



Lethal effect of high concentrations of Parecoxib and Flunixin meglumine on the *in vitro* culture of bovine embryos

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Abstract

Since cyclooxygenase (COX) inhibitors have been pointed out as potential treatments to increase pregnancy rates after embryo transfer, the present experiment aimed to evaluate the effects of flunixin meglumine (FM) and parecoxib (P), a COX-1 and 2 or COX-2 specific inhibitor, respectively, on the development of bovine embryos until the hatched blastocyst stage. *In vitro* produced bovine embryos were cultured in media with different concentrations of FM (0.14; 1.4; 14; 140 or 1400 µg/ml) or P (0.09; 0.9; 9; 90 or 900 µg/ml) and the production rates were evaluated. Concentrations of FM ≤14 µg/ml and P ≤90 µg/ml did not impair embryo development, although compiled data from non-lethal FM concentrations (≤14 µg/ml) indicated a toxic effect enough to decrease the hatching rate of blastocysts. Concentrations of FM at 140 and 1400 µg/ml and P at 900 µg/ml were lethal as no cleavage was detected on presumptive zygotes.

Keywords: blastocyst, cyclooxygenase inhibitors, drug concentration, embryotoxicity, nonsteroidal anti-inflammatory drugs.

Introduction

The relevance of the steroidal hormone progesterone (P4) on the establishment and maintenance of gestation is well documented (Wilmot *et al.*, 1985; Binelli *et al.*, 2001), as is the relationship between plasma P4 concentrations and the corpus luteum (CL; Lamming *et al.*, 1989; Binelli *et al.*, 2004). Briefly, when the CL is functionally active in the ovary (luteal phase), the concentration of P4 is high and the uterus is prepared for the establishment of pregnancy (Lamming *et al.*, 1989; Shelton *et al.*, 1990). The conceptus must signal its presence via interferon- τ production, thus preventing luteolytic prostaglandin F 2α (PGF 2α) pulses, which results in the maintenance of the CL and the continued release of progesterone (Binelli *et al.*, 2001, 2004; Nogueira *et al.*, 2004). Additionally, reports indicate a negative relationship between PGF 2α concentrations and embryo quality (Schrick *et al.*, 1993;

Buford *et al.*, 1996; Scenna *et al.*, 2004a, b, 2005).

Cyclooxygenase (COX) inhibitors are nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit PGF 2α synthesis. The resulting antiluteolytic effect of NSAIDs make them potential tools for increasing pregnancy rates after embryo transfer (Binelli *et al.*, 2001, 2004; Schrick *et al.*, 2001). COX exists in two isoforms; COX-1, a constitutive enzyme present in many tissues, and COX-2, an inducible enzyme, mainly produced at the sites of inflammation, and is the key enzyme in the synthesis of PGF 2α by its precursor, arachidonic acid (Thatcher *et al.*, 2001). Flunixin meglumine (FM), a nonselective COX-1/-2 inhibitor, and parecoxib (P), a specific COX-2 inhibitor, could be appropriately used to block COX-2 in an attempt to increase pregnancy rates in the bovine species. However, authors disagree on the concentrations and administration pathways for FM (Buford *et al.*, 1996; Odensvik *et al.*, 1998; Geary *et al.*, 2005; Purcell *et al.*, 2005; Scenna *et al.*, 2005), and the potential side effects of P have yet to be elucidated (Ayar, 2007).

This study aimed to establish the embryotoxic threshold for concentrations of flunixin meglumine and parecoxib on the *in vitro* culture of bovine embryos. This approach was previously described to validate a model for pharmacological dose-response (Avery and Schmidt, 1995).

Materials and Methods

Unless otherwise mentioned, all chemicals were obtained from Sigma-Aldrich Laboratory (St. Louis, MO, USA).

Abattoir location and animals

Ovaries from the Nelore breed (*Bos indicus*) were collected from a local abattoir (Frigol, Lençóis Paulista, SP, Brazil, latitude 22°35'S, longitude 48°48'W) located 47 km from the laboratory (Botucatu, SP, Brazil). The animals came from several different commercial herds and the replicates were performed from January 2007 to June 2008.

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Recovery of cumulus oocyte complexes

Cumulus oocyte complexes (COCs) were aspirated from small follicles (3-8 mm in diameter) using an 18-ga needle attached to a 10 ml syringe. Oocytes were classified according to their morphology (Khurana and Niemann, 2000) and the ones with a uniformly granulated cytoplasm and surrounded by several (3-4) layers of compact cumulus cells were selected and washed three times with HEPES-buffered TCM-199 medium containing fetal calf serum (FCS, 10%; Gibco, BRL, Burlington, Ontario, Canada), pyruvate (2 μ l/ml, 100 mM solution) and gentamicin (75 μ g/ml) then washed once with maturation medium.

In vitro maturation

Fifteen to 25 COCs were incubated in groups. Each droplet (90 μ l) of maturation medium consisted of TCM-199 medium (Earle's salt) supplemented with sodium pyruvate (2 μ l/ml, 100 mM solution), gentamicin (75 μ g/ml), pFSH (20 μ g/ml, Pluset[®], Serovet, Roma, Italy) and pLH (2 IU/ml, Lutropin[®], Bioniche, Belleville, Ontario, Canada). The droplets were covered with mineral oil (3.5 ml) and pre-incubated under the maturation conditions for a minimum of 2 h (39°C, 5% CO₂ in air with 100% humidity) then incubated for 18 to 24 h once the COCs were added.

In vitro fertilization

In vitro fertilization (IVF) took place in droplets (90 μ l) containing tyrode albumin lactate pyruvate (TALP) supplemented with 6 mg/ml fatty acid free BSA, 100 mM sodium pyruvate, 75 μ g/ml gentamicin, 11 μ g/ml heparin and 44 μ g/ml PHE (2mM penicillamine, 1 mM hypotaurine and 250 mM epinephrine). Semen was thawed at 37°C for 20 s and spermatozoa were washed in a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) prepared by adding 2 ml of 45% Percoll to 2 ml of 90% Percoll in a 15 ml centrifuge tube (Corning[®], 900 g; 30 min). After removal of the supernatant, spermatozoa were resuspended in IVF medium (1 x 10⁶ cells/ml) and added into each droplet for incubation (39°C; 5% CO₂; 10 to 14 h).

In vitro culture

Cumulus cells were removed from presumptive zygotes by vortexing the embryos in conical tubes containing TCM-199 medium with HEPES for 4 min. Cumulus-free presumptive zygotes were washed twice in TCM-199 medium with HEPES and transferred to 90 μ l culture drops (15 to 25 zygotes per drop) under mineral oil. The culture medium

consisted of synthetic oviduct fluid with amino acids, sodium citrate and myo-inositol (SOFaaci; Holm *et al.*, 1999) plus FCS (5%; Gibco[®]), BSA (5%), and sodium pyruvate (0.2%), which was used to cultivate the embryos. A submarine incubation system, based on a previous experiment by Satrapa *et al.* (2011), was used. Briefly, Petri dishes containing embryos were placed in plastic bags (25 x 15 cm) containing a gas mixture of 5% O₂, 5% CO₂ and 90% N₂ (White Martins, São Paulo, SP, Brazil). The culture medium was partly replaced with 50 μ l of fresh medium at 48, 96, 144 and 192 h after fertilization (Satrapa *et al.*, 2011). Before each medium replacement, cleavage, morula, blastocyst and hatched blastocyst yields were evaluated.

Drug concentrations

Presumptive zygotes were cultured as described before in the absence (control group) or presence of parecoxib (P; Bextra[®], injectable, 40 mg, Pfizer Pharmaceuticals, Barceloneta, Puerto Rico) or flunixin meglumine (FM; Banamine[®], injectable, 500 mg, Schering-Plough, Rio de Janeiro, Brazil). Standard concentrations were estimated by the usual dosage for embryo transfer in the bovine species (14 μ g/ml FM; Geary *et al.*, 2005; Purcell *et al.*, 2005) and based on the maximum daily dosage of the prescription for a human being (*i.e.*, 80 mg of P, according to drug information contents). The approach to define the P concentration for the bovine species was attempted using the average body weight of a human being (70 kg) and bovine (450 kg). Metabolic weights of humans (MWH = 70^{0.75}) and the bovine (MWB = 450^{0.75}) species were calculated (Keeseey and Hirvonen, 1997) and the proportional concentration of P to MWH (80 mg/24.2 kg) was calculated for 97.7 kg of MWB (*i.e.*, approximately 323 mg of P). Bovine blood volume was calculated based on an average body weight of 450 kg and an average of 8% blood volume (l) relative to body weight, that is, 36 l of blood (Reynolds, 1953; Aird *et al.*, 2006). Finally, P serum concentration in the bovine species was calculated by dividing total dosage (323 mg) by blood volume (36000 ml). The calculated P serum concentration (approximately 9 μ g/ml) was used as the standard, and it could be the approximate P concentration of the uterine environment that the bovine embryo would be exposed to under real conditions of drug administration. For both drugs, decreased (x0.1 and x0.01) and increased (x10 and x100) concentrations were calculated relative to the standard P and FM concentrations (0.09; 0.9; 9; 90 and 900 μ g/ml of parecoxib; 0.14; 1.4; 14; 140 and 1400 μ g/ml of FM). During IVC, each culture dish had medium drops with all drug (P and FM) concentrations along with a control group (without the drug). When the medium was partially changed, the drug concentrations were maintained the same as the original drop concentration.



A pilot experiment (4 replicates) was performed to evaluate the embryo toxicity of the drug diluent itself (NaCl 0.9%) on bovine blastocyst and hatched blastocyst production. Only the diluents were added to the IVC medium, as the same volume would be used in the several different concentrations and IVC was done the same way as the P, FM and control groups.

Statistical analyses

Data were compared among concentrations (drug concentrations and respective control, *i.e.*, intradrag comparison) by a Cochran-Armitage Trend Test (Armitage, 1955; Agresti, 2002) and by clustering non-lethal concentrations of the same drug to compare hatched blastocyst rates with their respective control group using Chi-square analysis (Yates correction for continuity). Significance was considered when $P < 0.05$.

Results

The results show data from 11 replicates for FM and 14 for P. However, for the FM concentrations of 140 and 1400 $\mu\text{g/ml}$ and P concentration of 900 $\mu\text{g/ml}$, data are from 4 replicates only, since a lethal effect (*i.e.*, arrested embryo development) was observed in these groups.

Parecoxib

Cleavage, morula, blastocyst and hatched blastocyst rates of all P concentrations are summarized in Table 1. A concentration of 900 $\mu\text{g/ml}$ was lethal for embryo development as early as the first cleavages (48 h post insemination). The other concentrations (0.09; 0.9; 9 and 90 $\mu\text{g/ml}$) showed no significant differences in blastocyst and hatched blastocyst rates when compared to the control group.

Table 1. Number of oocytes (n) and cleavage (CLE), morula (MO), blastocyst (BL) and hatched blastocyst (HB) rates after treatment with increasing concentrations of parecoxib or the control.

Parecoxib ($\mu\text{g/ml}$)	Oocytes n	CLE n (%)	MO n (%)	BL n (%)	HB n (%)
Control (0)	247	197 (79.8)	148 (59.9)	88 (35.6)	42 (17.0)
0.09	223	171 (76.7)	127 (57.0)	60 (26.1)	32 (14.4)
0.9	208	158 (76.0)	110 (52.9)	52 (25.0)	22 (10.6)
9	219	156 (71.2)	109 (49.8)	53 (24.2)	31 (14.2)
90	205	146 (71.2)	99 (48.3)	49 (24.0)	22 (10.7)
900	45	0 (0.0)	0 (0.0)	NA	NA
P- value for trend test*		0.1	0.05	0.16	0.19

*Cochran-Armitage Trend Test was performed only with concentrations ≤ 90 $\mu\text{g/ml}$. NA (not applicable) since after evaluation of morula rate, the presumptive zygotes were morphologically dead (dark color and/or fragmented and/or no visible cytoplasmic membrane) and were removed from the culture drops.

Flunixin meglumine

Cleavage, morula, blastocyst and hatched blastocyst rates of all FM concentrations are summarized in Table 2. The two highest FM

concentrations (140 and 1400 $\mu\text{g/ml}$) were lethal for embryo development as early as the first cleavage evaluation. However, the other concentrations had no significant difference when compared to the control group.

Table 2. Number of oocytes (n) and cleavage (CLE), morula (MO), blastocyst (BL) and hatched blastocyst (HB) rates after treatment with increasing concentrations of flunixin meglumine or the control.

Flunixin Meglumine ($\mu\text{g/ml}$)	Oocytes n	CLE n (%)	MO n (%)	BL n (%)	HB n (%)
Control (0)	124	104 (83.9)	80 (64.5)	49 (39.5)	37 (29.8)
0.14	123	94 (76.4)	66 (53.7)	35 (28.5)	18 (14.6)
1.4	122	94 (77.1)	66 (54.1)	35 (28.7)	18 (14.8)
14	117	87 (74.4)	70 (59.8)	34 (29.1)	25 (21.4)
140	44	0 (0.0)	0 (0.0)	NA	NA
1400	44	0 (0.0)	0 (0.0)	NA	NA
P - value for trend test*		0.25	0.73	0.45	0.85

*Cochran-Armitage Trend Test was performed only with concentrations ≤ 14 $\mu\text{g/ml}$. NA (not applicable) since after evaluation of morula rate, the presumptive zygotes were morphologically dead (dark color and/or fragmented and/or no visible cytoplasmic membrane) and were removed from the culture drops.



Pooled data

The first analysis comparing non-lethal drug concentrations showed no significant difference among themselves (0.09; 0.9; 9 and 90 µg/ml for P, and 0.14; 1.4 and 14 µg/ml for FM). Hence, these concentrations were clustered in groups (non-lethal concentrations; nlc) for each drug (nlcP and nlcFM). Control groups from both experiments (Control P and Control FM) remained as the original control group for each drug and hatched blastocyst rates were compared to the nlcP and nlcFM groups, respectively. There was a statistical difference ($P < 0.003$) between hatched blastocyst rates from the nlcFM (16.9%; 61/362) and Control FM (29.8%; 37/124) groups. There was no difference in hatched blastocysts rates ($P = 0.09$) between the nlcP (12.5%; 107/855) and Control P (17.0%; 42/247) groups.

Discussion

Some concentrations (FM: 0.14; 1.4 and 14 µg/ml; P: 0.09; 0.9; 9 and 90 µg/ml) did not affect cleavage and blastocyst production. However, higher concentrations (FM: 140 and 1400 µg/ml; P: 900 µg/ml) were lethal, as no presumptive zygotes cleaved under these conditions.

Attempts of FM administration to increase bovine pregnancy rates have already been reported. However, there was no consensus among these studies since in every experiment, neither the dosage, time nor the route of administration were consistent (Buford *et al.*, 1996; Odensvik *et al.*, 1998; Elli *et al.*, 2001; Geary *et al.*, 2005; Purcell *et al.*, 2005; Scenna *et al.*, 2005). Additionally, there is insufficient published information regarding the pharmacological threshold of FM and P concentrations that may be toxic to bovine embryos. The *in vivo* use of any drug to modulate embryo development should first be tested for the embryotoxicity threshold using an *in vitro* model, similar to this study, to facilitate the usage of safe concentrations *in vivo*. The time that the embryos were exposed to drugs in this study (a maximum of 178 h on culture) may be considered an extreme condition compared with the shortest time (12 to 24 h) of regular treatment in cattle (Buford *et al.*, 1996; Odensvik *et al.*, 1998). Regardless, and despite the unknown bioavailability of these drugs in the bovine uterine lumen, the present assay provides useful information for future *in vivo* experiments with intramuscular treatments of both FM and P in the bovine species. The analysis of pooled data from the non-lethal concentrations of P resulted in no decrease in the hatching rate (178 h of culture) compared with the control group, whereas with the non-lethal concentrations of FM, there was a significant difference

between hatched blastocysts compared with the control group (16.9 and 29.8%, respectively). This finding could be interpreted as being due to the long time that the embryos were exposed to the drug until the hatching stage, and mainly due to the drug itself (*i.e.*, its pharmacological properties) because this effect was not observed ($P = 0.09$). In a pilot experiment (results not shown), the vehicle used to dilute each drug was tested to evaluate its embryotoxicity, and no apparent harmful effects of the P or FM vehicle were observed. Since individual FM non-lethal concentrations did not differ from the control, it is difficult to infer which concentrations were the main effectors of potential embryotoxicity observed in the pooled data. This presumptive embryotoxicity could have arisen when concentrations were clustered, resulting in a robust sample large enough to produce a statistical difference between pooled FM data and the control. Moreover, the system of bovine IVP itself is a source of great variation in inter replicates (age, body condition, health status of animals), thus, both factors could explain the statistical difference observed only in the pooled FM data.

As previously described, this dose-response study could be the first screening for potential bovine embryotoxic effects of new drugs (usage of P in bovine embryo transfer). However, embryonic safety needs *in vivo* validation since unexpected *in vivo* effects could not be ruled out solely based on an *in vitro* model of embryotoxicity.

A flunixin meglumine concentration of 14 µg/ml (already employed *in vivo* as a strategy for increasing pregnancy rates in bovine recipients) was not toxic enough to inhibit the development of cultured bovine embryos, even after a long time of exposure. In addition, high concentrations of P (900 µg/ml) and FM (140 and 1400 µg/ml) were lethal as early as the first cleavage, *i.e.*, under the effect of a few hours of drug exposure.

In conclusion, bovine embryo development was completely impaired with concentrations of 900 µg/ml parecoxib and 140 and 1400 µg/ml flunixin meglumine in the culture medium. When the non-lethal concentrations were compared, we inferred that parecoxib had a lower potential toxicity to decrease hatching rates after a long time of drug exposure (178 h) during culture.

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