Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(6): 1468-1470

Received: 15-08-2020
Accepted: 07-10-2020

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Superovulatory response and embryo yield in buffaloes (*Bubalus bubalis*)

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Abstract
Murrah graded pluriparous buffaloes (n=10) were subjected for three superovulatory treatments. Treatment I (Control; n=20): Follicle Stimulating Hormone (600 mg) was initiated on the Day 10 for a period of five days. Treatment II (SO-OS; n = 7): Superstimulatory treatment was initiated after sychnronization of oestrous cycle by Ovsynch protocol. Gonadotrophin treatment for superovulation was initiated on Day 6 of synchronized cycle. Treatment III (SO-FWS; n = 7): GnRH (Inj. Receptal; 10 µg i.m.) was administered to all the animals on Day 6 of the cycle (Day 0: natural oestrus) and FSH treatment was initiated 96 h after GnRH (Day 10). Animals were inseminated thrice with frozen thawed proven during superovulatory oestrum and embryos/ova were recovered non-surgically on Day 5.5 of the superovulatory cycle. Six animals (30.0%) in control group did not respond for the treatment, while all the animals (100%) in SO-OS and SO-FWS groups responded for superovulation treatment. The mean number of CL is significantly ($P < 0.05$) higher in SO-OS group than the other groups. The control and SO-FWS groups had significantly ($P < 0.01$) greater number of anovulatory follicles (AFs). More percentage of transferrable quality embryos were recovered from SO-OS group, while more number of unfertilized ova and poor quality embryos were recovered from control and SO-FWS groups. It could be concluded that superovulatory response, embryo yield and embryo quality are better in buffaloes superovualted after the Ovsynch protocol.

Keywords: Buffaloes, superovulation, superovulatory response, embryo yield, embryo quality

Introduction
Buffaloes constitute a significant part of the domestic stock and contribute very significantly in the rural economy, especially in South and South-East Asia. Multiple ovulation and Embryo transfer technique (MOET), a proven tool for faster multiplication of desired genetic resource has been satisfactorily used for cattle and is being adopted for buffalo. The ovarian response of buffaloes to superstimulatory treatment has been less than one third of that reported in cattle. Following very limited research in buffalo, the viable embryo production has increased significantly from less than 1 per flushing to 2.5-3.0 in general and over 4 in isolated cases [6]. Poor response to superovulation and low embryo recovery is attributed to low primordial follicle pool in buffaloes as compared to cattle and high rate of follicular atresia. It was hypothesized that superovulatory response and embryo yield could be improved by initiating the gonadotrophin treatment after synchronizing the follicular wave emergence. Hence the present study was designed to study the effect of protocols combined with follicular wave synchronization (FWS) on superovulatory response (SOR) and embryo yield in buffaloes.

Materials and methods
Healthy Murrah graded pluriparous buffaloes (n=10), aged 5-6 years, maintained at Centralized Embryo Biotechnology Unit, Department of Animal Biotechnology, Madras Veterinary College, Chennai – 600 051, Tamil Nadu were utilized and were maintained under uniform managerial conditions throughout the study. They were fed with adequate concentrates, green fodder, paddy straw and *ad libidum* water. They were monitored regularly for oestrus symptoms and cyclicity of the animals was confirmed by frequent rectal examination. All the animals were subjected for three superovulatory treatments, as mentioned below with an interval of two months each.

i) Treatment I: Conventional (Control)
Superstimulatory treatments with Follicle Stimulating Hormone (600 mg NIH-FSH-P1 Folltropin-V; Bioniche Animal Health Inc., Athens, GE, USA) were initiated on the Day 10 of

(anticipated time of second follicular wave emergence) in twice daily tapering doses at 12 h interval for a period of five days (100mg:100mg; 80mg:80mg; 60mg:60mg; 40mg:40mg and 20mg:20mg). Superovulatory oestrus was induced with administration of Inj. Lutalyse (Dinoprost tromethamine: 25mg each, i.m.; Pfizer Manufacturing Belgium NV, Belgium) twice, 72 and 84 h after first FSH injection.

ii) Treatment II: Superovulation after Ovsynch (SO– OS)

Superstimulatory treatment was initiated after sychnronization of oestrous cycle by Ovsynch protocol. All the animals were injected with GnRH (Inj. Receptal; 10 µg i.m.), followed by PG administration after 7 days and injected with GnRH (10 µg i.m.) 48h later (GnRH – PG – GnRH). Gonadotrophin treatment for superovulation was initiated on Day 6 of synchronized cycle i.e. 6 days after the second GnRH, the expected day of follicular wave emergence. Superovulation schedule was followed as described in control group.

iii) Treatment III: Superovulation after follicular wave synchronization (SO-FWS)

GnRH (Inj. Receptal; 10 µg i.m.) was administered to all the animals on Day 6 of the cycle (Day 0: natural oestrus) and FSH treatment was initiated 96 h after GnRH (Day 10) [7]. Superovulation and other procedures were followed as described previously.

A total of 34 superovulation programmes (Control: 20; SO-OS:7 and SO-FWS: 7) were conducted.

All the treated buffaloes were observed for oestrus signs for 30 min at six hourly interval starting from 24h after PGF2α injection. Simultaneously rectal palpation was also done to confirm oestrus. The exhibition of first oestrus sign after the PGF2α injection was considered as onset of oestrum. Animals were inseminated thrice with frozen thawed proven buffalo bull semen at 12h interval, starting from 36h after $PGF_2\alpha$ injection in all the groups.

Embryos were recovered non-surgically on Day 5.5 after the onset of superovulatory oestrum (SOE), from the uterine horns with Dulbecco's Phosphate Buffered Saline (BIOLIFE Advantage, Agtech Inc., USA) by intermittent gravity flow through two-way, round tip Foley catheter. On the day of embryo collection, the SOR was assessed by estimating the number of corpora lutea (CL) and anovulatory follicles (AF) by rectal palpation and confirmed by ultrasound scanning (Sonovet 600, Universal Medical Systems equipped with a 7.5 MHz rectal probe). Based on which animals were categorized as either responders (animals having > 2 CL) or non-responders (animals having $\leq$ 2 CL) [12].

The flushed media was filtered through 75 µm EmCon embryo filter (Agtech Inc., USA). About 30-50ml of the flushed medium was transferred to Petri dishes and screened under a zoom stereomicroscope (Nikon) for the presence of embryos. Subsequently embryos were transferred to the holding media containing DPBS + 0.4% Bovine serum albumin (BSA fraction V) and evaluated. Numbers of embryos, unfertilized ova and transferable embryos collected were recorded for each animal.

The embryos were morphologically scored for quality, color and developmental stage [5]. The embryos were classified into four grades (1 = excellent; 2 = good; 3 = fair; 4 = poor). The grade 1 and 2 embryos were considered as transferable, while grade 3 and 4 were non-transferable quality embryos. Apart from the unfertilized oocytes (UFO), embryos in earlier developmental stages than morulae were categorized as 'arrested or degenerated'.

Mean and standard error values were arrived for SOR and embryo recovery rate. The data were analysed statistically as per the standard procedures [11].

Results and Discussion

The SOR and embryo yield in superovualted buffaloes subjected to various protocols were studied.

Superovulatory response

The data on SOR is represented in the Table1. Six animals (30.0%) in control group did not respond for the treatment and they were not flushed, while all the animals (100%) in SO-OS and SO-FWS groups responded for superovulation treatment. The mean SOR was non-significantly ($P > 0.05$) higher in SO-OS group than the other two groups.

Gonadotrophins stimulate growth of multiple follicles and induce multiple ovulations; however, the follicular response remains variable within and between the animals. In the control group only 70 per cent of the animals responded to the gonadotrophin stimulation. In general, thereduced response could be associated with low antral follicle populations and high levels of follicular atresia [2]. One of the reasons for variability is the number of gonatrophin responsive follicles present in the ovary at the time of superovulation treatment and the other is the number of follicles that actually ovulate subsequent to superovulation [6]. Studies confirmed that follicular development in buffalo also occurs in waves [1,8]. The latter author explained that the dominant follicle (DF) of anovulatory waves remained in the static phase for a significantly increased more period than in crossbred cows, which indicated early loss of gonadotropin receptors in the DF of buffaloes. Thus, a wide variation in SOR observed with the conventional treatment could be attributed to animal-to-animal and cycle-to-cycle variations in follicular status at the onset of superstimulatory treatment [9].

Significantly ($P < 0.01$) more number of AFs were recorded in control and SO-FWS groups when compared to SO-OS group. Aberrations in the follicular maturation process after FSH stimulation might be a reason for poor ovulatory response in superovulated heifers [3]. According to Duchens *et al.* [4], high P_4 concentrations on the day of SOE inhibited the E_2 induced LH surge and thus the ovulation was prevented. However in the present study we have not conducted any endocrinological study, hence the reason for incidence of AF could not be ruled out precisely.

Embryo recovery rate

The data on embryo recovery rate is depicted in the Table1. The embryo recovery rate was significantly ($P < 0.05$) high in SO-OS group when compared to Control and SO-FWS groups. The poor recovery rates in the latter groups could be attributed to increased number of AFs on the day of collection. As suggested by Sato *et al.* [10], abnormally high levels of oestradiol (E_2) secreted by AFs which were found persisting from the day of SOE to the day of embryo collection might have hindered the ova or embryo transport in the oviducts.

Embryo developmental stage and quality

The developmental stages and quality of ova/embryos recovered from all the groups were represented in Table 3. A total of 27 embryos were recovered from superovulated buffaloes. By day 5.5 post onset of superovulatory oestrus, majority of the embryos were in morula stage. Significantly more percentage of transferrable quality embryos were recovered from SO-OS group. In both the control and SO-FWS groups, 50.0 per cent of the recovered ova were

unfertilized (UFO). Increased incidence of UFO and poor quality embryos in these two groups could be correlated to high E_2 concentrations from more number of AFs which would have severely compromised the fertilization and / or subsequent embryo development adversely. On the contrary to the findings in the crossbred cattle [9], superovulation after follicular wave synchronization did not yield good results in buffaloes. Hence further studies should be conducted in standardizing the GnRH induced synchronization of follicular wave emergence in buffaloes.

Table 1: Superovulation and embryo recovery rate in Murrah graded buffaloes

S. No	Treatment group	No. of animals programmed	No. of animals responded and flushed (%)	Mean Superovulatory response		Mean number of embryos recovered	Average embryo recovery rate (%)
				CL	AF		
1	Control	20	14 (70.0)	2.57 ± 0.27^a	3.47 ± 0.22^b	0.86 ± 0.14^a (12)	26.78 ± 3.18^a
2	SO-OS	7	7 (100.0)	3.43 ± 0.30^a	1.14 ± 0.26^a	1.29 ± 0.18^b (9)	36.67 ± 2.41^b
3	SO-FWS	7	7 (100.0)	3.14 ± 0.14^a	2.85 ± 0.14^b	0.86 ± 0.40^a (6)	25.00 ± 10.43^a
Significance				NS	**	*	*

Values within the column with different superscripts differ significantly
NS- Not signignificant; * $P < 0.05$; ** $P < 0.01$

Table 2: Embryo quality in superovulated Murrah graded buffaloes

S. No	Treatment group	Stages of embryos	Quality of embryos				
			I	II	III	IV	Transferrable embryos
1	Control (12)	Morula (6)	3	1	1	1	6 (50.00%)
		Early Blastocyst (1)	--	1	--	--	
		Blastocyst	--	--	--	--	
		Unfertilized (5)	5				
2	SO-OS (9)	Morula (5)	4	1	--	--	8 (88.89%)
		Early Blastocyst (3)	3	--	--	--	
		Blastocyst	--	--	--	--	
		Unfertilized (1)	1				
3	SO-FWS	Morula (4)	2	1	--	1	4 (66.67%)
		Early Blastocyst	--	--	--	--	
		Blastocyst	--	--	--	--	
		Unfertilized (2)	2				

Conclusion

Perusing the findings of the study it could be concluded that SOR, embryo yield and embryo quality are better in buffaloes superovualted after the Ovsynch protocol. The findings indicated that the synchronization effected by Ovsynch protocol would have regularized the cycle with more homogenous group of follicular inventories at the time of initiating the superovulation treatment.

Acknowledgement

The research work was carried out under the ICAR-NAIP funded project on 'Transcriptional Level of Developmentally Important Genes in Buffalo Preimplantation Embryos'.

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