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Effect of pretreatment with insulin on the response of buffaloes with inactive ovaries to gonadotrophin-releasing hormone agonist treatment in summer

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Abstract. The aim of the present study was to evaluate the effect of a pretreatment with insulin on the response of buffalo cows with inactive ovaries to gonadotrophin-releasing hormone agonist (GnRHa) treatment during hot summer months (July and August). Thirty-six Egyptian buffalo cows with inactive ovaries were randomly allocated into three groups: (1) group treated with GnRHa (G1, $n = 16$) in which each buffalo received an intramuscular injection of 250 mg of GnRHa (Day 0; gonadorelin, Fertagyl); (2) group treated with insulin before the GnRHa injection (G2, $n = 8$) in which each buffalo received a subcutaneous injection of biphasic insulin at a dose of 0.25 IU kg^{-1} bodyweight once daily starting at Day −3 for 3 consecutive days, followed by an intramuscular injection of 250 mg of GnRHa on Day 0; and (3) the control group (G3, $n = 12$) in which each buffalo cow received an intramuscular injection of 2.5 mL of sterile saline on Day 0. The ovaries of all animals were examined by trans-rectal ultrasonography (5 MHz) on Days −7, −3 and 0 and continued thereafter at 4-day intervals until oestrus or the end of the experiment. On the same days that ultrasound examinations were performed blood samples were collected to measure the progesterone concentrations. Administration of insulin for 3 days before GnRHa injection (G2) significantly ($P < 0.05$) increased the diameter of the largest follicle from 6.85 ± 0.64 to 12.4 ± 0.88 mm. There was a significant ($P < 0.01$) increase in the oestrous induction rate in G2 compared with both G1 and G3 during the first 12 days after the treatment interval. It is concluded that pretreatment with insulin for 3 days before GnRHa injection increases the diameter of the dominant follicle and therefore the oestrous induction rate of acyclic buffaloes.

Introduction

Breeding efficiency in buffaloes is reduced during the hot season from March to August with longer daylength, when ~80% of non-pregnant buffaloes have quiescent ovaries (Razdan 1988; Manik *et al*. 2002; Nanda *et al*. 2003). Heat stress reduces the luteinising hormone (LH) pulse frequency and therefore follicle maturation and oestradiol production in buffaloes (Palta *et al.* 1997; De Rensis and Scaramuzzi 2003). Gonadotrophin-releasing hormone (GnRH) agonist (GnRHa) treatment, which normally induces oestrus in cattle, failed to do so in summer acyclicity in buffalo (Sadasivarao and Rao 1984; Nanda *et al.* 1991) as the response rate was 0 (Khurana *et al.* 1982) and 11% (Singh *et al.* 1984). The limited half-life of GnRH analogue, which does not exceed 5–6 h in buffalo, renders the response of acyclic buffaloes unsatisfactory as it depends on the size and stage of ovarian follicles at the time of GnRHa treatment (Nanda *et al.* 1991; Palta and Madan 1995; Rastegarnia *et al.* 2004). In cattle, the presence or absence of a dominant follicle at the time of GnRHa administration is critical for the induction of ovulation (Roche *et al.* 1992) as the GnRHa injection causes ovulation of the largest (dominant) and luteinisation or atresia of smaller follicles (Thatcher *et al.* 1993; Twagiramungu *et al.* 1994; Wolfenson *et al.* 1994). In cattle, exposure to high temperature and humidity reduces the diameter of the dominant follicle, which may decrease the chance of ovulation in response to GnRHa treatment (Badinga *et al.* 1993; Wolfenson *et al*. 1995).

Insulin has been reported to influence LH release by the anterior pituitary and to play a role in regulating ovarian responsiveness to gonadotrophins in cattle (Spicer and Echternkamp 1995; Mognet and Martin 1997; Poretsky *et al*. 1999). Increased serum insulin concentration promotes the differentiation and maturation of the dominant follicles so that they become ready for ovulation in response to the LH surge (Beam and Butler 1997, 1999). Administration of insulin in cows increases steroidogenic capacity and the diameter of the largest follicle (Simpson *et al*. 1994). In buffaloes with inactive ovaries, increasing the energy density of the diet promotes an increase in the serum concentration of insulin, which is associated with an improvement in the

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oestrous resumption rate in response to GnRHa treatment (Bakr and Ramoun 2000). The aim of the present study was to test if the administration of insulin before GnRHa injection has an enhancing effect on the ovulatory response of acyclic buffalo cows.

Materials and methods

Animals and management

Thirty-six acyclic buffalo cows (>180 days post-partum), 4–7 years in age, were used to conduct the present study. The body condition score of the animals was 3.71 ± 0.40 (scale 1 = thin to 5 = fat; Bhalaru *et al.* 1987). The experimental period extended from July to August when the relative humidity was 60–70% and the ambient temperature was 34–40°C. The animals were kept in Mahallet Mousa Research Station belonging to the Animal Production Research Institute, where 50% of the yard area was sheltered and the animals had free access to water. They were fed on a diet calculated to met both their maintenance and milk production requirements according to the recommendation of the Animal Production Research Institute (1997, unpublished data).

Animal grouping and treatment

The animals were considered to suffer from ovarian inactivity when no corpora lutea were detected by two rectal palpations of the ovaries (on Days −12 and −3), two ultrasound examinations (on Days −7 and −3) and when serum progesterone concentration did not exceed 0.5 ng mL^{-1} (Quintans *et al*. 2004) in two blood samples taken simultaneously at the time of rectal palpation.

The animals ($n = 36$) were randomly assigned into three groups: (1) group treated with GnRHa (G1, $n = 16$); (2) group treated with insulin plus GnRHa (G2, $n = 8$); and (3) control group treated with saline (G3, $n = 12$). At Day 0, each buffalo cow in G1and G2 received an intramuscular injection of 250 mg gonadorelin (Shah *et al.* 2002) (2.5 mL Fertgyl; Intervet International, B. V. Boxmeer, the Netherlands) whereas each one in G3 received an intramuscular injection of 2.5 mL sterile saline. Before GnRHa in G2, each buffalo received a subcutaneous injection of insulin at a dose of 0.25 IU kg^{-1} bodyweight (Simpson *et al.* 1994) in the form of Mixtard 30 HM (Nova-Nordisk, Bagsvaerd, Denmark) at Days −3, −2 and −1 (Day 0 = day of GnRHa injection). Mixtard 30 HM is a biphasic isophane insulin injection 30/70 in which each mL contains 30 IU of soluble and 70 IU of isophane biosynthetic human insulin.

Ultrasound examination of ovaries

The diameter of the largest follicle was measured using a real-time B-mode scanner (Ultrascan 900; Alliance, Quebec, Canada) equipped with a 5-MHz linear transducer (length 118 mm × diameter 23 mm). Transrectal scanning of the ovaries was conducted in all animals on Days −7, −3 and 0. The ultrasound scanning on Day 0 was performed to judge the effect of insulin on the follicular size in G2 and in comparison to G1 and G3. The ultrasound scanning was continued at 4-day intervals after treatment in all groups until oestrus or the end of the experimental period. It aimed to detect the development of preovulatory-sized follicles and predict the occurrence of oestrus.

Blood sampling and progesterone assay

Blood samples were collected by jugular vein puncture in all animals three times before and seven times after treatment simultaneously with the ultrasound examination of the ovaries. The collected blood samples were centrifuged at 1500*g* for 15 min and the harvested serum samples were stored at −20°C until progesterone was analysed.

Serum progesterone assay

Serum progesterone levels were measured by means of radio immunoassay according to El-Bana and Gamal (1987) in the laboratories of the Endocrinology Research Unit, Atomic Energy Authority, Cairo, Egypt. Standard points were made in buffalo hormone-free serum. The sensitivity of the assay was 80 pg tube^{-1} and the intra- and interassay coefficients of variation were 5.8 and 13.7%, and 7.9 and 5%, respectively, for high-quality (7 ng mL^{-1}) and low-quality (0.4 ng mL^{-1}) controls. The accuracy was tested by standard curves made in buffer matrix, and the two curves were superposed indicating a high degree of accuracy at all concentrations of progesterone.

Reproductive management and fertility status

Buffaloes were observed twice daily (at 0600 hours and 1800 hours) by the herdsman for at least 1 h each time for signs of oestrus, especially the acceptance of buffalo bull teaser by the buffalo cows (Vale *et al*. 1990). The oestrous induction rate (EIR) was determined based on the results of visual observations for oestrous signs, teasing results and changes in serum progesterone concentrations for each group. Buffalo cows were assumed to resume cyclicity when the serum progesterone level was 0.5 ng mL^{-1} (Quintans *et al*. 2004).

Twelve hours after heat detection, buffaloes were inseminated by an experienced inseminator using fertile semen. Pregnancy was diagnosed by ultrasound examination 35–40 days after insemination and the conception rate was calculated.

Statistical analysis

Fisher's exact test was used to compare both oestrous induction and conception rates among the three groups. The data concerning the body condition score and the largest follicle size were analysed using the general linear model procedure for calculation of least square means (SAS 1990). The least square means calculated for all groups on Days −3 and 0 were compared within each group and among groups using Dunican's multiple range test to study the effect of 3 days of insulin injections on subsequent follicular growth. Means of the serum progesterone levels of buffalo cows before (on Days −7, −4 and 0) and after (Days 4, 8 and 12) GnRH injection (only in responded buffalo cows) were compared among the three groups.

Results

Oestrous induction rate

Pre-GnRH injection of acyclic buffaloes with insulin for 3 days in summer improved their response to GnRH treatment. There was a significant increase in the EIR in G2 (Table 1) compared with G1 ($P < 0.01$) and G3 ($P < 0.05$).

Table 1. Oestrous induction rate and conception rate in buffalo treated with gonadotrophin-releasing hormone agonist (GnRHa) alone (G1), insulin plus GnRHa (G2) and the control group (G3)

Group	Oestrous induction rate (frequency) after GnRHa injection				Conception rate (frequency)		
	Days 1–4	Days 5–8	Days 9–12	Total	Induced oestrus	First spontaneous oestrus	Total
G1 ($n = 16$)	0	4/16	2/12	6/16[a]	0/6	4/6	4/6
G2 ($n = 8$)	4/8	4/4		8/8[b]	2/8	2/6	4/8
G3 ($n = 12$)	0	3/12	0	3/12[a,c]	0/3	0/3	0/3

[a,b] $P < 0.01$; [b,c] $P < 0.005$.

Table 2. Diameter of the largest follicle (mm) in buffalo treated with gonadotrophin-releasing hormone agonist (GnRHa) alone (G1), insulin plus GnRHa (G2) and the control group (G3)

Least squares means ± s.e. Uppercase letters are used for the comparison between values in the same row (within the groups). Lowercase letters are used for comparison between values in the same column (among groups) ($P < 0.05$). Day −3 = the day at which the diameter of the largest follicle was measured in G1 and G3, and immediately before the injection of insulin in G2. Day 0 = the day at which the diameter of the largest follicle was measured in G1 and G3, and after 3 days of insulin injection (Days −3, −2 and −1) and before gonadotrophin-releasing hormone agonist injection in G2

Group	Diameter of the largest follicle (mm)	
	Day −3	Day 0
G1	6.56 ± 0.62^{aA}	6.85 ± 0.64^{bA}
G2	6.71 ± 0.87^{aB}	12.4 ± 0.88^{aA}
G3	6.67 ± 0.71^{aA}	6.84 ± 0.69^{bA}
Total	6.65 ± 0.43^{B}	8.72 ± 0.43^{A}

Conception rate

Whereas none of the animals that resumed cyclicity in the control group conceived, four out of six and four out of eight in G1 and G2, respectively, conceived (Table 1). Conception rate was higher ($P < 0.05$) in G2 compared with G3. All of the pregnant buffaloes in G1 conceived at the second oestrus after treatment (first spontaneous) whereas 50% of the pregnant animals in G2 conceived at the first oestrus after treatment (induced) and the other 50% conceived at the second oestrus after treatment (first spontaneous) (Table 1).

Size of the largest follicle

The ultrasound examination of the ovaries at the day −3 (i.e immediately before the first injection of insulin in G2) revealed non-significant differences in the size of the largest follicle among all groups. However, administration of insulin to animals of G2 induced a significant ($P < 0.05$) increase in the diameter of the largest follicle compared with the pretreatment in the same group and in G1 and G3 also (Table 2).

Serum progesterone level

Serum progesterone concentrations were $<0.5\ ng\ mL^{-1}$ before and after treatment in non-responder buffaloes, as well as in the pretreatment samples in the responder buffaloes, ensuring acycility. In contrast, the overall means of progesterone concentration increased ($P < 0.05$) after treatment ($>0.5\ ng\ mL^{-1}$) in the responder animals in all groups indicating resumption of ovarian activity (Table 3).

Discussion

Available literature dealing with the effect of insulin on reproduction in buffalo is scarce. The results of the present study suggest that the negative effect of heat stress on the reproductive axis is in part due to low concentrations of insulin, as pretreatment with insulin before GnRHa injection improved the fertility response of buffaloes. Post-partum anoestrus is caused by a variety of environmental factors, such as nutrition and heat stress, that alter the positive feedback mechanism between oestradiol and the LH surge, through the reduction of circulating concentrations of metabolic hormones (e.g. insulin and insulin-like growth factor-1), which increases the senitivity of follicles to gonadotrophins (Schallenberger and Prokopp 1995; De Rensis and Scaramuzzi 2003). Bakr and Ramoun (2000) found that increasing the energy density of the diet increased the response of buffaloes with inactive ovaries to GnRHa treatment, and increased serum insulin concentrations. This finding suggests that insulin may modulate the effect of energy on the ovaries so that follicles become responsive to GnRHa treatment.

In the present study, the significant increase in EIR in buffaloes treated with insulin for 3 days before GnRHa injection may be attributed to:

(1) The direct effect of insulin in enhancing growth of the largest follicle (Table 2), thus preparing a follicle to induce oestrus and ovulation after GnRHa treatment. This explanation is supported by our results as the size of the largest follicle increased significantly after insulin administration. Our results are in agreement with previous findings suggesting that insulin promotes differentiation and maturation of the dominant follicle, increasing its chance to ovulate in response to GnRHa (Beam and Butler 1997, 1999; Rhodes *et al*. 2003).
(2) The effect of insulin in enhancing the release of LH through the positive feedback effect of high levels of oestradiol produced by the largest follicles. A previous study in cattle showed that administration of insulin increased oestradiol production by the follicles (Simpson *et al.* 1994). Moreover it is proposed that LH released in G2 in response to the insulin-induced increase in serum oestrogen level throughout the 3 days of insulin injection simulates LH released physiologically in spontaneous oestrus (3–4 days of

Table 3. Serum progesterone concentrations (ng mL^{-1}) in buffalo treated with gonadotrophin-releasing hormone (GnRH) agonist (GnRHa) alone (G1), insulin plus GnRHa (G2) and the control group (G3)

Means ± s.d. Before treatment = before GnRH (G1 and G2) or saline (G3) injections (blood samples taken on Days −7, −3 and 0). After treatment = after GnRH (G1 and G2) or saline (G3) injections at 4-day intervals. Lowercase letters are used for comparison between values ($P < 0.05$)

Group	Before treatment				After treatment			
	Day −7	Day −3	Day 0	Overall	Day 4	Day 8	Day 12	Overall
G1 ($n = 16$)	0.20 ± 0.05^{a}	0.15 ± 0.03^{a}	0.16 ± 0.02^{a}	0.17 ± 0.03^{a}	0.39 ± 0.17^{b}	1.86 ± 0.46^{b}	0.94 ± 0.90^{b}	1.06 ± 0.05^{b}
G2 ($n = 8$)	0.18 ± 0.03^{aA}	0.18 ± 0.04^{aA}	0.24 ± 0.12^{aB}	0.20 ± 0.07^{a}	1.10 ± 1.26^{b}	2.55 ± 2.87	0.81 ± 0.95^{b}	1.48 ± 1.97^{b}
G3 ($n = 12$)	0.14 ± 0.01^{a}	0.17 ± 0.02^{a}	0.15 ± 0.01^{a}	0.15 ± 0.01^{a}	0.57 ± 0.12^{b}	0.57 ± 0.11^{b}	1.95 ± 0.27^{b}	1.03 ± 0.17^{b}

[A]Progesterone values measured in the blood samples before insulin injection on Days −7 and −3.
[B]Progesterone values in the blood samples taken after the end of a 3-day course of insulin treatment and just before the GnRH injection at the Day 0.

pro-oestrus phase) in both amplitude and frequency. But in G1 animals, injection of GnRHa may only result in a short-lived release of LH (5–6 h, half-life period of LH; Palta and Madan 1995).

The non-significant difference in EIR between buffaloes treated with GnRHa and the control group may be attributed to high temperature and humidity, which may interfere with the response of the pituitary gland to GnRHa treatment. This explanation may be supported by Nanda *et al*. (1991), who found that the LH response to exogenous GnRHa was suppressed in summer anoestrous buffalo. Even if the administration of GnRHa resulted in a short-lived release of LH, it would not find a large preovulatry follicle (G1; Table 2) necessary to start the sequence of events occurring before the reinitiation of cyclicity.

That heat stress reduces the degree of dominance of selected follicles is well known and is supported by our findings as the size of the largest follicle in both G1 and G3 did not exceed 7 mm before GnRHa injection, whereas it increased to 12.4 ± 4.6 mm following the 3 days of insulin injection in G2 (Table 2; Badinga *et al*. 1993; Wolfenson *et al*. 1995; Vasconcelos *et al*. 1998; Wilson *et al*. 1998; Roth *et al*. 2001; De Rensis and Scaramuzzi 2003). The EIR obtained in G1 (37.5%) and G3 (25%) are comparable with the results reported previously for buffalo treated with GnRHa (27.5%) and non-treated buffalo (18%; Sadasivarao and Rao 1984; Narasimha Rao and Venkatramiah 1991).

The significant increase in the diameter of the largest follicle promoted by the insulin treatment in G2 may be attributed to the stimulatory effect of insulin on follicular growth. Simpson *et al*. (1994) found that exogenous administration of insulin significantly increased the diameter of the largest follicle in insulin-treated compared with control cows. The stimulatory effect of insulin on follicular growth may be attributed to the direct effect of insulin on its own receptors present in the ovary (Poretsky and Kalin 1987) or indirectly through increasing the sensitivity of the follicle to follicle stimulating hormone (Simpson *et al*. 1994).

The lack of significant difference in conception rates among groups may be attributed to the low number of animals used in this study. The non-significant increase in conception rates in G1 and G2 indicated that both treatments may counteract the suppressing effect of heat stress on the follicles in the responder animals. This effect may be achieved by turnover of the heat-stressed follicles and replacement with healthy ones in G1. In this group the replacement of heat-stressed follicles with healthy follicles may occur through ovulation of the dominant follicle or luteinisation of smaller ones present at the time of GnRHa injection (Twagiramungu *et al.* 1994; Wolfenson *et al.* 1994). This may explain why all animals in G1 conceived at the first spontaneous oestrus but none at the induced oestrus. In G2, insulin has been suggested to stimulate the development, maturation and steroidogenic capacity of heat-stressed follicles, preparing them to ovulate in response to GnRHa injection. The optimum development of granulosa cells resulted in the secretion of large quantities of oestrogen, oocyte maturation and ovulation leading to successful fertilisation and maintenance of corpus luteum (Singh 2003). The ovulation of the insulin-induced large follicles in G2 animals resulted in development of well-functional corpora lutea as suggested by the higher progesterone concentration in G2 compared with G1 (Spicer and Echternkamp 1995; Webb *et al.* 1999). An increase in the conception rate was obtained after feeding on a diet that increased insulin concentrations in post-partum cows (Gong *et al.* 2002). The beneficial effect of insulin may explain why 50% of buffalo cows in G2 conceived at the first oestrus compared with 0% of buffaloes in the G1 and G3 groups.

We conclude that pretreatment with insulin for 3 days before GnRHa injection increases the size of the largest follicle and the oestrous induction rate in buffaloes suffering from summer acyclicity.

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