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Segment–Specific Expression of MMP/TIMP in the Oviduct of Llama (*Lama glama*) and Gelatinolytic Activity in the Oviductal Fluid

Expresión Segmento Específica de MMP/TIMP en el Oviducto de Llama (*Lama glama*) y Actividad Gelatinolítica en el Fluido Oviductal

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SUMMARY: Oviductal molecules have the potential to improve the reproductive biotechnologies. In camelids, knowledge and assessment of the oviductal environment are necessary to successfully develop species-specific reproductive technologies, especially because of the camelids reproductive particularities. Among the oviductal factors, the matrix metalloproteinases/tissue inhibitor of matrix metalloproteinases system (MMPs/TIMPs) should be investigated more thoroughly due to their participation in reproductive processes. Consequently, the current study assayed gene and protein expression of MMPs throughout the llama oviduct. MMPs zymogen and active forms in the oviductal fluid were also characterized. MMP2 and MMP9 transcripts were detected in ampulla, isthmus, utero-tubal junction and papilla, being MMP2 and MMP9 2.15 and 1.10 folds higher in papilla than in ampulla, respectively. In addition, differences in immunolocalization of MMP2 and MMP9 between the epithelial mucosa layers of the oviductal segments were observed. The presence of MMPs in the epithelium suggests their secretion into the oviductal lumen. Coincidently, bands of 62 and 94 kDa, corresponding to MMP2 and MMP9 were detected by zymography in the oviductal fluid. Treatment with an exogenous activator (APMA) suggests that they are present as proMMPs. TIMP2 and TIMP1, the specific inhibitors of MMP2 and MMP9, respectively, were expres sed in each oviductal segment, indicating a well-regulated control of MMP proteolytic activity in the oviduct. These findings prove that the llama oviduct produces and secretes MMPs into the oviductal lumen, suggesting that these enzymes may have an unknown role in the preparation of the oviductal environment for gametes, fertilization and early embryo development in camelids.

KEY WORDS: Oviduct; Llama; MMPs; TIMPs; Gene expression levels.

INTRODUCTION

Within the female reproductive system, the oviduct and its secretions provide an optimal microenvironment necessary for the reproductive events that precede implantation, including gamete transport, sperm capacitation, fertilization, and early embryonic development (Avilés *et al.*, 2010). A thorough study of how the oviductal environment is regulated and organized has the potential to improve reproductive biotechnologies, enabling to mimic biochemical composition of tubal fluid in the synthetic media for in vitro maturation, in vitro fertilization or embryo culture.

Regarding South American camelids (SACs), interest in applying reproductive technologies has increased in the last decade because they have become internationally known due to their productive characteristics. Nevertheless, assisted reproduction in camelids is still challenging compared with other livestock because of their special anatomical and physiological characteristics. Female ovulation is induced by mating and during copulation the male deposits the semen deep inside the uterine tubes. Ovulation occurs 26–42 h late, only when a growing follicle is larger than 7 mm in diameter (Brown, 2000).

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As in other mammalians, the camelids oviduct comprises four segments with different roles: utero-tubal junction (UTJ), isthmus, ampulla and infundibulum with the particularity that UTJ forms a kink-like anatomical constriction ending in a small papilla that is projected into the uterus. These segments present a specific regional micro-morphology and function, which involves adjustments in the biochemical composition of the oviductal fluid. At this time, only a few molecules were identified in SACs oviduct (Zampini *et al*., 2014; Apichela *et al*., 2015). Among the oviductal factors, matrix metalloproteinases (MMPs) and their inhibitors (TIMPs: tissue inhibitors of matrix metalloproteinases) are worth to examine. MMPs are a large family of proteolytic enzymes, able to degrade extracellular matrix (ECM) components or activate growth factors and cytokines (Fowlkes & Winkler, 2002). MMPs activity is mainly regulated by TIMPs. There are four TIMPs (TIMP1, TIMP2, TIMP3 and TIMP4) that are able to inhibit MMPs by forming non-covalent 1:1 stoichiometric complexes (Visse & Nagase, 2003). The MMPs-TIMP system has been involved in reproductive events such as cumulus expansion, fertilization (Gabler *et al*., 2001), semen fluidization (Tentes *et al*., 2007) and early embryonic development (Avilés *et al*.), occurring in the oviduct.

In a previous study, we detected gelatinolytic activity corresponding to MMPs in the llama (*Lama glama*) oviductal fluid (OF) and presence of MMP2, MMP9, TIMP1 and TIMP2 transcripts in the oviduct (Zampini *et al*.). In order to make a step forward to a better understanding of oviductal MMPs in llama, the aims of the present study were to establish how MMP2, MMP9 and their main inhibitors (TIMP2 and TIMP1, respectively) were displayed in each oviductal segment: ampulla, isthmus, UTJ and papilla, by the quantification of their mRNA expression levels and the proteins localization. Considering that MMPs are synthesized and secreted as inactive proenzymes (zymogens), a metalloproteinase-specific activation test was applied to differentiate active forms of MMPs and MMP precursors in OF.

MATERIAL AND METHOD

Animals and Samples. Non-lactating, non-pregnant, 5–8 years old fertile female llamas (*Lama glama*) were used in this study (n = 7). The llamas belonged to the Campo Experimental de Altura, Instituto Nacional de Tecnología Agropecuaria (INTA), located in Abra Pampa (22 °S 65 °O, Jujuy, Argentina) at 3,484 m altitud.

Llama females which showed ovaries with follicles smaller than 7 mm (follicular phase) were chosen for the study. The reproductive tracts were obtained immediately after animals were slaughtered, in accordance with protocols approved by local institutional animal care. Oviducts were separated and OF was obtained by perfusion with 100 mL of 10 mM Tris-HCl, 150 mM NaCl (pH 7.4) at 4 ºC; the liquid was subsequently centrifuged to remove cellular debris. Next, oviducts were sliced and separated into ampulla, isthmus, UTJ and papilla segments. The tissues were fixed with 10 % formaldehyde in PBS (pH 7.4) for histological and immunohistochemical analysis or placed in RNA later solution (Ambion, Austin, TX, USA), according to the manufacturer's instructions, for expression analysis.

RNA isolation and cDNA synthesis. Total RNA from oviductal tissue segments (ampulla, isthmus, UTJ and papilla) was isolated using the SV total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. RNA was quantified spectrophotometrically at 260 nm, and RNA integrity was examined by electrophoresis on 1.5 % agarose gels, stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA).

Table I. Specific primers used in qPCR.

	Primer sequences (5´-3´)	GenBank accession number	Amplicon size (bp)
MMP2 forward	CATGATGGAGAGGCTGACAT	GQ244429.1	148
MMP2 reverse	GCTCATCGTCATCAAAGTGG		
MMP9 forward	GTTCGATGTGAAGACGCAGA	GU207475.1	175
MMP9 reverse	GTCCACCTGGTTCACCTCAT		
TIMP1 forward	GTGGCTCCCTGGAACAGTC	KC425456.1.	143
TIMP1 reverse	TCGGTCCACAAGCAATGAGT		
TIMP2 forward	GCACCACCCAGAAGAAGAGC	KC425455.1	117
TIMP2 reverse	CCATCCAGAGGCACTCATCC		
ACTB forward	GCGGGACCACCATGTACC	XM_006210388.1	183
ACTB reverse	ACTCCTGCTTGCTGATCCAC		
HPRT forward	TGACACTGGCAAAACAATGCA	XM_006215984.1	94
HPRT reverse	GGTCCTTTTCACCAGCAAGC		

Briefly, 1 µg of RNA from ampulla, isthmus, UTJ and papilla was reverse-transcribed with M-MLV reverse transcriptase (Promega) and oligo-dT primer in a 25 mL reaction mixture according to the manufacturer's instructions. Reactions were performed by incubating the mixture in a thermal cycler at 42 ºC for 90 min followed by a reverse-transcriptase inactivation at 94 ºC for 5 min.

Quantitative PCR of MMPs and TIMPs in oviductal segments. Specific primers based on llama MMP and TIMP sequences previously identified were designed with Primer3 software (Table I) and used to analyze expression in the oviductal segments with qPCR. ACTB (b-actin) and HPRT (hypoxanthine phosphoribosyltransferase) primers, previously designed using predicted *Vicugna pacos* nucleotide sequences, were employed as internal control of qPCR (reference genes).

Real-time qPCR was performed on a CFX96 real-time PCR detection system (Bio-Rad, CA, USA), using iTaq Universal SYBR Green Supermix (Bio-Rad) and specific primers. Parallel reactions with total RNA, which were not reversely transcribed (–RT), and reactions without templates (NTC) were also performed. Ampulla, isthmus, UTJ and papilla cDNA samples (n = 7) were amplified in a total volume of 20 µL using iTaq Universal SYBR Green Supermix (Bio-Rad) and 250 or 300 nM of specific primers for each sample; all amplifications were carried out in triplicate. A two-step protocol (98 ºC for 15 s, 63 ºC for 20 s) was repeated during 50 cycles, followed by a melting curve starting at 70 ºC and subsequently increasing to 95 ºC at a transition rate of 0.2 ºC/s to ensure single product amplification and exclude possible interference with primer dimers. Relative expression levels were quantified using the ddCt method using CFX Manager Software, version 3.0 (Bio-Rad). Data were normalized to the geometric means of ACTB and HPRT.

Reactions were carried out following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (Bustin *et al.*, 2009).

Immunohistochemistry. The fixed tissues were subjected to standard immunohistochemistry protocols. Briefly, oviduct sections (5 µm) were mounted on poly-L-lysine-coated microscope slides and endogenous peroxidase activity was eliminated by incubation with 3 % H_2O_2 in PBS, pH 7.4, for 30 min. After washing with PBS, sections were blocked with 5 mg/mL milk-PBS solution for 30 min at room temperature. Sections were incubated overnight at 4 ºC with rabbit anti-human MMP2 polyclonal antibodies (dilution 1:100, AB19167, Millipore, Bedford, MA, USA) or rabbit anti-mouse MMP9 polyclonal antibodies (dilution 1:100, ab38898, Abcam, Cambridge, MA, USA). Then, sections were incubated for 1 h with a 1:200 dilution of the biotinylated anti-mouse/rabbit IgG antibody (BA-1400, Vector, Burlingame, CA, USA). Sections were washed with PBS before use of the Vectastain Elite ABC Kit detection system (Vector, Burlingame, CA, USA). The images were obtained with

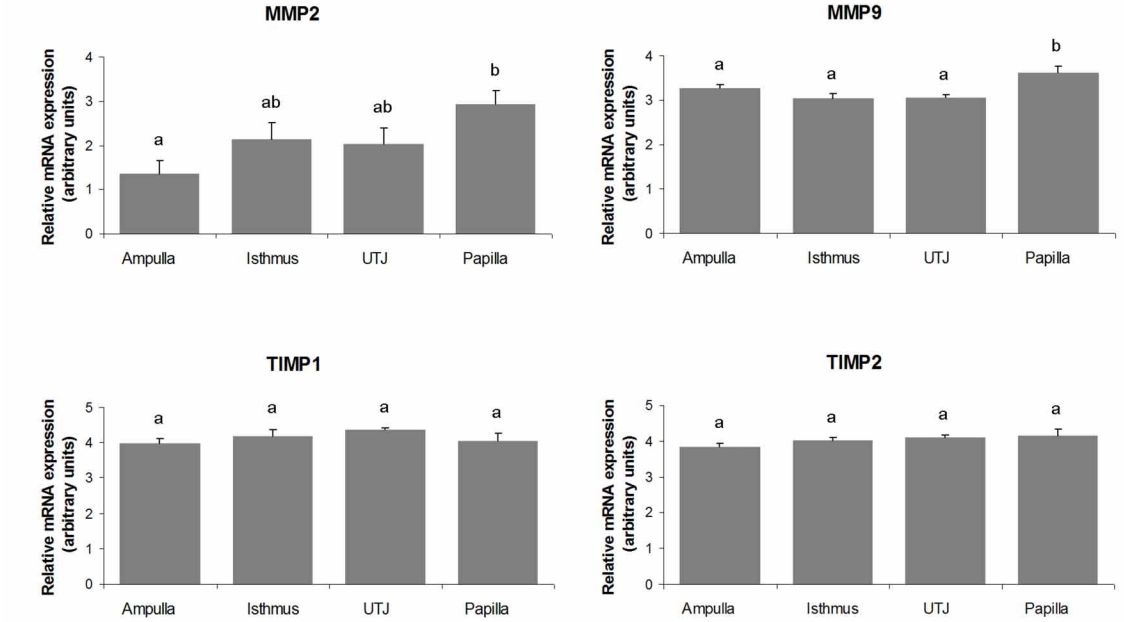

Fig. 1. Messenger RNA (mRNA) expression levels of MMP2, MMP9, TIMP1 and TIMP2 in llama oviductal tissue from ampulla, isthmus, UTJ and papilla. Data are shown as mean mRNA levels (normalised to the geometric mean of ACTB and HPRT references genes) ± standard error of the mean (SEM).

a digital Leica DCC-380x camera attached to a trinocular microscope (Leica, DM4000B Led) at x100 and x400 magnification using LASZ Leica Inc. Software

Gelatine Zymography. In order to confirm the character of oviductal MMPs, OF was incubated in the presence of APMA (aminophenylmercuric acetate) to activate the latent zymogen and induce the transition of proMMP to MMP, and analysed by zymography. Total protein was determined using a Micro BCA kit (Thermo Fisher Scientific, Rockford, USA). A OF pool with 30 µg of total proteins was incubated for 4 h at 37 °C in the presence of 1 mM APMA (diluted from a 20 mM stock in 80 mM NaOH). Then, the treated sample and pooled OF without APMA treatment were each separated under non-reducing conditions on 8 % polyacrylamide gels containing 1 mg/mL gelatine as described by Zampini *et al.*

Statistical analysis. In order to correct the inherent variability of biological samples, standardization of real-time PCR gene expression data was done following MIQE guide according to Willems *et al.* (2008). In this case, only log transformation of the normalized relative gene expression levels was performed; this makes the data distribution more symmetric. Statistical analysis was performed with SigmaStat 3.5 software (Systat Software, Richmond, CA, USA). One way Analysis of Variance (ANOVA) was used to analyse relative gene expression and densitometry of gelatinase bands. When ANOVA showed differences, Fisher's LSD test was used to determine the level of significance. Results were considered statistically significant at $P < 0.05$.

RESULTS

Expression of MMP2, MMP9, TIMP1 and TIMP2 in llama oviductal segments. Transcripts of MMPs and TIMPs were detected in the different oviductal segments analyzed. The expression level of MMP2 were significant differences between ampulla and papilla ($P < 0.05$), being increased 2.15 folds in papilla. Concerning MMP9, papilla expression levels were the highest when comparing with the ampulla, isthmus and UTJ ($P < 0.05$) (Fig. 1).

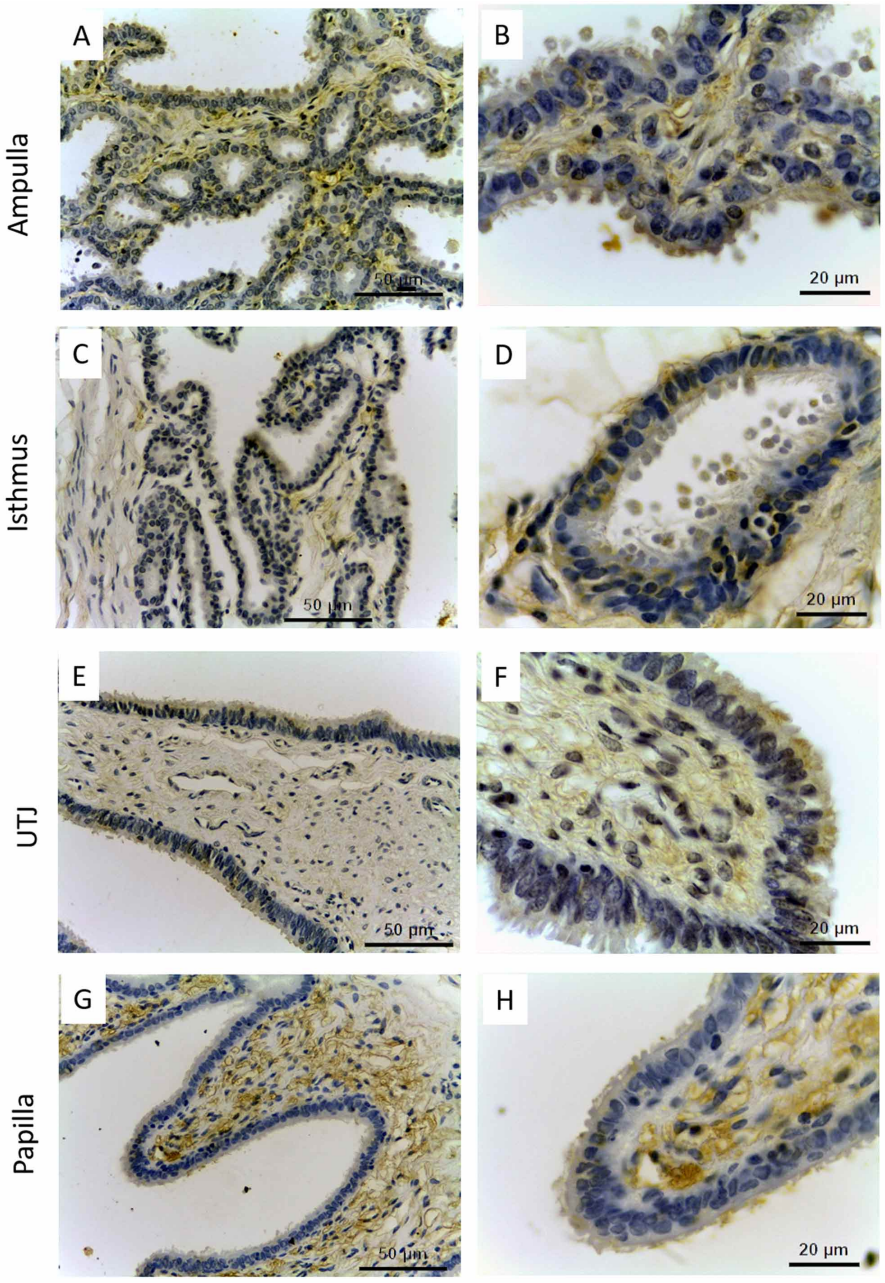

Fig. 2. Immunohistochemical localisation of MMP2 in llama oviductal segments: ampulla (A, B), isthmus (C, D), UTJ (E, F) and papilla (G, H). Tissue sections were counterstained with Mayer's haematoxylin.

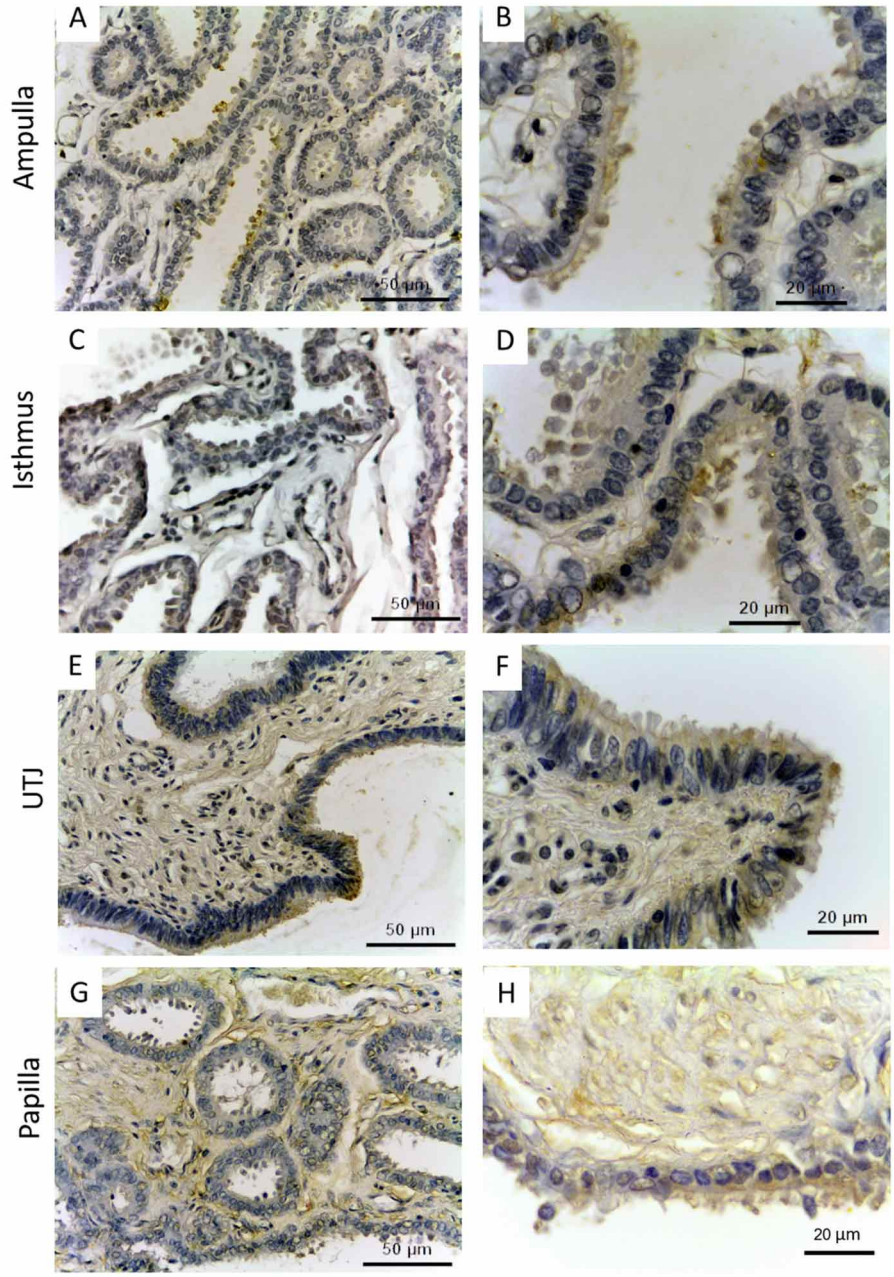

Fig. 3. Immunohistochemical localization of MMP9 in llama oviductal segments: ampulla (A, B), isthmus (C, D), UTJ (E, F) and papilla (G, H). Tissue sections were counterstained with Mayer's haematoxylin.

Regarding matrix metalloproteinase inhibitors, both TIMP1 and TIMP2 expression showed steady-state levels throughout the oviduct (Fig. 1).

Immunolocalization of MMP2 and MMP9 in the llama oviduct. Protein distribution of MMP2 and MMP9 in the llama oviduct was assayed by immunohistochemistry. Both enzymes were observed in all four oviductal segments assayed, but with notable differences between the different layers of the epithelial mucosa. Furthermore, MMP2 and MMP9 displayed a distinctive localisation pattern, between them. As shown in Fig. 2A and B, in ampulla a mild immunostaining of MMP2 was found in the subepithelial connective and in vesicular projections of ampulla epithelium (apical cytoplasm of epithelial cells) with an irregular distribution. Isthmus sections showed a weak and irregularly distributed signal in the subepithelial connective and epithelium (Fig. 2C, D). In UTJ, MMP2 was also

localized in both the apical cytoplasm of epithelial cells and subepithelial connective as a moderate and irregular signal (Fig. 2E, F). In contrast, papilla sections only showed a mild, regular, positive signal in the subepithelial connective, but no signal was observed in the epithelium (Fig. 2G, H).

Regarding MMP9, moderate, irregularly distributed staining was observed only in the vesicular projections (apical cytoplasm of epithelial cells) of ampulla and isthmus epithelium but no signal was observed in the subepithelial connective of these sections (Fig. 3A, B, C, D). While, in UTJ and papilla, MMP9 was detected in both epithelium and subepithelial connective, showing a mild regular signal (Fig. 3E, F, G, H).

Control slides incubated without primary antibodies were negative for immunostaining (Fig. 4).

Active/inactive MMPs in llama oviductal fluid. ProMMPs and their active forms (MMPs) were detected in the llama OF after incubation with or without 1mM APMA prior to zymography. OF samples without APMA incubation showed two gelatinolytic bands corresponding to 62 and 94 kDa, assumedly proMMP2 and proMMP9, respectively. The 62 kDa band was more prominent, whereas the 94 kDa band only showed weak activity. As shown in Fig. 5, APMA treatment revealed the disappearance of the 94 kDa gelatinase band and a 1.9-fold decrease in the 62 kDa gelatinase band intensity ($P < 0.05$). Furthermore, four additional bands of 79, 72, 59 and 56 kDa were observed. These results indicate that 94 kDa band corresponds to a proMMP, which after activation generated at least two active isoforms of 79 and 72 kDa. The 59 and 56 kDa bands could be active MMP isoforms originated from the 62 kDa band.

DISCUSSION

The oviduct is a dynamic organ comprising different anatomical and functional regions that may show differences in mRNA expression levels and in the synthesis and secretion of various proteins (Maillo *et al*., 2016). Thus, the present study focuses on the expression of MMP2, MMP9, TIMP1 and TIMP2 in the llama oviductal segments and the active and inactive MMP forms present in the OF.

Both metalloproteases, MMP2 and MMP9, showed patterns of mRNA expression and protein distribution that varies according to the oviductal segments. Regarding MMP2, statistically significant differences were observed between ampulla and papilla, being MMP2 expression

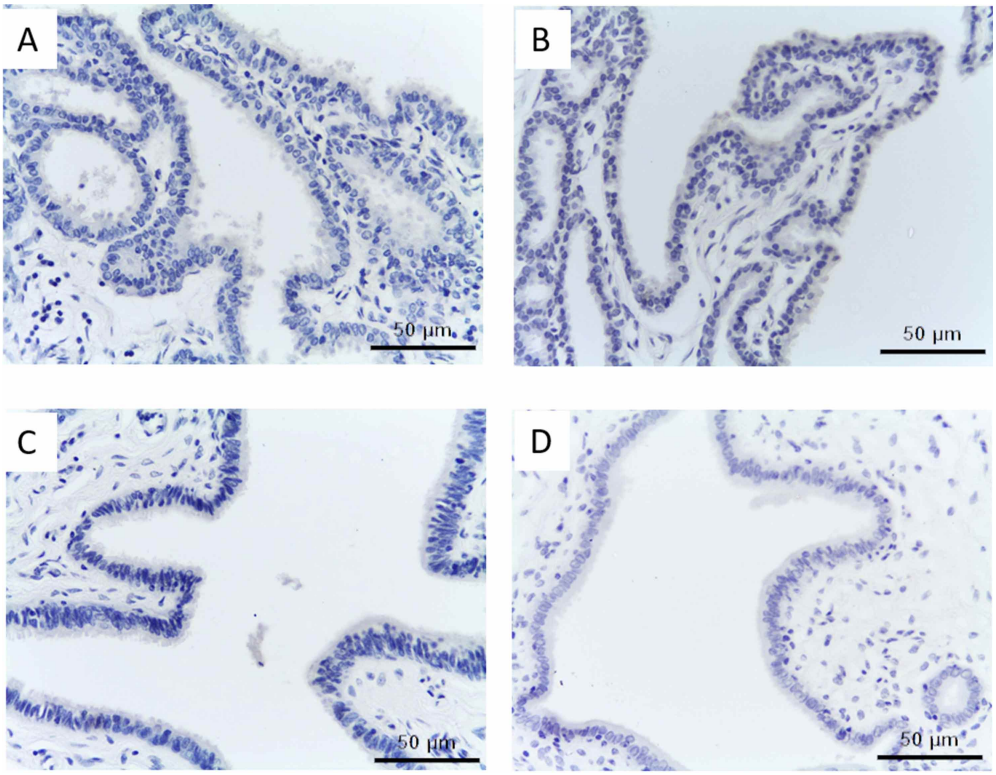

Fig. 4. Negative immunohistochemical controls without primary antibody for ampulla (A), isthmus (B), UTJ (C) and papilla (D). Tissue sections were counterstained with Mayer's haematoxylin.

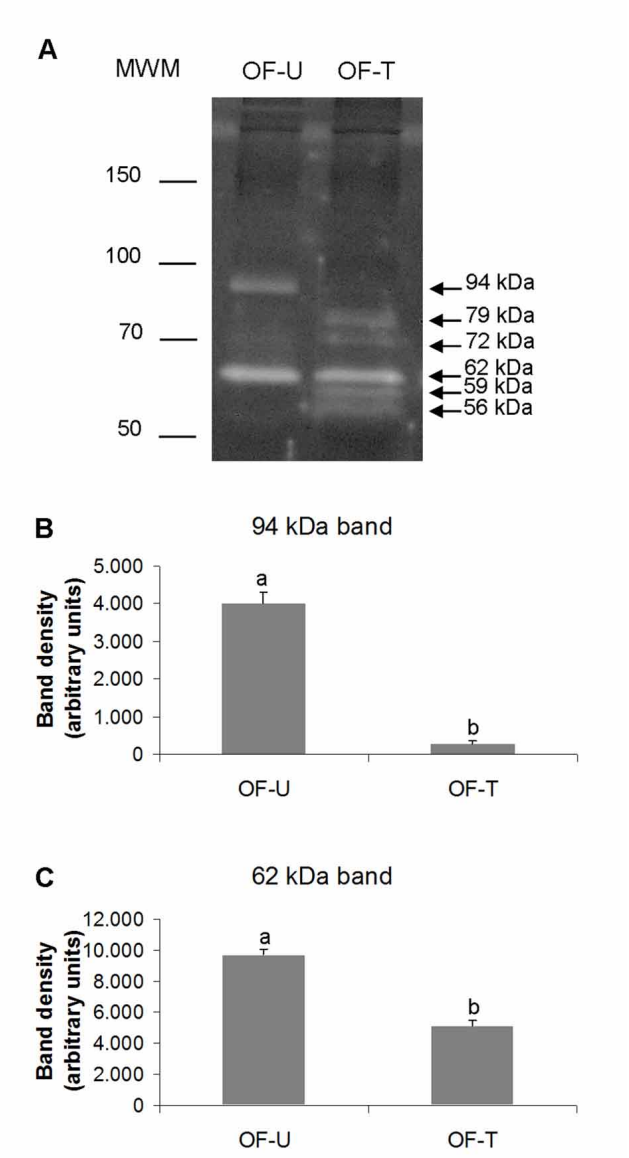

Fig. 5. Gelatine zymography of llama OF (A). Arrows indicate the position of bands with gelatinase activity and the corresponding calculated molecular weight. Densitometric analysis of 94 kDa (B) and 62 kDa (C) protein bands. Different letters above bars indicate significant differences ($P < 0.05$) between treatments. OF-T: oviductal fluid treated with 1 mM APMA. OF-U: untreated oviductal fluid, MWM: molecular weight marker (kDa).

levels increased in the papilla; moreover, the distribution pattern of the protein was also distinctive. MMP2 was detected in the epithelium and lamina propia of ampulla, while in papilla was detected only in the subepithelial connective tissue not so in the luminal epithelium. Considering that epithelial cells play an active role in the secretion of components of the oviductal fluid, the presence of MMP2 in the secretion vesicles of luminal epithelium suggests that this protein is synthesised and released in the oviductal lumen. Most likely, the papilla segment does not contribute to the secretion since epithelial MMP2 protein was not detected in this segment.

The hypothesis that oviductal epithelium secretes MMP2 is reinforced by previous results in llama (Zampini *et al*.), bovine (Gabler *et al*.) and human OF (Kim *et al*., 2003). Interestingly, after incubation of OF with APMA, a marked decrease in the 62 kDa protein and appearance of other proteolytic bands of lower molecular weight were observed, indicating that the 62 kDa protein corresponds to the inactive form of llama MMP2. Similarly, in bovine OF, only the latent form of MMP2 was detected (Gabler *et al*.). Different to other MMPs, proMMP2 is not readily activated by general proteinases. The main activation of proMMP2 is mediated by MT-MMPs (membrane-type MMPs), and takes place on the cell surface. Particularly, MT-MMP1 (MMP14) mediated activation of proMMP2 has been studied extensively, and it requires the assistance of TIMP2. Consequently, TIMP2 is required for both, activation and inhibition of MMP2. In concordance with this; we found that TIMP2 mRNA is expressed in the ampulla, isthmus, UTJ and papilla in llama. These findings seem to indicate that the activity of MMP2 in the llama oviduct is strictly regulated.

We also detected MMP9 in the llama oviduct in all the segments, being highly expressed in the papilla. In the ampulla and isthmus, MMP9 was mainly detected in the vesicle projections of epithelial cells, suggesting its active secretion into the oviductal lumen. In UTJ and papilla, MMP9 was observed in both epithelium and subepithelial connective tissue. Consequently, the oviduct may be responsible for synthesis and secretion of MMP9 in the lumen of the oviduct. The current study confirmed the zymogen nature of the 94 kDa protein after treatment with APMA. After activation, the 94 kDa proMMP generated at least two novel proteases of 79 and 72 kDa. In our opinion, the 79 and 72 kDa proteins are active MMP9 isoforms. These data are in agreement with a previous study that reported the presence a 94 kDa band in llama OF (Zampini *et al*.) and by Roy & Ghosh (2010) who described two MMP9 active isoforms in buffalo uterine fluid, that are coincident with the molecular weight observed in llama OF. Similar to llama MMP2, these findings suggest that MMP9 is not continuously active in the oviductal lumen; probably, it experiments a temporal and site-specific activation. Various proteases, such as plasmin and uPA (urokinase plasminogen activator) can activate latent proMMP9 (Visse & Nagase). The expression of members of the plasmin-generating system have been extensively studied in mammalian oviduct (Gabler *et al*.; Roldán-Olarte *et al*., 2005) and they could be essential for oviductal proMMP9 activation. Once activated, MMP9 enzymatic activity is regulated by

TIMPs. Between them, TIMP1 binds with high affinity to MMP9 (Van den Steen *et al*., 2002). Even though, llama TIMP1 expression was detected throughout the llama oviduct, no statistically differences were observed among the oviductal segments. Peng *et al*. (2015), reported that TIMP1 is expressed in the goat oviduct promoting epithelial cells proliferation and participating of gametes transport.

In the current study we examined MMPs/TIMPs expression in the llama oviduct at a follicular stage during which the proteases could intervene the extracellular matrix (ECM) turnover, in cell signalling by activation and/or release of cytokines and growth factors located in the ECM or the OF. Indeed, Diaz *et al*. (2012), suggest that proteins related to extracellular matrix remodelling could participate in the physiological changes that occur in the uterine tube throughout the menstrual cycle. In addition, MMP2 and MMP9 activate several cytokines and growth factors: fibroblast growth factor (FGF), tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) (Fowlkes & Winkler), all present in the mammalian oviduct and OF (Zhao *et al*., 1994; Viuff *et al*., 1995; Pushpakumara *et al*., 2002; Wijayagunawardane & Miyamoto, 2004). Consequently, oviductal MMPs could regulate the bioavailability of these factors.

Another reproductive process that occurs in the female llama at the follicular stage is the copulation, since SACs are induced ovulated, the pre-ovulatory LH peak, ovulation and corpus luteum formation happen 24-36 h after male insemination. Thus, MMPs could also be involved in sperm-oviduct interaction and/or liquefaction of the SACs high visco-elastic semen, allowing sperm to acquire progressive motility for fertilisation (Zampini *et al*.). Interestingly, MMP2 present an augmentation in the llama oviduct after mating and previous the ovulation (Data unpublished), indicating a possible role and regulation during this period. However, further studies are needed to define MMPs functions in the llama oviduct.

CONCLUSIONS

Considering all the information above, to our knowledge our findings are the first to describe and characterize a differential expression pattern for MMPs and TIMP among the oviductal segments in llama. The specific distribution MMP2 and MMP9 in ampulla, isthmus, UTJ and papilla highlights a possible participation of MMPs/TIMPs in the reproductive process; also provides information that could be useful to improving different aspects of reproductive assisted techniques use in domestic and endangered wildlife SACs species.

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ZAMPINI, R.; APICHELA, S.; SARI, L.; ANGIONO, G.; LOMBARDO, D.; MICELI, D. & ARGAÑARAZ, M. Expresión segmento específica de MMP/TIMP en el oviducto de llama (*Lama glama*) y actividad gelatinolítica en el fluido oviductal. *Int. J. Morphol., 35(2)*:615-623, 2017.

RESUMEN: Las moléculas oviductales tienen el potencial para mejorar las biotecnologías reproductivas. En los camélidos, debido a sus peculiares características reproductivas, el conocimiento del ambiente oviductal constituye una herramienta útil para el desarrollo de tecnologías reproductivas específicas para estas especies. Entre los factores oviductales de interés se encuentran las metaloproteasas de matriz (MMPs) y sus inhibidores específicos (TIMPs), los cuales han sido involucrados en diferentes procesos reproductivos. Por estas razones, en este trabajo se caracterizó la expresión génica y proteica de MMP2 y MMP9 en el oviducto de llama. Además, se analizó la presencia de las formas activas e inactivas (zimógenos) de estas enzimas en el fluido oviductal. Se observó que todos los segmentos oviductales, ámpula, istmo, unión útero-tubal y papila, expresan MMP2 y MMP9, siendo los niveles de expresión de MMP2 y MMP9 más elevados en papila respecto a ámpula; 2,15 y 1,10 veces respectivamente. Asimismo, se observaron diferencias en la distribución de las MMPs a nivel de la mucosa entre los segmentos oviductales. Consecuentemente, bandas con actividad gelatinolítica de 62 y 94 kDa, se detectaron en el fluido oviductal, las cuales corresponderían a las formas inactivas de la MMP2 y la MMP9, respectivamente. Los inhibidores específicos de MMP2 y MMP9; TIMP2 y TIMP1, también se detectaron en los segmentos oviductales, indicando su probable participación en la regulación de la actividad proteolítica de las MMPs en el oviducto de llama. En conjunto, los datos de este trabajo demuestran que el oviducto de la llama produce y secreta MMPs al lumen oviductal; sugiriendo que estas enzimas pueden participar en la preparación del ambiente oviductal para la recepción de los gametos, la fecundación y el desarrollo embrionario temprano en camélidos.

PALABRAS CLAVE: Oviducto; Llama; MMPs; TIMPs; Expresión génica.

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