Administration of the nonsteroidal anti-inflammatory drug tolfenamic acid at embryo transfer improves maintenance of pregnancy and embryo survival in recipient mice

Geraldine Schlapp · Lucía Goyeneche ·
Gabriel Fernández · Alejo Menchaca · Martina Crispo

Received: 6 August 2014 / Accepted: 27 October 2014 / Published online: 6 January 2015
© Springer Science+Business Media New York 2014

Abstract

Purpose To evaluate the effect of the nonsteroidal anti-inflammatory drugs tolfenamic acid and flunixin meglumine in pregnancy rate and embryo survival of recipient mice subjected to embryo transfer.

Methods A total of 142 recipient females were transferred with 2,931 embryos and treated with a single injection of tolfenamic acid (1 mg/kg; $n=54$ females with 1,129 embryos), flunixin meglumine (2.5 mg/kg; $n=46$ females with 942 embryos), or bi-distilled water (10 mL/kg) as control group ($n=42$ females with 860 embryos). Pregnancy was checked 2 weeks after embryo transfer, delivery was registered on the due date, and litter size was recorded on Day 7 after birth.

Results Pregnancy rate of tolfenamic acid treated females was significantly higher than flunixin group ($P<0.05$) and showed a tendency to be higher when compared to the control group ($P=0.06$). The number of pups born from transferred embryos in pregnant females was significantly higher for both treatment groups compared to controls ($P<0.05$). Number of pups from total transferred embryos was higher for both treatment groups ($P<0.05$) when compared to controls.

Conclusion The use of tolfenamic acid at the time of embryo transfer improves both pregnancy rate and number of live pups in recipient mice, with optimal effects observed with flunixin meglumine. We suggest that the use of tolfenamic

acid has beneficial effects on the maintenance of pregnancy and embryo survival in recipient mice, which should be taken into account for further studies in other mammalian females.

Keywords Flunixin meglumine · Prostaglandin · Corpus luteum · Pregnancy rate · Birth rate

Introduction

Pain relief is essential in surgical procedures since it optimizes postoperative care and well-being of animals [1]. In this sense, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) includes anti-inflammatory, analgesic and antipyretic effects, via inhibition of cyclooxygenase (COX) enzyme, particularly COX-2, which plays a major role in prostaglandin (PG) synthesis [2, 3]. During embryo transfer, manipulation of the reproductive tract produces an inflammatory process which could be induced by the embryo itself, by transfer media acting as foreign material and/or by microorganisms introduced into the uterus during surgery [4]. It has been shown that embryo transfer causes release of PGF2 α which has deleterious effects on luteal function and embryo survival [5, 6]. Since PGF2 α is the main hormone that triggers luteolysis [7], it could indirectly cause embryonic loss before maternal recognition of pregnancy. Furthermore, addition of PGF2 α to the culture medium has shown to inhibit in vitro development of rabbit [8] and bovine embryos [9]. Thus, in mammalian females the inhibition of endometrial PGF2 α release or the blockage of its action contributes to maintain luteal function, embryo survival and establishment of pregnancy.

Flunixin meglumine, a commonly used NSAID, has been previously used to reduce the inflammatory process during embryo transfer in some species. Koblishke et al. determined that administration of flunixin meglumine or meclofenamic acid to recipient mares produced a significant reduction of

Capsule Tolfenamic acid administration at embryo transfer in recipient mice improves pregnancy rate and number of live pups, suggesting beneficial effects on the maintenance of pregnancy and embryo survival.

G. Schlapp (✉) · L. Goyeneche · G. Fernández · M. Crispo
Unidad de Animales Transgénicos y de Experimentación, Institut Pasteur de Montevideo, Matajojo 2020, 11400 Montevideo, Uruguay
e-mail: gschlapp@pasteur.edu.uy

A. Menchaca
Instituto de Reproducción Animal Uruguay, Fundación IRAUy,
Montevideo, Uruguay

COX-2 positive cells and polymorphonuclear cell recruitment in the endometrium, and also a reduction of PGF2 α concentration in plasma was observed when meclofenamic acid was used. These phenomena were accompanied by a lower incidence of luteolysis [4]. Also, Santos et al. reported that inhibition of PGF2 α synthesis with flunixin meglumine interferes with luteolysis and leads to prolonged luteal activity in mares [10]. Flunixin meglumine have also been proposed to improve pregnancy in farm animals. Koblishke et al. detected higher pregnancy rates of mares receiving flunixin meglumine and vedaprofen simultaneously when compared to untreated mares [5]. In cattle, administration of flunixin meglumine at the time of embryo transfer or before maternal recognition of gestation increased pregnancy rates according to Scenna et al. [6] and Guzeloglu et al. [11] although, similar studies did not show beneficial effects of flunixin meglumine treatment in bovines [12, 13]. In mice, it has been shown that flunixin meglumine has no detrimental effects on embryonic survival and implantation [14], but possible beneficial effects of flunixin meglumine or other NSAIDs in pregnancy have not been reported yet.

Tolfenamic acid is a NSAID that has never been reported in recipient females. This drug differs from flunixin meglumine in their chemical structure: flunixin meglumine is an amino nicotinic acid derivate and tolfenamic acid belongs to the anthranilic acid subgroup, also known as fenamates. These NSAIDs also show some differences in the COX isoform selectivity. Tolfenamic acid preferentially inhibits COX-2 in dogs [15] and goats [16], whereas flunixin meglumine does not show specific inhibition [17]. Tolfenamic acid not only inhibits COX but also 5-lipoxygenase, both enzymes are involved in the pathways of arachidonic acid metabolism [18, 19]. Fenamates are also low-potency modulators of a diversity of ion channels and enzymes, exhibiting either activator or inhibitory effects [20]. The use of tolfenamic acid in recipient females at embryo transfer to improve the maintenance of the pregnancy has not been proposed in any specie.

The objective of this study was to evaluate the effect of tolfenamic acid and flunixin meglumine administration on pregnancy rate and embryo survival after embryo transfer surgery in recipient mice used in a transgenic animal facility.

Materials and methods

Animals

A total of 2,931 embryos produced during a 2 year period at the Transgenic and Experimental Animal Unit of Institut Pasteur de Montevideo were microinjected ($n=2,427$) or rederived ($n=504$). Experimental protocols were opportunely approved by the institutional Animal Ethics Committee, in

accordance with national law 18.611 and international animal care guidelines (Guide for the Care and Use of Laboratory Animal) [21] regarding laboratory animal's protocols. Mice were housed under Specific Pathogen Free conditions, in individually ventilated cages (Tecniplast, Milan, Italy), with 40–60 % RH, 20 ± 1 °C temperature, light:dark cycles of 14:10 h, autoclaved food (Labdiet 5K67, PMI Nutrition, IN, USA) and ad libitum water.

Embryo collection

Ovarian superstimulation was induced in 3–4 week-old B6SJL F1 or C57BL6/J donor females with a single intraperitoneal (i.p.) injection of 5 IU of equine chorionic gonadotrophin (eCG; Novormon®, Syntex, Buenos Aires, Argentina) and 5 IU of human chorionic gonadotrophin (hCG; Chorulon®, Intervet International B.V., Boxmeer, Holland) 46 h later. Soon after hCG treatment, females were mated with adult male mice in 1:1 ratio. For microinjection, donor females were euthanized by cervical dislocation 12 h after mating, oviducts were excised and one-cell embryos were collected from the swollen ampulla in M2 medium containing 0.3 mg/ml hyaluronidase to remove cumulus cells. For rederivation, females were euthanized by cervical dislocation 36 h after mating and embryos collected by oviduct flushing. All embryos were cultured in M16 medium microdrops under embryo tested mineral oil, in 5 % CO₂ in air at 37 °C [22]. Unless otherwise indicated, chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA).

Pronuclear microinjection

Pronuclear microinjection was performed using an inverted microscope (TE 2000, Nikon, NY, USA) and mechanical micromanipulators (Transferman NK2, Eppendorf, Hamburg, Germany) using embryo holding pipettes (35° bent angle, opening diameter of 20–25 μ m; Biomedical Instruments, Zöllnitz, Germany) and home-made injection pipettes. Since the embryos were produced for the establishment of different transgenic lines in our laboratory, different linearized DNA vectors were injected at 2 ng/ μ l diluted in microinjection buffer (5 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) [22]. For each microinjection session, 100–150 zygotes were loaded in groups of 30 in glass depression slides containing M2 medium under mineral oil. DNA solution was injected into the male or female pronucleus, until the pronuclear membrane visibly swelled. After microinjection, embryos were maintained in the aforementioned culture conditions for 30 min to evaluate survival rate before being transferred to foster mothers. Surviving zygotes were defined according to standard criteria [23].

Embryo transfer

All foster mothers (Swiss and B6D2 F1) were mated with vasectomized adult males with proven sterility to induce pseudopregnancy and were checked for copulatory plugs the day of embryo transfer. Embryo transfer was performed within 30 min after microinjection or 2–3 h after oviduct flushing for the rederived embryos. The recipient females were anesthetized with a mixture of ketamine (100 mg/kg; Vetanarcol®, König, Buenos Aires, Argentina) and xylazine (10 mg/kg; Seton®, Calier, Barcelona, Spain) via i.p. [24]. The embryos were loaded into a pulled glass pipette and placed through the infundibulum into both oviducts, transferring in average 15 rederived or 23 microinjected embryos per female. For surgery and recovery procedures, females were placed over a warm pad to avoid hypothermia [22].

Experimental design

A total of 142 Swiss and B6D2 foster females at 0.5 dpc were transferred with microinjected or rederived embryos. For each embryo transfer session recipients were randomly assigned to one of the following three groups: tolferamic acid (TA) group ($n=54$ females, 1,129 embryos), flunixin meglumine (FM) group ($n=46$ females, 942 embryos), or control group ($n=42$ females, 860 embryos). The treatment was performed in a single subcutaneous injection of tolferamic acid (1 mg/kg of body weight, Tolferdine®, Vetoquinol, Lure, France), flunixin meglumine (2.5 mg/kg of body weight, Niglumine®, Calier, Barcelona, Spain), or sterile bi-distilled apyrogenic water (10 mL/kg of body weight), immediately after embryo transfer and before the foster female came out of anesthetic plane. Pregnancy was determined by visual inspection by an experienced animal caretaker 2 weeks after embryo transfer, delivery rate was registered on the due date, and litter size was recorded on Day 7 after birth.

Statistical analysis

Pregnancy rate (pregnant/transferred recipients), delivery rate (parturition/pregnant recipients), birth rate (live pups/transferred embryos) in pregnant females and birth rate in total females were compared among three experimental groups by logistic regression (SPSS version 18.0). The analysis included the effect of the source of the embryos (i.e. microinjection or rederivation) and data are showed pooled since the results were not affected by this factor. Differences were considered significant for P value <0.05 and <0.1 was assumed as tendency.

Results

The administration of the NSAID tolferamic acid at the time of embryo transfer showed a tendency ($P=0.06$) to improve

pregnancy rate and improved birth rate in pregnant females ($P<0.05$) and the number of live pups from total transferred embryos ($P<0.05$) in comparison to control females (Table 1). The administration of flunixin meglumine did not have a significant effect on pregnancy rate, but improved the birth rate in pregnant females ($P<0.05$) and the birth rate in total transferred females ($P<0.05$) in comparison to control females. Interestingly, pregnancy rate was greater in TA group than FM group ($P<0.05$), but birth rate in pregnant females was greater in FM than TA group ($P<0.05$). The number of live pups from transferred embryos in total females did not show statistical differences with the administration of tolferamic acid or flunixin meglumine.

Discussion

In this study, the administration of NSAIDs to mice at the time of embryo transfer significantly improved embryo survival producing greater birth rate than control females. In addition, differences were found between NSAID treatments since tolferamic acid was more effective than flunixin meglumine in maintaining pregnancy, but flunixin meglumine was more effective in producing live pups in pregnant females.

The effect of tolferamic acid in recipient females had not been reported yet. However, the use of other NSAIDs at embryo transfer has shown interesting results in some species: the administration of one dose of flunixin meglumine in cows [6], a single dose of ibuprofen lysinate in heifers [25], and five doses of flunixin meglumine or meclofenamic acid in mares [4] produced higher pregnancy rates than untreated females. In the studies performed in cows and mares it was demonstrated that NSAIDs reduced PGF2 α release after embryo transfer. Moreover, meclofenamic acid, chemically similar to tolferamic acid, was more effective than flunixin meglumine to inhibit PGF2 α release in recipient mares. In this sense, our study shows greater pregnancy rate in tolferamic acid treated females than flunixin meglumine and is the first report to demonstrate an effect of this NSAID on maintenance of the pregnancy in mammalian females.

The different results obtained for flunixin meglumine and tolferamic acid could be explained by COX isoform selectivity, taking into account that flunixin meglumine has no specific inhibition whereas tolferamic acid shows a stronger specificity for COX-2, which has been shown to mainly produce inflammatory responses. In the corpus luteum (CL), different immune mediators and cells are recruited during its lifespan. There is a growing list of potential factors that may be involved in the luteolytic process, including endothelial-derived proteins, immune cell-derived cytokines, reactive oxygen species, neuropeptides and lipophilic molecules. Bidirectional communication between luteal and immune cells

Table 1 Reproductive outcomes of female mice treated with tolafenamic acid or flunixin meglumine at the time of embryo transfer

	Pregnancy rate (pregnant/ transferred recipients)	Delivery rate (parturition/ pregnant recipients)	Birth rate (live pups/transferred embryos) in pregnant females	Birth rate (live pups/transferred embryos) in total females
Control	47.6 % (20/42) ^{ab}	100 % (20/20) ^a	17.2 % (75/435) ^a	8.7 % (75/860) ^a
Flunixin meglumine	45.6 % (21/46) ^a	100 % (21/21) ^a	35.3 % (146/414) ^c	15.5 % (146/942) ^b
Tolfenamic acid	66.7 % (36/54) ^b	100 % (36/36) ^a	25.3 % (195/772) ^b	17.3 % (195/1129) ^b

Different superscripts indicate statistical differences ($P < 0.05$)

may thus facilitate hormonally regulated survival and demise of the CL [7, 26]. Both tolafenamic acid and flunixin meglumine could inhibit luteolysis by blocking PG synthesis but tolafenamic acid could also have effects over the immune responses and the survival of CL.

The beneficial effect of NSAIDs at embryo transfer does not only seem to be associated to the anti-luteolytic effect of the treatment. Women undergoing in vitro fertilization (IVF) receiving a daily oral dose of 100 mg of the NSAID acetylsalicylic acid had better ovarian response, uterine and ovarian blood flow velocity, implantation, and pregnancy rates than women receiving placebo [27]. Similar results were found in women undergoing IVF and receiving 75 mg of aspirin daily from the day of embryo transfer until pregnancy test in which higher pregnancy rates were reported [28]. Moreover, treatment of women with piroxicam, an oxicam kind of NSAID, administrated 1–2 h before embryo transfer increased implantation and pregnancy rates, in both fresh and frozen-thawed embryo transfer cycles [29]. The mechanisms involved in these effects are still not clear.

We suggest that tolafenamic acid could improve pregnancy rates in recipient mice by preventing early luteolysis, but also by reducing embryonic loss in those females getting pregnant, an effect probably associated with inflammatory process elicited by uterine manipulation. In addition, flunixin meglumine had positive effect on embryo survival, inducing greater birth rate from pregnant females, almost doubling the values of the control group (i.e. 35.3 % vs. 17.2 %, respectively). Thus, the reduced embryonic loss observed for tolafenamic acid was even more evident for flunixin meglumine.

The positive effect of NSAIDs on embryo survival showed in this study could also be associated with a local effect by the blocking of PG production in the endometrium. It has been demonstrated that development of in vitro produced bovine embryos to blastocyst stage was reduced by addition of PGF2 α (1 ng/ml) in the culture media, and that addition of PGF2 α to the culture media of in vivo derived bovine embryos in compact morulae stage did not affect development to blastocyst, but reduced hatching rates [9]. In other study, cows were administered with flunixin meglumine at the time of embryo transfer, and pregnancy rates were evaluated by quality score of the embryos. Pregnancy rate with Grade 2 embryos, but not with Grade 1 embryos, was improved following

administration of flunixin meglumine [6]. These findings suggest that compromised quality embryos are most susceptible to deleterious effects of PGF2 α , an effect that could be prevented by NSAID administration. Scenna et al. proposed that even though release of PGF2 α during embryo transfer may not result in luteolysis, embryonic survival may be compromised by the presence of small concentrations of PGF2 α in the uterine lumen, creating a hostile environment for embryonic development [6]. In our study, both tolafenamic acid and flunixin meglumine had positive effects on birth rate which could indicate that both NSAIDs could improve uterine environment for embryo development and surviving, although the mechanism of action is not clearly explained.

In conclusion, this study reports for the first time that the use of tolafenamic acid in recipient females at embryo transfer improves the maintenance of pregnancy and embryo survival. In addition, flunixin meglumine improves birth rate but not pregnancy rate, suggesting that its effect is mainly associated with embryo survival instead of luteal maintenance. Overall results show that both NSAIDs, and tolafenamic acid in particular, represent a promising treatment for embryo transfer programs in mammalian females.

Acknowledgments The authors wish to thank Juan José Goyeneche for statistical support. This project was financially supported by FOCEM (MERCOSUR Structural Convergence Fund), COF 03/11. AM and MC are fellows of Sistema Nacional de Investigadores (SNI).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The manuscript does not contain clinical studies or patient data. Experimental animal protocols were opportunely approved by the institutional Animal Ethics Committee, in accordance with national law 18.611.

References

1. Gaertner D, Hallman T, Hankenson F, Batchelder M. Anesthesia And Analgesia in Rodents. In: Fish R, Brown M, Danneman P, Karas A, editors. Anesthesia and analgesia in laboratory animals. 2nd ed. London, UK: Academic; 2011. pp. 239–82.
2. Brideau C, Van Staden C, Chan CC. In vitro effects of cyclooxygenase inhibitors in whole blood of horses, dogs, and cats. Am J Vet Res. 2001;62:1755–60.

3. Cashman J. The mechanisms of action of NSAIDs in analgesia. *Drugs*. 1996;52:13–23.
4. Koblishke P, Kindahl H, Budik S, Aurich J, Palm F, Walter I, et al. Embryo transfer induces a subclinical endometritis in recipient mares which can be prevented by treatment with non-steroid anti-inflammatory drugs. *Theriogenology*. 2008;70:1147–58.
5. Koblishke P, Budik S, Muller J, Aurich C. Practical experience with the treatment of recipient mares with a non-steroidal anti-inflammatory drug in an equine embryo transfer programme. *Reprod Domest Anim*. 2010;45:1039–41.
6. Scenna FN, Hockett ME, Towns TM, Saxton AM, Rohrbach NR, Wehrman ME, et al. Influence of a prostaglandin synthesis inhibitor administered at embryo transfer on pregnancy rates of recipient cows. *Prostaglandins Other Lipid Mediat*. 2005;78:38–45.
7. Pate JL, Johnson-Larson CJ, Ottobre JS. Life or death decisions in the corpus luteum. *Reprod Domest Anim*. 2012;47 Suppl 4:297–303.
8. Maurer RR, Beier HM. Uterine proteins and development in vitro of rabbit preimplantation embryos. *J Reprod Fertil*. 1976;48:33–41.
9. Scenna FN, Edwards JL, Rohrbach NR, Hockett ME, Saxton AM, Schrick FN. Detrimental effects of prostaglandin F2alpha on preimplantation bovine embryos. *Prostaglandins Other Lipid Mediat*. 2004;73:215–26.
10. Santos VG, Beg MA, Bettencourt EM, Ginther OJ. Role of PGF2alpha in luteolysis based on inhibition of PGF2alpha synthesis in the mare. *Theriogenology*. 2013;80:812–20.
11. Guzeloglu A, Erdem H, Saribay M, Thatcher W, Tekeli T. Effect of the administration of flunixin meglumine on pregnancy rates in Holstein heifers. *Vet Rec*. 2007;160:404–6.
12. Geary TW, Ansotegui RP, MacNeil MD, Roberts AJ, Waterman RC. Effects of flunixin meglumine on pregnancy establishment in beef cattle. *J Anim Sci*. 2010;88:943–9.
13. von Krueger X, Heuwieser W. Effect of flunixin meglumine and carprofen on pregnancy rates in dairy cattle. *J Dairy Sci*. 2010;93:5140–6.
14. Goulding DR, Myers PH, Goulding EH, Blankenship TL, Grant MF, Forsythe DB. The effects of perioperative analgesia on litter size in Crl:CD1(ICR) mice undergoing embryo transfer. *J Am Assoc Lab Anim Sci*. 2010;49:423–6.
15. Ricketts AP, Lundy KM, Seibel SB. Evaluation of selective inhibition of canine cyclooxygenase 1 and 2 by carprofen and other nonsteroidal anti-inflammatory drugs. *Am J Vet Res*. 1998;59:1441–6.
16. Sidhu PK, Landoni MF, Lees P. Pharmacokinetic and pharmacodynamic interactions of tolafenamic acid and marbofloxacin in goats. *Res Vet Sci*. 2006;80:79–90.
17. Southwood LL. Practical guide to equine colic. Wiley-Blackwell; 2012.
18. Kankaanranta H, Moilanen E, Vapaatalo H. Comparison of in vitro effects of flunixin and tolafenamic acid on human leukocyte and platelet functions. *Inflammation*. 1993;17:417–25.
19. Lees P, McKellar QA, Foot R, Gettinby G. Pharmacodynamics and pharmacokinetics of tolafenamic acid in ruminating calves: evaluation in models of acute inflammation. *Vet J*. 1998;155:275–88.
20. Garg P, Sanguinetti MC. Structure-activity relationship of fenamates as Slo2.1 channel activators. *Mol Pharmacol*. 2012;82:795–802.
21. NRC. Guide for the care and use of laboratory animals. In: Service PH, editor. Institute for laboratory animal research. 7th ed. Washington (DC): National Academies Press; 1996.
22. Nagy A, Gertsenstein M, Vintersten K, Behringer R. Production of Transgenic Mice. In: Nagy A, Gertsenstein M, Vintersten K, Behringer R, editors. Manipulating the mouse embryo. A laboratory manual. 3rd ed. New York: Cold Spring Harbour Laboratory Press; 2003. pp. 289–358.
23. Nagy A, Gertsenstein M, Vintersten K, Behringer R. Summary of mouse development. In: Nagy A, Gertsenstein M, Vintersten K, Behringer R, editors. Manipulating the mouse embryo. A laboratory manual. 3rd ed. New York: Cold Spring Harbour Laboratory Press; 2003. pp. 31–137.
24. Flecknell PA. Anaesthesia of animals for biomedical research. *Br J Anaesth*. 1993;71:885–94.
25. Elli M, Gaffuri B, Frigerio A, Zanardelli M, Covini D, Candiani M, et al. Effect of a single dose of ibuprofen lysinate before embryo transfer on pregnancy rates in cows. *Reproduction*. 2001;121:151–4.
26. Pate JL, Toyokawa K, Walusimbi S, Brzezicka E. The interface of the immune and reproductive systems in the ovary: lessons learned from the corpus luteum of domestic animal models. *Am J Reprod Immunol*. 2010;64:275–86.
27. Rubinstein M, Marazzi A, Polak de Fried E. Low-dose aspirin treatment improves ovarian responsiveness, uterine and ovarian blood flow velocity, implantation, and pregnancy rates in patients undergoing in vitro fertilization: a prospective, randomized, double-blind placebo-controlled assay. *Fertil Steril*. 1999;71:825–9.
28. Waldenstrom U, Hellberg D, Nilsson S. Low-dose aspirin in a short regimen as standard treatment in in vitro fertilization: a randomized, prospective study. *Fertil Steril*. 2004;81:1560–4.
29. Moon HS, Park SH, Lee JO, Kim KS, Joo BS. Treatment with piroxicam before embryo transfer increases the pregnancy rate after in vitro fertilization and embryo transfer. *Fertil Steril*. 2004;82:816–20.