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Effects of flunixin meglumine, recombinant bovine somatotropin and/or human chorionic gonadotropin on pregnancy rates in Nelore cows

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Abstract

The objective was to compare pharmacological strategies aiming to inhibit prostaglandin F2 alpha ($PGF_{2\alpha}$) synthesis (flunixin meglumine; FM), stimulate growth of the conceptus (recombinant bovine somatotropin; bST) and progesterone (P_4) synthesis (human chorionic gonadotropin; hCG), as well as their combinations, regarding their ability to improve pregnancy rates in beef cattle. Lactating Nelore cows ($N = 975$), 35 to 70 days postpartum, were synchronized and inseminated by timed artificial insemination (TAI) on Day 0. On Day 7, cattle were allocated into eight groups and received one of the following treatments: saline (S) on Days 7 and 16 (Group Control); S on Day 7 and FM on Day 16 (Group FM); bST on Day 7 and S on Day 16 (Group bST); bST on Day 7 and FM on Day 16 (Group bST + FM); hCG on Day 7 and S on Day 16 (Group hCG); hCG on Day 7 and FM on Day 16 (Group hCG + FM); bST and hCG on Day 7 and S on Day 16 (Group bST + hCG), or bST and hCG on Day 7 and FM on Day 16 (Group bST + hCG + FM). The aforementioned treatments were administered at the following doses: 2.2 mg/kg FM (Banamine®; Intervet Schering-Plough, Cotia, SP, Brazil), 500 mg bST (Boostin®; Intervet Schering-Plough), and 2500 IU hCG (Chorulon®; Intervet Schering-Plough). Pregnancy diagnosis was performed 40 days after TAI by transrectal ultrasonography. Pregnancy rates were not significantly different among treatments. However, there was a main effect of hCG treatment to increase pregnancy rates (63.0 vs. 55.4%; $P = 0.001$). Concentrations of P_4 did not differ significantly among groups on Day 7 or on Day 16. However, consistent with the higher pregnancy rates, hCG increased P_4 concentrations on Day 16 (10.6 vs. 9.6 ng/mL, respectively; $P = 0.05$). We concluded that hCG treatment 7 days after TAI improved pregnancy rates of lactating Nelore cows, possibly via a mechanism leading to induction of higher P_4 concentrations, or by reducing the luteolytic stimulus during maternal recognition of pregnancy.

Keywords: Luteolysis; Embryo development; Conceptus; Prostaglandin; Progesterone

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1. Introduction

In cattle, fertilization rates can reach 100% [1–3] whereas the rates of live births after a single insemination are only 53% [4]. There are reports indicating that the majority of the reproductive losses during gestation

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occur between Days 8 and 16 of pregnancy [2,5]. During maternal recognition of pregnancy, at approximately Day 16 in cattle, the conceptus secretes molecules that signal to the endometrium to block prostaglandin F2 alpha ($PGF_{2\alpha}$) synthesis, preventing luteolysis [6,7]. Therefore, pharmacological strategies aiming to inhibit the synthesis of $PGF_{2\alpha}$ are used to reduce embryonic mortality.

According to the cellular model for $PGF_{2\alpha}$ biosynthesis proposed by Burns et al. [8], free arachidonic acid (AA) is converted to prostaglandin H_2 by the cycloxygenase 2 enzyme (COX-2). Nonsteroidal anti-inflammatory drugs, such as flunixin meglumine (FM), inhibit $PGF_{2\alpha}$ synthesis by inhibiting the COX-2 enzyme, and have positive effects on pregnancy rates when administered between Days 14 and 17 post-AI [9,10]. Other studies, however, reported no effect of FM on pregnancy rates [11].

There is a positive correlation between the size of the conceptus and the amount of interferon (IFN)-τ, a potent $PGF_{2\alpha}$ synthesis inhibitor, secreted during maternal recognition of pregnancy [12]. Consistently, smaller conceptuses secreted less IFN-τ and were associated with higher rates of pregnancy loss. Recombinant bovine somatotropin (bST) has been associated with faster conceptus growth and increased pregnancy rates [13–16]. However other studies have observed no effect of bST on the aforementioned end points [17–19].

Human chorionic gonadotropin (hCG) has LH-like activity, and when given to cattle between 4 and 7 days post-estrus, induces formation of an accessory CL, thereby increasing progesterone (P_4) concentrations [20]. Numerous studies reported the positive effect of higher systemic P_4 concentrations on early embryonic development in cattle [21–24]. Cows with higher endogenous P_4 had an increased rate of embryonic growth and consequently greater IFN-τ production [25]. There are reports of higher pregnancy rates in cattle treated with hCG [26,27], whereas other studies detected an increase in systemic P_4 concentrations, but no effect on pregnancy rates [28,29].

There are limited data regarding the benefits of the aforementioned strategies in beef cattle, particularly in Nelore (*Bos taurus indicus*). Moreover, no assessment of the effects of FM, bST, and hCG, and their combinations, on pregnancy rates has been conducted in a single study with contemporaneous treatments and a large sample size. Therefore, the overall aim of this study was to test the effects of FM, bST, and hCG on plasma P_4 concentrations on Days 7 and 16 after timed artificial insemination (TAI) and pregnancy rates of Nelore cows on Day 40 post-TAI. Our hypothesis was that treatment with FM, bST, and/or hCG leads to higher pregnancy rates, potentially by directly (hCG) or indirectly (FM and bST) stimulating and/or maintaining P_4 production.

2. Materials and methods

2.1. Location, animal facilities and animal management

The experiment was performed on two farms. One farm was located in São Félix do Araguaia, in the state of Mato Grosso (latitude 11°37’02’’ S, longitude 50°40’10’’ W, altitude 195 m), whereas the other farm was located in Andradina, in the state of São Paulo (latitude 20°53’ S, longitude 51°22’ W, altitude 387 m). Nine hundred seventy-five Nelore cattle (*Bos taurus indicus*) were used (244 heifers and 731 cows). These cattle were between 35 and 70 days postpartum on the day they received the intravaginal P_4 device, with an average body condition score (BCS) of 2.8 ± 0.2 (range, 1 to 5). Females were kept on pasture composed of Brachiaria brizantha cv. Marandu, Panicum maximum cv. Tanzânia and Panicum maximum Jacq cv. Colonião with mineral supplementation and water ad libitum. Animal handling protocols were approved by the Ethics and Animal Experimentation Committee from UNESP – Dracena, São Paulo, Brazil (Process # 002/2008).

2.2. Timed artificial insemination (TAI)

Estrus synchronization was started by inserting an intravaginal device containing 1 g of P_4 (DIB®; Intervet Schering-Plough, Cotia, SP, Brazil), along with an im injection of 2 mg of estradiol benzoate (Gonadiol®; Intervet Schering-Plough). The day the P_4 device was inserted was considered Day –10. On Day –2, devices were removed and animals received an im injection of 112.5 μg of D-cloprostenol (Preloban®; Intervet Schering-Plough), an im injection of 300 IU of eCG (Folligon®; Intervet Schering-Plough), and 1 mg of estradiol cypionate (ECP®; Pfizer, Chácara Santo Antônio, SP, Brazil). Forty-eight hours after the last injection, cattle were bred by TAI (Day 0). One of the farms used semen from two Simmental and two Nelore bulls, whereas the other farm used semen from 15 Nelore bulls. Bulls were distributed homogeneously among experimental groups and each farm used their own technicians to perform TAI.

2.3. Experimental design

After TAI, females (N = 975) were allocated into eight groups. Postpartum status, BCS and semen source were homogeneously represented among experimental groups. Starting on Day 7, each group received one of the following treatments: saline (S) on Day 7 and S on Day 16 (Control Group; N = 124); S on Day 7 and FM on Day 16 (Group FM; N = 122); bST on Day 7 and S on Day 16 (Group bST; N = 119); bST on Day 7 and FM on Day 16 (Group bST + FM; N = 121); hCG on Day 7 and S on Day 16 (Group hCG; N = 124); hCG on Day 7 and FM on Day 16 (Group hCG + FM; N = 124); bST and hCG on Day 7 and S on Day 16 (Group bST + hCG; N = 120), or bST and hCG on Day 7 and FM on Day 16 (Group bST + hCG + FM; N = 121). Saline (2.5 mL), hCG (Chorulon®; Intervet Schering-Plough; 2500 IU), and FM (Banamine®; Intervet Schering-Plough; 2.2 mg/kg) were given as im injections. Recombinant bovine somatotropin (Boostin®; Intervet Schering-Plough; 500 mg/animal) was given as a sc injection. On Days 7 and 16 after TAI, approximately 8 mL of blood were collected from the coccygeal vessels into vials containing 175 μL of 30% sodium citrate. Blood was centrifuged at 1000 X g for 30 min at 4 °C. After centrifugation, plasma was stored at –20 °C. Due to the large number of cattle used in the study and high costs associated with hormone assays, 44% of animals in each group were selected randomly and plasma P_4 concentrations were measured by RIA (Count-a-Count®, DPC, Diagnostic Products Corporation; Los Angeles, CA, USA) on blood samples collected from these animals on both Days 7 and 16. Progesterone determinations were obtained in three assays. To determine intra- and interassay variability, high P_4 reference samples (HPR) and low P_4 reference samples (LPR) were used. The HPR reference samples had 1.6%, 1.6%, and 1.4% intra-assay CV, for assays 1, 2, and 3, respectively, whereas LPR reference samples had intra-assay CV of 0.1%, 0.05%, and 0.03%, for assays 1, 2, and 3, respectively. Interassay CV was 5.3% for HPR and 3.0% for LPR. Assay sensitivity for assays 1, 2, and 3 were 0.005, 0.005, and 0.008 ng/mL, respectively. Pregnancy was diagnosed by transrectal ultrasonography 40 days after TAI (Aloka Ultrasound Diagnostic Equipment, Model SSD-500, Tokyo, Japan with a 5 MHz, linear-array transducer).

2.4. Statistical analysis

The general assumption for binary variables such as pregnancy rate on Day 40 after TAI is that they have a Bernoulli distribution, which is a particular case of general linear models. Supposing that Yi, $i = 1, 2. . .$, in which i represents one of the measured variables, $Yi \sim$ Bernouille (¶i), and ¶ is the probability of success (pregnancy probability), the probability function of Yi, is calculated by:

$$P\left(Yi = yi\right) = \P yii\left(1 - \P i\right)1 - yi,\ yi = 0, 1$$

Pregnancy rate at Day 40 after TAI was analyzed by logistic regression using the GLIMMIX procedure from SAS (Version 9.1 of the SAS System for Windows, SAS Institute Inc., Cary, NC, USA). The independent variables initially included on the statistical model were the effects of farm, BCS, FM, bST, hCG, and their interactions. To obtain the final statistical model, the explanatory variables were sequentially removed based on the statistical criterion of Wald, using a P value cut off of $P > 0.2$. The explanatory variables included in the final model were farm and hCG treatment.

Progesterone concentrations on Days 7 and 16 matched the criteria for normally distributed residuals (Shapiro-Wilk test; $P > 0.01$) and homogeneity of variances (F test; $P > 0.01$), according to the function Guided Data Analysis from SAS, and were analyzed by ANOVA using the PROC GLM procedure from SAS. Data were analyzed using two independent models. In the first model, data were analyzed as a completely randomized design and the independent variable was treatment; in the second model data were analyzed as a $2 \times 2 \times 2$ factorial design and the independent variables were hCG, bST, FM, and all second and third degree interactions. Continuous variables were expressed as mean ± SEM. A significance level of 5% was used for all data analyses.

3. Results

3.1. Pregnancy rates on Day 40 post-TAI

Pregnancy rates were not different among groups ($P > 0.05$; Table 1). Cattle receiving hCG had a 7.6% higher pregnancy rate ($P = 0.001$). In contrast, none of the main effects of bST and FM, nor any interactions, were significant.

3.2. Effects of treatments on P_4 plasma concentrations measured on Days 7 and 16

There was no significant difference among groups for plasma P_4 concentrations on Days 7 or 16 (Table 2). However, there was an 11.1% average increase in P_4 concentrations on Day 16 (1.07 ng/mL) for cows receiving hCG compared with cows not receiving (hCG

Table 1
Pregnancy rates on Day 40 (timed AI was done on Day 0) in Nelore cows that received saline (Control), flunixin meglumine on Day 16 (FM), bovine somatotropin (bST) on Day 7 (bST), bST on Day 7 and FM on Day 16 (bST + FM), human chorionic gonadotropin (hCG) on Day 7 (hCG), hCG on Day 7 and FM on Day 16 (hCG + FM), bST and hCG on Day 7 (bST + hCG), or bST and hCG on Day 7 and FM on Day 16 (bST + hCG + FM), and the main effects of hCG, bST, or FM treatments.

Treatment	N	Pregnancy rates % (N)
Control	124	57.3 (71)
FM	122	47.5 (58)
bST	119	60.5 (72)
bST + FM	121	56.2 (68)
hCG	124	59.7 (74)
hCG + FM	124	64.5 (80)
bST + hCG	120	63.3 (76)
bST + hCG + FM	121	64.5 (78)
		P = 0.4995
hCG untreated	486	55.4 (269)
hCG treated	489	63.0 (308)
		P = 0.001
bST untreated	494	57.3 (283)
bST treated	481	61.1 (294)
		P = 0.210
FM untreated	487	60.2 (293)
FM treated	488	58.2 (284)
		P = 0.460

main effect, $P < 0.05$). Progesterone concentrations on Days 7 and 16 were not affected by bST, FM, or interactions among hCG, bST, and FM ($P > 0.05$).

4. Discussion

In an effort to determine whether FM, bST, or hCG affected pregnancy rates by preventing luteolysis or improving P_4 concentrations, our work has, for the first time, assessed the effects of these compounds, and their combinations, in a single study. Furthermore, the present study used a substantial number of experimental units (approximately 120 per experimental group), in the same reproductive stage (lactating cows/heifers) from the same beef breed (Nelore). Our main findings were that: (1) hCG treatment (2500 IU/animal) on Day 7 post-TAI induced greater pregnancy rates and increased plasma P_4 concentrations on Day 16 after TAI; (2) a single dose of bST (500 mg/animal) on Day 7 did not significantly affect pregnancy rates; and (3) a single dose of FM (2.2 mg/kg) on Day 16 did not significantly affect pregnancy rates.

Although pregnancy rates were not significantly different among all eight groups, there was a main effect of hCG to increase pregnancy rates (7.6%; $P < 0.01$). Other studies in cattle had already reported positive effects of hCG treatment on pregnancy rates when it was given between Days 4 and 7 post-AI [26,27,30]; this was attributed to the ability of hCG to increase P_4 plasma concentrations [26,27]. It is well known that treatment with GnRH, LH, or hCG given between 4 and 7 days postestrus stimulates ovulation of the dominant follicle from the first wave of follicular growth, leading to the formation of an accessory CL [26,31]. Another activity associated with hCG is its ability to stimulate the original CL by acting on small and large luteal cells [32] to result in greater P_4 output. It is well known that hCG has a longer half-life than GnRH or LH and a lower rate of dissociation from the ligand-receptor complex. These particular hCG characteristics promoted a more prolonged stimulus of the LH receptors on target cells [33], leading to an increase in CL weight [34]. Therefore, the positive effects of hCG on P_4 concentrations were likely due to its effects on the original CL, as well as on the formation of an accessory CL. In the present experiment, and in agreement with our initial hypothesis, hCG treatment 7 days after TAI increased P_4 concentrations and this was associated with a greater pregnancy rate. We propose that greater

Table 2
Plasma progesterone concentrations on Days 7 and 16 (timed AI was done on Day 0) in Nelore cows that received saline (Control), flunixin meglumine (FM) on Day 16 (FM), bovine somatotropin (bST) on Day 7 (bST), bST on Day 7 + FM on Day 16 (bST + FM), human chorionic gonadotropin (hCG) on Day 7 (hCG), hCG on Day 7 and FM on Day 16 (hCG + FM), bST and hCG on Day 7 (bST + hCG), or bST and hCG on Day 7 and FM on Day 16 (bST + hCG + FM), and the main effect of hCG, bST, or FM treatments.

Treatment	P_4 Concentration (ng/mL) on Day 7 (mean ± SEM)	P_4 Concentration (ng/mL) on Day 16 (mean ± SEM)
Control	4.73 ± 0.33	8.66 ± 0.72
FM	5.15 ± 0.32	9.11 ± 0.65
bST	5.48 ± 0.38	11.39 ± 0.85
bST + FM	5.19 ± 0.39	9.02 ± 0.74
hCG	5.64 ± 0.45	10.80 ± 0.78
hCG + FM	5.07 ± 0.43	10.46 ± 0.88
bST + hCG	5.18 ± 0.33	10.70 ± 0.73
bST + hCG + FM	5.18 ± 0.37	10.53 ± 0.77
	P = 0.8425	P = 0.1110
hCG untreated	5.14 ± 0.18	9.56 ± 0.38
hCG treated	5.27 ± 0.20	10.63 ± 0.39
	P = 0.6326	P = 0.0490
bST untreated	5.15 ± 0.19	9.76 ± 0.38
bST treated	5.26 ± 0.19	10.41 ± 0.39
	P = 0.6863	P = 0.2312
FM untreated	5.26 ± 0.19	10.41 ± 0.39
FM treated	5.15 ± 0.19	9.78 ± 0.38
	P = 0.6898	P = 0.2624

P_4 concentration stimulates endometrial glandular secretions that make the uterine microenvironment more suitable for conceptus development. Larger conceptuses have higher capacity to produce and secrete IFN-τ and block $PGF_{2\alpha}$ synthesis by the endometrium, which will ultimately lead to a greater probability of maintaining pregnancy.

However, it is important to consider potential mechanisms induced by hCG that are independent of P_4. The pattern of changes in pregnancy rates in response to increasing concentrations of P_4 has not been determined for postpartum Nelore females, such as those used in this study. It is not known whether there is a threshold of response to increasing P_4. For lactating dairy cows, with much lower P_4 concentrations, there was a clear, linear increase in pregnancy rates as plasma P_4 increased [35]. However, such a positive relationship may be different for beef cows and the 1 ng/mL increase in P_4 induced by hCG may not indeed improve pregnancy rates, especially because cows that did not receive hCG had relatively high (9.56 ng/mL) P_4 concentrations. Giving hCG on Day 7 likely caused ovulation of the first wave-dominant follicle and reprogrammed follicular development, hastening emergence of the second wave. Because timing of luteolysis is regulated by follicular estradiol [36], such reprogramming may delay luteolysis. Indeed, based on previous data from our group [20], we inferred that hCG delayed luteolysis an average of 1.6 days compared with noninjected cows. Perhaps a delay in luteolysis increased the odds of embryo survival, because it would give the conceptus more time to elongate and secrete enough IFN-τ to trigger an antiluteolytic response in the dam.

It was shown previously that cows given bST during estrus had greater pregnancy rates than controls [13]. Receptors for bST and insulin-like growth factor (IGF)-1 are expressed in the bovine endometrium, specifically in the endometrial glands [37]. It is possible that an increase in IGF-1 concentrations in response to bST treatment increases the secretory activity of the endometrial glands, leading to a uterine environment more favorable to the development of the conceptus, facilitating maintenance of pregnancy [13]. Indeed, in dairy cattle, bST treatment increased IFN-τ concentrations in uterine secretions [38].

Administration of bST on Day 7 failed to increase pregnancy rate in the current study. Although the dose of bST used was identical to that used in studies that showed a positive effect of bST on pregnancy rate [13,39,40], the absence of a significant effect in the present study may have been due to the day of treatment. Indeed, administration of bST at estrus increased blood concentrations of bST and IGF-1 concentrations for 14 days [41] and pregnancy rates [13]. It may be speculated that bST and IGF-1 affect events that take place in the preovulatory follicle, oviduct, endometrium and/or the early conceptus within the interval between estrus and Day 7 postestrus to result on an environment more conducive to the establishment of pregnancy. For example, treatment with bST increased IGF-1 concentrations, which in turn is important for absorption of lipoproteins and steroidogenesis of granulosa cells, thus stimulating P_4 synthesis on the newly-formed CL [42]. Conversely, in the beef cows of the present experiment, which have greater blood IGF-1 concentrations than dairy cows during lactation, exogenous bST may not be sufficient to cause the expected effects, because blood concentrations of bST and IGF-1 are already elevated [43].

In the present study, FM affected neither pregnancy rates nor P_4 concentrations. The recommended treatment with FM consists of two 1.1 mg/kg of body weight injections, given 12 h apart [10]. In the present study, due to the difficulties imposed by behavioral issues of *Bos taurus indicus* cows and to accommodate animal management, we chose to minimize handling by giving a single injection of 2.2 mg/kg. Flunixin meglumine is a potent nonsteroidal anti-inflammatory compound that inhibits COX-2 conversion of AA into prostaglandin H2 [44]. Intramuscular injection of FM decreases PGFM plasma concentrations in nonlactating cows within 12 h [45]. The mechanism of action described above and reports indicating increased pregnancy rates after AI [9] or embryo transfer (ET) [46,47] in response to FM treatment led to the hypothesis that the use of FM increases pregnancy rates. The present results were not consistent with a previous report of higher pregnancy rates in cows given FM 14 days after AI [9]. However, that experiment involved exposing animals to stress, which in combination with FM treatment might have accentuated differences between treated and untreated animals, because stressful handling of animals post-AI induced embryonic mortality [48]. Other studies reported an increase in the interval between ovulation and luteolysis, suggesting that FM could delay the latter process [44,49]. Delaying luteolysis might allow more time for the embryo to develop and secrete enough IFN-τ to induce maternal recognition of pregnancy [49]. Although the conceptus is able to secrete considerable amounts of IFN-τ by Day 14 of pregnancy, it was suggested that pulses of $PGF_{2\alpha}$ reached their maximum amplitude at approximately

Days 15 and 17 [6,50]. Considering that the half-life of FM is 24 h [45], the timing of treatment in the current study may have not been optimal, as the luteolytic mechanisms could have started in some cows before Day 16. In order to better define conditions that are more favorable to the improvement of pregnancy rates upon FM treatment, more studies involving timing of administration, dose, and length of treatment are necessary.

Numerous studies have reported that higher P_4 concentrations during early pregnancy modulate the uterine environment, generating more favorable conditions for the embryonic development, higher IFN-τ production, and pregnancy rates in cattle [21–24,51]. However, timing and magnitude of the P_4 increase postestrus is critical. For example, Geisert et al. [25] observed that cows treated with P_4 had embryos with increased embryonic growth rates and higher production of IFN-τ. Carter et al. [51] also reported that higher P_4 concentrations during the immediate post-AI period were associated with increased embryonic growth rate, IFN-τ production, and higher pregnancy rates. Consistently, Wathes et al. [52] suggested that a delay in the rise of milk P_4 concentrations was associated with low pregnancy rates. Therefore, we inferred that higher P_4 concentrations within the first week of pregnancy were associated with higher pregnancy rates. Thus, treatments designed to stimulate a quick elevation of P_4 concentrations should be beneficial to conceptus development. In the present study, strategies were designed to stimulate P_4 production after Day 7, which may at least partially explain the limited beneficial effects observed. Strategies that aim to increase rate of P_4 increase between ovulation and Day 7 are promising and warrant further investigation.

As a final point of discussion, it is important to speculate on the factors limiting pregnancy rates in postpartum *Bos taurus indicus* cattle raised in the tropics under grazing conditions. Understanding such limitations will lead to the development of new strategies that will lead to higher reproductive efficiency. In the present study, strategies aimed essentially at favoring conceptus growth and inhibiting luteolysis, but success was limited to administration of hCG. This overall result may be related to the fact that all cattle received eCG during the synchronization protocol. It is noteworthy that eCG increases growth of the preovulatory follicle and ovulation rate, P_4 production by the resulting CL, and pregnancy rates in beef cattle [53]. Thus, pregnancy rates for cattle in the present study were not likely limited by insufficient P_4. In addition to modulating endometrial glandular secretions that regulate conceptus growth [54], dos Santos and coworkers [55] reported that elevated P_4 concentrations stimulated synthesis of $PGF_{2\alpha}$. Thus, strategies aiming to retard or block luteolysis may be useful to increase pregnancy rates in situations where P_4 is not limiting. Such strategies may include reprogramming ovarian follicular development (to hasten the turnover of the second-wave dominant follicle), thereby reducing blood estradiol concentrations during the periluteolysis period, as probably achieved by hCG injections during the present experiment. Another possibility is to use anti-inflammatory agents, e.g., FM, to block $PGF_{2\alpha}$ synthesis, as attempted in the present study. To that end, further studies are necessary to optimize timing and doses of such compounds, in an attempt to increase pregnancy rates.

In conclusion, hCG, regardless of cotreatments, apparently rescued embryos from early death, as pregnancy rate improved 7.6%. We speculate that such effects were due primarily to the ability of hCG to reprogram follicle growth to delay the onset of luteolysis and, ultimately, enhance embryo survival.

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