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## Asian Pacific Journal of Reproduction

journal homepage: [www.apjr.net](http://www.apjr.net)

Original research <http://dx.doi.org/10.1016/j.apjr.2016.04.010>

### Effect of clomiphene citrate and human chorionic gonadotropin (hCG) on ovulation induction in prepubertal Sahiwal heifers

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#### ARTICLE INFO

##### Article history:

Received 14 Nov 2015

Received in revised form 23 Jan 2016

Accepted 1 Mar 2016

Available online 16 Apr 2016

##### Keywords:

Clomiphene citrate

Ovulation induction

Sahiwal heifers

#### ABSTRACT

**Objective:** To evaluate the ovulation inducing effect of clomiphene citrate along with human chorionic gonadotropin (hCG) in prepubertal Sahiwal heifers. Clomiphene citrate (an anti-estrogen) causes gonadotropin secretion from pituitary gland by removing the negative feedback effect of estrogen. This causes more FSH secretion which ultimately stimulates follicular growth and upon hCG administration (luteinizing hormone activity) ovulation occurs.

**Methods:** Twelve prepubertal Sahiwal heifers were divided into experimental and control groups, with six animals in each group. The grouping of animals was made on the basis of plane ovary. The status of ovarian puberty was checked through rectal palpation and history of previous estrus signs. Experimental heifers were given clomiphene citrate at 300 mg/heifer for 9 d orally, while heifers in control group were given placebo tablets. The dose of clomiphene citrate was selected on the basis of previous literature. On day 10, hCG (IVF-C 5000 iu (hCG) LG Life Sciences, Korea) was given to all heifers of both groups at 2 500 IU/heifer through intravenous route. Two base line blood samples were collected before start of the treatment at each rectal palpation examination for selection of animals. Subsequently, during and after treatment three blood samples per week (on alternate days) for 4 weeks were collected for measuring the progesterone (P4) in serum. The concentration of progesterone was measured through enzyme linked immunosorbent assay. For the comparison of variables, student *t*-test was applied.

**Results:** The P4 concentrations in five heifers (83.3%) in the experimental group with (P4 > 1 ng/mL) and one heifer (16.7%) in the control group along with corpus luteum on ovary showed ovulation. One heifer in control group ovulated that might be due to hCG. Mean progesterone concentration was significantly higher ( $P < 0.05$ ) in treated heifers than in control heifers.

**Conclusion:** Administration of clomiphene citrate and hCG can induce ovulation induction in prepubertal Sahiwal heifers.

## 1. Introduction

Clomiphene citrate is a tissue-selective estrogen receptor modulator [1] and it is a non steroidal synthetic hormone with estrogenic effect, has been the initial therapy for ovulation induction in women having anovulatory infertility and in couples with unexplained infertility [2,3]. Clomiphene citrate is part of the triphenylethylene family of compounds. It comprises of two isomeric forms, *cis* and *trans*, which in the current nomenclature

corresponds to zuclomiphene and enclomiphene, respectively [4]. The accumulation action of zuclomiphene due to its long half-life was the main force in the ovulation inducing action by clomiphene citrate, and thus that the clomiphene citrate's action was mainly caused by zuclomiphene [5]. Contrarily, it was stated that the major isomer for ovulation induction was enclomiphene, and that zuclomiphene was unable to do ovulation induction. Moreover it was determined that enclomiphene's anti-estrogenic action inhibits ovulation, and there was the need of administering hCG for ovulation induction [6,7].

Because of structural resemblances of clomiphene citrate to estrogen, it binds competitively with nuclear receptors of estrogen. Through decreasing the negative feedback of estrogen, it

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Peer review under responsibility of Hainan Medical College.

stimulates mechanism that alters the releasing trend of GnRH, which enhances pituitary gonadotropin hormones. This mechanism finally stimulates ovarian follicles to develop [8].

Puberty is initiated in heifers by increased LH secretion during peripubertal period [9]. The peripubertal increase in LH secretion comes from decreasing negative feedback of estrogen on GnRH/LH production [10]. Increased LH secretion stimulates growth of dominant ovarian follicles [11], leading to increased systemic estradiol concentrations, which stimulates the preovulatory LH surge. Puberty is the release of functional gametes, when regular estrous cycles start and make reproduction possible. Moreover, onset of puberty is an important factor influencing production efficiency and lifetime productivity of a female [12]. Heifers that achieve puberty and undergo multiple cycles before breeding season, have ability for increased the first service conception [13], earlier pregnancy [14] and increased lifetime productivity [15]. Clomiphene citrate medication causes depletion of estrogen receptors at pituitary and hypothalamic level, inhibiting the negative feedback exerted by estrogen normally. Because of this, the GnRH secretion is increased and stimulates pituitary for FSH production, which in turn stimulates growth and maturation of follicles with emergence of 1 or more follicles [16].

Sahiwal is well known tropical breed of Pakistan due to its unique tick resistant abilities [17] and performs well under the tropical conditions of Pakistan but its average of first calving is much higher as compared to dairy of developed countries. Average age of puberty in cow heifers is 34 months [18]. To reduce the age at first calving in this breed, this study was designed to use clomiphene citrate and hCG for stimulation of follicular growth and ovulation in prepubertal Sahiwal heifers.

## 2. Materials and methods

### 2.1. Experimental animals

Twelve prepubertal Sahiwal heifers having age of 30–36 months were selected. This study was approved by university supervisory committee. Heifers were not found in estrous activity before the start of experiment. Heifers were housed at the Proca Livestock Farm, University of Agriculture, Faisalabad, had free access to water and were fed green fodder and wheat straw. Housing and management was optimum. Rectal palpation was conducted to evaluate uterine development, ovarian size and presence of palpable corpus luteum [19]. The second rectal palpation was performed to check the prepubertal status. Blood was collected on both rectal palpations and serum was separated and estrous activity was checked again by measuring serum progesterone (P4) level which was below 1 ng/mL. Out of these selected Sahiwal heifers, six heifers were assigned to treatment group and other six to control group. Identification tags were allotted to each heifer.

The mean age of treated group [(34.17 ± 1.83) months] differed non-significantly compared with control group [(33.00 ± 2.10) months]. Similarly mean weight of both groups [(178.33 ± 8.76) kg vs (169.33 ± 8.29) kg] was also not significantly different.

### 2.2. Experimental design

Treated heifers were given clomiphene citrate at 300 mg/heifer orally (Ovafin<sup>®</sup>, OBS Pharmaceuticals Ltd Karachi

Pakistan, six tablets per heifer, each tablet contains 50 mg of clomiphene citrate) for 9 d, at the same time animals of control group were given placebo tablets. On the day 10, hCG was administered 2 500 IU to all heifers through intravenous route. Two base line blood samples were collected before start of the treatment at each rectal palpation examination for selection of animals. Subsequently, during and after treatment three samples per week (on alternate days) for 4 weeks were collected. Blood samples were taken from jugular vein with disposable syringe. About 5 mL blood was collected from each animal, serum was immediately separated by centrifuging at 2 000 r/min for 10 min and stored at –20 °C for hormonal assay. The 3rd rectal palpation was performed on day 10 of treatment and the fourth rectal palpation a week later to detect CL.

### 2.3. Hormonal assay

Serum samples were analyzed for progesterone concentration via enzyme immune assay. Progesterone level was measured using EIA PROGESTERONE (BioCheck<sup>®</sup> Inc. USA). Intra assay coefficient of variation was 7.1% and inter assay coefficient of variation was 12.6%. Detection range was 0.05–50 ng/mL.

### 2.4. Statistical analysis

Mean ± SEM of serum progesterone were calculated for experimental and control heifers. For the comparison of variables, student *t*-test was applied [20] using online software GraphPad (URL [www.graphpad.com/quickcalcs](http://www.graphpad.com/quickcalcs)). Difference was considered significant when *P* < 0.05.

## 3. Results

In the present study, clomiphene citrate treatment had an accurate response on treated heifers. Five of six treated heifers experienced ovulation (83.3%), they had progesterone concentration above 1 ng/mL of serum. Control heifers remained prepubertal except one who undergone ovulation and luteal phase (16.7%). In rest heifers, progesterone concentration remained below 1 ng/mL. Behavioral signs of estrous were not expressed by any heifer (Figure 1).

Mean progesterone level was similar between treated and control group prior to treatment and also during treatment (*P* > 0.05) and was below 1 ng/mL. After hCG injection in treated group, mean progesterone level rose and mean progesterone level was above 1 ng/mL on the day 7 after hCG injection and remained above 1 ng/mL for 4 d indicating average length of

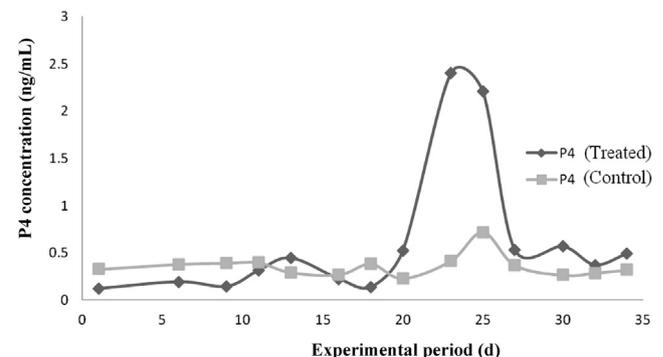


Figure 1. Mean progesterone profile during experiment.

luteal phase. On day 9 after hCG injection mean progesterone level in experimental heifers was on its peak and was  $(2.40 \pm 0.58)$  ng/mL and this was considerably higher ( $P < 0.05$ ) than control mean progesterone  $(0.42 \pm 0.22)$  ng/mL.

#### 4. Discussion

Delayed puberty is one of most common problem of dairy industry of Pakistan due to shortage of fodder and severe climatic conditions. This older age of puberty is responsible for the poor reproductive performance of cattle. Response of Sahiwal heifer in terms of serum progesterone was significant. Five of six treated heifers experienced ovulation (83.3%), they had progesterone concentration above 1 ng/mL of serum. The result of ovulation induction with the same treatment protocol was 66% when clomiphene citrate was used in prepubertal crossbred (Sahiwal  $\times$  Fresian) heifers. Out of six treated heifers four experienced ovulation [21]. Increase in small and medium sized follicles were observed in clomiphene citrate treated animals than in control animals [22]. Indian researchers consistently reported the efficacy of clomiphene in induction of estrous in cattle and buffaloes when clomiphene citrate was administered at the dose of 300 mg/animal orally [23].

The results of our study showed that clomiphene citrate treatment significantly increased the follicular growth in treated heifers and rate of ovulation induction was 83% in treated prepubertal Sahiwal heifers, *i.e.*, out of six treated heifers five experienced ovulation. In heifers which experienced ovulation, manifested short luteal phase and this is confirmed with other studies which revealed that luteal phase after the first ovulation in most of heifers is shorter [24]. The higher progesterone concentration was seen after clomiphene citrate treatment in infertile women which is due to developed CL or combined production of more than 1 CL [25,26]. Similar results like increase in progesterone concentration were observed in this study. All heifers used in this experiment were above average age of puberty. Possible reason can be the poor management to achieve the optimum weight at proper time [27,28]. Rectal palpation was unable to detect CL properly, most probably because of shorter life span of CL during experiment. Also some researchers observed that palpation may not be a better option for detecting presence of young and old CL [29]. Number of other studies in boys supports our findings in which age of puberty was reduced due to use of clomiphene citrate [30]. Tamoxifen (anti-estrogen), treatment accelerated puberty in both cockerels [31] and hens [32]. Early spermatogenesis was also evident at 9 weeks of sold chicks due to use of tamoxifen [33]. Similarly, in male turkeys and Muscovy drakes, treatment with tamoxifen advanced onset of semen production by 4 and 5 weeks, respectively [34,35].

In this study, rate of ovulation induction was 83% in treated prepubertal Sahiwal heifers, *i.e.*, out of six treated heifers five experienced ovulation. So the measurement of estrogens and use of ultrasonography can help to understand the ovarian activity after clomiphene citrate treatment.

The results achieved in this study are in favor of hypothesis made. So it may be proposed that clomiphene citrate and hCG can be used to induce ovulation hence puberty in prepubertal heifers. Further studies are advised to use this protocol of treatment on buffalo to induce the ovulation under the climatic conditions of Pakistan.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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