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Presence, distribution and steroidogenic effect of the peptides orexin A and receptor 1 for orexins in the testis of the South American camelid Alpaca (*Vicugna pacos*)

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ABSTRACT

The orexins A (oxA) and B are peptides discovered in the rat hypothalamus and successively found in some peripheral organs of the mammalian body. They binds two protein G-coupled receptors defined receptor 1 (ox1r) and 2 for orexins, the first of which is highly specific for oxA while the second binds both the peptides with equal affinity. This work aimed to detect the presence of oxA and ox1r in the testis of the South American camelid alpaca (*Vicugna pacos*) and investigate the role played by them on Leydig cell steroidogenesis. The species alpaca acquired, in the last years, increasing zootechnical interest for the quality of the wool produced and its breeding spread from the country of origin to USA, Australia and Europe. Immunohistochemistry allowed us to detect oxA in Leydig and Sertoli cells, spermatogonia, resting spermatocytes, round and oval spermatids. Ox1r-immunoreactivity was found in Leydig cells and round, oval and elongated spermatids. The expression of the two peptides in tissue extracts was established by using Western blotting technique. Such results demonstrated that in the alpaca testis exists in a cellular complex able to produce and/or internalize oxA. Finally, the effect of oxA on steroidogenesis was investigated by means of *in vitro* cultured thin testis slices which were added with oxA or/and Müllerian Inhibiting Substance (MIS), a steroidolitic agent basally produced by the Sertoli cell. OxA evoked increase of testosterone production while MIS a decrease. The consecutive addition of oxA and MIS, or vice versa, highlighted an antagonistic interplay between the two substances which has been thought to be the main molecular event at the basis of the oxA-stimulated steroidogenesis mechanism.

1. Introduction

The orexins A (oxA) and B (oxB) are peptides discovered two decades ago in the rat hypothalamus [5,20] and more recently found in the gastrointestinal and genital tract of some mammalian species [14,7,30,2,17,25,26]. They derive from the proteolitic cleavage of the common precursor molecule prepro-orexin, and bind two protein G-coupled receptors defined receptor 1 and 2 for orexins. The first receptor is specific for oxA while the second equally binds both the peptides.

The hypothalamic orexins have been shown to influence many functions of the mammalian body such as food intake [20], sleep/wake cycle [18], blood pressure and heart rate [21], sexual behavior and arousal [9], and plasma corticosterone levels [15].

An increasing amount of evidence suggests that the male genital tract is influenced by the orexin complex of the body which regulates its functions through a double modality of action. The first way is indirect and takes place in the hypothalamus where gonadotropin-containing neurons are stimulated to release their hormones by the input received from orexinergic nerve fibers found close in contact with them [3,23]. Such linkage partially constitutes the first step of the so called hypothalamic/gonadal axis. The second modality is direct because played in the genital tract by cytotypes able to synthesize orexins and release them towards targets containing the cognate receptors. The neuroendocrine cells of cattle urethra, the principal cells of rat epididymis, the Sertoli cells of rat testis provide a source of oxA in this tract [2,19,25,26]. Moreover the presence of ox1r mRNAs has been demonstrated in the seminal vesicles, penis and epididymis of humans [13], in the testis of sheep [30], chicken [16] and rat [1,12], in the cattle prostate [19] and in the rat epididymis [25].

In this work we applied the study of the orexinergic system to the testis of a South American animal species, the alpaca (*Vicugna pacos*), aimed by two reasons. Firstly, in our knowledge, the orexins and their receptors were never investigated in the genital tract of

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non conventional animals of zootechnical interest. The alpaca breeding, which plays an important socio-economic role in the country of origin for meat and wool production, in the last years has been extended in USA, Australia and Europe where the characteristics of the wool produced are greatly appreciated [22]. At present 38 alpaca farms have been officially recognized in Italy by the Italian association of alpaca breeders (Italpaca), but it is probable that the number of those really existing is superior. Furthermore we were intrigued by the difference existing, under an evolutionistic point of view, between alpaca and rat; this latter is the lonely species which drew, at present, sufficient attention on the argument treated in this work [1,2,12,26].

The alpaca is a camelid belonging to the genus *Vicugna* together with vicuna (*Vicugna vicugna*), and phylogenetically similar to llama (*Lama glama*) and guanaco (*Lama guanicoe*). All species, as known, are widely diffused in the Andean regions of South America, from Ecuador to Southern Chile. The physiology of alpaca reproduction is still poorly known and thus the breeding management is founded mainly on practice as far as mating and selection. Interestingly, it seems that the transfer from the cold Andean altitudes to regions having more temperate climate enhanced the fertility of the species which, outside its original environment, became increasingly richer of non-seasonal breeders [24].

Here, we investigated the presence of oxA and its specific receptor 1 in the alpaca testis by means of immunohistochemistry and assessed the tissue expression of the peptides by western blotting. Furthermore, the effect of oxA on testosterone (T) synthesis was studied on *in vitro* cultured thin slices of testis which were added with the peptide or/and with the steroidolitic compound Müllerian Inhibiting Substance (MIS).

2. Material and methods

2.1. Antibodies and chemicals

Peroxidase conjugated goat anti-rabbit (AP 132P) and rabbit anti-goat (PI-9500) IgG were purchased, respectively, from Millipore Corporation (Billerica, MA, USA) and Vector Laboratories (Burlingame, CA, USA); goat polyclonal anti-oxA (sc-8070) and anti-ox1r (sc-8072) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit polyclonal anti-prepro-orexin antibody (AB3096) from Millipore Corporation (Billerica, MA, USA); biotinylated secondary antibodies and avidin–biotin complex (PK-6105) from Vector Laboratories (Burlingame, CA, USA); enhanced chemiluminescence kit (RPN 2109) from ECL Amersham (Little Chalfont, Buckinghamshire, UK). The peptide oxA (003-30) was obtained from Phoenix Pharmaceuticals, MIS (sc-111265) and ox1r (sc-8073P) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); enzyme immunoassay kit for T determination from Adaltis (Bologna, Italy); marker proteins from Prosieve quadcolor (London, UK).

2.2. Animals

The animals employed in this work belonged to an officially recognized Italian breeding farm where they were kept in optimal nutritional and sanitary conditions. Four healthy males of about seven years of age were chosen for castration. All were lacking of preputial adhesions, which is a signal of sexual maturity [27]. The surgical operation was performed according to Fowler [8]. After removal the testes were finely cut in small samples which were either fixed in Bouin's fluid for immunohistochemistry or frozen in liquid nitrogen and stored at -80 °C until used for western blotting analysis. To perform *in vitro* experiments other freshly collected material was treated as described beyond.

2.3. Immunohistochemistry

The fixed material was processed for paraffin embedding in vacuum and cut at a thickness of 5–7 μm. The avidin–biotin immunohistochemical method was performed using a commercially obtained kit. Briefly, the sections were deparaffinized in xilene, hydrated in graded ethanols and incubated for 30 min in 3% hydrogen peroxide in water in order to quench endogenous peroxidase activity. Then they were transferred for 30 min in 0.01 M phosphate buffered saline containing 1.5% normal rabbit serum to block non specific binding of secondary antibodies. Goat policlonal anti-oxA and goat policlonal anti-ox1r primary antibodies were applied to the sections at dilution of 1:200 in a moist chamber overnight at 4 °C. The sequent day, the sections, after washing in PBS, were incubated for 30 min at room temperature in 1:200 diluted biotinylated anti-goat IgG. Then they were washed and again incubated for 30 min in freshly prepared ABC reagent. A diaminobenzidine solution was used as a final staining. The specificity of the immunoreaction was tested by replacing the primary antibody with buffer or absorbing the same with an excess (100 μg/ml) of the relative antigen. Control sections resulted always negative. The preparations were observed by the light microscope Nikon Eclipse E-600 and microphotographs were taken by a Coolpix 8400 digital camera.

2.4. Western blotting analysis

Frozen tissues were homogenized in buffer (50 mM Tris HCl, pH 7.0; 150 mM NaCl; 2% Triton; 5 mM EDTA; 10 mg/ml leupeptin; 0.1 U/ml aprotinin; 1 mM PMSF) using an Ultra-Turrax homogenizer and centrifuged at 16,000*g* for 20 min at 4 °C. Aliquots of the supernatant were subjected to electrophoresis on 15% sodium dodecyl sulfate–polyacrylamide (SDS PAGE) (Bio-Rad, Hercules, CA, USA) gel which successively was transferred to nitrocellulose by using a semidry apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The membrane was incubated for 1 h at 42 °C in 5% BSA containing TBST buffer (150 mM NaCl; 20 mM Tris HCl, pH 7.4; 0.3% Tween-20), washed in the same buffer and again incubated for 2 h at room temperature with rabbit polyclonal anti-prepro-orexin and goat polyclonal anti-ox1r antisera. These latter were diluted 1:500 in 2.5% BSA containing TBST. After washing three times in TBST, the membrane was incubated in 1:2,000 diluted peroxidase conjugated goat anti-rabbit and rabbit anti-goat secondary antibodies for 1 h at room temperature. Proteins were visualized by an enhanced chemiluminescence kit. Marker proteins were used to estimate the molecular weight of each band.

2.5. In vitro tests

To study the effect of oxA and MIS on T secretion, incubations of tissue were carried out as follows. The removed testes were decapsulated and cut with a thin blade into pieces of approximately equal size (mean weight/piece: 250 ± 7 mg). Slices were distributed in multi-well plates (250 mg/well) and incubated in 1 ml Krebs–Ringer bicarbonate buffer (pH 7.4) containing 10 mM glucose, 100 μM bacitracin, 0.1% ascorbic acid and 0.1% bovine serum albumin (KRB). The samples were preincubated for 60 min at 37 °C in an atmosphere of 95% O_2/5% CO_2 constantly shaking at 60 cycles per min. In the first set of experiments the medium was substituted with 1 ml of fresh KRB containing 1 nM oxA or 1.7 μmol SB-408124, an highly selective antagonist of the oxA specific receptor ox1r. The incubations lasted 90 min and, at this time, some of them were prolonged up to 180 min with or without the addiction of 1 nM oxA. Controls were incubated in medium alone. In the second experiment, parallel sets of suspensions were incubated with 1 nM oxA or 1 nM MIS for 6 h. At this time some wells containing

oxA were added with 1 nM MIS and, conversely, others containing MIS were added with 1 nM oxA. The relative incubations lasted 6 h still, up to a final time of 12 h. These conditions were chosen on the basis of preliminary tests performed using increasing concentrations of oxA (0–2 nM) or MIS (0.5–2.0 nM), at different incubation times (0–24 h). Moreover, in order to verify the tissue vitality at every stage of the experiments, testis slices were absorbed with LH and the T production monitored in the time range of 90–720 min. T was measured as reported below. The results were expressed as normalized values per g of incubated tissue. The experiments were performed in triplicate and data were expressed as means ± SD. Statistically significant differences were set at $p < 0.05$ and $p < 0.01$.

2.6. Testosterone determination

Determination of T levels in the incubated medium was carried out using an enzyme immunoassay kit. The following limits of detection were observed: sensitivity 6 pg, intra-assay variability 5.3%, inter-assay variability 7.5%. The medium was mixed vigorously with ethyl ether (1:10, v/v) and the ether phase was withdrawn after centrifugation at $3{,}000 \times g$ for 10 min. Three extractions were performed. Pooled ether extracts were dried by evaporation, and the residue was dissolved in 0.5 ml sodium phosphate buffer (0.05 M, pH 7.5) containing 10 mg/ml BSA. The determination was performed as reported in Di Fiore et al. [6]. The rate of T recovery from testis was about 80%.

2.7. Statistical analysis

Data coming from *in vitro* tests were compared by analysis of variance followed by Duncan's test for multi-group comparison and Student's *t*-test for between-group comparison. All data were expressed as means ± S.D. (standard deviation). The level of significance was taken at $p < 0.01$ and $p < 0.05$.

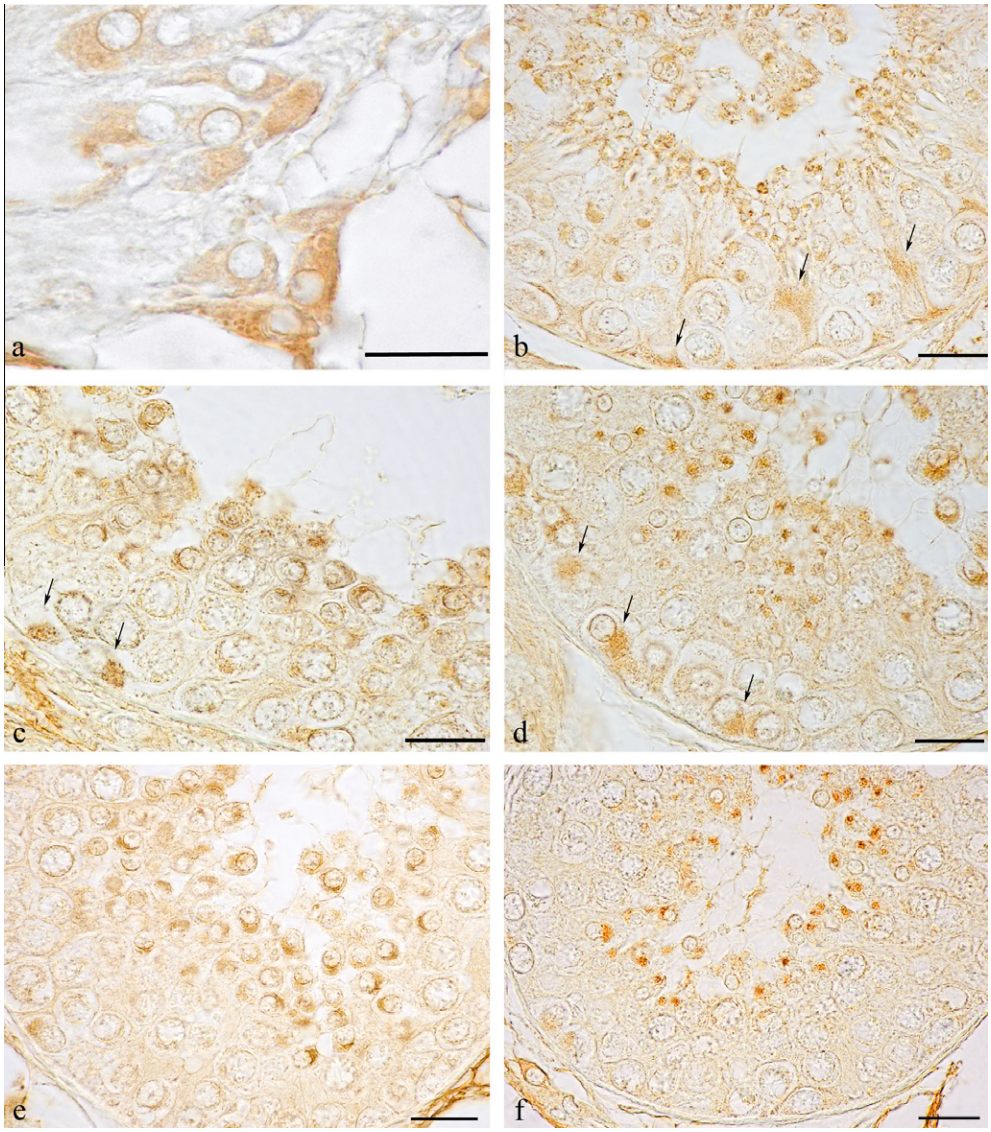

Fig. 1. OxA-immunoreactive cytotypes in the alpaca testis. (a) a small cluster of oval shaped Leydig cells whose cytoplasm is completely filled with granular, positive material. (b) weakly stained secretory granules contained in the basal cytoplasm of three adjacent Sertoli cells (arrows). (c and d) oblonged subtype of spermatogonia (arrows) (c) and round shaped resting spermatocytes (arrows) (d) are aligned along the tubular membrane and show red/brown stained positivities clustered in their tipically pale cytoplasm. (e and f): round (e) and oval (f) spermatids contain in their acrosomes positive material which appears as a perinuclear halo in the younger cytotype and as a peripheral, small cluster in the more developed one. Avidin–biotin immunohistochemical technique. Bars: 20 μm.

3. Results

3.1. OxA- and ox1r-immunoreactivity in testicular cytotypes

OxA-immunoreactivity has been found in Leydig and Sertoli cells, spermatogonia, preleptotene (resting) spermatocytes, round and oval spermatids. The reactive material showed granular aspect and cytoplasmic localization. Positive Leydig cells (Fig. 1a) were numerous, round or oval in shape and often organized in small groups composed by differently stained elements. Sertoli cells and spermatogonia were the rarest reactive cytotypes even found. The positivities of the first (Fig. 1b) appeared as small, scarcely reactive granules often scattered in the whole cytoplasm. The spermatogonia of the alpaca testis were round or oval in shape and only the second subtype was found to be, sometimes, positive. In this case (Fig. 1c), they showed a clearly visible cluster of reactive granules in one of the two extremities of the cytoplasm. Preleptotene spermatocytes were round in shape and aligned in brief rows along the basal membrane. When positive (Fig. 1d), these cells showed a cluster of stained granules in perinuclear position. In spermatids, the reactive material was always contained in the acrosome body and assumed shape and localization of this latter during the evolutive cycle of the cell. Thus, round (immature) spermatids showed semilunar and perinuclear positivities (Fig. 1e), which progressively became punctiform and peripheral in the oval (developing) cells (Fig. 1f).

Ox1r-immunoreactivity was observed in Leydig cells (Fig. 2a and b) and in round (Fig. 2c), oval (Fig. 2d) and oblonged (mature) (Fig. 2e) spermatids. Quantity, aspects and staining intensity of the positivities found in these cells were similar to those just described for the same cytotypes containing oxA.

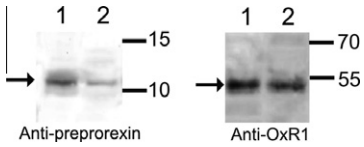

Fig. 3. Prepro-orexin and ox1r expression by Western blot analysis. Lane 1: rat brain tissue. Lane 2: alpaca testis tissue. Molecular weight markers are expressed in kDa.

3.2. Expression of prepro-orexin and ox1r in tissue extracts

Tissue extracts of rat brain were chosen as positive controls because the brain is an organ known to contain high quantity of oxA and ox1r [12]. Tissue extracts of alpaca testis and rat brain reacted with the anti-prepro-orexin and anti-ox1r antisera which recognized two protein bands weighting 16 and 55 kDa, respectively. Such molecular weights correspond to those assigned to prepro-orexin and ox1r usually found in the mammalian body. The data reported above are shown in Fig. 3.

3.3. T levels in oxA and MIS treated testis slices

The effect of oxA and MIS on alpaca testis steroidogenesis has been studied here in a series of *in vitro* experiments. The compounds were absorbed with thin sections of tissue separately or contemporaneously. Fig. 4 shows the results from an *in vitro* experiment in which 1 nM oxA, alone or in competition with 1.7 μmol SB-408124, was added to a testis slice containing medium for a time ranging from 0 to 180 min. It is evident that oxA significantly stimulated basal T secretion. Specifically, after 90 min the synthe-

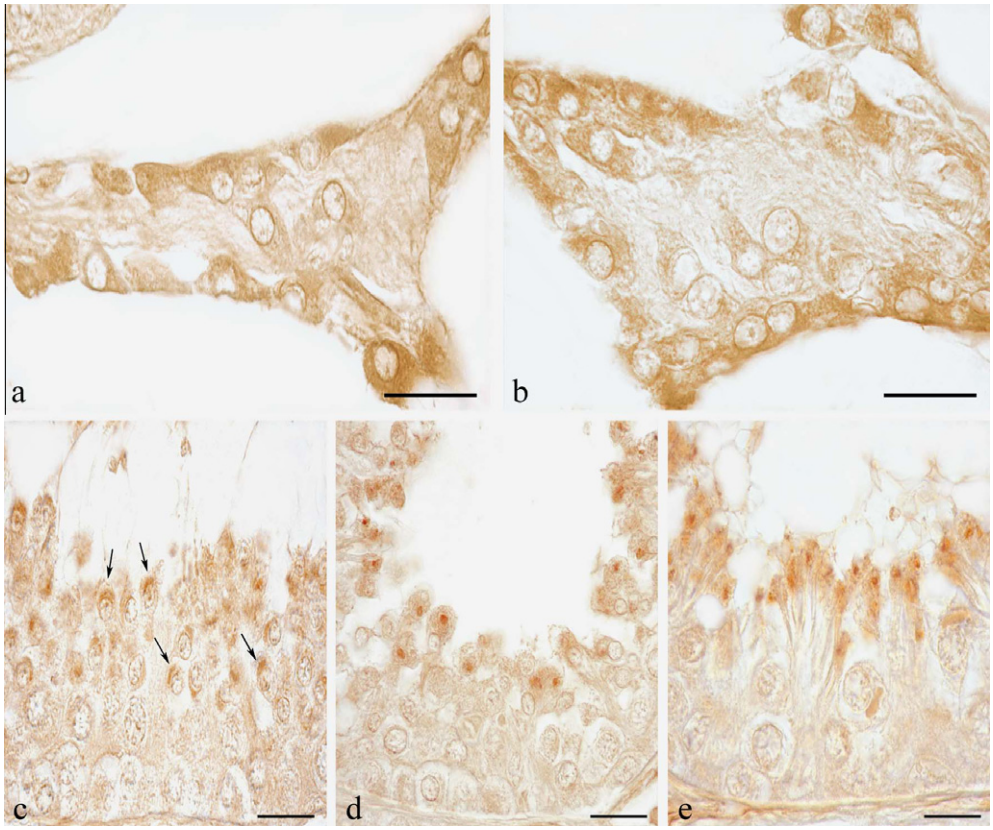

Fig. 2. Ox1r-immunoreactive cytotypes in the alpaca testis. (a and b): irregularly shaped Leydig cells show a prominent nucleus and variable staining intensity of cytoplasmic positivities. (c–e) the acrosomal bodies of round (c), oval (d) and elongated (e) spermatids contain positive material and progressively change shape and cytoplasmic localization during the evolutive cycle. Avidin–biotin immunohistochemical technique. Bars: 20 μm.

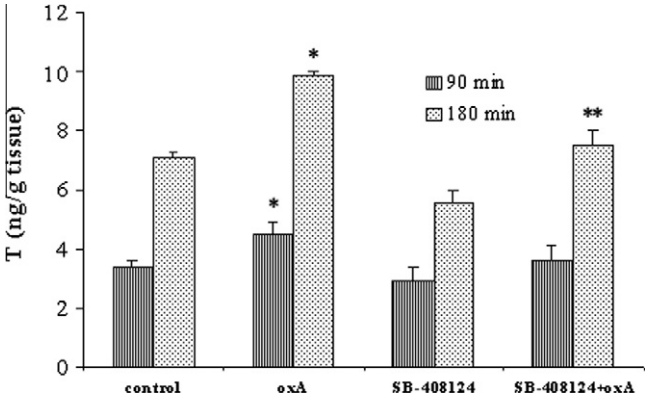

Fig. 4. Stimulation of T secretion *in vitro* by oxA. Testicular slices were incubated with oxA and the ox1r antagonist SB-408124, separately or contemporaneously. T level in the media was monitored after 90 and 180 min. Values are normalized per g of incubated tissue. Data are expressed as mean ± SEM (9 samples/group). *, $p < 0.01$ *versus* control; **, $p < 0.01$ *versus* oxA alone (ANOVA followed by Student test).

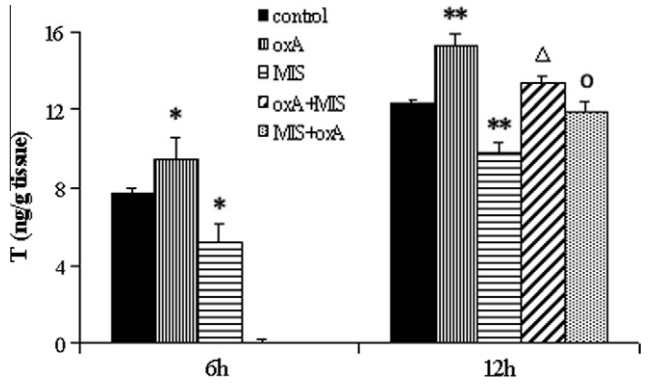

Fig. 5. OxA and MIS effects on T secretion. Testis slices were incubated with oxA or MIS, and T level in the media was monitored after 6 h. At this time some incubations were added with MIS or oxA, respectively, and the time prolonged up to 12 h. Values are normalized per g of incubated tissue. Data are expressed as mean ± SEM (9 samples/group). *, $p < 0.05$ *versus* control; **, $p < 0.01$ *versus* control; $^{\Delta}$, $p < 0.01$ *versus* oxA alone; $^{\circ}$, $p < 0.01$ *versus* MIS alone (ANOVA followed by Student test).

sis rate was higher than control ($p < 0.01$), while the antagonist alone remained ineffective. After 180 min the stimulation was confirmed ($p < 0.01$ oxA *versus* control) as it was the inactivity of the antagonist. On the contrary this latter strongly decreased the effects of the peptide when both the compounds were present in the incubation medium ($p < 0.01$ *versus* oxA alone). Fig. 5 shows the results of the second set of experiments in which testis slices were incubated with 1 nM oxA or 1 nM MIS for 6 and 12 h. Sometimes samples, after an initial treatment with each substance for 6 h, were incubated with MIS or oxA, respectively, up to 12 h. OxA increased T rate at 6 and 12 h ($p < 0.05$ and $p < 0.01$ *versus* control, respectively). On the contrary MIS decreased T rate at same times ($p < 0.05$ and $p < 0.01$ *versus* control, respectively). The addition of MIS to incubation with oxA significatively decreased the expected oxA-dependent T increase ($p < 0.01$ *versus* oxA alone). Similarly the addition of oxA to incubations with MIS partially abolished the MIS-dependent T inhibition ($p < 0.01$ *versus* MIS alone). The tissue vitality was verified by LH stimulation (500 ng/g tissue) at 90, 180, 360 and 720 min. Fig. 6 shows that the rate of T synthesis, expressed as T production for g tissue, was significant at every stage of the experiment.

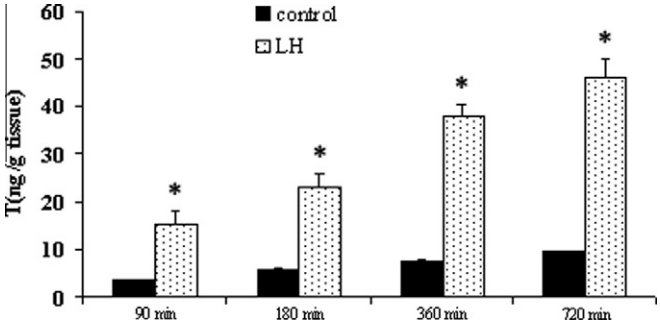

Fig. 6. Tissue vitality assay. Testis slices were incubated with or without LH (500 ng/g tissue) and T level in the media was monitored after 90, 180, 360 and 720 min. Values are normalized per g of incubated tissue. Data are expressed as mean ± SEM (9 samples/group). *, $p < 0.01$ *versus* corresponding control (ANOVA followed by Student test).

4. Discussion

In this work immunohistochemistry allowed us to describe the presence of oxA- and ox1r-containing cytotypes in the testis of the South American camelid alpaca. Western blotting supported such findings demonstrating in tissue homogenates the existence of both the peptides investigated.

The effect of oxA on Leydig cell steroidogenesis has been studied in rat testis by Barreiro and coworkers [1,2]. Absorbing thin slices of testis with oxA these authors found, in two separate *in vitro* experiments, an increase of T production and a marked decrease of mRNA codifying for MIS synthesis. MIS is a compound basally produced by the Sertoli cells of the mammalian testis showing a potent down regulation of T synthesis in fetal, neonatal and mature Leydig cells [26,28]. On this basis, the mechanism of action of the oxA-induced T synthesis was hypothesized to be an antagonistic crosstalk between oxA and MIS taking place in the tubular compartment of the gonad [2]. As MIS, in fact, oxA and its specific receptor 1 have been thought to be produced by the Sertoli cell [1,2,25].

In the *in vitro* experiments here performed we absorbed slices of alpaca testis with oxA and MIS, separately or contemporaneously, and obtained results consistent with the previous findings: while oxA enhanced T production, the addition of MIS significantly decreased it. Moreover, the steroidogenic activity of a 12 h long absorption of oxA resulted to be higher than that obtained when a preliminary 6 h long absorption of oxA was followed by a same time long addition of MIS. On the other hand, the steroidolitic effect of 12 h long absorption of MIS resulted to be much higher than that observed when a preliminary 6 h long absorption of MIS was followed by a same time long addition of oxA. These findings provide definitive evidence that oxA and MIS really play an antagonistic role in the determinism of oxA-induced testis steroidogenesis. In such a mechanism the oxA specific receptor ox1r is surely involved because, as described above, the addition of its antagonist SB-408124 to oxA treated slices strongly decreases the steroidogenic effect of the peptide. The responsiveness of the tissue used in our *in vitro* settings is warranted by the use of the T synthesis stimulator LH which showed significant production of the hormone all along the length of the experiments.

In the rat testis oxA-immunoreactivity was found in resting, leptotene, zygotene and pachytene spermatocytes [2] as well as in Sertoli cells and spermatids gradually maturing from the VIIth stage of the germ evolutive cycle up to the XIVth [26]. In the same species ox1r-immunoreactivity has been detected in leptotene and pachytene spermatocytes and immature spermatids [our unpublished findings]. Still in the rat ox1r mRNA was present in all stages of the germ cycle, as stated in a study performed on *in vitro* cul-

tured fragments of seminiferous tubules [1]. In the alpaca testis oxA-immunoreactivity was detected in all cytotypes of the tubular epithelium, from Sertoli cells to elongated spermatids, with the exception of II spermatocytes which notoriously have a brief life. In the same compartment ox1r was found in the whole arch of the spermatid development, from the round, immature phase up to the elongated shape of terminal cell.

Taking together these findings, we are induced to think that the seminiferous tubule of the mammalian testis contains a cellular complex able to produce and/or internalize oxA. The main component of this complex could be the Sertoli cell, already thought as the principal source of oxA in the rat testis [2]. In *in vitro* cultured slices from this organ oxA strongly decreases the production of Stem Cell Factor (SCF) mRNA [2]. SCF is a compound produced by the Sertoli cell and when released in the intercellular spaces stimulates spermatogonia proliferation and consequently germ epithelium development [10,29]. Thus, the intratubular synthesis of oxA could indirectly decrease germ cell proliferation inhibiting SCF production [2]. On the other hand a direct effect of oxA on the germ epithelium cannot be excluded because many components of this latter contain, as described above, the relative receptor 1.

In our knowledge, only another endocrine-like substance has been described in the alpaca testis: the epidermal growth factor (EGF) and the cognate receptor EGFR [11]. In post-pubertal subjects both the peptides are widely diffused in interstitial and tubular cytotypes and, as reported above for oxA, have been thought to influence both steroidogenesis and spermatogenesis. This study also highlights a streaking similarity between the orexinergic cytotypes of the alpaca testis and those described in the rat testis. The consideration that the two animal species are different under a phylogenetic point of view led us to retain that a complex of oxA- and ox1r-containing cells could be present also in the testis of many other mammals. Finally, it is known that environmental factors such as temperature, humidity and light influence the reproductive behavior of an animal species. The camelids llamas and alpacas were adapted to survive on the altitudes of the Andean regions and, when transferred in more temperate climates, became more fertile because, among others, were given optimal nutrition at least along the major portion of the year [8].

4.1. Conclusions

This research provides evidence of a wide diffusion of oxA and its specific receptor 1 in the alpaca testis. The peptides have been thought to be involved in the regulation of the main functions of the male gonad, steroidogenesis and spermatogenesis. Particularly the oxA-induced steroidogenesis is a phenomenon still poorly known but it is probable that in the alpaca, as in the rat [2], a critical role of its determinism is played by an antagonistic crosstalk between oxA and the steroidolitic compound MIS.

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Further reading

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