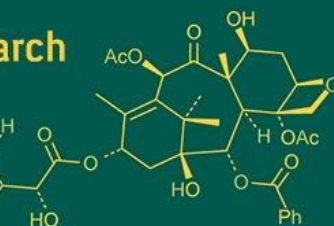


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Ashman Sidhu
Department of Zoology,
College of Basic Sciences and
Humanities, Punjab
Agricultural University,
Ludhiana, Punjab, India

Dr. Nisha Vashishat
Department of Zoology,
College of Basic Sciences and
Humanities, Punjab
Agricultural University,
Ludhiana, Punjab, India

Mohit Pun
Department of Zoology,
College of Basic Sciences and
Humanities, Punjab
Agricultural University,
Ludhiana, Punjab, India

Corresponding Author:
Dr. Nisha Vashishat
Department of Zoology,
College of Basic Sciences and
Humanities, Punjab
Agricultural University,
Ludhiana, Punjab, India

Effect of quinestrol on biochemical markers in female albino rats

Ashman Sidhu, Nisha Vashishat and Mohit Pun

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Abstract

Various measures are being taken to check the uncontrollable increase in human population over the decades, devising various contraceptive methods is one of them. Quinestrol (synthetic estrogen) is used in formulation of many contraceptive pills. These hormonal pills have a very high success rate if used as prescribed. Present study was formulated to evaluate the effect of quinestrol on biochemical markers in female albino rats. Female albino rats were treated with 2 mg/kg body weight of quinestrol for a period of 10 days. Quinestrol treatment showed no significant effect on the body weight and organ weight of treated rats. However, uterus of all the treated rats showed severe oedema. Biochemical markers performed on rat ovarian tissue homogenate showed significant decrease in total lipid content, phospholipid content and total soluble proteins. Whereas, cholesterol levels increased significantly.

Keywords: Contraceptive, quinestrol, female albino rats, biochemical markers

Introduction

The past few decades have witnessed an overwhelming rise in the human population, from 2.5 billion in 1950 to 7.2 billion in 2013, and it is expected to reach 10.9 billion by 2100 (Bongaarts 2015) [3]. This population increase is attributed to enormous developments in medical sciences, which have lowered average mortality rates. Nevertheless, there arises an alarming need to keep these numbers in check. Contraception thus helps in the management of uncontrolled and rapid population growth. Various contraceptive methods have been devised to address this issue. One such method includes hormonal methods, which include oral contraceptive pills comprising different compositions of two hormones: estrogen and progesterone. These are proven to be 99% effective if consumed as prescribed. The mode of action of these pills can vary from preventing the release of an ovum to thickening cervical mucus or by interfering with the hypothalamo-hypophyseal-gonadal axis (Trussell J 2018) [28]. Also, oral contraceptives lead to the inhibition of follicular development, ovulation, and hence, corpus luteum formation, as a consequence of which, a reduction in estradiol and the absence of progesterone are witnessed and reported.

Quinestrol (synthetic estrogen), which is C3-cyclopentyl ether of ethinyl estradiol (17 alpha-ethynyl estradiol), is widely used in hormonal contraceptives. However, it does not have estrogenic activity of its own; instead, it is a prodrug of ethinyl estradiol. It has proven to be more efficacious than the direct administration of ethinyl estradiol due to its long half-life (>120 hours/5 days), which is attributed to the fact that it is stored in adipose tissue, leading to its slow release over time and hence tackling the problem of frequent ingestion. It has been witnessed that quinestrol mimics estrogen, and the production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary is influenced by a feedback mechanism involving ovarian hormones. Therefore, the negative influence of quinestrol on mRNA levels led to its effect on pituitary hormones (Nett *et al.* 2002) [19]. Levels of estrogen receptor-alpha (ER-α) mRNA increased in the anterior pituitary, which are major receptors for estrogen binding. Since quinestrol is released gradually, it was found that its concentration kept increasing in the brain, giving an inhibitory signal to the pituitary for the production of FSH and LH (Lv *et al.* 2012) [16].

Kader *et al* (1997) [30] also showed that quinestrol administration leads to significant alterations in plasma lipid profiles, characterized by reductions in free fatty acids and cholesterol, alongside increases in phospholipids and triglycerides.

These findings point to the potential of quineestrol to alter lipid and protein content, particularly in reproductive tissues, with implications for metabolism and steroidogenesis. The effects of quineestrol on various biochemical parameters, including lipid content, phospholipid content, and total soluble proteins, have been examined in several studies. In contrast, Lalithamma and Changamma (2013) ^[11] explored the effects of estradiol valerate, which shares a similar mechanism of action with quineestrol, on lipid and phospholipid content in the ovarian tissue of aged rats. Their study showed a significant increase in lipid concentration, which may be associated with altered steroidogenic activity. However, the phospholipid content remained unaltered with estradiol valerate treatment, indicating that quineestrol and similar compounds might impact lipid metabolism while leaving phospholipids unaffected. Sidhu *et al.* (2020) ^[27] investigated the impact of quineestrol on male bandicoot rats and found that treatment with quineestrol at concentrations of 0.02% and 0.03% resulted in an increase in total soluble protein levels in the testes. This suggested an alteration in protein metabolism or synthesis in response to quineestrol exposure. The main objective of this study was to determine the effect of quineestrol on biochemical markers in female albino rats and their functioning.

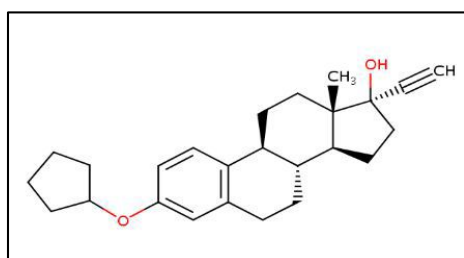
Methods

Experimental Animals

Fourty adult female albino rats (*Rattus norvegicus*), aged 8-10 weeks and weighing 100-150 g, were obtained from the Disease Free Small Animal House (DFS AH), Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana, after approval from The Institutional Animal Ethics Committee (IAEC) vide letter no. GADVASU/2022/IAEC/63/12. Rats were kept in the small animal house, Punjab Agricultural University, Ludhiana. All IAEC guidelines for animal care were followed. Rats were housed in polypropylene cages with rice husk bedding in the animal house, which had environmentally controlled conditions with temperatures of 25±2 °C and humidity of 70±5%. Rats were fed cracked wheat, and water was provided via feeders (glass bottles with rubber stoppers). Rats were acclimatized for 15 days before the commencement of treatment.

Test Chemical

Quineestrol, synthetic estradiol (17 alpha-Ethinylestradiol 3-cyclopentyl ether, C₂₅H₃₂O₂, 364 g/mol), was procured commercially from CDH Chemicals and used in this research. The rats were orally intubated to study its effects.



Structure of quineestrol

Grouping and Treatment

Adult female rats were divided into two groups with n=20 rats per group. Group 1 comprised rats that were kept as

controls, and the rats of group 2 were treated with 2 mg/kg body weight of quineestrol via oral intubation. The treatment course lasted for 10 days. Experiments were carried out in the Animal physiology lab, Department of Zoology, Punjab Agricultural University, Ludhiana.

Effect on Body Weight

After acclimatizing the rats for 15 days, they were weighed at regular intervals to examine any significant change in body weight during the experiment, and the data was recorded at Day 0, Day 5, and Day 10.

Autopsy of Animals

After 10 days of treatment, the rats were dissected. Blood was drawn from the heart's ventricle and transferred into heparinized vials. The reproductive organs (uterus and ovaries) were dissected out and weighed to assess any differences in weight or morphology between the control and treated groups.

Effect on Organ Weight

After the 10-day treatment period, the rats were sacrificed, and the reproductive organs (uteri and ovaries) of rats from both groups were dissected out and weighed. After weighing, the ovaries were homogenized for further procedures.

Preparation of Ovarian tissue homogenate

After completing the 10-day treatment period, the rats were dissected, and the required tissues (uterus and ovaries) were collected. Ovarian tissue homogenate was prepared by pooling 1.5 ml of PBS solution for 0.5 g of tissue in a centrifuge tube. Homogenization was performed until the tissue was completely disintegrated and a homogenous mixture was obtained. This was followed by centrifugation at 3000 rpm for 10 minutes. The supernatant was then collected in Eppendorf tubes (2 ml) and stored at -20 °C in a deep freezer. This solution was used for further biochemical analysis.

Biochemical Studies

Total lipid extraction and estimation

Total lipids were extracted and estimated following the method of Folch *et al.* (1957) ^[8]. Tissue homogenate was mixed with 20 times its weight in chloroform:methanol (2:1 v/v) and allowed to stand for 3-4 hours. The mixture was then filtered, and the filtrate washed with 0.85% saline (0.25% of the filtrate volume) in a separating funnel, and left overnight. The bottom layer was collected in a pre-weighed crucible, the top layer rinsed with chloroform, and the bottom layer collected again in the same crucible. The combined layers were evaporated to dryness at 60 °C, the crucible weighed, and the lipid aggregate dissolved in 2-5 ml of chloroform for storage at 4 °C, protected from light. Total lipid content was calculated as (Final weight of crucible - Initial weight of crucible) mg/g tissue wt.

Estimation of total phospholipids

Phospholipid content was estimated using the method of Ames (1966) ^[2]. 0.5 ml of the lipid sample and phosphate standards were evaporated to dryness at 100 °C. 0.18 ml of 10% magnesium nitrate in 95% ethanol (reagent 1) was added, and the samples heated over a flame until brown fumes ceased. After cooling, 1.8 ml of 0.5N HCl (reagent 2)

was added, vortexed, and refluxed in a boiling water bath for 15 minutes. 4.2 ml of a freshly prepared 6:1 mixture of 0.42 g ammonium molybdate in 1N sulfuric acid (reagent 3) and 10 g ascorbic acid in water (reagent 4) (reagent 5) was added, the samples incubated at 45 °C for 20 minutes, and the absorbance measured at 820 nm. Phospholipid content was expressed in mg/g tissue wt.

Estimation of total cholesterol

Total cholesterol was estimated following the method of Chiamori and Henry (1959). 0.5 ml of the lipid sample was evaporated to dryness at 60 °C. 5 ml of 0.05% ferric chloride in glacial acetic acid (reagent 1) was added and vortexed. 3 ml of concentrated sulfuric acid (reagent 2) was then added, vortexed again, cooled for 10 minutes, and the absorbance measured at 560 nm against a blank. Cholesterol concentration was calculated and expressed in mg/g tissue weight.

Estimation of total soluble proteins

Total soluble protein content was determined using the method of Lowry *et al.* (1951) [14]. 0.5 ml of tissue homogenate was brought to a final volume of 1 ml with distilled water. 5 ml of a freshly prepared 50:1 mixture of 2% sodium carbonate in 0.1N NaOH (Lowry A) and 0.3% copper sulfate in 1% sodium citrate (Lowry B) (reagent 4) was added and vortexed. After 10 minutes, 0.5 ml of a 1:1 dilution of Folin-Ciocalteu reagent (reagent 5) was added, vortexed, and allowed to stand for 30 minutes. The absorbance was measured at 520 nm against a blank. Total soluble proteins were calculated (mg protein/g wet tissue) using a BSA standard curve (20-200 µg/ml) with the formula: (Conc. of standard / O.D. of standard) × (O.D. of sample / Vol. of sample) × (Total vol. / Wt. of sample).

Statistical Analysis

All values are expressed as mean ± S.E. The Statistical Package for the Social Sciences (SPSS) version 16 for Windows was used to determine significant differences between the control group and the group treated with 2 mg/kg body weight of quinestrol. An independent samples t-test was used, where a p-value < 0.05 indicated a statistically significant difference. Two-way analysis of variance (ANOVA) with Tukey's post hoc test was also performed. At a significance level of 0.05, values with different alphabetical superscripts were considered significantly different.

Results Body Weight

Body weights were recorded at 5-day intervals (Day 0, Day 5, and Day 10) and are summarized in Table 1 and Figure 1. Control group rats exhibited a normal increase in weight gain. However, rats treated with quinestrol showed a non-significant decrease in body weight over the treatment period. This suggests that quinestrol did not have any significant adverse effects on the body weight of the rats.

Organ Weight

Uteri and ovaries from both control and treated rats were weighed after dissection at the end of the treatment period. The average organ weights, expressed as g/100 g b.wt., are presented in Table 2 and Figure 2. There was no significant difference in ovary weight (p = 0.093) or uterus weight (p =

0.157) between the control and treated groups. However, uterine edema (Figure 3) was observed in all treated rats.

Biochemical Studies

Various biochemical parameters were analyzed (Table 3, Figure 4).

Total Lipid Content

Total lipid content was measured in ovarian tissue homogenate of the both groups. Control rats had a total lipid content of 32.48±1.36 mg/g tissue weight. A significant decrease (p = 0.013) in total lipid content was observed in the quinestrol-treated group (2 mg/kg b.wt.), with a value of 27.60±0.79 mg/g tissue weight.

Phospholipid

Phospholipid content was measured in ovarian tissue homogenate of the both groups. The treated group showed a significantly lower (p = 0.004) phospholipid content of 3.95±0.04 mg/g tissue weight compared to the control group's 4.15±0.04 mg/g tissue weight.

Total Cholesterol

Total cholesterol levels were measured in ovarian tissue homogenate of the both groups. 0.57±0.02 mg/g tissue weight in the control group and 0.71±0.02 mg/g tissue weight in the treated group was reported. This increase in the treated group was statistically significant (p = 0.002).

Total Soluble Proteins

Total soluble protein levels were measured in ovarian tissue homogenate of the both groups. 4.52±0.32 mg/g tissue weight in the control group and 3.35±0.04 mg/g of tissue weight (p = 0.004) was reported. This decrease in the treated group was statistically significant.

Discussion

Quinestrol, a synthetic estrogen, is stored in body fat after administration and slowly released over time. This stable estradiol analog acts as a long-term contraceptive by suppressing the hypothalamo-hypophyseal-gonadal axis and gonadotropin release, disrupting normal reproductive function. Its high estrogenic potency (8-10 times that of its base compound) can lead to imbalances in estradiol levels, further inhibiting GnRH release and follicular development. Due to its potent antifertility effects, quinestrol has been used as a long-term contraceptive in women.

In the present study, treatment with quinestrol showed that the control group gained weight normally but quinestrol-treated group showed a non-significant decrease. These findings indicate that quinestrol did not significantly impact the rats' body weight. Studies on the effects of quinestrol and levonorgestrel on body weight in various rodent species show inconsistent results. Massawe *et al.* (2004) [17] found that quinestrol, alone or in combination with levonorgestrel, reduced body mass in *Meriones natalensis*, with a greater decrease at higher concentrations. Lv and Shi (2011, 2012) [15, 16] corroborated these findings with *Meriones unguiculatus* (Mongolian gerbils) for quinestrol, but not levonorgestrel. However, Liu *et al.* (2013) [13] observed no significant body weight change in *Rattus nitidus* treated with quinestrol, possibly due to reduced bait consumption.

In contrast, Shi *et al.* (2020) [26] reported that quinestrol, but not levonorgestrel, significantly lowered body weight in

Lasiopodomys brandtii (Brandt's voles). Similarly, Kang *et al.* (2022) [10] found no significant body weight reduction in *Eospalax baileyi* (Plateau zokors) treated with quinestrol, levonorgestrel, or a combination, attributing the slight reduction to potential appetite suppression due to oral gavage administration and observing reduced food consumption in the quinestrol and combination groups. These conflicting findings highlight the complexity of hormonal effects on body weight and the influence of factors like administration method and species-specific responses.

Regarding reproductive organ weights, the findings are more uniform. Studies consistently report no significant changes in uterine or ovarian weights following quinestrol and levonorgestrel treatment (Massawe *et al.*, 2004; Shi *et al.*, 2020) [17, 26]. However, the presence of uterine oedema, a common physiological response to estrogenic compounds, is a recurring observation (Meli *et al.*, 1963; Wan *et al.*, 2006; Huo *et al.*, 2006; Lv and Shi, 2011; Shi *et al.*, 2020) [18, 9, 26, 15]. This oedema, along with observed thinning of uterine walls (Shi *et al.*, 2020) [26], suggests potential disruptions to the uterine environment, potentially interfering with implantation and early pregnancy. The importance of normal endometrial growth for successful gestation underscores the potential antifertility effects of these hormonal compounds.

Oxidative stress (OS), a state of imbalance between pro-oxidants and antioxidants, emerges as a significant factor in reproductive health and is potentially influenced by exposure to quinestrol and related compounds (Cindrova-Davies *et al.*, 2007; Ruder *et al.*, 2009; Al-Gubory *et al.*, 2010) [6, 23, 1]. Estrogen administration, including via oral contraceptives, is known to elevate lipid peroxide levels (Ciavatti and Renaud, 1991; Subakir *et al.*, 2000; Pincemail *et al.*, 2007) [5, 24, 20], indicating increased oxidative activity. While reactive oxygen species (ROS) play essential roles in normal cellular processes (Chandra *et al.*, 2009) [7], an overabundance of ROS can overwhelm the body's

antioxidant defenses and compromise female reproductive function (Al-Gubory *et al.*, 2010) [1].

ROS-mediated damage to lipid metabolism can disrupt cell membrane integrity, leading to the release of lipids and impaired metabolic regulation (Robertson *et al.*, 2001) [22]. Quinestrol-induced lipid peroxidation may contribute to the observed decrease in phospholipid levels (Li *et al.*, 2014) [12], as this autocatalytic process can ultimately result in cell death (Sidhu *et al.*, 2020) [27]. The concurrent increase in ovarian cholesterol levels (Obisesan *et al.*, 2002; Lalithamma and Changamma, 2013) [11] may be a consequence of estrogen's influence, given that cholesterol is not directly involved in ovarian follicle dynamics. Furthermore, oxidative stress can impair steroidogenesis, the process of steroid hormone synthesis, by interfering with the conversion of cholesterol to steroid hormones. This can lead to cholesterol accumulation (Sidhu *et al.*, 2020) [27] and potentially disrupt the hypothalamic-pituitary-gonadal axis. Estrogen administration can further exacerbate this effect by reducing the activity of key enzymes involved in steroidogenesis, such as 17 beta-hydroxysteroid dehydrogenase (17 β -HSD) and aromatase. The observed reduction in ovarian protein content suggests a decrease in protein synthesis, which could be a consequence of oxidative stress-induced damage to cellular components, including cell membranes, DNA, and proteins (Shen *et al.*, 2011) [25]. Crucially, this reduction in protein does not suggest an increased risk of ovarian cancer (Lalithamma and Changamma, 2013) [11].

Table 1: Average body weight (g) of female albino rats exposed to 2 mg/kg body weight of quinestrol

Groups Days	Control	Treatment
Zero (0)	139.13 $\pm$ 6.58 ^{ab}	139.00 $\pm$ 8.23 ^{ab}
Five (5)	139.50 $\pm$ 6.02 ^{ab}	138.25 $\pm$ 8.63 ^{ab}
Ten (10)	140.50 $\pm$ 5.98 ^{ab}	137.50 $\pm$ 8.03 ^{ab}

*All values are expressed as mean $\pm$ S.E.; n= 6/group

*Values with different alphabetical superscripts differ significantly

*Values are significant at 5% level of significance

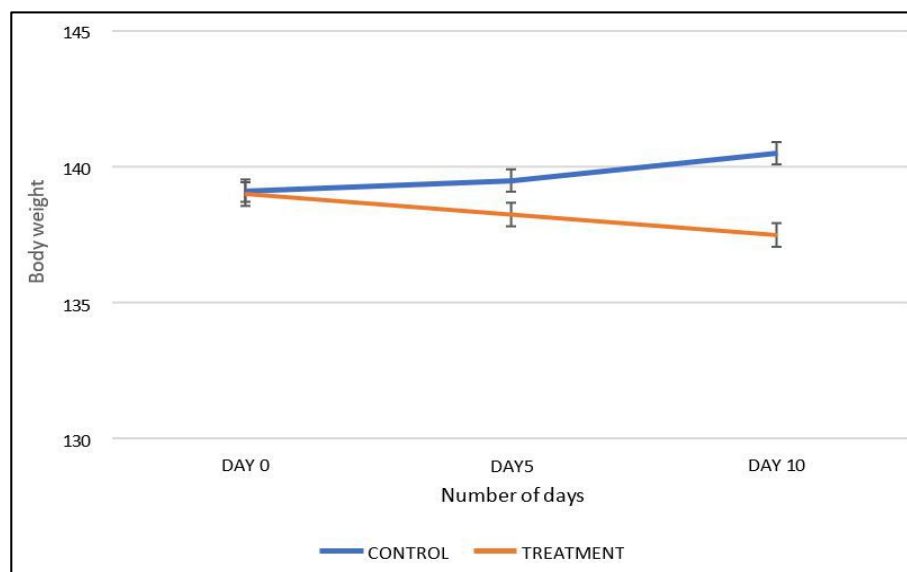


Fig 1: Average body weight (g) of female albino rats of control group v/s treated group

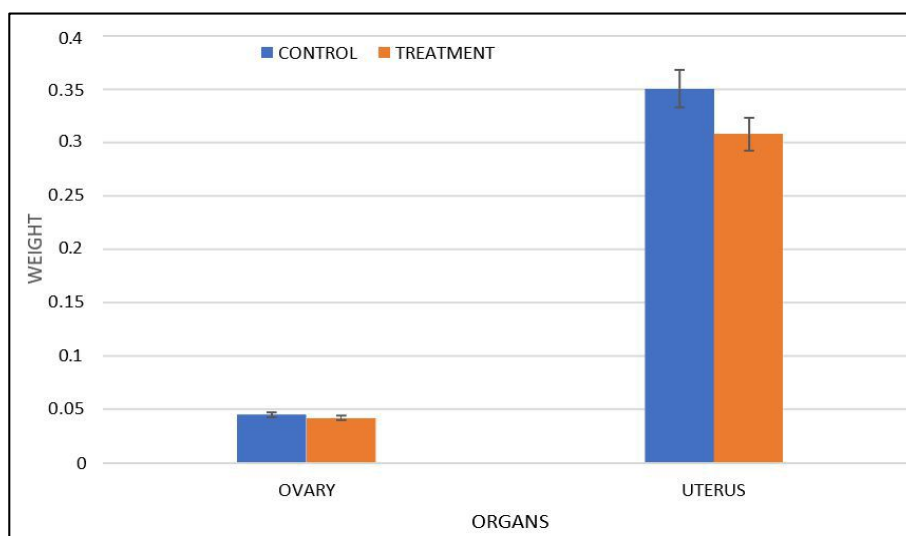
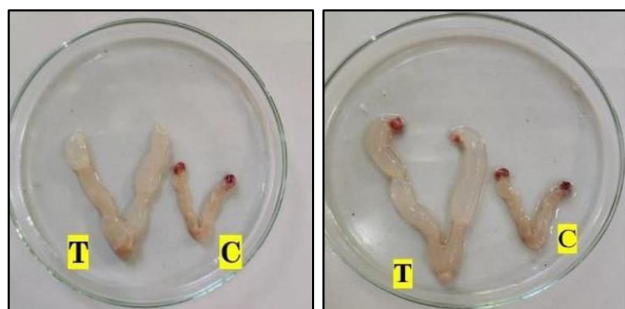
Table 2: Organ weight (g/100 g b.wt.) of female albino rats exposed to 2 mg/kg b.wt. of quineestrol

Organs Groups	Control	Treatment	Statistical analysis (t-test)
Ovary	0.045±0.003	0.042±0.004	p= 0.093
Uterus	0.350±0.04	0.308±0.02	p= 0.157

*All values are expressed as mean ± S.E.; n=6/group

*Value of p < 0.05 depicts significant difference

*Values are significant at 5% level of significance

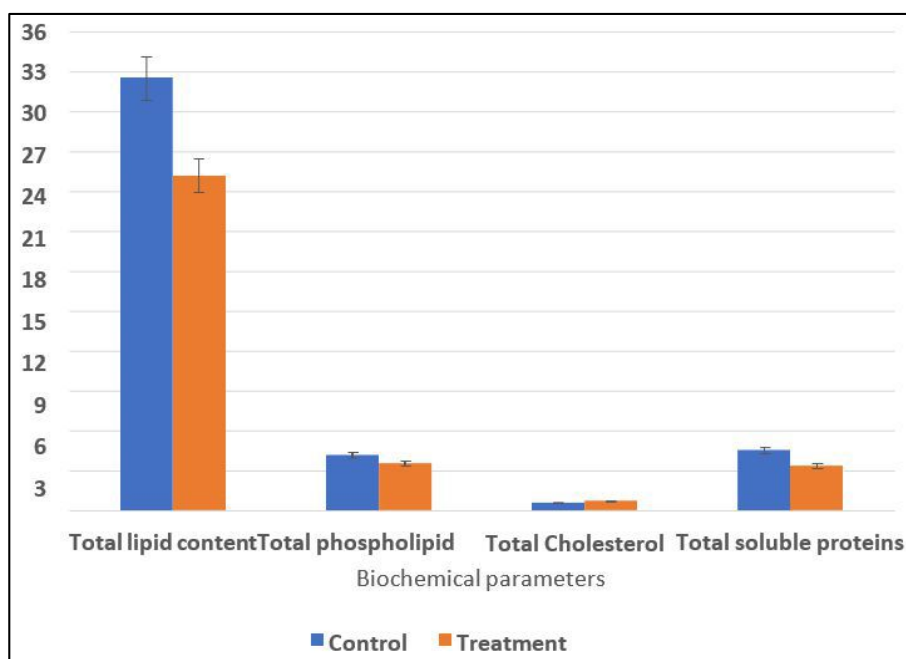
**Fig 2:** Organ weights (g/100 g b.wt.) of female albino rats of control group v/s treated group**Fig 3:** Uterine oedema in rats of treated group (T)**Table 3:** Biochemical markers (mg/g wet weight) of ovarian tissue homogenate of albino rats exposed to 2 mg/kg body weight for 10 days

Groups Biochemical parameters	Control	Treatment	Statistical analysis (t-test)
Total lipid content	32.19±1.49	27.60±0.79	p= 0.013
Total phospholipids	4.15±0.04	3.95±0.03	p= 0.004
Total cholesterol	0.57±0.02	0.71±0.06	p= 0.002
Total soluble proteins	4.52±0.32	3.35±0.04	p= 0.004

*All values are expressed as mean ± S.E.

*Value of p < 0.05 depicts significant difference

*Values are significant at 5% level of significance

**Fig 4:** Comparison of different biochemical markers of ovarian tissue homogenate of female albino rats of control group v/s treated group

Conclusion

In conclusion, this study provides valuable insights into the effects of quinestrol on the female reproductive system. While the minimal changes in body and organ weights might initially seem reassuring, the significant alterations in ovarian biochemistry, particularly the evidence for oxidative stress and its impact on lipids, cholesterol, and proteins, raise important questions about the potential long-term consequences of quinestrol exposure on female reproductive health. Future research should focus on elucidating the precise molecular mechanisms underlying these biochemical changes and investigating the potential impact on fertility and reproductive function.

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