EMBRYOS PRODUCTION BY IVF AND ICSI IN ALPACA *Vicugna pacos*

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ABSTRACT

The development of reproductive biotechnologies in South American camelids could shorten the time between generations and, therefore, be used in breeding programs. In alpacas, the number of in vitro fertilization protocols is still limited, and even more so in procedures such as ICSI (intracytoplasmic sperm injection). Therefore, the general objective of the present study was to produce alpaca preimplantation embryos in vitro using the ICSI procedure as well as conventional in vitro Fertilization. The biological samples were collected in the municipal slaughterhouse of Huancavelica and transported in a 0.9% NaCl solution at 4 ° C within approximately 20 to 22 hours. In the laboratory, the cumulus - oophorus complexes (CCO) were isolated and cultured at 38.5 ° C, 5% CO2 and 100% relative humidity for 30 to 36 hours. We worked with sperm isolated from the caudal area of the epididymis, these were selected by the method of Percoll and Swim up. The in vitro fertilization was carried out with a concentration of 2x106 esp / mL in HAM medium supplemented with heparin and PHE (Penicillamine-Heparin-Hipotaurine) as capacitor agents for 18 hours, later they were evaluated and cultured in KSOM development medium supplemented with SFB, sodium pyruvate and gentamicin for 7 days. On the other hand, the ICSI procedure was performed in the Global Total Environment with Hepes with the help of the inverted microscope and its accessories for micromanipulation, later this protocol was complemented with a chemical activation of the injected oocytes that consists in the incubation in 5uM ionomycin for 5 minutes followed by a second incubation of 3 hours in 6-DMAP, which is necessary for some species according to the literature. When evaluating the results, 17 embryos were obtained by IVF and 5 embryos by ICSI, that is, 36.9% and 31.3%, respectively. No significant difference was found between the groups evaluated ($p>0.05$)

KEYWORDS

Alpaca, fertilization, intracytoplasmic injection, *in vitro* fertilization, *Vicugna pacos*.

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INTRODUCTION

During the last decades, the world demand for South American camelids has increased due mainly to the fact that they are a source of high quality fiber for the textile industry, and also because of their value as exotic pets. However, the characteristics inherent to the reproductive physiology of these species have made it difficult to apply technologies that allow the development of more efficient productive systems (Sansinena et al., 2003), while the deficiencies in these reproductive management schemes have contributed to a deterioration of the quality of the animals (Huanca, 2012).

The development of reproductive biotechnologies in South American camelids, could shorten the time between generations and, therefore, could be used in breeding programs, thus allowing the propagation of genetically superior animals, especially those with fine fiber and of natural colors (Miragaya et al., 2006; Sansinena et al., 2007; Conde et al., 2008).

The technique of in vitro fertilization is one of the most developed technologies in recent years and involves the control of the maturation and interaction mechanisms of the male and female gametes in an artificial environment (Torres, 2017). However, at the present time studies in relation to the application of this and other techniques such as intracytoplasmic sperm injection (ICSI) for the production of embryos in these species is still very limited (Pérez et al., 2007), being still It is necessary to overcome many deficiencies in in vitro maturation, fertilization and embryo culture to standardize an IVF protocol consisting of these species (Ruiz, et al., 2017).

Intracytoplasmic sperm injection, on the other hand, is an important technology of assisted reproduction. Since the first report of ICSI in hamsters the intracytoplasmic injection of sperm has also provided a valuable tool in the study of cell cycle control, the mechanisms underlying the activation of sperm-induced oocytes and the need for greater chemical activation after ICSI (Sansinena, et al., 2007).

The general objective of the present study was to produce pre-implantable embryos of alpaca in vitro using the ICSI procedure, as well as conventional in vitro fertilization.

MATERIALS AND METHODS

The biological samples, testicles and ovaries were collected in the city of Huancavelica, Peru (4050 masl, average annual temperature 8 ° C - 10 ° C) (Figure 05), and were transported at 4 ° C in a solution of NaCl 0.9% for approximately 24 hours to the Laboratory of Physiology of Reproduction (LFR) of the Faculty of Biological Sciences of the UNMSM (Lima, 100 masl, temperature 15 ° C - 27 ° C). Ovaries and testes were transported in 0.9% saline solution was used at 4ºC of temperature using gel-packs and a thermo-insulating box.

Isolation and selection of oocytes

The ovaries were washed twice with running water, followed by washes with phosphate buffered saline or phosphate buffered saline (BFS) plus penicillin 0.075g / L and streptomycin 0.075g / L at 38.5 ° C.

Oocyte isolation was performed using two techniques: aspiration and cuts. Aspiration of follicles of 2 mTabla 2. Embriones producidos mediante FIV

Collection and transfer of the sample

Groups	N° Oocyte with PB	%Embryos
Group 1	38	14 (36.8%)
Group 2	8	3 (37.5%)

Embryo production using ICSI

Of 27 mature oocytes injected, 5 embryos were obtained; 16 injected mature oocytes were exposed to a chemical activation treatment (Group 2), while the rest were only injected and immediately put into development medium (Group 1) (Table 3)

Tabla 3. Embriones producidos mediante ICSI

Groups	N° Microinyected oocytes	% Embryos
Group 1	11	0/11 (0%)
Group 2	16	5/16 (31.3%)

Comparison of embryos obtained through IVF and ICSI

The embryos produced by both methods: FIV, 17/46 (36.9%) and ICSI, 5/16 (31.3%) are described in Table 4, while the comparison of their stages is described in Table 5. It was taken in counts the total number of oocytes that arrived or passed through that stage. Note that the maximum development stage was morula.

Table 4. Comparison of embryos by means of IVF and ICSI

Grupos	**N° oocyte with PB**	**% Embryos**
FIV	46	17/46 (37%)
ICSI/Activation	16	5/16 (31.3%)

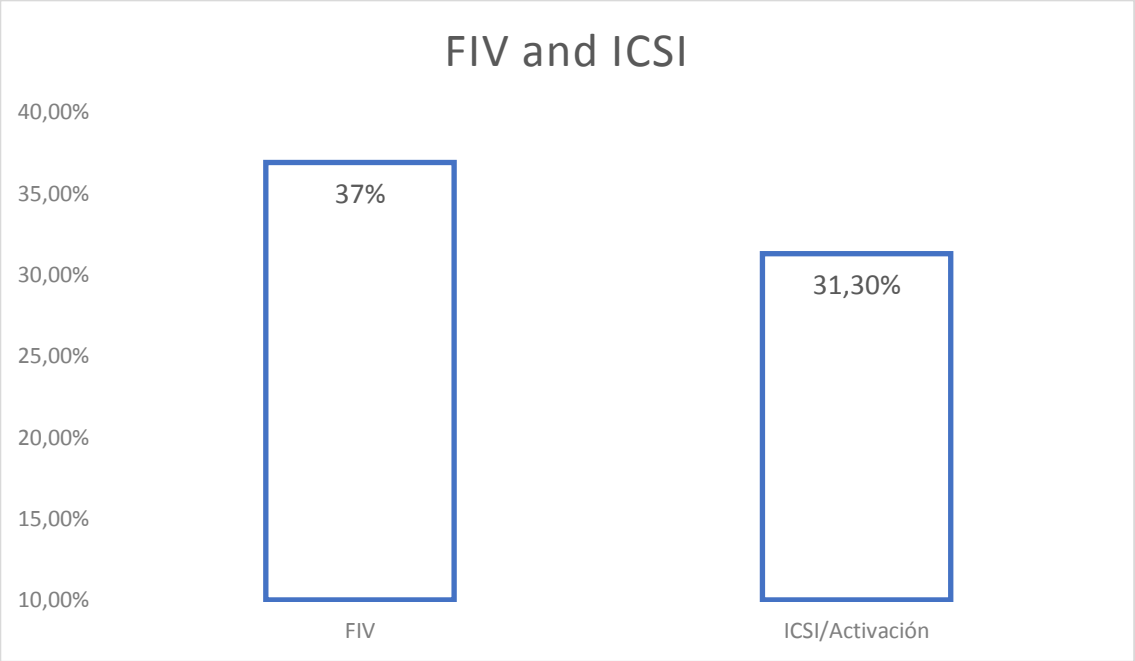

Figure 11: Comparison of embryo production using IVF and ICSI

Tabla 5. Development stages by FIV and ICSI

% Estadios de desarrollo

Group	N° Embryos	Fertilizated /Pronucleo	2 cell (Day 2)	4 cell (Day 3)	8 cell (Day 4)	Morule (Day 5)
FIV	46	17(37%[a])	9(19.6% [b])	7 (15.2% [c])	3 (6.5% [d])	2 (4.3% [e])
ICSI/Activaction	16	5 (31.3% [a])	4 (25% [b])	2(12.5% [c])	2(12.5% [d])	2(12.5% [e])

[a,b,c,d,e] Superíndices iguales dentro de columnas indican que no hay diferencia significativa (p>0.05)

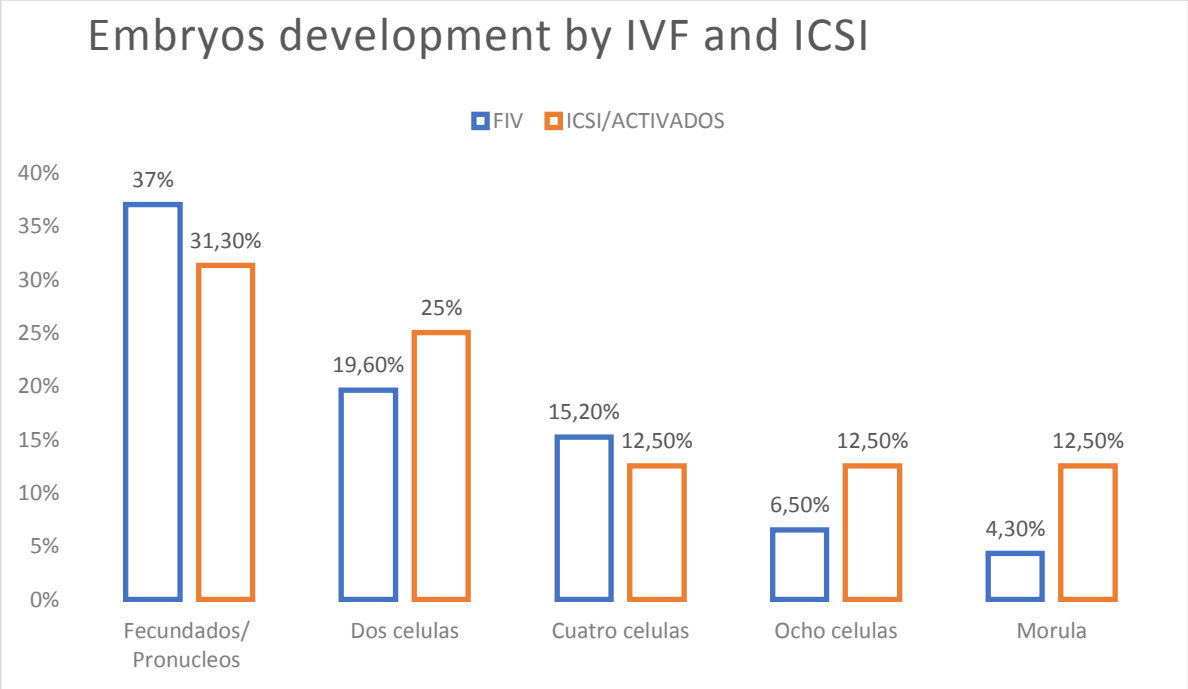

Figure 12: Embryonic development stage for embryos obtained through IVF and ICSI

The number of oocytes that fertilized for both methods was compared, considering the pronucleus stage onwards. Being 37% for IVF and 31.3% for ICSI. No significant difference was found (p>0.05) for both methodologies.

			FECUNDACION		
			FECUNDADOS	NO FECUNDADO	Total
METODO	FIV	Recuento	17	29	46
		% dentro de METODO	37.0%	63.0%	100.0%
	ICSI	Recuento	5	11	16
		% dentro de METODO	31.3%	68.8%	100.0%
Total		Recuento	22	40	62
		% dentro de METODO	35.5%	64.5%	100.0%

Oocyte count fertilized by both methods of in vitro fertilization

The number of oocytes that fertilized for both methods was compared, considering the stage of two cells onwards. Being 19.6% for FIV and 25% for ICSI. No significant difference was found (p>0.05) for both methodologies.

			CLIVADOS		
			CLIVADOS	NO CLIVADOS	Total
METODO	FIV	Recuento	9	37	46
		% dentro de METODO	19.6%	80.4%	100.0%
	ICSI	Recuento	4	12	16
		% dentro de METODO	25.0%	75.0%	100.0%
Total		Recuento	13	49	62
		% dentro de METODO	21.0%	79.0%	100.0%

Figure 14: Counting of cleaved oocytes, from two cells onwards, by both methods of in vitro fertilization

Se comparó el número de embriones de cuatro células, obteniéndose 15.2% para FIV y 12.5% para ICSI. No se encontró diferencia significativa (p>0.05) para ambas metodologías.

			CUATRO_CELULAS		
			EMBRION DE CUATRO CELULAS	NO DESARROLLO	Total
METODO	FIV	Recuento	7	39	46
		% dentro de METODO	15.2%	84.8%	100.0%
	ICSI	Recuento	2	14	16
		% dentro de METODO	12.5%	87.5%	100.0%
Total		Recuento	9	53	62
		% dentro de METODO	14.5%	85.5%	100.0%

Figura 15: Recuento de embriones de 4 células por ambos métodos de fecundación in vitro

Comparison of 8 cell embryos by IVF and ICSI

The number of embryos of eight cells was compared, obtaining 6.5% for IVF and 12.5% for ICSI. No significant difference was found (p>0.05) for both methodologies.

			OCHO_CELULAS		
			EMBRION OCHO CELULAS	NO DESARROLLADO	Total
METODO	FIV	Recuento	3	43	46
		% dentro de METODO	6.5%	93.5%	100.0%
	ICSI	Recuento	2	14	16
		% dentro de METODO	12.5%	87.5%	100.0%
Total		Recuento	5	57	62
		% dentro de METODO	8.1%	91.9%	100.0%

Figure 16: Embryo count of 8 cells by both methods of in vitro fertilization

Comparison of 8 cell embryos by IVF and ICSI

The number of embryos of eight cells was compared, obtaining 6.5% for IVF and 12.5% for ICSI. No significant difference was found (p>0.05) for both methodologies.

			MORULA		
			MORULA	NO DESARROLLO	Total
METODO	FIV	Recuento	2	44	46
		% dentro de METODO	4.3%	95.7%	100.0%
	ICSI	Recuento	2	14	16
		% dentro de METODO	12.5%	87.5%	100.0%
Total		Recuento	4	58	62
		% dentro de METODO	6.5%	93.5%	100.0%

Figure 17: Morula stage embryo count by both methods of in vitro fertilization

Fluorescence microscopy evaluations using the Hoechst 33342 dye allowed us to discern the embryonic stage, as shown below, whose main contribution was to identify the oocytes fertilized by the presence of the pronuclei (Figure 18.A) since these do not show any cleavage .

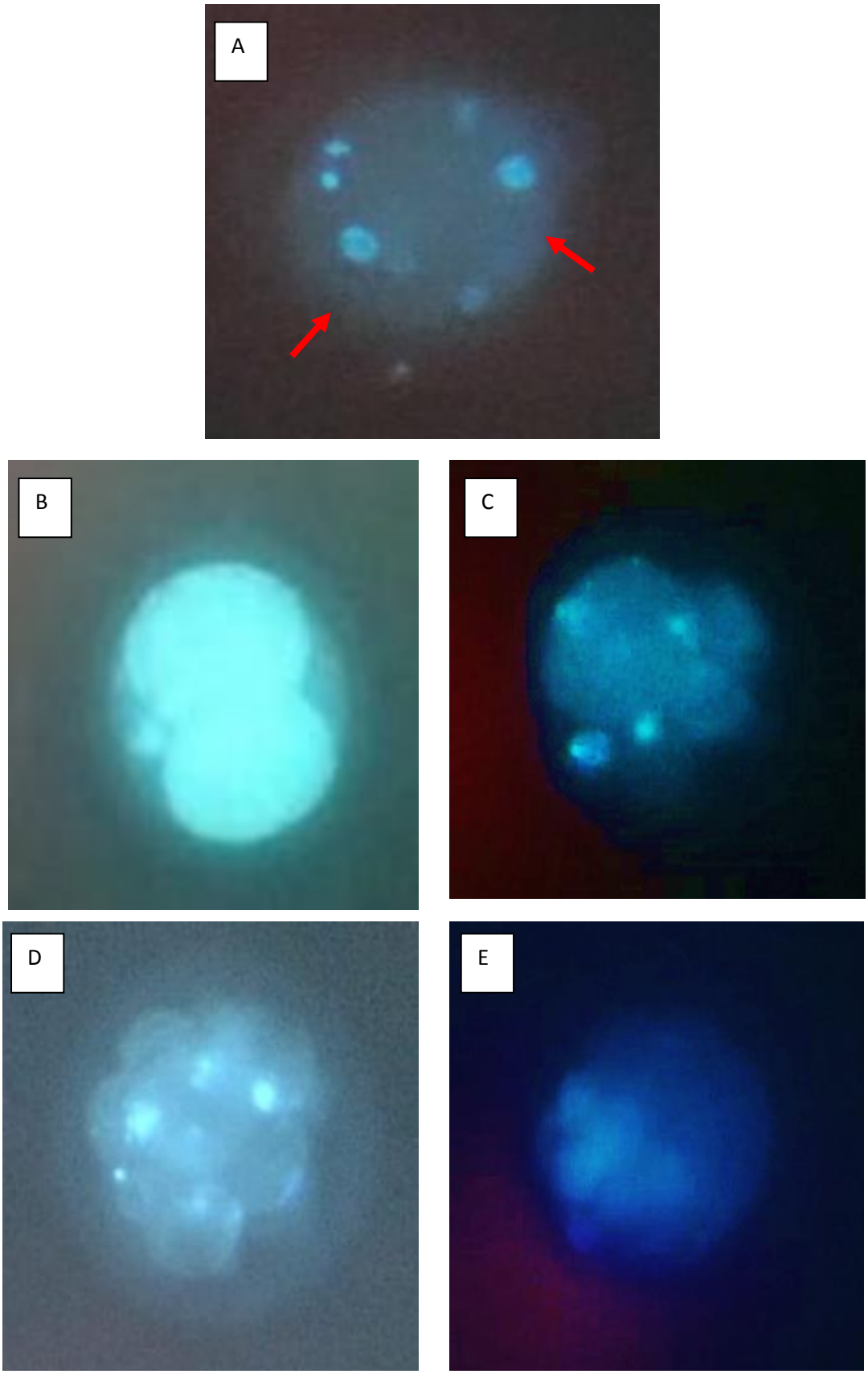

Figure 18: Embryo analysis by fluorescence microscopy using Hoechst 33342 dye. (A) Fertilized oocyte, pronucleus staining (arrows) (B) Embryo of two cells. (C) Embryo of 4-8 cells. (D) Embryo of 8-16 cells. (E) Embryo in a morula stage.

DISCUSSION

In the present work, conventional in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) were used to produce embryos. Both techniques were designed to solve fertility problems, while ICSI allows obtaining good reproductive results in cases in which IVF does not, its success rates vary greatly depending on the cause of infertility, characteristics of the gametes, etc. There is a limited number of studies that report the in vitro production of embryos for South American camelids. Therefore, the objective of this study was the production of pre-implantation embryos of Vicugna pacos, alpaca.

Fertilization rates obtained were 37% (17/46) and 31.3% (5/16) for IVF and ICSI, respectively. These values do not present significant differences ($p>0.05$) as shown in Figure 13. Studies in the production of Camelus dromedarius embryos report a 21.2% cleavage rate for in vitro fertilization using epididymal sperm, and a cleavage rate of 23 % for the ICSI methodology (El-Sayed et al., 2015) comparable with the results obtained in the present work.

The oocytes obtained through cuts or aspirations were fertilized in vitro, 17 fertilized oocytes were obtained from 46 mature oocytes. (Table 2). The injected oocytes (ICSI) were in total 27, divided into two groups; one of them was taken to culture immediately after the intracytoplasmic injection where no embryo was obtained (0/11), while in the second group, which was exposed to a chemical activation, 5 embryos were obtained (5 / 16), that is, 31.3%. (Table 3)

Damage to the membrane level of the sperm after ICSI has been shown to be related to the time of onset of calcium oscillations, oocyte activation and pronucleus formation. (Kasai T. et al., 1999, Morozumi K. et al., 2006) On the other hand, the complete injection of the sperm, including the plasma membrane, as well as the acrosome, prevents decondensation and the formation of the male pronucleus (Garcia-Rosello E et al., 2006)

In this study, injection followed by chemical activation of oocytes promoted the development of alpaca embryos, the use of ionomycin / DMAP for activation is widely used due to its effect on calcium channels in oocytes. (Yanagimachi et al, 1994)

During fertilization, the sperm directs a series of calcium oscillations in the inner membranes of the endoplasmic reticulum and mitochondria and can last for hours (Carroll and Swann et al., 1992, Kline and Kline, 1992, Miyazaki et al., 1993). These oscillations are responsible for a cascade of events such as the reaction of the cortical granules (Miyazaki et al., 1990), the reaction of the zona pellucida (Yanagimachi et al., 1994) and the escape of the arrest of metaphase II (Whitaker and Irvine et al., 1984). The protocols are experimental for each species being those taken into account for this work, those applied in close species such as other South American camelids (llamas), camelids of the old continent (camels, camels), etc.

Some studies in alpaca have reported an increase of 29% to 63% (Sansinema et al, 2007) in the increase of embryos by this technique, as well as in camels. (Wani et al, 2018). In this case, no embryos were obtained more than in the group in which the oocytes were activated after intracytoplasmic injection (31.3%). On the other hand, it is observed that in flames have been reported data on different activation techniques, only ICSI reported 29% (4/14), while those activated with ionomycin and DMAP were 63% (15/24) (Sansinema et al., 2007) which correlates with the results found.

The culture conditions in both methods were the same: 38.5 ° C temperature, 5% CO2 and 100% relative humidity; It is known that if the conditions resemble the natural microenvironment of the uterus, this could be positively reflected in the number of embryos that develop. For example, in this work we controlled the percentage of CO2 but not the other gases such as nitrogen or oxygen. Since the oxygen tension in the female reproductive tract is low, cases have been reported where a greater success is shown in the results when the oxygen tension is 5% or 10% during the embryo culture. (Harvey et al., 2007)

The number of fertilized oocytes, as well as that of clivated embryos, did not present significant differences ($p>0.05$) (Figure 13, 14). Like the analysis performed in comparison of the stages of 4 cells, 8 cells and morulae. However, it should be noted that a greater number of embryos reached more advanced stages such as morula (12.5%) in the group fertilized by ICSI / Activation compared to those obtained by IVF (4.3%)

No embryo that reached the blastocyst stage was reported in the results (Table 5, Figure 12). Some studies relate blastocyst production to the effect of oxygen tension, as well as the use of different somatic cells during culture. On the other hand, low oxygen tension may have a relationship or be beneficial when individual oocytes are cultured but not a group with somatic cells.
The observation and analysis of DNA by fluorescence microscopy is proof of the presence of nuclei and the subsequent classification of embryos by their cleavages and stages, as reported by other authors (Conde et al., 2008)
The protocols proposed by the literature indicate that both the means used and the culture conditions for this procedure are of vital importance for its success. The quality of the sample and the treatment from the collection to its arrival to the work area are crucial for the maturation of the gametes, as well as for fertilization, mainly if they come from a slaughterhouse (Klumpp, 2004; Arriaga et al., 2014). That is to say, the long periods of storage, as well as the transport temperature, are relevant as reported by several studies in which the quality, number and development of embryos are related. (Ratto, et al., 2005, Chileno & Cainzo, 2014, Chuquitaype, 2015, Ruiz, et al., 2017 and Torres, 2017) Samples used arrived from 22 to 24 hours after slaughter, at 4 ° C . Work in bovines has reported that damage is possible because the lipid membrane undergoes changes at temperatures below 20 ° C (Arab et al, 1996), as well as lesions in the spindle and hardening of the zona pellucida. (Wlodarczyk et al, 2009)
However, these lipid analyzes have not been evaluated in alpacas. Studies carried out on species such as llamas or dromedaries indicate that the maturation time is in the range of 32 to 44 hours (Abdoon et al., 2001, Wani et al., 2005), but it is also known that the performance of aged oocytes is less in relation to fertilization and embryo development, since there is a probability of damage to cortical granules and microtubules (Long CR et al., 1994, Hunter RHF et al, 1989). These observations are related to the quality of gametes In this study, oocytes were evaluated at 31 hours followed by the application of in vitro methods: IVF and ICSI. The conclusion is that the production of alpaca preimplantation embryos is possible using both the IVF and the ICSI technique plus chemical and physical activation.

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