Effect of *Punica granatum*, *Citrus limon* and their combinations on the plasma Gonadotropins in female rabbits

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Abstract: Fruits produce revitalizing effects, hence the impact of *Punica granatum*, *Citrus limon* and their combinations have been investigated on the plasma levels of gonadotropin, testosterone and sexual development capacity in female rabbits. Ninety female rabbits were randomly assigned into nine groups, each comprising of ten animals. One group was given saline and designated as control. Three groups were given *P. granatum* 2mL/kg, 5mL/kg, 8mL/kg, other three groups received *C. limon* 0.2mL/kg, 0.4mL/kg, 0.6mL/kg respectively, remaining groups received *C. limon* and *P. granatum* in combination i.e. 0.4mL/kg *C. limon* + 5mL/kg *P. granatum* and 0.2mL/kg *C. limon* + 8mL/kg *P. granatum*. Juices were administered once daily from day 0 of pups delivered to postnatal day 15. Blood samples were gathered from ear vein at day 11 and day 15. There was a significant increase in follicle stimulating hormone by *P. granatum* at 5 and 8mL/kg on day 11 and 15, by *C. limon* at 0.4 and 0.6mL/kg on day 11, 0.4mL/kg at day 15, by combination doses of *C. limon* and *P. granatum* 0.4 + 5mL/kg at day 11, 0.4 + 5mL/kg and 0.2 + 8mL/kg at day 15. There was also a significant increase in luteinizing hormone by *P. granatum* at 2, 5 and 8mL/kg and by *C. limon* 0.4mL/kg at day 11. There was a highly significant increase on day 11 in LH at combination doses of *C. limon* and *P. granatum* 0.4 + 5mL/kg. There was a significant increase in testosterone level by *P. granatum* at 2, 5 and 8mL/kg on day 11 and 5mL/kg on day 15 and highly significant increase at 0.4 mL/kg on day 11, 0.2 and 0.6mL/kg on day 15 and highly significant increase at 0.4mL/kg on day 15. Whereas combinations doses of *C. limon* and *P. granatum* at 0.4 + 5mL/kg caused highly significant increase in testosterone level as compared to control. Results of present study revealed increase in plasma gonadotropin and testosterone levels showing increase in sexual capacity of female rabbits which could be mainly accounted for high vitamin C and flavonoids contents of these juices.

Keywords: *Citrus limon*, Follicle stimulating hormone, Luteinizing hormone, *Punica granatum*, Testosterone.

INTRODUCTION

Follicle stimulating hormone (FSH) plays an important role to retain the normal function of the ovary in producing oocytes and hormones in all mammalian species. When there is lack of FSH, follicular growth is detained and female becomes infertile (Findlay and Drummond, 1999). Luteinizing hormone (LH) is the key supervisor for the functions of Leydig cells and studies suggest that FSH can affect Leydig cell indirectly through Sertoli cell derived factors (Sriraman and Rao, 2004). Many factors have been suggested to be the mediators among these two cell types such as cytokines, growth factors and steroids (Saez and Lejeune, 1996). Testosterone (TS) is well known for its actions in reproduction as well as in the regulation of emotionality, reinforcement and memory processes (Buddenberg et al., 2009; Topic et al., 2007).

The ultimate goal of infertility therapy is to achieve pregnancy in the harmless, effective and suitable way, using the highest purity agent’s available (Latash et al., 2004). Though recent technology is rapidly developing recombinant LH and FSH preparation yet, there is a need to develop oral and transdermal gonadotropin agonists to eradicate the use of injections.

Phytoestrogens i.e. flavonoids, synthesized by plants and mostly found in fruits are associated with the cure of specific diseases of the reproductive organs (Pedersen et al., 2000; Gutendorf and Westendorf, 2001; Wang et al., 2002). They have a significant role as suppressor of free radicals and act as antioxidants (Grassi et al., 2010; Lee et al., 2016). Hence alterations in synthesis of Reactive oxygen species (ROS) in gonads stimulate the oxidation and DNA damage of cells (Sikka, 1996; de Lamirande et al., 1997; Sanocka and Kurpisz, 2004; Henkel, 2005).

*P. granatum* contains substantial amounts of flavonoids such as quercetin, kaempferol, ellagitannins, gallotannins and anthocyanins (Fischer et al., 2011). Moreover presence of minerals, electrolytes and vitamin C increases its nutritional activity (Turk et al., 2008; Mirdeghan et al., 2007). Testosterone, estrogen and estradiol in *P. granatum* make it useful in variety of ailments (Gil et al., 2000; Langley, 2000; Lansky and Newman, 2007).

Flavonoids found in *C. limon* includes rutin, hesperidin, quercitrin, eriocitrin, narirutin, didymin and naringin.
Numerous combination studies of fruit juices, reported their synergistic effect in many system of health (Riaz et al., 2010; Riaz et al., 2014). Thus, in presence of various evidences regarding use of fruit juice as dietary substances current study was planned to examine the hormonal effect of C. limon, P. granatum and their combinations on gonadotropins, testosterone and sexual behavior in female rabbits.

MATERIALS AND METHODS

Animals

Ninety healthy white female rabbits with mean body weight of 1300±50grams were randomly distributed in different groups each comprising of ten rabbits. Individual rabbits were accommodated in stain less steel cages, under particular condition of temperature 23±2°C and humidity 50-60%. Animals were preserved during the experiment on a 12/12h light and dark cycle with free contact to rabbit chow and tap water. The use of animals during the study was in accordance with the National Institute of Health (NIH) guide for the care and use of Laboratory Animals (Council, 1996) and permitted by the Board of Advance Studies and Research University of Karachi.

Administration of Juices

Citrus Limon

C. limon was procured from local market and recognized by plant conservation center, University of Karachi. The coupon specimen no C.L 11-11 was placed in department of Pharmacognosy, University of Karachi. Fresh juice of C. limon was obtained by pressing the fruit by hand which was filtered and administered in three doses i.e. 0.2, 0.4 and 0.6ml/kg according to body weight through oral route and was considered as CL-1, CL-2 and CL-3 respectively.

Punica granatum

P. granatum was procured from local market, recognized by plant conservation center, University of Karachi. The coupon specimen no P.G 11-12 was placed in department of Pharmacognosy, University of Karachi. Fresh juice of P. granatum was obtained by pressing the fruit by hand which was filtered and administered in three doses i.e. 2, 5 and 8ml/kg according to body weight through oral route and was considered as PG-1, PG-2 and PG-3 respectively.

Combinations of C. Limon and P. granatum

C. limon and P. granatum were given orally in two combination doses i.e. 0.4ml/kg CL+5ml/kg PG and 0.2ml/kg CL+8ml/kg PG and was abbreviated as CP-1 (0.4+5ml/kg) and CP-2 (0.2+8ml/kg). All doses were given once daily through oral route.

Design of experiment

Female rabbits were kept with male rabbits until pregnancy was induced. Ninety female rabbits were distributed into nine groups, each containing ten animals. Control group was given normal saline, three groups were given C. limon and designated as CL-1, CL-2, CL-3, three groups on P. granatum were labeled as PG-1, PG-2, PG-3 and two groups received combination doses of CL and PG mentioned as CP-1, CP-2. This study was scheduled for fifteen days. Dosing was started once daily orally, from day of pups birth designated as postnatal day0 and continued up to the postnatal day15 (day15). Entire study was completed under NCCL guideline (Wayne, 1998). Blood samples were gathered from ear vein in gel tubes at day11 and day15 after completion of dosing period.

Determination of FSH, LH and TS

Serum FSH, LH and TS levels were determined using Elisa kits (Human, Germany) as per manufacturer instructions. The activity levels of FSH, LH and TS in the samples were measured photometrically and their intensity of color was directly proportional to concentration in ng/ml.

STATISTICAL ANALYSIS

Data was entered and analyzed using by SPSS version 17 and represented as mean ± S.E.M with 95% confidence interval. ANOVA followed by post hoc was used for comparisons of values with control. Values of p≤0.05 were considered significant and p≤0.005 as highly significant.

RESULTS

Effect on FSH

Fig. 1 reveals the effect of P. granatum on FSH. There was significant increase in FSH level in both animal groups of P. granatum i.e. PG-2 and PG-3at day 11 and day 15, as related to control animals.

Fig. 2 reveals the effect of C. limon on FSH. Significant increase in FSH was observed by both CL-2 and CL-3, however increase by CL-2 was on day 11 and 15, but increase by CL-3 was only on day 15 as compare to control.

Fig. 3 reveals the effect of combination doses of CL and PG on FSH. Significant increase in FSH was observed by CP-1 on both days 11 and 15 as compare to control,
whereas CP-2 resulted in significant increase in FSH on day 15 only as compared to control.

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\text{Plasma FSH level (ng/ml) Day 11}
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n=10, Average values ± S.E.M. Columns with (A) differ significantly, P ≤ 0.05 related to control; cont = control; PG-1 = P. granatum 2 mL/kg; PG-2 = P. granatum 5 mL/kg; PG-3 = P. granatum 8 mL/kg

Fig. 1: Effect of P. granatum on FSH

**Effect on LH**

Fig. 4 reveals the effect of PG on luteinizing hormone (LH). Significant increase in LH was observed by all three doses at day 11 as compared to control.

Fig. 5 shows the effect of CL on LH. There was significant increase in LH at day 11 by CL-2 as compared to control.

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\text{Plasma FSH level (ng/ml) Day 15}
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n=10, Average values ± S.E.M. Columns with (A) differ significantly, P ≤ 0.05 related to control; cont = control; CL-1 = C. limon 0.2mL/kg; CL-2 = C. limon 0.4mL/kg; CL-3 = C. limon 0.6mL/kg

Fig. 2: Effect of C. limon on FSH

**Effect on TS**

Fig. 7 shows the effect of PG on testosterone level. Significant increase in testosterone level on day 11 was observed at all three doses as compared to control. However on day 15 there was highly significant rise in testosterone by PG-1 and PG-3 and significant increase by PG-2 as compared to control.

Fig. 8 reveals the effect of CL on testosterone. There was
significant increase in testosterone level by CL-2 on day 11 and highly significant increase on day 15 as compare to control, while at day 15 testosterone level was significantly increased by CL-1 and CL-3 as compare to control animals.

n=10, Average values ± S.E.M. Columns with (A) differ significantly, P ≤ 0.05 related to control

Fig. 3: Effect of combination doses of C. limon and P. granatum on FSH

Fig. 9 reveals the effect of combination doses of CL and PG on testosterone. There was highly significant rise in testosterone by CP-1 and CP-2 on day 11. While at day 15 there was highly significant rise in testosterone by CP-1 and significant increase by CP-2 as compare to control.

DISCUSSION

Flavonoids are naturally occurring molecules abundantly found in fruit and vegetables, number of studies have suggested an inverse association between flavonoids intake and various disorders, due to their antioxidant properties. Since flavonoids as antioxidant remove and prevent the formation of ROS and lipid per-oxidation. Hence alterations in synthesis of ROS in gonads kindle the oxidation and DNA damage of cells (Sanoeka and Kurpisz, 2004; Henkel, 2005; Grassi et al., 2010). Present study specifically evaluates the therapeutic effectiveness
of flavonoids rich fruits *C. limon* and *P. granatum* along with their combinations (CP) on plasma gonadotropins.

![Graph of Plasma LH (ng/ml) Day 11](image1)

![Graph of Plasma LH Level (ng/ml) Day 15](image2)

In present study rabbits were selected, since biochemical and histopathological changes produced in rabbits are comparatively similar to humans (Irena et al., 1979). Moreover sufficient amount of blood samples can easily be obtained twice at Post natal days (PND) 11 and 15. These treatment paradigms were chosen so as to collect blood during the rise (day11) and during the peak (day15) of plasma FSH in female rabbits (Wilson et al., 1998).

PG is a vital source of anthocyanins, hydrolysable tannins punicalagin and punicalin, ellagic and gallic acids and vitamin C. Their antioxidant and free radical searching activity have been reported. Results of present study showed significant increase in plasma FSH and testosterone level by PG, this might be due to the presence of testosterone and estrogen like compounds in PG (Kim et al., 2004; Lansky et al., 2005). Dihydrotestosterone (DHT) can excite FSH secretion in vitro responsible for infantile surge of FSH and its responsiveness to GnRH even if there is ovarectomy (Tohei and Kogo 1999). Report of Wilson et al., (1998) showed relationship of estrogen with FSH. According to this report estrogen receptor antagonists can suppress FSH secretion.

![Graph of Plasma level of LH (ng/ml) Day 11](image3)

![Graph of Plasma level of LH (ng/ml) Day 15](image4)

Result of present study also showed significant increase of serum FSH and TS by CL. This might be due to abundant flavonoids present in CL, which are reported to influence all steroidal function by increase in protein synthesis via cAMP-dependent protein kinase A (PKA).
pathway, since interaction of FSH with its receptors activates adenyl cyclase (AC) that leads to the production of cAMP, which in turn activates PKA (Yu et al., 2003). Another possible mechanism by which serum FSH and TS level may increase is probably the result of the loss of direct central nervous inhibition of FSH secretion by GnRH, that promotes the synthesis and secretion of FSH and LH in female rabbits and speeds up the development of ovary and follicles in female rabbits (Wi et al., 2012; Kerr and Sharpe, 2013).

Results of present study also demonstrated significant increase in FSH and TS by CP-1 almost similar to PG and CL. Hence it could be postulated that estrogen like components in PG and flavonoids in CL have activates steroidal receptors, giving estrogen like effect (Wilcox et al., 1999; Kim et al., 2002) are by CP-1 on FSH and TS. Progesterone in PG has been shown to modulate levels of FSH, signifying a role for progesterone in the regulation of FSH synthesis, hence it may be suggested that alterations in gonadotropins secretion of the female rabbits during sexual development is due to these essential components (Lansky and Newman, 2007). Thus PG is used as a natural symbol of fertility in many cultures (Mahdihasan, 1984; Fawole and Opara., 2013).

Fig. 7: Effect of *P. granatum* on Testosterone

Results of current study revealed significant increase in LH by PG, CL and CP at PND 11, which is consistent with the result of Wilson and Handa (1997) who revealed that LH was inhibited by GnRH antagonist at PND 11, suggesting the role of progesterone as GnRH agonist.
increasing level of LH (Lansky and Newman, 2007). While result observed at PND 15 in present study by PG, CL and CP matches with the report of Wilson and Handa (1998). According to this report no significant change was observed at PND 15, indirectly support our results and suggest that LH secretion altered in treated animals from PND 12-15.

Report of Cho et al., (2013) suggests that antioxidant action of flavonoids affect gonadotropins. They modulate antioxidant enzyme activity or interrupt signaling cascade and plays important role in blocking oxidative hormonal injury. Therefore it may be suggested that flavonoids richly present in C. limon and P. granatum may be responsible for favorable effect of these fruits on gonadotropins.

Overall data suggests that testosterone and estrogen like compounds in these fruits act on FSH and LH in early infantile period in a positive fashion (Sreeja et al., 2012; choi et al., 2006). This increase in FSH and LH level may also be due to chloride presence in these fruits (Waheed et al., 2004; Gonzalez et al., 2010), since there are several studies which demonstrates the possible role of chloride in the regulation of both FSH and LH (Dutt et al., 1978; Ramalingam et al., 2003). Hence it may be concluded that concomitant administration of PG, CL and CP to female rabbits improves the reduced fertility. Therefore, it seems logical to advise infertile patients to include PG and CL juices in their diet to minimize or prevent the female fertility and avoid adverse effect of drugs taken for this purpose.

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REFERENCES


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