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Systemic Immunosuppression by Methylprednisolone and Pregnancy Rates in Goats Undergoing the Transfer of Cloned Embryos

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Contents
The presence of the zona pellucida has been perceived as a requirement for the oviductal transfer of cloned embryos at early stages of development while protecting the embryo from an immune system response. We hypothesized that steroid hormone therapy could reduce a potential cellular immune response after the transfer of zona-free cloned embryos into the oviduct of recipient female goats. In Experiment 1, seven does were used to study the systemic immunosuppressant effect of the methylprednisolone administration (for 3 days) on blood cell counts. Whole blood was collected prior to treatment with methyprednisolone and then on Days 1, 2, 3, 4, 7, 14, 21 and 28 after the first dose of methylprednisolone for the analysis of haematological parameters. Methylprednisolone treatment significantly reduced circulating white blood cells and neutrophils in comparison with pre-treatment levels, demonstrating a systemic immunosuppressant effect. In Experiment 2, a group of 58 does were used as recipient females to study the effect of administration of methylprednisolone for 3 days on the establishment of pregnancies after the transfer of zona-free cloned embryos into the oviducts. No effects on pregnancy rates on Day 30 were observed regarding the distinct treatment groups (control *vs.* methylprednisolone), the source of oocytes (*in vivo*- vs *in vitro*-matured) or the presence or absence of the zona pellucida in embryos. In summary, methylprednisolone was effective at inducing a systemic immunosuppressed state in goats, but the treatment prior to embryo transfer did not affect pregnancy rates. Moreover, pregnancy rates were similar between zona-free and zona-intact goat cloned embryos.

Introduction

Many factors can influence the outcome of somatic cell nuclear transfer (SCNT) procedures, which also include immune response and inflammation. A variety of inflammatory imbalances can increase the chance of pregnancy loss in animals (Hill and Chavatte-Palmer 2002) and humans (Turi et al. 2010). In addition, complex *in vitro* embryo manipulation procedures, such as cloning by SCNT, can reduce the number of successful pregnancies in livestock (Keefer et al. 2001; Ueno et al. 2007). Goats are a particularly physiologically more demanding species to use as a model as cloned goat embryos are typically surgically transferred after only 1 or 2 days in culture (Wan et al. 2012). Moreover, the zona pellucida (ZP) is often perforated (Willadsen 1986; Wilmut et al. 1997) or removed from the embryos (Booth et al. 2001; Nasr-Esfahani et al. 2011). Pelvic surgical procedures are known to illicit inflammation and cause adhesions, which can also lead to low fertility (Schindler 2004). This increased inflammation in conjunction with the removal or perforation of the ZP so early in development may leave the embryo more susceptible to the maternal immune system (Ueno et al. 2007). Nonetheless, the transfer of zona-broken cloned embryos into the uterine tube has been a common procedure for several mammalian species, including sheep (Willadsen 1986; Wilmut et al. 1997), goats (Baguisi et al. 1999; Keefer et al. 2001), pigs (Polejaeva et al. 2000; Xin et al. 2013), rabbit (Chesne et al. 2002) and mule (Woods et al. 2003).

The presence of the ZP has been perceived as a requirement for the oviductal transfer of cloned embryos at the early stages of development, as the ZP allows greater blastomere aggregation during successive early cleavages while protecting the embryo from an immune system response (Ueno et al. 2007; Fujiwara et al. 2009). A dogma that embryos would be attacked by the immune system if not surrounded by an intact ZP or by any protective nutrient-permissive barrier has not been clearly stated in the literature. Most procedures performed so far with early-stage goat embryos transferred to the oviduct took into consideration the presence of the ZP. In early times, cloned embryos were transiently *in vivo*-cultured into the oviduct of surrogate females after agar embedding during early cleavage stages, for subsequent retrieval and transfer of morula or blastocyst stage embryos into the uterus (Willadsen 1986; Lu et al. 1987; Wilmut et al. 1997). More recently, the transfer of zona-broken cloned embryos directly into the oviduct without any artificial protective layer has become a common and very successful practice (Baguisi et al. 1999; Polejaeva et al. 2000; Keefer et al. 2001; Reggio et al. 2001; Woods et al. 2003; Baldassarre et al. 2004; Xin et al. 2013), demonstrating that development into the oviduct is not impaired if the ZP is not intact.

An alternative technique to conventional cloning is the micromanipulation procedure without the ZP. The micromanipulation without the ZP is envisioned as procedurally easier than with ZP, enabling the production of a large number of embryos per procedure (Booth et al. 2001). This can be advantageous when only a rather small number of good quality oocytes are available, such as in the Brazilian semi-arid region. However, the loss of the ZP during the SCNT process can be a negative immune-related issue, as better results are obtained after embryo transfer (ET) into the oviduct of recipient does (Chavatte-Palmer et al. 2013). In spite

of this, we have already demonstrated the feasibility of transferring zona-free handmade cloned pig embryos into the oviducts of recipient females (Ohlweiler et al. 2009). Nonetheless, even conventional cloning may reduce the overall efficiency after ET, as Ueno et al. (2007) found a decrease in embryo yield and an increase in the number of degenerated embryos after the transfer of artificially unprotected zona-broken pig embryos as compared with zona-intact counterparts. Curiously, the authors described the presence of macrophages inside the ZP of zona-broken embryos after a few days of *in vivo* culture, which provides support for the cell immune response theory. Consequently, the transfer of zona-free embryos into the oviduct appears to be a procedure associated with low survival, unless a cell-mediated immune response can be minimized or blocked to circumvent the attack over zona-free early cleavage stage embryos.

The use of anti-inflammatory drugs could be an alternative to induce immune suppression in the surrogate female prior to the transfer of zona-free embryos, thus minimizing the cell-mediated immune response. Anti-inflammatory steroids such as prednisone can be used as anti-inflammatory and immunosuppressive drugs that may improve the intrauterine environment and increase implantation rates (Carp et al. 2012). Prednisone is a common anti-inflammatory drug used to treat inflammatory, autoimmune and allergic disorders, and when used prior to organ transplants, the drug has a 'lymphocidal' effect, that is, it aims to kill mature lymphocytes, for a quick response and prevention of rapid and acute rejection (De Bosscher and Haegeman 2009). Many T-cell subtypes have different sensitivities and responses to glucocorticoids. In pro-inflammatory T cells, glucocorticoids have an immunosuppressive effect, while such steroidal hormones stimulate regulatory T-cell (Treg) activity (Zen et al. 2011). Prednisone also has opposing effects on different subsets of neutrophils, sometimes reducing apoptosis (Schleimer 2004) and other times increasing phagocytosis by macrophages (Wigenstam et al. 2012). In women with autoimmune disease, Turi et al. (2010) demonstrated that prophylactic therapy with prednisone following induced ovulation was associated with a significantly higher pregnancy rate (33%) than placebo (8%). Up to this point, little has been done to investigate whether glucocorticoid anti-inflammatory therapy could increase reproductive success in agricultural species undergoing SCNT, particularly in procedures utilizing zona-free embryo reconstruction and surgical embryo transfer into the oviduct.

We hypothesize that steroid hormone therapy will reduce or block a potential cellular immune response after the transfer of zona-free early cleavage stage cloned embryos into the oviduct of surrogate recipient females, mimicking the systemic immunosuppression-induced state attained prior to organ transplants, resulting in pregnancy rates with zona-free embryos at least at the same rate as with zona-intact controls. Basically, the corticoid treatment, at the dose and rate of administration, should be 'lymphocidal', destroying mature circulating lymphocytes, thus reducing or blocking the rejection of the allograft (i.e. zona-free embryos). Thus, the aims of this study were to assess the effects of short-term methylprednisolone (a prednisone derivative) treatment on circulating immune cell populations and to assess pregnancy rates after the transfer of zona-free 1-cell stage embryos into the oviducts of synchronous recipient goats treated with methylprednisolone.

Materials and Methods

All chemicals and reagents were from Sigma-Aldrich Co. (St. Louis, USA), unless stated otherwise.

Animals

Two groups of pubertal adult female crossbred goats were used in two experiments, as follows: (i) Experiment 1: a group of seven does were used for the study on the systemic immunosuppressant effect of the methylprednisolone administration and on blood cell count profile for a period of four weeks, and (ii) Experiment 2: a group of 58 does were used as recipient females for the study of the effect of methylprednisolone on the establishment of pregnancies using zona-free cloned goat embryos.

Methylprednisolone treatment and blood collection

Adult pubertal does were treated with daily 500mg of methylprednisolone (MPD) doses (Solu-Medrol®, Novaplus, LLC, Brazil) given IM for three consecutive days. All animals were cared for in conditions approved by the University of Fortaleza animal use ethics committee and were observed and examined by experienced veterinarians a minimum of once a day throughout the study.

Experiment 1

Whole blood was collected from seven does in EDTA tubes from the jugular vein prior to the administration of methylprednisolone (Day 0) and on Days 1, 2, 3, 4, 7, 14, 21 and 28 after the onset of the methylprednisolone administration, as illustrated in Fig. 1. Blood samples were analysed on a BC-2800 Auto Hematology Analyzer (Mindray, Shenzhen, China) along with manual counts of blood smears. A total of 19 haematological parameters were investigated: relative and absolute values for monocytes, lymphocytes, eosinophils, basophils, segmented neutrophils and rods, and white blood cells (WBC), red blood cells (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC).

Experiment 2

Fifty-eight female recipients were segregated into four groups: (UN-ZI) MPD-untreated does receiving zona-intact embryos (n = 27), (b) (UN-ZF) MPD-untreated does receiving zona-free embryos (n = 11), (MPD-ZI) MPD-treated does receiving zona-intact embryos (n = 6) and (MPD-ZF) MPD-treated does receiving zona-free embryos (n = 14). The MPD treatment

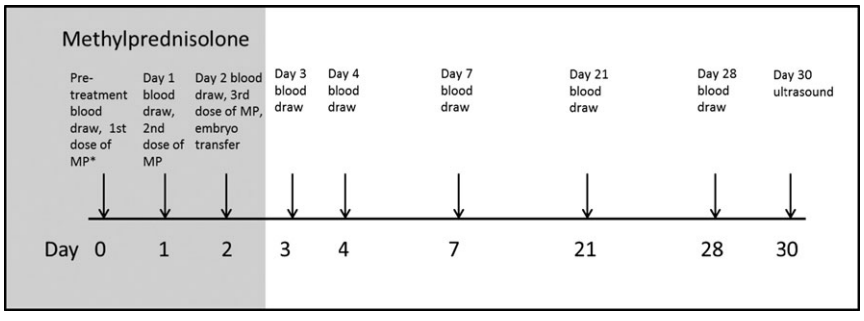

Fig. 1. Timeline for the administration of the methylprednisolone doses, blood drawings, embryo transfer and ultrasound examination for pregnancy diagnosis. *MP indicates methylprednisolone

consisted on the administration of 500 mg methylprednisolone intravenously to the female recipients for 3 days, starting 2 days prior to the transfer of cloned goat zona-free or zona-intact embryos.

Goat cloning by somatic cell nuclear transfer (SCNT)

Goat nucleus donor cells

The cells used during the SCNT procedures were isolated from goats transgenic for the human lysozyme gene (hLZ, University of California at Davis, CA, USA; Maga et al. 2006). Briefly, mesenchymal stem cells (MSCs) were isolated from the bone marrow of a female neonate, foetal fibroblasts (FF) were isolated from a female foetus at 40 days of gestation, and adult fibroblasts (FA) were isolated from the auricular cartilage upon a biopsy from a puberal female. After *in vitro* expansion, cells were frozen and stored in liquid nitrogen according to Gerger et al. (2010). Cells were imported to Brazil in the cryopreserved form, upon the approval by the Brazilian National Biosafety Committee (CTNBio). Cells were thawed 48–120 h prior to their use for cloning procedures. On average, MSCs were used at 70% confluence and passage 4, FF cells at 80% confluence and passage 4 and FA cells at 95% confluence and passage 3.

Collection and preparation of oocytes

Both *in vivo*- and *in vitro*-matured oocytes were collected and processed according to Reggio et al. (2001), with minor modifications. Following *in vivo* collection and processing from FSH-stimulated females or after *in vitro* maturation for 22 h from non-stimulated slaughterhouse females, cumulus cells were removed and oocytes were selected for the presence of the first polar body (PB).

Enucleation and nuclear transfer

Matured oocytes were segregated in zona-intact (ZI) and zona-free (ZF) oocytes for enucleation and embryo reconstruction, following procedures by Keefer et al. (2002) and Booth et al. (2001), respectively, with minor changes. In the ZF group, and prior to enucleation, oocytes were briefly exposed to 0.5% protease in TCM-HEPES + 0.01% PVA for ZP removal and rinsed in TCM-HEPES + 30% FCS. Both ZI and ZF oocytes were incubated for 15 min in TCM-199 supplemented with 5.0 μg/ml cytochalasin B (C6762) and 5.0 μg/ml Hoechst 33342 (B2883), followed by enucleation by micromanipulation. Then, reconstruction in the ZF group was carried out by adhering enucleated zona-free cytoplasts, quickly exposed to 500 μg/ml phytohaemoagglutinin in TCM-HEPES, to one donor goat cell, whereas in the ZI group, a single donor goat cell was transferred into the perivitelline space of each enucleated oocyte, by means of micromanipulation, in 5 μg/ml cytochalasin B (C6762) in TCM-HEPES medium.

Electrofusion

Cell–cytoplast couplets were fused immediately after embryo reconstruction. Couplets were manually aligned between electrodes into a 320-μm gap fusion chamber (BTX 453, BTX Instruments, Genetronics, San Diego, CA, USA). For membrane fusion in the ZI group, two fusion pulses were given at 2.0 kV/cm for 20 μs, whereas in the ZF group, two 1.0-kV/cm DC pulses were used for 20 μs (BTX Electro Cell Manipulator 200, Biotechnologies & Experimental Research Inc., San Diego, CA, USA). Fusion assessment was performed 30–60 min after fusion. Non-fused structures were subjected to re-fusion pulses as described previously.

Embryo activation

Two hours after fusion, cloned embryos were exposed for 5 min to a solution containing 5 μM ionomycin (I0634), followed by a 4-h incubation in 2 mM 6-DMAP (D2629) in M199 medium, at 38.5°C and 5% CO_2. Then, activated cloned embryos were *in vitro*-cultured for 18 h prior to embryo transfer in mSOFaa medium (Holm et al. 1999) supplemented with 10% FBS and 1% ITS, at 38.5°C, 5% CO_2, 5% O_2 and 90% N_2.

Synchronization of female recipients

For oestrous cycle synchronization, female recipients received a progesterone insert (Eazi-Breed CIDR®, Laboratórios Pfizer Ltda., Brazil) on Day 0. On Day 10, the progesterone insert was removed and 0.075 mg d-cloprostenol (Prolise, ARSA S.R.L., Argentina) and 200 IU eCG (Folligon, MSD Saúde Animal, Brazil) were given IM. A dose of 0.025 mg gonadorelin acetate (Gestran®, ARSA S.R.L., Argentina) was given IM 36 h after. Embryo transfer was performed on Day 14 from the onset of the synchronization protocol, on Day 1 of embryo development (Day 0 = embryo activation).

Embryo transfer (ET)

Between 12 and 15 (13.5 ± 0.4) zona-free or zona-intact embryos were surgically transferred to the oviduct ipsilateral to the ovulation-bearing ovary of each recipient doe, in four recipient groups: (i) zona-intact (UN-ZI) or (ii) zona-free embryos (UN-ZF), both groups transferred to untreated females, and (iii) zona-intact (MPD-ZI) or (iv) zona-free (MPD-ZF) embryos, both groups transferred to methylprednisolone-treated recipients. On the 4th day after embryo transfer, a progesterone insert (Eazi-Breed CIDR®, Laboratórios Pfizer Ltda., Brazil) was inserted intravaginally in each recipient and remained in until pregnancy diagnosis, performed on Day 30 by means of rectal ultrasonography. Visualization of the embryo, amniotic vesicle and presence of a heartbeat were used as positive indicators of pregnancy.

Data analysis

Data analyses were performed using the SAS statistical software (SAS, Cary, NC, USA). For the haematological data, the Tukey's test was used to determine p-values and standard errors comparing each distinct time point to the pre-treatment values. For pregnancy data, the chi-square or the Fisher's test was used to compare the pregnancy outcomes between prednisone-treated and untreated groups, and the presence or absence of the ZP in embryos (ZI *vs*. ZF embryos), also comparing the source of oocytes (*in vivo*- vs *in vitro*-matured).

Results

Haematology

A total of six (WBC, segmented neutrophils, RBC, HGB, HTC and MCHC) of the 19 haematological parameters investigated from the seven methylprednisolone-treated goats from Experiment 1 were significantly different from the pre-treatment at one or more of the time points (Figs 2 and 3), demonstrating a systemic immunosuppressant effect of the steroid hormone treatment on goat blood cell values. The total number of WBC was significantly reduced ($p < 0.05$) from the pre-treatment control amount on Days 3, 4, 7,

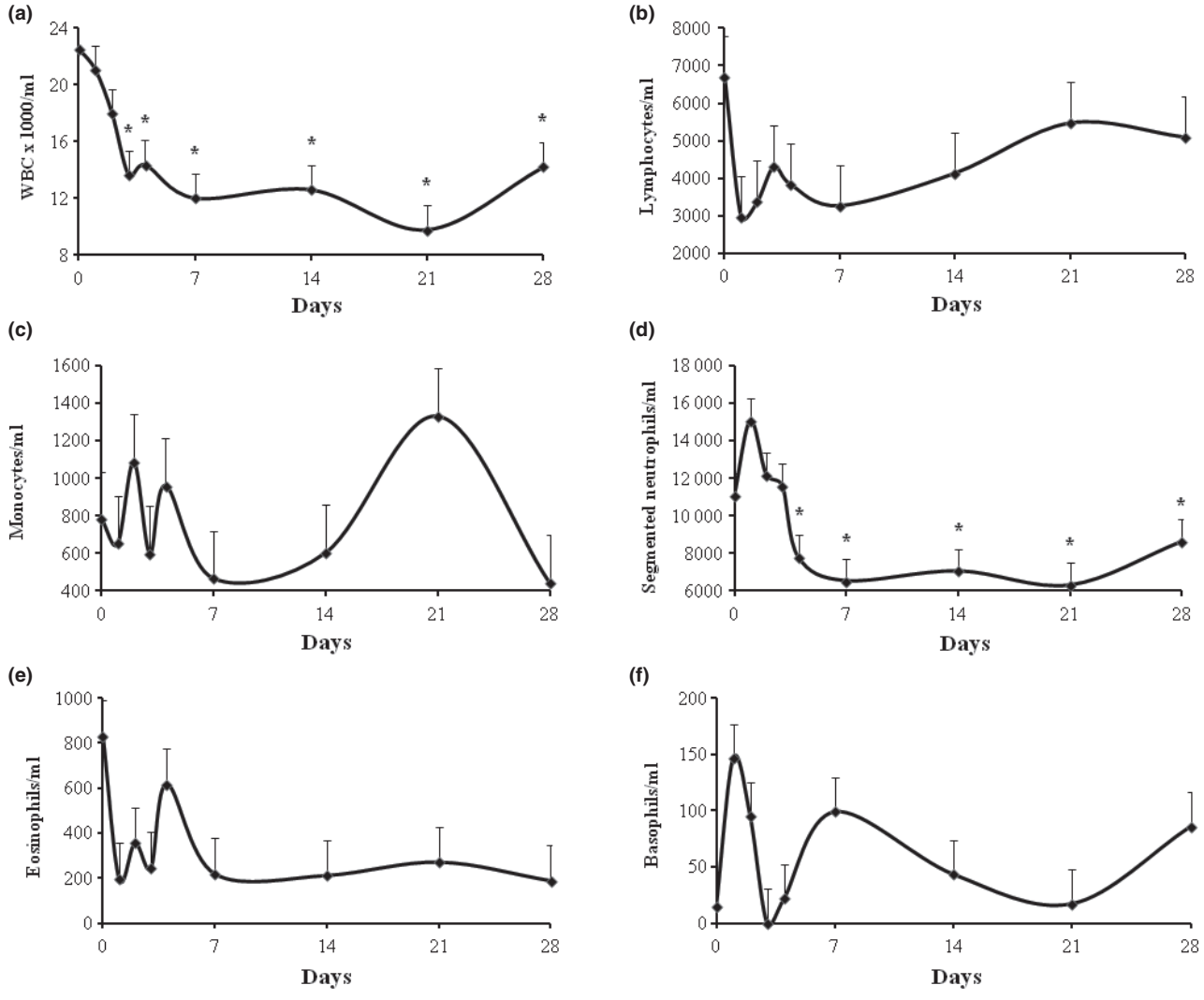

Fig. 2. Total circulating (a) white blood cells (WBC x 1000/ml), (b) lymphocytes/ml, (c) monocytes/ml, (d) segmented neutrophils/ml, (e) eosinophils/ml and (f) basophils/ml in the blood of methylprednisolone-treated goats. The reduction in total white blood cells can be mostly attributed to a significant decrease in circulating segmented neutrophils. All value presented as mean ± SEM. *Significantly different ($p < 0.05$) relative to pre-treatment control values ($t = 0$ Days)

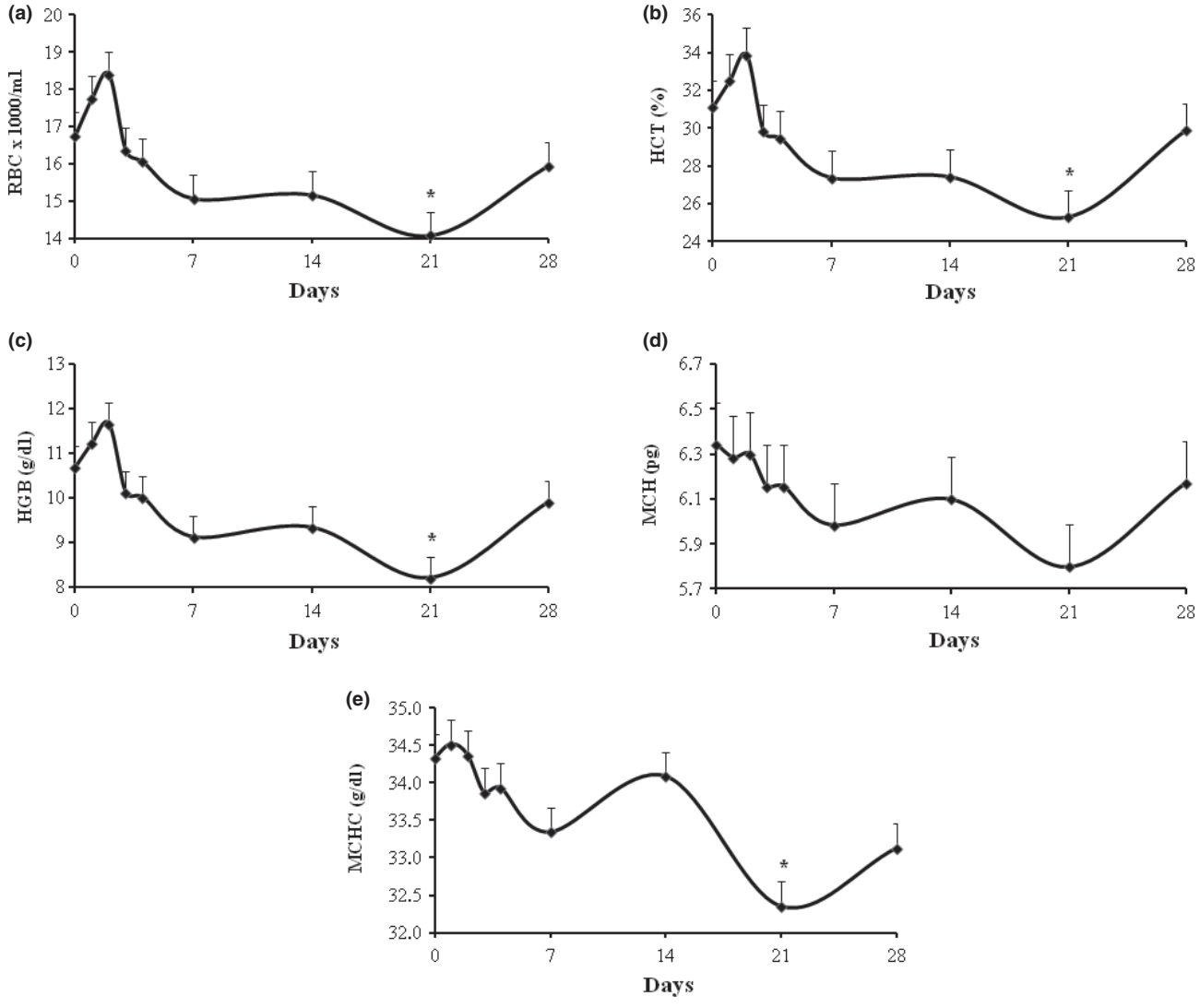

Fig. 3. (a) Total red blood cells (RBC x 1000/ml), (b) haematocrit (HCT%), (c) haemoglobin concentration (HGB, g/dl), (d) mean cell haemoglobin (MCH, pg) and (e) mean cell haemoglobin concentration (MCHC, g/dl) in the blood of methylprednisolone-treated goats. All value presented as mean ± SEM. *Significantly different ($p < 0.05$) relative to pre-treatment control values ($t = 0$ Days)

14, 21 and 28. Numbers of total circulating segmented neutrophils were also significantly reduced on Days 4, 7, 14, 21 and 28 (Fig. 2). On Day 21, total RBC, HGB, HTC and MCHC were also significantly reduced ($p < 0.05$) when compared with the pre-treatment levels (Fig. 3).

Pregnancy rates

Based on the visualization of the embryo proper and heartbeat on Day 30 of pregnancy, no effects were observed between experimental subgroups regarding the distinct treatment groups (control vs methylprednisolone), the source of oocytes (*in vivo*- vs *in vitro*-matured) or the presence or absence of the ZP in embryos (ZI *vs.* ZF embryos), as depicted in Tables 1 and 2. Collectively, neither oocyte nor donor cell types had any detectable effect on the results observed in this study, after using distinct data analysis approaches. A pregnancy rate of 80.0% and 76.3% was observed in goats treated with methylprednisolone and control goats, respectively, which was not statistically significant. When separated by group (Tables 1 and 2), results were also not statistically significant, with only 2 and 3 animals from the methylprednisolone-treated and the control goats, respectively, having pregnancies with visible heartbeats. All pregnancies were lost before or at the late embryonic phase (prior to Day 45 of pregnancy).

Discussion

his study was part of a wider programme for the production of transgenic animal models by cloning procedures in our laboratory. For such reason, we designed studies to produce transgenic goats seeking procedures that would improve the overall cloning efficiency. Specifically in this study, our focus was on the effect of the absence of the zona pellucida on pregnancy outcome after the transfer of zona-free cloned goat embryos into the oviduct of synchronous recipients and the potential beneficial effects of the use of an methylprednisolone on pregnancy rates and circulating

Table 1. Pregnancy rate between groups of recipients subjected or not to methylprednisolone treatment (MPD-untreated *vs*. MPD-treated recipients), considering the presence or removal (ZI vs ZF) of the zona pellucida and oocyte source (*in vivo*- vs *in vitro-matured*)

Experimental group	Zona pellucida	Oocyte source	Embryos n	Recipients n	Pregnancy (Day 30)*			
					Embryo proper		Heartbeat	
					n	%	n	%
MPD-Untreated	ZI	*In vivo*	84	6	4	66.7	0	0
		In vitro	280	21	17	81.0	2	9.5
	ZF	*In vivo*	79	6	5	83.3	0	0
		In vitro	69	5	3	60.0	1	20.0
MPD-treated	ZI	*In vivo*	26	2	1	50.0	1	50.0
		In vitro	55	4	4	100.0	0	0
	ZF	*In vivo*	52	4	4	100.0	0	0
		In vitro	137	10	7	70.0	1	10.0

MPD - methylprednisolone group; ZI - zona-intact embryos; ZF - zona-free embryos.
*Pregnancy rates denote the number of recipient females detected pregnant by ultrasonography on Day 30, by the visualization of either an embryo proper or a heartbeat, based on the total number of recipients (column *Recipients*) that received cloned embryos (column *Embryos*).

Table 2. Pregnancy rate by subgroups of recipients subjected or not to methylprednisolone treatment (MPD-untreated vs MPD-treated recipients), considering the presence or removal (ZI vs ZF) of the zona pellucida and the oocyte source (*in vivo*- vs *in vitro-matured*)

Experimental subgroups		Embryos n	Recipients n	Pregnancy (Day 30)*			
				Embryo proper		Heartbeat	
				n	%	n	%
Zona pellucida	ZI	445	33	26	78.8	3	9.1
	ZF	337	25	19	76.0	2	8.0
Oocyte source	*In vivo*	241	18	14	77.8	1	5.6
	In vitro	541	40	31	77.5	4	10.0
Treatment group	MPD-Untreated	512	38	29	76.3	3	7.9
	MPD-treated	270	20	16	80.0	2	10.0
Total		782	58	45	77.6	5	8.6

ZI - zona-intact embryos; ZF - zona-free embryos; MPD - methylprednisolone.
*Pregnancy rates denote the number of recipient females detected pregnant by ultrasonography on Day 30, by the visualization of either an embryo proper and/or a heartbeat, based on the total number of recipients (column *Recipients*) that received cloned embryos (column *Embryos*).

leucocytes in goats undergoing the transfer of zona-intact and zona-free cloned embryos. This study provided novel concepts, based on our working hypotheses, that pregnancies can be established in goats after the transfer of zona-free embryos into the oviducts in early development and that immunosuppressant agents may be beneficial as a means to improve chances for *in vivo* development of zona-free cloned embryos after transfer to the oviduct of female recipients. As much as the latter idea did not show clear positive effects (nor any detectable negative effects as well) in this study, the former demonstrated that pregnancies can indeed be established under the model proposed, irrespective of the immunosuppressant treatment. We consider that such results *per se* are of biological significance, as historically (and anecdotally), zona-free embryos could not develop into the oviduct in early development, likely due to the immune response from the dam.

Pregnancy presents a unique challenge for the immune system as conceptus growth and development depends on the immunological lack of response to non-self-antigens that are present on the conceptus (Carp et al. 2012; Chen et al. 2012). Within the uterus, the trophoblast has an immune-privileged status that allows it to develop in such close proximity to the maternal immune system (Carp et al. 2012). During pregnancy, there are many alterations made to immune cells to provide this immune-privileged area, including M2 polarization of macrophages, progesterone stimulating a Th2 response, proliferation of Tregs in the uterus and chemokine decoy receptors (Chen et al. 2012). These changes decrease inflammatory cytokines, repress allogenic responses, predispose the immune system to anti-inflammatory actions and overall help maintain the immune suppressive environment (Chen et al. 2012). When this immune suppressive environment is disrupted, it can lead to pregnancy complications and pregnancy loss (Carp et al. 2012).

Methylprednisolone is a glucocorticoid commonly used to treat inflammatory disorders and in organ transplants. In women with autoimmune and inflammatory diseases, clinical trials have demonstrated the efficacy of glucocorticoid treatment to increase pregnancy rates (Turi et al. 2010). In agricultural animals, inflammation can also reduce fertility; therefore, glucocorticoids could improve pregnancy rates in livestock species as well. In this study, goats treated with methylprednisolone had reduced circulating white blood

cell, indication of a systemic immunosuppressive effect during the 4-week period after the methylprednisolone protocol. However, pregnancy rates were not different in goats treated with methylprednisolone prior to embryo transfer of reconstructed embryos with or without ZP, also being similar for ZF or ZI embryos, regardless of the steroid hormone treatment.

While the main targets for methylprednisolone in circulation are white blood cells, in this study, we found that methylprednisolone treatment also affected parameters associated with the erythrocytes, significantly reducing RBC, HGB, HTC and MCHC 21 days after the first dose compared with pre-treatment values. Nevertheless, all four parameters were still well within the reported reference interval for goats (Casas-Díaz et al. 2008). Thus, it is unclear whether these changes had any biological significance for the treated group of animals.

The short-term treatment with methylprednisolone also had significant and lasting effects on the population of circulating leucocytes. By Day 3 of treatment, the total white blood cell count was significantly reduced from the pre-treatment level and this reduction persisted until Day 28. The main subset of leucocytes affected was the class of circulating segmented neutrophils, which were significantly reduced from pre-treatment levels on Day 4, persisting lower until Day 28. This is a very interesting result because a previous report has shown that glucocorticoids actually protected neutrophils from apoptosis in humans (Schleimer 2004). However, recent studies have shown that in certain murine models, glucocorticoids reduce neutrophils (Roumestan et al. 2007; Wigenstam et al. 2012). It has been demonstrated in healthy humans and horses that treatment with glucocorticoids (prednisolone or dexamethasone) has an inhibitory effect on pro-inflammatory gene expression in neutrophils, and this effect is very similar to that seen in other leucocytes (Hirsch et al. 2012).

In this study, goats had an average of 21.8×10^3/ml and 14.1×10^3/ml white blood cells and total segmented neutrophils, respectively, at the start of methylprednisolone treatment. The previously reported reference interval for goat white blood cells and neutrophils are between 8.7 to 21.2×10^3 and 3.2 to 9.8×10^3 cells/mm^3 (Casas-Díaz et al. 2008), respectively, indicating that, on average, the goats from this study had slightly increased white blood cell counts. All the other leucocytes were within normal reference interval ranges before treatment with methylprednisolone. This indicates that the goats under the methylprednisolone treatment were experiencing neutrophilia at the onset of the study and that treatment with methylprednisolone reduced neutrophil counts. Neutrophilia can be caused by a number of diseases; however, it is possible that such animals had subclinical bacterial infections, as proposed by others (Higino et al. 2012). Interestingly, animals with subclinical infections also experience increased inflammation that can reduce fertility (Schrick et al. 2001; Higino et al. 2012).

The rather high pregnancy rates observed in this study with Day 1 cloned embryos (45/58, 77.6%) were similar to results obtained in parallel experiments in our laboratory (unpublished data) using *in vivo*-produced goat embryos (7/9, 77.7%), for which, most pregnancies went to term (83.3%). Nonetheless, despite the similar high pregnancy rates, conceptus viability was low for cloned embryos in this study. Even though embryonic vesicles were visualized from Day 23 after ET in each of the experimental groups, visible heartbeats were not observed in most cases. This ultrasound pattern suggests that in some cases, these vesicles were likely formed only by trophoblast tissue (moles), as typical embryonic structures common to this period of gestation were not observed. These embryonic structures remained *in utero* often until Day 50 of gestation, when the progesterone implant was withdrawn. Then, after a few days, the embryonic structures were no longer detected. These findings corroborate with those found by Baguisi et al. (1999) and Zhang et al. (2010). Baguisi et al. (1999) found that more than 2/3 of the pregnancies from clones were not viable. According to the authors, these embryonic structures were visualized in the uterus until Day 55 of gestation. In our experiment, out of the 45 embryonic structures visualized, only five resulted in positive detection of an embryonic heartbeat. Several factors may contribute to these findings, as for instance, faulty placentation or failure in the activation of the embryonic genome (Baguisi et al. 1999).

In addition to potential failures, the high pregnancy rates observed in this study may also be related to the use of the progesterone insert on the 4th day after ET, which may have prevented return to natural oestrus, rescuing less viable embryos that nonetheless would not have been able to trigger the maternal recognition of pregnancy, an event already hypothesized by Bertolini et al. (2002a, 2002b) for *in vitro*-derived bovine embryos. In fact, an unpublished pilot study using progesterone supplementation (intravaginal inserts) on Day 4 after artificial insemination of goats as a means to increase pregnancy rate was performed by our group, resulting in increased pregnancy outcome in the progesterone-treated group (8/10; 80%) when compared with controls (5/12; 42%). Such pilot study indicated the potential benefit of the use of progesterone treatment to improve fertility in goats, but in this study, as *in vitro*-manipulated embryos have been shown to be smaller than normal since early stages (Bertolini et al. 2002b; Martin et al. 2007), it is quite possible that the rescuing of less viable embryos may have occurred (Bertolini et al. 2002a,b), resulting in pregnancy rates twice as high as reported in the literature for cloned goat embryos (Chavatte-Palmer et al. 2013).

One disadvantage of the ZF procedure is the fact that the embryos are devoid of ZP, which theoretically would prevent its transfer to the oviduct during the initial stages (D1–D2) of development. However, no differences were observed in pregnancy rates between groups for embryos with and without ZP, regardless of the hormone treatment or the source of oocytes used for cloning. More studies should be conducted as live births produced by the ZF technique were originated from embryos cultured *in vitro* and transferred in advanced stages of development into the uterus. The birth of an animal from a ZF embryo transferred into the oviduct may overturn a dogma in reproductive biology, as there is a virtual consensus that the ZP is essential for *in vivo*

survival in the early days of development. However, pregnancies were obtained with zona-free embryos after the transfer into the oviduct, which raises questions regarding the need for an immune suppression, especially with regard to the proposed rejection response by the recipient′s immune system in early embryo development (Fujiwara et al. 2009).

In summary, results from this study demonstrated that methylprednisolone was effective at inducing a systemic immunosuppressed state in treated goats for a period of up to 4 weeks. However, the methylprednisolone treatment prior to embryo transfer did not improve or worsen pregnancy rates on Day 30 compared with non-treated control goats. Moreover, pregnancy rates with ZF goat cloned embryos was similar to results obtained with zona-broken goat cloned embryos. Further studies are needed to investigate the effects of the zona removal on pregnancy rates when ZI embryos are used as controls.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

Cristiano Feltrin was involved in embryo production, cell culture, embryo transfer, ultrasound and analysed data; Caitlin A. Cooper drafted paper and analysed data; Nuradilla Mohamad-Fauzi contributed to cell culture and freezing; Victor Hugo V. Rodrigues contributed to blood collection and blood analysis; Luiz Henrique de Aguiar was involved in embryo production, cell culture and embryo transfer; Saul Gaudencio Neto was involved in embryo production, cell culture and embryo transfer; Leonardo Tondello Martins contributed to embryo production, cell culture and embryo transfer; Carlos Enrique Méndez Calderón was involved in embryo transfer and ultrasound; Arthus S. Morais contributed to cell culture and blood collection; Tales M. de Almeida was involved in blood analysis; Isaac N. G. da Silva contributed to blood analysis; José Luiz Rodrigues designed study and drafted paper; Elizabeth A. Maga designed study and drafted paper; James D. Murray designed study and drafted paper; Alexandre B. Libório designed study Luciana Relly Bertolini designed study and drafted paper Marcelo Bertolini designed study, drafted paper, embryo transfer, ultrasound and analysed data.

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