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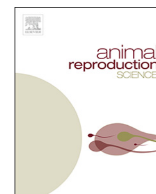

Effects of orexins on 17β-estradiol synthesis and P450 aromatase modulation in the testis of alpaca (*Vicugna pacos*)

Giovanna Liguori[a,1], Alessandra Pelagalli[b,c,1], Loredana Assisi[d,*], Caterina Squillacioti[a], Anna Costagliola[a], Nicola Mirabella[a]

[a] *Department of Veterinary Medicine and Animal Productions, University of Naples "Federico II", Via Delpino 1, 80137, Naples, Italy*
[b] *Department of Advanced Biomedical Sciences, University of Naples "Federico II", Via Pansini 5, 80131 Naples, Italy*
[c] *Institute of Biostructures and Bioimages, National Research Council, Via De Amicis 95, 80131 Naples, Italy*
[d] *Department of Biology, University of Naples "Federico II", Via Mezzocannone 6, 80134 Naples, Italy*

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ABSTRACT

The steroidogenic enzyme P450 aromatase (ARO) has a key role in the conversion of testosterone (T) into estrogens (E), expressed as 17β-estradiol. The presence and localization of this key enzyme have not been described before in the South American camelid alpaca (*Vicugna pacos*). In our previous studies of the expression and biological effects of orexin A (OxA) and OxB on the alpaca testis demonstrated that OxA, *via* its specific receptor 1 (OX1R), stimulated T synthesis. In order to extend these findings, we presently explored the presence and localization of ARO in the alpaca male gonad, and the possible correlation between ARO and the orexinergic complex. Western blotting and immunohistochemistry demonstrated the presence of ARO in tissue homogenates and its localization in the tubular and interstitial compartments of the alpaca testis, respectively. The addition of OxA to fresh testicular slices decreased the 17β-estradiol E levels. This effect was annulled by the sequential addition of the selective OX1R antagonist, SB-408124. OxB incubation did not have any effect on the biosynthesis of E. Furthermore, the OxA-mediated down-regulation of E secretion could be ascribed to ARO inhibition by exogenous OxA, as indicated by measurement of ARO activity in tissue slices incubated with OxA. Overall, our findings suggest that locally secreted OxA interacting with OX1R could indirectly inhibit ARO activity, disabling the conversion of T to E, and consequently lowering E biosynthesis and increasing the production of T in mammalian testis.

1. Introduction

In the male gonad, sexual steroids occupy a central role in spermatogenesis and steroidogenesis regulation. Testosterone (T) secreted by the interstitial (Leydig) cells is critical for several reproductive functions (McLachlan et al., 1996). In mammals, also estrogens (E) are involved in several testicular phenomena (Hess et al., 1997, Hess and Carnes, 2004; O'Donnell et al., 2001; Carreau et al., 2007; O'Donnell et al., 2001). The biosynthesis of E is catalyzed by the irreversible aromatization of androgens by P450 aromatase (ARO) (Carreau, 2003; O'Donnell et al., 2001; Carreau et al., 2007, 2011). ARO is an enzymatic complex encoded by the CYP19A1 gene and is characterized by two proteins: a ubiquitous NADPH-cytochrome P450 reductase and a cytochrome P450 aromatase (Carreau et al., 2011). ARO is localized in the cellular endoplasmic reticulum in different body districts and in the male

* Corresponding author.
E-mail address: assisi@unina.it (L. Assisi).
[1] GL and AP equally contributed to this work.

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gonad (Carreau et al., 2001). The presence of ARO in the testes has been described in numerous species (Menad et al., 2017; Carreau et al., 2006, 2011; Carpino et al., 2001; Nitta et al., 1993; Levallet et al., 1998).

Orexin A (OxA) and OxB are two peptides of hypothalamic origin, derived from the proteolytic cleavage of a common precursor called prepro-orexin (Sakurai et al., 1998; de Lecea et al., 1998). The biological effects of these peptides are mediated by binding with two G coupled receptors called orexin 1 (OX1R) and OX2R for orexins. OX1R first is specific for OxA, while OX2R can bind the two peptides with equal affinity. In the male genital apparatus, prepro-orexin, OxA, and OX1R were investigated in the testis and epididymis of rat (Barreiro et al., 2004, 2005; Assisi et al., 2012; Tafuri et al., 2009, 2010), alpaca (*Vicugna pacos*) (Liguori et al., 2012, 2014), mouse testis (Joshi and Singh, 2016, 2017), the urethro-prostatic complex of cattle (Russo et al., 2008), and in the normal, hyperplastic and neoplastic prostate of human beings (Alexandre et al., 2014; Valiante et al., 2013, 2015). Moreover, OxB and OX2R distribution were described in the testis of rat (Liguori et al., 2017a) and alpaca (Liguori et al., 2017b), and OX2R mRNAs were detected in several male genital organs (Karteris et al., 2004) and prostate (Malendowicz et al., 2011) of humans.

Alpaca is a South American camelid that is phylogenetically comparable to llama (*Lama glama*) and guanaco (*Lama guanicoe*). The breeding of alpaca has always been important in the Andean culture for their wool and meat, and currently, has spread to Europe, including Italy. Alpacas have adapted to survive in the high-altitude Andean regions, and when transferred in more temperate climates, became more fertile because of optimal nutrition for the majority of the year (Fowler, 1998).

The main aim of this study was to provide the first evidence of ARO in the alpaca testis and the effects of orexins on this enzyme and E biosynthesis (expressed as 17β- estradiol). For this purpose, we investigated the presence and localization of ARO in the alpaca testis by means of western blotting and immunohistochemical analyses, respectively. Furthermore, we explored the relationship between orexins and ARO, focusing our attention on 17β- estradiol synthesis *in vitro* experimentally and on cultured testicular slices. Considering the previous demonstration of the effect of OxA on T production in alpaca testis (Liguori et al., 2012), we presently addressed whether this effect also involved E biosynthesis and/or ARO modulation.

2. Material and methods

2.1. Antibodies and chemicals

The OxA (003-30) and OxB (003-32) peptides were obtained from Phoenix Pharmaceuticals Inc. (Karlsruhe, Germany). The OX1R antagonist, SB-408124, luteinizing hormone (LH) from sheep pituitary (L5269), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The estradiol ELISA kit (DKO003) was purchased from Diametra (Perugia, Italy). Rabbit polyclonal anti-cytochrome P450 (aromatase) antibody (ABIN3023082) was purchased from Antibodies-online.com. Biotinylated goat anti-rabbit (BA-1000) secondary antibody and VECTASTAIN ABC kit (PK-6105) and 3,3′-diaminobenzidine tetra-hydrochloride (DAB) solution were from Vector Laboratories (Burlingame, CA, USA). Qproteome Formalin-Fixed Paraffin-embedded (FFPE) Tissue Kit was purchased from Qiagen (Hilden, Germany). Peroxidase-AffiniPure goat anti-rabbit IgG secondary antibody (111-035-003) was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The DC protein assay kit from Bio-Rad Laboratories (Hercules, CA, USA). Enhanced chemiluminescence (ECL, Western Bright ECL) was purchased from Advansta, (Menlo Park, CA, USA). The marker proteins were from Prosieve QuaColor (London, UK).

2.2. Animals and tissue collection

The animals for this work were kept in sanitary, nutritional, and semi-ranged environments at the “Domus Alpaca” farm (Pratola Peligna, Italy). Five healthy sexually matured males with no preputial adhesions detected underwent orchiectomy as previously described (Fowler, 1998). The protocol followed the International Guiding Principles for Biomedical Research Involving Animals in Italy and were approved by Italian laws (D. Lgs 26/2014). Animal care was guaranteed during the surgical procedures and the experimental research was approved by the Ethical Animal Care and Use Committee of the University of Naples Federico II, Department of Veterinary Medicine and Animal Production, Naples, Italy (no. 0024889). The owner of the animals gave verbal consent to perform surgical procedures, collection of the samples and animals were not involved in any clinical trials or treatments. Immediately after the orchiectomy procedure, testis samples were carefully cut into small pieces and rapidly fixed in Bouin’s fluid for FFPE western blotting and immunohistochemical techniques as previously described (Liguori et al., 2013). Other samples for *in vitro* experiments were frozen at -80 °C.

2.3. Protein extraction and Western blotting analysis

The Qproteome FFPE Tissue Kit was used for protein extraction from sections obtained by cutting paraffin-embedded SSS samples (Becker et al., 2007). Sections 15 μm in thickness were deparaffinized in xylene and rehydrated in a series of descending alcohol solutions. Then, 100 μL of extraction buffer (EXB) along with β-mercaptoethanol and 1x protease and phosphatase inhibitor cocktail were added to the hydrated material. After cooling on ice for 5 min, samples were heated at 100 °C for 20 min and successively at 80 °C for 2 h with continuous shaking. Total proteins were recovered after centrifugation at 14,000 × *g* at 4 °C for 15 min, and their concentration was determined by the Bradford protein assay using bovine serum albumin (BSA) as the standard. Western blotting was performed as described elsewhere (Pelagalli et al., 2016; D’Angelo et al., 2016). Nitrocellulose membranes were probed overnight at 4 °C with anti-ARO antibody (diluted 1:400 in 2.5% BSA), washed three times in Tris-buffered saline containing Tween 20 (TBS-T) and incubated with peroxidase-affiniPure goat anti-rabbit IgG antibody (diluted 1:5000 in 2.5% BSA) for 1 h. Then, the membranes

were treated with ECL and the obtained signals were detected using a C-Digit blot scanner (LI-COR Biosciences, Lincoln, NB, USA). Prosieve QuadColor proteins were used as the standard.

2.4. Immunohistochemistry

After Bouin's fluid fixation, the specimens were dehydrated in a series of ascending alcohols and embedded in Paraplast. Successive 7 μm-thick sections were obtained. After deparaffinization and hydration procedures, the sections were immersed in citric buffer (pH 6.0) and antigen retrieval was performed (Arcamone et al., 2014). Tissue sections were stained with the ABC method as described elsewhere (De Luca et al., 2014; Liguori et al., 2015). Sections were covered with DAB solution until the desired intensity of staining was achieved and then were counterstained with hematoxylin to better identify the correct cytotypes. The slides were observed using a model DMRA2 microscope (Leica, Wetzlar, Germany). Negative controls were obtained by omitting the primary antiserum used.

2.5. In vitro Test for 17β- estradiol determination

Testis samples were de-capsulated, cut into small pieces (250 mg each) and distributed into sample tubes (one piece per tube). After addition of 2 mL of KRB buffer (10 mM glucose, 100 μM bacitracin, 0.1% ascorbic acid, and 0.1% BSA), the tubes were incubated for 60 min at 37 °C in 95% O_2 and 5% CO_2 with shaking at 60 cycles/min. The medium was then substituted with 2 mL of fresh KRB buffer and 1 nM of each substance, depending on the group. The first group was the control incubated with KRB buffer alone. In the second group, LH was added to verify tissue vitality. In the third group, OxA was added. In the fourth group, OxA was added followed by the OX1R antagonist, SB-408124. The last group included only OxB. All sample tubes were incubated at 37 °C for 12 h. After incubation, ethyl ether was added to each sample and shaken vigorously. The samples were stored at −18 °C for 10 min and the supernatants were collected. These supernatant-containing tubes were dried overnight at room temperature. The residue in each tube was dissolved in 0.5 mL of 0.05 M sodium phosphate buffer, pH 7.5, containing 10 mg/mL BSA. The 17β-estradiol level was determined by the 17β-estradiol ELISA kit according to the manufacturer instructions. The experiments were performed in triplicate and the limits of detection were identified; the sensitivity, intra-assay variability, and inter-assay variability were 4 pg, 4.9%, and 6.8%, respectively. The rate of E recovery from the male gonad corresponded to 85%.

2.6. ARO activity assay

ARO activity was measured by evaluating the *in vitro* conversion rate of T to 17β-estradiol in fresh tissue. Testicular slices were distributed in each well of a multi-well plate and the aforementioned substances were added. Finally, each well was incubated with T (35 μM) dissolved in 100 μmL of NADPH solution (3 mg/mL). The suspensions were incubated in a shaking bath as described above and then rapidly frozen. The well contents were extracted three times with ether. Solvents were pooled and dried in air. 17β-Estradiol determination was carried out on the residues using the enzyme-linked immunoassay (ELISA) kits as previously reported. The results are expressed as the 17β-estradiol concentration produced per g of tissue and per h.

2.7. Statistical analyses

The results obtained in the *in vitro* experiments were statistically processed by Duncan's test for multi-group comparison and Student's t-test for between-group comparison. All statistical results were defined as mean ± S.D. (standard deviation). A P-value < 0.01 and $P < 0.05$ were indicative of significance depending on the experiment.

3. Results

3.1. Expression of ARO in tissue homogenates

Western blot analysis using a rabbit polyclonal P450 ARO antibody revealed a positive signal having a molecular mass of 55–60 kDa in protein extracted from alpaca testis (Fig. 1, lane 1). These data were confirmed in rat testis, used as a positive control.

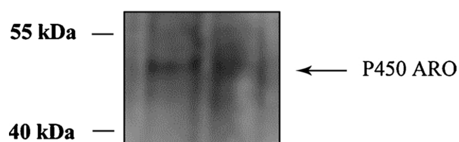

Fig. 1. ARO expression in tissue extracts. Western blotting of ARO production in testis from alpaca (lane 1) and rat (lane 2; positive control). A specific band was observed at 51 kDa by comparison with co-migrating size markers. Western blot analysis was performed using anti-CYP19 (P450 ARO) antibody. Representative data from three independent experiments are shown.

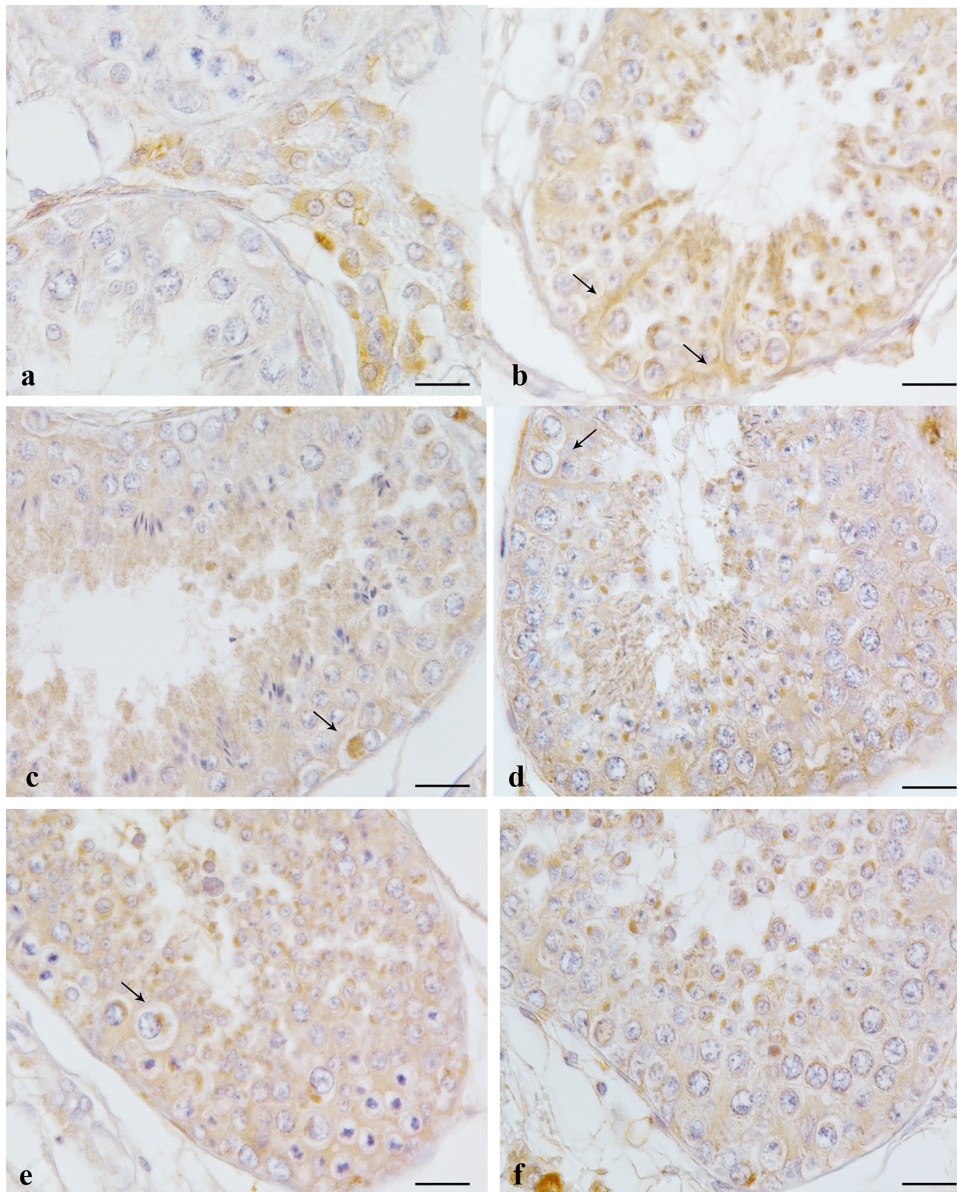

Fig. 2. ARO-IR in cytotypes of alpaca testis. (a): Large group of Leydig cells containing immunoreactive material in their cytoplasm. (b) immunopositive material entirely filled the cytoplasm of a few Sertoli cells, defining their profile from the basal membrane to the lumen (arrows). (c–e) An oblonged subtype of spermatogonia (arrow) (c), preleptotene or resting spermatocytes (arrow) (d), and pachytene spermatocytes (arrow) (e) were positive and particularly in this latter cytotype, the immunoreactive material appeared as a single roundish granule close to the nuclear membrane. (f) Round spermatids contained intensely stained acrosomal/semilunar positive material with a perinuclear localization. An avidin-biotin immunohistochemical technique was used. Bars denote 20 μm.

3.2. Immunohistochemical detection of ARO in alpaca testis

Analysis of tissue sections showed the presence of ARO- immunoreactivity (IR) in Leydig (Fig. 2a) and Sertoli (Fig. 2b) cells, oval spermatogonia (Fig. 2c), in resting or preleptotene (Fig. 2d) and pachytene (Fig. 2e) spermatocytes and in round (immature) spermatids (Fig. 2f). Positive Leydig cells (Fig.2a) were intensely stained and the cytoplasm was almost completely filled by immunoreactive granules that were sometimes condensed. Few positive supporting (Sertoli) cells (Fig. 2b) were characterized by the immunoreactive material describing the entire profile of these cytotypes. In oval spermatogonia (Fig. 2c) and resting spermatocytes (Fig. 2d), the reactive granules clustered largely in one extremity of the cytoplasm. Couples of positive resting spermatocytes originating from the spermatogonia division were often described in this species (Fig. 2d). In pachytene spermatocytes, the positive material was described as a single roundish cytoplasmic granule close to the nuclear membrane of the cells. Only round (immature)

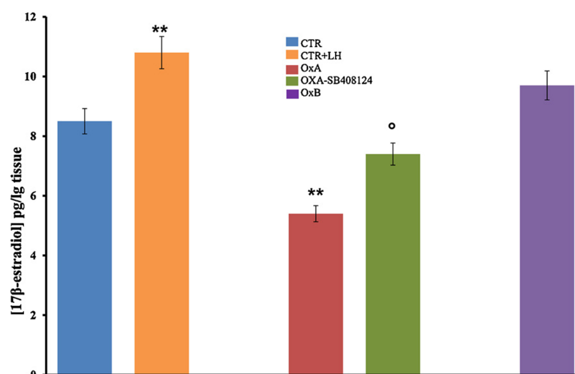

Fig. 3. Regulation of 17β-estradiol secretion*in vitro* in adult male alpaca tissue. Testicular slices were incubated with OxA alone, OxA and the OX1R antagonist SB-408124, OxB, or LH (1 nM). The 17β-estradiol level in the medium was monitored after 12 h. Values were normalized per g of incubated tissue. Data are expressed as mean ± S.D. (n 5 samples/group). *P < 0.05 **P < 0.01 *vs.* control, °P < 0.05 °°P < 0.01 *vs.* OxA (ANOVA followed by Student test).

spermatids were positive to ARO (Fig. 2f), and the immunoreactive material, semilunar in shape, was localized in a perinuclear position (probably corresponding to the acrosome complex).

3.3. 17β-Estradiol levels in tissue samples

OxA and OxB effects on testicular 17β-estradiol secretion *in vitro* were assessed in adult male alpaca. Fig. 3 shows the results from an *in vitro* experiment in which 1 nM of OxA alone or OxA and OX1R antagonist SB-408124, or OxB were added to alpaca testicular slices. OxA significantly decreased the basal level of 17β-estradiol secretion. Specifically, after 12 h, the 17β-estradiol level was lower than that of the control (from 8.5 ± 0.3 to 5.4 ± 0.4 pg/g tissue, $p < 0.01$ *versus* control). Conversely the OX1R antagonist SB-408124 seemed to nullify this effect (from 5.4 ± 0.4 to 7.4 ± 1.5 pg/g tissue, $p < 0.05$ *versus* OxA). OxB did not produce any effects. The LH steroidogenic effect (from 8.5 ± 0.3 to 10.8 ± 0.8 pg/g tissue, $p < 0.01$ *versus* control) guaranteed the tissue vitality.

3.4. Expression of ARO in tissue samples

ARO activity was demonstrated in alpaca testis tissue slices incubated in the presence of T, OxA alone, OxA and the OX1R antagonist, or OxB (Fig. 4). ARO activity was evaluated on the basis of 17β-estradiol production from exogenous T (35 μM) conversion. In response to OxA, the ARO activity decreased (from 1.19 ± 0.04 to 0.59 ± 0.05 pg of 17β-estradiol/g tissue/h, $p < 0.01$ *versus* control). In contrast, the simultaneous presence of the OxA and OX1R antagonist, SB-408124, removed this effect (from 0.59 ± 0.05 to 1.08 ± 0.1 pg of 17β-estradiol/g tissue/h, $p < 0.01$ *versus* OxA). Additionally, in this case, OxB did not produce any effects.

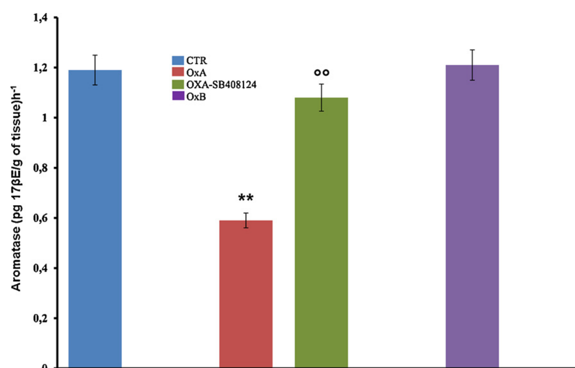

Fig. 4. Regulation of ARO activity*in vitro* in adult male alpaca tissue. Testicular slices were incubated with OxA alone, OxA and the OX1R antagonist SB-408124, or OxB (1 nM). ARO activity evaluated as the conversion rate of testosterone to 17β-estradiol in the medium was monitored after 12 h. Values are normalized as 17β-estradiol concentration produced per g of tissue and per h. Data are expressed as mean ± S.D. (n 5 samples/group). *P < 0.05 **P < 0.01 *vs.* control, °, P < 0.05 °°P < 0.01 *vs.* OxA (ANOVA followed by Student test).

4. Discussion

The goal of this research was to investigate the presence of ARO in the alpaca testis, and the possible effects of OxA and OxB on this enzyme, and the biosynthesis of E, expressed as 17β-estradiol. At first, western blotting analysis was performed to demonstrate the presence of ARO in the tissue extracts. Immunohistochemistry localized ARO both in the interstitial and tubular compartments of the alpaca testis. The location, amount, and length of action of ARO are all vital for normal testicular development (Haverfield et al., 2011).

Although in many domestic species environmental factors such as temperature, humididy and photoperiod appear to affect male gonadal physiology (Albrizio et al., 2013), alpacas transferred in more temperate climates, became more fertile because, among others, were given optimal nutrition at least along the major portion of the year (Fowler, 1998).

Previous studies performed by our research group demonstrated the presence and expression of the orexinergic complex in the male gonad of the adult male alpaca (Liguori et al., 2012, 2017b). Particularly, OxA and OxB were seen in both the interstitial and tubular compartments and their receptors were found only in the Leydig cells and developing spermatids (Liguori et al., 2012, 2017b). The immunohistochemical co-localization between the orexinergic complex and ARO in most testicular cytotypes can be ascribed to a functional relationship between these substances in the tubular and interstitial compartments.

The regulation of ARO expression cannot be ignored in the testicular development. The detection of ARO-IR in the tubular compartment of the testis during the different phases of the reproductive cycle strongly suggests that ARO controls the proliferative and meiotic phases of gametogenesis of mammals (Carreau et al., 2007). Thus, we can hypothesize a particular role played by ARO alone or in combination with orexins during spermatogenesis. Alternatively, there could be a modulation of ARO activity by orexins.

Leydig cells immunoreactive to the orexin complex and ARO serve as important sites for activating the downstream signaling of steroidogenesis. The balance between expression of ARO and sex hormone titer is quite interesting. Previous findings demonstrated the effects of OxA on steroidogenesis in the alpaca testis. A significant increase of T synthesis was observed after the addition of OxA in testis slices. The countereffect was discovered when the steroidogenic compound Müllerian Inhibiting Substance (MIS) was added to OxA, and *vice versa*. The overlying principle is most probably the OxA-induced decrease of MIS expression (Liguori et al., 2012). Hence, MIS no longer inhibited T synthesis and an elevated T level was detected. These findings in the alpaca testis provide clear evidence that OxA truly plays an antagonistic role in MIS-inhibited T synthesis. These data confirm the findings from a previous study involving rat testis (Assisi et al., 2012). The unique finding regarding the OxB effect on steroidogenesis was obtained from rat testis, and suggested that OxB had no discernable influence on steroidogenesis. (Liguori et al., 2017a). To elucidate and deepen our previous data, we presently investigated the effects of OxA and OxB on E biosynthesis expressed as 17β- estradiol, and the consequent effects on ARO modulation. ARO is considered the principal source of E in the mammalian testis. According to Raven et al. (2006), ARO inhibition is associated with lowered E levels and subsequently elevated levels of gonadotropins and T, through the E-sensitive male hypothalamus-pituitary-gonadal axis, which potentially stimulates sperm production. In the assessment of orexins on testicular E secretion, OxA decreased the level of E, particularly 17β-estradiol. This effect vanished upon the addition on the OX1R antagonist, SB-408124. On the contrary, OxB did not affect the basal E secretion. This OxA-mediated down-regulation of E secretion may be ascribed to ARO inhibition by exogenous OxA. Concerning the OxA-induced down-regulation of E, the lowered E level may be caused by reduced ARO activity, perhaps due to the inhibition of its genetic expression. This result was confirmed by the addition of SB-408124, the specific antagonist for OX1R (the specific receptor for OxA). This addition significantly aborgated the inhibitory effect of OxA, suggesting that OxA may act *via* OX1R. In this regard, a study revealed that postmenopausal women not on E replacement therapy displayed significantly higher plasma OxA levels and significantly lower E levels (El-Sedeek et al., 2010). Moreover, the injection of orexins significantly decreased the mean hypothalamic ARO mRNA level in androgenized female rats compared to androgenized control group (Salimi et al., 2016). In addition, the influence of OxA was described in the porcine uterus steroidogenesis during pregnancy (Kiezun et al., 2017).

Taken together, the findings imply that OxA may indirectly regulate ARO expression causing a decrease in E levels and consequently stimulate T production. The steroidogenic effect of OxA and its indirect modulation on ARO activity led us to hypothesize a possible role of this peptide as a therapeutic target for pathological conditions associated with steroidogenic alterations.

5. Conclusions

In summary, although the role of the orexinergic complex in testicular development and function is still regarded as being nonessential, evidence that questions this assumption is accumulating. Concerning the influence of orexins on steroidogenesis, OxA induced T secretion by interacting with OX1R, presumably by reducing the gene expression of the innate T inhibitor MIS, thereby increasing T synthesis (Liguori et al., 2012). On the other hand, OxA was also involved in down-regulating E secretion. This was most likely due to the diminution of ARO activity, which further disabled the conversion of T to E and consequently increased the OxA-mediated synthesis of T. OxB did not affect steroidogenesis. Orexin-induced steroidogenesis is a poorly-understood phenomenon. Increased knowledge could provide a basis for developing therapeutic treatments for both animals and humans.

Declarations of interest

The authors have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper entitled:” Effects of orexins on 17β-estradiol synthesis and P450 aromatase modulation in the testis of alpaca *(Vicugna pacos)”.*

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