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# Influence of prostaglandin $F_{2\alpha}$ analogues on the secretory function of bovine luteal cells and ovarian arterial contractility in vitro

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ABSTRACT

Although prostaglandin (PG) $F_{2\alpha}$ analogues are routinely used for oestrus synchronisation in cattle, their effects on the function of the bovine corpus luteum (CL), and on ovarian arterial contractility, may not reflect the physiological effects of endogenous $PGF_{2\alpha}$. In the first of two related experiments, the effects of different analogues of $PGF_{2\alpha}$ ($aPGF_{2\alpha}$) on the secretory function and apoptosis of cultured bovine cells of the CL were assessed. Enzymatically-isolated bovine luteal cells (from between days 8 and 12 of the oestrous cycle), were stimulated for 24 h with naturally-occurring $PGF_{2\alpha}$ or $aPGF_{2\alpha}$ (dinoprost, cloprostenol or luprostiol). Secretion of progesterone (P4) was determined and cellular $[Ca^{2+}]_i$ mobilisation, as well as cell viability and apoptosis were measured.

Naturally-occurring $PGF_{2\alpha}$ and dinoprost stimulated P4 secretion ($P < 0.05$), whereas cloprostenol and luprostiol did not influence P4 synthesis. The greatest cytotoxic and pro-apoptotic effects were observed in the luprostiol-treated cells, at 37.3% and 202%, respectively ($P < 0.001$). The greatest effect on $[Ca^{2+}]_i$ mobilisation in luteal cells was observed post-luprostiol treatment (200%; $P < 0.001$).

In a second experiment, the influence of naturally-occurring $PGF_{2\alpha}$ and $aPGF_{2\alpha}$ on ovarian arterial contraction in vitro, were examined. No differences in the effects of dinoprost or naturally-occurring $PGF_{2\alpha}$ were found across the studied parameters. The effects of cloprostenol and luprostiol on luteal cell death, in addition to their effects on ovarian arterial contractility, were much greater than those produced by treatment with naturally-occurring $PGF_{2\alpha}$.



## Introduction

Prostaglandin (PG) $F_{2\alpha}$ is considered the major luteolytic factor in domestic ruminants (McCracken et al., 1999; Weems et al., 2006), and is released from the uterus in a series of pulses at the end of the luteal phase, reaching the ovary via counter-current transfer in the utero-ovarian broad ligament (Niswender et al., 2000). Naturally-produced $PGF_{2\alpha}$, along with its synthetic analogues ($aPGF_{2\alpha}$), and progestagens, oestrogens and gonadotropin-releasing hormones, are used for oestrus synchronisation in cattle (Berardinelli and Adair, 1989; Wright and Malomo, 1992; Nebel and Jobst, 1998; Jemmeson, 2000; Ahuja et al., 2005; Repsi et al., 2005; Thatcher and Santos, 2007). Although pharmacological manipulation of the oestrous cycle is a useful tool used in animal breeding, it has been suggested that use of $PGF_{2\alpha}$ or $aPGF_{2\alpha}$ result in lower conception rates and impaired fertility (Diskin et al., 2002; Ahuja et al., 2005). Artificially shortening the oestrous cycle with $aPGF_{2\alpha}$ could impair follicle selection and ovulation, or the formation of a functional corpus luteum (CL) (Hansen et al., 1987).

Oestrus synchronisation by $PGF_{2\alpha}$ or $aPGF_{2\alpha}$ leads to a reduction in progesterone (P4) concentrations, and modulates steroidogenesis in both bovine (Hansen et al., 1987; Xu et al., 1997; Santos et al., 2004) and ovine (Wierzchos et al., 1995) CLs. In an in vitro study, significantly lower P4 secretion by bovine CL was observed in $aPGF_{2\alpha}$-synchronised oestrus cows, compared to controls (Skarzynski et al., 2009). At day 8 of the oestrous cycle, oestrus synchronisation using 'double injections' of three different $aPGF_{2\alpha}$ (cloprostenol, luprostiol, and dinoprost), resulted in lower basal and $PGE_2$- and luteinising hormone (LH)-stimulated P4 secretion by the CL (Skarzynski et al., 2009). These data suggest that oestrus synchronisation, depending on the method and $aPGF_{2\alpha}$ used, decreases basal, $PGE_2$- and LH-stimulated secretion of P4 by the bovine CL in the subsequent oestrous cycle.

The effect of $PGF_{2\alpha}$ on the CL is not simply limited to luteal steroidogenic cells, but also impacts on endothelial cells and those of the immune system (Korzekwa et al., 2008). Interestingly, CL morphology and cell composition varies, features dependent on the

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naturally-occurring or $PGF_{2\alpha}$-synchronised oestrus cycle: the number of large and small steroidogenic CL cells is lower in cows with $PGF_{2\alpha}$-synchronised oestrus, compared to controls with spontaneous cycles (Hansen et al., 1987). It has been suggested that poor post-ovulatory P4 secretion is due to inadequate post-ovulatory development of a CL, rather than delayed ovulation and follicle formation (Starbuck et al., 2006). Thus, pharmacological manipulation of the oestrous cycle potentially impairs granulosa and theca cell function, which are ultimately responsible for the production of healthy oocytes, ovulation, and CL formation and function. Sudden increases in local blood flow initiate luteolysis in response to $PGF_{2\alpha}$ (Miyamoto et al., 2005). Given that the morphology of the CL is not homogeneous, the direct effects of $aPGF_{2\alpha}$ on the function of the bovine CL, as well on ovarian blood flow (as reflected in the contractility of the ovarian artery), may not reflect the physiological action of endogenous $PGF_{2\alpha}$.

We established experiments to examine the direct effects of $aPGF_{2\alpha}$ on the function of the bovine CL, and on the contractility of the ovarian artery, comparing the influence of naturally-occurring $PGF_{2\alpha}$ and various $aPGF_{2\alpha}$ (i.e. dinoprost, cloprostenol, and luprostiol) on the secretory function and apoptosis of cultured bovine luteal cells, as well as on the contractility of the ovarian artery.

## Materials and methods

### *Animal selection, oestrus synchronisation and tissue collection*

Ovaries, between days 8 and 12 of the oestrous cycle, of normally cycling Polish Holstein cows were obtained from a local abattoir. The animals had been culled from dairy herds at agricultural research institutes at Baranowo and Cieszymowo, Poland, because of low milk production. Oestrus was synchronised in the cows by the administration of two injections of $aPGF_{2\alpha}$ (dinoprost, Dinolytic, Upjohn-Pharmacia) using an 11–14 day interval, as described previously (Skarzynski et al., 2009). All cows were non-lactating and 390–450 days post-partum. Follicular development and CL formation were assessed by a veterinarian by ultrasound examination (USG) per rectum (MyLab 30VET Gold Colour Doppler Diagnostic Ultrasound System, ESOATE Pie Medica). The onset of oestrous was confirmed by experienced farm staff based on the production of vaginal mucus and animal behaviour. Oestrus was considered day 0 of the cycle. One day prior to slaughter, the animals were transported to the local abattoir and the ovaries and associated vascular plexi were collected within 10–15 min of slaughter, placed in ice-cold saline and transported to the laboratory.

All procedures relating to the animals and tissue collection were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (23/2004/N and 06/2007/N), and have been described previously (Bah et al., 2006; Skarzynski et al., 2009).

### *Experiment one: Effect of $PGF_{2\alpha}$ and its analogues on cultured bovine luteal cells*

#### *Cell isolation and culture*

Enzymatic dissociation of CL, and the culture of luteal cells were performed as previously described (Pate and Condon, 1982), with some modifications (Davis and Pate, 2007). Cell viability was >85%, as assessed using the trypan-blue exclusion assay. The cell suspension used in every experiment contained approximately 40–45% steroidogenic CL cells (25% small, and approximately 20% large, luteal cells) and 55–60% non-steroidogenic CL cells (accessory cells: i.e. mostly endothelial cells and some fibroblasts and smooth muscle fibres), in an attempt to maintain the physiological distribution and conditions of cells in the intact bovine CL (Lei et al., 1991; Hojo et al., 2009). The cells distributed as $1.0 \times 10^5$/mL in Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium (DMEM/Ham's F-12; 1:1[v/v]), supplemented with 10% calf serum and 20 μg/mL gentamicin (all Sigma Aldrich). The cells were cultured for 24 h (pre-incubation) on culture plates (Sarstedt) in a humidified incubator at 38.5 °C (in 5% $CO_2$ and 95% air).

After 24 h of pre-incubation, the cells were washed twice with serum-free DMEM and the medium was replaced with fresh medium containing DMEM/Ham's F-12 supplemented with 0.1% (w/v) bovine serum albumin (BSA), 0.5 mmol ascorbic acid, 5 ng/mL of sodium selenite, 5 μg/mL of holo-transferrin (all Sigma Aldrich), and 20 μg/mL of gentamicin. Simultaneously, the cells were treated for 24 h with 1 μg/mL of $PGF_{2\alpha}$ (Cayman Chemical) or its analogues, namely, dinoprost (1 μg/mL; Dinolytic, Pharmacia), cloprostenol (1 μg/mL; Oestrophan, Leciva), luprostiol (1 μg/mL; Prosolvin, Intervet), as well as luteinising hormone (positive control; 100 ng/mL; Sigma Aldrich), cytokines (tumour necrosis factor [TNF]-α and interferon [IFN]-γ; positive control, each 50 ng/mL; Dainippon Pharmaceutical), or phorbol myristate acetate (PMA; $10^{-7}$ M; Sigma Aldrich). The doses used for cell stimulation were based on previous studies (Korzekwa et al., 2004, 2008), and validated in preliminary pilot experiments.

#### *Effects of $PGF_{2\alpha}$ and its analogues on progesterone secretion by bovine luteal cells*

After 24 h of culture, the medium was replaced with incubating medium. The cells were then exposed to $PGF_{2\alpha}$, its analogues, or LH. After 24 h incubation, the concentration of P4 in the medium was measured using a direct enzyme immunoassay (EIA) (Skarzynski et al., 2003). Antiserum to P4 (anti-P4, code SO/91/4; kindly donated by Dr. S. Okrasa, Warmia-Mazury University, Olsztyn, Poland) had been characterised previously (Ciereszko et al., 2001), and was used at a final dilution of 1:100,000. Horseradish peroxidase (HRP)-labelled P4 was used at a final concentration of 1:75,000. The standard curve ranged from 0.39 to 100 ng/mL, and the effective dose for 50% inhibition (ED 50) was 4.5 ng/mL. The intra- and inter-assay coefficients of variation (CV) were 5.5% and 8.5%, respectively.

#### *Effects of $PGF_{2\alpha}$ and its analogues on calcium ion mobilisation in luteal cells*

Mobilisation of $[Ca^{2+}]_i$ was evaluated using the cell-permeable form of the fluorescent $[Ca^{2+}]_i$ indicator fura-2 AM (Dojindo), as described previously (Korzekwa et al., 2006). After pre-incubation, the cells were washed with calcium-free Hank's buffered salt solution (HBSS). Fura-2 AM, the acetoxymethyl ester of the fluorescent calcium probe fura-2, was dissolved in DMSO to form a 1 mM stock solution. Next, 10 μL of fura-2 were added to each culture well to a final concentration of 5 μM in order to pre-load the cells with dye. The cells were then incubated at 38 °C for 40 min, and then washed four times in calcium-free HBSS. The cells were next incubated in calcium-free HBSS supplemented with 0.1% BSA for 30 min at 38 °C to allow the hydrolysis of cytoplasmic fura-2 to occur. The cells were washed three times in calcium-free HBSS.

Changes in the intracellular concentrations of $[Ca^{2+}]_i$ were monitored using an inverted microscope equipped with a fluorescent lamp and a fura-2 filter (Olympus IX71). Every 5 s, the intensity of fluorescence and the area occupied by the fluorescing cells were measured, from 15 s before, to 45 s after, treatment with $PGF_{2\alpha}$ and its analogues. PMA was used as the positive control for $[Ca^{2+}]_i$ efflux in both cell types (at 45 s time-point of the experiment). Digital interpretation of a computer-supported $[Ca^{2+}]_i$ visualisation method was achieved using the density green and density mean of the examined cells in each photograph. Changes in intracellular $[Ca^{2+}]_i$ following treatments were displayed on the graph as arbitrary units of intensity, as assessed by the software (Micro Image 4.0; Olympus).

#### *Effects of $PGF_{2\alpha}$ and its analogues on cell viability, DNA defragmentation and caspase-3 activity in luteal cells*

After 24 h of culture, the medium was replaced with incubating medium, and the cells were exposed to $PGF_{2\alpha}$, its analogues, or to the test cytokines. After 24 h of stimulation, the cells were washed three times with PBS, and cell viability was measured using a commercially available kit, TOX-1 (Sigma Aldrich), according to the manufacturer's instructions,[1] and as detailed previously (Okuda et al., 2004; Korzekwa et al., 2006). Caspase-3 activity was measured using colourimetric assay kits (Sigma Aldrich), according to the manufacturer's instructions,[2] and as described previously (Okuda et al., 2004). For Tunnel staining, the cells growing on the glass slide inserts were washed with PBS and fixed in PBS containing 4% paraformaldehyde followed by washes in PBS prior to permeabilisation with 0.5% Triton X-100 (Sigma Aldrich) in PBS. The cells were then briefly washed in PBS and incubated in fluorescein-conjugated dUTP and TdT (Tunel reagents; Dead-end fluorimetric TUNEL System, Promega). The cells were observed under fluorescent illumination using 470 nm excitation, and a 515 nm absorption, filter. Numbers of cells with fragmented DNA were counted, and a proportion of 500 DNA-fragmented cells was analysed as described previously (Korzekwa et al., 2006). The data were graphed as arbitrary units of fluorescent intensity using Micro Image (version 4.0) software.

### *Experiment two: Effects of $PGF_{2\alpha}$ and its analogues on the contractility of the bovine ovarian arterial explants*

The possible in vitro effects of $PGF_{2\alpha}$, and its analogues, on the contractility of the ovarian artery during the mid-luteal phase (days 8–12 of cycle; $n = 6$), were determined as described previously (Barszczewska and Jaroszewski, 2004). The contractility of the arterial explants was measured using Hugo Sachs Electronik Apparatus (March-Freiburg) equipped with an isometric transducer (type 372, HSE). Primary data registered by the transducer were analysed using HSE-ACAD computer software. During the 30–60 min pre-incubation period, the basic voltage (10 ± 1.0 mV) was assessed, and the spontaneous, regular contractions of the arteries were observed (stabilisation period). Following this stabilisation period, the

[1] See: http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/TOX1.
[2] See: http://www.sigmaaldrich.com/sigma/bulletin/casp3cbul.pdf.