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Superovulatory response to gonadotrophin FSH/LH treatment and effect of progestin supplement to recipients on survival of transferred vitrified embryos in goats

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ABSTRACT

Two experiments were carried out in goats to evaluate the effects of the FSH/LH ratio during treatment on ovarian response and embryo production (experiment 1) and the efficiency of progestin supplementation on pregnancy and the survival of vitrified embryos (experiment 2). In experiment 1, 30 goats were synchronized and allocated to 2 groups (n = 15) corresponding to the following superovulatory treatments with p-FSH (250 IU, over 3 days) having different doses of purified FSH and LH: (group A) control, FSH/LH ratio of 1, kept constant during treatment; (group B) FSH/LH ratio of 2 and daily FSH/LH ratio of 5.0:1.0:0.3 for the first, second, and third days of treatment, respectively. Ovarian response and embryo production were assessed 7.5 days after estrus. In experiment 2, 46 vitrified blastocysts from p-FSH–superovulated donors were transferred to 26 recipients (2 blastocysts per goat) 7.5 days after estrus. The recipients were synchronized with donors and allocated to 2 experimental groups (n = 13). Group C received progestin supplement as fluorgestone acetate (FGA) inserted into the vagina at the time of embryo transfer, replaced with a new one 16 days later, and maintained until the 45th day of pregnancy; group D, no treatment (control). Pregnancy was diagnosed by transrectal ultrasound scanning on Days 30 and 45 after estrus and followed to term. The results indicated that the increase in FSH/LH ratio from 1 to 2 with decreasing daily FSH/LH (treatment B) did not improve the superovulatory response. Superovulatory treatment A (control) advanced ($P < 0.05$) the onset of estrus and showed a higher ovulation rate compared to group B (14.9 vs. 10.9; $P < 0.05$). Fertilization rate, embryo yield, and mean number of transferable embryos in group A (7.5) were higher ($P < 0.05$) than those in group B (3.2). Recipient goats receiving progestin supplementation (group C) showed a higher ($P < 0.05$) pregnancy rate and embryo survival (kids born per embryos transferred; 69.3% and 73.1%) than the controls (group D; 23.3% and 19.2%). In conclusion, regimen A with FSH/LH ratio of 1 kept constant during the treatment gave the best ovarian response and embryo production. The progestin supplementation as FGA-pessary administered at embryo transfer time to the 45th day of pregnancy improved the pregnancy rate, kidding rate, and embryo survival of transferred vitrified embryos. Intravaginal progestin supplement has the potential to reduce the incidence of pregnancy losses during early pregnancy.

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1. Introduction

Multiple ovulation and embryo transfer (MOET) is widely used to increase genetically superior offspring produced from selected females. However, the variation in the superovulatory response of the donor makes the MOET technique rather unreliable [1], limiting its application in

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field conditions. The use of gonadotrophic pituitary extracts, commonly of porcine origin (p-FSH), results in a greater superovulatory efficiency than obtained using eCG [1,2], likely due to its half-life (10–15 hours) [3]. FSH is usually administered twice daily for 3 to 4 days because of its short half-life (5 hours in cows) [4].

The high variability in the biological activity of FSH and LH in the pituitary extracts [5], which are considered to contribute to the variability in responses to superovulatory treatment, has led to the use of more purified preparations with a defined biological activity for both FSH and LH fractions [6]. The daily FSH/LH ratio during treatment also appears to be important in the superovulatory response [1,6,7], taking into account the physiological hormonal pattern from the luteal regression and preovulatory peak in LH [8]. The results on the amount of LH needed to produce a higher ovulatory response and embryo production are rather contradictory.

In MOET biotechnology, embryo cryopreservation is useful for storing embryos in cases in which there is an insufficient number of recipient animals, and it enables them to be easily transported. Among the various procedures, vitrification has been found to be a reliable method for the cryopreservation of embryos [9,10].

In sheep and goats, it has been estimated that during the first 3 weeks of pregnancy, 30% to 40% of fertilized eggs are lost [11]. It is believed that most embryonic deaths occur within the peri-implantation period and may be attributable to inadequate circulating progesterone (P4) concentrations and the subsequent downstream consequences on uterine function or failure of the conceptus to develop appropriately, on signal pregnancy recognition and/or implantation and placentation [12,13].

Luteal insufficiency is an important cause of the reduction in pregnancy rate after embryo transfer (ET) [14] and is especially useful when frozen embryos are being transferred because of their lower survival rate compared to fresh embryos [15]. Systems to enhance embryo survival during early pregnancy involve direct supplemental progesterone or induction of a supplementary CL. Progesterone supplementation before the maternal recognition of pregnancy, either with a progesterone-releasing device or by the induction of accessory CL through the administration of hCG and GnRH, has been used in cows and sheep [12,16]; however, the results are controversial. In goats, little information is available on the effects of progesterone or progestin supplementation to recipients on pregnancy rate and embryo survival of transferred embryos [17].

The aim of the present study was to evaluate in goats (1) the effects of the FSH/LH ratio on superovulatory response and *in vivo* embryo production and (2) the efficiency of progestin supplementation, *via* fluorgestone acetate (FGA)–vaginal sponges, administered to recipients at ET time to the 45th day of pregnancy, on pregnancy rate and survival of vitrified transferred embryos.

2. Materials and methods

The experiments were conducted according to protocols approved by the Italian Ministry for Scientific Research in accordance with European Commission regulations.

2.1. Animals and general information

The study was carried out in southern Italy (41°N latitude) in October during the breeding season [18]. A total of 56 adult nonlactating goats (3- to 4-year old; body weight, 43.9 ± 1.6 kg) and 10 bucks (3- to 4-year old) of Ionica breed were used. The goats were allocated to the experimental groups described in the following sections, through random stratification according to body weight and age. Bucks of proven fertility were used in turn for hand mating (n = 6) of the superovulated does (experiment 1) or as aproned teaser (n = 4) to check for estrus recipient does (experiment 2).

2.2. Experiment 1: effect of FSH/LH ratio regimen on embryo production

The experiment investigated the effects on ovarian response and embryo production of superovulatory treatment with FSH/LH ratio of 2, decreasing daily during the treatment, compared to the regimen of FSH/LH ratio of 1:1 kept constant during the treatment. This design was established on the basis of previous studies in which the superovulatory regimen with FSH/LH of 1 kept constant during the treatment provided a higher transferable embryo yield than the decreasing daily FSH/LH ratio [19], also compared to the regimen of FSH/LH ratio of 2:1 kept constant during the treatment [10].

A total of 30 goats were used. Estrus was synchronized by a progestin (17α-acetoxy-9-fluoro-11βhydroxyl pregn-4-ene-3,20-dione; FGA 45 mg, in vaginal sponges; Intervet, Milan, Italy) [20], for 9 days, and PGF2α (50 μg intramuscularly [im]; ICI cloprostenol, Estrumate; Schering-Plough, Milan, Italy) on the Day 7.

For the treatment, all goats received a total superovulatory dose of 250 IU purified p-FSH (Laboratorios Calier, Madrid, Spain) supplemented with different purified p-LH (contamination 0.1%; Laboratorios Calier) in relation to the experimental FSH/LH ratio. The treatment was performed after a regimen of gonadotrophic administration over 3 days (2 injections im per day, 12 hours apart), starting on the Day 7 of FGA treatment. The goats were allocated to the following 2 treatment groups (n = 15). In group A (control), the FSH/LH ratio of gonadotrophic preparation was 1.0 (250 IU p-FSH:250 IU p-LH) kept constant during the 3 days of treatment; the preparation was administered in 6 decreasing doses as follows: 71.5-71.5, 35.7-35.7, and 17.8-17.8 IU. In group B, the FSH/LH ratio was 2.0 (250 IU p-FSH:125 IU p-LH), decreasing during the treatment as follows: 5.0 (Day 1), 1.0 (Day 2), and 0.3 (Day 3). In the 3 days of treatment, the preparation was administered in 6 decreasing doses as follows: 100.0-20.0, 20.0-20.0, and 6.0-20.0 IU, respectively. The does were checked for estrus with the aid of teaser bucks, every 6 hours beginning 12 hours after sponge removal, and hand mated every 10-12 hours until the end of estrus.

Ovarian response (CL and preovulatory follicles >4 mm) and embryo production were assessed 7.5 days after estrus using a semilaparoscopy procedure under general anesthesia [21]. Briefly, 2 stab incisions were made 3 to 5 cm on either side of the midline about 10 to 12 cm anterior to the

udder: the first for the laparoscope and the second for the insertion of uterine grasping forceps. The number of CL (number of ovulations) was counted through the laparoscope. Each uterine horn was then exteriorized and flushed with modified PBS [22]. The medium was collected through a Foley catheter (8–10 inch; [21]) into a Petri dish. The flushing medium was examined under a phase-contrast microscope (X 225; Diavert Leitz, Wetzlar, Germany) to search for oocytes and embryos, taking cleavage as evidence of fertilization. Embryos were judged to be of excellent and good quality on the basis of the development stage in relation to estrus time and their morphologic features, corresponding to grades I and II of International Embryo Transfer Society conventions [23]. Such embryos were then considered transferable.

2.3. Experiment 2: effect of progestin supplementation on pregnancy and vitrified transferred embryo survival

This experiment investigated whether treatment of recipients with a progestin supplement, such as FGA-vaginal sponge, from ET time until 45 days after estrus improves pregnancy rate and survival in vitrified warmed embryos. The estrous cycles of the 26 recipient does used in this trial were synchronized with donors using FGA (45 mg, in vaginal sponges) for 9 days, and prostaglandin cloprostenol (50 μg im) on the Day 7, and 400 IU im of eCG (Folligon Intervet, Milano, Italy) at FGA sponge removal. All recipient does were teased with the aid of 4 aproned bucks as described previously for donors. Does were then allocated to 2 experimental treatment groups (n = 13): group C, treatment with progestin supplement given as a first FGA sponge (45 mg) inserted into the vagina at transfer of vitrified warmed embryos (7.5 days after estrus), replaced with a new one 16 days later, and maintained until 45 days after estrus; and group D, no treatment (control).

2.3.1. Cryopreservation of embryos

Only excellent and good quality embryos at blastocyst to expanded blastocyst stage obtained from p-FSH–superovulated donor goats (experiment 1) were cryopreserved by the vitrification method [24]. Briefly, the embryos were exposed to cryoprotectants (glycerol [GLY], ethylene glycol [EG]) in 3 steps at increasing concentrations (GLY 1.4 M; GLY 3.4 M + EG 1.4 M; GLY 4.6 M + EG 3.4 M). Embryos were loaded in groups of 2 into 0.25-mL plastic straws and were directly plunged in liquid nitrogen. Thawing consisted of rapidly warming the straws in a 25 °C water bath. The contents were then emptied into a Petri dish and transferred into 0.25 M of sucrose solution to remove the cryoprotectants. The embryo quality was then evaluated under a phase-contrast microscope (X 225) on the basis of the morphologic features, and all degenerated embryos were eliminated.

2.3.2. Embryo transfer and pregnancy diagnosis

Forty-six vitrified warmed good quality blastocysts were transferred into recipient goats (n = 2 blastocysts per goat), on Day 7.5 of the estrous cycle (Day 0 = day of estrus). A semilaparoscopic procedure under general anesthesia was used [21]. The ovaries were inspected laparoscopically to confirm whether the recipient had CL present on the ovaries. Through a 3-cm incision, the uterine horn ipsilateral to the ovary displaying CL was grasped with the aid of a blunted forceps, and a loop of 3-4 cm was exteriorized. For each recipient, 2 embryos were aspirated with 20-mL PBS in a capillary connected to a 1-mL syringe and were introduced surgically into the top of the uterine horn, about 4 cm from the uterotubal junction. After repositioning the uterus, the incision was closed with a single suture. Pregnancy was diagnosed at Days 30 and 45 after estrus, using an ultrasound scanner (Agroscan ALR 575; Foschi, Ozzano, Italy), with a transrectal 5-MHz linear-array probe, and by observation of kidding at term.

2.4. Statistical analysis

For experiment 1, the effect of superovulatory treatment on onset of estrus, ovulation rate, global ovulatory response, embryo production, and transferable embryos was analyzed using the generalized linear model procedure of the Statistical Analysis Software (SAS) [25]. Least-square means were compared by *t* test with the predicted difference (pdiff) option of generalized linear model. Percentages were analyzed by the chi-square test [25]. For experiment 2, the percentages of pregnant goats and embryo survival were compared using the chi-square test [25].

3. Results

3.1. Experiment 1

Estral and ovarian responses and embryo production are reported in Table 1. Superovulatory treatment performed with different FSH/LH ratios influenced ($P < 0.05$) the onset of estrus. In the control group, the occurrence of estrus was

Table 1
Effects of superovulatory treatment with gonadotrophic preparations having different FSH/LH ratios during the 3 days of treatment on estrus, ovarian response, and embryo production.

Daily FSH/LH	Group A (control)[c]	Group B[d]
	1.0:1.0:1.0	5.0:1.0:0.3
Treated goats, n	15	15
Onset of estrus, h	25.2 ± 2.2[a]	31.6 ± 2.2[b]
Ovulations (CL)	14.9 ± 1.9[a]	10.9 ± 1.9[b]
Large follicles (LF >4 mm)	1.2 ± 0.3[a]	3.9 ± 0.3[b]
Global ovarian response (CL + LF)	16.1 ± 2.0	14.8 ± 2.0
Embryo production	8.4 ± 1.6[a]	4.0 ± 1.5[b]
Fertilization rate, %	82.8[a]	44.2[b]
Transferable embryos	7.5 ± 1.5[a]	3.2 ± 1.5[b]

Values are means ± standard error.
[a,b]Within a row, means with different superscripts differ ($P < 0.05$).
[c] Total FHS/LH ratio = 1:1 (250 IU p-FSH:250 IU p-LH) and daily FSH/LH ratio of 1.0:1.0:1.0, for the first, second, and third days of treatment: The p-FSH doses administered 12 hours apart, beginning 48 hours before sponge removal in 6 decreasing doses, 71.5-71.5, 35.7-35.7, and 17.8-17.8 IU for the first, second, and third days of treatment, respectively.
[d] Total FHS/LH ratio = 2:1 (250 IU p-FSH:125 IU p-LH) and daily FSH/LH ratio of 5.0:1.0:0.3, respectively, for the first, second, and third days of treatment: The p-FSH doses administered 12 hours apart, beginning 48 hours before sponge removal in 6 decreasing doses, 100.0-20.0, 20.0-20.0, and 6.0-20.0 IU for the first, second, and third days of treatment, respectively.

advanced ($P < 0.05$) compared to group B (25.2 ± 2.2 vs. 31.6 ± 2.2 hours). All the goats ovulated and exhibited a superovulatory response (>3 CL). The ovulatory response and embryo yield were affected by the treatment. The ovulation rate was higher ($P < 0.05$) in group A than that in group B (14.9 vs. 10.9). The mean number of large unovulated follicles was higher ($P < 0.05$) in group B than that in group A. There was no significant difference between groups A and B in total ovarian response.

Treatment with total FSH/LH of 1 and daily FSH/LH ratio of 1 (group A, control) resulted in a higher ($P < 0.05$) mean embryo yield, fertilization rate, and mean number of transferable embryos (8.4 ± 1.6, 82.8%, and 7.5 ± 1.5, respectively) than those in group B treated with FSH/LH of 2:1 and a decreasing daily FSH/LH ratio of 5.0:1.0:0.3 (4.0 ± 1.5, 44.2%, and 3.2 ± 1.5, respectively).

3.2. Experiment 2

No differences in ovulation rate were observed between the recipient groups C and D at ET time (2.1 ± 1.1 and 2.3 ± 1.2 CL, respectively; data not shown). Table 2 compares the pregnancy and embryo survival rates after vitrified-warmed ET between treated and control recipients.

Nine of the 13 recipients (69.2%) treated with FGA (group C) were diagnosed as pregnant on Days 30 and 45 after estrus and went to term, whereas only 3 of the 13 recipients (23.3%) were pregnant and went to term in the control group (group D). The difference was significant for $P < 0.05$. The embryo survival (kids born per embryos transferred) was higher ($P < 0.05$) for the recipients in group C than in group D (57.7%, 15 of 26 vs. 15.4%, 4 of 26).

4. Discussion

The first objective of this study was to detect a suitable FSH/LH regimen in the superovulatory treatment for *in vivo* embryo production in goats. The results demonstrated that the superovulatory control treatment with the total dose p-FSH (250 IU) with a ratio FSH/LH of 1.0, kept constant in the 3 days of treatment, provided a better ovarian response and embryo production. The ovulation rate and transferable embryo yield obtained were in agreement with the results (6–8 embryos/donor) reported in other studies [3,6,14]. In the present study, the increase in total FSH/LH from 1 to 2 did not improve the superovulatory response. This is inconsistent with the results of previous studies carried out in cattle, which report that more purified FSH extracts are preferable for superovulation [26]. The regimen of gonadotrophin with daily FSH/LH of 5.0:1.0:0.3 (group B) also did not increase the ovulation rate and provided only a higher number of unovulated large follicles. The reasons for this are difficult to explain. The superovulatory response is believed to reflect the follicular population present at the initiation of the gonadotrophin treatment [1]. It has been reported that the increase in ovulation rate could be due to a longer time for follicular recruitment [27] or, alternatively, due to an increase in the pool of small antral follicles in the gonadotrophin-dependent stage, while preventing large (dominant) follicles on the ovary at the onset of superstimulation [1,27]. In addition, massive ovarian stimulation can reduce the proportion of ovulating follicles because of a reduction in their responsiveness to LH [28].

Again, compared to the control, the treatment of group B resulted in a lower fertilization rate, embryo yield, and transferable embryo production. This may also be due to the failure of the sperm to fertilize ovulated oocytes and/or failure of oocyte fertilization. The poor ova fertilization might be associated with high levels of estradiol because of a high prevalence of unovulated follicles [29]. This can give rise to an impairment of sperm transport to the uterus and oviduct through the cervix [30] and the failure of fertilization in superovulated does [1]. It is also possible that the prevalence of LH on FSH from the second day of treatment in group B could have caused a premature stimulation of the oocytes, thus preventing their normal fertilization and subsequent development. In sheep, a positive effect of the addition of pLH in the last round of injections (FSH/LH = 0.3, 0.4, and 0.8) has been observed during the nonbreeding season [1,7].

The results of the second experiment indicate that the FGA treatment improved the pregnancy rate by 69.2%. This is in agreement with the positive effect of progesterone supplement on pregnancy found in recipient cows [31] and women [32] of transferred embryos. How this progestin supplement acts is uncertain because the steroid was filled in the vagina. However, the progesterone vaginal administration seems to be superior to other routes for luteal support in humans [33]. Its influence may have been mainly to increase the concentration of progesterone in the systemic circulation.

The lower pregnancy rate observed in this study for the untreated goats (−46%) may be attributable to early pregnancy loss. Most embryonic mortality is early and has been reported as a very significant cause of pregnancy loss [11,13]. The positive effect of the progestin supplement as FGA-vaginal sponges reflected the probable effect of hormonal treatment on reducing early embryonic mortality, which is mainly caused by luteal insufficiency [11,13].

Possible explanations of progestin action include enhanced luteotrophic or antiluteolytic actions resulting in improved luteal maintenance in recipients or enhanced

Table 2
Effects of treatment with FGA-vaginal sponges on pregnancy rate and embryo survival in recipient goats after transfer of vitrified embryos.

Items	FGA-Group[c]	Group D (control)
Recipient, n	13	13
Pregnant,[d] n	9	3
Pregnancy rate, %	69.2[a]	23.3[b]
Recipient kidding, %	69.2[a]	23.3[b]
Transferred embryos, n	26	26
Kids born[e], n	19	5
Embryo survival rate, %	73.1[a]	19.2[b]

Abbreviation: FGA, fluorgestone acetate.
[a,b]Within a row, means with different superscripts differ ($P < 0.05$).
[c] Treatment with progestin supplement given as FGA-impregnated sponge inserted into the vagina at the time of embryo transfer (7.5 days after estrus = Day 0), replaced for a new one 16 days later, and maintained until Day 45 of pregnancy.
[d] At 23 and 45 days after transfer and at term.
[e] Kids born per embryos transferred.

signals to the endometrium involved in the process of implantation (placental attachment) [13,32]. It has been reported that progestative treatment increases circulating progesterone levels, which stimulate endometrial secretions, thus enhancing the development of the embryo, which stimulates higher interferon-τ (IFNT) secretion [13]. The effect of this stimulation seems to be more important in the early to midluteal phase.

In cows, progesterone supplementation from Days 5 to 9 increased embryo development and IFNT production [34]. In the present study, it is possible that progestin supplementation as FGA at ET time in group C recipients, before the maternal recognition of pregnancy, could have supported the transferred embryos in IFNT secretion, thus improving the pregnancy rate. It has been reported that caprine IFNT secreted between Days 16 and 21 of gestation abrogates the luteolytic mechanism from preventing pulsatile release of luteolytic PGF and extends the lifespan of the CL that ensures the continued production of P4 [13]. All this would have reduced the embryo mortality, thus increasing the pregnancy rate and leading the pregnancy to term.

It has also been recognized that pregnancy maintenance and embryo and fetus survival are dependent on luteal progesterone for a long time in goats [11]. This thus justifies the higher kidding rate (+46%) observed in this study in the FGA-treated recipients than in the control groups. The embryo survival rate (kids born per embryos transferred) was improved by the FGA treatment of the recipients (73%) and was higher (+59.3%) than in the control. It was also higher compared to the survival rates of vitrified blastocyst transfer, without P4 supplement, reported by other authors with values of 14% to 35% [9], 51.4% [35], 64% [36], or 70% [37].

We believe that the present study is the first to show that progestin supplementation results in a substantial improvement in pregnancy and survival of embryos in transferred vitrified embryos in recipients goats. In another study, the treatment with a progestin norgestomet implant in transferred frozen demiembryo–recipient goats resulted in a pregnancy rate and embryo survival of 13% and 6%, respectively, compared to 0% in the control group [17]. The difference with our results could be explained by the lower survival rate of demiembryos than that of intact embryos [38], together with the additional impact of freezing and thawing to demiembryos [39]. The findings of the present study may have a potential application in MOET programs in goats. Further studies are needed on a larger number of transferred vitrified embryo recipients.

4.1. Conclusions

For *in vivo* embryo production in goats, a satisfactory superovulatory response and transferable embryos resulted using a gonadotrophic preparation with FSH/LH of 1 kept constant during the 3 days of treatment. The regimen of FSH/LH of 2 and daily FSH/LH ratio of 5, 1.0, and 0.3 did not improve the superovulatory response but led to a lower ovulation rate, fertilization rate, embryo yield, and transferable embryo production.

The progestin supplementation to recipients as FGA-vaginal sponges at ET time to 45 days after estrus improved pregnancy and survival rates of transferred vitrified embryos in goats and should be considered to prevent early pregnancy losses.

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