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Leptin and IGF1 receptors in alpaca (Vicugna pacos) ovaries

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ABSTRACT

Folliculogenesis and ovulation are regulated by gonadotrophins and other factors such as Insulin like growth factor 1 (IGF1) and leptin. In various species the presence of IGF1 receptor (IGF1R) and leptin receptor (ObR) has been detected in the ovary, but not in the alpaca. Thus, the aim of the present study was to evaluate the presence of these receptors in this tissue and analyze if the presence of these receptors in the ovary is related to the presence of a corpus luteum (CL) and if abundances, as determined by immunostaining intensity vary with follicle size. The IGF1R and ObR were identified in primary and secondary follicles, granulosa and theca interna cells of tertiary follicles and in CL. There were greater abundances of IGF1R in granulosa cells of tertiary follicles of ovaries without compared with those with CL. In both groups, the immunostaining of granulosa cells was greater than in theca interna cells. The abundance of ObR was greater in primary and secondary follicles, and theca interna cells of tertiary follicles in ovaries with than those without CL. Immunostaining of granulosa cells was greater than theca interna cells only in ovaries without CL. There were no differences in the abundance of ObR and IGF1R between primary and secondary follicles and granulosa cells of tertiary follicles, neither in ovaries with or without CL. The abundance of IGF1R was not correlated with abundance of ObR neither in ovaries with or without CL. These results indicate a possible role for IGF and leptin in ovarian function. Furthermore, these receptors could be regulated by ovarian steroid hormones because abundance of these receptors in ovaries varies depending on whether there is a CL present in the ovary.

1. Introduction

Camelids have some particular reproductive characteristics in comparison with other domestic animals, some of which may lead to poor reproductive efficiency). Furthermore, reproductive efficiency is affected by early embryonic death, which may be as great as 35%–60% (Fernandez Baca et al., 1970; Tibary et al., 2001; Vaughan and Tibary, 2006; Van Saun, 2008). Factors responsible for this poor reproductive efficiency are unknown but hormonal imbalances are one of the possible causes (Knight et al., 1995; Vaughan and Tibary, 2006). The lack of ovulation and marginal follicular development are also physiological functions that contribute to the poor reproductive efficiency in camelids (Bravo, 1994; Vaughan and Tibary, 2006).

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Alpacas are induced ovulators meaning there needs to be copulation when there is a fully developed follicle present to induce ovulation (Bravo et al., 1991; San-Martín et al., 1968). In this species, there are waves of ovarian follicular growth and regression. During each wave of follicular development, one follicle becomes dominant, grows to maturity and subsequently regresses (Vaughan et al., 2004). Folliculogenesis and ovulation are regulated by gonadotrophins and other factors such as Insulin like growth factor 1 (IGF1) and leptin (Schams 2002; Lange-Consiglio et al., 2013; Rodriguez et al., 2015). In various species IGF1 stimulates proliferation and differentiation of granulosa cells as well as steroideogenesis and vascularization (Monget et al., 2002; Schams et al., 2002; Rodriguez et al., 2015). Also, in cattle, abundance of the IGF1 receptor (IGF1R) varies depending on the size of follicles indicating that abundance of the receptor varies with ovarian tissue type, thus, may be related to ovarian function (Schams et al., 2002). Leptin is a hormone of 16 kDa that is encoded by the LEP gene and is secreted mainly by adipose tissue (Zhang et al., 1994; Chilliard et al., 2005). In various species, leptin and its receptor (ObR) are present in the granulosa, theca interna, and luteal cells as well as in oocytes (Batista et al., 2013; Lange-Consiglio et al., 2013). In addition, it has been reported that the abundance of ObR in the ovary varies with stage of the oestrous cycle (Duggal et al., 2002; Craig et al., 2004). It therefore, has been proposed that leptin is involved in oocyte maturation, steroideogenesis, angiogenesis and corpus luteum (CL) function and development; although the mechanisms of regulation have not been fully elucidated (pigs Ruiz-Cortés et al., 2003; rats: Roman et al., 2005; cattle: Sarkar et al., 2010; goats: Batista et al., 2013). Leptin and IGF1 are thought to interact to stimulate ovarian function (pigs: Gregoraszczuk et al., 2004; cattle: Sarkar et al., 2010; monkeys: Suter et al., 2000). Furthermore, IGF1 stimulates the production of ObR in prepubertal pig ovaries (Gregoraszczuk et al., 2006).

There are few reports about the functions of IGF1 and leptin in the reproductive system of camelids. In llamas, leptin secretion is decreased as a consequence of dietary restriction which is associated with development of a smaller CL and less progesterone secretion, suggesting that this hormone might influence ovarian function in this species (Norambuena et al., 2013). Furthermore, leptin supplementation in camelids led to greater CL vascularization (Norambuena et al., 2017). In contrast, there have been no reports regarding to the effects of IGF1 on ovarian function in camelids.

The abundance and localization of IGF1R and ObR in alpaca ovaries has not been previously studied. Advances in the understanding of regulatory mechanisms of ovarian function may provide further knowledge that could improve reproductive management and fertility of camelids. Thus, the aim of this study was to evaluate the abundances and localization of IGF1R and ObR in alpaca ovaries and analyze if abundances are related to the presence of CL.

2. Materials and methods

2.1. Tissue collection and follicle classification

This study was conducted in compliance with regulations set by the Veterinary Faculty, UBA. The ovaries of 14 non-pregnant, non-lactating, sexually mature alpacas (without CL: $n = 6$, with CL: $n = 8$) were obtained in the spring-summer season from animals housed at the Estación Experimental de altura de Chuquibambilla (Universidad Nacional del Altiplano, Puno, Perú) located at 14 °S, 70 °W (3970 msnm). All females were clinically healthy, with a healthy reproductive status, and in good nutritional condition with a mean body condition score of 3 (body condition score from 1 = thin to 5 = obese) (Van Saun, 2009). Females were randomly mated with males of the herd, and at 7 to 9 days post-mating the animals were slaughtered. In those alpacas that developed a CL, uterine flushing and observation of the recovered fluid using a stereoscope was performed to ascertain if embryos were present. Only females that were not pregnant were included in the study.

After evisceration of the carcass, ovaries were collected for immunohistochemical analysis. The samples were fixed in 4% buffered formol, washed in phosphate-buffered saline, and then dehydrated in an ascending series of ethanol, cleared in xylene, and embedded in paraffin.

Follicles were classified microscopically as being in a primary, secondary or tertiary stage according to the criteria previously described in the Nomina Histologica (1994) and by Priedkalns and Leiser (2006). Briefly, primary follicles were composed of an oocyte surrounded by simple cuboidal epithelial follicular cells. Secondary follicles were composed of an oocyte surrounded by a stratified epithelium of polyhedral follicular cells (granulosa cells). Tertiary follicles were composed of an oocyte surrounded by a stratified epithelium of granulosa cells and by a multilaminar layer of specialized stromal cells (theca interna and externa)with the follicle having a fluid-filled cavity (antrum).

2.2. Immunohistochemistry

2.2.1. IGF1R

An immunohistochemical technique was performed as previously described by Rodriguez et al (2015) in cattle ovaries, with minor modifications. Sagittal sections (5 μm thick) were mounted on silanised slides and subsequently rehydrated by placing these in xylol and a graduated series of alcohol solutions, and subsequently there was placement in phosphate buffered saline (PBS, 0.01 M, pH 7.4). The extravidin–biotin immunoperoxidase method (CytoScan™ HRP detection system, Cell Marque, CA, USA) was subsequently used. Briefly, after deparaffinization, the antigen was exposed by incubating the sections in 0.01 M citrate buffer (pH 6.0) in a microwave oven at 800 W. Endogenous peroxidase activity was inhibited using 3% (v/v) H_2O_2 in methanol and non-specific binding sites were blocked (CytoScan™ HRP detection system, Cell Marque, CA, USA). Slides were incubated with polyclonal rabbit anti-IGFR1 diluted 1:300 (sc-712, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 18 h at 4 °C and then for 30 min at room temperature with a biotinylated link (CytoScan™ HRP detection system; Cell Marque, CA, USA). The IGFR1 was visualized using the

CytoScan™ HRP detection system, and 3, 3-diaminobenzidine (liquid DAB-Plus substrate kit; Invitrogen) was used as chromogen. All sections were processed during the same session and had the same development time. The slides were subsequently washed in distilled water and counterstained with activated haematoxylin (Biopur, Rosario, Argentina), dehydrated and mounted. To verify the immunoreaction specificity, adjacent control sections were subjected to the same immunohistochemical method, replacing primary antibody with rabbit non-immune sera. As a positive control, the alpaca liver was used. Also, human liver and ovary paraffin archived blocks were used, as previously described by Rodriguez et al. (2015).

2.2.2. ObR

Sagittal sections (5 μm thick) were mounted on silanised slides and treated using the same protocol as previously described with minor changes. Non-specific binding sites were blocked using 10% (v/v) normal horse serum for 30 min (Vectastain ABC-Kit, Elite, PK-6102, Burlingame, CA, USA). Sections were incubated in a humidified chamber overnight at 4 °C with a 1:20 dilution of the anti-ObR primary antibody (mouse monoclonal, sc-8391, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS. After being washed with PBS, sections were incubated for 1 h with a 1:100 dilution of the biotinylated anti-mouse antibody (BA-200, Vector, Burlingame, CA, USA). Sections were washed with PBS before use of the "Vectastain Elite ABC Kit" detection system (Vector, Burlingame, CA, USA). The tissue sections were subsequently incubated for 45 min with a horseradish peroxidase–avidin–biotin complex (Vectastain Elite ABC-kit, Vector Laboratories, Cat No. PK-6100). The site of the bound enzyme was visualized by the application of 3,3´-diaminobenzidine (DAB kit; Dako, Cat No. K 3468). The sections were counterstained with haematoxylin and dehydrated before cover slips were applied with mounting medium (Biopack, Argentina). Negative controls were produced by replacing the primary antibody with normal mouse IgG at an equivalent concentration (Santa Cruz, Cat No. sc-2025, CA, USA). As a positive control, alpaca liver was used.

2.3. Image analysis

A subjective image analysis was performed to estimate the abundance, as indicated by intensity of immunostaining for IGF1R and ObR, as previously described for other receptors (Sosa et al., 2004; Bianchi et al., 2013). The evaluation was performed by two independent observers. Ten fields were analyzed for each CL and for each follicle type (primary, secondary or tertiary) at a magnification of 1000 × . The total area of positively stained cell cytoplasm (brown reaction product) was measured and expressed as a ratio of the total area of cell cytoplasm (brown reaction product + blue haematoxylin) (Bianchi et al., 2015). The staining was scored as being negative (−), faint (+), moderate (++) or intense (+++) and the staining of each cell type was in proportion on a scale of 0–10 (Thatcher et al., 2003). The average staining was calculated as $= 1 \times n1 + 2 \times n2 + 3 \times n3$, where n = proportion of cells per field that were faintly (1), moderately (2) and intensely (3) stained (Boos et al., 1996). Thus, abundance of IGF1R and ObR was evaluated as the average of total positive area (percentage of the immunoreactive area) and average staining intensity.

2.4. Statistical analysis

The percentage of the area that was positively stained and that had average amounts of staining were analyzed using a Student`s *t*-test (Graph Pad 5, USA), comparing ovaries with or without CL, while follicular types in each group were compared using a one way analysis of variance (ANOVA) followed by conducting a Bonferroni test. Pearson's correlations were calculated to study the relationship between receptors. The values were expressed as the mean ± SD. Statistical significance was set at $P < 0.05$.

3. Results

3.1. IGF1R immunostaining

Based on immunostaining results, the IGF1R were present in all follicle types, both in granulosa and theca interna cells, and also in luteal cells of all ovaries (Fig. 1). The IGF1R positive immunostaining area in primary follicles was 82% and 84% in ovaries with and without CL, respectively ($P = 0.7$). The positive immunostaining area for IGF1R in secondary follicles was about 75% in both groups ($P = 0.95$). Greater immunostaining was observed in granulosa cells of tertiary follicles of ovaries without CL compared with ovaries with CL (94% and 64%, respectively; $P = 0.009$; Fig. 2; Fig. 3). There were no differences in positive immunostaining for IGF1R in theca interna cells between groups (22% and 14% with and without CL, respectively, $P = 0.11$). In both groups, the immunostaining of granulosa cells was greater than in theca interna cells ($P = 0.001$). There was IGF1R immunostaining detected in most of luteal cells (89% of positive area; Fig. 1).

There were no differences in the positive area for immunostaining for IFG1R among primary follicles, secondary follicles and granulosa cells of tertiary follicles in ovaries with or without CL ($P = 0.24$).

Immunostaining intensity for IGF1R was greater in granulosa cells of tertiary follicles without CL than with CL ($P = 0.006$). Also immunostaining intensity for IGF1R was greater in granulosa cells than in theca interna cells of ovaries with and without CL ($P = 0.002$ and $P < 0.0001$, respectively). There were no differences in the intensity of immunostaining for IGF1R in primary or secondary follicles between ovaries with and without CL ($P = 0.2$ and $P = 0.7$, respectively). There were also no differences in IGF1R immunostaining between follicular types in ovaries with or without CL ($P = 0.5$; Fig.4).

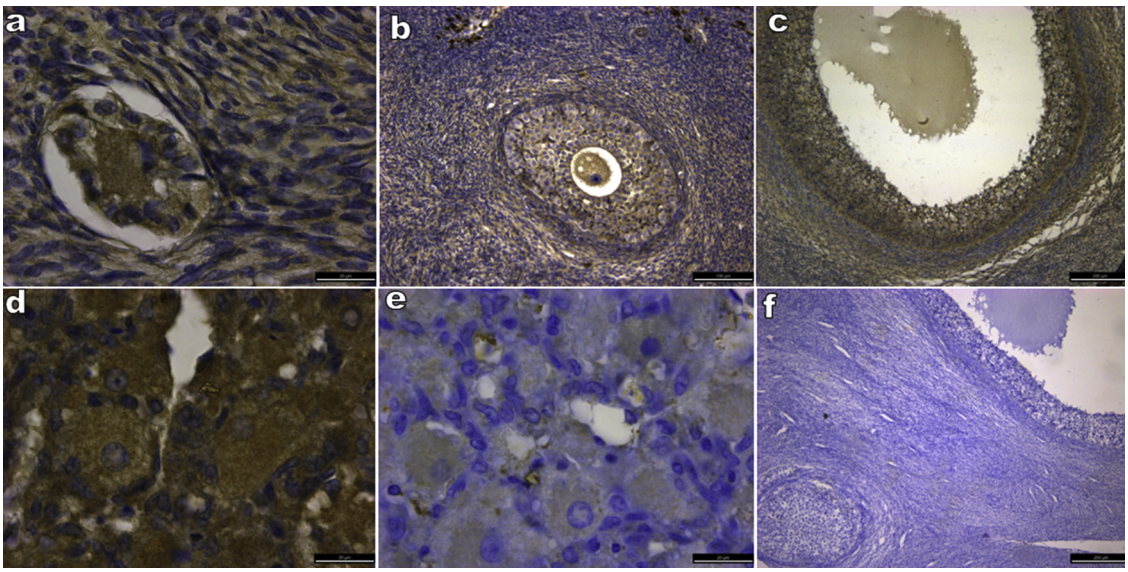

Fig. 1. Immunostaining intensity for IGF1R in a primary follicle (a), secondary follicle (b), tertiary follicle (c) and corpus luteum (d) of alpaca ovaries; Negative controls (e, f); Scale bar indicates 20 μm (a, d, e), 100 μm (b) and 200 μm (c).

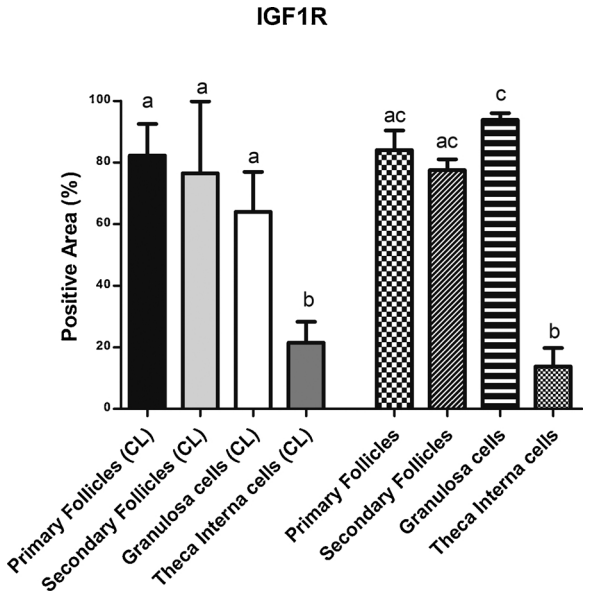

Fig. 2. Positive immunostaining area of IGF1R in alpaca ovaries with CL (left side) and without CL (right side); Bars with different letters are different ($P < 0.05$); It can be observed that granulosa cells have a greater positive immunostaining area than theca interna cells, and that in ovaries without CL the granulosa cells have a greater positive immunostaining area than in ovaries with CL.

3.2. ObR immunostaining

There was ObR immunostaining in all follicle types, both in granulosa and theca interna cells, and also in luteal cells of all ovaries (Fig. 5). The area of ObR positive immunostaining in primary follicles was 91% and 73% in ovaries with and without CL, respectively ($P = 0.16$). In secondary follicles, the positive area for ObR immunostaining was of 74% and 62% in ovaries with and without CL, respectively ($P = 0.34$). There were no differences in positive areas of immunostaining for ObR in granulosa cells of tertiary follicles between groups (88% and 86% in ovaries with and without CL, respectively; $P = 0.6$; Fig. 6). There, however, were differences for ObR immunostaining in theca interna cells between ovaries with and without CL (86% and 42%, respectively; $P = 0.001$; Figs. 6 and 7). Immunostaining for ObR in granulosa cells was greater than in theca interna cells in ovaries without CL (86% and 42% respectively; $P < 0,0001$) but not in ovaries with CL (88% and 86% respectively; $P = 0.5$). The ObR immunostaining was observed in most of luteal cells (94% positive area; Fig. 5).

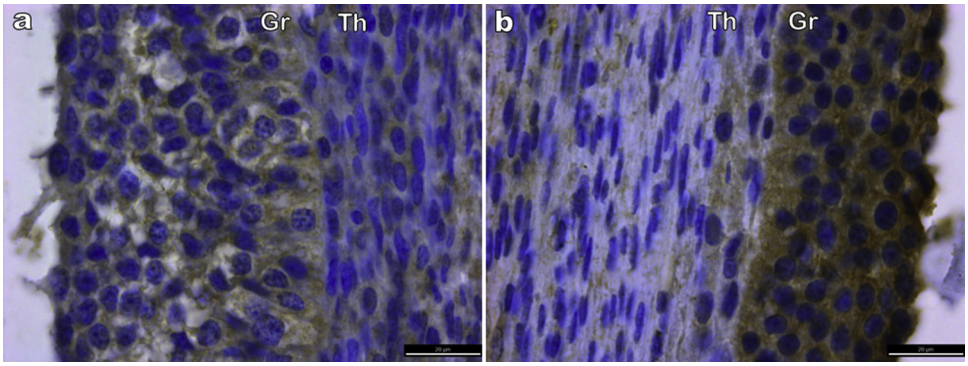

Fig. 3. IGF1R immunostaining intensity in granulosa cells (Gr) and theca interna cells (Th) of alpaca ovaries with CL (left side) and without CL (right side); Scale bar indicates 30 μm; Positive area and immunostaining intensity of granulosa cells is greater in the ovary without CL (right side).

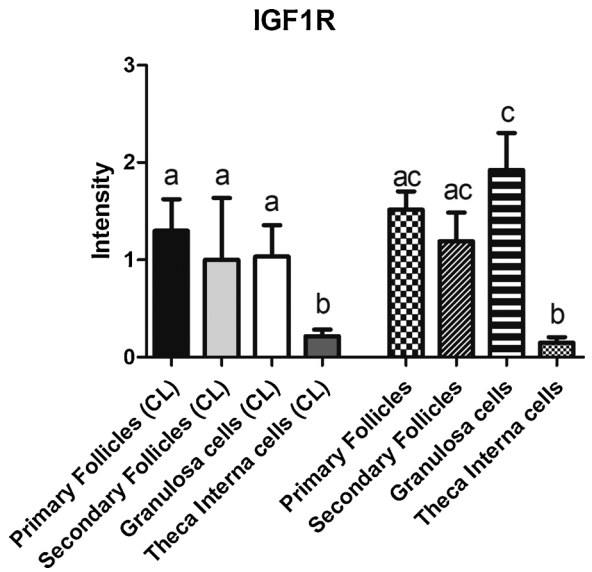

Fig. 4. Average immunostaining intensity for IGF1R in alpaca ovaries with CL (left side) and without CL (right side); Bars with different letters are different ($P < 0.05$). Granulosa cells have a greater immunostaining intensity than theca interna cells, and in ovaries without CL granulosa cells have a greater immunostaining intensity than in ovaries with CL.

There were no differences in positive immunostaining areas for ObR among primary follicles, secondary follicles or granulosa cells of tertiary follicles in ovaries with and without CL ($P = 0.22$; Fig. 6).

Immunostaining intensity for ObR was greater in primary follicles, secondary follicles and in theca interna cells of tertiary follicles of ovaries with than without CL ($P = 0.007$, $P = 0.04$ and $P = 0.006$, respectively). There were no differences in the average areas of immunostaining of granulosa cells of tertiary follicles between ovaries with and without CL ($P = 0.87$). Granulosa cells of tertiary follicles had a greater immunostaining for ObR than theca interna cells in ovaries without CL ($P = 0.0003$) but not in ovaries with CL ($P = 0.07$). There were no differences in immunostaining for ObR among follicular types in ovaries with or without CL ($P = 0.8$; Fig. 8).

3.3. Correlation analysis

There was no correlation of values for immunostaining between IGF1R and ObR neither in ovaries with CL or without CL ($r = 0.49$; $P = 0.06$ and $r = 0.39$, $P = 0.055$, respectively).

4. Discussion

To the best of our knowledge, this is the first study where IGF1R and ObR were identified to be present in alpaca ovaries. Immunostaining for both receptors was detected in primary and secondary follicles, granulosa and theca interna cells of tertiary follicles and in CL. In other species, these receptors have been observed both in granulosa and theca cells as well as in luteal cells, and

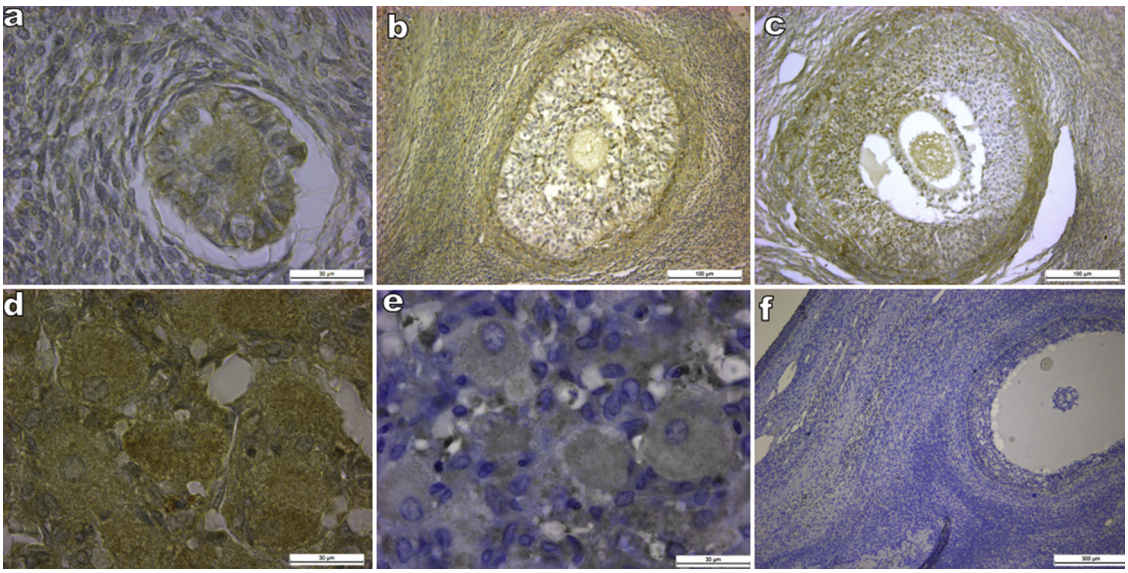

Fig. 5. ObR immunostaining intensity in a primary follicle (a), secondary follicle (b), tertiary follicle (c) and corpus luteum (d) of alpaca ovaries; Negative controls (e, f); Scale bar indicates 30 μm (a, d, e); 100 μm (b,c) and 300 μm (f).

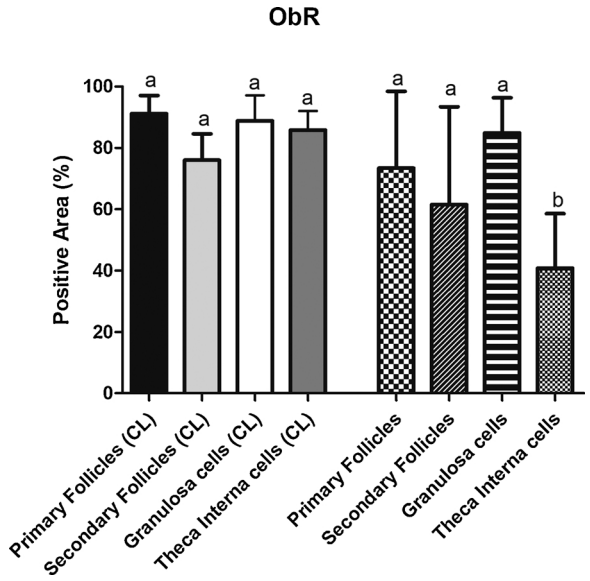

Fig. 6. Positive immunostaining area for ObR in alpaca ovaries with CL (left side) and without CL (right side); Bars with different letters are different ($P < 0.05$); Theca interna cells have a greater positive immunostaining area in ovaries with CL than without CL; Granulosa cells also have a greater positive immunostaining area than theca interna cells in ovaries without CL.

it has been proposed that IGF1 and leptin have functions in the ovary by binding to and activation of these receptors (cattle: Schams et al., 2002; human/sheep/cattle/pigs: Silva et al., 2009; goats: Batista et al., 2013). Likewise, the presence of ObR and IGF1R in alpaca ovarian follicles and in CL, indicates a possible role of IGF1 and leptin in ovarian function of camelids.

It has been reported in various domestic animals such as pigs, cattle and sheep that IGF1 stimulates both proliferation and differentiation of granulosa cells, as well as ovarian steroideogenesis (sheep/pigs: Monget et al., 2002; cattle: Schams et al., 2002; cattle: Fortune et al., 2004). In the present research, there was greater immunostaining for IGF1R in granulosa cells than in theca interna cells. Thus, IGF1 may have a predominant role in proliferation, differentiation and hormone synthesis by granulosa cells. Considering there was a greater immunostaining for IGF1R in granulosa cells of tertiary follicles as compared with immunostaining intensity in CL (greater positive area and intensity), there may be regulation of ovarian steroid hormone synthesis in these cells by IGF1, as has been reported in other species (Panner et al., 1991; Spicer and Echternkamp, 1995; Sosa et al., 2010). Furthermore, considering there were IGF1R detected in most of luteal cells in the present study, this finding supports the supposition that IGF1 may be involved in steroid synthesis in camelids. There could be functions of IGF1 in the CL by binding to its receptor and contributing to

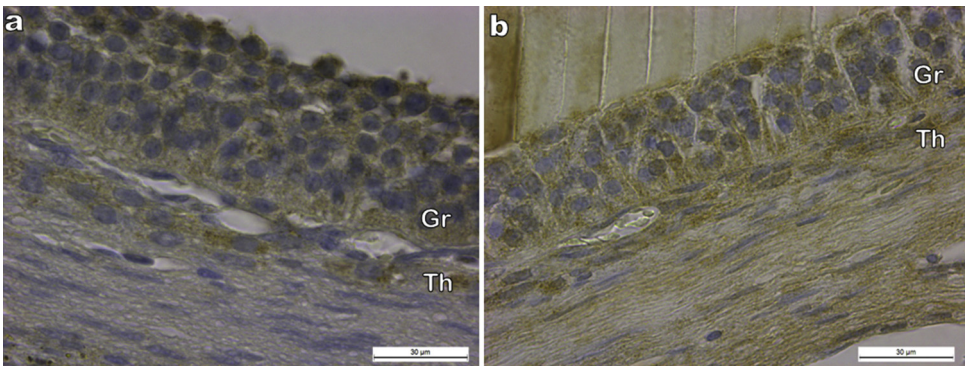

Fig. 7. ObR immunostaining in granulosa (Gr) and theca interna cells (Th) of tertiary follicles of alpaca ovaries without CL (a) and with CL (b); Scale bar indicates 30 μm.

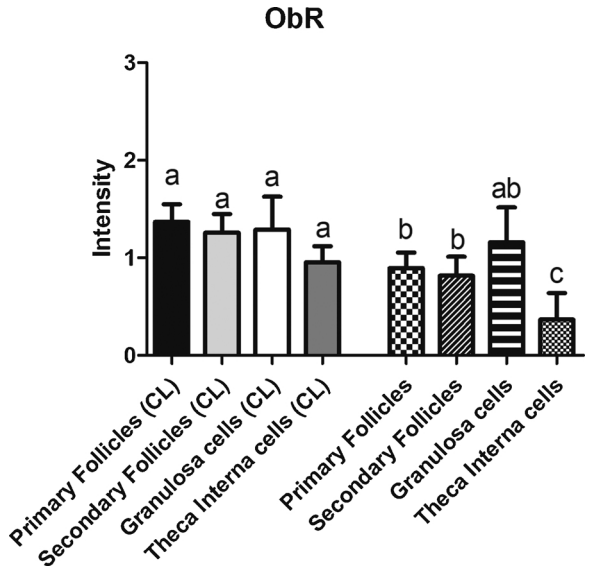

Fig. 8. Average immunostaining for ObR in alpaca ovaries with CL (left side) and without CL (right side); Bars with different letters are different ($P < 0.05$); Primary follicles, secondary follicles and theca interna cells have a greater immunostaining intensity in ovaries with CL than without CL; Granulosa cells have a greater immunostaining intensity than theca interna cells only in ovaries without CL.

the regulation of angiogenesis or steroideogenesis, as has been reported to occur in pigs and cattle, among other species (Sarkar et al., 2010; Balogh et al., 2018).

The presence of leptin and its receptor has been reported in sheep, cattle and goat ovaries, among other species (Sarkar et al., 2010; Batista et al., 2013). It has been proposed that by binding to its receptor, leptin stimulates ovarian angiogenesis, steroideogenesis and CL function (Ruiz-Cortés et al., 2003; Sarkar et al., 2010; Balogh et al., 2012). The presence of ObR in alpaca ovaries suggests leptin could have a similar role in this species. In fact, in llamas (another South American camelid) relatively lesser concentrations of leptin are associated with a smaller CL and less progesterone secretion, while leptin supplementation leads to greater CL vascularization (Norambuena et al., 2013, 2017). In the present study, there was greater ObR immunostaining in theca interna cells of tertiary follicles (greater positive area and intensity) and in primary and secondary follicles (greater intensity) of ovaries with CL, which may be related to an enhanced ovarian steroid synthesis when the IGF1R is activated because of IGF1 binding to this receptor. This possibility is supported by research findings in other species where it has been reported that estradiol regulates the abundance of ObR in different tissues, including the ovaries (Duggal et al., 2002; Thorn et al., 2007; Sosa et al., 2010).

It has previously been reported that leptin and IGF1 interact to stimulate ovarian function (Suter et al., 2000; Gregoraszczuk et al., 2004; Sarkar et al., 2010). These two hormones are thought to have synergic effects on ovarian steroids synthesis (Ruiz-Cortés et al., 2003; Sarkar et al., 2010). Furthermore, it has been reported that IGF1 functions to increase the abundance of ObR in prepubertal pig ovaries (Gregoraszczuk et al., 2006). In the present study, there was not any correlation between immunostaining intensity values for the two receptors. It, however, is of interest that the abundance of ObR was greater in ovaries with CL, while the abundance of IGF1R was greater in ovaries without CL. This could be related to the effects of leptin and IGF1 on these receptors through autocrine or paracrine actions, or because of differential regulation of each receptor by sex steroids (Thorn et al., 2007; Di Yorio et al., 2008;

Sarkar et al., 2010; Batista et al., 2013).

Although in ruminants and rats it has been reported that both IGF1R and ObR are of differential abundance depending on follicle size (Duggal et al., 2002; Schams et al., 2002; Sarkar et al., 2010; Batista et al., 2013), there were no differences in abundances of these receptors in the different follicle types of alpaca ovaries. These differences may not have existed in the alpaca, due to the relatively greater estradiol concentrations that persist during the follicular ovarian wave as compared to what occurs in other species (Bravo, 1994; Brown, 2000).

5. Conclusion

In conclusion, based on immunostaining results, IGF1R and ObR are present in alpaca ovarian follicles and CL, suggesting a possible role for IGF and leptin in follicular development and CL function in this species. Furthermore, these receptors could be regulated by ovarian steroid hormonesbecause the abundance of these receptors in alpaca ovaries varies depending on whether there is or is not a CL in the ovaries. Although further studies are needed, the findings in the present study provide foundational knowledge about the roles of IGF1 and leptin in ovarian functions of alpacas.

Conflict of interest

None of the authors have any conflict of interest to declare.

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