Original Article

Effect of Fluoxetine hydrochloride on the hormonal profile

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Keywords: *Hormone, Fluoxetine, Depression*

Abstract

While the world if fighting with several diseases and waiting for their complete prognosis, one of the diseases taking centre stage in death drama is depression. Depression is a psychological-physiological disorder involving intense feeling of sadness, lack of energy, feelings of helplessness, hopelessness and despair. Depression affects mood, thoughts, body and work efficiency. By the year 2020, depression will be the 2nd most common health problem in the world (WHO & Harvard University).Fluoxetine (trade name Prozac) is an antidepressant drug belonging to the class of the selective serotonin reuptake inhibitors (SSRI). Fluoxetine is approved for the treatment of major depression, obsessive-compulsive disorder, bulimia nervosa, panic disorder and premenstrual dysphoric disorder. Despite the availability of newer agents, it remains extremely popular due to its reduced side effects as compared to other tricyclics anti-depressants. The two main objectives of the treatment for such a disorder are complete prognosis and avoidance of the relapse. The main aim of the current investigation was to find the efficacy of the drug and test its side effects on the reproductive milieu using the albino rats and extrapolate the results of the same to human. Estimation of hormones related to reproduction in order to find the effect of the drug on Hypothalamus-pituitary-gonadal axis. In all the overall objective of the work was to find the effect of the drug on hormonal levels.

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Introduction

Depression is associated with low levels of testosterone in some individuals due to the complex interactions between hormones and emotion ([1]). Low testosterone levels have also been implicated to alter serotonin neurotransmission ([2,3]). Studies on various animal models supports the association of low testosterone levels with dysfunction of serotonin neurotransmission ([4, 5, 6]). Testosterone has also been shown to modulate the expression of different serotonin 5-Hydroxy tryptophan (5-HT) receptors in various regions of the brain ([7, 8, 4, 9]), as well as the function of the 5-HT system ([10, 11, 12]). Further, various studies have shown that age-related decline in free plasma testosterone in some men is associated with changes in mood and mental state ([13, 14]). It has been also reported that under the periods of mental or extreme physical stress, testosterone is reduced and cortisol is increased. And when stress subsides, Testosterone rises and cortisol levels decrease ([15]).

Thus it is evident that mental stress and depression is associated with low testosterone. Since testosterone modulates serotonin neurotransmission, any decrease in testosterone levels that is associated with depression may further affect the mood and mental state through its interaction with the serotonergic neurotransmission. One of the objectives of the project was to study the role of fluoxetine treatment on the testosterone levels as a part of its anti-depression mechanism. This would further help to analyse the role of fluoxetine treatment on reproductive functioning of the animal.

The other hormone under the investigation was estrogen. Estrogen is the steroid hormone involved in the reproductive function and is also known to regulate mood. Various studies have reported the effects of estrogen treatment ranging from depressant to anti-depressant ([16, 17]). Estrogen has also been associated with the increase in serotonin transporter (SERT) expression in the Dorsal raphae nuclei (DRN) of gonadectomized rats, implicating its role in serotonin reuptake dynamics ([18, 19]). George et al. ([20]) have reported that estrogen can also affect the serotonin receptor (5 Hydroxy tryptophan 2A receptor)5-HT2AR by directly affecting the 5-HT2AR gene, or by indirectly affecting the 5-HT2AR gene by way of a transynaptic mechanism, or by a direct non-genomic mechanism. Further, serotonin receptor 5-HT2AR has also been shown to be associated with serotonergic neurotransmission and depression ([20]). Thus it is evident that estrogen can affect serotonergic neurotransmission by interacting with various molecular mechanisms. Estrogen can also induce the spontaneous surge of gonadotropin releasing hormone (GnRH) and thereby pituitary gonadotropins ([21, 22]). Any change in the level of estrogen in body is likely to affect the GnRH and the other hormones that are induced in response of GnRH.

Fluoxetine interacts with serotonergic neurotransmission system to exert its anti-depressant effect. Fluoxetine could therefore affect estrogen levels via its interaction with serotonergic neurotransmission. This would further have its impact on the reproductive functioning. One of the objectives of the project was to study the effect of fluoxetine on the levels of estrogen in blood.

Progesterone was also considered for the investigation. Progesterone is produced and secreted in the circulatory system by both the ovaries and the adrenal glands ([23]). It is considered to be a neurosteroid when it is synthesized and released in the brain or in peripheral nerves ([23]). Earlier studies have reported that the plasma-derived progesterone can serve as a precursorin the brain for the synthesis of potent neuroactive steroidswhich can affect Gamma (γ)-aminobutyric acid (GABA)-mediated neurotransmission ([24]). It was also reported that allopregnanolone is the positive allosteric modulator of the action of GABA at GABA receptors and maintains the local levels of GABAergic neurotransmission efficacy at the required neurophysiological level ([25, 26, 27]).

Fluoxetine and other psychotropic drugs have been shown to affect the metabolism of neurosteroids such as progesterone and allopregnenolone in the brain ([28]). Several lines of evidence support the concept that a down-regulation of brain allopregnanolone biosynthesis plays a role in the development and maintenance of GABA receptor neurotransmission deficits leading to abnormal emotional behaviors, including impulsive aggression, posttraumatic stress disorders, anxiety, and depression ([29, 30, 31, 32]).

If fluoxetine treatment alters the progesterone levels, then the subsequent synthesis of neuroactive steroids would also be affected. These neurosteroids through its effect on GABA mediated neurotransmission would further affect mood and mental status. To study the effect of fluoxetine treatment on progesterone levels was one of the objectives of the project.

Materials and Methods

Test sample: Fludac capsules (manufactured by Cadila 1389, Dholka-387810, Dist-Ahemadabad.), containing Fluoxetine hydrochloride was used for the treatment. The stock solution was prepared in the sterile distilled water. The drug was administered intraperitoneally. The concentration of the drug was 20 mg per kg of the bodyweight of the rat .The drug was administered in the morning hours throughout the study period.

Animal procurement and management: Fresh stock of the male albino rats (*rattusnorvegicus*) of wistar strain (weighing 150-180 grams and 5-8 weeks of age) were used for all the experimental work. All the animals were procured from Haffkine Bio Pharma Corporation Ltd., Parel, Mumbai. All the animals were weighed and their health was verified. Animals were acclimatized to the experimental environment for a minimum period of eight days prior to the commencement of the study.

All the animal experiment was conducted in accordance to the guidelines of CPCSEA (Ministry of SJ & E Govt. of India).

Housing: All the animals were housed in polyurethane cages with wire mesh tops and rice husk bedding. The rice husk bedding was changed every day. Food and water was provided to the animal ad-libitum. Water was provided in an amber coloured glass bottle. A standard laboratory rat feed with balanced nutrition (crude protein 20-21%, crude fibre 4%, calcium1.2%, phosphorus 0.6%) AMRUT FEEDS, supplied by Excel Agencies, Mumbai, was provided to the animals.

The temperature of the animal house was maintained at 28°c (+/- 2°c). The animal house was provided with an artificial light at a sequence of 12 hrs light and 12 hrs dark cycles. Humidity of animal house was not controlled. The humidity as recorded on humitherm was between 50-77% RH during the period of experiment.

Animals were treated for the period of 28 days during which there bodyweight is measured to find any effect on bodyweight. Then they were sacrificed by cervical dislocation, blood was collected by cardiac puncture and tested for levels of testosterone, estrogen, progesterone as described below.

Principle for Radioimmunoassay: The Coat-A-Count procedure is a solid-phase radioimmunoassay, wherein ^{125}I-labelled hormones (testosterone, estrogen, progesterone) competes for a fixed time with hormones (testosterone, estrogen, progesterone) in the serum sample for antibody sites. Because the antibody is immobilised to the wall of a polypropylene tube, simply decanting the supernatant suffices to terminate the competition and to isolate the antibody- bound fraction of the radio labelled hormones (testosterone, estrogen, progesterone). Counting the tube in a gamma counter then yields a number, which converts by way of a calibration curve to a measure of the hormones (testosterone, estrogen, progesterone) present in the serum sample.

Materials required for testosterone, estrogen, progesterone estimation:

Testosterone: Total Testosterone Antibody-Coated tubes:Polypropylene tubes coated with antibodies to testosterone.

^{125}I Total Testosterone:Iodinated testosterone.

Total Testosterone Calibrators: Six vials of testosterone calibrators. The zero calibrator vial A contains 4 ml; the remaining vials B through F contain 1.0 ml each.

The calibrators represent, respectively, 0, 20, 100, 400,800, and 1,600 nanograms of testosterone per decilitre (ng/dL) in processed human serum; Equivalently: 0, 0.7, 3.5, 14, 28, and 55 nanomoles per liter (nmol/L).

Estrogen: Estradiol Antibodies-Coated tubes: Polypropylene tubes coated with rabbit antibodies to estradiol.

^{125}I Estradiol: Iodinated estradiol.

Estradiol Calibrators: One set of seven vials, labelled A through G, of estradiol calibrators, with preservatives. The zero calibrator vial A contains 5 ml; the remaining vials B through G contain 2 ml each. The calibrators represent, respectively, 0, 20, 50, 150, 500, 1800 and 3600 picograms of estradiol per millilitre (pg/ml); in processed human serum. Equivalently: 0, 73, 184, 551, 1,836, 6,608 and 13,216 picomoles per litre (pmol/L).

Progesterone: Progesterone Antibodies-Coated tube 100(200,500,1000): Polypropylene tubes coated with rabbit antibodies to progesterone.

^{125}I Progesterone: One vial (two vials, five vials, ten vials) of iodinated progesterone, with preservatives, supplied in liquid form, ready to use.

Progesterone Calibrators: One set (two sets, three sets) of seven vials of progesterone calibrators, ready to use, in processed human serum, with preservative. The zero calibrator vial A contains 4 ml; the remaining vials B through G contain 2 ml each. The calibrators represent, respectively, 0, 0.1, 0.5, 2, 10, 20 and 40 nanograms of progesterone per millilitre (ng/ml); Equivalently: 0, 0.3, 1.6, 6.4, 31.8, 63.6 and 127.2 nanomoles per litre (nmol/L).

Miscellaneous: Gamma counter compatible with standard 12x75 mm tubes, Vortex mixer, and Radioimmunoassay-Plain 12x75 mm polypropylene tubes-for use as non- specific binding (NSB) tubes, Micropipettes: 50µL and 1.0mL, 100µL and 1000µL. Water-bath capable of maintaining 37^0C- required only for the alternate procedure. Foam decanting rack, Logit-log graph paper, a tri-level, human serum-based immunoassay control, containing known hormones (testosterone, estrogen, progesterone) as one of over 25 assayed constituents.

Procedure:

All components must be at room temperature ($15\text{-}28^0$C) before use.

1. Plain Tubes: Label four plain (uncoated) 12x75 mm polypropylene tubes T (total counts) and NSB (nonspecific binding) in duplicate.
2. Coated Tubes: Label twelve Total Testosterone Antibodies-Coated Tubes A (maximum binding) and B through F in duplicate. (For estrogen estimation: Label fourteen estradiol Antibodies-coated Tubes A (maximum binding) and B through G in duplicate. For progesterone estimation: Label fourteen progesterone Antibodies-coated Tubes A (maximum binding) and B through G in duplicate.) Label additional antibody-coated tubes, also in duplicate, for controls and serum samples.
3. Pipet 100µL of the zero calibrator A into the NSB and A tubes, and 100µL of each calibrators B through G (for estrogen and progesterone) (for testosterone B through F) into correspondingly labelled tubes. Pipet 100 µL of each control and serum sample into the tubes prepared. Pipet directly to the bottom.
4. Serum samples expected to contain estradiol concentrations greater than the highest calibrator (3600 pg/ml) should be diluted in the zero calibrator before assay. Serum samples expected to contain testosterone concentrations greater than the highest calibrator (1,600ng/ml) should be diluted in the zero calibrator before assay. Serum samples expected to contain progesterone concentrations greater than the highest calibrator (40 ng/ml) should be diluted in the zero calibrator before assay.
5. Add 1.0 ml of ^{125}I hormone (^{125}I testosterone, ^{125}I estradiol, ^{125}I progesterone) to every tube. Vortex.
6. No more than 10 minutes should elapse during the dispensing of the tracer. If a repeating pippetor with a plastic syringe is used, rinse twice with tracer before dispensing. Set the T tubes aside for counting (at step 6); they require no further processing.
7. Incubate for 3 hours at room temperature ($15\text{-}28^0$C) for estrogen and progesterone. For testosterone the incubation is for 3 hours at 37^0C.
8. Decant thoroughly. Removing all visible moisture will greatly enhance precision. Using a foam decanting rack, decant or aspirate the contents of all tubes (except the T tubes) and allow them to drain for 2 or 3 minutes. Then strike the tubes sharply on adsorbent paper to shake off all residual droplets.
9. Count for 1 minute in gamma counter.

Calculation of Results([33]):

To obtain result in terms of concentration from a logit-log representation of the calibration curve, first calculate for each pair of tubes the average NSB-corrected counts per minute (CPM):

Net Counts = Average CPM – Average NSB CPM

Then determine the binding of each pair of tubes as a percent of maximum binding (MB), with the NSB-corrected counts of the A tubes taken as 100%.

Percent bond = (Net Counts/ Net MB counts) x 100

(The calculation can be simplified by omitting the correction for nonspecific binding; samples within the range of calibrators yield virtually the same results when percent bound is calculated directly from Average CPM.)

Using logit-log graph paper, plot Percent Bound on the vertical (probability) axis against Concentration on the horizontal (logarithmic) axis for each of the non-zero calibrators, and draw a straight line approximating the path of these points. Results for the un-

knowns may then be read from the line by interpolation.

Result

Body weights of male and female rats treated with fluoxetine were monitored for a period of 28 days. The results of body weights of control as well as treated group of male and female rats are shown in Fig.1. and Fig.2 respectively. The body weights of male as well as female rats showed a significant decrease ($P \leq 0.05$) on treatment on treatment with fluoxetine for a period of 28 days. Two–way ANOVA (Analysis Of Variance) was used to detect significant differences between control and treated group of male and female rats.All values of the body weights for male and female rats are expressed in "grams".

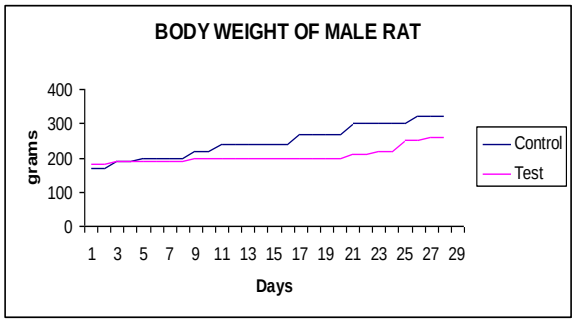

Fig.1 Body Weight of Male Rat treated with Fluoxetine for 28 days

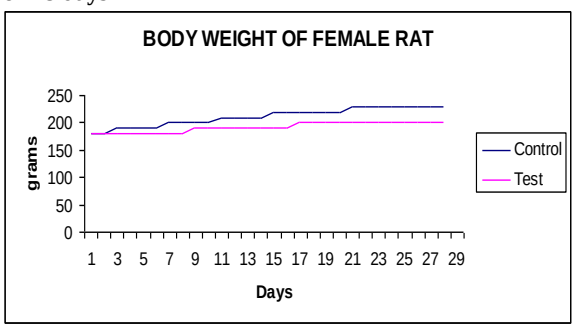

Fig.2 Body Weight of Female Rat treated with Fluoxetine for 28 days

Testosterone, Estrogen, Progesterone, levels from blood in Male Rats treated with drug for 28 days were determined by Radio Immune Assay. The results of the blood testosterone level of control as well as treated group are shown in Fig3. The blood testosterone levels showed a non-significant decrease ($P \leq 0.01$) on treatment on treatment with fluoxetine for a period of 28 days. Student's t-Test was used to detect significant differences between control and treated group rats.The total content of testosterone in blood of control as well as treated animals is expressed as ng/ml (Nanograms/Millilitre).

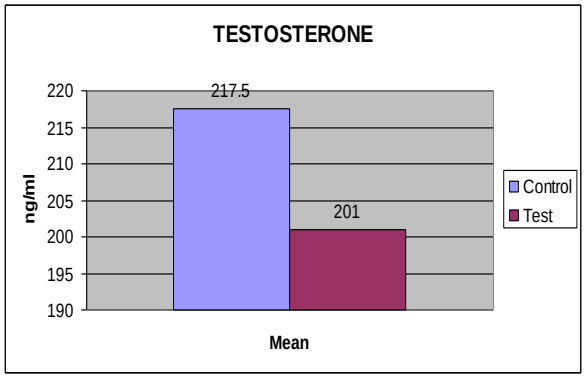

Fig.3 Effect of fluoxetine on Testosterone Level in blood of Male Rats

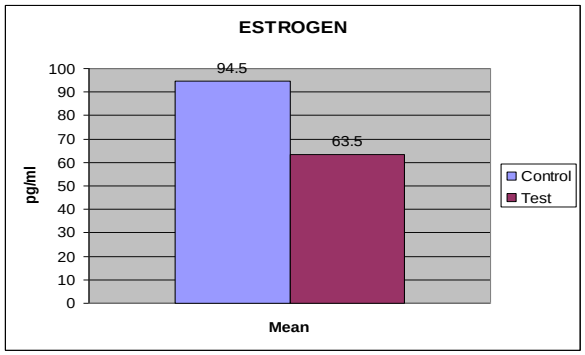

Fig.4 Effect of Fluoxetine on Estrogen Level in blood of Female Rats

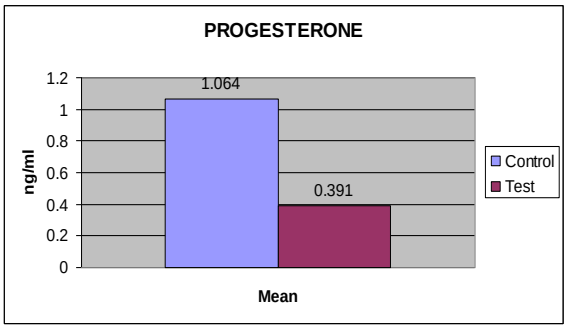

Fig.5 Effect of Fluoxetine on Progesterone Level in blood of Female Rats

The results of the blood Estrogen level of control as well as treated group are shown in Fig.4. The blood estrogen levels showed a significant decrease ($P \leq 0.01$) on treatment on treatment with fluoxetine for a period of 28 days. Student's t-Test was used to detect significant differences between control and treated group rats. The total content of estrogen in blood of

control as well as treated animals is expressed as pg/ml (Picograms/Millilitre)

The results of the blood Progesterone level of control as well as treated group are shown in Fig.5. The blood progesterone levels showed a significant decrease ($P \leq 0.01$) on treatment on treatment with fluoxetine for a period of 28 days. Student's t-Test was used to detect significant differences between control and treated group rats. The total content of progesterone in blood of control as well as treated animals is expressed as ng/ml (Nanograms/Millilitre).

Discussion

Treatment of animal with fluoxetine for 28 days resulted in a non- significant decrease of testosterone levels in the blood. Earlier investigations on the effect of Fluoxetine on testosterone have shown varying result. Anderson et al. ([34]) have reported that a significant decrease in testosterone levels occurs after chronic high dose of 60 mg/day fluoxetine treatment. In another report on animal study, fluoxetine was not found to affect testosterone levels in rats ([35]). In human studies, a study utilizing a single dose of 80 mg fluoxetine in non-depressed controls found no effect on testosterone levels ([36]). The study did not find an overall change in testosterone levels after four weeks of fluoxetine treatment in the 14 patient groups taken as a whole. Earlier study conducted by Unnikrishnan ([37]) has reported a significant decrease in the levels of testosterone in depression induced rats on fluoxetine treatment. In the current study there was a non-significant decrease in the levels of testosterone on fluoxetine treatment which is not in agreement with the previous results observed in the same laboratory. The differences in the observation can be well attributed to the differences in the treatment regimen.

Jørgensen ([38]) have reported that serotonin increases the synthesis of corticotropin-releasing hormone (CRH) in para ventricular nucleus (PVN) and proopiomelanocorticotropic (POMC) region in the anterior pituitary lobe via the activation of 5-HT1A, 5-HT2A, 5-HT2C and 5-HT1B. In the current investigation fluoxetine treatment that leads to the increase in serotonin levels could have resulted in the increased synthesis of corticotropin-releasing hormone (CRH). Fabbri et al. ([39]) have reported that CRH inhibits the testosterone production by Leydig cells by autocrine suppression. Further, Norman et al. ([40]) have reported that CRH is one of the factors responsible for the stress induced inhibition of LH. Thus increase in the level of serotonin induced by flouxetine treatment may have increased the CRH in the circulation. This increased CRH could have been one of the reasons for the decrease in the level of testosterone in the blood of treated animals.

Investigations conducted by Fink et al. ([5]) have shown that testosterone and estrogen affect the density of 5-HT2AR sites in regions of the rat brain which in the human are concerned with cognition, mood and mental state. Further, the 5-HT2AR sites have been implicated in depression ([41, 42, 43]). Testosterone or estrogen has also shown to improve mood by increasing the density of 5-HT2AR in cerebral cortex ([41, 44]). The decrease in testosterone and estrogen levels on fluoxetine treatment as observed in the current investigation would therefore further affect the mood and mental state via its effect on 5-HT2AR sites.

Thus fluoxetine treatment decreases the testosterone levels non-significantly in the current investigation. Increase in serotonin levels and subsequent increase in CRH could be the probable reason for decrease in testosterone. Low testosterone levels would further affect mood and mental status through its interaction with 5-HT2AR sites. Further the reproductive impairment due to the low testosterone levels is another side effect associated with the use of drug.

28-days treatment of animal with fluoxetine resulted in decreased estrogen levels in the blood. Decreased estrogen levels would have an impact on mood and mental status of animal because estrogen is involved in the maintaining mood and mental status of animal. Earlier investigations have shown that estrogen can induce an increase in serotonin receptor 5-HT2AR mRNA in dorsal raphae (DR) and subsequent increase in the 5-HT2AR binding sites in various regions of the brain concerned with control of mood, mental status and cognition ([45, 46, 47]). Further, 5-HT2AR sites have been reported to be involved in depression ([41, 42, 43]). Various studies suggests the involvement of effects of estrogen on 5-HT2AR sites ([45, 47, 48]) for the depression in women that occurs due to fall in levels of estrogen before menstruation ([49, 50, 51]) or around menopause ([52, 53, 54, 55]). The decrease in estrogen levels on treatment with fluoxetine as observed in the current investigation may therefore have an impact on mood and mental status. It is probable that the reduction in estrogen may have resulted in the reduction of 5-HT2AR sites which are in turn responsible for maintenance of normal level of estrogen.

Rocha et al ([56]) have shown that estrogen's antidepressant actions are mediated through estrogen recep-

tor β (ERβ). ERβ have been found to mediate estrogen's regulation of mood by regulating oxytocinergic or serotonergic neurotransmission ([57]). The positive effects of estrogen on mood appear to be largely via its actions at ERβ and a dynamic interplay between the stress and oxytocinergic or serotonergic systems in the brain ([57]). Any decrease in estrogen levels is likely to negate its antidepressant action. Decrease in estrogen levels associated with fluoxetine treatment is likely to have an impact on mood and mental status by its effect on 5-HT2AR sites and ERβ.

The 28 day treatment of animal with fluoxetine resulted in significant decrease of progesterone levels in blood. The decrease in progesterone is likely to have an impact on the neurologic function. Earlier reports suggest that progesterone increases the myelin-specific proteins and it also enhances the g–aminobutyric acid (GABA)-induced chloride current ([58, 59]). Paul et al. ([60]) have reported that progesterone metabolites such as 3a -hydroxy-5a -pregnan-20-one (allopregnanolone) and 3a ,5a –tetrahydrodeoxycorticosterone act as positive allosteric modulators of GABA type A receptors, and thereby reduce brain excitability and elicit sedative-hypnotic, anxiolytic, and anticonvulsant effects. Any decrease in the progesterone levels as observed in the current investigation is likely to affect the neurologic functioning of GABA.

Earlier investigations have reported that fluoxetine competitively inhibits the 21-hydroxylation of Progesterone and Allopregnenolone mediated by Cytochrome P450-2D4 (CYP2D4) and Cytochrome P450-2D6 (CYP2D6) ([61]). It increased both Km (Michaelis-Menten constant) and Vmax (maximum velocity) values of CYP2D6- mediated Progesterone 21-hydroxylation ([61]). Thus fluoxetine treatment is likely to affect the metabolism of progesterone and allopregnanolone. This in turn would affect the neurologic functioning of GABA.

The decrease in the progesterone levels on treatment with fluoxetine as observed in current investigation would have its effect on the neurologic functioning of the brain by affecting the functioning of GABA and thereby affecting mood and mental status.

Conclusion

In the current invistigation it is evident that the fluoxetine brings about non-significant reduction in testosterone and significant reduction in estrogen and progesterone. The reduction in estrogen and testosterone can be well attributed to the reduced levels of leutinising hormone (LH), while the reduction in LH is caused probably due to the increased CRH via serotonin on fluoxetine treatment. Thus it can be concluded that the observed reduction in levels of testosterone, estrogen as well as progesterone is due to the fluoxetine treatment.

Abbrevations:

5-HT: 5-Hydroxy tryptophan.
DRN: Dorsal raphae nuclei.
GnRH: Gonadotropin releasing hormone.
5-HT2AR: 5 Hydroxy tryptophan 2A receptor.
GABA : gamma amino butyric acid.
CRH: Corticotropin-releasing hormone.
PVN: Para ventricular nucleus.
POMC: Proopiomelanocorticotropic.
CRH: Corticotropin-releasing hormone.
CYP2D4: Cytochrome P450-2D4.
LH: Leutinising hormone.

Acknowledgements

None.

Funding

None.

Competing Interests

None declared.

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