SUPEROVULATORY RESPONSES AND EMBRYO RECOVERY IN GERMPLASM CONSERVATION OF SEMI WILD TODA BUFFALOES OF NILGIRIS

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ABSTRACT

As a breed conservation measure in Toda buffaloes, a unique, ferocious, semi wild breed of Nilgiris district of Tamilnadu, whose population is dwindling over the years, an attempt has been made towards the collection and cryopreservation of embryos. Toda buffalo cows (10) and Toda bulls (3) used for the study were representative samples of the breed sourced from Toda hamlets in the breeding tract and maintained under organized farm conditions. The response for superovulation was 93.33% (28/30) and 82.14% (23/28) of the animals were flushed. A total of 39 embryos were recovered, of this 26 viable embryos were cryopreserved. The viable embryo recovery rate per animal flushed was 1.13. buffaloes administered with 400 mg of FSH responded higher than those administered with 600 mg. Buffaloes with bigger superovulatory corpus luteum (SOV CL) had significantly higher ovulation rate (3.50 ± 0.26) and viable embryo recovery (1.46 ± 0.49) than animals with medium or small SOV CL. The viable embryo recovery rate was higher on 10th day SOV (1.35 ± 0.36) than on 9th day (0.75 ± 0.75) of starting of FSH treatment. Donors in standing estrum yielded a significantly higher number of total embryos (2.00 ± 0.41 vs 0.60 ± 0.30) and viable embryos (1.33 ± 0.36 vs 0.40 ± 0.40) than the donors with non standing estrum.

Keywords: buffaloes, *Bubalus bubalis*, Toda buffalo, conservation, superovulation, embryo cryopreservation

INTRODUCTION

The Toda buffaloes, a unique breed traditionally maintained by the Toda tribes of Nilagiri district, has a long history as it was first described by Finicio in 1603 (cited by Rivers, 1906). Usually a herd of Toda buffaloes consists of a few females with rarely one or two males. Toda bulls are known to stay in dense forests and will come out during the breeding season. Toda buffaloes are known for a very high fat (8.00%) content of milk (Karthikeyan, 1995). Toda buffaloes are very hardy and they survive and flourish on pasture alone, without additional feed/fodder. They are well adapted to harsh climate of Nilgiris.

The breed is associated with the Toda tribals economically and socially. With the change in the living pattern of the Todas, the breed has lost its economic importance but still it has a cultural / religious relation with their owners. A recent census (2013) indicated that the Toda buffalo population in Nilgiris district has decreased from 3314 in 1994 to 1320 in 2013. In addition the natural habitats of Toda buffaloes are fast disappearing; the grass land

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have come down by a factor of six from 29,875 ha in 1849 to about 4700 ha and in few areas of the district there is 100 percent reduction in grass land (Kumar, 1997). Hence intense species management becomes highly essential for the Toda buffaloes. The oocyte and embryo related technologies for enhancing reproduction has been used significantly in livestock, laboratory animals and humans (Farstad, 2000; Sharkey *et al*., 2001; Squires *et al*., 2003; Baldassarre and Karatzas, 2004 and Devroey and Van Steirteghem, 2004). However, embryo transfer and IVF had a negligible impact on conservation of endangered and wild animals. This project with the aim of conservation of germ cell in the form of embryos in Toda buffaloes was carried out as a collaborative project between Sabarmati Ashram Gaushala-SAG (managed by National Dairy Development Board- NDDB) and Sheep Breeding Research Station, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai.

MATERIALS AND METHODS

Toda buffaloes

Characteristically, the Toda buffaloes are medium size animals; body is fairly long with a broad and deep chest. They have short, strong and sturdy legs. They have two characteristic white to light brown chevron markings, one around the jowl and other anterior to the brisket. The horns are typically long, set wide apart curved outward, slightly downward and upward with the points recurved inward forming a crescent shape or semi circle. They have chromosomal complement of 50 and the morphology resembles (first 5 pairs were submetacentric and the remaining 19 pairs were acrocentric) that of the river buffaloes (Murali *et al*., 2009).

Selection of animals

Ten buffalo cows with typical Toda buffalo characteristics were purchased from Toda hamlets in the breeding tract. The animals have calved at least once and have completed 1-4 lactations. All the animals were checked per-rectally before purchase to ascertain the normalcy of the reproductive organs and all the animals were found to have normal reproductive organs. All the cows were dry and non pregnant at the time of purchase.

Three Toda bulls at an age of 4 to 5 years were purchased by transfer on closure of an earlier conservation project of Toda buffaloes by Indian Council of Agricultural Research (ICAR) - National Bureau of Animal Genetic Resources (NBAGR) Karnal. The bulls were maintained in the research station from the age of 10 to 12 months under the above project.

Housing and management

All the animals were housed at Sheep Breeding Research Station, Sandynallah, The Nilgiris district, Tamil Nadu, India. During the day time animals were allowed for grazing on natural pastures of farm land and were housed in pucca sheds during night hours. The females were ferocious as they have been brought up under isolation. Initially it was very difficult to handle the female herd as they were never used to be tethered or handled (except milking). They have been applied with halter and few of them were applied with nose rope for better control and were trained for handling and casting in trevis. Towards the end of the project period, most the buffaloes could easily be handled. Few nervous animals were tranquilized during the flushing procedure. Although these animals are ferocious in nature, they never attacked any of the handler or the scientist during the procedure.

Feeding

The female Toda buffaloes being purchased from Toda tribes have been maintained under zero concentrate feeding. Hence, they were very reluctant to take concentrate feed. But, gradually they were accustomed to concentrate feeding and were fed with 2 kg concentrate / day. The feeding rate was increased to 3 kg of concentrate ration during the superovulation and embryo collection programmes. The bulls were reared from a young age and were accustomed to concentrate feeding and handling. The bulls were fed with 2 kg concentrate and supplemented with 3 to 4 kg of paddy straw.

Estrous synchronization and Superovulation

The females were routinely observed for exterior symptoms of regular estrum. The estrum was synchronized with either a single or two injections of Prostagladin (PG:- inj. Iliren – Dinoprost, 0.750 mg, Hoechst, India - i/m). The animals were checked per-rectally at 72 h for presence of follicle on the ovary, uterine tone and discharge. The animals reporting for estrum were selected for superovulation and were checked for the presence and quality of corpus luteum (CL) before FSH treatment. The animals were superovulated using either 300 mg or 400 mg or 600 mg of NIH-FSH-p (Folltorpin-V, Vetrepharm, Ontario, Canada). The superovulatory treatment was started on day 9 or 10 of the estrus cycle. FSH was given in 10 tapering doses for 5 days at 8.30 a.m and 8.30 p.m. Luteolysis was induced with two PG injections along with 7^{th} and 8^{th} FSH dose. All the animals reporting to estrum (at 48 h post PG) were bred by the bulls. In the second and subsequent programmes, GnRH 5 ml was injected along with 10^{th} dose of FSH to increase the ovulation.

Embryo collection and cryopreservation

The superovulated animals were flushed on day 5.5 or 6 after breeding. The number of corpora lutea was counted per-rectally before flushing. Flushing was carried out as per the standard procedures (Misra *et al*., 1990) using 18 gauge Rusch catheter (Minitub, Germany) and DPBS media (IMV, France). All the recovered embryos were evaluated as per standards given by International Embryo Transfer Society Manual (IETS) and viable embryos were frozen in 1.4 M Glycerol (Misra *et al*., 1992) using Planer R-204 cell freezer (Planer products Ltd, Sunbury-on-Thames, England).

Statistical analysis

The mean and standard error for all variables were calculated and presented. Difference between the batches of flushing (I, II, III and IV) was tested by Student 't" test. Similarly the effect of dose rate of FSH (300, 400 and 600 mg), size of SOV CL (Big, Medium and Small), day of starting of SOV (9^{th}, 10^{th}, 11^{th} and 12^{th} day) and quality of estrus (Standing and Non-standing) on the ovulation rate, total embryo and viable embryo recovery was assessed by 't" test.

RESULTS AND DISCUSSION

Conservation of Toda buffaloes, a semi wild and endangered animal in the form of cryopreservation of embryos was attempted in this study. During this project 30 animals (repeated flushings) were programmed, 93.33 percent (28/30) of the animals responded and 82.14% (23/28) of the animals were flushed. Total embryo recovery was 39 and out of this 26 viable and good quality embryos were cryopreserved. The total embryo recovery rate was 60.00% (39/65) when calculated based on the number of CL observed in these animals. The

viable embryo recovery rate per animal flushed was 1.13. There were 9 unfertilized ova (UFO) and empty zonas and four degenerated embryos.

Total embryo recovered and viable embryo recovered per flush in the present study was 1.70 and 1.13 respectively, which were well below the response in cattle. Most of the embryo collection studies in buffaloes throughout the world have observed a lower superovulatory response than cattle (reviewed by Perera, 2008). The lower response was attributed to the fewer number of recruitable follicles in ovary (Madan *et al*., 1996 and Manik *et al*., 2000) and relatively low rate of transfer of oocytes to the oviduct and impaired transport of ova and embryo in the reproductive tract (Baruselli *et al*., 2000).

The response for superovulation was 93.33 percent (28/30). One of the two animals that has not responded had a small SOV CL and was programmed on 12th day of the estrous cycle. The follicular wave not matching to the day of start of superovulation may be the reason for the animal not responding.

Only 23 animals could be flushed out of the 28 animals superovulated because there was handling damage to the cervix in four animals and one animal was highly non-cooperative. Few of the animals which were known to be non-cooperative and furious were given tranquilizer Xylazine hydrochloride (Xylaxin, Indian Immunologicals) at the dose rate of 10-15 mg per animal just before the start of flushing, still there were sudden violent movements during flushing and hence one animal was not flushed during the first batch of flushing. The embryo recovery rate (60.00%) observed in this study was in agreement with the finding of Misra *et al*. (1994). Out of the total embryo recovered (39) one third of them were not useful, in which seven were empty zona. In addition there were 10 embryos at blastocyst stage during this programme, which showed early ovulations or fast development of embryos in the superovulated animals. Further investigations are needed to clarify the problem.

Batch of flushing

The total embryo recovery rate was higher in II and IV batch of flushing (Table 1). First batch of flushing was during peak winter (January-having inadequate and poor quality pasture), when the condition of most the animals were poor and also the animals were subjected to synchronization and superovulation for the first time. The II and IV batch of flushing were carried out after giving a rest for 20 to 25 days from I and III batch of flushing. Hence the increase in the embryo recovery rate during the II and IV batch may be due to the availability of active CL of previous flushing.

Dose of FSH

Total number of CL, total follicles and total embryo recovery were similar in Toda buffaloes treated with 300 mg, 400 mg or 600 mg of FSH. But there was significant increase in viable embryo recovery in animals administered with 400 mg than 600 mg of FSH. In buffaloes a higher dose of FSH (600 mg) has been widely used for superovulation (Misra *et al*., 1994 and Madan *et al.,* 1996). However, results from the present study suggest that lower dose rates of FSH can be successfully administered in Toda buffaloes with better results. Similar results were obtained in Pandharpuri buffaloes at SAG, Gujarat (personnel communication).

The quality of SOV CL and day of start of SOV

The quality of SOV CL had significant effect on the ovulation rate and viable embryo recovery. Buffaloes with big SOV CL had

significantly higher ovulation rate (3.50±0.26) and viable embryo recovery (1.46±0.49) than animals with medium (1.79±0.29) or small SOV CL (1.75±0.31) (Table 2). Similarly the day of SOV also had significant difference in the viable embryo recovery at 9th and 10th day compared to 11th day. Although there was response to superovulation started on day 11 or more, the embryo recovery rate was zero. Out of three animals started SOV on 11th day, two responders could not be flushed due to very thin cervix which got damaged while handling, while one animal of 12th day SOV didn't respond to FSH.

Nature of estrum

The group of animals reporting to standing SOV estrum showed a non significant increase in the total ovulations and total follicles than in animal having non standing estrum. However, there was significant increase in total embryo recovery (2.00 ± 0.41 vs 0.60 ± 0.30) and viable embryos (1.33 ± 0.36 vs 0.40 ± 0.40) in animals showing standing estrum than non standing estrum.

The Toda buffaloes though known to be semi wild and ferocious can be tamed and reared under semi intensive system of management under farm conditions, if they were trained from a young age. Superovulation results shows that with the improved protocols like giving GnRH injections prior to SOV and inclusion of LH during SOV heat (Tatham, 2000), still better results can be achieved. On 6th day embryo flushing procedure adopted in the present study, more number of blastula stage embryo / empty zona was recovered. Hence, for arriving at the correct time of ovulation specific to this breed,

Table 1. Flushing report of Toda buffaloes.

Sr. No.	Particulars	I Batch (Jan-06)	II Batch (Mar-06)	III Batch (Sep-06)	IV Batch (Oct-06)	Cumulative
1	No. of animals selected for programming	8	8	8	9	33
2	No. of animals Programmed	7 (87.5%)	8 (100%)	8 (100%)	7 (77.8%)	30 (90.90%)
3	No. of animals responded	7 (100%)	8 (100%)	7 (87.5%)	6 (85.7%)	28 (93.33%)
4	No. of animals flushed	5 (71.4%)	7 (87.5%)	5 (71.4%)	6 (100%)	23 (82.14%)
5	Total no. of CL (Includes all responders)	14 (2.0)	25 (3.13)	16 (2.29)	20 (3.33)	75 (2.69)
5a	Total no. of CL (Flushed animals)	11 (2.2)	22 (3.14)	12 (2.4)	20 (3.33)	65 (2.83)
6	Total Follicles/ Cysts (Includes all responders)	8 (1.14)	8 (1.00)	11 (1.57)	9 (1.5)	36 (1.29)
6a	Total Follicles/ Cysts (Flushed animals)	6 (1.2)	8 (1.00)	10 (2.0)	9 (1.5)	33 (1.43)
7	Total Embryo Recovery	5 (1.0)	14 (2.00)	8 (1.6)	12 (2.0)	39 (1.70)
8	Viable Embryo Recovery	3 (0.6)	10 (1.43)	5 (1.0)	8 (1.33)	26 (1.13)
9	UFO / Zona	1	4	2	2	9
10	Degenerated Embryos	1	0	1	2	4
11	Total Embryos Frozen	3	10	5	8	26

flushing studies with more number of animals are required.

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Table 2. Mean (± SE) of superovulatory response and embryo recovery in female Toda buffalo.

Details	No. of animals flushed (responded)	Average no. of CL (Total)	Average no. of follicle (Total)	Embryo recovered (Total)	Viable embryo recovered (Total)
Batch					
Batch I	5 (7)	2.00±0.30 (14)	1.14±0.32 (8)	1.00±0.77 (5)	0.60±0.60 (8)
Batch II	7 (8)	3.13±0.43 (25)	1.00±0.22 (8)	2.00±0.69 (14)	1.43±0.72 (10)
Batch III	5 (8)	2.13±0.30 (17)	1.38±0.38 (11)	1.60±0.51 (8)	1.00±0.45 (5)
Batch IV	6 (7)	3.00±0.39 (21)	1.29±0.24 (9)	2.00±0.94 (12)	1.33±0.67 (8)
Dose					
300 mg	3 (3)	2.67±0.60 (6)	1.00±0.48 (3)	1.67±1.20 (5)	1.00±1.00^{a} (3)
400 mg	10 (13)	2.77±0.34 (36)	1.00±0.16 (13)	2.20±0.63 (22)	1.80±0.55^{a} (18)
600 mg	10 (14)	2.36±0.19 (33)	1.43±0.26 (20)	1.20±0.39 (12)	0.50±0.22^{b} (5)
SOV CL					
Big	13 (14)	3.50±0.26^{a} (49)	1.43±0.25^{a} (20)	2.08±0.58 (27)	1.46±0.49^{a} (19)
Medium	5 (8)	1.79±0.29^{b} (14)	1.00±0.26^{ab} (8)	1.60±0.24 (8)	1.20±0.37^{b} (6)
Small	5 (8)	1.75±0.31^{b} (7)	1.00±0.22^{b} (5)	0.80±0.37 (4)	0.20±0.20^{b} (1)
SOV Day					
9	4 (5)	2.00±0.42 (10)	1.40±0.42 (7)	2.00±0.91^{a} (8)	0.75±0.75^{b} (3)
10	17 (20)	2.85±0.24 (57)	1.25±0.19 (25)	1.76±0.42^{a} (30)	1.35±0.36^{a} (23)
11	2 (3)	2.33±0.43 (7)	1.30±0.30 (4)	0.50±0.50^{b} (1)	0^{b} (0)
12	0 (2)	1.5±0.29 (3)	-	-	-
Nature of Estrum					
Standing	18 (20)	2.75±0.24 (55)	1.45±0.19 (29)	2.00±0.41^{a} (36)	1.33±0.36^{a} (24)
Non Standing	5 (10)	2.20±0.27 (22)	0.70±0.21 (7)	0.60±0.40^{b} (3)	0.40±0.40^{b} (2)

Means in the same column within categories with different superscript differ significantly (P<0.05).

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