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Effects of gonadotropin releasing hormone conjugate immunization and bioenhancing role of Kamdhenu ark on estrous cycle, serum estradiol and progesterone levels in female *Mus musculus*

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Abstract

Background: Active immunization with gonadotropin releasing hormone conjugate (GnRH-BSA) manipulates the fertility axis and thus alters the reproductive cyclicity, serum estradiol and progesterone levels. While the application of Kamdhenu ark increases the efficacy of GnRH-BSA.

Objective: This experimental investigation is aimed to evaluate the modulatory effects on estrous cycle, serum estradiol and progesterone levels in female mice after Kamdhenu ark and GnRH-BSA immunization.

Materials and Methods: Sixty sexually mature female mice were divided into three groups of twenty each. Group I served as control, while group II was immunized with GnRH-BSA conjugate (50µg/animal) for 120 days. However, group III was supplemented with Kamdhenu ark (100 ppm) orally along with GnRH-BSA conjugate immunizations and their vaginal estrous cyclicity, serum estradiol and progesterone levels were estimated after 30, 60, 90 and 120 days of intervals.

Results: GnRH-BSA immunized females showed regular estrous cycle initially but after 13^{th} day animals started showing irregular and prolonged estrous cycle with a complete diestrus stage after 65^{th} day onwards. In connection to this, GnRH-BSA + Kamdhenu ark supplemented animals also showed regular cyclicity initially but later they showed more interrupted cycle with complete diestrus stage after 55^{th} day. Besides this, the serum estradiol and progesterone levels lowered significantly in all the experimental groups as compared to control animals. The more severe decrease in hormonal levels was noticed in later part of the experiment especially in the group supplemented with Kamdhenu ark along with GnRH-BSA immunizations.

Conclusion: All these observations suggest that the GnRH-BSA conjugate has a deleterious effect on the reproductive hormones and estrous cycle of female mice; and Kamdhenu ark acts as a bioenhancer in immunization efficacy to modulate these effects.

Key words: *GnRH-BSA immunization, Kamdhenu ark, Estrous cycle, Female Mus musculus.*

Introduction

The gonadotropin releasing hormone (GnRH) is a key regulator of reproductive functions in mammals, acting mainly at the level of hypothalmo-hypophysis axis. The decapeptide GnRH is synthesized in the neurons of the hypothalamus and released into the portal circulation where it interacts with GnRH receptors on the gonadotrope cells in the anterior pituitary (1). Stimulation of the GnRH receptor is essential

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for the secretion of LH (luteinizing hormone) and FSH (follicle stimulating hormone), which, in turn, are required for steroidogenesis, gametogenesis and cyclicity (1).

Because of this central role in reproduction, GnRH peptide analogs have found therapeutic applications in controlling fertility, cryptorchidism, polycystic ovarian syndrome, leiomyomata, endometriosis, acute intermittent porphyria, and breast and prostate cancer (2-3), and show promise as new generation contraceptives for males and females. There is a need for alternative and cost-effective approaches to regulate gonadal activity, particularly in wild and domestic animals and in chronic diseases in man.

Active immunization of various mammals to GnRH, has been shown to lead in the case of males to testicular regression, reduction of testosterone secretion and cessation of spermatogenesis, while in the case of females, loss of cycling and ovarian regression (4-5).

Immunoneutralization of GnRH by vaccination with synthetic peptides is effective in regulating fertility in animals (6,7) and in the treatment of prostate cancer in males (8) and they have therapeutic potential in sex hormone-dependent neoplasms in females (9). Kamdhenu ark (distilled cow urine) has been reported as a strong immunomodulator and bioenhancer by various workers (10, 11).

In present investigation our aim is to evaluate the impacts of immunization with GnRH-BSA conjugate on estrous cycle and reproductive hormones (estradiol, progesterone) in female mice and the modulatory role of Kamdhenu ark after the immunization.

Materials and methods

Sixty sexually mature disease free female mice weighing 30±5 gr were used for this experimental investigation. The animals were divided into three groups of twenty each. Group I served as control, fed with standard mice feed and water ad libitum, while the animals of group II were immunized four times (Day 1, 28, 56, 84) with 50 µg GnRH-BSA conjugate (Sigma Aldrich) [100 µl phosphate buffered solution (0.01 N) and emulsified with an equal volume i.e., 100 µl Freund´s adjuvant] up to 120 days. However, group III was supplemented with Kamdhenu ark (100 ppm) orally along with GnRH-BSA conjugate immunizations. Vaginal smears were prepared daily in the afternoon in all the groups from day 1st to 120th day and stained with Giemsa solution to observe different stages of estrous cycle (12). Estradiol and progesterone levels in blood serum were estimated after 30, 60, 90 and 120 days along with control by using ELISA reader (Thermo multiskan lab system) adopting the methodology of Wisdom (1976) (13). Five animals from each group at different intervals were sacrificed and blood was collected through cardiac puncture and processed for serum preparation.

Statistical analysis

Results of the experiment were expressed as mean and standard error of mean of different groups.

The differences between the mean values were evaluated by Student's t test. The values for $p<0.001$ were considered as highly significant.

Results

Irregular and prolonged estrous cycle was observed in all the experimental groups as compared to control group. The normal estrous cycle lasts for 4-5 days which was observed in the control group (Figure 1). But the animals immunized with GnRH-BSA showed regular estrous cyclicity initially up to 13th day and then started showing irregular and prolonged estrous cycle with a complete diestrus stage after 65th day onward (Figure 2). While, the animals supplemented Kamdhenu ark with GnRH-BSA also showed regular cyclicity initially i.e. up to 19th day but in later they showed more interrupted estrous cycle with a complete diestrus stage after 55th day of treatments (Figure 3). Along with this, it has been noticed that estradiol and progesterone levels were lowered significantly throughout the experiments i.e. 30, 60, 90 and 120 days in immunized and Kamdhenu ark treated animals as compared to control group. While, these effects were more prominent and severely observed in the later part of the experiment especially supplemented with Kamdhenu ark (Table I; Figures 4 and 5).

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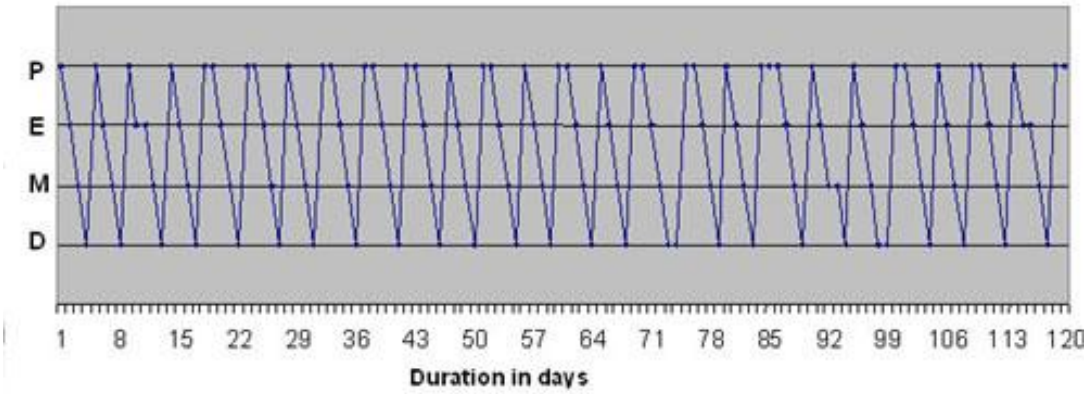


Figure 1. Estrous cycle in control female mice (*Mus musculus*). [Phases of estrous cycle: P= Proestrus; E= Estrus; M= Metaestrus; D= Diestrus]

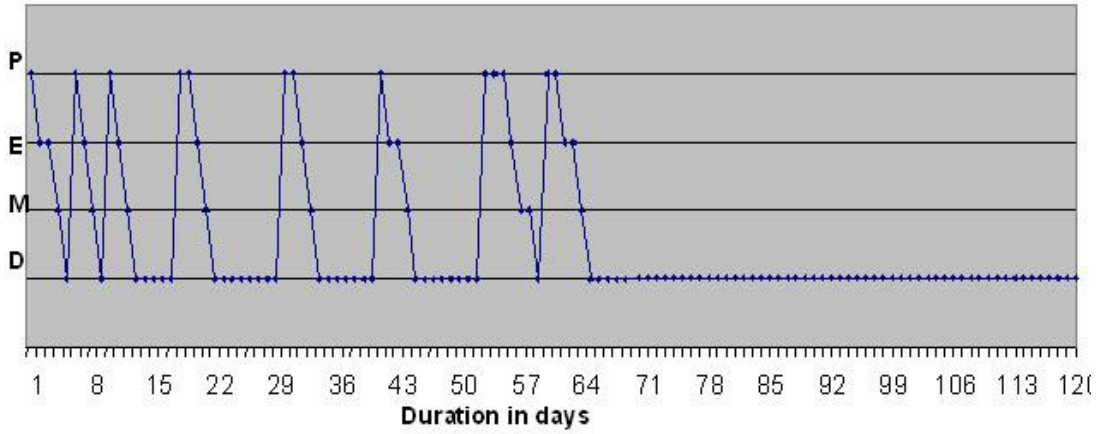


Figure 2. Estrous cycle in GnRH-BSA immunized female mice (*Mus musculus*). [Phases of estrous cycle: P= Proestrus; E= Estrus; M= Metaestrus; D=Diestrus]

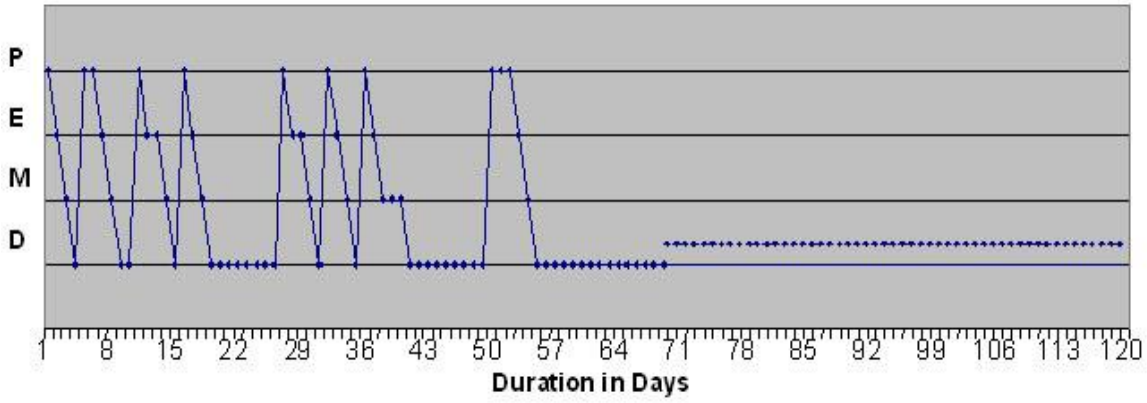


Figure 3. Estrous cycle in GnRH-BSA immunized and Kamdhenu ark supplemented female mice (*Mus musculus*). [Phases of estrous cycle; P= Proestrus, E=Estrus, M= Metaestrus, D= Diestrus]

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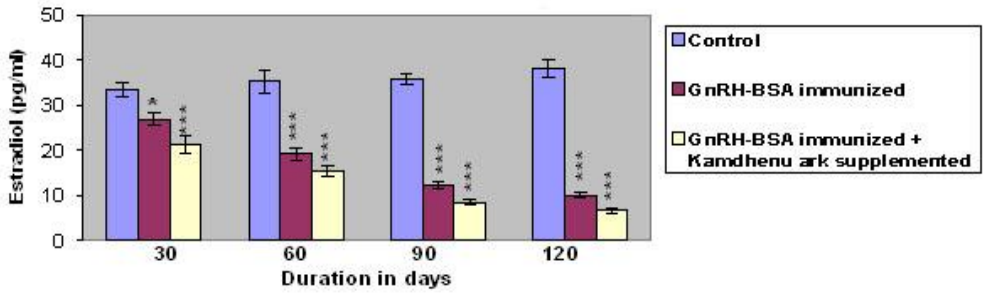


Figure 4. Estradiol estimation (pg/ml) in serum of GnRH-BSA immunizwd, GnRH-BSA immunized+Kamdhenu ark supplemented and control female mice (*Mus musculus*).
± SEM of five animals.
*= Significantly different (p< 0.05) from the control by Student's 't' test.
*** = Highly significant (p< 0.001) from the control by Student's 't' test.

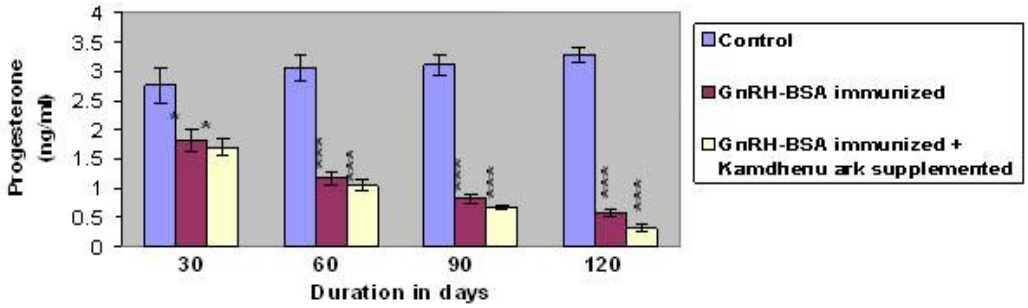


Figure 5. Progestrone estimation (ng/ml) in serum of GnRH-BSA immunized, GnRH-BSA immunized + Kamdhenu ark supplemented and control female mice (*Mus Musculus*).
± SEM of five animals.
*= Significantly different (p< 0.05) from the control by Student's 't' test.
*** = Highly significant (p< 0.001) from the control by Student's 't' test.

Table I. Hormonal estimations in GnRH-BSA immunized, GnRH-BSA+kamdhenu ark supplemented and control female mice, *Mus musculus*.

Parameters	Groups	30Days	60Days	90Days	120Days
Estradiol (pg/ml)	Control	33.38±1.55	35.24±2.56	35.89±1.20	38.19±1.98
	GnRH- BSA immunised	26.94±1.50*	19.17±1.25***	12.22±0.80***	10.06±0.59***
	GnRH-BSA+ kamdhenu ark supplemented	21.32±1.82***	15.46±1.04***	8.52±0.67***	6.58±0.44***
Progestrone (ng/ml)	Control	2.76±0.31	3.06±0.23	3.11±0.17	3.28±0.13
	GnRH- BSA immunized	1.81±0.20*	1.17±0.12***	0.82±0.08***	0.57±0.06***
	GnRH-BSA+ kamdhenu ark supplemented	1.70±0.15***	1.05±0.09***	0.68±0.03***	0.32±0.05***

± SEM of five animals.
*= Significantly different (p< 0.05) from the control by Student's 't' test.
*** = Highly significant (p< 0.001) from the control by Student`s `t` test.

Discussion

In the pituitary, GnRH binds to the GnRH receptors on the gonadotropic cells to stimulate the release of FSH and LH to the circulation. The pulsatile secretion pattern of GnRH induces the cyclic release of LH and to a lesser extent of FSH. In female mammals, FSH induces follicle growth and subsequently estradiol and inhibin secretion by the granulose cells. After ovulation the luteinised granulosa and the theca cells start to produce the high levels of progesterone. Because of relatively lower immunogenicity of the GnRH peptide, a number of adjuvants, carrier proteins, recombinant peptides and immunomodulators have been designed to modulate the immunogenecity of the GnRH peptide (14-16). Production of antibodies to

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gonadotropin releasing hormone was found to be associated with gonadal atrophy in mammals after GnRH immunization (17-19). Long term effects of GnRH immunization in white tailed female deer including reduced fawning rates, altered estrous behavior and reduced concentration of progesterone has been reported by Lowell *et al* (2003) (20).

Tshewang *et al* (2008) also reported suppressed ovarian activity along with irregular estrous behavior and decreased progesterone and androstenedione concentration in Australian stock horse fillies after GnRH immunization (21). Kamdhenu ark has been reported to enhance the immunogenecity of different antigens in mammals by various workers (22-23). Besides this, Kamdhenu ark acts as a bioenhancer and increases the efficacy of the antibiotics against infection agents. In our study it has been noticed that the GnRH-BSA conjugate modulate the hormonal levels in female mice. GnRH-BSA immunizations led to irregular estrous cyclicity and lowered estradiol and progesterone concentrations. These effects were more effective in later part of the experiment modulated through Kamdhenu ark supplementation.

All these results suggested that the efficacy of antibodies were raised after GnRH-BSA immunizations against the biologically active GnRH was more after supplementation of Kamdhenu ark resulted declined hormonal levels and alterations of the estrous cycle in female mice.

Conclusion

Our results concluded that the GnRH-BSA conjugate has a deleterious effect on the reproductive hormones and estrous cycle of female mice and Kamdhenu ark acts as a bioenhancer in immunization efficacy to modulate these effects.

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