Journal of Animal Research: v.6 n.6, p. 1047-1052. December 2016

DOI: 10.5958/2277-940X.2016.00152.2

Effect of Supplementation of *Aegle marmelos* and *Murraya koenigii* on the Follicular Development in Delayed Pubertal Sahiwal Heifer

Brijesh Kumar[1,2*], Vikas Sachan[1], Anuj Kumar[1], Vijay Singh[1], Goutam K. Das[3] and Atul Saxena[1]

[1]Department of Veterinary Obstetrics and Gynaecolog (DUVASU) Mathura, U.P. INDIA
[2]ICAR Research Complex for NEH region, Sikkim Centre, Tadong, Gangtok Sikkim, INDIA
[3]Division of Animal Reproduction, IVRI, Izatnagar, U.P. INDIA

**Corresponding author: B Kumar; Email: drbrijeshvet02@gmail.com*

Received: 09 June, 2016 **Accepted:** 14 September, 2016

ABSTRACT

The present study was conducted to study the effect of *Aegle marmelos* and *Murraya koenigii* on the follicular development in delayed pubertal Sahiwal heifers. A total of 14 anoestrus Sahiwal heifers with a mean age of 32.85 ± 0.86 months and were divided in to two groups *viz*. Group-1 (G-1) (n=8) and Group-2 (G-2) (n=6). Heifers in G-1 were treated with shade dried grinded leaves in combination in concentrate mixture for 9 days after dose extrapolation from the effective 50% ethanolic extract dose in rats (*i.e.* 1000 mg/ Kg b. wt.) showing optimum ovarian function. Heifers in G-2 were not given any treatment. All the heifers were monitored by a trans-rectal USG scanner in alternate day for 10 occasions. At pretreatment, the difference in the mean number of total follicles and diameter of Largest follicles (LFs) between the groups remained (P>0.05) similar. However, on herb supplementation, the mean size of LFs (12.8 mm) increased (P<0.001) significantly as compared to control (8.70 mm). The growth rate of LF was faster (P< 0.05) in herbs treated (0.55 mm/day) than control (0.25 mm/day) heifers. The mean number of total follicles prior to treatment and each day of scanning remained similar between the groups (P> 0.05). Results revealed that in the delayed pubertal heifers, the follicular development continues and the number of total follicles at any day remained constant. It was concluded that supplementation of *Aegle marmelos* and *Murraya Koenigii* can influence the growth processes of LF by the attainment of dominance, accelerated growth rate, preovulatory size and the process of ovulation.

Keywords: Heifers, delayed puberty, herbs, follicular development

Reproductive efficiency is the key determinant influencing productivity of all the livestock enterprises. It is adversely affected by many infertility conditions including anoestrus. In dairy heifers, delayed puberty *i.e.* pubertal anoestrus has been recognized as one of the most common form of infertility conditions (Iyer, 1978; Shrivastava and Kadu, 1992).

The incidence of pubertal anoestrus is reported to vary between 38.7 to 64.1% and 12.92 to 52.50 % under farm and field condition, respectively (Luktuke *et al*., 1978; Shrivastava and Kadu, 1992). Anoestrus in dairy heifers is multi-factorial in nature and remained the subject of investigations over the decades (Deshpande and Velhankar, 1982; Behera *et al*., 1993; Abeygunanwardena and Damatawewa, 2004; Das *et al.*, 2016).

Sahiwal is a native cattle breed of India, has recently drawn planners attention because of their high milk production potential (Khune *et al*., 2016), heat and disease resistance as well as wider adaptability. Hence, Government of India has launched a promising scheme *Gokul Mission* to promote and propagate raring of high yielding indigenous breed of cattle with special attention to Sahiwal. However, the breed has some inherent limitations including shy in breeding with poor behavioural signs of estrus. Like other breeds, the delayed puberty is also a problem reported elsewhere in current literature (Kumar *et al.*, 2012). Many attempts are made to understand the causes of pubertal anoestrus in heifers during last few decades even then the persistence of the condition both at farm and field level indicates our inadequate knowledge about the pathogenesis of the condition. Similarly, many research investigations

involving both non-hormonal and hormonal treatments have also been carried out to develop the cost effective and suitable therapy to combat the condition by reducing the extent of loss due to anoestrus in heifers (Khade *et al*., 2011; Lone *et al*., 2012). But due to high cost involvement, inconsistent results and poor availability in rural pockets, most of the synthetic drugs and exogenous hormones have been failed to become popular to the poor dairy farmers. Recently, a number of herbal plants have drawn research attentions for their medicinal properties besides neutraceutical values. Accordingly, they brought future promise as an alternate therapeutic to treat reproductive disorders (Kadu *et al.*, 2001; Mehrotra, 2011).

Further, in some reports, two medicinal plants namely *Aegle marmelos* and *Murraya koenigii* have drawn researcher's attention for their promising estrus induction and fertility response in anoestrus animals (Dutt, *et al*., 2010; Kumar *et al.*, 2012; Das *et al.*, 2016). The estrus induction response after such herbs treatment is reported to be mediated through the increase in the number of medium and large follicle in anoestrus goats (Dutt, *et al.*, 2010). But effect of such herbs supplementation on the follicular development in anoestrus heifers is still lacking to the best of our knowledge (Das *et al*., 2016). Although in preliminary trial/ report, it has been found that supplementation of *Aegle marmelos* and *Murraya koenigii* increases the size of the largest follicle in the ovaries (Kumar *et al.*, 2012). However, a detailed description of the follicular growth pattern following treatment with those herbs is essential to understand the mechanism of action of such herbs on the ovary of responded heifers and also the pathogenesis of anoestrus in heifers those remained non-responded. In the present investigation, we have examined the follicular growth and development in anoestrus Sahiwal heifers after supplementation with *Aegle marmelos* and *Murraya koenigii* and to understand the changes in terminal follicular growth using ultrasonography.

MATERIALS AND METHODS

Location of study

The study was conducted at the Instruction Dairy Farm of Deen Dayal Upadhyaya Veterinary and Animal Sciences University, Mathura located at an altitude of 287 meters above sea level latitude: 27° 30' 0" North, and longitude: 77° 41' 0" East. The experimental trial was conducted during winter months between December and February.

Experimental animals and management

Fourteen Sahiwal heifers of mean age above 32.85 ± 0.86 months with mean body weight of 224.29±9.25 kg were used for the present study. All the heifers were subjected to daily bull parading by a morning evening schedule for detection of estrus. As per the record, the animals did not exhibited estrus prior to start of the treatment and declared as delayed pubertal anoestrus, which was further supported by trans-rectal clinical examination ten days apart showing true anoestrus condition. During entire period of study the heifers were confined to a barn with small covered and rest uncovered area with free movement inside enclosure. The daily feed consisted of adequate chaffed green fodder, wheat straw, concentrates (@ 2 kg/ animal/day, mineral mixture (@2%) and *ad lib* water.

Grouping of the animals

A total of 14 animals were allocated into two groups *i.e.* Group-1 (G-1, n= 8) and Group-II (G-II, n= 6). The heifers in G-I were supplemented with grinded shade dried leaf powder of *Aegle marmelos* and *Murraya koenigii* in combination mixed with concentrate mixture for 9 days per oral. And, heifers in group G-II were kept as untreated control.

Collection of herbal leaves and dose preparation

The green leaves of *Aegle marmelos* and *Murraya koenigii* were collected from in or around Indian Veterinary Research Institute, shade dried and grinded from local market. The dose in the form of powder for each heifer was calculated by dose extrapolation from rat (50% ethanolic extract effective @1000 mg/ kg for augmentation of ovarian function) to cattle using dose equivalent system (Van Miert, 1986) and subsequently converted in to powder form by the method described previously (Mehrotra, 2002; Jondhale, 2007; Dutt *et al*. 2010).

Ultrasonographic study of follicular development

All the heifers were subjected for real-time B mode trans

rectal ultarasonography (Mindray, DP-6600 Vet, China) with a frequency 7.5-MHz on alternate occasion to monitor the follicular development for a period of 22 days starting from pre-treatment till exhibition of estrus during the treatment cycle. Ovarian sketch were prepared for each animal per pair of ovary basis. The sizes (≥ 3 mm in diameter) and locations of all follicles on each ovary were recorded on a sketch of each ovary and retrospectively analyzed. Ovaries were scanned on lateromedial and dorsoventral planes (Ginther, 1993) for complete and accurate study of each ovarian follicle. Images of follicles were frozen at the maximum cross-sectional area, and the internal ultrasound caliper was used to measure the length and width of those structures. The diameter was determined by calculating the average of the length and width of each follicle (Zeitoun *et al.,* 1996).

Within the scanning period, each heifer showing the largest diameter of follicle in either of the ovary was recorded and the growth rate of the largest follicle was calculated by subtracting the minimum diameter from the maximum diameter and dividing this by the growth period in days.

Statistical analysis

Data on follicular parameters were analyzed by the Student 't' test for comparison of mean values for size of LFs pretreatment, maximum size of LFs, days taken to attain maximum size, growth rate of the LFs, number of total follicles at each day of scanning. Further, the difference in the number of total follicles among the days of scanning between control and treated groups was analyzed by two ways ANOVA (Snedecor and Cochran, 1994). Numerical data are represented as mean ± SEM, and differences were considered to be significant at $P < 0.05$, 0.01 and 0.001 as fit appropriate.

RESULTS AND DISCUSSION

The results pertaining to pattern of growth of ovarian follicles following treatment and untreated heifers are presented in Table 1 and Table 2. Prior to treatment, the mean number of total follicles varied between 9.16±0.40 and 9.87±0.78 in control and treatment group, respectively, and the difference was non-significant (P>0.05). Irrespective of the day of scanning, the mean number of total follicles varied between 7.9±0.31 and 10.6±0.67 in control and 8.6±0.42 and 10.3±0.30 in treatment group with an overall mean number of 9.08±0.39 and 9.45±0.25 in control and treatment group, respectively (Table 2).

The differences in the mean numbers of total follicles detected at each day of scanning between the groups was non-significant (P>0.05). Further, the difference in the number of total detected follicles among the days of scanning within each animal, among the animals within and between groups also remained similar (P>0.05) in this study. Furthermore, the difference in the mean number of total detected follicles irrespective of the days of scanning within and between the groups did not differ significantly (P>0.05) in this study. The number of total antral follicles remained constant among the scanning days within the same heifer in both the groups indicates that antral follicle development during a wave is a continuous process even during anoestrus and the number of follicles that are recruited in each wave emergence remained constant at any day of wave during anoestrus heifers like in cycling heifers. Further, characteristic growth and regression pattern of follicular development of this study suggests a similar wave like growth pattern perhaps exists in anoestrus heifers as observed cycling heifers and in other species reported elsewhere (Ravindra and Rawlings, 1997; Evans *et al.,* 2001; Das *et al.,* 2009; Dutt *et al.,* 2010; Ghuman *et al.,* 2010).

Prior to treatment, at a random day of wave, the mean diameter of the largest follicle ranged from 5.75 mm to 7.44 mm in control and herb treated heifers, respectively (Table 1). But the difference in the mean diameter of the largest follicles between the groups remained non-significant (P>0.05) in this study. The LFs in untreated control heifers reached above 8.0 mm in size, ranging from 6.1 to 10.2 mm, with a mean diameter of 8.7 ± 0.62 mm. However, on herb supplementation, the LFs reached above 12 mm in size, ranging between 12.5 and 13.2 mm, with a mean diameter of 12.8 ± 0.08 mm in treatment group. The difference in the mean diameter of largest follicles between the groups differed significantly (P<0.001). In control group, only in two heifers the LF reached up to a diameter of 10 mm, the size of DF, but majority of them had LF between 6.0 and 9.0 mm in size. At present there is no available report regarding the follicular dynamics of heifers in any Indian native breeds for comparison. However, report on Nellore heifers from Brazil suggested that the maximum diameter of DF can vary between 10

Table 1: Follicular attributes in delayed pubertal heifers in control and treatment groups

Treatment/ Group	No. of heifers scanned	Mean diameter of LF prior to treatment (mm)	Maximum diameter attained by the LF (mm)	Days taken to attain maximum diameter (days)	Growth rate (mm/day)
Control	6	5.75±0.72[a] (range-3.3-8.3)	8.7±0.62[b] (range-6.1-10.2)	12.7±1.50[a] (range-7-18)	0.25±0.06[c] (range-0.07-0.59)
Herb	8	7.44±0.91[a] (range-4.8-10.7)	12.8±0.08[a] (range-12.5-13.2)	9.6±4.67[a] (range-3-14)	0.55 ± 0.06[d] (range-0.34-0.83)

Means bearing different superscripts (column wise) differ (a: b=P<0.001; c:d =P <0.05) significantly

Table 2: Surface follicles turnover in delayed pubertal heifers after treatment with medicinal herbs *A. marmelos* and *M. Koenigii*

Group	Day of ovarian scanning									
	1	2	3	4	5	6	7	8	9	10
Control	9.16 ± 0.40	8.83±0.30	9.33±0.88	10.16±1.16	8.16±0.54	8.83±0.30	9.16±0.65	9.0±0.25	9.50±0.42	8.66±0.33
Herbs	9.87±0.78	9.5±0.32	10.00±0.56	9.25±0.52	9.12±0.54	9.00±0.37	9.50±0.37	9.62±0.42	9.37±0.46	9.25±0.41
	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

to 12 mm with an average diameter of 10.2 mm, which is lesser than the pre-ovulatory size (Figueiredo *et al.*, 1997).

Thus comparing the previous report and our present finding, it can be infered that in some of the delayed pubertal heifers (two in the present study) the LF could attain the DF size but failed to reach pre-ovulatory size and some may fail to attain even the DF size. Previously, in *Bos indicus* heifers, other than Sahiwal, the selection of DF was reported to occur at a mean diameter of 7-8 mm. In the present study, the LFs in 5 untreated heifers reached a diameter above 8.0 mm and one maximally reached up to 6 mm. This further suggests that the largest follicles in majority (five heifers) of the untreated delayed pubertal heifers attained the selection process, but 3 (8.3, 8.4 and 9.1mm) of them failed to progress further to reach a DF size *i.e.*10 mm or above.

However, in two control heifers, the LF crossed the process of selection and progressed further to the process of dominance by attaining a diameter 10 mm ((10.1 and 10.2 mm), but subsequently failed to proceed to reach the preovulatory size, unlike the herbs treated group. The LF in the heifer that could reach up to 6 mm diameter perhaps failed to cross the process of selection. Therefore, comparing the previous report and our present findings, we can speculate that in delayed pubertal heifers, the follicular growth may be a continuous process but may be interrupted at any stages of their terminal development, firstly, follicle may fail to attain the size at the point of selection as DF, secondly some may cross the point of selection but failed to attain a size of dominant follicle and the process of dominance and thirdly, in case dominance is attained, they may subsequently fail to reach preovulatory size. Studies pertaining to temporal relationship between endocrine profiles and follicular dynamics needs further investigation to unravel the underlying mechanism.

Interestingly, in herbs supplemented group, the LF attained the size above 12.0 mm diameter with an average of 12.8 mm in all the heifers in this study, which is well comparable with the DF size (10.2 mm), reported in Nellore heifers (Figueiredo *et al.*, 1997). Thus, the size attainment of LF above 12 mm diameter in herbs treated heifers indicates that they crossed both the previous processess *i.e.*, attainment of D.F. size, process of dominance and pre-ovulatory size unlike most of the control heifers of this study. Therefore, it is reasonable to speculate that the principles present in the herbs have the crucial roles to overcome the above process. It is not unclear, which particular phytochemical steroids of the plants, play active role in those processes needs future investigation.

At present, no literature pertaining to the follicular dynamics of Indian native breeds of heifers is available for comparison in respect to follicular growth pattern with

anoestrus heifers in general and Sahiwal in particular. Also, reports comparing the follicular dynamics after treatment with any medicinal plants in ailing condition like delayed puberty are not available. However, with a similar herb treatment, earlier it has been shown that the size of the largest follicles increased significantly in anoestrus goats (Dutt *et al.,* 2010). Present finding thus strengthens the earlier observation of Dutt *et al*., 2010) and support the view that the *Aegle marmelos* and *Murraya Koenigii* have the beneficial role during the terminal growth stage of ovarian follicle during the wave. But the exact phytocostituents responsible and the mechanism by which the effect occurred are still unknown to us.

The mean duration that the largest follicle took to reach from its first detected size to its largest size was 9.6 ± 4.67 days (ranging between 3 and 14 days) and 12.7 ± 1.50 days (ranging between 7 and 18 days) in herbs treated and control group, respectively. However, the difference in the mean duration taken by the LFs to attain its largest size from the first detected size to the DF size failed to approach (P>0.05) significance in this study.

Another interesting finding of this study was growth rate of the largest follicles in both the groups. The growth rate of the future morphologically dominant follicle ranged between 0.37 mm/ day to 0.83 mm/ day with a mean growth rate of 0.55±0.06 mm/ day in the herb treated heifers while in control heifers it ranged between 0.07 mm/ day and 0.59 mm/ day with a mean growth of 0.25±0.06. However, the difference in the mean growth rates of the future morphologically dominant follicles between the groups differed (P<0.05) significantly, being faster in the herb treated group than the control group. In Nellore heifers, the growth rate of DF was reported to vary between 0.73 and 1.06 mm/day (Figueiredo *et al.,* 1997). In contrast to the aforesaid study, the growth rate recorded in this study was lower in delayed pubertal Sahiwal heifers and the heifers treated with the two medicinal plants. Reasons are unknown to us. However, unlike herb group, relatively slower growth rate of LF in control animals perhaps causes lower estradiol production and longer persistence of LF in the wave and more tendencies to become atretic. On the other hand, the higher estradiol production by the LFs in herb treated animals (Dutt *et al.,* 2010) perhaps promoted the process of dominance and attainment of pre-ovulatory size in this study.

CONCLUSION

From the present study, it can be concluded that slower growth rate of follicles, failure to attain dominant size, process of dominance or preovulatory size may be the possible reason for pathogenesis of delayed puberty or pubertal anoestrus in Sahiwal heifers. And the supplementation of medicinal plants like *Aegle marmelos* and *Murraya Koenigii* has the beneficial role to overcome the above said processes. However, the principal phyto-consitituent that is responsible for triggering the final cascade of event leading to attainment of preovulatory size and ovulation warrant future investigation.

ACKNOWLEDGEMENTS

Authors are very thankful to Dean College of Veterinary Sciences DUVASU, Mathura for providing necessary facilities to carry out the research. The support received from dairy workers and other staffs are dually acknowledged.

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