigmentation and vacuolization. Therefore, 5 µM was selected to perform larger replicates and evaluate the behavior for greater data accuracy (SILVA, 2021).

Regarding the timing of supplementation, fertilization was chosen based on the results of Yuan et al. (2020), as they supplemented the fertilization media with 10 mM Nicotinamide for different periods. They found that when supplementation continued throughout fertilization or during the last five hours (7 to 12 hours), lower blastocyst rates were found. There was a better response to supplementation when it continued only in the final third (9 to 12 hours).

Thus, two experimental groups were created considering the addition (SFN group) or not (Control group) of 5 µM sulforaphane in the final period of *in vitro* fertilization. For the SFN group, the plate was removed from the incubator and, with the aid of a micropipette, 5 µM sulforaphane was added to the drops; then, the plate was returned to the incubator. Sulforaphane was added 9 h after *in vitro* fertilization and remained for 3 h (totaling 12 h of the fertilization process).

In vitro culture

After the fertilization period, the likely zygotes were removed from the fertilization medium drops and underwent a process of denudation (removal of cumulus cells) by swirling. Then, they were bathed in drops of TL semen and culture medium and then transferred to a plate containing microdrops (150 µL) of culture medium. During the entire culture period, embryonic development

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was evaluated to determine the cleavage, morula, blastocyst, and hatching rates.

Statistical analysis

The data obtained in the experiment were previously analyzed for normality using the Shapiro-Wilk test and for homogeneity using the Bartlett test. Subsequently, each variable (cleavage rate, morula rate, blastocyst rate, and hatching rate) was analyzed using the Chi-square test (X2) at a 95% confidence level, using the R Core Team statistical program (2023).

RESULTS

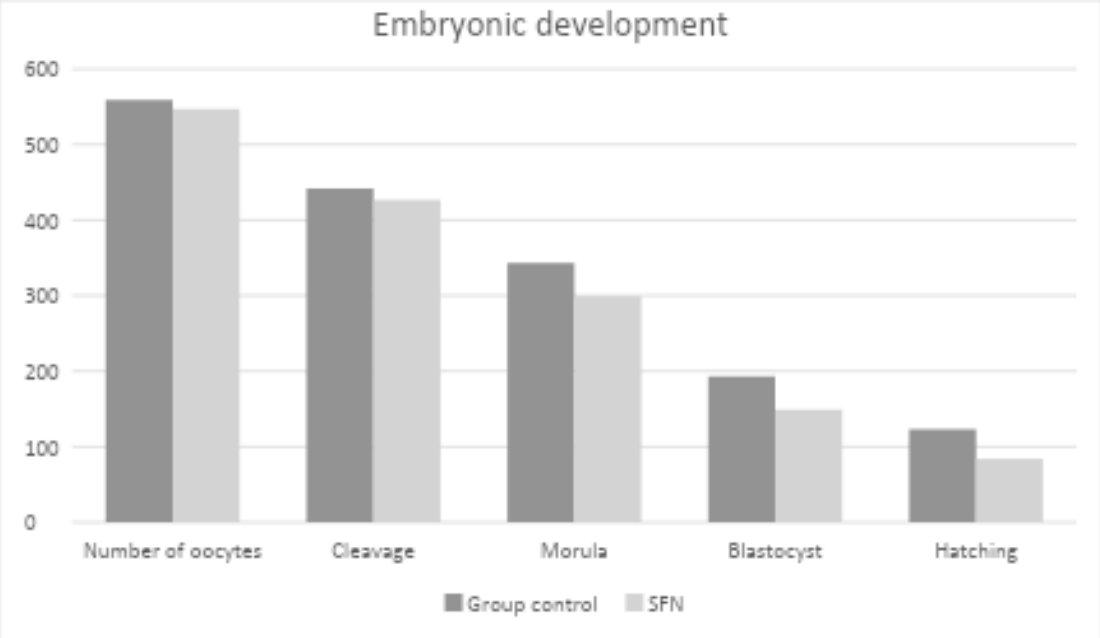
The embryo production rate was within the expected range for *in vitro* production of bovine embryos. No significant differences were observed in the cleavage and morula rates between the Control and SFN groups. Significant differences were found in the blastocyst and hatching rates (Table 1).

Table 1. Cleavage, morula, blastocyst and hatching rates of groups treated (SFN) or not (Control) with 5 µM of sulforaphane.

	Number of oocytes	Embryonic Production			
		Cleavage (%)	Morula (%)	Blastocyst (%)	Hatching (%)
Group					
Control	559	442 (79,07) [a]	343 (61,36) [a]	193 (34,53) [a]	123 (22,0) [a]
SFN	547	427 (78,06) [a]	299 (54,66) [a]	149 (27,24) [b]	84 (15,36) [b]

Values with different superscript letters in the same column are significantly different by the Wilcoxon test ($p<0.05$).

Graphic 1. Embryonic development of groups treated (SFN) or not (control) with the use of 5 µM Sulforaphane.



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DISCUSSION

Studies on antioxidant supplementation of *in vitro* culture media are increasing. For example, Sá *et al*. (2019) demonstrated that supplementation of the *in vitro* maturation medium with the compound anetol promotes higher rates of cleavage and embryonic development, in addition to increasing the average number of total cells in blastocysts and improving the quantity and quality of *in vitro*-produced bovine embryos. These data differ from the findings in the present study, in which the addition of 5 µM sulforaphane in the IVF medium promoted a decrease in embryo production rates.

In the search for improvements in the *in vitro* production of bovine embryos, researchers have evaluated the use of different antioxidants at all stages of the technique. For instance, Moraes (2018) obtained a higher percentage of blastocysts (64.6%) in groups treated with cysteamine during the maturation phase and quercetin during embryo culture. These data differed from those obtained with the use of sulforaphane, with which the rate of blastocysts obtained (27.24%) was lower than that observed in other studies using other antioxidants.

Antioxidants play crucial roles in the survival of cells and maintenance of cell function and integrity under oxidative stress. Hidaka *et al*. (2018) observed a positive correlation between oocyte glutathione concentration during maturation and embryonic development. Additionally, the authors used cysteine to stimulate an increase in the concentration of intracellular glutathione in bovine oocytes and obtained a blastocyst rate of 38.7%, which was higher than that observed in the present study.

In the study by Hidaka *et al*. (2018), the hatch rate with cysteine (13.8%) was similar to that obtained in the present study using sulforaphane (15.36%). Thus, the antioxidant did not compromise total embryonic development during cultivation.

To date, no studies have reported the use of sulforaphane in the *in vitro* production of embryos; thus, the results obtained in the current research can only be compared with the cultivation of other types of cells from different animal species. Therefore, the effect of sulforaphane on embryo culture should be investigated at other stages of the process, such as at the expression of antioxidant factors and embryo inoculation.

Sulforaphane has exceptional antioxidant characteristics and has already been tested in different forms, at different concentrations, and in different cell groups. For example, data obtained by Priya *et al.* (2011) demonstrate that sulforaphane stimulates glutathione synthesis in cells and regulates the occurrence of oxidative stress in culture systems.

The endoplasmic reticulum (ER) plays an essential role in protein folding and calcium homeostasis, and its dysfunction is responsible for inducing cellular stress, the accumulation of misfolded proteins, and the activation of apoptotic and inflammatory pathways. In this context, sulforaphane (SFN), derived from the hydrolysis of glucoraphanin, has been widely described as a bioactive compound with antioxidant, anti-inflammatory, antitumor, and antiangiogenic properties. It also exhibits low toxicity and good tolerability in experimental models and humans. Evidence suggests that part of its protective effects is related to the modulation of ER stress pathways, making it a potential candidate for the management of several diseases, including cardiovascular, neurological, metabolic, hepatic, and reproductive disorders (HAJIMOHAMMADI *et al*., 2024).

In addition to its recognized antioxidant and anti-inflammatory potential, sulforaphane (SFN) has recently been explored as a promising nutraceutical in sports nutrition. Studies in animal models and humans indicate that SFN supplementation can attenuate exercise-induced inflammation and oxidative stress by modulating cellular signaling pathways involved in the response to muscle damage and tissue recovery. Although human research is still limited, preliminary results suggest beneficial effects that may be enhanced when SFN is combined with other bioactive compounds with similar properties (RUHEE; SUZUKI, 2024).

It was hypothesized that the addition of sulforaphane to the fertilization medium could contribute to an increase in antioxidant enzyme expression in cells and, consequently, increase the production of blastocysts, reducing the losses inherent to the *in vitro* production process. However, the practical application of this antioxidant in the *in vitro* production of bovine embryos under the conditions of the current research was limited by a decrease in embryo production rates, and its mechanism of action in this type of culture system has not been elucidated.

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A large number of studies using sulforaphane have shown positive results regarding the antioxidant action of this compound. For example, Qin et al. (2021) suggested that pretreatment with sulforaphane significantly alleviates the negative effects of oxidative stress in human epithelial cells via the activation of the nuclear factor erythroid type 2 (Nrf2) pathway. Moreover, Yang et al. (2019) demonstrated that sulforaphane alleviates the deleterious effects of oxidative stress in mouse Leydig cells by modulating the expression of genes linked to Nrf2. Corroborating these studies, Langston-Cox et al. (2020) showed a protective effect of only 1 µM sulforaphane on the mitochondrial activity of placental cells affected by oxidative stress. In the present study, 5 µM sulforaphane caused a decrease in embryo production under an *in vitro* culture system.

The antioxidant concentration largely depends on the experimental setup and study objectives. For Visalli *et al*. (2017), the dosage of 5 µM sulforaphane allowed the maximum benefit of its adaptive response to stressors and, at higher doses, sulforaphane can be toxic for certain groups of cells. However, Sohel *et al.* (2018) demonstrated that the concentration of 10 µM sulforaphane in the culture of bovine granulosa cells effectively protects these cells against the effects of oxidative stress induced by H_2O_2.

In the present research, 5 µM sulforaphane was used in an *in vitro* culture system for bovine embryonic cells; however, there was no evidence of beneficial effects of the antioxidant at this dosage for this type of cell culture. Thus, the concentration used in the present work might not have been sufficient to induce an effective response or even too high for this type of cultivation system. Thus, new approaches are needed to adjust the concentration of sulforaphane added to *in vitro* bovine embryo production media.

Although SFN was used at a concentration of 5 µM, it is plausible that the observed effects may be related to a dose-response pattern not yet established for this system. Lower concentrations could exert a cytoprotective effect without compromising embryonic development, while higher levels could accentuate redox imbalance, favoring the occurrence of cellular damage. Therefore, future studies with different doses of SFN are needed to more precisely define its safety and efficacy range.

Furthermore, the reduction in blastocyst and hatching rates observed in the present study may have relevant biological implications, since these stages are directly associated with embryonic competence, interaction with the uterine environment, and implantation success. Alterations in redox homeostasis and ROS-dependent signaling pathways can compromise fundamental processes of cell expansion and differentiation, impacting embryonic viability and potentially subsequent gestational development.

During fertilization, an appropriate concentration of ROS is essential for sperm hyperactivation, capacitation, and acrosome reactions. Thus, antioxidants must be used with caution, and adequate adjustment of their concentrations is necessary.

In the present experiment, sulforaphane was not added at the beginning of the fertilization process to avoid compromising the metabolic functions of oocytes, as well as spermatozoa, via the action of ROS. The antioxidant was added only in the final third (3 h) of the fertilization stage, assuming that from that point on, the medium was overloaded owing to the death of sperm that did not fertilize the oocytes, starting the framework of oxidative stress.

The use of antioxidants in fertilization media is complex. Ali *et al*. (2003) supplemented a fertilization medium with cysteine and observed a significant decrease in the cleavage rate (below 60%); however, antioxidant supplementation did not improve the proportion of morulae and blastocysts. These data are similar to those obtained in the current study, in which sulforaphane interfered with the developmental competence of oocytes, both in the initial stages of cleavage and morula formation and in the formation of blastocysts.

A possible explanation for the decrease in production rates with the inclusion of sulforaphane could be that low levels of ROS are required for IVF. Thus, our results suggest that a low concentration of ROS is favorable for optimal fusion between oocytes and sperm and that sperm capacitation is reduced by the addition of sulforaphane.

Yuan *et al*. (2020) added 10 mM nicotinamide to a fertilization medium for different treatment durations to assess the effect of time on the response to this antioxidant during IVF. In this study, we observed that when the antioxidant remained in the medium during the entire phase (12 h) or the final 5 h (from 7 to 12 h), the rate of blastocyst formation decreased. However, when the same

concentration was used during the final 3 h of fertilization (9–12 h), nicotinamide promoted antioxidant and anti-apoptotic actions, thereby improving *in vitro* development.

Sulforaphane did not significantly affect the hatching rate of embryos. The hatching of embryos in an *in vitro* culture system assumes that the medium provides favorable conditions for their development during the incubation period.

Studies using sulforaphane for the *in vitro* production of bovine embryos are scarce; therefore, the action of this compound in this type of culture system is still unknown. Thus, future research is needed to obtain data regarding the measurement of antioxidant and oxidative stress markers in embryo cultures with sulforaphane to unravel the mechanism of action of this compound in this culture system.

CONCLUSION

The addition of sulforaphane during the final stage of *in vitro* fertilization significantly decreases the blastocyst and hatching rates of bovine embryos.

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