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The effect of angiotensin-converting enzyme inhibition throughout a superovulation protocol in ewes

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ABSTRACT

Many studies identified new components of the renin–angiotensin system (RAS), such as Angiotensin-(1-7) [Ang-(1–7)] and Angiotensin-converting enzyme type 2 (ACE2), in mammalian ovaries. We previously showed Angiotensin-Converting Enzyme (ACE) inhibition, which increases the level of Ang-(1–7), stimulated ovarian estradiol output in ewe after estrous synchronization. Considering that Ang-(1–7) stimulates ovarian function and elevated estradiol before ovulation is associated with increased chance of achieving pregnancy, the present study investigated whether ACE inhibition throughout a superovulation protocol in ewe might improve ovulation outcome. At first, immunohistochemistry in ovaries of nonpregnant ewes revealed localization of Angiotensin II (Ang II), Ang-(1–7) and ACE2 in theca cells of antral follicles and in corpus luteum. Ang II and Ang-(1–7) were also detected in follicular fluid (FF) by Radioimmunoassay (RIA). Enalapril treatment throughout the superovulation protocol decreased 17β-estradiol (E2) output and raised progesterone:estradiol (P4:E2) ratio without a direct influence on ovulation and quality of embryos.

1. Introduction

The systemic action of the RAS on blood pressure and body fluid homeostasis is well-established (Fyhrquist and Saijonmaa, 2008; Skrbic and Igic, 2009). In recent decades, all components of the RAS have been described in different tissues, giving rise to the concept of local RAS (Paul et al., 2006). The presence of prorenin, angiotensinogen, ACE, Ang II and Ang II receptors (AT_1 and AT_2) has also been reported in the ovary, suggesting the involvement of RAS in the ovarian physiology. Specifically, RAS components regulate follicular development, steroidogenesis, ovulation, atresia and oocyte maturation (Ferreira et al., 2011; Herr et al., 2013; Yoshimura, 1997).

In the past few years, the classical RAS has undergone some changes. In particular, other components of the RAS have been identified (Santos et al., 2013), such as: Ang-(1–7), which is generated from Ang II by hydrolysis of C-terminal amino acid or directly from Ang I by neutral endopeptidase 24.11 (NEP) and prolyl-endopeptidase (PEP); ACE2, an important Ang-(1–7)-forming enzyme, which is not blocked by ACE inhibitors (Donoghue et al., 2000; Tipnis et al., 2000); and the receptor Mas, a specific Ang-(1–7)-G protein-coupled receptor (Santos et al., 2003). These components were also described in the ovaries of rats (Costa et al., 2003; Pereira et al., 2009), rabbits (Viana et al., 2011), cattle (Tonellotto dos Santos et al., 2011) and women (Reis et al., 2011). Previous studies showed that the ACE2/Ang-(1–7)/Mas axis was also upregulated by gonadotrophins, suggesting its involvement in the ovulatory process. In rat ovaries, levels of Ang-(1–7) were raised during proestrus and estrus when the gonadotropin surge happens (Costa et al., 2003). Furthermore, injection of equine chorionic gonadotrophin (eCG), which exhibits follicle-stimulating hormone (FSH)-like effects, increased Ang-(1–7), Mas and ACE2 expression in rat ovaries (Honorato-Sampaio et al., 2012; Pereira et al., 2009). In addition, in vitro studies showed that Ang-(1–7) stimulates steroidogenesis and ovulation in rabbit ovaries (Viana et al., 2011) and oocyte maturation in rat preovulatory follicles (Honorato-Sampaio et al., 2012).

ACE inhibition is widely used as a therapeutic strategy for hypertension. During the treatment with ACE inhibitors, an upregulation of multiple angiotensin metabolites occurs, highlighting Ang-(1–7) (Campbell et al., 1994; Castro-Moreno et al., 2012; Widdop et al., 1999). Considering the stimulatory effect of Ang-(1–7) in the ovary, we previously evaluated the effect of enalapril, an ACE inhibitor, on ovarian steroidogenesis after estrous synchronization in ewes. Our results showed that enalapril

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increased estradiol output in synchronized animals before ovulation (Costa et al., 2014). It is well known that elevated estradiol before ovulation is associated with good follicular growth, reduction of atresia and enhancement of cytoplasmatic maturation of oocyte, increasing the chance of achieving pregnancy (Revelli et al., 2009; Tsonis et al., 1984). Previous studies also demonstrated that Ang-(-1–7) activates the phosphoinositide 3-kinase/protein kinase B (IP3K–Akt) through receptor Mas (Giani et al., 2007; Sampaio et al., 2007), which is the same pathway used by many growth factors to prevent apoptosis e atresia (Hu et al., 2004; Krysko et al., 2008; Quirk et al., 2004). So, our hypothesis is the increase in Ang-(1–7) levels induced by ACE inhibition might improve the ovulation outcome. Thus, we evaluated the effect of enalapril throughout a superovulation protocol on steroidogenesis, ovulation and quality of embryos in ewes. In addition, we described the specific localization of Ang II, Ang-(1–7) and ACE2 in ovaries of nonpregnant ewes for the first time.

2. Materials and methods

2.1. Animals

To determine the presence and distribution of Ang II, Ang-(1–7) and ACE2 in ewe ovary, all tissue samples were collected from healthy animals routinely killed in a local slaughterhouse (Teresina, Piauí, Brazil) in compliance with all regulations for animal care of the Ministry of Agriculture, Livestock and Food Supply. Tissue samples were collected from intact ovaries of nonpregnant ewes. The ovaries were discriminated between animals bearing a corpus luteum ≥1 cm in at least one ovary (n = 5) and animals with antral follicles ≥4 mm (n = 6 animals). The macroscopic appearance of ovarian structures was according to Camp et al. (1983).

To evaluate the effect of ACE inhibition throughout a superovulation protocol, the experiment was conducted at an experimental farm of the Agricultural College, which belongs to the Federal University of Piauí (UFPI), located at 09°04′07″ south, 44°21′27″ west. Fifteen ewes (2 years-old; weighing 35–45 kg) were fed with grass and supplemented with sheep chow (16–18% CP). The animals had free access to water and mineral salt.

2.2. Experimental protocol

The superovulation protocol was designed according to Fonseca et al. (2011). The experimental protocol and surgical procedure were approved by the Ethics Committee on Animal Experimentation of the UFPI. Briefly, all ewes received intravaginal sponges containing 60 mg of medroxyprogesterone acetate (Promone-E®, Rhodia, Santo André, SP, Brazil). On the 7th day, all ewes received an intramuscular injection of 100 μg cloprostenol (Sincrocio®, Ourofino, Cravinhos, SP, Brazil). Following 12 days after insertion of sponges, the animals received intramuscular injections of FSH (Folltropin®, Tecnopec, São Paulo, SP, Brazil) separated in six applications with intervals of 12 h (60 mg, 60 mg, 40 mg, 40 mg, 25 mg and 25 mg). On the 14th day, the sponges were removed. Twenty-four hours after device withdrawal, rams with proven fertility were joined to the ewes.

To evaluate the effect of ACE inhibition, ewes were randomly divided into two groups: 1) animals (n = 8) that received daily subcutaneous administration of enalapril maleate (Pharlab, Lagoa da Prata, MG, Brazil) in the following doses: 1–2 days = 0.125 mg/kg/day; 3–4 days = 0.250 mg/kg/day; 5–14 days = 0.325 mg/kg/day; 2) and a control group (n = 7) that received saline. Blood samples were collected by puncture of the vena jugular using heparinized vacutainers in days 1, 11 and 14 after the insert of sponges. The blood samples were centrifuged and plasma was frozen at −20 °C.

Surgical recovery of embryos was performed on day 7 after the copulation. The animals were fasted for 24 h and anesthetized with simultaneous intravenous administration of xylazine hydrochloride (0.1 mg/kg) (Dorcipec®, Vallée, Montes Claros, MG, Brazil) and ketamine (15 mg/kg) (Dopalen®, Vetbrands, Goiania, GO, Brazil). Laparotomy was performed through an incision of 10 cm in the midline (cranial to udder). The reproductive tract was carefully exteriorized to assess the number of corpora lutea (CL). Embryos were recovered by flushing the uterus with saline. The recovered embryos were morphologically evaluated using a stereoscopic microscope and their quality was scored on a scale of 1 to 5 according to the guidelines of the International Embryo Transfer Society (Stringfellow, 2007).

2.3. Immunohistochemistry

Ovarian fragments of nonpregnant ewes were fixed in 10% buffered formaldehyde and embedded in paraffin. Sections of 5 μm thick were mounted on gelatin-coated slides and stained using the avidin–biotin–peroxidase method with the Vectastain Universal Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) as previously described (Honorato-Sampaio et al., 2012). Briefly, sections were dewaxed in xylene, rehydrated, and treated with 1% hydrogen peroxide in methanol for 30 min followed by normal equine blocking serum for 30 min. The sections were incubated with primary antibodies at room temperature in a humid chamber for 2 h. The primary antibodies used were: rabbit polyclonal anti-ACE2 (1:500) (Abcam Inc., Cambridge, MA, USA), rabbit polyclonal anti-Ang-(1–7) (1:1000) and anti-Ang II (1:500) as previously described (Botelho et al., 1994; Simões et al., 2004). The slices were incubated with biotinylated universal antibody (1:50) plus normal horse serum (1:50) for 30 min followed by avidin–biotin peroxidase complex (1:50) for 30 min. The immunostaining was visualized with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO, USA) and counterstained with Hematoxylin. Negative controls were obtained by preadsorption of the primary antibody with antigens or by its replacement with nonimmune serum.

2.4. Hormonal dosages

Ang II and Ang-(1–7) were quantified in FF of antral follicles obtained from nonpregnant ewes. The fluid was collected by ovarian aspiration and mixed 1:1 with a protease inhibitor mix containing: 10^{-5} mol/L phenylmethylsulfonylfluoride, 10^{-5} mol/L pepstatin A, 10^{-5} mol/L ethylenediaminetetraacetic acid (EDTA), 10^{-5} mol/L p-hydroxymercuribenzoate and 9×10^{-4} mol/L orthophenanthroline (all from Sigma-Aldrich Corp., St. Louis, MO, USA) diluted in 1 0.1 N acetic acid. The FF samples were thawed and processed for peptide extraction through Bond-Elut cartridges (Analytichem International, Harbor City, CA, USA), then lyophilized on a speed vacuum concentrator, and reconstituted to 65% of the original volume of assay buffer. Ang II and Ang-(1–7) was quantified in the reconstituted samples by RIA as previously described for FF (Reis et al., 2011). The polyclonal antibodies anti-Ang II and anti-Ang-(1–7) have been described elsewhere (Simões et al., 2004). The anti-Ang II antibody exhibited 100% cross-reactivity with the angiotensin fragments, Ang-(2–8), Ang-(3–8), and Ang-(4–8), and <0.001% with Ang I and Ang-(1–7). The anti-Ang-(1–7) antibody cross-reacted <0.001% with Ang I and Ang II, <0.01% with Ang-(2–7) and Ang-(3–7), and <0.08% with Ang-(4–7).

Testosterone, progesterone (P4) and E2 concentrations in plasma of ewe submitted to the superovulation protocol were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using commercial kits (DRG Diagnostics, Marburg, Germany). The assays were performed according to the manufacturers' instructions.

2.5. Statistical analysis

Data were expressed as mean ± SEM. To analyze time- and treatment-dependent differences in the steroidogenesis, we used two-way ANOVA for repeated measures followed by Bonferroni post hoc