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Effect of embryotrophic factors at different oxygen tension for *IN VITRO* culture on the embryonic development of alpacas up to the blastocyst stage

Efecto de factores embriotróficos a diferentes tensiones de oxígeno en cultivo *IN VITRO* sobre el desarrollo embrionario de alpacas hasta la etapa de blastocisto

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Abstract

The alpaca is the most important South American domestic camelid for Peru, a country that regionally has 87% of these animals. Since traditional forms of reproduction do not guarantee its genetic quality, *in vitro* reproduction is an alternative for its improvement. This study evaluated the influence of embryotrophic factors of epidermal development (EGF) and insulin-like growth factor (IGF-1) and oxygen tension on the *in vitro* development of alpaca oocytes up to the blastocyst stage. From ovaries of sacrificed animals, oocytes were obtained and placed in TCM-199 medium, supplemented with sodium pyruvate, glutamine, estradiol (E2), follicle stimulating hormone (FSH), luteinizing hormone (LH), embryotrophic factor (EGF or IGF-1), 5% fetal bovine serum and gentamicin (10μL/mL) for 32 hours, at 38.5 °C, with 5% CO_2 and relative humidity greater than 95%, with O_2 tensions between 6 and 20%. Subsequently, the oocytes were fertilized with fresh semen and cultured in KSOMaa medium for 48 hours. Cultures were differentiated by growth factors (EGF and IGF-1) and O_2 tensions (6% and 20%), plus the control group without EGF or IGF-I supplementation, to assess oocyte and blastocyst division rates from oocytes. By cluster analysis, significant differences were established between treatments with $\alpha = 0.05$ for each response variable, with the highest rate of oocyte divisions (24.8%) with EGF at 6% O_2 and the highest blastocyst/oocyte production (18.4%) with IGF-1 at 6% O_2. It is concluded that the addition of embryotrophic factors and low O_2 tension are favorable for *in vitro* embryo development in alpacas.

Keywords: Alpaca, camelids, cleavage, fertilization, oocytes, reproduction.

Resumen

La alpaca es el camélido sudamericano doméstico de mayor importancia para el Perú, país que a nivel regional cuenta con el 87% de estos animales. Ya que las formas tradicionales de reproducción no garantizan su calidad genética, la reproducción *in vitro* es una alternativa para su mejoramiento. Este estudio evaluó la influencia de los factores embriotróficos de desarrollo epidérmico (EGF) y de crecimiento insulínico (IGF-1) y tensiones de oxígeno en el desarrollo *in vitro* de ovocitos de alpaca hasta la etapa de blastocistos. A partir de ovarios de animales sacrificados, se obtuvieron ovocitos que se colocaron en medio TCM-199, suplementado con piruvato de sodio, glutamina, estradiol (E2), hormona folículo estimulante (FSH), hormona luteinizante (LH), factor embriotrófico (EGF o IGF-1), 5% de suero fetal bovino y gentamicina (10μL/mL) durante 32 horas, a 38,5 °C, con 5% de CO_2 y humedad relativa mayor de 95%, con tensiones O_2 entre 6 y 20%. Posteriormente, los ovocitos fueron fecundados con semen fresco y cultivados en medio KSOMaa durante 48 horas. Los cultivos fueron diferenciados por factores de crecimiento (EGF e IGF-1) y tensiones de O_2 (6% y 20%), más el grupo control sin suplementar con EGF o IGF-I, para valorar las tasas de división de ovocitos y blastocistos a partir de ovocitos. Mediante análisis de conglomerados, se establecieron diferencias significativas entre los tratamientos con $\alpha = 0{,}05$ para cada variable de respuesta, observándose la mayor tasa de divisiones de ovocitos (24,8%) con EGF a 6% de O_2 y la mayor producción de blastocistos/ovocito (18,4%) con IGF-1 a 6% de O_2 . Se concluye que la adición de factores embriotróficos y una baja tensión de O_2 son favorables para el desarrollo embrionario *in vitro* en alpacas.
Palabras clave: Alpaca, camélidos, clivaje, fertilización, ovocitos, reproducción.

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1 Introduction

The breeding of domestic camelids such as llamas (*Lama glama*) and alpacas (*Vicugna pacos*) in South America is part of an ancestral culture, especially in Peru and Bolivia, which account for 98.89% of the total alpaca population (87.9% Peru and 10.9% Bolivia) and 93.4% of total llamas (60.8% Bolivia and 32.5% Peru), according to figures reported by the Ministry of Agriculture and Irrigation of Peru (MINAGRI, 2015). Peru reports a population of 3 685 516 alpacas and 1 257 000 llamas, hence genetic and reproductive studies of these species are paramount for the country, due to their great productive and commercial potential, especially alpaca based on the production of fiber, meat, skin and manure as organic manure, as well as its use as an animal for recreation and production of therapeutic solution (MINAGRI, 2019). According to the last agricultural census of Peru carried out in 2012 by the National Institute of Statistics and Information Technology (INEI), the regions with the highest density of alpacas are Puno with 39.6%, Cusco with 14.8% and Arequipa with 12.7%; the Huacaya variety represents 80.4% of the total population (INEI, 2013).

Despite the importance of alpacas in Peru, their reproduction mainly occurs by natural mating, without considering genetic variability and improvement of the species for commercial purposes, which has led to a decrease in genetic quality (Huanca, 2012). *In vitro* reproduction is shown as a viable alternative for genetic improvement and increased productivity of alpaca, since this technique is highly developed in buffalo, cattle, sheep and pigs (Liang et al., 2020; Javvaji et al., 2020; Dubeibe et al., 2019; Gonella Diaza et al., 2013; Rodrigues et al., 2013). The reproduction feasibility of camelids by artificial insemination, embryo transfer and *in vitro* fertilization has also been reported (Ruiz, 2018; Pérez et al., 2017; Pacheco et al., 2016), being necessary to study the conditions that allow the successful development of the reproduction of alpacas by *in vitro* techniques in Peru since there are no specific protocols for it.

Since *in vitro* production of embryos is limited due to the reduced survival in both embryonic and fetal and the high frequency of fetal, placental or neonatal abnormalities, alternatives are sought to improve the production of embryos with this technique, focused on growth factors that regulate the processes of cellular mitogenesis, differentiation, and apoptosis under *in vivo* conditions (Block, 2007; Kane et al., 1997).

The growth factors or embryotrophic factors more used in experimental studies to increase the maturation efficacy of oocytes and *in vitro* production of embryos are the insulin growth factor (insulin growth factor IGF), because of their effects on growth, follicular development and maturation induced by gonadotrophins (Lenz et al., 2007) and contribution to the pre-implantation and development of the embryo in cattle (Lima et al., 2006; Stefanello et al., 2006; Block, 2007), the epidermal growth factor (EGF), which stimulates cell proliferation and differentiation (Adams, 1999), and which is related to oocyte maturation (Harper and Brackett, 1993), the transforming growth factor (TGF), and the growth factor derived from platelets (Platelet-derived growth factor, PDGF) (Block, 2007).

On the other hand, the best yields of embryonic cultures are obtained with O_2 concentrations lower than atmospheric, mainly due to the reduction of the generation of O_2-free radicals, reducing their deleterious effect (Legge and Sellens, 1991; Noda et al., 1991; Umaoka et al., 1992). In addition, the reduced intrauterine O_2 tension reported in vivo studies would imply protection for the preimplantation of blastocyst (Clark et al., 2006). Thus, a better development of matured bovine oocytes in 5% of O_2 has been reported (Hashimoto et al., 2000; Van Blerkom et al., 1997).

Therefore, the aim of the research was to establish the ideal conditions for alpaca embryonic development by evaluating the influence of the embryotrophic factors EGF and IGF-1 and the oxygen tension at 6% and 20% on the division rate of oocytes after the first embryonic culture at 48 h in KSOMaa medium, and the blast rate from oocytes after seven days of second embryonic culture in SOFaa.

2 Materials and Methods

2.1 Study area

The research was carried out at the Quimsachata Research and Production Center (CIP), of the agri-

cultural experimental station ILLPA-Puno, National Institute of Agricultural Innovation-INIA -Peru, located between Santa Lucia and Cabanillas, provinces of Lampa and San Roman in Puno, 15° 44′ 00″ South Latitude and 70° 41′ 00″ West Longitude, in the agro-ecological area known as a dry puna, with an average altitude of 4300 masl, and a temperature that fluctuates between 2°*C* (May to July) and 15°*C* (September to December) (Díaz, 2013).

2.2 Collection of ovary simples

The collection of ovaries was made from animals at the slaughtering livestock in Nunoa, with random sampling that did not consider the reproductive status of alpacas. The ovaries collected were placed and transported in a thermos between 35 and 37°*C*, immersed in 0.9% saline and supplemented with gentamicin (10μL/mL).

2.3 Processing of oocytes

Using the modified Slicing method (Lorenzo et al., 2015), 1051 oocytes were collected, selected in categories I and II. For their maturation, oocytes were placed in TCM-199 medium, supplemented with sodium pyruvate, glutamine, estradiol (E2), follicle stimulating hormone (FSH), luteinizing hormone (LH), embryotrophic factor (EGF or IGF-1) at concentrations between 10 and 50 ng/ml, 5% fetal bovine serum and gentamicin (10h/ml) for 32 hours at 38,5°*C* with 5% CO_2, and relative humidity higher than 95%, with O_2 of 6% or 20%. Table 1 shows the distribution of oocytes based on inclusion of embryotrophic factors and O_2.

Table 1. Distribution of alpacas oocytes.

Embryotrophic Factor	Oxygen tension %	Number of Oocytes
EGF	6	205
	20	219
IGF-I	6	206
	20	210
Control	6	211
	20	211

After maturation, the oocytes were transferred to a fertilization medium (FER-TALP supplemented with 0.25 mM of sodium pyruvate, 6mg/mL of BSA, and 50μg/mL of gentamicin), in which they were washed three times. At the same time, sperm preparation was done by washing it in Sperm-TALP supplemented with 1.0 mm of sodium pyruvate, 3 mg/ml of BSA fraction V and 50μg/ml of gentamicin with 4 μL of heparin and 30 μL of PHE/ (penicillamine, hypotaurine and epinephrine) and centrifuged at 1500 rpm/10 min. The pellet formed was resuspended in 1ml of the FERT-TALP medium. *In vitro* fertilization was done with sperm from a fertile male, which after being prepared were transferred to a 80 μl drop from the fertilization medium and placed in an incubator for 10 hours.

2.4 Embryo development

At the end of the fertilization period, the possible zygotes were removed from the fertilization drops and introduced into multiwell plates with 500 μl KSOM-AA culture medium, where EGF or IGF-1 (10-50 10 and 50 ng/ml) were added at 38,5°*C*. Maximum relative humidity $> 95\%$, CO_2 voltage 5% and 6 or 20% O_2 voltage. The zygote division rate was evaluated 48 hours after fertilization and then transferred to the SOFaa culture medium, adding EGF or IGF-1 under the above conditions. On day seven after fertilization, blastocyst stages were observed.

2.5 Statistical data treatment

The experiment combined EGF IGF-1 embryotrophic factors and a control group with two O_2 levels (6% and 20%). Euclidean distance cluster analysis was used for close neighbors with a 95% confidence level to establish differences and similarities in the joint application of treatments on oocyte and blast cell division rates obtained from oocytes (blasts/oocytes). This technique allows the grouping in clusters of variables according to their distances, where there is no significant difference according to what is established in the analysis, since they are exclusive with respect to factors that do not belong to the group, not establishing hierarchies, but statistically differentiated groups (Cuadras, 2020). The InfoStat version 2018 statistical package was used for all statistical analyzes.

3 Results and Discussion

3.1 Oocyte division rate

Once the experiment was conducted, it was observed that the lower O_2 (6%) produced the highest rate of oocyte division and blastocyst formation per oocyte, compared with the control group and 20% of O_2 (Table 2).

Table 2. Oocyte and blastocyst division rates per oocyte after application of EGF and IGF-I with two O_2 tensions.

Embryotrophic Factor	O_2 tension (%)	Oocyte Divisions (%)	Blastocysts/ Oocytes (%)
EGF	6	51(24.9)	29(14.1)
	20	13 (5.9)	12 (5.5)
IGF-I	6	42(20.4)	38(18.4)
	20	24(11.4)	14(6.7)
Control	6	33(15.6)	6 (2.8)
	20	33(15.6)	6 (2.8)

Similarly, the percentage of oocyte divisions (24.9%) was higher with the embryotrophic EGF factor with 6% of O_2, lower than the reported by Benavides et al. (2015), who, when analyzing the influence of oxygen tension on bovine embryonic development, obtained 69.7% of oocyte divisions with 5% of O_2. However, these authors do not assess the effect of EGF; while Ahumada (2011), when adding EGF, obtained 74.15% of cleavage in bovine oocytes grown at 5% of O_2.

In Figure 1, the dendrogram for oocyte divisions presents three clusters as groups that differ significantly when the cut is taken according to the result of the cophenetic distance calculated in 0.72. A cluster with EGF and IGF-1 treatments with 6% of O_2 stands out, which, when separated from the control group with different O_2 would indicate that EGF and IGF-I significantly increase oocyte division, although there were no differences between embryotrophic factors with this O_2. On the other hand, that IGF-I with 20% of O_2 form a cluster with the control group, and with both O_2 it would suggest that IGF-I, under these conditions, does not affect the division of oocytes. Meanwhile, EGF treatment with 20% of O_2 would have an inhibitory effect on this variable.

These results are consistent with those reported by Delgado (2018), who observed a greater division of oocytes with 2% of O_2 and an improvement in the quality of bovine embryos than with 5% and 20% of O_2 in the culture. Likewise, Arias et al. (2007) reports similar results to current research applied to bovine embryos, under conditions of high (20%) and low (7%) O_2. In this regard, studies in sheep and swine have concluded that the absence of O_2 promotes the ability to activate, and improves the parthenogenesis of oocytes *in vitro* cultures (Iwamoto et al., 2005; Loren et al., 2016; Yao et al., 2019).

In contrast, He et al. (2020) reported that the excision rate of yak oocytes was significantly lower ($P < 0{,}05$) at 5% of O_2 concentration than at 10% and 20% concentrations, improving the maturation and competition of oocyte development. Rodrigues et al. (2013) found that the division of canine oocytes was not affected by O_2 of 5% or 20%. The differences between these results are probably explained by the characteristics of the embryonic development of these species.

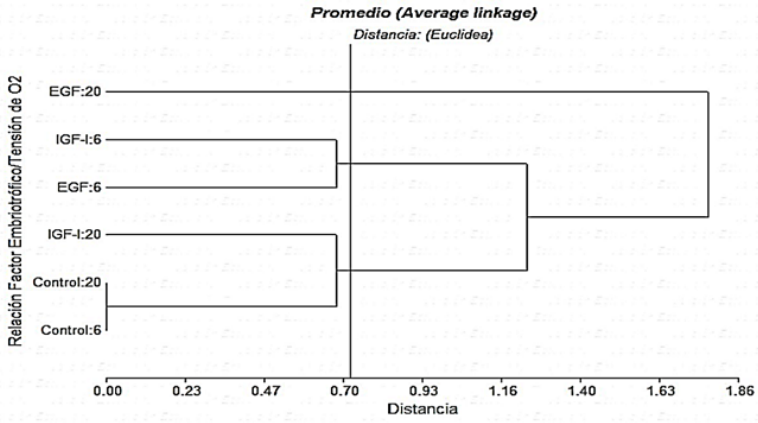

Figure 1. Conglomerate dendrogram for the response variable number of oocyte divisions.

3.2 Blastocyst rate from oocytes

Figure 2 shows the dendogram of cluster analysis with four well-defined clusters with the cut-off according to the cophenetic distance of 0.48, indicating that there are significant differences between the experimental treatments and the control group with both O_2, i.e., that the use of EGF and IGF-1 with 6 and 20% of O_2 increases the formation of blastocysts. On the other hand, the percentage of blastocysts/oocyte obtained is higher than the 14.0% reported by Soto-Martínez et al. (2019), also with bovine embryos evaluated in sequential synthetic oviductal liquid, avoiding the accumulation of embryotoxic substances at a maximum of 5% of O_2.

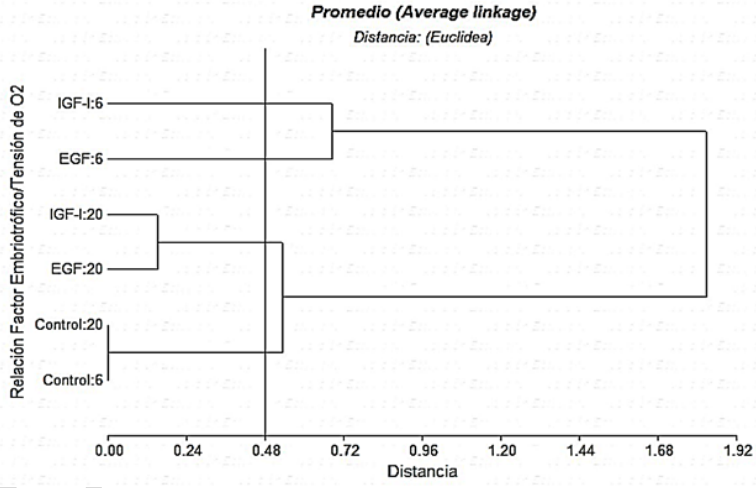

Figure 2. Cluster dendrogram for the variable response number of blastocysts per oocyte.

However, the number of blast cells per oocyte was not modified by EGF and IGF-I when using an O_2 of 20% so they were located in the same cluster. Whereas the 6% IGF-1 embryotropic factor of O_2 showed the highest number of blasts per oocyte (18.4%). This result is similar to that reported by Sirisathien and Brackett (2003), who obtained a higher number of blast cells per oocyte with IGF-1 than with EGF in cattle; i.e., similar proportions of parthenogenetically activated oocytes became blastocysts than inseminated oocytes (28.8%). Yong et al. (2017), highlighted the importance of growth

factor treatment for *in vitro* maturation of porcine oocytes, which is consistent with the results of this study.

Regarding the effect of IGF-1, Javvaji et al. (2020) report that the addition of this factor significantly improved the maturation of oocytes in ovine compared with untreated oocytes by regulating PI3K/Akt expression and apoptosis signaling, which are related to the activation of oocytes in ovines. Finally, the addition of epidermal growth factor (EGF) to the maturation medium stimulates oocyte maturation, but only EGF supplementation increases embryonic and blastocyst development. This evidence is consistent with Richani and Gilchrist (2018), who determined that the EGF also dominates the translation of maternal transcripts into the inactive oocyte, a phase that is necessary to the competition of the oocyte. In addition, it is similar with the study of Salgado et al. (2013), showing that there was a significant difference ($p < 0{,}05$) of the EGF over the proportion of oocytes, explaining the highest proportion of oocytes under treatment with 50 ng/mL.

4 Conclusions

In vitro division rate of alpaca oocytes grown in KSOM-AA medium and blastocyst formation in SOFaa medium, both with embryotrophic factors (EGF and IGF-1), was favored by low oxygen (6 %), with significant results in the control groups, observing a higher percentage of oocyte divisions with EGF treatment and in oocyte blastocysts with IGF-1 treatment.

Although the use of the embryotrophic factors EGF and IGF-1 and the low O_2 resulted in an increase in the number of divisions and the number of blasts per oocyte, indicating that under the conditions of this study they can be used for an improvement *in vitro* embryonic development in alpacas, it is necessary to have additional information to clarify the mechanisms of action of embryotropic factors in order to optimize the procedure and achieve a viable *in vitro* alpaca reproduction that leads to the genetic improvement of the species.

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