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Original Research Article

Effect of a single administration of FSH delivered in hyaluronic acid on oocyte competence and hormonal concentrations in Italian Mediterranean buffaloes undergoing ovarian stimulation prior to ovum pick-up

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ABSTRACT

This study evaluated the effectiveness of a single intramuscular administration of follicle-stimulating hormone (FSH) dissolved in 1 % hyaluronic acid (HA) for ovarian stimulation in Italian Mediterranean buffaloes (IMB) undergoing ovum pick-up (OPU). The aim was to simplify hormonal protocols, reduce handling stress, and improve oocyte competence. In Experiment 1, multiparous IMB (n = 24) were enrolled in a crossover design comparing the standard six-dose FSH protocol (FSH-6) with a single FSH-HA injection. Transrectal ultrasonography was used to record the number and the size of follicles, in vitro embryo production to assess oocyte developmental competence, and an in-house radioimmunoassay (RIA) method to measure plasma cortisol levels. In Experiment 2, plasma FSH profile during follicular growth was evaluated in IMB (n = 12) subjected to FSH-HA, FSH-6, or no stimulation (control) protocols. The FSH-HA treatment resulted in a higher ($P < 0.05$) proportion of medium-sized follicles compared to FSH-6 (3.4 ± 2.7 and 2.0 ± 2.3, respectively) as well as a significantly greater number of cleaved oocytes (3.3 ± 0.4 and 2.4 ± 0.4 respectively), although the total embryo yield remained similar (30.1 ± 7.2 and 29. 2 ± 4.5, respectively). Cortisol concentrations increased after OPU in all groups, but the FSH-HA group showed a trend ($P < 0.10$) toward lower stress levels compared to FSH-6. In Experiment 2, FSH-HA maintained higher plasma FSH concentrations for a longer period, with peak values observed between 30 and 72 h post-administration, suggesting the importance of optimizing the timing of OPU in HA-based protocols.

1. Introduction

The buffalo (*Bubalus bubalis*) is a globally widespread species, mainly known for its contribution to meat and milk production. [1]. The importance of buffalo in the global economy is evident from the steady growth of its population worldwide, as reported by the FAO, with numbers rising from approximately 201 million in 2017 to 206.6 million in 2018, marking a 2.8 % increase within a year [2]. The Italian Mediterranean buffalo (IMB) is in great demand for its marked milk-producing attitude [3]. The use of reproductive biotechnologies such as artificial insemination (AI), multiple ovulation and embryo transfer (MOET), and ovum pick-up and in vitro embryo production (OPU and IVEP) combined with embryo cryopreservation is, now, the only way to accelerate the genetic development in dairy farms and the spread of valuable germplasm around the world. As MOET is not commercially feasible in buffalo [4–6], using OPU + IVEP is the only method to accelerate genetic progress through the maternal line in buffalo [7]. The reduced blastocyst production per OPU session, observed in buffalo compared to cattle, is primarily attributed to the lower number of follicles that develop during each estrous cycle, resulting in a diminished quantity of oocytes retrieved per session [7]. This is associated with the smaller reserve of primordial follicles

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characteristic of the species [7]. Various factors such as season, management, and nutrition further limit gamete availability and oocyte competence in this species, resulting in higher embryo production costs [7–9]. It is possible to select the best donors based on the antral population, but this often results in the exclusion from programs of buffalo cows with high genetic merit [10]. Follicular selection is crucial for determining which follicles will continue to develop, becoming competent for ovulation or, in the case of OPU, for in vitro maturation. If this phase is inadequate, a reduced number of oocytes, often with suboptimal quality, is recovered. In the absence of natural selection, hormonal priming becomes essential to stimulate follicular growth and compensate for the lower number of recruited follicles, a well-known characteristic of the buffalo species [7]. For this purpose, gonadotropin-based protocols are used, aiming not only to increase the number of retrievable oocytes but, more importantly, to enhance their developmental competence [11–13]. Although some trials have been conducted on ovarian stimulation prior to OPU in buffalo, there is still a paucity of data regarding the best choice in terms of cost/efficiency/-safety and manageability. It has been demonstrated that the use of the follicle-stimulating hormone (FSH) has a positive effect on the number of medium-large follicles available for aspiration and oocyte competence [14,15]. More recently, Petrovas et al. [13] demonstrated that the treatment with FSH, in the presence of Progesterone (P4), with a coasting time of 28–32 h improves the efficiency of OPU-IVEP in buffaloes in terms of blastocyst yield. There is growing interest in developing ovarian stimulation protocols that are more manageable and less harmful for animals. Undoubtedly, the need for multiple injections of FSH is a limitation, implying a higher risk of failure due to mishandling and treatment errors, and undue stress in donor animals, potentially leading to reduced ovarian response. In cattle, hyaluronic acid (HA) has been used with varying degrees of success as a vehicle for the slow release of FSH in both MOET and OPU programs [16,17]. Similar embryo yields have been obtained from cows superovulated with a single intramuscular injection of FSH diluted in HA (20 mg/mL) vs the standard twice-daily intramuscular injections of FSH [18,19]. Likewise, a single FSH injection in HA has been successfully used to prime bovine donors before OPU [17]. To our knowledge, no data are currently available in the literature regarding the use of HA as a vehicle for gonadotropins in buffalo. Therefore, the overall aim of this experiment was to improve the stimulation protocol of buffalo donors undergoing OPU-IVEP, making it more practical for field application and less harmful for the animals. More specifically, the objectives of the work were: 1) to compare the efficacy of a single intramuscular injection of pituitary extracts dissolved in a 1 % HA (FSH-HA group) and the standard protocol requiring twice-daily injections over 3 days (FSH-6 group) on follicular growth and oocyte developmental competence in IMB (Experiment 1); 2) to evaluate whether the replacement of six intramuscular drug injections with just one shot may reduce the stress levels associated with the procedure, by measuring plasma cortisol concentrations (Experiment 1); 3) to evaluate plasma profile of endogenous FSH during the follicular growth phase in buffaloes stimulated with a single shot (FSH-HA), multiple doses (FSH-6) compared to unstimulated animals (control, CTR; (Experiment 2).

2. Materials and methods

If not otherwise stated, all chemicals and reagents were purchased from Merck KGaA (Darmstadt, Germany).

2.1. Animals

The Ethical Animal Care and Use Committee of the University of Naples Federico II (Naples, Italy) approved the experimental design and animal treatments (PG/2023/0148747 of November 28, 2023). In both experiments, multiparous IMBs (Bubalus bubalis), kept under controlled nutrition and housed in barns on a farm in Caserta, Italy, were included. Before the start of the experiments, all animals were evaluated, and only healthy, cyclic buffaloes with no genital tract pathologies were selected. In Experiment 1, IMBs (n = 24) with average age, parity, and days in milk of 6.9 ± 2.77, 4.0 ± 2.34, and 91.0 ± 49.62, respectively, were enrolled. In Experiment 2, IMBs (n = 12) with average age, parity, and days in milk of 10.2 ± 3.7, 6.9 ± 3.3, and 198.4 ± 64.8, respectively, were used.

2.2. Experimental design

In Experiment 1, each animal was subjected to two consecutive OPU sessions, with a washout period of two weeks between sessions in a cross-over design. In particular, the animals were randomly divided into two stimulation groups: the standard six injections protocol (FSH-6, n = 12), and one injection of FSH dissolved in 1 % HA solution (FSH-HA, n = 12); after the first OPU session, the treatments were inverted to stimulate all the animals with both the protocols reducing the individual variability. For each OPU session, the number of follicles divided by size category, i.e. small (diameter <0.5 cm), medium (diameter between 0.5 and 1 cm), and large (diameter >1 cm), the recovery rate, the total number of cumulus-oocyte complexes (COCs), the number of COCs suitable for in vitro embryo procedures (grade A + B + C), as well as cleavage and embryo rates were recorded. During both sessions, to assess plasma cortisol concentrations, blood samples were collected by venipuncture of the lateral tail vein one day before the dominant follicle removal (DFR, Day −1; T0) and on OPU day, just before the beginning of the procedure (T1) and 10 min after the end of the OPU procedure (T2). The blood samples were centrifuged (1500 *g* for 15 min) and stored at −80 °C until analysis.

In Experiment 2, IMB (n = 12) were split into three groups: unstimulated animals (CTR, n = 4), stimulated with a standard 6 injections protocol (FSH-6, n = 4), and stimulated with one injection of FSH dissolved in HA (FSH-HA, n = 4) before being subjected to OPU. Blood samples were collected every 6 h from Day 2 up to Day 5 to assess the plasma FSH profile during the follicular growth. The samples were centrifuged (1500 *g* for 15 min) and stored at −20 °C until analysis.

2.3. Stimulation protocols

In both experiments, after dominant follicle removal (Day 0), an intravaginal progesterone-releasing device (PRID Delta 1.55 g, Ceva Animal Health Ltd., Amersham, Buckinghamshire, United Kingdom) was inserted according to the manufacturer's instructions. Starting on Day 2, six consecutive injections of 70 IU of porcine pituitary-derived FSH (Pluset, Laboratorios Calier, Les Franqueses del Vallès, Barcelona, Spain) were injected twice/day (every 12 h) in the FSH-6 protocol. For each animal of the FSH-HA group, a 15 mL solution of 1 % HA containing 420 IU of Pluset was prepared and administered intramuscularly on Day 2. The solution was prepared by dissolving 150 mg of HA (Altergon Italia s.r.l. Avellino, Italy) in 6.6 mL and left to cool. The HA solution was then mixed with 8.4 mL of Pluset (420UI) previously reconstituted accordingly to the producer's instructions and vigorously mixed. A schematic representation of the stimulation protocols is reported in Fig. 1.

2.4. Ovum pick-up (OPU) and in vitro embryo production

OPU was conducted as previously described [20]. In brief, a 10 MHz micro-convex probe was used to scan ovaries (Dramiński ® blue, Olsztyn, Poland). All antral follicles with a diameter ≥2 mm were aspirated and classified according to size: small (diameter <0.5 cm), medium (diameter between 0.5 and 1 cm), and large (diameter >1 cm)follicles. Oocytes collected into 50 mL conical tubes (Falcon, Corning Science, Reynosa, México), maintained at 37 °C, were recovered and selected according to their morphology [21]. Only grade A + B + C COCs were chosen, and in vitro matured, fertilized, and cultured as previously

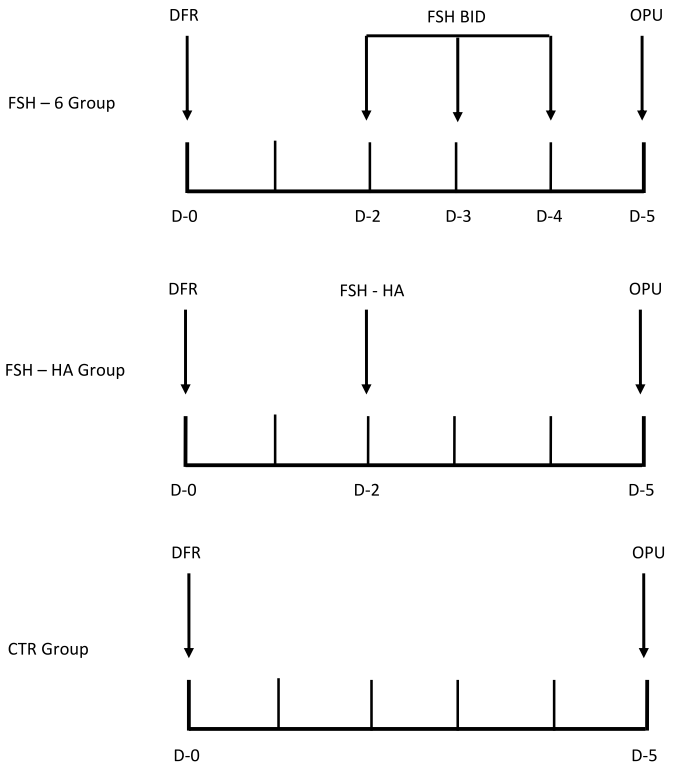

Fig. 1. Schematic representation of the experimental procedure for animals stimulated with the standard follicle-stimulating hormone (FSH) six-injection protocol (FSH-6) or single intramuscular administration of FSH dissolved in 1 % hyaluronic acid (HA) protocol (FSH-HA) and unstimulated animals (CTR group). DFR = dominant follicle removal; FSH BID = Twice/day injection of 70 IU of FSH (Pluset, Laboratorios Calier, Les Franqueses del Vallè, Barcelona, Spain); FSH-HA, single administration of 420 IU Pluset dissolved in 1 % HA; OPU = Ovum pick-up.

described [20]. On Day 5 (Day 0 = start of in vitro fertilization), the cleavage rate was assessed, and embryos were transferred into fresh droplets of the same medium for a further two days of culture. The final embryonic outcome, measured as the number of morulae and blastocysts (TM + BL), was assessed on Day 7 of culture.

2.5. Hormone assays

Plasma samples were extracted, and cortisol concentrations (ng/ml) were measured using an in-house radioimmunoassay (RIA) method as previously described for buffaloes by Cotticelli et al. [22]. The cross-reactivities of the anti-cortisol antibody with other steroids were as follows: cortisone 4.3 %, corticosterone 2.8 %, 11-deoxycorticosterone 0.7 %, 17-hydroxyprogesterone 0.6 %, dexamethasone 0.1 %, progesterone, 17-hydroxypregnenolone, DHEA-S, androsterone sulphate, and pregnenolone <0.01 %. The sensitivity of the assay was 16.8 pg/mL. The intra- and inter-assay coefficients of variation were 3.7 % and 10.1 %, respectively.

The concentrations of FSH in plasma were determined by a commercial ELISA kit (Bovine follicle-stimulating hormone (FSH) ELISA Kit, code CSB-E15856B, Cusabio, Wuhan, China) according to the manufacturer's protocols and validated for the buffalo species. The kit's detection range is 2 mIU/mL-800 mIU/mL, with a sensitivity of 2 mIU/mL. Each validation test involved different pools, each constituted of five plasma samples that were analyzed by quintuple. The parallelism test consisted of determining the deviation from the standard curve of a series of samples containing known amounts of FSH, that were prepared by serial dilution of plasma samples from animals that showed high concentrations of FSH. Linear regression was used to determine if plasma samples and the standard FSH curve deviated from parallelism. The recovery test was conducted to evaluate the system response to an increasing amount of FSH standard added to a plasma sample with low FSH concentrations. The precision of the assay was estimated by assessing intra and inter-assay variation, which was expressed as the coefficient of variability (CV). The intra-assay precision was evaluated by testing 20 replicates of a plasma sample with a known medium FSH concentration in the same assay. The inter-assay precision was evaluated by running the same plasma sample in 20 assays in duplicate over different days.

2.6. Statistical analysis

Statistical analyses were carried out using SPSS (29.0.1.0) for Windows 10 (SPSS Inc., Chicago, IL). The normal distribution and the homogeneity of variances of data were verified using the Shapiro–Wilk and Levene test, respectively, for both Experiments 1 and 2. In Experiment 1, differences in follicular, oocyte, and embryo parameters between groups were evaluated by multivariate analysis of variance (ANOVA; general linear model) with treatment as a fixed factor. Cortisol concentrations were log-transformed and were compared between groups through ANOVA for repeated measures (generalized linear mixed model) with sampling time as repeated measures, treatment as a fixed factor, and the post hoc comparison between the sampling times was adjusted using the Bonferroni correction.

In Experiment 2, FSH concentrations were log-transformed and compared between the three groups by analysis of variance (ANOVA) for repeated measures (generalized linear mixed model) with sampling time as repeated measures and treatment as a fixed factor. The Bonferroni test was used for post hoc multiple comparisons between the sampling times. An additional statistical analysis was conducted with the R vers. 3.4.0 for Windows 11 and the packages pROC, ggplot2 were used. The area under the curve (AUC) of plasma FSH concentrations log-transformed was calculated by the trapezoid method, and DeLong's test was computed for multiple comparisons. A statistically significant difference was accepted at $P < 0.05$, and a tendency was discussed at $P < 0.10$.

3. Results

3.1. FSH ELISA kit validation

The validation of the commercial ELISA kit for FSH in buffalo plasma involved the parallelism between the dilution curves and the standard one that indicated that plasmatic FSH and standard FSH reacted identically to the antibody because of the high correlation ($r = 0.99$) observed between the concentrations obtained and those expected; the relationship between FSH concentrations and the standard FSH curve was given by the equation $y = 1.07x\text{-}5.55$. The recovery test aimed to evaluate the reaction of the system to an increasing amount of FSH standards and revealed a recovery rate of 124.9 ± 20.2 % (mean ± SD). In repeated determinations, FSH samples showed intra- and interassay coefficients of variation of 9.5 % and 15.1 %, respectively. Despite the suboptimal CVs recorded during the validation test, plasmatic FSH and standard FSH reacted identically to the antibody, as highlighted by the parallelism, with a correlation of 0.99 between the obtained concentrations and the expected ones.

3.2. Experiment 1

As shown in Fig. 2, in the FSH-HA group, the percentage of medium-sized follicles increased ($P < 0.05$) compared to the FSH-6 group, while no differences were recorded for small and large follicles between groups (Fig. 2). The total number of follicles did not differ between groups (9.1 ± 4.3 and 9.9 ± 4.6 in FSH-6 and FSH-HA, respectively). The FSH-HA treatment also increased ($P < 0.05$) the number of medium follicles (3.4 ± 2.7 and 2.0 ± 2.3 in FSH-HA and FSH-6, respectively).

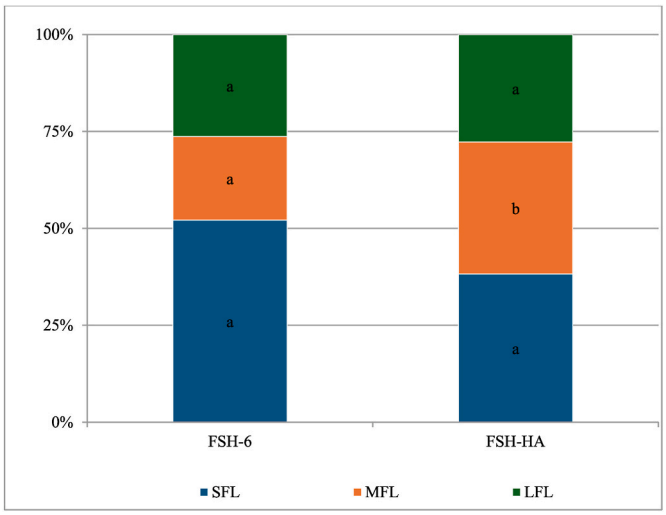

Fig. 2. Percentages of Italian Mediterranean buffalo follicles according to size class in the standard six FSH injections protocol (FSH-6) and one injection of FSH dissolved in a 1 % in a hyaluronan (HA) solution (FSH-HA) SFL = Small size follicles (<5 mm diameter); MFL = Medium size follicles (0.5 mm- < 0.9 mm diameter); LFL = Large size follicles (≥0.9 mm diameter). [a,b] Values with different superscripts within follicle categories are significantly different; $P < 0.05$.

However, the treatment did not affect the number of small (4.7 ± 2.5 and 3.8 ± 2.1 in FSH-6 and FSH-HA, respectively) and large follicles (2.4 ± 1.6 and 2.8 ± 3.0 in FSH-6 and FSH-HA, respectively).

The parameters related to COC's number, morphology, and competence are shown in Table 1. The FSH-HA treatment increased ($P < 0.05$) the number of cleaved oocytes compared to the FSH-6 treatment. However, cleavage and blastocyst rates, as well as the recovery rate, were not affected by the treatment. Additionally, FSH-HA treatment did not affect the total number of COCs nor the number and percentage of superior quality COCs (A + B) and COCs suitable for IVEP (A + B + C).

Regardless of the treatment, plasma cortisol concentrations were higher at T2 (10 min after OPU, $P < 0.01$) compared to T1 (immediately before OPU) and T0 (Fig. 3).

According to the sampling time and the experimental group, plasma cortisol concentrations increased ($P < 0.05$) at T2 compared to T1 in the FSH-HA group (Fig. 3). Interestingly, FSH-6 showed a tendency ($P < 0.10$) to have higher plasma cortisol concentrations compared to FSH-HA at T1 and lower ($P < 0.10$) plasma concentrations at T2 (Fig. 4).

Table 1
Effect of FSH stimulation delivered in hyaluronan solution (FSH-HA) and with standard six-injections protocol (FSH-6) on oocyte quality and developmental competence parameters. The data are reported as mean ± Standard error.

Parameters	FSH-HA	FSH-6
TOT COC	6.6 ± 0.7	5.9 ± 0.8
TOT A + B	2.8 ± 0.3	2.4 ± 0.5
TOT A + B + C	5.3 ± 0.6	4.7 ± 0.6
Recovery rate %	65.8 ± 4.8	62.1 ± 6.4
Cleavage %	60.5 ± 5.8	49.4 ± 4.6
Cleaved zygotes n	3.3 ± 0.4[a]	2.4 ± 0.4[b]
TM-BL %	30.1 ± 7.2	29.2 ± 4.5
TM-BL n	1.8 ± 0.4	1.3 ± 0.3

COC = Cumulus complex oocytes; A + B = superior quality COCs; A + B + C=COCs suitable for IVEP; TM-BL=Morulae and blastocysts, i.e. total embryos produced.
[a,b] Values with different superscripts within rows are significantly different; $P < 0.05$.

3.3. Experiment 2

In Experiment 2, regardless of sampling time, the average plasma FSH concentrations were lower ($P < 0.01$) in the FSH-6 group compared to the CTR and FSH-HA groups (Fig. 5). Furthermore, the AUC of plasma FSH concentrations of FSH-HA and FSH-6 groups differed significantly from the CRL group ($P > 0.01$). AUCs were 0.494, 0.898, and 0.892, respectively, in the CTR, FSH-6, and FSH-HA groups.

However, when FSH plasma concentrations were analyzed according to sampling time, FSH-HA showed higher values compared to FSH-6 at 18 and 84 h ($P < 0.05$) and compared to FSH-6 and CTR at 30 h ($P < 0.01$) (Supplementary Table 2 and Fig. 6). Additionally, FSH-6 showed lower concentrations ($P < 0.05$) compared to CTR at 72 h (Supplementary Table 2 and Fig. 6).

4. Discussion

To the best of our knowledge, this is the first report of the use of HA as a vehicle for gonadotropins for ovarian stimulation prior to OPU in IMB. Furthermore, it is the first assessment of cortisol concentrations associated with this procedure in the IMB. The use of HA as a vehicle for FSH did not negatively affect performance in terms of follicular population, oocyte number, and competence. Both groups showed similar results regarding the follicular population, with only a significant increase in the number of medium-sized follicles in the FSH-HA group compared to FSH-6. The increased proportion of medium-sized follicles compared to small ones is a positive outcome since their presence is associated with increased oocyte developmental competence [23,24]. An interesting finding is the higher number of cleaved oocytes over the total reported in the FSH-HA group compared to FSH-6; however, this increase does not translate into a higher embryo yield in the FSH-HA group. Considering that not only the FSH dose and stimulation duration but also the interval between FSH administration and oocyte retrieval are crucial for acquiring competence [13,25,26], and according to the FSH profile in experiment 2, it is hypothesized that optimizing the timing of oocyte collection when FSH is diluted in HA is essential for enhancing oocyte competence. Previous research in IMB demonstrated that, with a 6-injection protocol, the optimal interval between the final FSH injection and OPU is 28–32 h [13]. In this trial, however, the use of HA as a vehicle does not allow a priori prediction of when the effect of exogenous gonadotropin ceases. It can be speculated that the lack of an increase in embryo numbers, despite the higher number of cleaved zygotes in the FSH-HA group, may be due to a suboptimal FSH deprivation period in this group. These results are consistent with the findings of Ongaratto et al. [26] and Vieira et al. [17] who reported that embryo yield was not influenced by either single or multiple FSH administrations in bovine. Another objective of Experiment 1 was to assess the circulating cortisol concentrations associated with the two treatments and the OPU procedure. The confinement of animals in the containment unit and the handling associated with oocyte retrieval, although not harmful in the long term, constitute a stressful event, particularly for animals not accustomed to such procedures. Cortisol is an important parameter in the dairy industry; it is considered a valuable tool for investigating animal welfare, being also related to their immune system [27]. Evaluating cortisol concentrations is crucial not only because it is linked to the animal's stress response, but also because cortisol and other substances released during stressful conditions can negatively impact reproductive performance. It has been demonstrated that animals with a docile temperament, less inclined to suffer in stressful situations, have higher pregnancy rates [28,29] but also show a better response to hormonal stimulation, producing more viable embryos [30]. Indeed, glucocorticoids can induce apoptosis in ovarian cells and oocytes by activating the TNF-α system or by the FAS system, increasing oxidative stress in mice [31]. Furthermore, several studies have assessed the negative effect of cortisol during stressful situations on bovine reproduction [32,33]. Cortisol can inhibit the release of GnRH, thus affecting

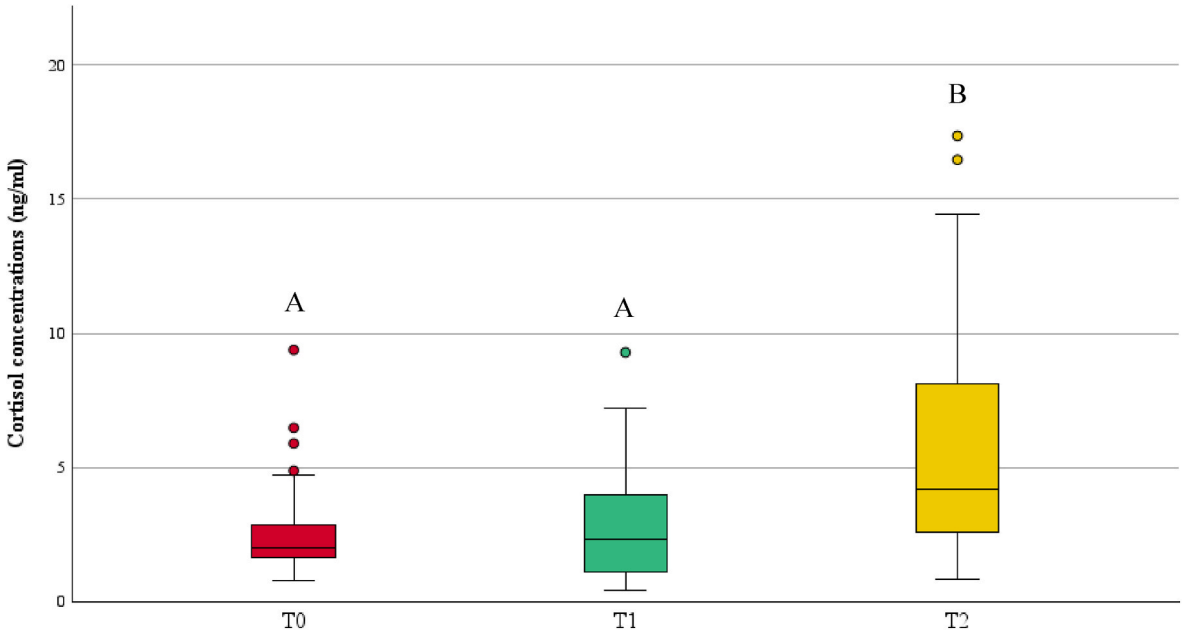

Fig. 3. Plasma cortisol concentrations (ng/ml) in Italian Mediterranean buffaloes one day before the dominant follicle removal (T0) immediately before Ovum pick-up (OPU; T1) and 10 min after OPU (T2). [A,B] Values with different superscripts among time points are significantly different; $P < 0.01$.

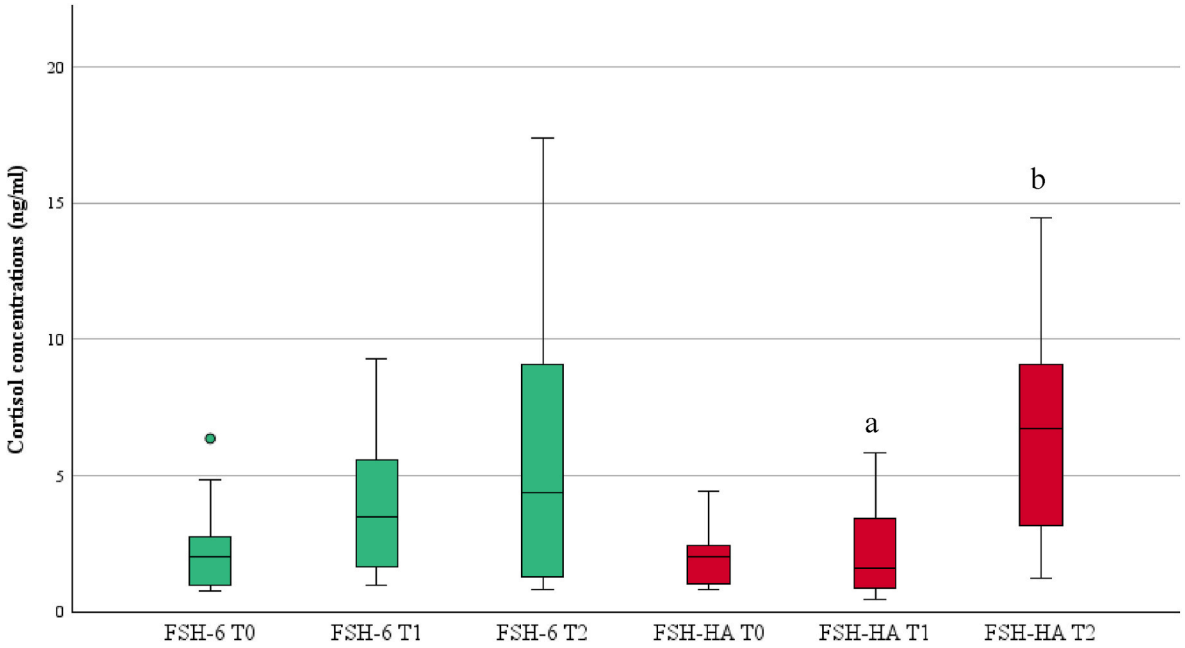

Fig. 4. Plasma cortisol concentrations (ng/mL) in Italian Mediterranean buffaloes subjected to ovarian stimulation with FSH delivered by six injections (FSH-6) or in a hyaluronan solution (FSH-HA) at three different time points: one day before the dominant follicle (T0) immediately before Ovum pick-up (OPU; T1) and 10 min after OPU (T2). The data are reported as mean ± standard deviation
[a,b] Values with different superscripts among time points are significantly different; $P < 0.05$.

the pre-ovulatory LH peak and, consequently, the functionality of the corpus luteum and progesterone concentrations, leading to a reduction in pregnancy rates [34]. To our knowledge there are no reports on OPU-associated variations in cortisol plasma concentration in buffalo, but, in a previous trial conducted by our research group it was demonstrated that repeated OPU procedures in buffalo did not affect welfare, as indicated by the low concentrations of haptoglobin (a reliable marker of acute stress), lysozyme, complement, and bactericidal capacity [35]. Despite this, an increase in cortisol concentrations associated with the OPU procedure has been previously reported in cattle [36] and sows [37]. Our experiment follows this pattern, showing elevated cortisol concentrations at T2 compared to both T0 and T1, regardless of the treatment. Even if no statistically significant differences were observed between groups, a lower variability of cortisol concentrations was observed at both T1 and T2 in the FSH-HA group compared to the same time points in the FSH-6 group. This is likely due to reduced manipulation during hormonal stimulation and aligns with a previous study by Biancucci et al. [16], which demonstrated that minimizing animal handling by using HA as a vehicle for gonadotropins could decrease the activation of the hypothalamic-pituitary-adrenal axis in a

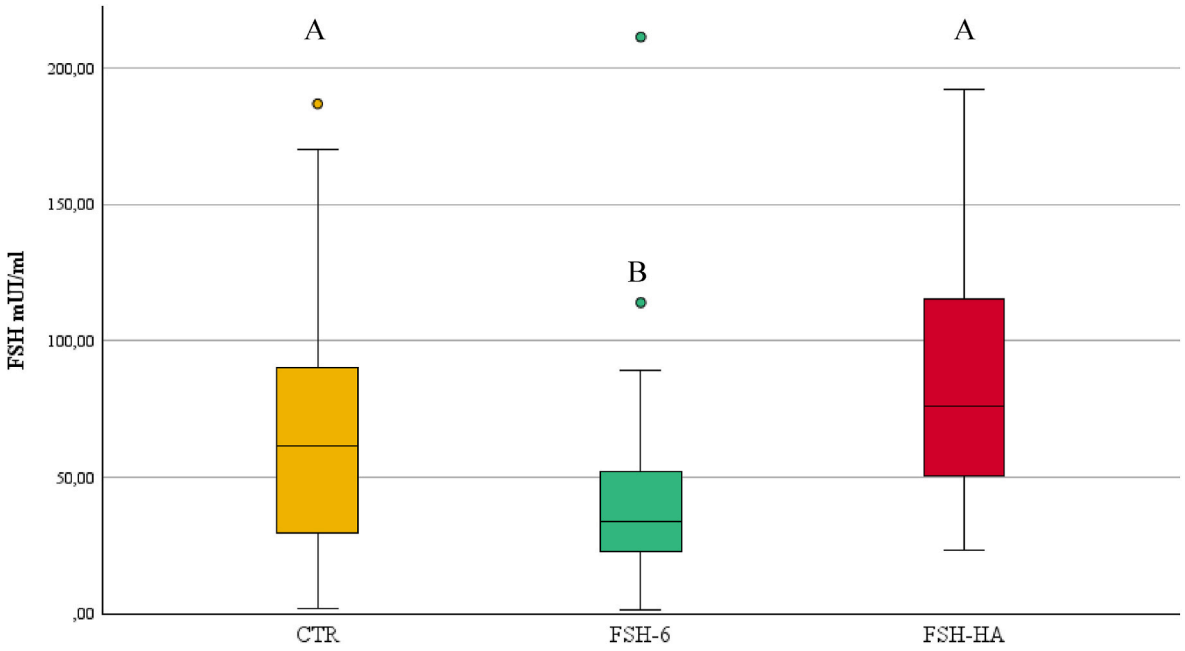

Fig. 5. Average concentrations (mean ± SD) of FSH in plasma collected in unstimulated buffaloes (CTR), stimulated with a standard 6-injections protocol (FSH-6), and stimulated with one injection of FSH dissolved in HA (FSH-HA)
[A,B] Values with different superscripts within groups are significantly different; $P < 0.01$.

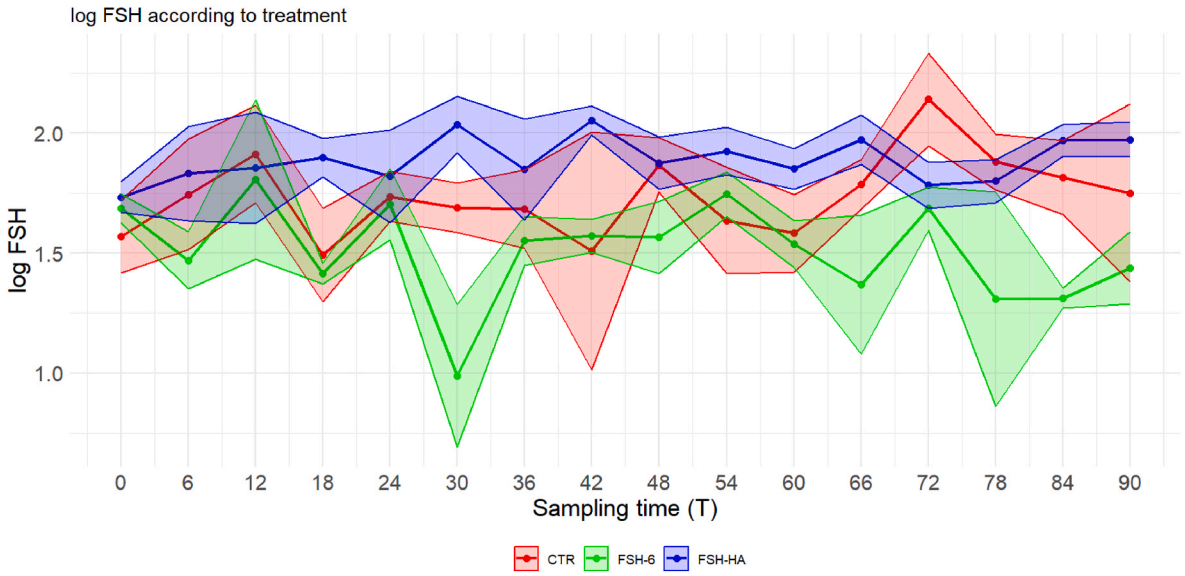

Fig. 6. Trend of FSH (log of average concentration mUI/ml + SE) plasma concentrations in unstimulated buffaloes (CTR, n = 4), stimulated with standard 6 injections protocol (FSH-6, n = 4), and stimulated with one injection of FSH dissolved in hyaluronan (HA) solution (FSH-HA, n = 4) collected every 6 h from day 2 after the dominant follicle removal to the moment of Ovum pick-up (OPU).

superovulation program in Marchigiana cattle, leading to an increase in the number of transferable embryos. In Experiment 2, the plasmatic profile of endogenous FSH during ovarian stimulation with porcine pituitary-derived FSH in IMB was assessed. To the best of our knowledge, this is the first report on endogenous FSH concentrations in the IMB during stimulation with exogenous gonadotropins. Additionally, in the unstimulated (CTR) group, this study provides the first indication of physiological FSH concentrations associated with the presumed emergence of a new follicular wave during IMB.The FSH concentrations reported in the present study should be interpreted with caution, as this represents the first validation of the specific bovine FSH immunoassay in the buffalo species and on a limited number of animals. Total average FSH concentrations in plasma differed among groups, with FSH-HA and CTR exhibiting higher mean concentrations compared to FSH-6. However, when the AUC was calculated as previously reported by Vieira et al. [17], FSH-HA and FSH-6 showed higher values compared to CTR, indicating the maintenance of constant plasma FSH concentrations. The multidose FSH-6 protocol induced the highest FSH concentrations compared to CTR and FSH-HA, only around 12 h after the start of stimulation. This partially contrasts with a similar experiment in bovine heifers, where FSH delivered in HA resulted in a higher short-term response, but an overall lower response compared to the multidose protocol [17]. However, it is worth noting that the experiment referenced above differs not only in species but also in the stimulation

protocol, as well as the concentrations of HA and FSH, compared to our study. We hypothesize that the lower FSH concentrations observed in the FSH-6 group may be attributed to a negative feedback on the hypothalamic-pituitary-gonadal axis. As follicular and oocyte population were similar between the two stimulation protocols, it is likely that in the FSH-6 group, the follicular growth is primarily driven by exogenous FSH. Conversely, as shown in Fig. 6 and Supplementary Table 2, the FSH-HA group exhibited sustained elevated FSH concentrations beginning 6 h post-administration, which persisted up to 66–72 h.

Additionally, administering FSH in HA allowed for a more gradual decrease in FSH concentrations, which returned to values similar to those observed during the initial sampling (0 h) between 72 and 78 h and rose again at 84 h. Based on these observations, it was hypothesized that the optimal timing for oocyte harvesting, when FSH is dissolved in HA, should likely occur between 72 and 78 h after FSH administration when endogenous FSH concentrations return to baseline.

In conclusion, it has been shown that a single administration of FSH in an HA solution is as effective as the multi-injection protocol in terms of embryo production. The use of HA resulted in higher circulating FSH concentrations compared to the six-injection protocol, although this did not lead to an increased embryo yield. This highlights the need for further research to better determine the optimal timing for oocyte retrieval in the presence of HA as a vehicle of gonadotropins. This would be possible by also investigating the relationship between FSH concentrations and follicular growth in future studies. Furthermore, the use of HA allowed us to reduce the number of manipulations to which the animals are subjected, which was reflected in a tendency to have lower cortisol concentrations, suggesting a positive impacts impact on animal welfare by reducing the stress associated with handling procedures.

CRediT authorship contribution statement

Michal Andrzej Kosior: Writing – original draft, Methodology, Investigation, Conceptualization. **Valentina Longobardi:** Writing – original draft, Methodology, Investigation, Conceptualization. **Chiara Del Prete:** Writing – review & editing, Data curation. **Riccardo Esposito:** Investigation. **Federica Piscopo:** Investigation. **Giorgio Antonio Presicce:** Methodology, Investigation. **Alessio Cotticelli:** Investigation, Data curation. **Tanja Peric:** Methodology, Conceptualization. **Natascia Cocchia:** Writing – review & editing, Methodology, Conceptualization.

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Declaration of interest statement

The authors of the manuscript “Effect of a single administration of FSH delivered in hyaluronic acid on oocyte competence and hormonal concentrations in Italian Mediterranean buffaloes undergoing ovarian stimulation prior to ovum pick-up” submitted to the International Journal “Theriogenology”, declare that:

There are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2025.117569.

Data availability

All data generated or analyzed during this study will be available on request.

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