Effect of chemical activators after intracytoplasmic sperm injection (ICSI) on embryo development in alpacas

Dionet Keny Bellido-Quispe [a,*], Fidel Rodolfo Mujica Lengua [b], Mijaíl Contreras Huamani [a], J. Manuel Palomino [c]

[a] *Instituto Nacional de Innovación Agraria, Estación Experimental Agraria Canaán, Laboratorio de Biotecnología Reproductiva, Ayacucho, Peru*
[b] *Universidad Nacional de San Cristóbal de Huamanga, Facultad de Ciencias Biológicas, Laboratorio de Biotecnología, Ayacucho, Peru*
[c] *Universidad Científica del Sur, Carrera de Medicina Veterinaria y Zootecnica, Lima, Peru*

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ABSTRACT

Low motility and low sperm concentration are characteristics of alpaca semen. Thus, the intra-cytoplasmic sperm injection (ICSI) technique represents an alternative to improve the repro-ductive capacity of the male. However, the effect of post-ICSI activation in alpaca is not yet known. The aim of the present study was to compare the effect of chemical activators on alpaca embryo development after ICSI. Alpaca ovaries were collected from a local slaughterhouse and transported to the laboratory. Category I, II and III oocytes were matured for 30 h at 38.5 °C. After ICSI, injected oocytes were randomly divided and activated as follows: i) 5 μM ionomycin for 5 min, ii) 7% ethanol for 4 min, iii) 5 μM ionomycin for 5 min, window period 3 h plus 7% ethanol for 4 min, iv) 5 μM ionomycin for 5 min, window period 3 h, a second ionomycin treatment for 5 min, followed by 1.9 mM 6-DMAP for 3 h, v) 10 mM $SrCl_2$ for 3 h. Culture was carried out for 5 days in SOFaa at 38.5 °C. The cleavage rate was the lowest in the $SrCl_2$ group, morula devel-opment was the lowest in the $SrCl_2$ and without activation groups, and blastocyst stage was not different between groups (P<0.05). The rates with $SrCl_2$ were lower in total embryos produced, whereas in transferable embryos they were lower with 2Io/6-DMAP and with $SrCl_2$ (P<0.05). In conclusion, alpaca oocyte activation is more efficient with ionomycin and ethanol to produce transferable embryos.

1. Introduction

Intracytoplasmic sperm injection (ICSI) is a technique that involves the direct injection of a single sperm into the ooplasm of a mature oocyte arrested in metaphase II (Palermo et al., 2017). This technique had satisfactory results in humans and animals giving live offspring after embryo transfer. The ICSI procedure has been shown to be sufficient to trigger all oocyte activation events in humans (Catt and Rhodes, 1995; Tesarik et al., 1994; Van Steirteghem et al., 1993), mice (Kimura and Yanagimachi, 1995), rabbit (Keefer, 1989), hamster (Uehara and Yanagimachi, 1976) and horse (Bedford et al., 2003; Galli et al., 2002); while in cattle (Suttner et al., 2000), sheep (Hernández-Pichardo et al., 2016), goats (Kharche et al., 2016), and pigs (Choi et al., 2004), activation is incomplete. Therefore, additional stimuli are required to increase activation rates which will increase the number of oocytes that continue their development into blastocysts (Ferrer-Buitrago et al., 2018). In this scenario, artificial oocyte activation (AOA) and

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sperm pretreatment are the most used strategies to help complete activation. AOA helps induce activation within the oocytes and sperm pretreatments help increase nuclear decondensation and the formation of the male pronucleus. Using these strategies, a significant increase of embryo development to blastocyst has been reported (Lee et al., 2015; Habsah et al., 2016; Arias et al., 2016).

In all cases, after the fusion of the sperm membrane with the oocyte, a process known as oocyte activation occurs (Sanders and Swann, 2016). This event is caused by a series of repeated increases in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) leading to a series of morphological and biochemical changes (Yanagida et al., 2001). These changes are consequence of exocytosis of cortical granules, resumption of meiosis, extrusion of the second polar body, rearrangement of cytoskeleton, recruitment of maternal mRNA (translation of maternal mRNAs), decondensation of sperm DNA, formation of pronuclei and the transition from meiosis to mitosis (Ajduk et al., 2006; Swann and Lai, 2016; Swann et al., 2004; Swann and Yu, 2004). On the other hand, the normal physiological processes of oocyte activation can be triggered by chemical and physical (mechanical and electrical) compounds (Kashir et al., 2022). From those, chemical activation is the most used strategy for ICSI (Ferrer-Buitrago et al., 2018).

Chemical activation can be done with activators that induce a single increase of $[Ca^{2+}]_i$, with inducers of dynamic oscillations of $[Ca^{2+}]_i$ or those independent of calcium (Ferrer-Buitrago et al., 2018). Examples of activators that induce a single increase in calcium are ethanol (EtOH) (Emuta and Horiuchi, 2001; Korkmaz et al., 2013; Lee et al., 2015), ionomycin (Io) (Bevacqua et al., 2010; Lee et al., 2015; Sansinena et al., 2007) and calcymycin (A23187, IoCa) (Habsah et al., 2016; Lee et al., 2015; Tasripoo et al., 2012). For inducers of calcium oscillations, we have strontium chloride (SrCl$_2$) (Bevacqua et al., 2010; Kim et al., 2014; Norozi-Hafshejani et al., 2018; Vanden Meerschaut et al., 2013); and for chemical compounds independent of calcium may be those of broad spectrum such as 6-dimethylaminopurine (6-DMAP) (Arias et al., 2016; Bevacqua et al., 2010; Castro-Modesto et al., 2022; Conde et al., 2008; Moulavi et al., 2021; Sansinena et al., 2007; Tasripoo et al., 2012), cycloheximide (CHX) (Arias et al., 2016; Tasripoo et al., 2012), anisomycin (ANY) (Arias et al., 2015, 2016) and puromycin (Lu et al., 2006); or those more specific compounds such as roscovitine (Rosc) (Suvá et al., 2015; Wani and Hong, 2018) and dehydroleucodine (DhL) (Vichera et al., 2010). Recently, zinc chelators have had greater relevance for oocyte activation because they can activate them without affecting calcium changes nor reducing the zinc content (de Macedo et al., 2019; Uh et al., 2019; Ferrer-Buitrago et al., 2020). These chelators include N, N, N′, N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), 1,10-phenanthroline (PHEN) and tris(2-pyridylmethyl)amine (TPA). The latest findings open a new alternative for the artificial activation of oocytes, both in single treatments and in combination with the most used compounds, which should be explored in the activation of alpaca oocytes.

Alpaca semen is characterized for its low volume, motility, and sperm concentration at the time of collection (Garnica et al., 1993). Therefore, the ICSI technique represents an alternative to improve the reproductive capacity of the male. However, the efficiency of *in vitro* production of alpaca embryos by ICSI is low because it depends, among other factors, on an additional step, referring to the adequate activation of the oocytes (Castro-Modesto et al., 2022). There are very few reports about the use of activators on injected oocytes in camelids. Among others, ionomycin/roscovitine (Wani and Hong, 2018), ionomycin and ionomycin/6-DMAP (Castro-Modesto et al., 2022; Conde et al., 2008; Sansinena et al., 2007) have been used in this species after ICSI. Furthermore, to the best of our knowledge, the ICSI is a recently developed technique in alpaca (*Vicugna pacos*); therefore, the results are still inconclusive compared to the knowledge that has been learned in other mammals. On this basis, the present research aimed to determine a suitable chemical activator that can be used after ICSI in the alpaca species.

2. Materials and methods

2.1. Facilities

The study was conducted at the Laboratorio de Biotecnología Animal, Estación Experimental Agraria "Canaán", Instituto Nacional de Innovacion Agraria (INIA). The experiment was carried out in accordance with Peruvian National Law No. 30407: "Animal Protection and Welfare State.".

2.2. Cumulus-oocyte complexes (COCs) collection and maturation

Alpaca ovaries were collected from the municipal slaughterhouse of Huancavelica (74°58'21.58" longitude west and 12°46'57.4" latitude south, Perú) and transported to the Laboratorio de Biotecnología Animal, Estación Experimental Agraria "Canaán", Instituto Nacional de Innovacion Agraria (INIA), in sterile 0.9% saline solution with 80 μg/mL of gentamicin at 4–10 °C. At the laboratory, COCs were recovered from follicles of 2–7 mm in diameter by slicing and classified according to Ratto et al. (2005). Subsequently, COCs categories I, II and III were incubated for 30 h at 38.5 °C, 5% CO$_2$ and high humidity in TCM-199 supplemented with 10% FBS, 0.6 mM pyruvate, 2.5 μg/mL FSH, 2.5 μg/mL of LH, 29.2 μg/mL of L-glutamine, 0.2 μg/mL 17-β estradiol, 10 ng/mL IGF-I, 10 ng/mL EGF and 50 μg/mL of gentamicin. This media was obtained and slightly modified from Huamani et al. (2023).

After 30 h of maturation, expanded COCs were denuded with 0.5% hyaluronidase and, under a stereomicroscope (Motic, model: SMZ-168) the polar corpuscles were searched. Only mature oocytes with uniform cytoplasm and intact zona pellucida were selected for injection. Expanded oocytes were maintained in the maturation medium until the moment of fertilization by ICSI at 38.5°C, 5% CO$_2$ and saturation humidity.

2.3. Sperm capacitation

Alpaca testes were collected from the municipal slaughterhouse of Huancavelica and transported at 4–10 °C to the laboratory of

reproduction (INIA). At the laboratory, the sperms were recovered from the tail of the epididymis and diluted with a commercial dilutor AndroMed®. After that, the dilution was stored under refrigeration at 4 °C for 30 h. Subsequently, the *Swim up* technique was used to select and capacitate the sperms. Briefly, 100 μL of diluted sperm were placed in 1 mL *Sperm* TALP, then centrifuged at 307 x *g* for 3 min. The sediment was placed in 1 mL of *Sperm* TALP to be centrifuged again in the same conditions. Finally, it pellet with sperms was incubated in 500 μL of *Sperm* TALP for 1 h at 45° with a temperature of 38.5°C, 5% CO_2 and saturation humidity.

2.4. Intracytoplasmic sperm injection (ICSI)

Injection was performed with an inverted microscope (Carl Zeiss/Axiovert 135, Oberkochen, Germany), micromanipulator (Kanetec, MB.PM) and two pneumatic injectors (IM-11–2; Narishige Inc). The sperms were manipulated with a micropipette for injection with ID: 5 μm (IC-5–30-S; GYNEMED) and oocytes with a Holding micropipette with ID: 20–25 μm and DE: 120–130 μm. Both micropipettes were handled with an angle of 30°. The Holding micropipette was fabricated using a Puller (PC-10 H, Narishige) and Microforge (MF2, Narishige).

Before ICSI, the injection plate was prepared. Briefly, in a 35×15 mm Petri dish, 3 drops of 20 μL of TL medium (Tyrode's lactate) supplemented with 2% FBS and 0.2 mM pyruvate were prepared. On each drop, 10–15 matured oocytes were placed. Also, 2 drops of 10 μL with 5% PVP was prepared on the same plate and on one drop, 2 μL of the capacitated spermatozoa solution was placed. All of the drops were covered with mineral oil.

To perform the injection, the pipettes (Injection and Holding) were connected to the injectors. Then, small amounts of mineral oil were aspirated by capillary action in the injection pipettes followed by small amounts of 5% PVP solution. After that, spermatozoa with progressive motility and normal morphology were immobilized by hitting their tail with the injection pipette and the bottom of the plate. Immotile sperm was aspirated through the tail into the pipette and the injection pipette was brought to the medium with oocytes. Subsequently, using the Holding pipette, an oocyte was held and oriented so that the spindle was not damaged. Then, around the spindle free area the injection pipette is introducing through the zona pellucida and the plasma membrane (oolemma). A small amount of cytoplasm was aspirated to confirm that the plasma membrane of the oocyte had been breached. The sperm was injected into the cytoplasm along with the removed cytoplasm and little amount of extracellular medium. Finally, the injection pipette was removed, and the oocyte was released (Fig. 1).

2.5. Oocyte activation

In this study, Ionomycin (Io, SC-263405, ChemCruz®), 6-dimethylaminopurine (6-DMAP, SC-203220, ChemCruz®), strontium chloride ($SrCl_2$, SC-258180, ChemCruz®) and ethanol (EtOH, 107017, Sigma) were used as activators. The injected oocytes were placed in TCM-199 supplemented with 12 mM HEPES and 10% FBS for 30 min for Io, Io/EtOH, 2Io/6-DMAP and $SrCl_2$ or 4 h for EtOH
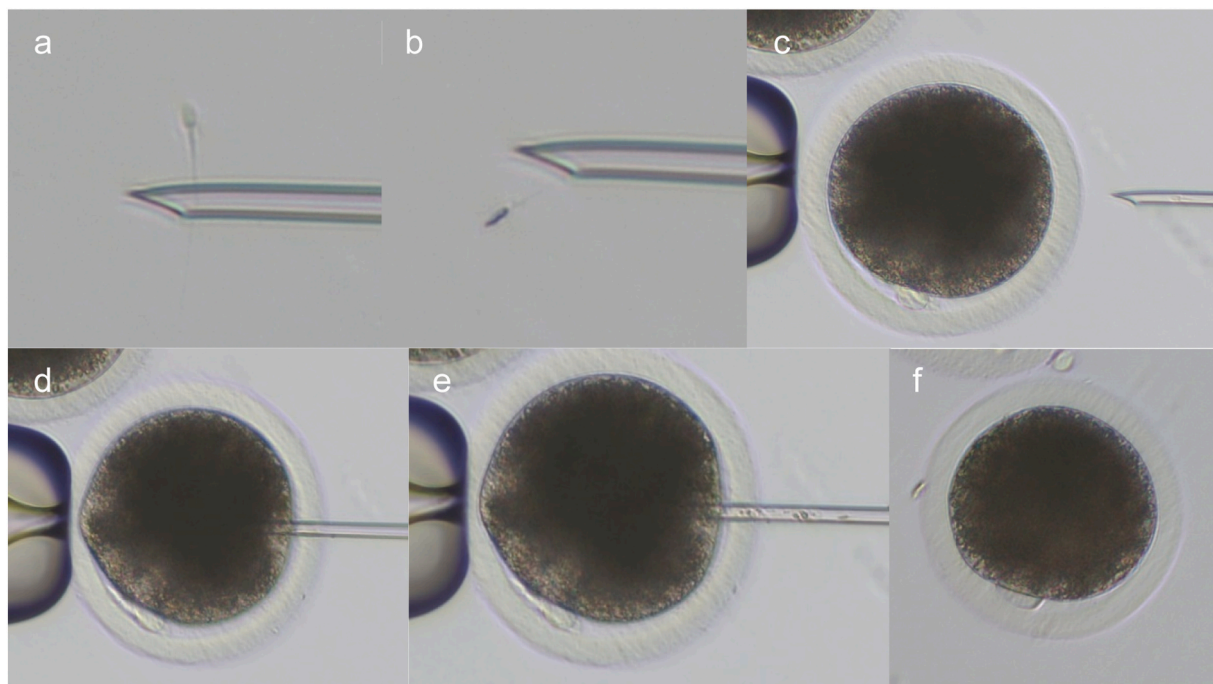

Fig. 1. *In vitro* fertilization by intracytoplasmic sperm injection (IVF-ICSI) in alpaca oocytes. a-b, immobilization, and aspiration of the sperm into the injection pipette. c-e, polar body positioning, oocyte retention, and sperm injection. f, oocyte injected with sperm.

at 38.5 °C and 5% CO_2, and the activation was carried out as described:

Ethanol: Activation was carried out according to Tasripoo et al. (2012) and Emuta and Horiuchi (2001), with some modifications. After 4 h postinjection, oocytes were treated with 7% ethanol for 4 min at room temperature in TCM-199 supplemented with 12 mM HEPES and 10% FBS.

Ionomycin: Activation was adapted from the calcium ionophore activation method (A23187) described by Tasripoo et al. (2012). Oocytes were exposed to 5 μM ionomycin for 5 min in TCM-199 supplemented with 12 mM HEPES and 10 % FBS at 38.5 °C, 5% CO_2 and humidity at saturation.

Ionomycin followed by ethanol: Activation was adopted from the method described by Bevacqua et al. (2010), with some modifications. Oocytes were exposed to 5 μM ionomycin for 5 min in TCM199 supplemented with 12 mM HEPES and 10% FBS at 38.5 °C, 5% CO_2 and humidity at saturation. After 3 h window period, oocytes were treated with 7% ethanol for 4 min at room temperature in TCM-199 supplemented with 12 mM HEPES and 10% FBS.

Ionomycin followed by 6-dimethylaminopurine (6-DMAP): Activation proceeded according to Bevacqua et al. (2010). Oocytes were exposed to 5 μM ionomycin for 5 min in TCM-199 supplemented with 12 mM HEPES and 10% FBS at 38.5 °C, 5% CO_2 and humidity at saturation. After a 3 h window period, they were exposed to a second ionomycin treatment under the same conditions, then treated with 1.9 mM 6-DMAP for 3 h in TCM-199 supplemented with 12 mM HEPES and 10% FBS at 38.5 °C, 5% CO_2.

Strontium chloride: Activation was carried out according to the protocols described by Kim et al. (2014), Norozi-Hafshejani et al. (2018) and Bevacqua et al. (2010), with some modifications. Oocytes were exposed to 10 mM $SrCl_2$ for 3 h in TCM-199 supplemented with 12 mM HEPES and 10% FBS at a temperature of 38.5 °C, 5% CO_2, 5% O_2, and 90% N_2.

2.6. In vitro embryo culture

After activation, oocytes were transferred to 500 μL of SOFaa culture medium (Trasorras et al., 2012). The culture was carried out in 4-Well plates with a gas mixture (5% CO_2, 5% O_2 and 90% N_2) and incubated at 38.5 ºC with an atmosphere of 5% CO_2 and 90% relative humidity. Cleavage rates were evaluated at 48 h and embryonic development (embryonic stages and embryonic quality) were evaluated at 120 h after onset of culture. Morphological evaluation of embryos was done using an inverted microscope at 40X (Carl Zeiss / Axiovert 135, Oberkochen, Germany) with a thermal stage and according to the criteria of the International Embryo Technology Society (IETS) guidelines (Stringfellow and Givens, 2010; Bó and Mapletoft, 2013).

2.7. Statistical analysis

Cleavage, morula, blastocyst, total embryos, and transferable embryos rates were compared by Fisher's exact test or Chi-square test using R software version 4.2.2 (R-4.2.2 https://cran.r- project.org/). Statistical significance was set up at $p < 0.05$.

3. Results

3.1. Embryo development after ICSI oocyte activation

The effects of activation with Io, EtOH, Io/EtOH, 2Io/6-DMAP, $SrCl_2$ and control group (without activation) post ICSI on embryonic development *in vitro* are shown in Table 1, Fig. 2 and Fig. 3. Cleavage rates with $SrCl_2$ were significantly lower than the Io, EtOH, Io/EtOH treatments, but similar compared to the 2Io/6-DMAP and without activation treatments (P<0.05). Additionally, the morula rates obtained from the treatments with $SrCl_2$ and without activation were the lowest compared to the rest of the treatments. There were no differences in the blastocyst rates among the treatments (P<0.05).

3.2. Total and transferable embryos

The effects of Io, EtOH, Io/EtOH, 2Io/6-DMAP, $SrCl_2$ and control group (without activation) on total embryos and transferable

Table 1

Effect of chemical activators after intracytoplasmic sperm injection on *in vitro* development of alpaca embryos.

Treatment groups	n	Preimplantation embryo development		
		Cleavage (%)	Embryo stages	
			Morula (%)	Blastocyst (%)
Io	21	17 (81.0)[a,c]	12 (57.1)[a]	3 (14.3)
EtOH	22	19 (86.4)[a,d]	13 (59.1)[a]	4 (18.2)
Io/EtOH	15	13 (86.7)[c,d,f]	7 (46.7)[a]	5 (33.3)
2Io/6-DMAP	19	11 (57.9)[a,e,f]	8 (42.1)[a]	3 (15.8)
$SrCl_2$	20	7 (35.0)[b,e]	3 (15.0)[b]	4 (20.0)
Without activation	18	10 (55.6)[c,e]	7 (38.9)[a,b]	2 (11.1)

Io: Ionomycin, EtOH: Ethanol, 6-DMAP: 6-Dimethylaminopurine, $SrCl_2$: Strontium chloride. n: Number of oocytes injected.
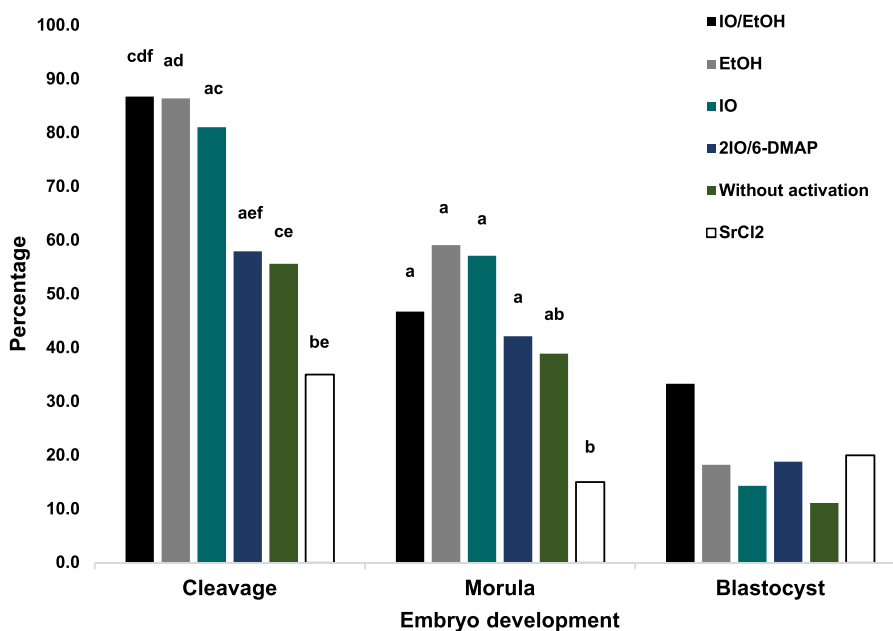Values with different letters in the same column differ (p<0.05).

Fig. 2. *In vitro* development of alpaca embryos after chemical activation. Cleavage at 48 h- and morula and blastocysts at 120 h of culture. The results are expressed in percentages of cleavage, morula and blastocyst of four replicates. Io: Ionomycin, EtOH: Ethanol, 6-DMAP: 6-Dimethylaminopurine, $SrCl_2$: Strontium chloride. Different letters on bars indicate statistically difference among treatments (p<0.05).

embryos are shown in Table 2, Fig. 4 and Fig. 5. Total embryos were considered all morulae and blastocysts present at the time of the evaluation. Transferable embryos were grades 1 and 2 homogeneous embryos with no blastomeres extruded. The classification was done using the IETS guidelines (Stringfellow and Givens, 2010). The $SrCl_2$ group was the lowest in total embryos but was not different to the without activation group. Whereas, Io and EtOH groups had higher transferable embryos compared to the 2Io/6-DMAP and $SrCl_2$ groups (P<0.05).

4. Discussion

In this study, we demonstrated that chemical activation after intracytoplasmic sperm injection improved the activation of alpaca oocytes, in terms of embryo development of cleavage and morula stages. Also, the activation affects the results of total embryo (morula and blastocysts) and transferable embryo (Grades 1 and 2 embryos) rates.

Our results showed that the chemical activation of oocytes with ionomicina (Io), EtOH, Io/EtOH, or the combination 2Io/6-DMAP increased cleavage rates after 48 h of *in vitro* culture compared to the activation with $SrCl_2$. In camelids, few reports are available on the use of chemical compounds to activate oocytes after ICSI and have variable results. The cleavage rates of 86.7% obtained in this study are superior to some previous reports. For instance, in llamas, cleavage rate was 33% with Io and 63% (Sansinena et al., 2007) and 36% (Conde et al., 2008) with Io/6-DMAP. In alpacas, cleavage rate was 43.8% with Io/6-DMAP (Castro-Modesto et al., 2022). In dromedaries, cleavage rate was 72.7% with Io/6-DMAP (Moulavi et al., 2021), and 58.9% with Ros/Io (Wani and Hong, 2018). Additionally, in cattle, the use of different chemical oocyte activators has also been investigated. The cleavage rates reported were 32.7% with IoCa (Tasripoo et al., 2012), 60.0% with 2Io, 64.7% with Io/EtOH (Bevacqua et al., 2010), 71.7% with 2Io/EtOH, 78.8% with 3Io/EtOH (Lee et al., 2015), and 81.2% (Arias et al., 2016) to 82.7% with 2Io/6-DMAP (Bevacqua et al., 2010). CHX resulted in a cleavage rate of 55.1% when used alone (Tasripoo et al., 2012), and of 83.4% when used in combination with Io (Arias et al., 2016). Therefore, we can speculate that, in alpacas, the individual or combined use of Io and EtOH is sufficient to trigger all activation events with minimal damage in alpaca oocytes, compared to other chemical compounds.

The cleavage rate of 35% with $SrCl_2$ in this study was lower than the one reported previously in humans (Fawzy et al., 2018; Kim et al., 2014; Norozi-Hafshejani et al., 2018) and cattle (Bevacqua et al., 2010). In humans, activation was done in a Ca^{2+} and Mg^{2+} free media and the exposure time was 60 min, while in this study, activation was done in media with Ca and Mg and the exposure time was 3 h. It is unknow if the prolonged exposure had negative effects on the oocytes or if the different media composition affected the results.

According to the results reported to date in the different species, it seems that the success of the methods of oocyte activations varies among species (Arias et al., 2016; Bevacqua et al., 2010; Moulavi et al., 2021; Castro-Modesto et al., 2022). Importantly, the variability of the mechanism of oocyte activation is believed to be species-specific. Therefore, finding a method for oocyte activation in a specific species is of vital importance for the ICSI technique. In this sense, zinc chelators were used as a new way to activate oocytes without causing changes in calcium concentration (Uh et al., 2022). In pigs, post-ICSI oocytes were activated with TPEN combined with Io,
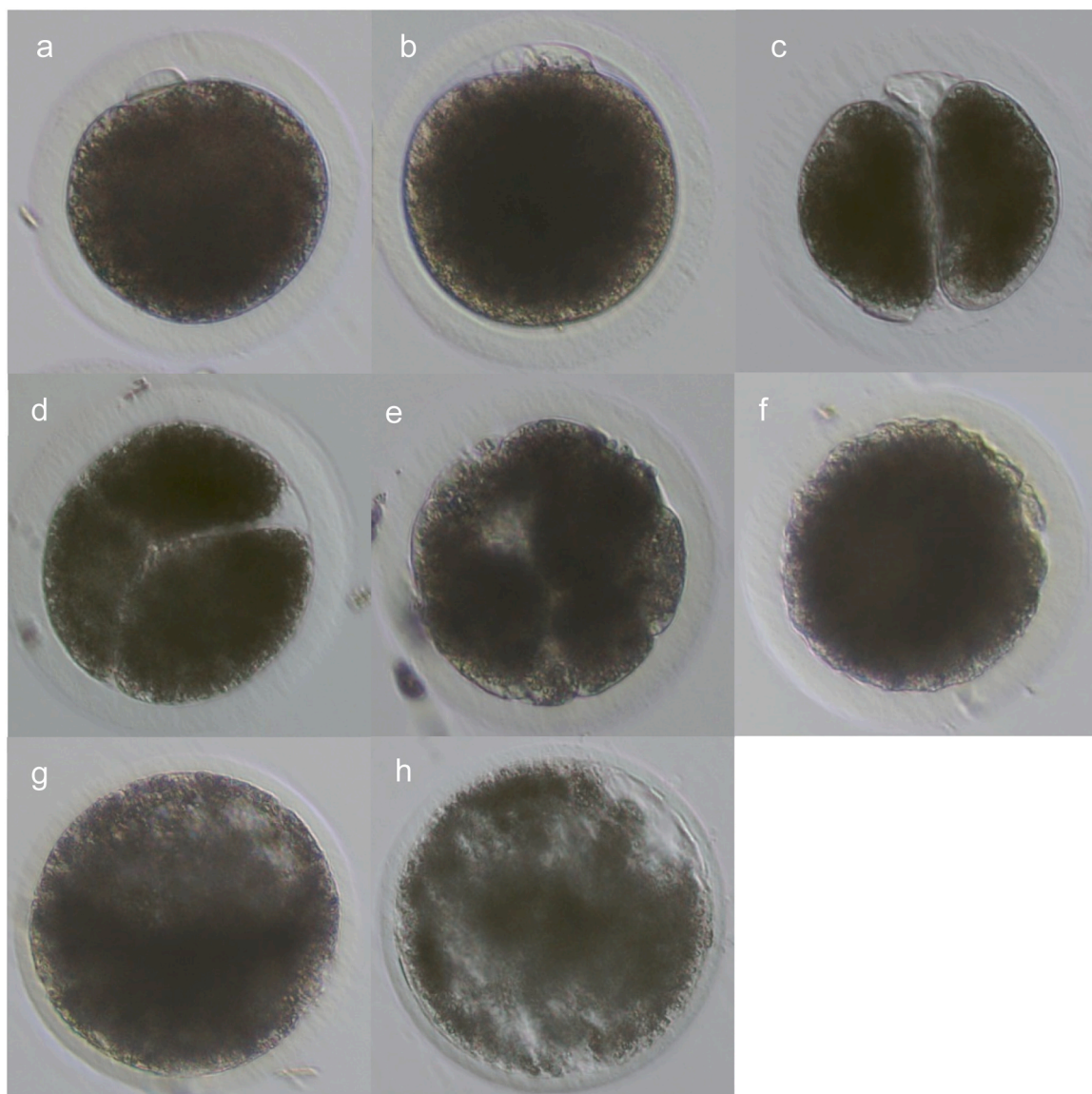
Fig. 3. Alpaca embryos obtained from ICSI after 5 days (120 h) of *in vitro* culture. (a) mature oocyte (MII); (b) zygote; (c) 2-cell embryo; (d) 4-cell embryo; (e) early morula; (f) compact morula; (g and h) blastocysts.

Table 2
Effect of chemical activators after ICSI on the production of total and transferable embryos in alpacas.

Treatment groups	n	Total embryos (%)	Transferable embryos (%)
Io	21	15 (71.4)[a]	12 (57.1)[a]
EtOH	22	17 (77.3)[a]	13 (59.1)[a]
Io/EtOH	15	12 (80.0)[a]	6 (40.0)[a, b]
2Io/6-DMAP	19	11 (57.9)[a]	4 (21.1)[b]
SrCl$_2$	20	7 (35.0)[b]	3 (15.0)[b]
Without activation	18	9 (50.0)[a,b]	7 (38.9)[a, b]

Io: Ionomycin, EtOH: Ethanol, 6-DMAP: 6-Dimethylaminopurine, SrCl$_2$: Strontium chloride. n: Number of oocytes injected.
Values with different letters in the same column differ ($p < 0.05$).

obtaining up to 75.2% cleavage (de Macedo et al., 2019) and with PHEN 72% (Briski et al., 2022). While, in mice, 62% was obtained with TPEN using knockout sperm for the PLCζ protein (Ferrer-Buitrago et al., 2020). In alpacas, there is a lack of knowledge of the use of these activators for oocyte in the ICSI technique. Furthermore, new insights into the role of zinc in improving complete activation
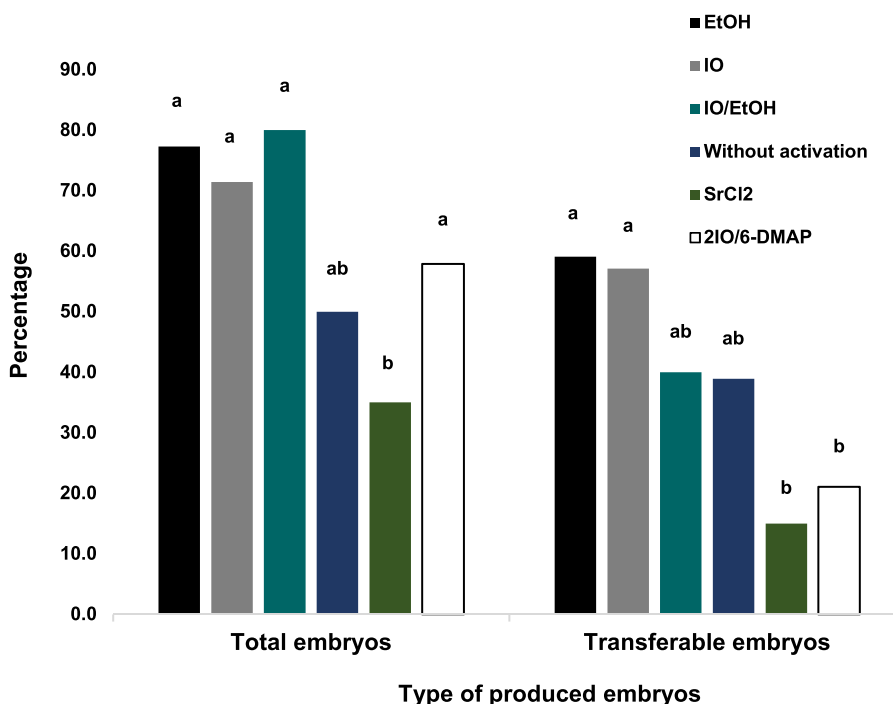
Fig. 4. Total embryos and transferable alpaca embryos produced after chemical activation and evaluated at Day 5 (120 h of culturing *in vitro*). The results are expressed in percentages of total embryo and transferable embryo of four replicates Io: Ionomycin, EtOH: Ethanol, 6-DMAP: 6-Dimethylaminopurine, SrCl$_2$: Strontium chloride. Different letters on bars indicate statistically difference among treatments ($p<0.05$).

efficacy need to be tested in this species.

Additionally, there are different causes of oocyte activation failure, in which both oocyte and sperm factors are thought to be involved. In oocytes, one of the most important factors is the poor cytoplasmic maturation caused by low Ca^{2+} concentration in the endoplasmic reticulum (ER), and by a low number of mitochondria; mitochondria play an important role in replenishing Ca^{2+} in the cytoplasm and the ER are crucial for energy production (Wang et al., 2018). In the sperm, the release of the sperm-borne oocyte activating factor (SOAF) plays a role in oocyte activation (Homa et al., 1993; Kline, 2000). Therefore, the use of poorly matured oocytes and the lack of release of SOAF to the cytoplasm after ICSI may reduce oocyte activation. In this regard, Korkmaz et al. (2013) used different oocyte qualities and different activators after ICSI, and reported poor cleavage and embryonic development rates when using low quality oocytes, which would have experienced inadequate cytoplasmic maturation.

Furthermore, success of oocyte activation may be compromised when care is not taken during the ICSI injection procedure whether in the aspiration of the oocyte cytoplasm, injection time (1–2 min per oocyte), positioning of the polar body, injection medium used (for the first increase in [Ca^{2+}]$_i$), and the concentration of polyvinylpyrrolidone (PVP) solution (Mansour et al., 1996; Blake et al., 2000; Tesarik and Mendoza, 1999; Ding et al., 2020).

In standard ICSI procedures, the polar body (PB) of oocytes are positioned at 6 or 12 o'clock to minimize spindle damage caused by direct sperm injection. Blake et al. (2000) evaluated the effect of different positions of the PB on fertilization and embryonic development, and found that the 7 or 11 o'clock positions during ICSI resulted in high rates of fertilization and the development of the greatest proportion of high-quality embryos. Position of PB; however, not always is a true guide of the location of the metaphase plate, therefore, oocytes should be injected as far away as possible from the PB. In this study, the PB were oriented between 6 and 7 or 11–12 o'clock positions during ICSI, and those did not seem to affect the activation of the oocytes in terms of embryo development.

In this study, the medium used for ICSI was TL medium (Tyrode's lactate) supplemented with 2% FBS and 0.2 mM pyruvate, and resulted in a greater activation of alpaca oocytes compared to that reported previously after using Global Total® with HEPES medium Ca^{2+}-Mg^{2+}-free (Castro-Modesto et al., 2022). There are variations in the chemical ingredients between G-TL™ and Global® (Morbeck et al., 2017) and these may explain some differences in embryo development. In other species, some frequent media used for ICSI are IVF-TALP (bovine; Korkmaz et al., 2013; Keller et al., 2017), TCM-199 with HEPES (equine; Agnieszka et al., 2021; Cheng et al., 2012) and TALP-H (feline; Moro et al., 2014).

To facilitate sperm handling, PVP has been used for ICSI in a variety of species to make the medium more viscous and immobilize individual sperm (Kato and Nagao, 2012). PVP, however, can harm sperm membranes and lower fertilization rate (Kato and Nagao, 2012). The use of a lower concentration of PVP solution for ICSI treatment has been found to significantly enhance the development of embryos; 5% is an optimal concentration (Ding et al., 2020). Therefore, in the present study, the PVP was used in a concentration of 5%, which differs from previous studies that used a concentration between 7% and 10%.

All chemical activators used in the present study in alpacas resulted in embryo development to the morula and blastocyst stages.
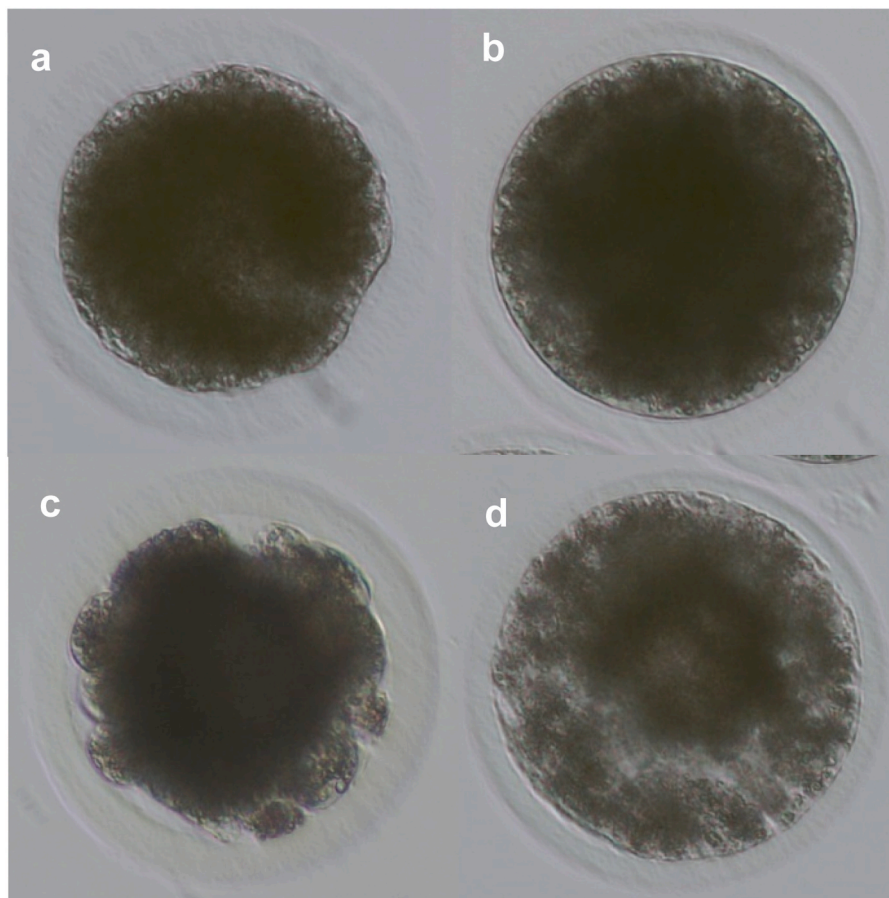
Fig. 5. Alpaca embryos evaluated at Day 5 (120 h of *in vitro* culture). Morulae: (a) and (c). Blastocysts: (b) and (d). Transferable embryos: (a) and (b). Non-transferable embryos: (b) and (d).

When comparing to other studies that used lo/6-DMAP, our results were superior to the morula rate reported in alpacas (12%, Castro-Modesto et al., 2022), and to the blastocyst rates reported in llamas (12%, Sansinena et al., 2007; 16%, Conde et al., 2008). But it was inferior to the blastocyst rate reported in dromedary camels (28.1%, Moulavi et al., 2021). Importantly, when using lo/EtOH, our morula and blastocyst rates were superior to all previous ICSI reports in camelids and dromedary camels. The blastocyst rate of this study (33%) was also superior to the one reported in dromedary camels (20.1%) when using lo and roscovitine, a potent inhibitor of the maturation-promoting factor or MPF (Wani and Hong, 2018).

No blastocysts were obtained when llama embryos were cultured in CR1aa medium (Sansinena et al., 2007), or when alpaca embryos were cultured in KSOM medium (Castro-Modesto et al., 2022). In the present study, embryos were cultured in SOFaa medium. In bovine, it was reported that SOFaa supports higher development to blastocyst stage than KSOMaa and CR1aa (Sagirkaya et al., 2006). There is limited number of IVP studies in camelids, but as in other species, there is a need for more in-depth evaluation of the effect of culture media on embryo development.

In ICSI, sometimes occurs an abnormal fertilization (3PN or 1PN) due to lack of extrusion of the second PB, alterations of spindle microtubules, parthenogenetic activation, premature fusion of the pronuclei (maternal and paternal), and asynchrony in pronuclear formation or appearance at the time of evaluation (Bevacqua et al., 2010; Macas et al., 1996; Wani and Hong, 2018). It is known that some embryos that come from abnormal fertilization have the capacity of become blastocysts; but the majority have reduced embryo development (Lee et al., 2015; Li et al., 1999; Wani and Hong, 2018). We can speculate that parthenogenetic embryos in alpacas can reach 30% in 8-cell stages as it has been reported in cattle (Li et al., 1999). Therefore, it is important for future studies to determine the number of pronuclei in zygotes fertilized by ICSI, with or without activation, to estimate the production of parthenogenic embryos.

In this study, the use of 6-DMAP and SrCl$_2$ resulted in similar number of transferable embryos. In other species, 6-DMAP is widely used to activate oocytes but it has been postulated that its broad-spectrum phosphorylation inhibition may inhibit or permanently destroy a number of unknown kinases important for early nuclear remodeling and reprogramming (Alexander et al., 2006). In addition, it has also been documented that 6-DMAP could bypass the metaphase stage in some nuclei and resume division in S phase; cause chromosomal anomalies such as mixoploidy, polyploidy, and abnormal zygote bodies; and prevent the second polar body extrusion (Bhak et al., 2006; Ock et al., 2003; Oikawa et al., 2005). By affecting metabolic pathways in the oocyte, and producing

chromosomal changes in the embryos, 6-DMAP may impair embryonic development potential or viability (Oliveira et al., 2014). Unlike 6-DMAP, there is no much information of the effects of $SrCl_2$ in the oocytes or embryos. While $SrCl_2$ has been shown to induce calcium oscillations and oocyte activation in mouse models (Ma et al., 2005). It is unknow if $SrCl_2$ induces calcium oscillation in other species, if the oscillations differ from physiological conditions, or if its use on oocyte activation carries potential risk (Liu et al., 2023). A major increase or accumulation of Ca^{2+} in mitochondria can alter mitochondrial morphology, redox status and ATP production, leading to oxidative stress and apoptosis (Wakai and Fissore, 2013; Zhang et al., 2020).

Currently, there are many challenges in the *in vitro* production of embryos in alpacas and llamas, including but not limited to the low number of oocytes available for embryo production and the long distance from animals' pastures to handlings and research facilities. For instance, in previous studies in camelids, were used only between 14 and 24 llama oocytes per treatment group (Sansinena et al., 2007) and 16 alpaca oocytes per treatment group (Castro-Modesto et al., 2022). Despite using a similarly low number of oocytes per treatment group, the present study is the first to use multiple chemical compounds for oocyte activation following ICSI in alpacas.

5. Conclusion

In conclusion, the results of the present study show that the activation of alpaca oocytes is better when Io, EtOH or Io/EtOH is used for *in vitro* production of embryos by ICSI. Further studies, with a larger number of oocytes, are needed to determine an appropriate protocol for artificial oocyte activation in the *in vitro* production of alpaca embryos using ICSI. Finally, there are no reports of offsprings produced using ICSI in camelids. Therefore, it is necessary to evaluate *in vivo* studies with ICSI embryos to be able to rescue and produce elite animals that are of interest to alpaca producers.

CRediT authorship contribution statement

Dionet Keny Bellido-Quispe: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing, **Fidel Rodolfo Mujica Lengua:** Funding acquisition, Investigation, Project administration, Resources, Writing – review & editing, **Mijaíl Contreras Huamani:** Data curation, Investigation, Methodology, Writing – review & editing, **J. Manuel Palomino:** Formal analysis, Visualization, Writing – review & editing.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

Data availability

None of the data were deposited in an official repository. The data that support the study findings are available from the authors upon request and after authorisation by all authors.

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