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Original Article

The Effect of Roaccutane on Development of Ovarian Follicles and Uterine Changes in Adult NMRI Mice Strain

Arghavan Fayazmanesh[1], Mohammad Nasehi[1,2], Gholamhassan Vaezi[3], Ramezan Khanbabaee[4]

[1]Department of Biology, Faculty of Advanced Science and Technology, Islamic Azad University, Tehran medical sciences, Tehran, Iran
[2]Cognitive and Neuroscience Research Center (CNRC), Amir-Almomenin Hospital, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran
[3]Department of Biology, Faculty of Basic Sciences, Islamic Azad University, Damghan Branch, Damghan, Iran
[4]Department of Biology, Faculty of Basic Sciences, Islamic Azad University, Qaemshahr Branch, Qaemshahr, Iran

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Correspondence
Mohammad Nasehi
Email: nasehi@iricss.org

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Abstract

Introduction: Roaccutane, Acuten, and Isotretinoin are derivatives of the vitamin A naturally found in human body. Since vitamin A is a fat-soluble vitamin, it is prescribed through controlling the amount of skin oil by forcing low-secreted sebaceous glands to treat severe skin acne with the risk of permanent scarring. Thus, we aimed to investigate the effects of Roaccutane on evolution of ovarian follicles and uterine, and possible attendant liver changes in adult NMRI mice.

Materials and Methods: Roaccutane was orally administered by gavage at the doses of 0.5, 10, and 20 mg/kg for 21 days. Then, the mice were dissected, and the uterine, ovarian, and liver tissues were separated. The levels of Estradiol (E2), Follicle-stimulating hormone (FSH), Alanine transaminase (ALT), Aspartate transaminase (AST), and Alkaline phosphatase (ALP) were examined by ELISA test and chlorometric biochemical method.

Results: Increase in the Roaccutane dose led to the damage to endometrium layers, and there were no significant changes in myometrium and perimetrium. Observations of the tissue of ovaries indicated the maturity of them. Significant reduction in the number of hepatocytes and rise of glands and blood vessels were the results of the liver damage. High level of liver enzymes (ALT, AST, and ALP) was the important reason for the liver damage. Hormonal findings through the increase of E2 and FSH also showed tissue damage.

Conclusion: The results revealed the harmful effect of Roaccutane on evolution of ovarian follicles and uterine and liver changes of adult laboratory NMRI mice (either tissue or hormone).

Keywords: Roaccutane, Isotretinoin, NMRI mice, ovarian follicle, E2, FSH, ALT, AST, ALP

1. Introduction

Isotretinoin is a retinoid that is derived from vitamin A and found naturally in small amounts in body [1, 2]. Oral isotretinoin is marketed as a gel capsule with various brands, the most famous of which are Acuten and Roaccutane from the Roche Pharmaceutical Factory [2, 3]. The topical form of the drug is also available in pharmacies under the brand name Isotrex or Isotrexin. Isotretinoin production in 1982 was a major breakthrough in the treatment of acne. Since 1982, Roaccutane has been commercialized. The drug was licensed in the United States until 1991, when the evidence of teratology was released [2, 3].

Today, treatment with isotretinoin begins if other methods fail. Acne can be treated with topical medications, followed by antibiotics, and finally isotretinoin [4]. This process is

selected due to lower side effects as well as lower cost. Acne vulgaris is an inflammatory disease, a self-limiting polymorphism seen from puberty to adulthood due to factors such as heredity, hormones, immune system defects, and bacteria. The skin has a natural flora, anaerobic, and gram-positive Propionibacterium acnes [5-7].

Non-invasive treatment with isotretinoin (oral or ointment) is followed by antibiotic therapy such as tetracycline, erythromycin, clindamycin, and spiramycin. Roaccutane prevents cell division and sebaceous cell differentiation. The important mechanism of the Roaccutane is its anti-inflammatory effect [8-10].

The paraffin used for medicinal purposes have certain characteristics, the most important of which is reducing the percentage of excess oils. In this study, paraffin was chosen as the solvent due to its neutral properties [11, 12].

Clinically, the behavior of liver enzymes in disorders can be divided into two general categories: hepatocellular pattern and cholestatic pattern. In hepatocellular pattern, acute or chronic liver damage raises serum levels of aminotransferases. ALT and AST are catalysts for the transfer of alpha-aminoase groups of aspartate and alanine to the alpha-ketoglutaric acid group, to produce oxaloacetic acid and pyruvic acid, respectively [13]. Concentrations of both enzymes in the liver are very high, but ALT is more specific. AST is found in the liver, heart, skeletal muscle, brain, kidneys, and red blood cells, and ALT is found in very small amounts in the kidneys and skeletal muscle and abundantly in the liver [13].

If Roaccutane increases the percentage of infertility, the mentioned problems will also increase. Therefore, the identification of an effective drug in the treatment of infertility and the application of the type of drug explains the significance of this research. Due to the frequent use of Roaccutane during and beyond puberty, its effect on the growth and development of ovarian follicles and uterine changes was the main goal of this research.

2. Materials and Methods

2.1. Animals

Forty healthy adult female NMRI mice weighing 25-30 g were purchased from the Pasteur Institute. They were stored on special plastic shelves at a temperature of 22±2 centigrade with a humidity of 50±5. The mice were fed through prepared plates and the required water from special flasks. The temperature and humidity of the mice room were determined by a thermometer and a hygrometer, and during the day and night, the air conditioner or heater prevented marked temperature changes. On the 22nd day, the mice were dissected and blood was taken from the heart; additionally, the uterine horn, ovaries, and a lobe of the liver (left lateral lobe) were separated.

2.2. Drugs

Twenty mg/kg Roaccutane gelatin tablet form was obtained from Roche Company. The oral paraffin liquid was selected as the appropriate solvent. The animals received gavage at the doses of 5, 10, and 20 mg/kg.

2.3. Blood sampling of mice and biopsy

The mice were anesthetized by intraperitoneal injection of 100 mg/kg Ketamine and 12.5 mg/kg Xylazine and the blood was taken from the left ventricle. With this method, about 1 milliliter of blood can be taken from the heart of each mouse. After the blood sampling, the uterus, ovary, and left lateral lobe of liver were carefully separated [14].

2.3.1. Hormonal measurement

Both FSH and E2 were measured by ELISA (Sandwich Enzyme Immunoassay/enzyme-linked immunosorbent assay) [15].

2.3.2. Measurement of liver enzymes

ALP, ALT and AST were studied based on the kits methods (the Frankel-Reitman method / endpoint) using biochemical methods.

2.4. Preparing tissue sections

All the necessary work was done to have a basic slide with haematoxylin and eosin (H&E) staining and then it was observed under a light microscope. The tissues of the sham group and the group1 ,2 and 3 were compared with the control group. Photographs taken for uterine tissue were enlarged 10 times, and for ovarian and liver tissues were enlarged 4 times. Measuring the width of the uterine layers, counting the total categories of ovarian follicles, examining the number of glands and blood vessels and hepatocytes were all done by enlarging 40 times, based on Junqueira's histology [16]. Measurements and counts were performed with the eye under a light microscope.

2.5. Statistical analysis

The results were statistically analyzed using SPSS software (SPSS version 21.0). One-way ANOVA and Tukey's were utilized. *P<0.05, **P< 0.01, and ***P<0.001 were considered to be significant levels. The corresponding diagrams were drawn using Excel software.

3. Results

Uterine

The statistical analysis indicated that following administration of higher dose of the drug, blood vessels and glands rose and also the diameter of the endometrium increased. The tissue results were compared with the control group considering the number of blood vessels, glands and width of three uterine layers. In the sham group, no significant changes were observed in the three layers of the uterus, diameter, blood vessels, and glands. In group 1 (Mice fed 5 mg/kg of dissolved drug), no noticeable changes were observed in overall diameter of the ovary and glands. Yet, relatively minor damage to the endometrial layer and the formation of blood vessels was recorded. With increase of dose in group 2 (Mice fed 10 mg/kg of dissolved drug), the layers of myometrium and perimeter were almost unchanged, but the width of the endometrial tissue showed diminution with dense tissue rich in blood vessels and glands, signifying tissue damage. In group 3 (mice fed 20 mg/kg of dissolved drug), the maximum endometrial layer width and growth in the number of glands compared to previous doses were observed, but the number of blood vessels was approximately equal to group 2. Finally, an increase in the dose of the drug resulted in destruction of the endometrial layer, abnormal enlargement of the glands and blood vessels. The myometrium and perimeter layers showed no significant changes, and a slight deformation was observed in the overall tissue of the uterus.

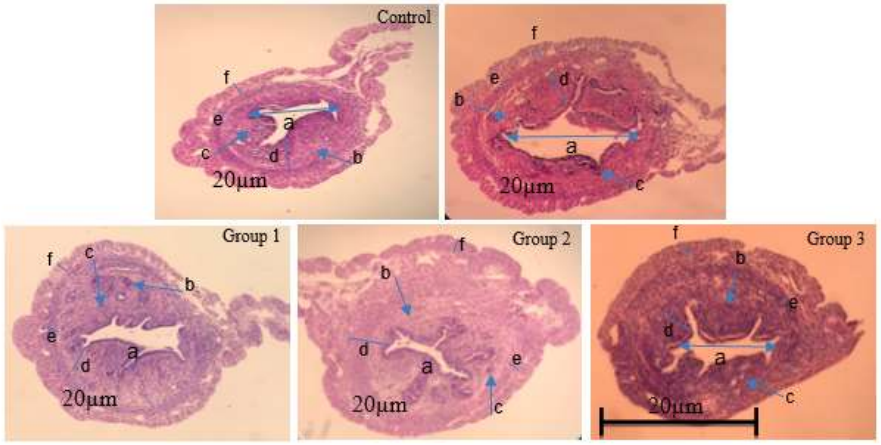

Figure 1. The uterus histopatological results in different groups. Significant increase in glands, blood vessels and endometrial layer following increase of drug doses (5, 10 and 20 mg/kg) and no significant increase in premetrium and myometrium. Glands and blood vessels are counted at 400 magnification under a light microscope. (a: lumen b: gland c: blood vessel d: endometrium e: myometrium f: premetrium, H&E staining, 100x, bar: 20µm)

Ovary

As the mice took more medication, the ovaries progressed to maturity.

Follicles were counted on the basis of primary, secondary (monolayer and bilayer simultaneously), graph, corpus luteum, and atretic. Figure 2 and 3 shows that, following an increase in dose of group 1 (lowest dose of the drug), the primary follicles were slightly reduced, but other follicles, glands, and blood vessels were observed with normal numbers and dimensions. In group 2 (medium dose of the drug), an increase in blood vessels was observed. The nuclei of the cells became abnormal, and both the corpus luteum and the atrial cells were raised. Primary follicle cells were difficult to see. In the group 3 (highest dose of the drug), the follicles were fully mature, with the primitive follicles being virtually invisible. Compared to group 2, most graphic follicles became atretic. Blood vessels were abundant. The nuclei of the cells were completely degenerated. The final result observed in ovarian tissue was ovarian maturation and a significant reduction in the number of primary follicles and an increase in yellow and atretic follicle.

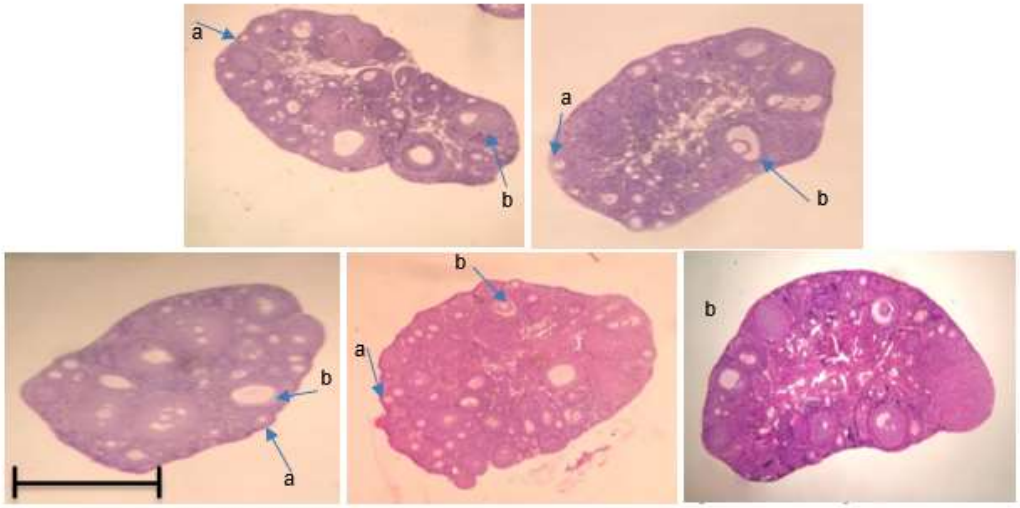

Figure 2. The ovarian histological results in different groups. Mice that received the highest dose of the drug had the most mature ovary. All follicles are counted at 400 magnification under a light microscope. (a: Primitive follicles b: Atretic follicles, H&E staining, 40x, bar: 50µm)

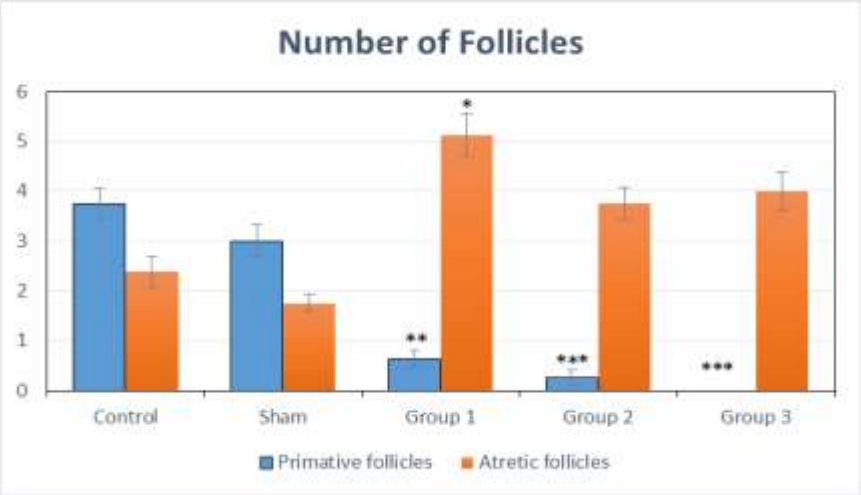

Figure 3. Significant fall in the number of primary follicles and significant rise in the number of atretic follicles following an increase in the dose of the drug (5, 10 and 20 mg/kg), *P<0.05, **p<0.01 and ***p<0.001. as compared to respective group)

Liver

Tissue destruction by increasing hepatocyte cells, the number of glands and blood vessels were seen in the mice receiving the drug, respectively 20, 10 and 5 mg/kg of drug.

Here, the number of blood vessels, glands, and hepatocytes were counted. Hepatic observations compared groups 1, 2 and 3 and sham group with the control group based on the liver damage. A significant reduction in the number of hepatocytes, and a rise in the number of glands and blood vessels were observed.

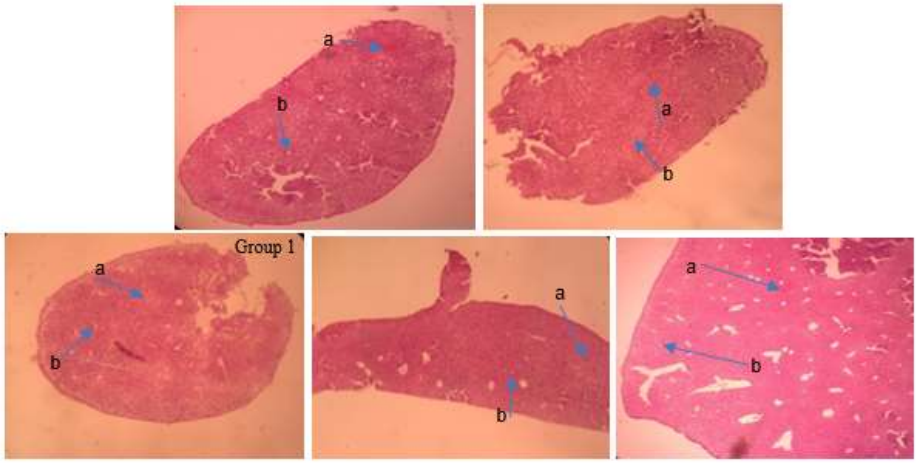

Figure 4. The liver histopatological results in different groups. A fall in the number of hepatocytes, a rise in the number of glands and blood vessels following an increase in drug dose (1.5-4 and 8 micro liters). Hepatocytes, glands and blood vessels are counted at 400 magnification under a light microscope. (a: gland b: blood vessel, 40x, H&E staining, bar: 50µm)

The hormonal and enzymatic measurement

E2 and FSH

Decrease in both hormones indicates premature menopause.

Significant decrease in estradiol level indicates sexual dysfunction, which in severe cases can lead to infertility. Due to the fact that we observed ovarian puberty, the mice in the middle dose and the highest dose, in comparison with the lowest dose, showed a significant reduction in estradiol, which means menopause. In general, since menopause has occurred and given the interpretation of the FSH test in female ovaries according to the stage of the menstrual cycle, this hormone has shown a significant decrease among the mice of the two groups.

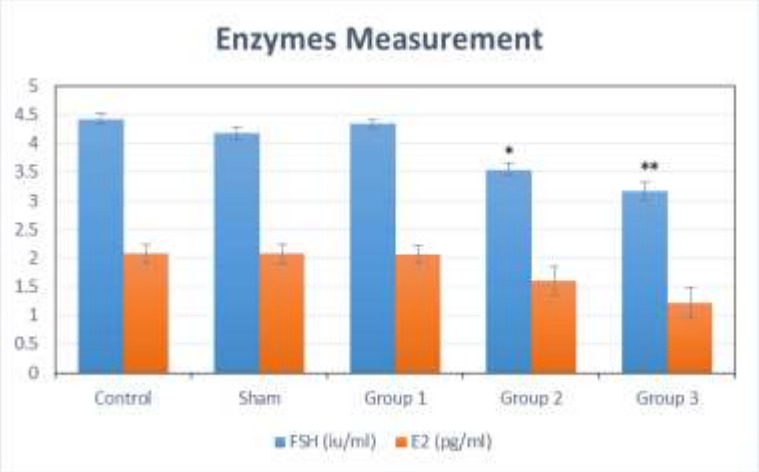

Figure 5. Reduction in E2 (pg / ml) and FSH (iu / ml) following the administration of drug (5, 10 and 20 mg/kg) in addition to the histopatological results will accentuate ovarian maturity and premature menopause. *P<0.05, **p<0.01 as compared to the respective group.

ALT, AST, ALP liver enzymes

ALP in a specialized way next to ALT and AST shows liver tissue damage. Figure 6 shows a significant increase referring to tissue damage. The rise in all three liver enzymes, especially the ALP which is more specialized than the other two, along with tissue evidence, indicates severe and significant damage in group 3 (20 mg/kg) and relatively severe damage in group 2 (10 mg/kg).

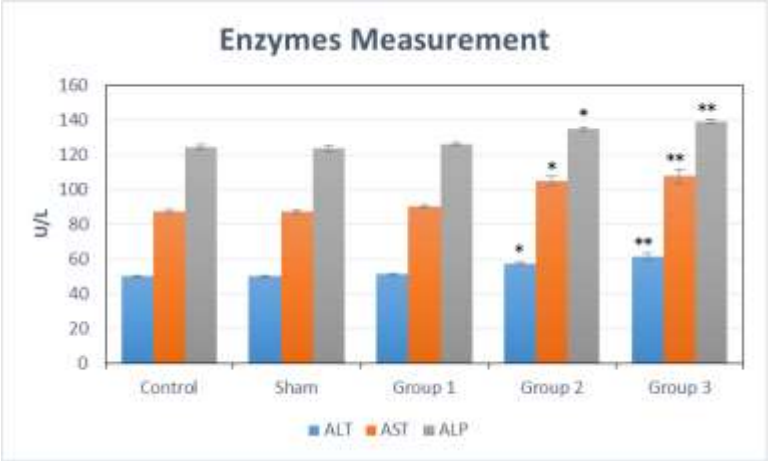

Figure 6. The rise in all 3 liver enzymes (u/l) following the increase in drug doses (5, 10 and 20 mg/kg), *P<0.05 and **p<0.01 as compared to the respective group.

4. Discussion

The purpose of this study was investigating the effect of Roaccutane on development of ovarian follicles and uterine changes in adult NMRI mice strain. The main findings of the present study, after examining the tissue evidence and measuring enzymes and hormones, are that Roaccutane affects the development of ovarian follicles and uterine. Sex hormones are mainly made from cholesterol in the gonads (ovaries and testicles) [17]. Estradiol is found in a large group of female hormones called estrogen. Estrogen in the hypothalamus reduces the release of sex-stimulating hormone and reduces the release of Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) from the pituitary gland [1, 17]. After menopause, almost no estrogen is produced in the body. E2 is produced in the ovaries. Women have a regression mechanism for this hormone, so that a decrease in E2 levels stimulates the hypothalamus and gonadotropin-releasing factors which, in turn, stimulate the pituitary gland and the secretion of FSH and LH hormones. These hormones stimulate the ovaries and produce E2. Estradiol is the most abundant estrogen in premenopausal [17]. This investigation showed that reduction in E2 and FSH after increase in the Roaccutane dose along with the histopatological results will precipitate ovarian maturity and premature menopause.

When any damage is done to the liver tissue, its endocrine part is activated and raises the level of liver enzymes in the blood above its specified amount [16]. The liver histopatological results in this study beside AST, ALT and ALP measurements indicated severe liver damage in all the groups 1-3. Patients with moderate to severe elevations in aminotransferases have acute liver damage [13]. ALP is routinely tested but GGT is evaluated under certain conditions because of its high sensitivity and low specificity. ALP originates in tissues such as the placenta, kidneys, intestines, white blood cells, and liver [13]. The most common causes of pathological increase in ALP levels are liver and bone diseases [13].

There are several studies demonstrating that Roaccutane induces adverse effects on different parts of body [18]. It has been shown that the use of retinoic acid at birth affects ovarian development [18]. Retinoic acid stimulates follicle formation. After studying the effect of taking the drug in pregnant mothers, scientists found that babies are born with problems such as lack of eyes, cohesive ear layer, cleft palate, and respiratory gap, which require multiple and heavy operations if they survive [18]. Pregnancy blood pressure was measured following the use of Roaccutane in 118 mothers, taking into account age and dose, of which only 11 were asymptomatic [19]. The use of retinoic acid after birth was found to affect the growth of the testis and the epithelium of its magnesium tubes. Retinoic acid affects the division of magnesium epithelial cells so that the number of germ cells is reduced by the effect of this substance [20]. The British Association of Dermatologists sought a way to safely introduce Roaccutaneto the organization's standards for improvement by 2012. The ovaries were in good condition due to the side effects of the drug and pregnancy tests that were taken from patients before, during, and after the treatment [21]. Scientists have concluded that the psychological impact of a drug should be investigated due to its large market size and availability [22]. According to the World Health Organization (WHO), the world's infertility rate has risen between 12 to 15 percent. In Iran, the Ibn Sina Research Center has issued warnings from all over the country, announcing statistics above the normal global level. One of the most common infertility disorders is ovulation problems. The prevalence of this disorder in infertile women is 30-40%. Due to the importance of fertility and the need for positive population growth in developed societies and the relatively positive trend for

developing societies, the sensitivity of Roaccutane complications to infertility in both men and women is high. Because isotretinoin is widely prescribed, a large number of risk reduction programs have been implemented to prevent drug use by pregnant women and to prevent women from becoming pregnant. The most recent and stringent of such programs is an Internet-based, performance-linked system called iPLEDGE which tries to ensure that the drug is dispensed only when there is documentary proof that the patient is not pregnant and she is using two forms of birth control[23]. Despite the implemented pregnancy prevention program (PPP), 51 out of every 10,000 pregnancies are exposed to isotretinoin in the Netherlands. Evidence from this study advises pregnant mothers (single or multiple) to avoid taking the drug for at least 30 days before pregnancy [24].

According to the Canadian pregnancy survey and pregnancy outcomes during isotretinoin treatment from 1996 to 2011, 90.4% of pregnancies terminated spontaneously or ended with medical intervention. Congenital malformations were observed in 9.3% biogenesis cases [19].In a pilot study, it was observed that isotretinoin administration improves sperm production in men with infertility from oligoasthenozoospermia [25].

5. Conclusion

The aim of this study was to examine the effect of Roaccutane on development of ovarian follicles and uterine changes in adult NMRI mice strain. Histological, enzymatic and hormonal studies show the destructive effect of Roaccutane on the uterus, ovaries and liver. Our experiments have shown that increasing the dose of the drug causes more harm to body. Unfortunately, the use of this drug during puberty is very high, which doubles its side effects. Further research in this area is suggested to gain more information about the advantages and disadvantages associated with this drug.

Conflict of interest

The authors declare no conflict of interest.

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