In vitro maturation (IVM) of Alpaca *(Vicugna pacos*) oocytes with follicular fluid

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ABSTRACT

Studies related to the application of assisted reproduction techniques in alpaca are very limited, there are still many deficiencies in the techniques of maturation, *in vitro* fertilization and embryo culture. The aim of the present work was to evaluate the effect of follicular fluid as a supplement in the maturation medium on *in vitro* maturation rates of alpaca oocytes. The biological samples were collected at a local slaughterhouse in Huancavelica and transferred in 0.9% NaCl solution at 4ºC. In the lab, the cumulus-oophorus complexes (COCs) were isolated from the follicles by two methods (aspiration and slicing) and the selected COCs were placed in a maturation medium supplemented with fetal bovine serum (FBS), follicular fluid (FF) or SFB + FF and cultured at 38.5 ºC, 5% CO_2 and 100% of relative humidity for 32 to 36 hours. The maturity of the oocytes was evaluated post-culture by the presence of the first polar body. To determine the ability of mature oocytes to complete the processes of fertilization and embryonic development, 4 IVF trials were performed. We worked with spermatozoa isolated from the caudal area of the epididymis, which were selected by the swim-up method. Fertilization was carried out with a concentration of $2x10^6$ esp/mL in HAM medium supplemented with heparin and PHE as capacitating agents. The sperm and oocytes were cultured together under conditions similar to those used for maturation during 18 hours, after which the oocytes were washed and cultured in KSOM medium supplemented with FBS, sodium pyruvate and gentamicin for 7 days. First, the average of COCs recovered by the two methods used was compared. An average of 2.1 COCs isolated by ovary was obtained by the aspiration method, while with the slicing method an average of 1.2 COCs was obtained per ovary, finding a significant difference between these results ($p < 0.05$). In relation to the rate of maturation, for oocytes isolated by the aspiration method, with the medium supplemented with FF 24.7% of oocyte matured was found and when the medium was supplemented with SFB and SFB + FF, the rates for maturation were 25% and 19.2% respectively. For the group of oocytes isolated by slicing, a rate of 26.1% were obtained for medium supplemented with FF and 33.2% and 9.6% for oocytes matured in the media supplemented with SFB and SFB + FF respectively, however, for none of the cases significant difference was found between the groups ($p > 0.05$). For embryo production, a rate of 14.2% were obtained for medium supplemented with FF, while for media supplemented with SFB and SFB + FF, rates of 27.5% and 29.2% respectively were obtained, however, no significant difference was found between the groups ($p > 0.05$). In conclusion, the results suggest that the aspiration method shows a higher proportion of optimal COC isolation compared to the slicing method, likewise it shows that FF can be used as a natural supplement equivalent to SFB for *in vitro* maturation media for alpaca oocytes.

KEYWORDS: Alpaca, oocyte maturation, follicular fluid, *in vitro* fertilization, *Vicugna pacos*.

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INTRODUCTION

The South American camelids present considerable economic importance in South American countries such as Peru, Bolivia, Chile and Argentina, where they represent one of the main sources for the textile industry and particularly in the high Andean region where they are a source of meat and wool for many families (Santiani et al., 2016).

In the last decade, interest has increased in the application of assisted reproduction technologies in South American camelids (Apichela, et al., 2015), resulting in an increase in the number of reports of its application in the literature. In 2014, for example, the effect of 3 variables (age of the female donor of the ovaries, size of the follicle from which the CCO were obtained and presence or absence of SFB in the maturing medium) on maturity rates was studied. in vitro of alpaca oocytes, reporting a positive effect when follicles larger than 2mm in diameter were used (36% maturation) compared to the use of follicles less than 2mm in diameter (8% maturation). Likewise, supplementation of the medium with SFB showed a positive effect in relation to the rate of oocytes that reached metaphase II (57.6%) compared to oocytes cultured in medium without SFB (18.2%) (Leisinger et al., 2014).

On the other hand, also in 2014, the effect of the cultivation time on the post-FIV maturation and division rates in alpaca was evaluated, observing a higher proportion of oocytes that reached the metaphase II (75.3%) when these were cultivated during 42hs , compared to the crop for 30hs (26.3%), 34hs (52.6%) and 38hs (68.5%). (Chileno et al., 2014). In another report, in addition to the maturation time (24, 28 and 32 hours), the effect of the sperm selection method used (swim-up vs percoll) and the oxygen tension (20% vs 5%) present during in vitro cultures in alpaca. A significantly higher percentage of mature oocytes was reported when the culture was performed for 32hs (65.1%) compared to cultures performed during 24 and 28 hours (46.1% and 50.3% respectively), while the rate of blastocyst production was not seen affected neither by the variation of O2 tension or by the sperm selection method (Ruiz et al., 2017).

Within the techniques of assisted reproduction, the use of in vitro fertilization, in particular, represents an alternative to achieve the propagation of genetically superior animals from the point of view of production, especially those with fine fiber and natural colors. (Miragaya et al., 2006), and for the preservation of wild camelids (Ruiz, 2011, cited by Torres, 2017). However, despite the increase in the number of reports in relation to the study and application of assisted reproduction technologies in these species, its validation and commercial application remains in the early stages of its development, and there are still many deficiencies in the techniques of maturation, in vitro fertilization and culture of embryos applied in alpaca and related species (Brown, 2000, Morton et al., 2007, Leisinger et al., 2014, Ruiz et al., 2017).

The in vitro maturation of oocytes represents the starting point of not only in vitro fertilization and embryo production, but also of other techniques of assisted reproduction such as cloning, production of transgenic animals and research in stem cells (Kakkassery et al., 2010). However, in alpaca and in general in all species, it is still a great challenge to develop in vitro culture systems capable of maintaining the long growth and maturation processes, through which the oocyte can acquire the capacity to complete the Meiotic maturation as to maintain embryonic development (Silva et al., 2016).

Among the alternatives used, for the improvement of the effectiveness of in vitro oocyte maturation systems, studies in pigs and cattle showed that supplementation of maturation media with follicular fluid (FF) can promote maturation and / or fertilization in vitro of the oocytes and their subsequent embryonic development (Algriany et al., 2004; Klumpp et al., 2004; Bijttebier et al., 2008; Ito et al., 2008; Agung et al., 2010).

Based on the above, the objective of this work was to evaluate the effect of FF as a natural supplement of the maturation medium for alpaca oocytes.

MATERIALS AND METHODS

Biological samples

The biological material (ovaries and alpaca testes of the Huacaya race) used for the present work was obtained from the municipal camal of the city of Huancavelica, Huancavelica region, Peru (4050msnm, average annual temperature 8 ° C - 10 °C), using a total of 412 ovaries and 4 epididymides. For the transport of the ovaries and testes 0.9% saline was used, which was maintained at 4 ºC by the use of gel-packs and a thermo-insulating box.

Methods

Collection and transfer of biological samples (ovaries)

The biological samples (ovaries of alpaca) were collected in the municipal slaughterhouse of Huancavelica during the months of May to October 2018 and transported in a 0.9% NaCl solution at 4ºC to the Laboratory of Reproduction Physiology. Faculty of Biological Sciences-UNMSM (the approximate time from the benefit of the animal until its arrival at

Follicular fluid isolation

The follicular fluid used in the present work was collected in 2 different periods of time. The first group of ovaries used for FF isolation was collected during the months of January to December 2017 and stored at -20ºC for up to its use. The second group of ovaries used for the same purpose was collected during the first days of July 2018 and in the same way they were stored at -20ºC for up to their use.

Prior to the start of the maturation trials, the stored ovaries were thawed for follicular fluid (FF) isolation. Briefly, the already thawed ovaries were washed with sodium chloride and with a No. 21G needle attached to a 10ml syringe the follicular fluid of the follicles between 2-8mm in diameter was aspirated. The collected follicular fluid was centrifuged at 1500 rpm for 15 minutes, then the supernatant was recovered and placed in microtubes and centrifuged again at 2500 rpm for 5 minutes. Finally, the supernatant was recovered and filtered with 0.22µm pore filters. The filtered FF was placed in aliquots and stored at -20 ° C until use.

Isolation and selection of oocytes

For the in vitro maturation assays, once in the reproductive physiology laboratory, the ovaries were washed 2 times with phosphate buffered saline or phosphate buffered saline (BFS) plus penicillin 0.075g / L and streptomycin 0.075g / L a 38.5ºC and the tissues adhered to the ovaries were dissected.

Isolation by the aspiration method

The follicles between 2-8mm in diameter were then aspirated with a 21G needle attached to a 10mL syringe containing Dulbecco's solution plus penicillin 0.075g / L and streptomycin 0.075g / L at 38.5ºC. The follicular fluid aspirated was placed in a 15mL tube containing Dulbecco's solution plus antibiotics and kept in an incubator at 38.5ºC. Once the aspiration of all the follicles was finished, it was left to rest for 10 minutes, and then with the help of a pipette, recover the pellet formed at the bottom of the tube. The recovered pellet was placed in plates containing Dulbecco's solution at 38.5 ºC and with the help of a stereoscope the isolation, selection and washing of the morphologically normal CO CCO Cs was carried out, according to the evaluation of the number and compaction of the cell layers of the cumulus and homogeneity of the cytoplasm, for in vitro maturation (classes I and II described by Sánchez et al., 2003). The selected CCOs were washed 3 times more in the maturing medium, and then placed in a culture dish containing 50uL drops of the maturation medium with the corresponding supplements.

Isolation by the CCOs method

For the isolation of the CCOs by the cut method, after the aspiration the ovaries were placed on Petri dishes containing Dulbecco's solution at 38.5 ºC and superficial cuts were made on the entire surface of the ovaries. Then, with the help of a stereoscope, the class I and II CCOs were isolated and selected according to Sánchez et al., 2003 and the same procedure used for the CCOs isolated by the aspiration method was followed.

All the ripening media used were prepared at least 12 hours in advance, covered with mineral oil and kept in a CO2 incubator at 38.5ºC for stabilization of the pH and temperature until use.

Selection of CCOs for culture in the middle of maturation

For the selection of the CCOs, the morphological classification of those described by Torres in 2017 was used as a reference. Based on these characteristics, the isolated CCOs were classified into one of the corresponding categories Class 1 (CCO with more than 5 complete layers of cumulus cells that completely surround the oocyte and homogenous dark cytoplasm), Class 2 (CCO with less than 5 layers of cumulus cells), Class 3 (CCO with layers of cumulus cells present, but less compact and

inhomogeneous cytoplasm) and Class 4 (Oocyte with heterogeneous or fragmented cytoplasm and with cumulus cells completely disintegrated or absent).

Evaluation of the vitality of the CCO

For the evaluation of this parameter, BCCs were used that did not enter the culture in vitro. Those that were kept at 38.5 ºC in Dulbecco's solution until their evaluation. To determine the vitality, the remaining CCOs were isolated and placed in drops containing 0.4% trypan solution, diluted in BFS and maintained at 38.5 ºC. Live BCCs were considered as those that did not acquire any staining, after exposure to the vital blue dye of trypan, while CCOs that took a dark blue color after exposure were considered dead.

In vitro maturation oocytes

The maturation medium was composed of 199 medium supplemented with FSH 1.5uL / mL, eCG 10UI / mL, estradiol 17β 0.1μL / mL, epidermal growth factor (EGF) 50ng / mL, 0.2mM sodium pyruvate, serum bovine albumin (BSA)) 4mg / mL, fetal Bovine serum (10%) and gentamicin 50μg / mL. In addition, 3 supplements were evaluated for the maturation medium: fetal bovine serum (SFB) at 10%, follicular fluid (FF) at 10% and fetal bovine serum + follicular fluid (SFB + FF) both at 10%.

The CCOs selected for culture, after being washed, were randomly placed in culture plates containing 50μL drops of the maturation medium with the corresponding supplements (SFB, FF or SFB + FF) and in groups of 10-12 CCO per drop. The culture was carried out at 38.5 ° C 5% CO2 and 100% relative humidity for 32 to 36 hours.

After the cultivation period, the CCOs were washed in Dulbecco solution maintained at 38.5ºC. The cumulus cells that were still attached to the surface of the oocyte were mechanically detached (denudation) by gentle aspirations and exhalations by means of a Pasteur pipette (pipetting). The maturation of the oocytes was evaluated by the presence of the 1st polar body observed under the inverted light microscope and by fluorescence microscopy.

Evaluation of the nuclear stage of the oocytes and the presence of fragmented DNA

After the evaluation of maturity, both mature and immature cataloged oocytes were evaluated by means of fluorescence microscopy to determine the stage of the present meiosis. For DNA staining the denuded oocytes were exposed to a solution of BFS-Hoechst 33342 at a concentration of 5μg / mL for 15-30 minutes covered with light. After this time, the oocytes were evaluated by using a fluorescence microscope with a UV excitation filter and an emission wavelength of 461 nm at an increase of 40X.

The stages of meiosis were identified by means of the evaluation of the degree of condensation of the DNA present in each of the oocytes. The stages of the germinal vesicle and metaphase I were considered immature, while those in the metaphase II stage determined by the presence of the first polar body and / or the metaphase plate were considered mature. Finally, the oocytes that presented fragmented regions of stained DNA were cataloged as degraded oocytes.

Sperm selection by the swim-up technique

The testicles were washed with BFS supplemented with penicillin (0.075g / L) and streptomycin (0.075g / L). The caudal area of the epididymides was dissected and separated from each testicle and cleaned until the epididymis became free of adipose tissue and blood vessels. Once clean, the epididymis were cut into small pieces and washed with 500μL of HAM medium at 38.5 ° C and the suspension was allowed to stand for 5 minutes. After the time the supernatant was recovered and proceeded to centrifuge at 1600rpm for 6 minutes at 38.5 ° C. After centrifugation the supernatant was discarded and the pellet was resuspended in 500 μL HAM medium supplemented with 15mg / mL of BSA, the centrifuge tube containing the suspension was left in incubation (tilting the tube 45º degrees) during 60 minutes in an incubator of CO2 at 38.5 ºC. After this time, 200μL of the middle region of the suspension was recovered and the concentration and mobility of the spermatozoa were evaluated.

IVF

Four in vitro fertilization tests were performed, using mature oocytes and sperm isolated from the caudal epididymis region and selected by the swim-up method.

Once the ripening time elapsed, the oocytes were washed in fertilization medium and kept at 38.5 ºC, 5% CO2 and 100% relative humidity until fertilization. IVF was performed in 50μL microdrops of HAM-F10 medium, containing a final concentration of 2 million sperm per milliliter, supplemented with PHE (2mM Penicillamine, 1mM Hipotaurine and 250μM Epinephrine) and 10μL / mL heparin, used as capacitor agents . Between 10-15 mature oocytes were placed for each microdrop and the culture

plate was covered with mineral oil. The oocytes together with the sperm were incubated at 38.5 ° C, 5% CO2 and 100% relative humidity for 18 hours.

Embryo culture

After 18 hours, the spermatozoa adhered to the surface of the presumed zygotes were separated by continuous pipetting and washed with the embryo culture medium at 38.5 ° C. After washing, 10 to 15 oocytes per drop were placed in 35x10mm plates containing 50 µL drops with KSOM medium supplemented with 10% SFB, 2mM sodium pyruvate and 50µg / mL gentamicin (embryo culture medium), and covered with mineral oil. The presumed zygotes were cultivated at 38.5 ° C, 5% CO2 and 100% relative humidity for 7 days.

Evaluation of embryonic development

On the 3rd, 5th and 7th day of the start of the culture, the embryonic development achieved by staining with the fluorescent dye Hoechst 33342 was assessed. The presumed embryos were exposed to a solution of BFS-Hoechst at a concentration of 5µg / mL for 15-30 minutes covered in light. After this time they were evaluated by using a fluorescence microscope with a UV excitation filter and an emission wavelength of 461nm at an increase of 40X for their classification according to

Statistical analysis

We analyzed the variables amount of CCO isolated by test and by ovary, expansion of cumulus cells, maturation of oocytes, presence of germ cell and metaphase I stages, oocyte degradation and embryo production. The software SPSS v.23 was used for the statistical analyzes. The determination of the normality of the evaluated data was carried out using the Shapiro-Wilk test. Likewise, comparisons were made between the means of the groups using the ANOVA statistic when the samples presented a normal distribution and the Kruskal-Wallis nonparametric test when the variables did not show a normal distribution. The values $p < 0.05$ were considered as statistically significant.

RESULTS

Average CCO isolated and vitality

For the development of the present research work, a total of 412 ovaries obtained in 16 in vitro maturation experiments were used. Table 1 shows that the average CCO, morphologically adequate for in vitro maturation, (classes I and II according to Torres, 2017) isolated was 63.5 CCO per shipment, while the average per ovary was 2.7. In addition, the vitality of the CCOs was evaluated by means of staining with the vital blue dye of trypan, with an average of 32.6% of live oocytes per shipment.

Table 01. Average oocytes isolated by ovary and vitality of oocytes.

	Characteristics evaluated Average (%) ± DE		
Nº of ovary (n)	*Mean of CCO I and II Class isolated*	*Mean of I and II Class isolated by ovary*	*Viability*
412	*63.5 ± 29.8*	*2.7± 1.1*	*32.6 ± 14.8*

CCO: Cumulus-oocyte complex; DE: standard deviation.

Comparison of Two methods of CCO isolation (by aspiration and by cuts)

Table 2 shows the results obtained when evaluating the total amount of CCO classes I and II isolated by means of the follicle aspiration method vs isolation by the cut method, as well as the comparison of the average of the CCO classes I and II obtained by ovary. It is observed that, on average, 52.6 CCO were isolated by the aspiration method, while by the cut method, an average of 34.9 CCO was isolated per experiment. On the other hand, when comparing the average of isolated CCOs for each worked ovary, it is observed that the average obtained by the aspiration method is 2.1 CCO per ovary, while by

the cut method, an average of 1.2 CCO per ovary was obtained. Statistical analysis showed a significant difference ($p < 0.05$) when comparing both methods of isolation for the number of CCO classes I and II isolated per shipment as well as for the average isolated by ovary.

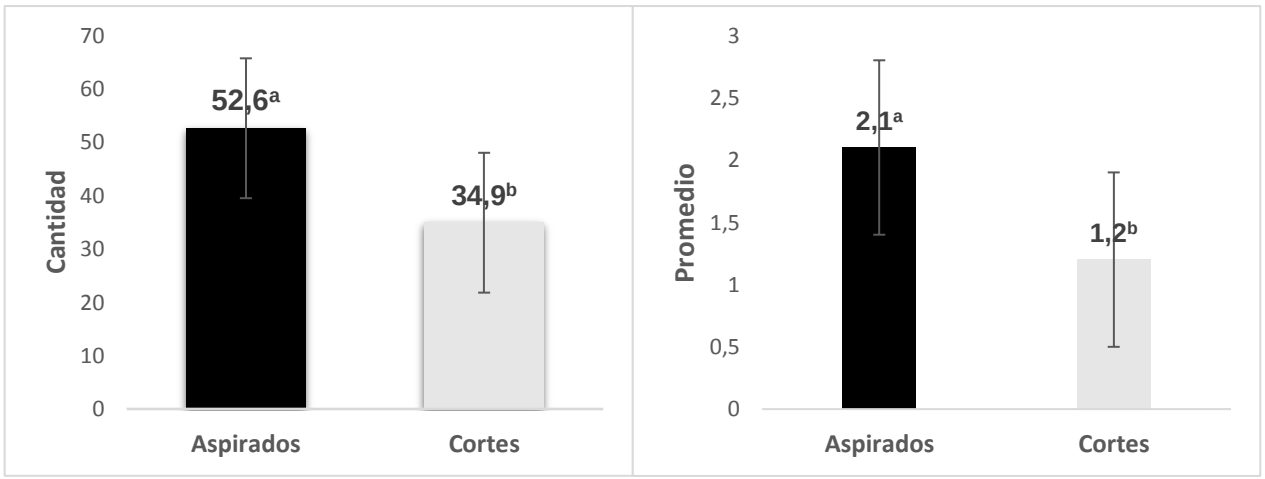
Table 02. Comparison of the recovery methods of the CCOs on the number of CCO I and II class isolated by aspiration by oocyte

Methods	**I and II** isolated oocytes **(%)**		
	n	I y II CCO class isolated By experiment	Mean of I y II class isolated By ovary
Aspiration	412	52.6±17.8 [a]	2.1±0.8 [a]
Cuts	255	34.9 ±13.1 [b]	1.2±0.7 [b]

Different letters showed significant difference ($p<0.05$); n: ovary numbers.

Figure 04. Comparison of the quantity and average of CCO classes I and II isolated by the methods of aspiration vs cuts.

** ($p<0.05$).*



I y II class isolated by assay

Mean of I and II class CCOs isolated by ovary

Effect of FF and SFB supplementation on in vitro maturation media on the cumulus expansion rates and maturation in CCO isolated by the aspirate method

Table 3 shows the results of comparing the 3 supplements used in the in vitro maturation medium: fetal bovine serum (SFB), follicular fluid (FF) and SFB + FF on the percentages of expansion of cumulus cells and maturation (evaluated by observation of the presence of the first polar body) in CCO isolated by the aspiration method and subjected to a protocol of in vitro maturation for 32 to 36 hours. An average expansion of the cumulus cells of 69.3% was observed for the CCO incubated in maturation medium supplemented only with SFB, while for the CCOs incubated in medium supplemented only with FF, a percentage of 78.7% was obtained, and for the CCO incubated in a medium supplemented with both SFB and FF, a 67.4% CCO was found with expansion of the cumulus cells at the end of the incubation period. Statistical analysis showed no significant difference between the groups evaluated ($p>0.05$). However, a higher percentage of expansion of the cumulus cells is observed for the CCOs incubated in the medium supplemented only with FF in comparison with the other 2 treatments.

Table 03. Percentages of maturation and expansion of cumulus cells for oocytes obtained by the post-32 aspiration method at 36 hours of culture in the maturation medium with the corresponding supplements

Suplements	Characteristics evaluated (%)		
	n	Oocytes with expanded Cumulus cells	Oocytes MII with PB
SFB	295	69.3±27.5 [a]	25.5±13.8[a]
FF	138	78.7±17.5 [a]	24.7±11.8 [a]
SFB+FF	361	67.4±8.0 [a]	19.2±12.9 [a]

(p>0.05). n: CCO number

Likewise, in Table 3 also shows the effect of the 3 supplements on the maturation of the oocytes. An average maturation of the oocytes of 25% was observed for the CCOs incubated in medium supplemented only with SFB, while for media supplemented with FF and SFB + FF, averages of 24.7% and 19.2%, respectively, were observed. In the same way as in the previous case, no significant difference was observed (p>0.05) between the treatments evaluated. However, a lower percentage of maturation can be observed for the oocytes incubated in the medium supplemented with SFB + FF compared to the percentages obtained when the supplements were used separately.

Effect of the supplementation of in vitro maturation media with FF and SFB on the expansion rates of cumulus cells and maturation in CCO isolated by the cut method

Table 4 shows the results of the comparison of the effect of the 3 supplements used on the expansion of cumulus cells and nuclear maturation in CCO isolated by the cut method and subjected to an in vitro maturation protocol under the same conditions as for the previous case. An average expansion of cumulus cells of 63.4% was observed for the CCO incubated in maturation medium supplemented only with SFB, while in the case of the CCO incubated in medium supplemented only with FF, a percentage of 77.9% was obtained and for the CCOs incubated in a medium supplemented with both SFB and FF, a percentage of 61.9% was found. The statistical analysis showed no significant difference between the groups evaluated (p>0.05). However, as in the case of CCO isolated by aspiration, a greater percentage of expansion of cumulus cells is observed for the CCOs incubated in the medium supplemented only with FF (77.9%).

Table 04. Percentages of maturation and expansion of cumulus cells for oocytes obtained by the method of post-32 cuts at 36 hours of culture in the maturation medium with the corresponding supplements

Suplements	Characteristics evaluated (%)		
	n	Oocytes with expanded Cumulus cells	Oocytes MII with PB
SFB	68	63.4±29.7 [a]	33.2±15.3 [a]
FF	62	77.9±23.2 [a]	26.1±18.4 [a]
SFB+FF	60	61.9±26.9 [a]	9.6±11.0 [a]

Letters indicated not significant difference (p>0.05). n: CCO number.

With regard to the effect on oocyte maturation, Table 4 shows an average maturity of 33.2% for the CCOs incubated in medium supplemented only with SFB, while for media supplemented with FF and SFB + FF, averages of 26.1 and 9.6% respectively. No significant difference ($p>0.05$) was found among the treatments evaluated. However, a marked decrease in the percentage of maturation is observed for the oocytes incubated in the medium supplemented with SFB + FF (9.6%).

Figure 05. Percentages of maturation and expansion of cumulus cells obtained in the 3 supplemented maturation media

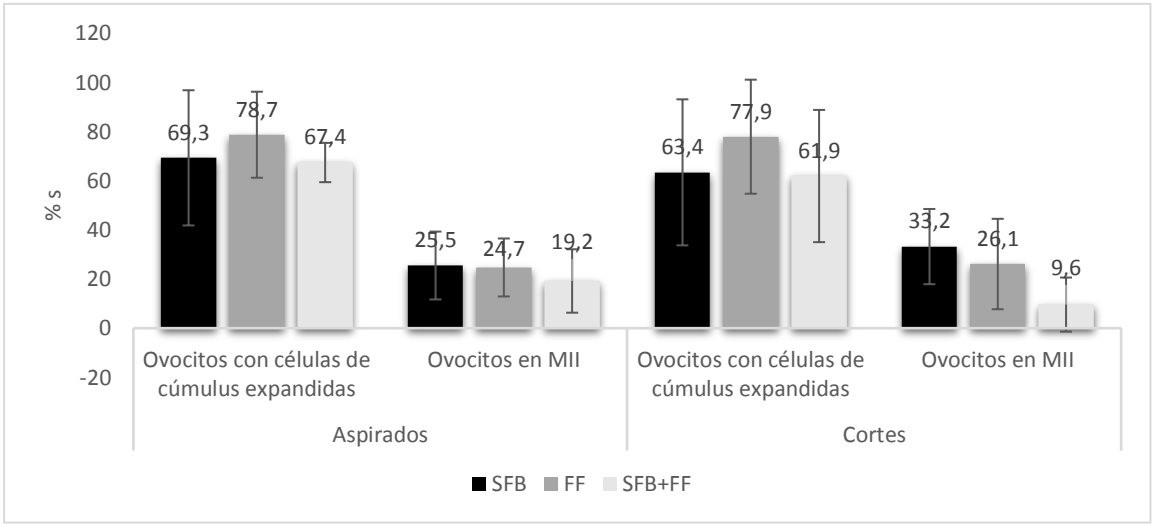


Table 5 and Figure 6 show the results referring to the nuclear stages present in the oocytes matured in the presence of the supplements evaluated. For the group of oocytes isolated by the aspiration method, no significant difference ($p>0.05$) was found between the percentages found for the stages of the germinal vesicle (34.5%, 22.4% and 22.3%, for media supplemented with SFB, FF and SFB + FF respectively) and metaphase I (13.1%, 13.1% and 4.3% for SFB, FF and SFB + FF respectively).
For the oocytes isolated by cuts, on the other hand, a significantly lower percentage ($p <0.05$) of oocytes in the stage of VG was found for the medium supplemented with SFB, in comparison with the oocytes in the same stage and matured the medium supplemented with SFB + FF (31.8%), while for metaphase I oocytes no significant difference ($p>0.05$) was found in the percentages found among the 3 evaluated.

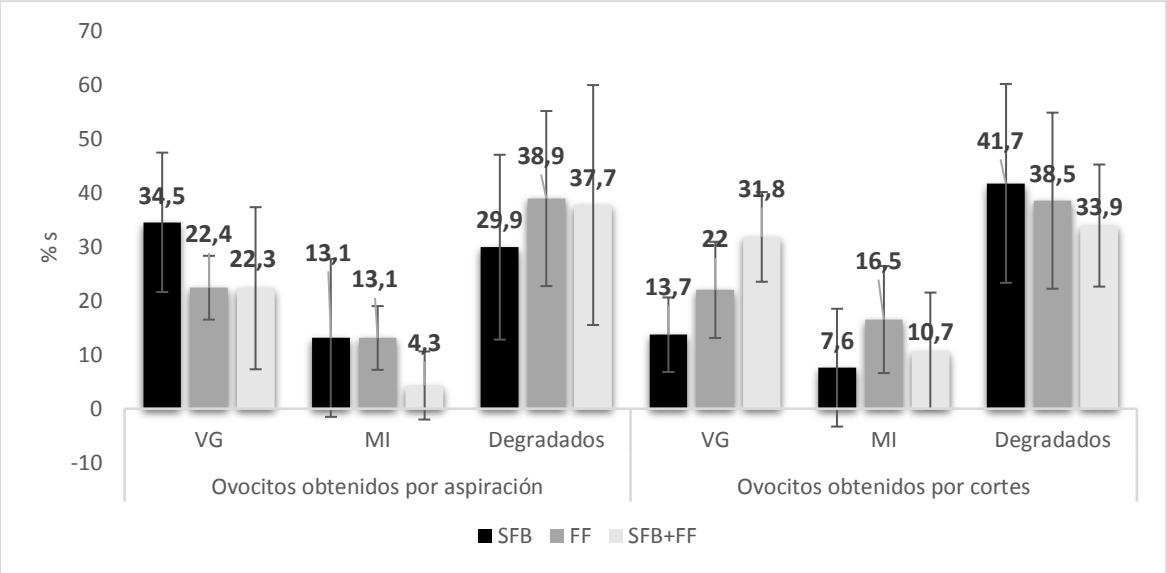
Table 05. Nuclear stage of immature post-culture oocytes in the presence of the corresponding supplements

	Nuclear stage**(%)**							
	Oocytes recovery by aspiration				Oocyte recovery by cuts			
	n	VG	MI	Degraded Oocytes	**n**	VG	MI	Degraded Oocytes
SFB	76	34.5±12.9 [a]	13.1±14.6 [a]	29.9±17.1 [a]	57	13.7±6.9 [a]	7.6±10.9 [a]	41.7±18.4 [a]
FF	81	22.4±5.9 [a]	13.1±5.9 [a]	38.9±16.2 [a]	43	22.0±8.9	16.5±9.9 [a]	38.5±16.3 [a]
SFB+FF	86	22.3±15.0 [a]	4.3±6.3 [a]	37.7±22.2 [a]	51	31.8±8.3 [b]	10.7±10.8 [a]	33.9±11.3 [a]

VG: Germinal vesicle MI: metafase I. a, b index showed significant difference ($p<0.05$); n: oocyte number.

Likewise, the results are shown with respect to the percentage of degraded oocytes (with the presence of fragmented DNA) after having been submitted to the in vitro maturation protocol, no significant difference being found (p>0.05) between any of the means with the 3 supplements for both the oocytes isolated by aspiration as by cuts.

Figure 06. Nuclear stage and presence of fragmented DNA in immature post-culture oocytes in the presence of the corresponding supplements



VG: Germinal vesiclel, MI: metafase I

Efecto de la suplementación del medio de maduración con FF y SFB y FF+SFB sobre el porcentaje de producción de embriones post FIV

Para el análisis de la producción de embriones, dado que el número de embriones totales fue pequeño, se analizaron en conjunto los datos obtenidos por medio de los 2 métodos de aislamiento de CCO empleados (aspiración + cortes).

La tabla 6 y la figura 7 muestran los porcentajes de producción de embriones, de los ovocitos madurados en presencia de los suplementos empleados y sometidos a un protocolo de fertilización *in vitro* (FIV). Para los ovocitos madurados en presencia de SFB un promedio de 27.5% de producción embrionaria, mientras que para los ovocitos madurados en presencia de FF y SFB+FF se obtuvieron tasas de producción embrionaria de 14.2% y 29.2% respectivamente, no encontrándose diferencia significativa (p>0.05) entre los grupos evaluados; sin embargo, se observa una tendencia a una menor producción de embriones para los ovocitos madurados en el medio suplementado solo con FF.

Tabla 06. Comparación de los porcentajes de producción de embriones entre los suplementos usados

Suplements	n	Embryo production (%)
		Oocytes recovery by aspiration +cuts
SFB	20	27.5±32.0 [a]
FF	20	14.2±18.9 [a]
SFB+FF	12	29.2±47.9 [a]

No significant difference. (p>0.05). n: oocytes maturity number.

Figure 07. Percentages of embryo production for matured oocytes in the presence of used supplements

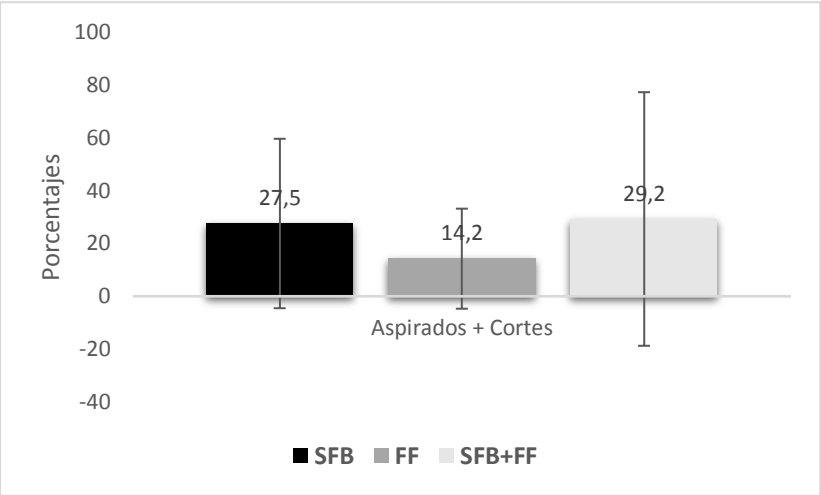


Figure 07. Percentages of embryo production for matured oocytes in the presence of used supplements

	Characteristics evaluated(%)			
Methods	**n**	% mature oocytes	**n**	% degenerated oocyte
Aspirates	794	22.9±13.1 [a]	243	35.5±17.4 [a]
Cuts	190	22.8±17.3 [a]	151	38.0±14.3 [a]

Not showed significant differences (p>0.05). noocytes number used.

On the other hand, when evaluating the percentages of oocytes with presence of fragmented DNA after culture, for the group of oocytes recovered by the aspiration method, 35.5% of degraded oocytes were observed, while for the cut method, 38 % of oocytes with presence of fragmented DNA. In a similar way to the previous case to the statistical analysis, there was no significant difference between the groups evaluated (p>0.05).

Figure 08. Comparison of the percentage of maturation and presence of fragmented DNA among the oocytes recovered by the different methods of DC isolation between of oocytes recovery with different methods.

Effect of supplementation of the maturation medium with FF and SFB and FF + SFB on the percentage of embryo production by spontaneous parthenogenesis

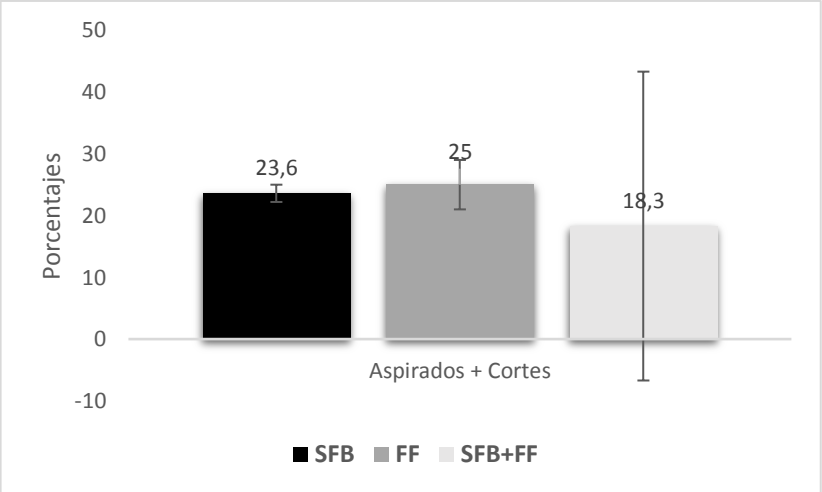
Activation by spontaneous parthenogenesis was recorded after a prolonged culture (approximately 120 hours) of the oocytes in the ripening media used. In total, a percentage of 22.7% of spontaneously activated oocytes (10/44) was obtained.

Table 8 and Figure 9 show the results of the comparison of the percentages of embryos produced by spontaneous parthenogenesis presented in the media with the supplements evaluated. Percentages of 23.6%, 25% and 18.3% were observed for oocytes matured in the presence of SFB, FF and SFB + FF respectively, and no significant difference was found between any of the groups evaluated.

Suplements	n	Partenogenesis oocytes
		Oocytes recovery by aspiration
SFB	17	23.6±1.4 [a]
FF	16	25.0±4.0 [a]
SFB+FF	11	18.3±25 [a]

Not significant differences (p>0.05). n: oocytes number

Figure 09. Percentages of embryos produced by spontaneous parthenogenesis in the media with the evaluated supplements



DISCUSSION

Effect of the supplementation of in vitro maturation media with FF and SFB on the expansion percentages of cumulus cells, maturation and embryo production.

Although in vitro oocyte maturation is currently part of the assisted reproduction protocols applied to various species, the effectiveness of this technique for many species is still low. This could be due to deficiencies in the culture media used (Combelles, et al., 2002).

Several studies have documented the benefits of in vitro maturation media supplementation with SFB in oocyte maturation and development rates. Although its composition is not yet fully understood, serum components that would benefit maturation systems include proteins, hormones and growth factors (Holzer et al., 2007; Leisinger et al., 2014).

On the other hand, studies in pigs and cattle report that supplementation of media with follicular fluid can promote maturation and / or fertilization of oocytes and their subsequent embryonic development (Algriany, et al., 2000, Tatemoto et al., 2004; Agung et al., 2010). However, it should be mentioned that most of these studies have been performed with media in the presence of serum or hormones, so the results obtained would not necessarily reflect the effect of the FF, which could be masked by the action of these other components. (Algriany et al., 2000).

In this work we compared the effect of FF on the expansion rates of cumulus cells, in vitro maturation of oocytes and embryo production compared to a medium supplemented with SFB and with both supplements (FFD + SFB). No significant difference ($p>0.05$) was found in the parameters evaluated after supplementation of the medium with FF compared to the other supplements used (SFB and SFB + FF) (Table 4, Figure 5). These results would indicate that the action of FF on the expansion rates of cumulus cells, maturation and subsequent development of oocytes is comparable to the effect of SFB.

However, although the effects of FF and SFB were evaluated separately, the means used were also supplemented with hormones and growth factors for all cases, which could leave in doubt whether the results obtained would be more related to the actual effect of both SFB and FF or are a product of the aforementioned masking derived from the hormones and growth factors present in the medium.

In this regard, studies in the maturation of alpaca oocytes in the presence of SFB as a supplement in the maturation medium with FSH (15ng / µL), LH (1µg / µL), estradiol 17β (0.1µg / µL), growth factor epidermal (50ng / µL) and 10mM cysteamine, found higher maturation percentages when the medium contained SFB (57.6%) compared to the medium without SFB (18.2% (Leisinger et al., 2014). benefit of the SFB in addition to that conferred by the hormones and growth factors (and not due to their masking), and therefore, based on our results that report a FF action comparable to that of the SFB (25.5% and 24.7% maturation for media supplemented with SFB and FF respectively), it is possible to infer that FF, like SFB, also confers an additional benefit to in vitro oocyte maturation systems.

In relation to the percentages of expansion of the cells of the cumulus, although as for the other parameters already mentioned, no significant difference was found ($p>0.05$), we observe a tendency towards higher percentages of expansion of the cumulus cells for matured oocytes when the medium was supplemented with FF for both the CCO isolated by aspirate and by cuts (Figure 5, tables 3 and 4). This result is in agreement with the previous reports that indicate an increase in the expansion rates of the

cumulus cells after a supplementation of the FF media (Algriany, et al., 2000, Agung et al., 2010). In addition, this finding would present a particular relevance since it has been reported that this characteristic is related to the improvement in fertilization rates and embryonic development capacity, even being considered as a critical process for the good performance of said processes and therefore a parameter used for the choice of oocytes that will follow in vitro fertilization protocols (Daen, 1994, Yoshino, et al., 2006).

Effect of supplementation of maturation media with FF and SFB on the nuclear stage present in immature post-culture oocytes

Table 5 and Figure 6 show the results of the evaluation of the nuclear stage present in oocytes classified as immature after culture. For the oocytes recovered by the aspiration method, when evaluating the presence of the germinal vesicle stage, no significant difference was found between the groups evaluated (34.5%, 22.4% and 22.3% for SFB, FF and SFB + FF respectively). In the same way, for the metaphase I stage, no significant difference was found between the groups (13.1%, 13.1% and 4.3% for SFB, FF and SFB + FF respectively). On the other hand, with respect to the oocytes recovered by the cutting method, the results showed a significant difference between the percentage of oocytes in the germinal vesicle stage for the group cultivated in the presence of SFB (13.7%) in comparison with the cultivated group in the presence of SFB + FF (31.8%), a result that could indicate a relationship between the presence of FF in the maturation media and higher rates of oocytes arrested in the germinal vesicle stage.

In pig several studies have reported that the supplementation of in vitro maturation media with FF increases the percentages of oocytes that reactivate meiosis, reach metaphase II stage and have better rates of embryonic development (Yoon, et al., 2000; Algriany et al., 2004¸ Tatemoto et al., 2004; Agung et al., 2010). These reports are contradictory to the results found in the present work, however, in the literature the effect of FF on the parameters evaluated has shown variable results, which are often associated with the size of the follicles from which the FF. Daen et al. (1994), for example, reported that supplementation with FF from follicles larger than 5mm in diameter reduces the penetration rate of sperm and the formation of the male and female pronuclei in pigs. On the other hand, Tao et al., (1995), found no difference when comparing the effect of FF from follicles between 2 and 5mm on nuclear maturation and blastocyst formation compared to FF from follicles of more than 5mm in diameter. These reports would reflect a variability in the effect of FF on oocyte maturation and embryonic development, variations that may be, in some cases, associated with the components present in the FF and that would vary according to the size of the follicle from which it was extracted.

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In cattle similar results have been associated with changes in the lipid membrane of oocytes when exposed to temperatures below 20 ° C (Arav et al., 1996 cited by Arriaga et al., 2014), as well as lesions of the meiotic spindle and hardening of the zona pellucida that directly affect fertilization (Wlodarczyk et al., 2009,), so these events could also be related to the susceptibility of alpaca oocytes to prolonged storage at low temperatures.
On the other hand, when evaluating the percentages of cleavage obtained after IVF protocols (Table 6, Figure 7) it can be seen that the percentages of embryo production obtained (30.6%, 18.6% and 33.3% for SFB, FF and SFB + FF respectively) are comparable to those reported in the literature (Arriaga et al., 2014, Chileno & Cainzo, 2014, Chuquitaype, 2015), so it can be inferred that although there was a decrease in the capacity of the oocytes To reach maturity, oocytes matured under these conditions maintained their competence for post-FIV development.
The recovery of the highest number of intact and good quality CCO is of great importance for the application of assisted reproduction techniques, due to its positive impact on the processes of in vitro maturation, fertilization and embryonic development (Pawshe et al., 1994 Shirazi et al., 2005). However, in alpacas the ovaries are much smaller than other species such as cattle or pigs, so the recovery of CCOs is more difficult and therefore it is necessary to study the best methods for the isolation of CCO of the classes I and II for the application of the diverse techniques of assisted reproduction.
In the results obtained from the comparison of the proportion of morphologically normal CCO (classes I and II) isolated by 2 methods (aspiration and cuts) a significantly higher proportion of CCO isolated by the method of aspiration compared to the cut method is observed ($p < 0.05$) for both the total number of CCO isolated by experiment and for the average CCO isolated by ovary (Table 2, figure 4). In contrast, several groups working on species such as sheep, goats and buffaloes reported a higher proportion of good quality CCO isolates using the cut method (Pawshe et al., 1994, Shirazi et al., 2005, Mehmood, et al., 2011). In alpaca, however, Leisinger et al. (2014), reported a significantly higher proportion of morphologically normal CCO recovered by the aspirate method compared to the cut method (55.5% vs. 29.6% respectively), while Turpo et al. (2015) found no significant difference between morphologically normal CCO averages recovered by both methods, but a tendency to a higher percentage of morphologically normal CCO recovered by the aspiration method compared to the cut method (71.1% and 61% respectively). These results would be concordant with the data obtained in the present work, which could indicate that the aspiration is a more adequate method for the recovery of the CCO in this species. With regard to maturation rates, the isolation of heterogeneous BCC groups and lower maturation rates for oocytes recovered by the cut method compared to those recovered by aspiration has been reported (Ratto et al., 2005; Mehmood, et al. al., 2011). In our work, however, no significant difference was found ($p > 0.05$) in the maturation percentages for the groups recovered by aspiration (22.9%) and cuts (22.8%). (table 7, figure 8). These results coincide with those found by Pawshe et al. (1994), working on goat oocytes, who did not report a significant difference between the rates of oocytes that reached the metaphase II stage for the

groups isolated by both methods (87.1% and 90.4% for the groups recovered by aspiration and cuts respectively) . Likewise, no significant difference was reported when evaluating the rates of embryonic production between both methods (17.1% of blastocysts produced for the group recovered by aspiration and 21.6% for the group recovered by cuts). On the other hand, when evaluating the oocyte rates that presented fragmented DNA after culture in the medium of maturation, our results show no significant difference between the oocytes coming from both isolation groups (35.5% and 38% for aspiration and cuts respectively).

These results could indicate that, although the aspiration method provides a greater amount of morphologically normal CCO, once selected, the CCOs from both methods would present an equivalent capacity to complete their maturation and allow embryonic development.

In the present work an embryo production by spontaneous parthenogenesis was reported (without the use of any chemical or physical activation protocol) of 22.7% after the oocytes were left in the maturing medium under the culture conditions detailed in the methodology , for a prolonged period of time (120 hours). In this regard it has been reported that oocyte aging is a very influential factor in spontaneous activation, finding that the longer it has passed since ovulation, the more likely it is that parthenogenesis occurs (Vallejo et al., 2003). On the other hand, it has been reported that physical agents, such as the manipulation used to isolate the oocytes may be sufficient to induce activation, although this may be dependent on the species. In mice, for example, Kaufman in 1978, reported rates of spontaneous parthenogenesis of between 45-65% after the simple manipulation necessary for the isolation of oocytes. In humans, however, this simple manipulation does not seem to be sufficient for the activation of oocytes (Abramczuk and Lopata, 1990). In our case, since the events of parthenogenesis only occurred after a prolonged culture in the maturation media, it is unlikely that manipulation of the oocytes played an important role in the activation. On the other hand, the oocyte aging time (120 hours) and the prolonged exposure to the agents present in the maturation medium (hormones, growth factors and supplements such as SFB and FF), could have played a key role in the activation of the oocytes.

Finally, with respect to the influence of the supplements evaluated (SFB, FF and SFB + FF) in Table 8 and Figure 9, the percentages of embryos produced by spontaneous parthenogenesis for each of the supplements used were shown, and no significant difference was found between the groups ($p>0.05$). FF can be used as a natural supplement equivalent to SFB in in vitro maturation media for alpaca oocytes since there is no difference in in vitro maturation percentages between matured oocytes in media supplemented with SFB, FF and SFB + FF .

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