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Ameliorative Efficacy Of *Tribulus Terrestris* Fruit Extract On Female Reproductive Hormones: An Interactive Study Of Co-Induced Diabetes And Cold Stress

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ABSTRACT
Diabetes existed as a global disease and people residing in cold temperature areas encounter synergetic effects resulting in exacerbation of diabetic impediments. In view of dearth in literature with respect to its synergistic actions, this study was intended to discover an interactive effect of co-induced diabetes and cold stress on female rat reproductive hormones and also to assess ameliorative role of both *Tribulus terrestris fruit* (TTF) extract. STZ induced (45mg/kg bw) diabetic rats were exposed to cold stress 4±2ºC to explore the alterations in female reproductive hormones. A group of experimental rats were supplemented with TTF and extract individually at doses of 150, 200, 250 mg/kg bw for 19 days respectively. Significant alterations occurred in the levels of gonadal hormones in experimental rats' advocates exacerbation in toxicity due to cold stress. Both diabetes and cold stress alone found to decrease FSH, LH and estrogen levels significantly ($P<0.05$), while progesterone levels increased significantly. Contrary to supra, cold exposure to diabetic rats caused augmentations in FSH, LH and progesterone levels with decreased estrogen levels. Supplementation of both TTF extract was found to be advantageous in reinstating the altered gonadal hormones as witnessed in cold stressed diabetic rats. The ameliorative efficacy of phytoextract could be due to the additive/individual ability of polyphenols, flavonoids and steroidal saponins which are known for hormone regulatory actions and also antidepressant actions suggesting their therapeutic role in both cold stressed and diabetic population.
Key words----STZ induced Diabetes, Cold stress, *Tribulus terrestris* fruit

1. Introduction

The adverse effects of diabetes on physiological systems are commonly recognized and have been extensively studied. Evidences from both animal and clinical research work have proved that diabetes mellitus is normally linked with altered gonadal functional abilities. Defects in carbohydrate metabolism with consistent efforts of the physiological system to correct the metabolic imbalance pose an over exertion on the endocrine system leading to the disruption of endocrine control. In general hyperglycemia exhibit an altered endocrine function, reflecting changes in the levels of thyroid and gonadal hormones in particular, increased circulating glucocorticoids in general. Deterioration of endocrine control exacerbates the metabolic disturbances by altering the glucose levels which lead to hyperglycemia (Bastaki, 2005).
Gonadal hormones include both steroid and peptide hormones and the major steroid hormones constitute estradiol and progesterone from ovaries and testosterone from testes. The pituitary secretions such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) that target the ovaries and control the production of estrogens. Both FSH and LH shown to influence in synthesizing ovarian hormones; mainly estrogens from follicular cells and progesterone from luteal

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cells (Everett, 1994). It is evident from literature that the female reproductive function greatly altered by diabetes causing alterations in the timing of the estrous cycle, and modifications in ovarian function, such as decrease or absence of ovulated oocytes (Bestetti *et al*., 1985). Likewise, insulin-dependent diabetes shown to affect the fertility by increasing embryonic resorptions, congenital abnormalities and perinatal mortality in female rats and mice (Diamond *et al*., 1989; De Hertogh *et al*., 1992). In addition, insulin resistance also reported to modify the expression of gonadotropins and poor ovarian response to gonadotropin stimulation which was found association with female infertility (Kirwan *et al*., 2002; Cai *et al*., 2007).

Hyperglycemia caused decrements in serum FSH and LH levels in different diabetic animal models have been reported (Bestetti *et al*., 1985; Ballester *et al*., 2004), accompanied by loss of ovarian cell sensitivity in diabetic rats (Katayama *et al*., 1984). The findings of Diamond *et al*., (1989) and De Hertogh *et al*., (1992) have confirmed the alterations in female diabetic rat reproductive ability which have been implicated as metabolic disturbances caused by hyperglycemia and lack of insulin, and further evidenced by termination of normal estrous cycles, loss of the preovulatory luteinizing hormone (LH) surge and infertility in STZ induced diabetic female rats (Bestetti *et al*., 1985; Farina *et al*., 1971). Confirming the loss of ovarian functions in female diabetic rats, Katayama *et al*., (1984) reported lack of hypothalamic gonadotropin releasing hormone (GnRH) as the mechanistic reason (s) behind loss of ovulation in STZ induced diabetic rats; in particular, the alterations in hypophysial/gonadal hormonal axis in diabetic rats as reflected by changes in ovarian follicular growth, oocyte maturation and estrous behaviour cause metabolic disturbances (Bestetti *et al*., 1985).

Profound sex differences have been documented in laboratory experiments, where inducing stress has led to impaired reproductive functions. For instance, female reproductive system is found to be highly sensitive to physiological stress and several studies have reported adverse effects of cold stress on different aspects of reproduction (Warren and Perloth, 2001; Thibier and Rolland, 1976; Gray *et al*., 1978; Schillo *et al*., 1978; Tache *et al*., 1978; Barb *et al*., 1982). High cold stress perception being a risk factor for polycystic ovarian syndrome (PCOS), have been shown to cause variety of ailments such as anovulation, severe premenstrual pain, and poor pregnancy outcome including preterm delivery and low birth weight, as well as postpartum depression and early onset of perimenopause (Greenberg, 2002). Preponderance of studies conducted on animal models indicate ill effects of chronic cold stress on gonads that impairs gonadal hormone secretion particularly LH and follicle development in rats (Rose, 1985; Akibami *et al*., 1996; Grey *et al*., 1978; Tache *et al*., 1978; Moberg, 1987). In addition, chronic intermittent stress in the form of cold exposure has been shown to alter ovarian follicular development in rats (Dorfman *et al.,* 2003; Christian and Lemunyan, 1958; Christian, 1971; Moberg, 1987). Rats exposed to chronic cold stress twice a day (1 hr duration) for four days shown to elevate progesterone with decreased estrogen (Andersen *et al*., 2004). Contrarily, findings of Marcelo *et al*., (2008) demonstrated high plasma estradiol concentrations in rats upon cold stress (at 4 °C for 3 hr), while progesterone, LH and FSH levels remained unaltered. Daniels and Berga, (1997) and Pastor *et al*., (1998) observed a higher surge in gonadotropins in rats upon exposure to cold stress which seems to alter the feedback regulation of the hypothalamus-pituitary-gonad axis, as the increased plasma levels of estradiol and testosterone were not found to accompanied by an alteration in gonadotropin secretion. Studies of Nakamura *et al*., (2008) reported alterations in cold stressed rats wherein suppressions in gonadal hormone levels with activation of sympatho-adrenomedullary system as well as oxidative stress are the mechanistic reasons shown to affect the reproduction.

Plants being virtual in exhaustible sources of structurally diverse and biologically active substances as selective plants reported to possess the ability to boost reproductive functions. Impaired carbohydrate metabolism being the task to manage diabetics when modern drug action fails. The available literature on phytochemicals suitable for diabetes reveal diverse actions. For instance, studies of Ercan and El, (2016) and Zhang and Feng, (2012) have reported TTF usage to decrease the levels of blood glucose by increasing insulin concentration in STZ induced diabetic rats. The

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mechanistic reason being that the isolated saponin components of *Tribulus terrestris* have been shown to inhibit α-glucosidase enzyme and bring reductions in the rate of digestion as well as absorption of glucose (Amin *et al*., 2006). *Tribulus terrestris* extract at dose 10 mg/kgbw for 14-days shown to induce follicular growth and formation of corpus luteum which led to early onset of puberty (Esfandiari *et al*., 2011). Root extracts of *Tribulus terrestris* shown to possess hormone regulatory activity wherein, its exposure ameliorated the changes induced by lead toxicity in testes of mice and showed significant improvement in serum testosterone, FSH and LH (Khairwal *et al*., 2015). Handfull of studies proved that *Tribulus terrestris* has an ability to improve reproductive function by increasing the concentration of hormones such as estradiol, testosterone (Tomova and Gyulemetova, 1978; Gauthaman *et al*., 2002). Wang *et al*., (2013) Saponin fractions available in *Tribulus terrestris* (at a dose of 0.75 and 2.25 g/kgbw) found to be beneficial to stressed rats, wherein reductions in serum cortisol could significantly prevent the abnormalities induced upon stress by its anti-depressant activity.

There is increasing evidence that stress affects endocrine system. Some of these changes are due to hormonal changes like FSH, LH and effects of stress depend on the nature of specific stress. Even though extensive studies are conducted on both human and animal model especially on aspects connected to cold stress on stress and reproductive hormones, there is a lack of information concerning the *in vivo* impairment caused by the chronic cold stress exposure in diabetic models. The present study was conducted with a view to assess the changes in endocrine function of diabetic rats exposed to chronic cold stress. Previous reports on regulation of endocrine function in diabetics and cold stressed subjects indicate a clear alteration in pathways and the outcome. The impaired endocrine function and its complication(s) in diabetics of cold region remain unclear. Thereby phytotherapy is an awful need to normalize the endocrine functioning ability. Although several antioxidants of chemical origin have been proposed to challenge the hypoglycemic effect in diabetic models, their exposure may cause side effects, hence phytotherapy is the best option. Phytoextracts like TTF, is also known to possess specific mechanism of action(s) in regulating the altered endocrine hormones and to exhibit their hormone regulating and anti-hyperglycemic activity. Therefore, the present study aimed to address the extent of hormonal imbalance occurred in cold stress exposed diabetic rats and the amelioration brought by ethanolic extract of *Tribulus terrestris* fruit exposure.

2. Methodology

2.1 Experimental Design

Experimental rats (Wistar strain, *Rattus norvegicus*) procured from Sri Raghavendra enterprises, Bangalore, and they were acclimatized to laboratory conditions with supplementation of water and food *ad libitum*. Upon confirmation of healthy animals, they were proceeded for experimentation. The sample size and experimental design as well as objectives of the study was approved by the Institutional Animal Ethical Committee, Bangalore University, Bangalore (CPCSEA No. 402, File No. 25/525/2009 dated 23.03.2011). The sample size was calculated based on the pilot studies (reported in previous chapter 2b). Accordingly, the number of animals in each group comprises six female rats. Group I comprises control animals and they were given normal tap water and Group II consists of STZ (45 mg/kgbw, ip) induced diabetic animals. Group III consists of cold stress (4 ± 2 °C, 3 hr/day 7-days) exposed animals. Group IV serves as positive control wherein, rats with hyperglycemic state (>200mg/dl) were exposed to cold stress (4 ± 2 °C, 3 hr/day for 7-days) and these group animals were further segregated into sub groups (Group V (A, B, C) & Group VI (A, B, C) to check the efficacy of TTF extract, exposed for a period of 19 days. While continuing the cold stress exposure, from 8th day onwards, group V and VI animals in A, B, C subgroups were given intra-gastrically TTF extract at a doses of 150, 200, 250 mg/kgbw/day. At the end of 26th day of TTF supplementation, blood samples were collected by cardiac puncture for serum collection, the same was used for gonadal hormonal assessments (progesterone LH, FSH and estradiol). All test animals along with control group were sacrificed by spinal dislocation.

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2.2 Hormone analysis

Estimation of serum FSH, LH, Estrogen and Progesterone

Serum concentrations of FSH, LH were measured by double antibody radioimmuno assay method. Estrogen and Progesterone estimates were assessed by chemiluminescence method using reagent kit provided by Thyrocare Technologies Ltd, Mumbai, India. Highly purified rat LH (rLH-4) and rat FSH (rFSH-1-8) were iodinated with 1mCi freshly prepared chloramine T. The limit of detection of the assay was 0.1 ng at 90% and the intra-assay coefficient of variation was 3.0%. NIDDK rLH-RP-3 was used for the assay. The limit of detection was rLH was 0.05 ng at 80%. For the FSH assay, NIDDK rFSH-RP-2 was used as a standard and NIDDK anti-rFSH-S-11 was used for the assay. The limit of detection was 0.04 ng at 98%. All the samples were assayed on the same day to avoid inter assay variation.

The concentrations of estradiol and progesterone in blood serum samples were determined using the PATHFAST analyzer with the thyrocare reagent kit for estradiol and progesterone. In brief, sample measurement by PATHFAST was performed using a single reagent cartridge containing 100 µl of blood serum sample. In the competitive assays, estradiol and progesterone in the samples were inhibited from forming an immune-complex by an alkaline phosphatase-conjugated antigen. The desired number of coated wells in the holder secured and dispensed 25 µl of standards, specimens and controls into appropriate wells followed by 50 µl of rabbit anti-estrogen and anti-progesterone reagent to each well separately. Gently mixed for 5 seconds and added 100 µl of working estrogen and progesterone-HRP conjugate reagent into each well. Further thoroughly mixed for another 30 seconds and incubated at room temperature (18-25 °C) for 90 minutes. Then it was followed by rinsing and flicking the micro wells 5 times with washing buffer (1X) and added 100 µl chemiluminescence substrate solution into each well. Finally, the reaction mixture was gently mixed for another 5 seconds and read wells with a chemiluminescence micro well reader 5 min later (between 5 and 20 min after adding the substrates). The resulting chemiluminescent reaction was measured as relative light unit (RLUs). An inverse relationship exists between the amount of estrogen and progesterone in the sample and the RLUs detected by the (architect i) optical system. Results were obtained by calculating the average read relative light units (RLU) for each set of reference standards, control, and samples (estrogen concentration in pg/ml and progesterone concentration in ng/dl).

3. Results

The findings of this study confirm the deleterious effect of diabetes and cold stress on the body glucose levels and hormones of female reproductive organs viz., uterus, ovary and oviduct.

3.1 Serum glucose levels

Supplementation of TTF extract to diabetic rats, the serum glucose levels decreased significantly ($P<0.05$) on the 19th day of extract administration and further remained constant till 30th days of TTF exposure, indicating the anti-hyperglycemic properties of the fruit extract of TT, as the substance with anti-hyperglycemic properties would be effective in the management of diabetes (Table 1). Statistical analysis reveal that the serum glucose levels reduced at a dose of 200mg/kg body weight suggesting the ameliorative role of TTF on the 19th day of exposure in extending protection to diabetic animals, compared to other doses. Hence, the present study demonstrates that TTF extract of 200mg/kg body weight dosage was found to be an effective dose (Table 1).

Table 1- Effect of *Tribulus terrestris* fruit ethanol extract of different doses (50-250mg/kg bw/day) on blood glucose level (mg/dl) in STZ induced diabetic female rats during the experimental period of 30 days. [Values are expressed as mean ± SD (n=6) from each group]

Groups	5th day	10th day	15th day	30th day

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Control	77±3.22#	76±2.68#	77±2.25#	77.66±4.13#
STZ	316.33±18.11* (-310.3)	333.66±12.40* (-338.9)	351± 3.09* (-355.8)	370 ±5.08# (-380.5)
STZ +TTF 50	231±8.53* (+26.9)	193.33±5.95*# (+42)	149.33±3.14*# (+57.4)	114.33±5.75# (+69.1)
STZ +TTF 100	230.66±5.08* (+27)	172±5.04*# (+48.4)	128±9.46*# (+63.5)	93±1.78# (+74.8)
STZ +TTF 150	218±7.32* (+31)	152±8.80*# (+54.4)	118±3.22*# (+66.3)	91.33±1.36# (+75.3)
STZ +TTF 200	219.66±17.89* (+30.5)	136.33±17.76*# (+59.1)	83±2.36# (+76.3)	89±1.78# (+75.9)
STZ +TTF 250	223±4.64* (+29.5)	147.66±11.80*# (+55.7)	94.33±2.25# (+73.1)	93.66±1.36# (+74.6)

STZ= Streptozotocin; TTF= *Tribulus terrestris* fruit.
P<0.05 as compared to * normal control rats; # diabetic control. Values in parenthesis indicate the % change & recovery; '+'sign indicates increase and '-'indicates decrease over the controls

3.2 *In vivo* Hormonal assays

Serum reproductive hormones

Data shown in Table (2) and Fig (1) represents the changes occurring in the reproductive hormones viz., FSH, LH, estrogen and progesterone as a consequence of STZ induced diabetes and cold stress and their co-exposure. From data, it is evident that both diabetes and cold stress alone found to decrease FSH, LH and estrogen levels significantly (P<0.05) while progesterone levels increased significantly. Contrary to supra, cold exposure to diabetic rats caused increments in FSH, LH and progesterone levels with decreased estrogen levels. Contrary to supra, induction of cold stress to diabetic rats during the experimental period significantly decreased serum oestrogen (-66.60%) and increased FSH (+44.84%), LH (+67.50%) and progesterone (+60.92%) levels when compared with the normal control group. Pre-treatments of diabetes-intoxicated and cold stress induced rats with TTF extract significantly (P<0.05) increased serum oestrogen (+139.63%) and decreased FSH (-23.65%) LH (-38.21%) and progesterone (-53.10%) levels when compared with the intoxicated control groups. Supplementation of TTF extract at a dose of 200mg/kgbw was found to be beneficial in restoring the altered FSH, LH, estrogen and progesterone levels.

Table 2. Dose-dependent effect of *Tribulus terrestris* fruit (TTF) extract on cold stress caused modulation in diabetic rats: Changes observed in female gonadal hormones.

Group	FSH (ng/ml)	LH (ng/dl)	Estradiol (pg/ml)	Progesterone (ng/dl)
Control	47.12±1.21	17.42±1.22	75.62±3.58	35.98±1.19
STZ	31.32±1.13* (-33.53)	16.81±1.31 (-3.50)	37.87±2.56* (-49.92)	49.23±1.14* (+36.83)
CS	39.43±.24* (-16.32)	16.21±1.21 (-6.95)	59.93±2.42* (-20.75)	48.21±1.25* (+34.00)
STZ+ CS	68.25±1.32* (+44.84)	29.18±1.43* (+67.50)	25.26±2.38* (-66.60)	57.90±1.33* (+60.92)
STZ+CS+TTF 150	62.04±1.13* b c (-9.09) ¥	18.19±1.06 b (-37.66) ¥	45.23±0.45*† b (+79.05) ¥	23.17±1.42*† b c (-59.98) ¥
STZ+CS+TTF 200	52.11±1.05† a (-23.65) ¥	18.03±1.04†b (-38.21) ¥	60.53±0.43*† a (+139.63) ¥	27.15±1.48† a (-53.10) ¥

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STZ+CS+TTF 250	60.16±1.07* b c (-11.85)¥	18.22±1.13 b (-37.56)¥	48.21±0.34*† b (+90.85)¥	21.31±1.34*† c (-63.19)¥

Abbreviations: STZ: Streptozotocin; CS: Cold stress; TTF: *Tribulus terrestris* fruit and numerical 150, 200 and 250 are dose(s) used in mg/kgbw as prophylactic treatment.]

Values are Mean ± SEM of six animals. *p<0.05 significantly different from control; †p<0.05 significantly different from positive control (STZ+CS) compared within each column by using Bonferroni post hoc. Different superscripts (a, b and c) within each column indicate significant (p<0.05) differences among antioxidant treatments compared to positive control. Values in parenthesis indicate percentage (%) change observed over control, while values in parenthesis with super script ¥ symbol indicate percentage (%) recovery, '+' sign indicates increase and '-' indicates decrease.

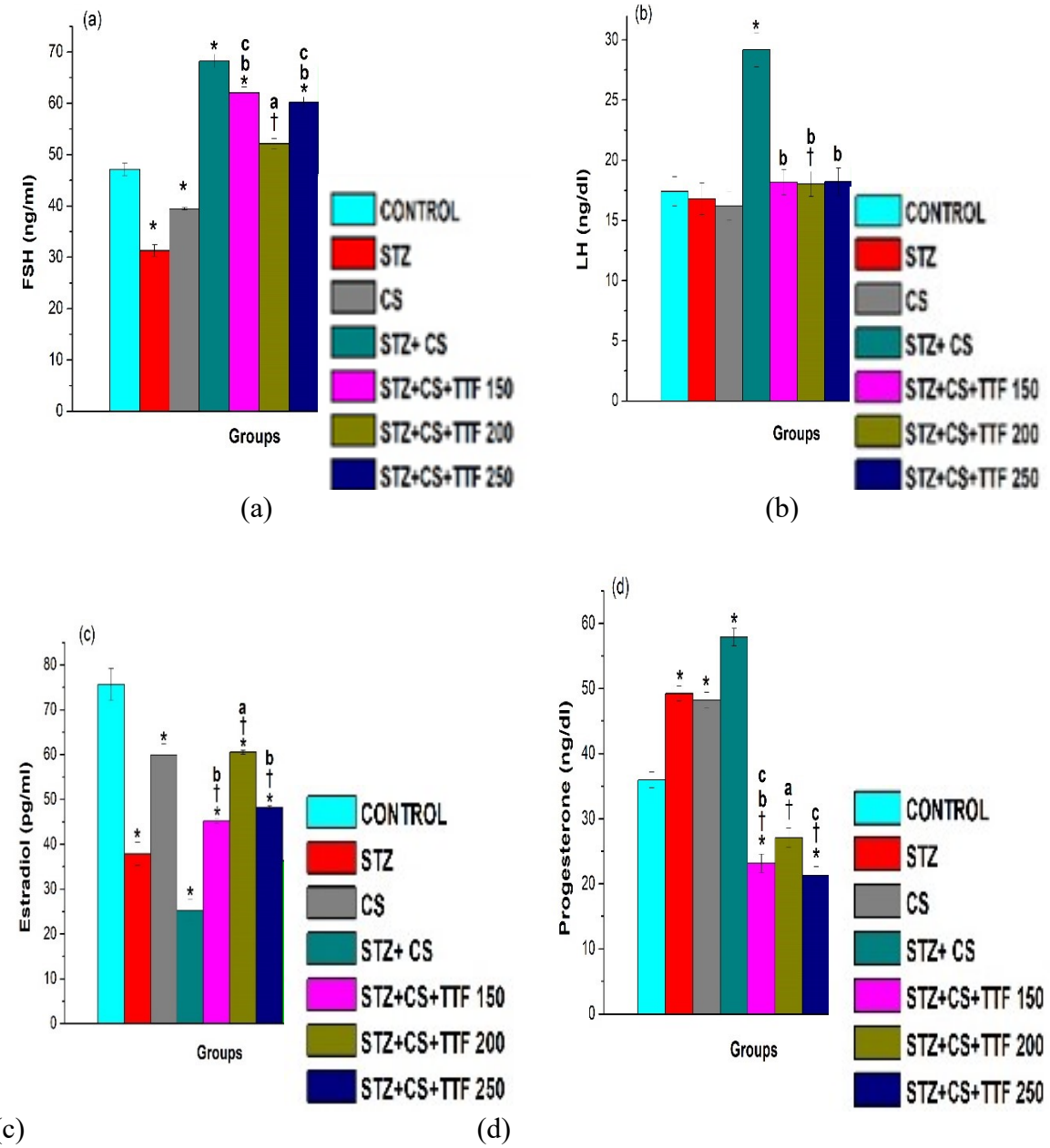

Figure 1. Dose-dependent influence of *Tribulus terrestris* fruit (TTF) extract on cold stress caused modulation in diabetic female rats: Changes observed in gonadal hormone levels (a) FSH (b) LH (c) Estradiol and (d) Progesterone.

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Results are Mean ± SEM of six animals. *p<0.05 significantly different from control; †p<0.05 significantly different from positive control (STZ+CS) compared with treatment groups. Different superscripts (a, b, and c) indicate significant (p<0.05) differences among antioxidant treatments compared to positive control.
[Abbreviations: STZ: Streptozotocin; CS: Cold stress and numerical 150, 200 and 250 are dose(s) used in mg/kgbw as prophylactic treatment.]

4. Discussion

4.1 Impact on Serum glucose levels

Increased glucose-oxidation, non-enzymatic glycation of proteins and their subsequent degradation cause unbalanced free-radical generation in diabetes Indeed, hyperglycaemia mediated advanced glycation of intracellular antioxidant defence enzymes results in hyper-susceptibility to the elevated oxidative stress due to lowered anti-oxidative protection. In the present study the elevated glucose level in all the experimental rats was significantly lowered upon TTF supplementation and may be through inhibition of α-glucosidase as well as by its antidiabetic effects and the results are in accordance with Lamba et al.[24]. It was also reported that saponin has the hypoglycemic effect and the fraction of it inhibited the activity of α-glucosidase in small intestines in rats and retarded the increase in postprandial blood glucose level in rats[37,38]. Our results indicated a decrease in body and reproductive organs weight of the diabetic and co-exposed rats in comparison to the normal control rats. The decrease in body and reproductive organs weight was as a result of loss of tissue proteins and muscle mass in diabetes[39]. It is known that glycosuria causes a significant loss of calories for every gram of glucose exerted and most likely, this loss results in severe weight loss in spite of increased appetite, particularly when it is coupled with muscle and adipose tissue due to excessive breakdown of the protein. In this study, supplementation of TTF extract at a 200mg/kg bw dosage level in STZ-induced diabetes intoxicated and co-exposed rats produced a significant protective effect against reproductive organs functional tissues toxicity and this effect characterized by increased weights of body and reproductive organs viz., uterus, ovary and oviduct, improved follicular quality and quantity. These findings are in accordance with those previously reported[40,27]. TT extract contents such as saponins (disgenin) and sterol (ß-siosterol, stigma sterol) which contain phytoestrogen and the metabolites of phytoestrogen exert an estrogenic effect on central nervous system which induces estrous and stimulates cell division and growth of genital tract of female animals[41]. On the other hand, in contrast Martino- Andrade *et al.*[42], who reported that low dose levels of TT purified extract given to castrated female rats for 28 days was unable to stimulate endocrine sensitive tissues such as uterus and vagina.

Profound sex differences have been documented in laboratory experiments, where inducing stress has led to impaired reproductive functions as well as gonadal tissue damage (Whirledge and Cidlowski, 2013). Though the exact mechanism(s) by which a hyperglycemia and cold stress induces organ damage is still a matter of discussion, the excitotoxic actions of glutamate through N-methyl-D-aspartate (NMDA) receptor, increases the potentiation of glucocorticoids that have been implicated in the pathogenesis of stress-induced organ injury (Kim *et al*., 2010). Increased oxidative stress has also been implicated due to elevated glucocorticoids (Shugaba *et al*., 2010; Pereira *et al*., 1999). Hence cortisol involvement enhances the toxicity of reactive oxygen species by decreasing the activity of glutathione peroxidase which results in a negative feedback mechanism that depletes GSH and exacerbates the apoptotic process (Macho *et al*., 1997; Patel *et al*., 2002).

4.2 Impact on Gonadal hormones levels

Follicular growth, oocyte maturation and estrous behaviour are regulated by FSH/estrogen hormones, whereas ovulation and maintenance of gestation (at early stages) are controlled by LH/progesterone system, although FSH plays a relevant role (Everett, 1994). The prevailing literature on female rat reproductive functions are greatly biased especially in diabetes and alterations as reported include variations in timing of the estrous cycle, modification in ovary functions such as decrements or absence of ovulated oocytes (Bestetti *et al*., 1985). In this study,

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decrements found in the level of gonadal hormones such as FSH, LH and estrogen while progesterone levels increased significantly. Thereby, results of the present study are in agreement with findings of Cecillia *et al*., (1990); Abeer *et al*., (2013); Ballester *et al*., (2007); Khaki *et al*., (2009) wherein, authors pronounced similar decrements in serum FSH, LH and estrogen levels in diabetic rats. In addition, studies of Dudley *et al*., (1981) have reported abnormality in estrogenic receptor translocation in hypothalamus and pituitary resulting in discrepancy of hormones. Besides, the proestrous LH surge was absent in diabetic rats which has been well documented in previous studies (Bestetti *et al*., 1985; Katayama *et al*., 1984). Later, studies evidenced that, STZ-treated female rats are anovulatory and attenuated LH response to ovariectomy (Blades *et al*., 1985; Steger *et al*., 1993; Tesone *et al*., 1986). Investigations of Cecillia *et al*., (1990) and Oksanen, (1975) confirmed insulin deficiency in female diabetic rat resulting in low FSH and estradiol as well as an elevated glucose levels. Since, FSH acts synergistically with LH in the stimulation of androgen synthesis, the reductions in gonadotropin, could play an important role in causing decrements of gonadal hormone (s) output in diabetic animals (Orth *et al*., 1979).

Stress impairs gonadal function and lowers concerned hormone levels (Akibami *et al*., 1996). Findings of Marcelo *et al*., (2008) demonstrate that when rats subjected to one session of acute cold stress (at 4°C for 3hr.) their plasma estradiol concentrations augmented while progesterone, LH and FSH levels remain unaltered. Contrary to above, this study results demonstrate that chronic intermittent cold stress able to induce a significant reduction in serum concentrations of FSH, LH and estrogen levels while increments in progesterone was evidenced upon cold exposure in rats. Changes as witnessed in reproductive hormones represent diminished reproductive performance associated with stress. Thus, the primary effect of cold stress appears to be in association with sympathetic nerves on the ovary, resultantly, their activation modifies ovarian follicular development in rats (Dorfman *et al*., 2003). In addition, studies of Lara *et al*., (1993) and Sotomayor *et al*., (2008) suggests mediation of nor-epinephrine (NE) in maintaining follicular and ovarian activity and the fact that ovarian NE activity seems to differ in cold stressed polycystic ovary due to trophic effect of estrogens, characterized by an increase in an ovarian NE activity that prevented all morphological and hormonal alterations induced by cold stress.

In this study a significant increase in the level of FSH, LH and progesterone along with decrements in estrogen level was observed as a result of cold stress exposure to diabetic rats. This could be due to interactive effect of cold stress in modulating the hormone levels as reflected in terms of increased serum concentrations of FSH, LH, progesterone levels and decreased levels of estrogen signifying the actions of cold stress as a state of allostasis, and showed synergistic interaction which was more significant. It can be said that chronic-intermittent cold stress as a physical stressor activates ovarian NE nerve terminals leading to insulin resistance in theca-interstitial cells of ovary accordingly may bring changes in an ovarian morphology and functions (Dorfman *et al*., 2009). In addition, neuroendocrine dysfunction underlies the abnormalities in female reproduction associated with diabetes and cold stress.

4.3 Ameliorative role of plant extract supplementation

Plants often contain substantial amount of antioxidants, including α-tocopherols (vitamin E), carotenoids, ascorbic acid (vitamin C), flavonoids, saponins and tannins (Larson, 1988). The bioactive compounds of phytoextracts has led to increased attention to their safety and efficacy in the treatment of diabetes as well as fertility issues. Female reproductive cycle as well as reproductive functions relay primarily by the interplay of hormones viz., luteinizing hormone (LH), follicle stimulating hormone (FSH), progesterone, estradiol and prolactin, *etc*. The integrity of female reproductive functions can be predicted by hormonal assessments (Bowman and Rand, 1980). In this study, supplementation of TTF extract found helpful in normalizing the altered gonadal hormones as witnessed in cold stressed diabetic rats. Rezaie *et al*., (2013) and Rajabi and Karimi, (2014) used *Tribulus terrestris* extract at different doses to assess cyclophosphamide induced stress wherein, amelioration was evident to normalize altered gonadal hormones in male and female rats. Steroidal saponins of *Tribulus terrestris* may be responsible to bring stimulative

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responsiveness on gonadal tissues such as the ovary and uterus (Postigo *et al*., 2016) and particularly protodioscin could increase endogenous androgen production by increasing luteinizing hormone (LH) from the pituitary gland (Martino *et al*., 2010) and also enhance the production of gonadal hormones in healthy subjects (Milanov *et al*., 1981).

In this study, supplementation of TTF found to be beneficial in normalizing gonadal hormones in cold stressed diabetic rats and its at moderate dose offered protection to gonadal hormones indicating dose-independent effect of extract and the highest amelioration found to be resided at the modest doses administered. With regard to dose-regimens tested individual exposures of TTF extract at a dose of 200 mg/kgbw respectively, is able to tackle the hormonal impairments caused by cold stress in diabetic rats. The ameliorative efficacy of phytoextract could be due to the additive/individual ability of polyphenols, flavonoids and steroidal saponins which are known for hormone regulatory actions and also antidepressant actions suggesting their ameliorative role in both cold stressed and diabetic population.

CONCLUSION

To summarize, glucoregulatory hormones viz., cortisol and gonadal hormones are altered in diabetic state. Besides, cold stress being stress factor added increased production of cortisol which in turn decreased glucose tolerance by increasing hepatic glucose production and impaired peripheral glucose utilization, moreover glucocorticoids reduce insulin sensitivity and responsiveness in peripheral tissues. It is presumed that reductions in glucose tolerance is further increased by altering thyroid hormone levels. The lowered estrogen, FSH, and LH as found in diabetic rats could be due to suppressions in hormone sensitivity, besides, progesterone may also produce similar effects in the absence of estrogens, but progestin's appear to antagonize the effects of estrogens. The influence of chronic intermittent cold stress in rats was found to increase the production of glucocorticoids which is partly volunteered by hypo thyroidal and hypo gonadal effect and a condition of exacerbated diabetes developed when the functional reserve of endocrine pancreas becomes limiting.

In conclusion both diabetic induction and cold exposure (4±2 °C) individually imposed mild effect on endocrine functioning, however their combined exposure has modulating effect on thyroid and reproductive hormones. Further, exposure to TTF extract at dose of 200 mg/kgbw/day for 19 days, offered protection normalizing hormonal imbalance that was witnessed in cold stressed diabetic rats.

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