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Effects of d-cloprostenol on different layers and regions of the bovine uterus during the follicular and luteal phases

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ABSTRACT

Introduction: D-cloprostenol is a synthetic $PGF_{2\alpha}$, commonly used in bovine reproduction, that increases myometral contractility. However, little is known regarding its contractile behaviour and how it depends on the reproductive state and uterine topography (regions and muscular layers).
Hypothesis and objectives: These aspects would affect the action of d-cloprostenol on the uterus. Therefore, we hypothesize a possible use of this drug at the time of artificial insemination, to improve conception rates and, in the *post-partum*, in order to accelerate uterine involution in dairy cattle. The purpose of the present study was to investigates the modulatory effect of d-cloprostenol on contractility of the bovine uterine region (horn and *corpus*) and their muscle layers (circular and longitudinal), in follicular and luteal phases. To our knowledge, motility effects of d-cloprostenol on different regions from healthy bovine uterus have not been described up to now.
Materials and methods: Uterine specimens were collected from uterine body and horn of cattle in the follicular (n = 28) and luteal phase (n = 32) of the estrous cycle at slaughter. Two strips were prepared from each regions corresponding to the circular and the longitudinal muscular layers, respectively. Samples were cultured in an organ bath, exposed to synthetic prostaglandin (1 μM d-cloprostenol) and their contractile activity was monitored for 10 min. The functionality of the strip throughout the experiment was tested by a dose of carbachol (10^{-5} M).
Results: The mean basal amplitude of contractions was higher in the follicular compared to the luteal phase in uterine horn samples, but not in muscles collected from the uterine body. The amplitude of contractions increased after d-cloprostenol administration in all tissues with a greater increase in samples from cattle in the follicular phase. The frequency of contractions increased after d-cloprostenol administration in longitudinal but not in circular fibrees.
Conclusion: The contractile responses to d-cloprostenol in both horn and *corpus* were strongest in the circular muscles but weak in the longitudinal muscles.

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1. Introduction

Uterine contractility plays, an important role, through a coordinated activity, during labour, *post-partum* involution and in sperm transportation after fecundation [1–3]. However, several studies revealed different behaviour of smooth muscle specimens depending on different patterns of regionally spontaneous activity, on cycle phase (estrus *vs* diestrus), on topography (horn *vs corpus*) on smooth muscular layers (circular *vs* longitudinal) [4,5]. Indeed, uterine motility is regulated by humoral, vegetative and paracrine mechanisms [6,7]. Among the hormones that stimulate contractility, an important role is played by prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$) [8]. It acts in a direct and indirect manner. Indeed, *in vitro* studies have demonstrated that $PGF_{2\alpha}$ can directly stimulate the electric and mechanical activity of the myometrium. The binding of $PGF_{2\alpha}$ with its membrane receptor activates, through the intermediation of the proteins G_q, a phospholipase C, which catalyzes the production of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), from phosphatidylinositol 4,5-bisphosphate (PIP_2) present in the plasma membrane [9]. Subsequently, the IP_3 binds to specific receptors present at the level of the sarco endoplasmic reticulum, resulting in

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the release of Ca^{2+} [10], while the DAG, together with Ca^{2+}, activates the protein kinase C responsible for the phosphorylation of specific proteins with target-promoting effects contractility [11]. Moreover, $PGF_{2\alpha}$ can stimulate contraction indirectly, by promoting the up-regulation of receptors for E_2 and oxytocin [12]. It is responsible for the lysis of the corpus luteum, the decrease in the concentrations of P_4, and also the increase in number of gap-junctions [13], too. Indeed, the action of $PGF_{2\alpha}$ generates synchronous contractions of high amplitude and frequency [10]. Such action justifies its use to induce cervical remodelling, childbirth and to modulate myometrial contractility during and after labour [14–16]. Therefore, the pharmacological control of myometrial contractions through the $PGF_{2\alpha}$ is extremely important. Indeed, the use of this molecule in reproduction is known and useful, not only to induce the delivery or to accelerate the involution of the uterus, but also to induce abortion or to treat pathological events, such as pyometra [17,18]. However, there are no studies that test the effectiveness of the contractile $PGF_{2\alpha}$ on the individual components that constitute the totality of contractile uterine unit.

On this basis, the aim of this work is to test *in vitro* the activity of d-cloprostenol, a synthetic $PGF_{2\alpha}$ commonly used in bovine reproduction. In particular, we test the effect of this hormone on contractility of bovine uterine strips from different regions (horn and *corpus*), muscular fibres (circular and longitudinal) and cycle phase (estrus and diestrus). Indeed, the distribution of receptors for prostanoids is not uniform in bovine species, but is affected by changes attributed to the muscular layers, to the uterine region and the phase of the cycle, parameters that could affect the effect of this drug, providing new clinical insights for d-cloprostenol.

2. Materials and methods

2.1. Preparation of uterine strips

A total of 60 uteri were obtained from Holstein Friesian cows aged 3–5 slaughtered at a local abattoir. All uteri were found without diseases and so were considered in our study: 28 from cows in the follicular phase and 32 from cows in the luteal phase. The estrus phase was recognized by antemortem and postmortem examinations. Antemortem, the phase of the estrous cycle and ovarian activity were determined through clinical examination, rectal palpation, and ultrasonography. At the same time, blood samples were collected from the coccygeal vein of each cow in prerefrigerated vacutainer glass tubes. After transporting blood samples to the laboratory, they were centrifuged at 1620xg for 10 min at 4 °C. The sera were subsequently frozen at −20 °C for later analysis of progesterone (P_4), which was conducted with a competitive immunoenzymatic colorimetric method (Progesterone EIA WELL; Radim SpA, Pomezia [Roma], Italy). The cross reactions between P_4 and steroid hormones were reported as follows: P_4 100%; 11-α OH-P_4 18%; 17-α OH-P_4 16%; 20-α OH-P_4 1%; estradiol less than 1×10^{-2}%; testosterone less than 1×10^{-2}%; cortisol less than 1×10^{-3}%; and cholesterol less than 1×10^{-3}%. The detection limit of the assay was 0.05 ng/mL. The intra-assay and interassay precisions had coefficient of variations of 2.9% and 4.8%, respectively. Cutoff values for estrus and diestrus were set at 1 ng/mL and greater than 2 ng/mL, respectively [19–21]. After stunning, the animals' genital tract and functional ovarian structures were visually examined for further identification of the phase of the estrous cycle and to exclude any pathologic conditions. For diagnosis of the cycle phase visual inspection of the ovaries *post mortem* was used by scoring the corpora lutea according to Ireland et al. [22] and Rizzo et al. [23]. Corpora lutea were annotated to stages I to IV and, furthermore, the presence of a Graffian follicle was recorded. Only the presence of corpora lutea stage II and III (day 5–17 of the estrous cycle) was classified as luteal phase; presence of corpora lutea stage IV (day 18–20) was classified as estrus if a Graffian follicle (>1.7 cm) was also present by Refs. [22,23]. The time from when the cows were slaughtered to when the uteri were collected was about 20 ± 10 min [19,20].

From each uterus, two circular portions, one of the middle part of the ipsilateral horn to the functional ovarian structure and one of the corpus, were excised and immediately placed in a flask containing prerefrigerated and oxygenated Krebs solution (NaCl 113 mM, KCl 4.8 mM, CaCl2·2H2O 2.2 mM, MgSO4 1.2 mM, NaH2PO4 1.2 mM, NaHCO3 25 mM, glucose 5.5 mM, and sodium ascorbate 5.5 mM), which was prepared daily. The flask was then immediately transported (15 ± 5 min) to the laboratory in an insulated box [19,20].

From each circular portion, a full-thickness (10-mm length and 3-mm width) uterine strips of each preparation (horn and *corpus*) were cut parallel to the circular muscular layers and to the longitudinal muscular layers, respectively, for recording of every combination (muscular layers × region). In this way, from every uterus we obtained four full-thickness uterine strips per cow (longitudinal horn, n = 1; circular horn, n = 1; longitudinal *corpus*, n = 1; circular *corpus*, n = 1).

2.2. Experimental design

Full-thickness uterine strips were immediately placed in an organ bath (10 mL) (model 4050; Ugo Basile, Milan, Italy) containing Krebs solution continuously bubbled with 95% O_2 and 5% CO_2. The pH was kept at 7.4, and the temperature was maintained at 37 °C. A silk thread was used to tie the myometrial strips to an isometric transducer (FORT25; AD Instruments, Castle Hill, NSW, Australia), in order to apply tension to longitudinal or circular layers, respectively. The contractile activities were recorded using a PowerLab 4/35 (AD Instruments acquisition software). After 1 h, stabilization strips were placed under tension of 2 g for about 30 min. Next followed the equilibration, carbachol (10^{-5} M), the esterified form of acetylcholine, was added to the bath. This concentration of carbachol had a selective and prolonged contractant effect and was subsequently removed with the wash (washout). Carbachol was repeated after 30 min, the time needed to ensure that the strip returned to the equilibrium condition. In the presence of a repeatable response with a deviation of 20% or less, calculated by the formula ($value_{maximum}$ - $value_{minimun}$/$value_{maximum}$) x 100, we proceeded to the experimental protocol, otherwise carbachol at the same concentration (10^{-5} M) was again administered. If the latter administration of carbachol was not repeatable, the strip was discarded [19,20].

With the start of the experimental protocol single doses (1 μM) of d-cloprostenol (Dalmazin®, Fatro, Italia) were added to the cuvette. This amount was chosen because it was known to elicit a response in bovine myometrium [3]. The *stock solution* (a known concentration solution of d-cloprostenol, which serves as the basis for preparing other solutions for dilution to lower concentrations) was added in ethanol at 10%. Previous studies from our group reported that ethanol has no effect on *in vitro* uterine contractility of the cow [19,20].

The dose (1 μM) of d-cloprostenol was left in the bath for a time of 10 min, after which we proceeded to remove them by *wash-out*.

Finally, a solution of carbachol (10^{-5} M) was used to test the functionality of the strips throughout the experiment. A variability ≤20% between tests was considered acceptable [19,20].

Contractile activity of the strips were computed as average amplitude (grams) and average frequency (number of contractions/10 min), before and after the administration of d-cloprostenol. For each administration, the percentage index of increase or decrease

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