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SPONTANEOUSLY ARISING DISEASE

Involvement of Matrix Metalloproteinases and their Inhibitors in Bovine Cystic Ovarian Disease

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Summary

The most important regulators of tissue remodelling during ovarian follicular growth, development, ovulation and atresia are gonadotropins, steroid hormones, growth factors and different proteolytic enzymes. Matrix metalloproteinases (MMPs) such as collagenase or gelatinase (i.e. MMP-1, -8, -2 and -9) and associated tissue inhibitors of metalloproteinases (TIMP-1, -2, -3 and -4) control connective tissue remodelling during follicular rupture. In this study, we hypothesized that an imbalance in the MMP–TIMP system may be an intra-ovarian component that contributes to the pathogenesis of cystic ovarian disease (COD) in cows. Taking into account that the control of MMP activity by TIMPs could determine their effects in both physiological and pathological conditions, MMP and TIMP mRNA and protein expression was examined by real-time polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC), respectively, in ovaries from control cows and cows with COD. Expression of mRNA encoding MMP-2, TIMP-1 and TIMP-2 was lower in follicular cysts than in control pre-ovulatory follicles, while the results by IHC showed this imbalance only for TIMP-2 protein expression. Additional analysis by zymography to evaluate the gelatinase activity of MMP-2 and MMP-9 demonstrated higher MMP-2 activity in follicular fluid (FF) of cysts than in FF of pre-ovulatory follicles. On the other hand, MMP-9 activity was increased in follicular cysts and absent in the FF of pre-ovulatory follicles. These findings suggest that the altered mRNA expression and activities of the MMP–TIMP system may be related to the failure in ovulation and follicular development observed in COD.

Keywords: cow; matrix metalloproteinase; ovary; tissue inhibitors of metalloproteinase

Introduction

Ovulation is a synchronized and complex inflammatory process controlled by endocrine and biochemical events that cause follicle rupture and extrusion of the oocyte (Espey, 1994; Bukovsky and Caudle, 2008; Amweg *et al*., 2013). During follicular development, ovulation and atresia, ovaries undergo continuous tissue remodelling. Specific components of the extracellular matrix (ECM) can be altered through cleavage by matrix metalloproteinases (MMPs), such as collagenase (i.e. MMP-1, -8 and -13) or gelatinase (i.e. MMP-2 and -9), whose activities are inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Smith *et al*., 2002). The extensive follicular remodelling is evident in the dissolution of the granulosa cell basement membrane that allows the oocyte release by fragmentation of the ECM of the follicular wall during ovulation (Richards *et al*., 2002; Bilbao *et al*., 2011). Moreover, the remodelling of the ECM through MMPs can affect multiple cellular processes such as proliferation, differentiation and apoptosis. Inhibition of MMPs by TIMPs can stabilize the

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components of the ECM and promote ECM deposition. The MMP:TIMP ratio may contribute to the control of many cellular processes associated with ovarian function. Additionally, TIMPs can act as growth factors through membrane-bound TIMP receptors (Smith *et al.*, 2002).

MMP-2 and -9 are identified by gelatin zymography as two distinct proteins of 72 and 92 kDa, respectively (McIntush and Smith, 1998; Curry *et al.*, 2001; Imai *et al.*, 2003). These enzymes are able to bind to and cleave gelatin and therefore degrade many constituents of basement membranes. Additionally, the activity of MMPs in the extracellular space is regulated by TIMPs.

TIMPs are strongly involved in cyclic ovarian processes (Curry and Osteen, 2003; Zhang *et al.*, 2003; Li *et al.*, 2004; Kliem *et al.*, 2007). TIMP-1, -2, -3 and -4, which differ in their regulation, enzyme specificity and mode of action, have been identified in different tissues of several species (Docherty *et al.*, 1985; Carmichael *et al.*, 1986; De Clerck *et al.*, 1989; Stetler-Stevenson *et al.*, 1989; Pavloff *et al.*, 1992; Greene *et al.*, 1996). TIMP-1 and -2 are the two most studied inhibitors. TIMP-1 can bind to the active forms of all known MMPs and the latent form of MMP-9 and may regulate steroidogenesis by stimulating progesterone production by rat granulosa cells. TIMP-2 is able to bind active MMPs and inhibit their protease activity (Boujrad *et al.*, 1995; Woessner, 2001; Zhang *et al.*, 2003). Although TIMP-2 has highest affinity for MMP-2, it may also be involved in proMMP2 activation, which indicates that this TIMP could have a dual function.

MMPs and TIMPs are differentially distributed in the ovary of rats, women and cows and the mRNA expression levels of MMPs and TIMPs change in association with gonadotropin-induced follicular development (Smith *et al.*, 1996; Duncan *et al.*, 1998; Simpson *et al.*, 2001). In this sense, the control of MMP activity by TIMPs could determine their effects in both physiological and pathological conditions (Curry and Osteen, 2003). However, in cattle, the role of MMPs in normal ovarian events, such as follicular growth, ovulation and/or atresia, is less known and there is no information about altered expression during disturbed repair/degradation mechanisms of the ECM in cystic ovarian disease (COD).

COD, which is considered one of the most important causes of reproductive failure in cattle, can result in significant economic losses to the dairy industry by delaying conception (Peter, 2004; Cattaneo *et al.*, 2014). This disease has been defined as the presence of one or more follicular structures in the ovary/ovaries, with a diameter of at least 20 mm, which persist for more than 10 days in the absence of luteal tissue, interrupting the normal reproductive cycle (Silvia *et al.*, 2002; Peter, 2004; Vanholder *et al.*, 2006). The pathogenesis of COD in dairy cattle is a complex process that involves dysfunction in folliculogenesis and ovulation, with an important intra-ovarian component (Ortega *et al.*, 2015). Based on this evidence, the aim of the present study was to determine the mRNA and protein expression patterns and activity of MMP-2 and MMP-9, and the expression of their inhibitors TIMP-1 and TIMP-2, in ovarian follicles from control cows and cows with COD. This study allowed us to test the hypothesis that an imbalance in the MMP−TIMP system may be a component of the pathogenesis of follicular persistence and ovulatory failure in this reproductive disorder.

Materials and Methods

Ethical Aspects

All procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (2010) and with the approval of the Institutional Ethics and Security Committee (Protocol N° 84/11, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Santa Fe, Argentina).

Animals

All cows were from local dairy farms. Twenty multiparous (66.2 ± 25.4 months old; 2.8 ± 1.3 lactations) Holstein cows, without reproductive disorders, were assigned to the control group ($n = 20$). Oestrous cycles were synchronized using the Ovsynch™ protocol, as previously described (Ortega *et al.*, 2008; Amweg *et al.*, 2013). Normal folliculogenesis was monitored daily by ultrasonography, using a real-time B-mode scanner equipped with a 5 MHz linear-array transrectal transducer (Honda HS101V, Toyohashi, Japan), through a complete oestrous cycle, to obtain normal growing follicles approximately on day 18 (Díaz *et al.*, 2015). The ovaries from 10 control cows were removed by transvaginal ovariectomy when the dominant follicle reached a diameter >10 mm, and the ovaries were macroscopically examined (Marelli *et al.*, 2014). The remaining 10 cows were subjected to follicular aspiration of dominant ovulatory follicles, using a digital ultrasound system Chison 8300vet equipped with a microconvex 5.0 MHz transducer (Chison Medical Imaging Co., Wuxi, China) mounted on a transvaginal probe for follicular fluid (FF) aspiration (Watanabe Applied Technology Limited., Sao Paulo, Brazil) (Marelli *et al.*, 2014).

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