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Original Paper

rbST Effects on *in Vitro* Embryo Production in Andean Cattle

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The efficiency of *in vitro* fertilization (IVF) in cattle depends on various factors, including the quality and quantity of oocytes collected. Recombinant bovine somatotropin (rbST) has been suggested to improve ovarian follicular development and oocyte recovery by enhancing insulin-like growth factor-1 (IGF-1) receptor activity. However, its effects on metabolic parameters and *in vitro* embryo production remain unclear, especially in high-altitude systems like the Andean region of Peru. This study aimed to determine the effects of rbST administration, in two different doses, within a hormonal protocol for follicular wave synchronization and *in vitro* embryo production in Brown Swiss cows in Andean region. Twenty-four cows were randomly assigned to three groups: T1 (500 mg rbST), T2 (250 mg rbST), and T3 (control, no rbST). Oocyte collection sessions were performed every 14 days, and variables such as follicle size, oocyte recovery, oocyte viability, β-hydroxybutyrate (BHB), glucose, total proteins, urea, oocyte maturation, and blastocyst rates were evaluated. Results showed that 500 mg rbST significantly increased ($P \leq 0.05$) the number of 2–4 mm follicles, as well as total and viable oocyte recovery. rbST also reduced BHB levels and increased circulating glucose ($P \leq 0.05$). However, no significant differences were observed in oocyte maturation or blastocyst rates across the treatments. In conclusion, 500 mg of rbST enhances the recovery of viable oocytes but does not significantly affect final embryo production *in vitro*. Further studies are needed to explore the long-term effects of rbST on reproductive performance in high-altitude cattle systems.

Keywords: rbST, β-hydroxybutyrate, ovarian follicles, *in vitro*, embryo

1 Introduction

The global application of *in vitro* fertilization (IVF) in lactating and non-lactating cows largely depends on oocyte sources, typically collected either from slaughterhouse ovaries or through ovum pick-up (OPU) techniques. Both methods provide viable and abundant sources of oocytes (Ferré et al., 2020). However, the process is highly complex, and its efficiency is influenced by various factors (Pérez Durand et al., 2022). Key factors that affect the quality and quantity of oocytes are critical for achieving high *in vitro* embryo production rates, which are closely linked to the number of antral ovarian follicles available (Ribeiro et al., 2020).

Several ovarian stimulation protocols have been evaluated to efficiently gather quality oocytes in adequate quantities. Hormone-based stimulation protocols have shown variable results, with some allowing the use of recombinant bovine somatotropin (rbST) due to its effects in increasing the number of ovarian follicles by upregulating insulin-like growth factor-1 (IGF-1) receptors, leading to enhanced follicular recruitment (Kozicki et al., 2005). Somatotropin is a hormone known to influence growth and lactation in animals, and it also plays a significant role in modulating energy and reproductive function (Schemm et al., 1990). mRNA for the somatotropin receptor has been identified in various reproductive tissues, including the hypothalamus, pituitary gland, corpus luteum, ovarian follicles, oviduct, endometrium, and placenta (Heap et al., 1996). Studies suggest that administering rbST leads to greater ovarian follicular development, with direct actions on somatotropin receptors located in granulosa cells and oocytes (Lucy, 2000).

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The *in vitro* embryo production system relies on selecting high-milk-producing donor cows, often shortly after parturition. However, this physiological-metabolic scenario is frequently associated with negative energy balance (NEB) (Rincón et al., 2019), which is linked to biochemical changes, such as increased plasma non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), insulin, and low glucose concentrations (Beam & Butler, 1999). *In vitro* studies have shown that elevated levels of these metabolic parameters and low glucose concentrations in follicular fluid are toxic to oocytes, resulting in poor maturation and compromised developmental competence (Leroy et al., 2006). rbST hormonal stimulation has been used in various cattle breeds, with mixed results (Gohary et al., 2015; Ramos et al., 2007). Given that *in vitro* embryo production efficiency depends on multiple factors, this study aims to contribute to the analysis of additional variables, such as the high-altitude environment and the adaptability of Brown Swiss cattle to semi-intensive systems in the Andean region. Environmental and management conditions in these herds may influence the measured variables, and there is limited information on the effects of rbST in oocyte donors of this breed. In this context, the objective of the study was to determine the effect of administering recombinant bovine somatotropin at two doses on ovarian follicular dynamics, metabolic parameters, and *in vitro* embryo production in Brown Swiss cattle raised in the Andean region of Peru.

2 Material and Methods

2.1 Study Location

The study was conducted at the "El Mantaro" experimental station, part of the Faculty of Animal Science at the Universidad Nacional del Centro del Perú. The *in vitro* fertilization protocols were carried out at the *Animal Reproduction* Research Laboratory, located in the Mantaro district, Jauja province, Junín department, Peru, at an altitude of 3,587 meters above sea level in the central Andes of Peru. The average ambient temperature was 13 °C, with a relative humidity of 65%.

2.2 Experimental Design

Twenty-four non-pregnant Brown Swiss cows, with 90 to 110 days of lactation, were used in this study. The cows were randomly assigned to three treatment groups, each consisting of 8 animals. The treatments were as follows: T1: Cows subjected to follicular wave synchronization with a base protocol + 500 mg of recombinant bovine somatotropin (rbST), T2: Cows subjected to follicular wave synchronization with a base protocol + 250 mg of rbST, T3: Cows subjected to follicular wave synchronization with a base protocol only (control).

The selection of these dosages was based on both conventional use and the specific hypothesis of this study. Historically, a 500 mg dose of rbST has been widely used to increase milk production and subsequently to improve reproductive performance in hormonal insemination protocols (Rivera, 2010). More recently, for *in vivo* and *in vitro* embryo production programs, various rbST dosages have been explored, with ranges reported between 143 mg and 500 mg (Putnam et al., 1999; Gulay et al., 2004). Since the stimulation protocols for follicular wave emergence used in *in vitro* embryo production are of short duration, our hypothesis was that a lower dose (250 mg), half of the conventional amount, could be appropriate for a 4-day stimulation protocol such as the one applied in this study. Therefore, this study aimed to compare the performance of this lower dose against the conventional 500 mg dose, given the limited information available, especially for cattle in high-altitude production systems.

Each cow underwent four oocyte collection sessions, with a 14-day interval between each session, resulting in 32 replicates per treatment. The follicular wave synchronization protocol lasted 4 days. It involved the insertion of a progesterone-releasing intravaginal device (0.6 g DISPOCEL® Von Franken), 2 mg of estradiol benzoate, and 0.526 mg of cloprostenol intramuscularly on day 0. The DISPOCEL® device was removed, and oocytes were collected using the ovum pick-up (OPU) technique on day 4 (Figure 1). Cows in the rbST groups received a subcutaneous injection of 500 mg or 250 mg of rbST (BOOSTIN-S® Battilana) on day -14 of the ovarian stimulation protocol and a second dose on day 0.

2.3 Ultrasound-Guided Oocyte Aspiration (OPU)

Oocyte aspiration was performed using an ultrasound device with a 7.5 MHz microconvex transducer. Oocytes were aspirated transvaginal with the aid of a follicular aspiration guide and an 18G needle. The needle was connected to a vacuum pump set at a pressure of 70 mmHg. The aspiration medium consisted of phosphate-buffered saline (PBS) supplemented with 1% fetal bovine serum (FBS) and 5 mg of heparin. Oocytes were visualized under a stereomicroscope at 20–40× magnification and immediately transferred to a 35 × 10 mm Petri dish (Falcon®), containing H-199® medium (Vitrogen Brasil), for classification under 40× magnification. Oocytes were evaluated based on morphology and classified into four categories (Sato et al., 2020; De Loos et al., 1989): Oocytes surrounded by ≥3 layers of cumulus cells with homogeneous cytoplasm, Oocytes partially surrounded by cumulus cells with irregular cytoplasm, Denuded

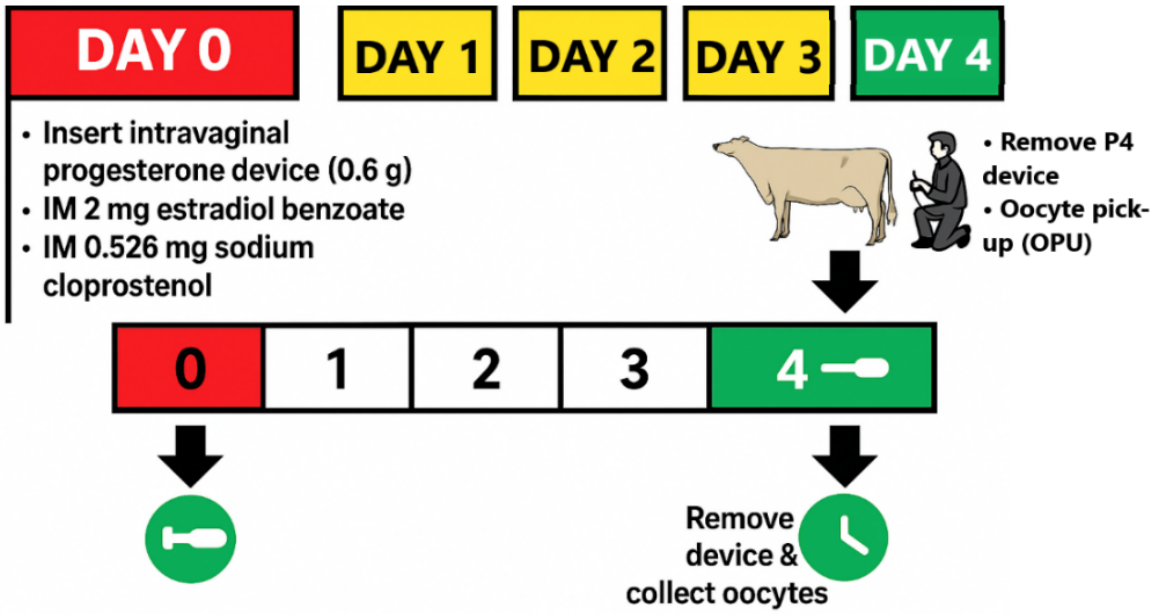

Figure 1 Ovarian stimulation protocol developed for the three treatments

oocytes and Oocytes surrounded by fibrin with a star-like appearance. Oocytes classified as grades A and B were considered viable, while those classified as grades C and D were considered non-viable.

2.4 In Vitro Oocyte Maturation (IVM)

Oocytes were matured in 70 µL microdroplets of IVM® medium (Vitrogen Brasil) covered with stabilized mineral oil (Sigma M3516), in 35 × 10 mm culture plates (Falcon®). Viable oocytes (grades A and B) were washed in H-199® medium and then in IVM® medium. Ten oocytes were placed in each microdroplet and incubated at 38.5 °C, 5% CO_2, and over 90% humidity for 22 hours.

2.5 In Vitro Fertilization (IVF) and Embryo Culture (IVC)

Frozen semen from four nationally recognized Brown Swiss bulls was used for fertilization. A total of 24 semen straws were used, with semen from all four bulls being equally distributed across treatments to avoid the male effect. Immediately after thawing, sperm motility was assessed at 37 °C under phase-contrast microscopy (×200); only samples displaying ≥50% progressive motility were included in IVF.

Sperm selection and capacitation were performed using a Percoll gradient (90/45). A final concentration of 2.0 × 10^6 spermatozoa per mL was standardized. Mature oocytes, identified by cumulus expansion, were washed in IVF® medium (Vitrogen Brasil) and transferred into groups of 10 to 70 µL microdroplets of IVF® medium, in 35 × 10 mm Petri dishes (Falcon®), covered with mineral oil. Sperm suspension (4–5 µL) was added to each droplet. The plates were incubated at 38.5 °C, 5% CO_2, and over 90% humidity for 18 hours. Eighteen hours post-insemination, oocytes were washed in IVC® medium (Vitrogen Brasil) and placed in 70 µL microdroplets (10 oocytes per droplet) of the same medium, covered with mineral oil. The 35 × 10 mm culture plates (Falcon®) were incubated at 38.5 °C, 5% CO_2, and 90% humidity. Medium changes were performed on days 2 and 4 post-insemination, replacing 50% of the medium. On day 7 post-insemination, embryos were evaluated and classified as blastocysts or expanded blastocysts.

2.6 Variable Evaluation and Metabolic Parameters

Follicular dynamics variables included the number of follicles measuring 2–4 mm and 5–8 mm in diameter, as assessed by ultrasonography before aspiration. Total oocytes recovered and total viable oocytes (grades A and B) were also evaluated through direct laboratory observation. Oocyte maturation rate was assessed 24 hours after oocytes were placed in the maturation medium, based on cumulus expansion. Embryo production was measured by the blastocyst rate, identified by the presence of a growing or expanded blastocoel on day 6 post-insemination. Blood samples were collected from the jugular vein using vacutainer tubes on day 4 of the synchronization protocol (day of oocyte collection). Samples were centrifuged at 1,000 × G for 10 minutes. β-hydroxybutyrate (β-HB) levels were measured using the TDM/β-hydroxybutyrate reagent (STANBIO® – USA). Glucose, blood urea nitrogen (BUN), and total protein concentrations were measured using commercial reagents from VALTEK® and analyzed by spectrophotometry.

2.7 Statistical Analysis

A non-parametric test was used to determine differences between treatments regarding the maturation rate and blastocyst rate. Analysis of variance (ANOVA) was conducted to identify differences between treatments for continuous numerical variables, with a completely randomized block design. Tukey's test was used for mean comparisons. Statistical analyses were performed using SAS v. 9.0 and SPSS v.25.

3 Results and Discussion

The results of this study demonstrate the significant effects of recombinant bovine somatotropin (rbST) administration on ovarian follicular dynamics, as detailed in Table 1. The treatments with rbST led to a higher total number of recovered oocytes compared to the control group ($P \leq 0.05$). However, no statistically significant differences were observed between the two rbST doses (500 mg and 250 mg). Regarding oocyte viability (grades A and B), the 500 mg rbST treatment produced a significantly higher number of viable oocytes ($P \leq 0.05$) than both the 250 mg rbST and control groups. Furthermore, cows treated with rbST exhibited a higher number of 2–4 mm diameter follicles ($P \leq 0.05$), although there were no significant differences between the 500 mg and 250 mg doses. In contrast, both rbST-treated groups differed significantly from the control. No significant differences were observed in the number of 5–8 mm diameter follicles between treatments.

These findings align with previous studies that have explored the role of rbST in follicular recruitment and development. Herrier et al. (1994), Ribeiro et al. (2020), Buratini et al.(2000) and Kassa et al., (2002) observed similar outcomes in dairy and beef cattle, where rbST administration resulted in an increased number of smaller follicles. The direct effects of rbST on follicular cells, as well as its influence on the synthesis and secretion of insulin-like growth factor-I (IGF-I), are likely mechanisms driving these changes. IGF-I is known to mediate nutrient redistribution, enhancing gluconeogenesis and increasing circulating glucose levels (Costa et al., 2020), which is consistent with the elevated glucose concentrations observed in rbST-treated cows in this study (Table 2). In the present study, the administration of a 500 mg dose of rbST was effective in increasing the number of recovered viable oocytes, an effect that was not significant with the 250 mg dose. This dose-dependent response is consistent with the broader literature where rbST dosages have been selected based on different production goals. For instance, the 500 mg dose has been conventionally used to enhance reproductive performance and ovulatory responses in dairy cows, our findings with this dose also align with Marques et al. (2009).

Moreover, rbST has been shown to prevent glucose oxidation (Kaminski et al., 2019), promoting its synthesis and allowing higher blood glucose levels. This mechanism could explain the inverse relationship observed between β-hydroxybutyrate (BHB) and glucose concentrations in our study, where lower BHB levels were detected in rbST-treated cows, suggesting a mitigation of negative energy balance (NEB) (Gohary et al., 2015; Lucy, 2000). These results corroborate the findings of Gohary et al.

Table 1 Effects of recombinant bovine somatotropin (rbST) administration on ovarian follicular dynamics in Brown Swiss cows

Variables	N	T1 (rbST 500 mg)	T2 (rbST 250 mg)	T3 (Control)	*P*-value
Total recovered oocytes	571	6.01 ±1.13^{a}	5.25 ±2.11^{ab}	4.8 ±2.05^{b}	0.034
Total viable oocytes (A and B)	416	5.02 ±1.25^{a}	3.7 ±1.56^{b}	3.1 ±1.11^{b}	0.021
Follicles (2–4 mm)	671	9.12 ±2.03^{a}	8.7 ±2.47^{a}	6.78 ±2.31^{b}	0.049
Follicles (5–8 mm)	243	1.89 ±1.05^{a}	1.88 ±1.69^{a}	2.04 ±1.19^{a}	0.902

Different letters (a, b) within the same row indicate statistically significant differences between treatments ($P \leq 0.05$)

Table 2 Effects of recombinant bovine somatotropin (rbST) administration on metabolic parameters in Brown Swiss cows

Variables	T1 (rbST 500 mg)	T2 (rbST 250 mg)	T3 (Control)	*P*-value
β-hydroxybutyrate (mM)	0.93 ±0.89^{a}	1.05 ±0.56^{a}	1.78 ±0.16^{b}	0.013
Glucose (mg.dL^{-1})	53.17 ±1.62^{b}	50.14 ±2.05^{b}	49.59 ±2.42^{a}	0.044
Blood urea nitrogen (mg.dL^{-1})	15.02 ±2.39^{a}	14.30 ±2.11^{ab}	12.7 ±2.64^{b}	0.019
Total proteins (mg.dL^{-1})	7.47 ±1.43^{a}	7.99 ±0.98^{a}	7.15 ±1.46^{a}	0.871

Different letters (a, b) within the same row indicate statistically significant differences between treatments ($P \leq 0.05$)

Table 3 Effects of recombinant bovine somatotropin (rbST) administration on *in vitro* embryo

Variables	T1 (rbST 500 mg)	T2 (rbST 250 mg)	T3 (Control)
Maturation rate (%)	59.8[a]	61.6[a]	58.1[a]
Blastocyst rate (% – day 7)	26.1[a]	25.4[a]	22.8[a]

Identical letters (a) within the same row indicate no statistically significant differences ($P > 0.05$)

(2015), who reported that while low doses of rbST may not prevent ketosis, higher doses over a longer period could reduce BHB and non-esterified fatty acid (NEFA) concentrations. The reduction in BHB levels observed in our study contrasts with some previous reports Dohoo et al. (2003), where rbST-treated cows experienced elevated BHB levels despite peak lactation. An interesting finding of this study is the increased number of viable oocytes collected from cows treated with 500 mg of rbST, which contrasts with the results reported by Ribeiro et al. (2020). This discrepancy may be explained by a potential protective mechanism of rbST, which could prevent the degeneration of atretic oocytes (Pavlok et al., 1996). If rbST can mitigate ketosis, as suggested by the lower BHB levels, it may stabilize protein-related parameters such as blood urea nitrogen (BUN) and total proteins. However, our study found no significant differences in total protein levels between treatments, which is consistent with the findings of Gallo and Block (1990), who reported no changes in these parameters in rbST-treated cows. Leroy et al. (2006) observed that elevated BHB concentrations negatively affect oocyte maturation both *in vivo* and *in vitro*, and when combined with low glucose levels, impair cumulus expansion and exert toxic effects during oocyte maturation, ultimately reducing blastocyst rates. In our study, rbST administration reduced BHB concentrations and maintained stable glucose levels, which may explain the similar oocyte maturation and blastocyst rates across all treatments, as shown in Table 3. The control group did not exhibit BHB or glucose levels outside the established ranges.

Previous studies, such as those by Songsasen et al. (1999), have reported a significant increase in transferable embryos following rbST administration. Similarly, Herrier et al. (1994) concluded that rbST increases both follicular and plasma IGF-I content, exerting profound effects on early follicular and embryonic development. However, Bols et al. (1998) noted no significant differences in blastocyst rates between cows treated with and without rbST. Our findings are consistent with the latter, as no differences were detected between rbST-treated cows and the control group in terms of blastocyst production.

The variability in results across studies highlights the complexity of *in-vitro* fertilisation success, which is influenced by numerous factors, including breed, management practices and environmental conditions. In the context of this study, the use of Brown Swiss cows in a semi-intensive system at high altitude presents additional variables that may contribute to the observed outcomes. Moreover, sub optimal *in-vitro* maturation (IVM) and embryo-culture (IVC) environments can mask follicular gains, because current media often fail to recreate the oocyte-somatic cell interactions and the precise composition of follicular fluid required for nuclear and cytoplasmic maturation (Sutton et al., 2002). Recombinant bST has been reported to improve mitochondrial oxidative activity and intracellular Ca^{2+} dynamics in bovine oocytes (Kuzmina et al., 2007), but these benefits may be offset when glucose, pyruvate and amino-acid availability during IVC does not match embryonic metabolic shifts, leading to diminished blastocyst yield (Ming et al., 2023). Defined media supplemented with antioxidants such as cysteamine can elevate intracellular glutathione and partially rescue developmental competence (Kobayashi et al., 2007); nonetheless, overall blastocyst rates remain below in-vivo levels, underscoring the challenges of fully mimicking the uterine milieu (Pfeifer & Corrêa, 2007). While rbST administration showed potential benefits in terms of follicular dynamics and oocyte viability, further research is needed to fully understand its long-term impact on *in-vitro* embryo production in different environmental and management settings.

4 Conclusions

The administration of recombinant bovine somatotropin (rbST) as part of a hormonal protocol for *in vitro* embryo production demonstrated clear benefits on ovarian follicular dynamics, particularly by increasing the number of smaller follicles (2–4 mm) and improving the recovery rate of viable oocytes. The use of rbST, specifically at a dose of 500 mg, was shown to significantly enhance the number of viable oocytes (grades A and B) while reducing the risk of subclinical ketosis, as evidenced by the lower β-hydroxybutyrate levels and increased glucose concentrations. Despite these positive effects, rbST administration did not have a significant impact on blastocyst rates, suggesting that while rbST improves early follicular development, it does not necessarily translate to enhanced *in vitro* embryo production.

Based on these findings, a recommended dose of 500 mg of rbST can be considered optimal for improving reproductive parameters such as follicle count and oocyte viability. Because rbST acts through conserved somatotropic and IGF-1 pathways, the protocol may be transferable to other breeds and production systems, but local validation of dose and metabolic responses is advised. However, it is important to note that no significant differences in blastocyst rates were observed between rbST-treated groups and the control, indicating that the final stages of embryo development may be influenced by additional factors beyond rbST administration. Further research is needed to investigate the long-term effects of rbST on *in-vitro* embryo production and subsequent reproductive performance (pregnancy establishment and calf viability), as well as to clarify the role of altitude-related oxidative stress and other environmental influences on embryo competence, in various environmental and management conditions, particularly in high-altitude systems and different cattle breeds.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author Contributions

Conceptualization: I.U.P.; Funding acquisition: I.U.P.; Investigation: I.U.P., F.A.V., C.Q.E.; Methodology: F.A.V., J.N.C., C.Q.E.; Supervision: F.A.V.; Validation: C.Q.E.; Data curation: J.N.C.; Writing – original draft: I.U.P., E.A.G.; Writing – review & editing: I.U.P.; Visualization: E.A.G.

AI and AI-Assisted Technologies Use Declaration

The following AI tools/AI-assisted technologies were used during the preparation of the manuscript: ChatGPT (OpenAI), which was used for improving readability and language. The authors take full responsibility for the content of the article.

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