

Effects of Clomiphene Citrate (CC) and Human Chorionic Gonadotropin (hCG) on hormonal profile, serum biochemical constituents, and oxidative stress in pre-pubertal Sahiwal heifers

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ABSTRACT

Clomiphene citrate (an anti-estrogen) has the abilities for the secretion of gonadotropin from the anterior pituitary gland. The present study was designed to investigate the effects of CC and hCG on female sex hormones (FSH, Estrogen) and serum biochemical constituents in pre-pubertal Sahiwal heifers. The twelve animals were randomly divided into a) treatment and b) control groups. These animals were grouped on the basis of reproductive status of heifers. The animals in treatment group were fed clomiphene citrate with dose of 300mg/ animal for 9 days. On 10th day of experiment, hCG (IVF-C 5000 iu (hCG) LG Life Sciences, Korea) was injected I/V to both groups. For the serum biochemical constituent's evaluation and oxidative stress (MDA), blood on day 0, 14 and 28 was collected. Similarly, for hormones evaluation three blood samples per week (on alternate days) for 9 days were collected. The concentrations of both hormones were assessed by ELISA. For the comparison of variables, student t-test was applied. Analysis of data revealed that levels of hormones were higher in treated animals as compared to control. There was significant ($p < 0.05$) increase in total cholesterol, triglycerides total protein and sugar. But there was nonsignificant ($p > 0.05$) effect on liver enzymes (ALT and AST) and MDA. It was concluded that use of CC and hCG increase the FSH and estrogen and few alterations in serum biochemical constituents in the pre-pubertal Sahiwal heifers.

Key words: Clomiphene citrate, FSH and estrogen, Sahiwal heifers, Serum biochemical constituents.

INTRODUCTION

The age of puberty is not dependent on the sufficient secretion of hypothalamus or adenohipophysis; rather it is the absent of high frequency of LH which ceases the commencement of puberty in heifers. Hypothalamus of prepubertal heifer is sensitive to negative feedback of estrogen, thereby the release of GnRH and LH remained low (Day *et al.*, 1984, 1998).

CC has the similar structure to estrogen, it blocks the receptors of estrogen and reduces the negative feedback of estrogen (Fritz *et al.*, 2011). The gonadotropin or anti-estrogens drug like clomiphene citrate (CC) is commonly used to function the ovaries of infertile women (Barbieri, R.L. 1999). Gonadotropins stimulate the steroidogenesis in follicles by regulating the pathway of cyclic adenosine 3',5'-cyclic monophosphate, thus stimulates the growth and maturation of follicles in mammalian ovaries. Further, gonadotropins play an important role in the stimulation of

PGE2 synthesis which stimulates the rupture of follicles (Duffy, and Stouffer, 2003 Duffy *et al.*, 2005). CC is very cheap drug and is commonly used for the ovulatory dysfunctions in women (Practice Committee USA, 2013). The treatment of CC causes the alterations in serum biochemistry of Albino rats (Wael, Al-Amoudi, 2012). Malondialdehyde (MDA) is considered as the important aldehyde from the lipid peroxidation during the process of various biological functions. It is considered as the main index during the process of lipid peroxidation (Draper and Hadley, 1990).

Limited information is available regarding the effect of clomiphene citrate and hCG on follicular growth in the form of the increase in hormones (estrogen and FSH) and serum biochemistry of pre pubertal cattle. The aim of present study was to observe the effects of these drugs on hormones (estrogen and FSH) and serum biochemical constituents.

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MATERIALS AND METHODS

Experimental design: The present study was conducted on 12 prepubertal Sahiwal heifers having the age of 30 to 36 months maintained at the Proca Livestock Farm, University of Agriculture, Faisalabad. These animals were randomly divided into two groups on the basis of no ovarian structures. Moreover, these animals have not exhibited estrus signs. The animals have the free excess to water and green fodder. The size of an ovary, corpus leuteum and uterus was verified through rectal palpation. The heifers in the treatment group were fed CC (Ovafin®, OBS Pharmaceuticals Ltd Karachi Pakistan,) with the dose of 300mg/animal/ day for 9 consecutive days. On the 10th day of an experiment, hCG was injected with dose 2500 IU to animals of both groups through I/V. One blood sample was collected on 0 day of experiment. Subsequently, three blood samples (on alternate days) for nine days were collected for the estimation of hormones. The blood was also collected on 14th and 28th day of experiment to find the concentrations of serum biochemical constituents. Blood samples were taken from jugular vein aseptically. About 5ml blood was collected from each animal, serum was immediately separated by centrifugation at 2000 rpm for 10 minutes and stored at -20 °C for hormonal assay.

Serum concentration of ALT and AST were measured utilizing commercially available colorimetric kits Fluitest company (Catalog #1180) and (Catalog #1622) respectively. Glucose and cholesterol were determined using the glucose kit (Breuer and Breuer Diagnostic Germany) and (FluitestHDL-D Biocon Germany) respectively. The absorbance of the samples and standards was measured using chemistry analyzer (BTS-330, Biosystems, Spain). Serum estrogen concentration was determined using a commercial kit ELISA, Catalog Number #:925-400 Mono bind USA with sensitivity 6.5 pg/ml. Follicle-stimulating concentrations in serum were determined through kit named as follicle-stimulating hormone (ELISA) test (Product Code; 425-500, Monobind Inc.). This test has the sensitivity of 0.006mIU well/1. For the determination of MDA, interaction with thiobarbituric acid (TBA) to form a pink complex with the absorption maximum at 535nm was used.

Statistical analysis: Mean \pm SEM of serum hormones and biochemical constituents were calculated for experimental and control heifers. Two-factor CRD was used for the analysis of data. For the comparison of variables, student t-test was applied (Snedecor and Cochran, 1994). The difference was considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

In the control group of animals, the concentration of estrogen and follicle stimulating hormone remained low showing the nonsignificant increase during the experiment (Fig 1 & 2). The mean concentration of estrogen and follicle stimulating hormones were 3.7ng/ml and

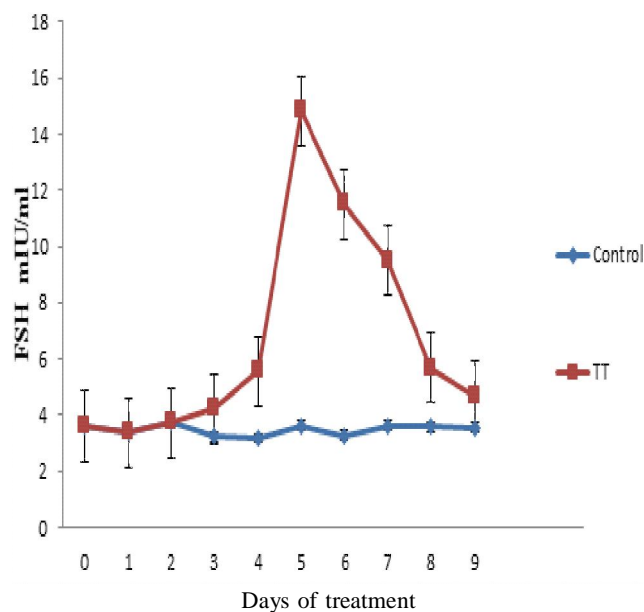


Fig 1: Mean follicle stimulating hormone profile during experiment; treated vs control

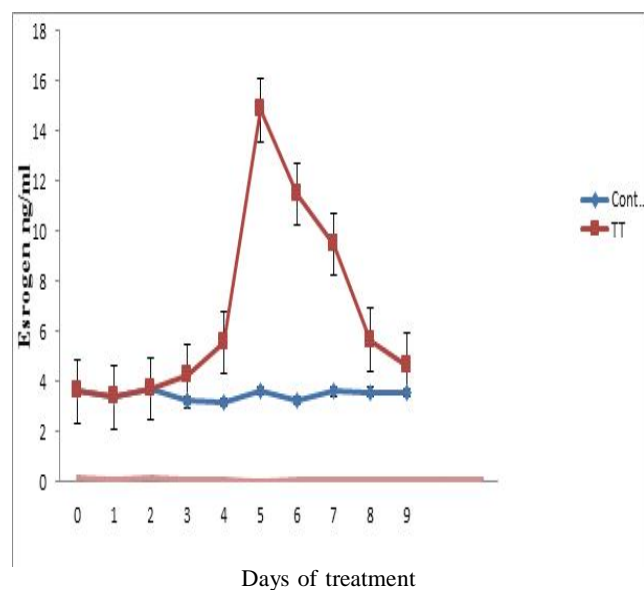


Fig 2: Mean estrogen hormone profile during experiment; treated vs control

3.4mIU/ml respectively prior to starting of the experiment. In control animals, mean E2 concentration remained below 4 pg/ml (mean 3.6 ± 1.00 ng/ml) throughout the experiment. In the treated group, mean E2 concentration increased during the last 3 days of treatment to a peak of 15.8 ± 5.7 ng/ml on a day of hCG injection as in Fig. 2. After hCG injection in the treated group, mean concentration of hormones estrogen and follicle stimulating levels increased indicating the ovulation.

Control heifers showed lower hormones as in Fig 1 & 2. Behavioral signs of estrous were not expressed by any heifer.

Analysis of serum biochemical constituents indicates that there was significant increase in mean values of total protein and albumin in treated animals as compared to control. Weekly analysis of data revealed that there was also significant rise of these parameters at 2nd and 4th week experiment. The mean values of cholesterol, triglycerides and sugar were significantly $P < 0.05$ increased in treated animals as compared to control. Likewise, Weekly analysis of data revealed that there was also significant increase in these parameters (Table 1). The mean serum level of ALT and AST was not increased and weekly analysis of data revealed that there was no effect on enzymes (Table 2). Analysis of data revealed that serum MDA was non-significantly increased in mean values as compared to control. Similarly, weekly analysis of data indicated that there was no significant increase in MDA (Table 3).

The level of hormones in serum of Sahiwal heifer was significant in the form of the increase in estrogen and FSH. This rise of hormones may be due to a removal of negative feedback of estrogen as well as the growth of follicles. The maturation of follicle may increase the FSH and estrogen. The use of CC increase level of both FSH and LH, then falls after the 5 days of treatment (Reyes *et al.*, 2000). The successful treatment of CC causes the recruitment and maturation of one or more follicle-raising the estrogen

which ultimately triggers the LH and subsequent ovulation (Berna *et al.*, 2008). The higher level of estrogen due to CC was reported in prepubertal crossbred (Sahiwal X Fresian) heifers (Rehman *et al.*, 2014). The dose of 300 mg/animal orally in buffaloes and cattle results in induction of estrus in India (Kankal *et al.*, 2008). The growth of follicles due to CC was also increased in Rahmani ewes (EL Sherry *et al.*, 2011). Other studies supporting our findings were the rise of FSH in male rats (AL-atabi and AL-diwan, 2014; Zamanet *et al.*, 2009; Ribeiro and Abucham, 2009). CC is used for successful ovulation in ART programs (Practice Committee USA, 2013).

The serum biochemical constituents are commonly used as reliable indices for the health of animals (Ohaeri and Eluwa 2011). In the present study, there was the significant rise in cholesterol and triglycerides. Similar kinds of findings were reported in albino rats (Wael, 2012). The findings of a present study are also in close harmony to results of Yasar and Ertugrul (2009) and Chaudhuri *et al.* (1990), who reported severe hypertriglyceridemia in women due to CC. The use of CC stimulates the synthesis of acetyl CoA, thus raising the precursor of cholesterol (Siedentop *et al.*, 1997; Haschek and Rousseau, 1998). The serum concentrations of total protein and albumin were increased significantly in treated animals. Contrary to present findings, reduced level of serum protein was present in the serum of rats (Su *et al.*, 1985). The concentrations of transaminase are known the key factor for the normal functions of liver (Sherlock, 1981).

Table 1: Mean values (\pm SE) of total protein, albumin, cholesterol, triglycerides and sugar in serum of control and treatment groups at different days of experiment

Total protein	0	14	28	Mean
Control	7.5000 \pm 0.8 B	7.4833 \pm 1.0 B	7.44 \pm 0.7 B	7.4917 \pm 0.8 B
Treated	7.5000 \pm 0.6 B	8.8571 \pm 0.6 A	8.91 \pm 1.0 A	8.1786 \pm 0.7 A
Albumin				
Control	2.1500 \pm 1.12 B	2.283 \pm 0.9 AB	2.33 \pm 0.54 AB	2.1667 \pm 0.8 B
Treated	2.1833 \pm 1.2 B	2.6974 \pm 0.7 A	2.65 \pm 1.4 A	2.4905 \pm 0.7 A
Cholesterol				
Control	64.833 \pm 0.7 B	65.940 \pm 0.7 B	64.88 \pm 0.5 B	64.917 \pm 0.62 B
Treated	65.000 \pm 0.2 B	97.186 \pm 0.4 A	97.4 \pm 0.8 A	81.563 \pm 0.5 A
Triglycerides				
Control	58.167 \pm 0.4 B	56.314 \pm 0.3 B	57.23 \pm 1.0 B	70.045 \pm 0.6 A
Treated	57.000 \pm 0.4 B	83.776 \pm 0.5 A	83.1 \pm 0.7 A	57.583 \pm 0.6 B
Sugar				
Control	52.833 \pm 1.2 B	52.038 \pm 0.7 B	51.8 \pm 0.6 B	52.500 \pm 0.9 B
Treated	52.167 \pm 0.2 B	61.115 \pm 0.8 A	61.2 \pm 0.6 A	56.577 \pm 0.7 A

Table 2: Mean values (\pm SE) of total ALT and AST in serum of control and treatment groups on different days of experiment

Parameter/group	Days	14	28	Mean
ALT				
Control	16.191 \pm 0.6 A	16.000 \pm 1.0 A	16.3 \pm 0.8 A	16.083 \pm 0.7 A
Treated	16.167 \pm 0.4 A	16.006 \pm 0.7 A	15.8 \pm 0.8 A	16.099 \pm 0.6 A
AST				
Control	43.167 \pm 1.0 A	44.971 \pm 0.9 A	43.22 \pm 0.8 A	42.417 \pm 0.8 A
Treated	41.667 \pm 1.2 A	41.878 \pm 1.4 A	41.23 \pm 1.0 A	41.772 \pm 1.3 A

Table 3: Mean of(\pm SE) malonaldehyde(nMol/ml) in serum of control and treated heifers on different weeks of treatments

Days	Control	Treated
0	34.0 \pm 3.02 ^{bc}	32.0 \pm 2.20 ^c
14	32.5 \pm 2.03 ^{ab}	33.75 \pm 3.42 ^{cb}
28	33.2 \pm 3.1 ab	34.34 \pm 2.1

Letters having different superscript are considered as significantly different whereas letters with similar superscript are nonsignificant.

The level of AST and ALT in were not increased or decreased significantly in present study. Contrary to our findings, increased serum concentrations were present due to CC in women (Shimono *et al.*, 1998).The rise in the activities of ALT and AST may be due to dose and species variation.Previous studies suggest that the CC causes the accumulation of ROS in female reproductive system of rat as well as human (Chaube *et al.*, 2005; Tripathi *et al.*,

2011).The extent of oxidative stress in ovarian along with uterine tissues was increased with the rising level of MDA and reduced antioxidants properties in the polycystic rats (Sasikala *et al.*, 2010).In the present study, ROS in the form of MDA in serum did not indicate any significant difference in control and treated animals. This may due to samples differences as well as dose and duration of treatment.

It may be concluded that clomiphene citrate and hCG can be used to increase the follicular growth in prepubertal heifers. Future studies are advised to use this drug to find out the conception rate and genomic studies under the climatic conditions of Pakistan in different species of livestock. This study also proposed to find out the harmful effects on ovarian structures.

Conflict of Interest;All the authors are willing for publication of this article.

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