ORIGINAL ARTICLE

Eastern Journal of Medicine
East J Med 29(1): 81-85, 2024
DOI: 10.5505/ejm.2024.37880

Investigation of Rotenone Effects on Ovarian Tissue Using Stereological Methods

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ABSTRACT

This study aims to stereologically investigate the effects of rotenone on rat ovarian tissue. Within the scope of the study, eighteen female rats were taken and divided into 3 groups as control group, to which physiological saline was administered, vehicle group to which sunflower oil was administered, and rotenone group to which rotenone dissolved in sunflower oil was administered at a dose of 2 mg/kg subcutaneously (s.c.). At the end of the experiment the right ovarian tissue was excised and removed, and kept in 10% formaldehyde for 72 hours. Then routine histological follow-up process were applied, and the tissues were embedded in paraffin. The sections of 5µm thickness were taken and stained with Haematoxylin-Eosin (H&E) and Masson's trichrome viewed under a light microscope and photographed. When the histological structure of the ovarian tissue in the rotenone group was examined, it was observed that there was a remarkably decrease in the number of follicles. When the total ovarian tissue volumes of the groups were compared stereologically, it was determined that the total ovarian tissue volume decreased in the rotenone group and this decrease was statistically significant ($p<0.05$). As a result, it was determined in our study that rotenone has harmful effects on the ovary, which is a part of the female reproductive system.

Keywords: Ovary, Rat, Rotenone, Stereology, Toxicity

Introduction

Rotenone, a native plant-derived matter with miticidal and insecticidal activity, is extensively used in farming (1). The World Health Organization (WHO) categorize rotenone as a moderately dangerous agent (a class II pesticide) (2). The common use of rotenone reason environmental soiling and neurotoxicity in humans and animals (1). It is lipophilic structure and thus readily crosses all biological membranes inclusive, the blood-brain barrier and does not require carriers. The mechanism of rotenone toxicity is still completely unknown (3). Since rotenone is a specific inhibitor of mitochondrial complex I, exposure to rotenone causes ATP manufacture lack, free radical formation, apoptosis, PINK1/PARKIN-mediated mitophagy, mitochondrial membrane permeability and mitochondrial membrane potential depolarization (1). The mitochondrial complex I enzyme (NADH: ubiquinone oxidoreductase), which is inhibited by rotenone, is the proximal constituent of the mitochondrial electron transport chain and produces the energy essential for all neuron and cell functions (4). It is known that by triggering ROS activity, it causes oxidative damage and changes in lipid, protein, and DNA structure through mitochondrial apoptotic pathways. DNA damage: it may play a role in the formation of important health problems such as immune system disorders, neurodegenerative diseases and cancer (3)

In addition, rotenone induces Parkinson's disease (PD) similar symptoms in animal models and humans. Exposure to rotenone also affects the female reproductive system, fertilization, oocyte maturation and ovulation (1). In men, it causes a decrease in the number of sperm cells, loss of sperm, abnormalities in the seminiferous tubules and degeneration of interstitial cells (5). Rotenone has an effect on public health through the food chain (6). We aimed to investigate the effects of rotenone, which is widely used and has negative effects on health on rat ovarian tissue using stereological methods in our study.

Materials and Methods

Experimental Animals: For this study, 18 adult Wistar albino female rats 200-250 g weighing were taken. Rats were fed ad libitum with standard pelleted rat chow (Bayramoğlu Feed and Flour Industry Trading Corporation, Erzurum, Turkey) and tap water and housed in standard plastic cages in rooms

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Received: 21.07.2023, **Accepted:** 23.01.2023

at 22 ± 2 °C, illuminated with a rhythm of 12 hours of light and 12 hours of dark, at Van Yuzuncu Yil University Experimental Medicine Research and Application Centre. All experiments in the study were performed with the approval of the Van Yuzuncu Yil University Animal Ethics Committee (2021/05-03). All experimental protocols applied on rats were accomplished in suitability with international guidelines.

Experimental Design: Sunflower oil was used as vehicle for rotenone. The animals to be used in the experiment were randomly divided into 3 groups, each consisting of 6 rats, as follows:

1. Control Group (n=6): Physiological saline (0.9% NaCl) at a dose of 0.1 mg/kg was administered to rats intraperitoneally (i.p.) for 21 days.

2. Vehicle Group (n=6): Sunflower oil at a dose of 0.1 mg/kg was administered to rats subcutaneously (s.c.) for 21 days.

3. Rotenone Group (n=6): Rotenone at a dose of 2 mg/kg dissolved in sunflower oil was administered to rats subcutaneously (s.c.) for 21 days (7).

At the end of the experiment rats were sacrificed and right ovary tissues were taken and prepared for analysis. The tissues were fixed in 10% formaldehyde for 72 hours. Routine light microscopic histological follow-up process was applied, and then the tissues were embedded in paraffin. The paraffin blocks in the form of sections with a thickness of 5 μm were taken with a microtome, stained with Haematoxylin-Eosin (H&E) and Masson's trichrome (8), and viewed and photographed under a light microscope (Olympus BX53, Tokyo, Japan). Ovarian structure and follicle number were evaluated in 3 randomly selected areas at different magnifications by taking an average of three sections per subject for histological evaluation.

Stereological Analysis: After choosing the first section randomly, every 25th section was taken. A modified method of the Cavalieri principle was used for stereological analysis (9). Afterwards, the total tissue volume ratios were measured using a point grid provided in the Shtereom 1.5 version package program (10, 11) (Figure 1). The groups values of coefficient of variation (CV) and coefficient of error (CE) were within the acceptable ranges (<0.05). CV and CE are calculated for each group in the stereological works (9).

Statistical Analysis: The Kruskal-Wallis test was used to compare the groups. Descriptive Statistics for the featured features are expressed as Mean, Median and Standard Deviation values. In the computations, the statistical significance level was taken as 5%. SPSS (ver.20) statistical package program was used for all computations.

Results

Histopathological Findings: The histological construction of the ovarian tissue of the control group was normal when examined under light microscopy. When the histological construction of the ovarian tissue of the vehicle group was examined, it was observed that the cortex tissue contained more follicles and thickened more than the other groups, and unlike the other groups, fat cells were observed in the medulla part. When the histological construction of the ovarian tissue in the rotenone group was examined, it was observed that there was a remarkably decrease in the number of follicles (primordial, primary, secondary), and that there were defects in the folliculogenesis stages, causing a remarkably decrease in the secondary follicle and then the formation of the corpus luteum structure, compared to the other groups (Figure 2,3).

Stereological Findings: When the groups were compared in terms of medulla and cortex volumes in the stereological evaluation, it was found that the cortex volume was higher in the vehicle group than those in the control and rotenone groups, which was statistically significant ($p<0.05$). It was determined that the medulla volume was higher in the control group than those in the vehicle and rotenone groups, which was statistically significant ($p<0.05$). When we compared the total ovarian tissue volume of the groups stereologically, it was determined that the total ovarian tissue volume decreased in the rotenone group compared to the control and vehicle groups, and this decrease was statistically significant ($p<0.05$) (Table 1).

Discussion

Because of its lipophilic structure, rotenone easily permeates the blood-brain barrier and induces free radical formation and cascade neurodegeneration in dopaminergic and non-dopaminergic neurons (6). Studies backup the hypothesis that rotenone damages central dopaminergic neurons in different parts of the brain in fish, altering behavior and reproductive activities (12). In addition, rotenone induces mitochondrial toxicity and thus damages the reproductive system by disrupt ovulation, oocyte maturation, and fertilization (1). It also causes deterioration in sperm quality and quantity in the male reproductive system (13). Jain et al. (2021) showed that rotenone impairs spermatogenesis by separating the basement membrane from germ cells in seminiferous tubules in mice and causes degeneration in Leydig cells and connective tissue (5).

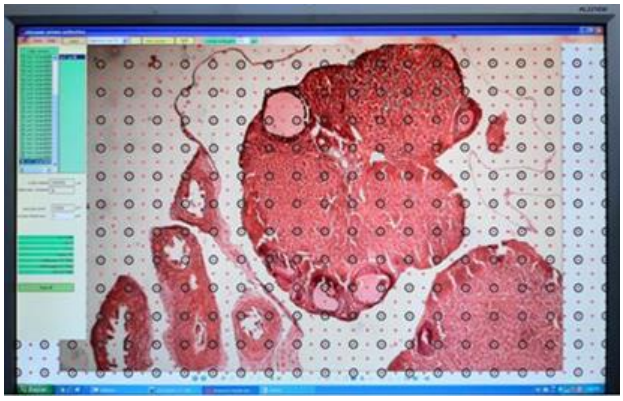

Fig. 1. Stereological analysis, ovarian tissue

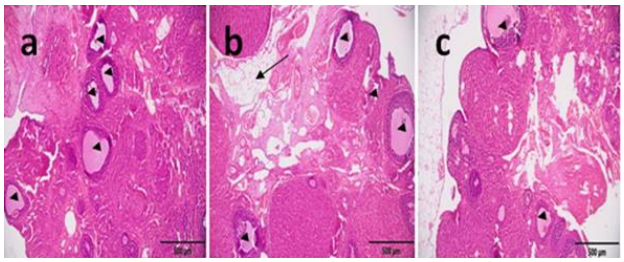

Fig. 2. a: Control group b: Vehicle group c: Rotenone group, black arrow: fat cells, arrowhead: follicles at different stages (Hematoxylin & Eosin, Scale bars: 500 µm)

The health of the mature oocyte and the resulting embryo is substantially dependent on the oocyte mitochondria, which are the most plenty of the organelle in the mature oocyte. Any deficiencies in mitochondria or in their functions affect the oocyte more easily after ovulation and are considered to be the major reason of female infertility and chromosomally aberrant conception (14). For these reasons, mitochondria are important, and rotenone causes deterioration in mitochondrial functions. In their study, Niu et al. (2020) investigated whether melatonin reduces the deterioration caused by rotenone exposure in embryo development with its mitochondrial protection effect. They found that melatonin removes rotenone-induced disruption in embryo development, mitochondrial dysfunction and ATP lack, and reduces apoptosis, oxidative stress significantly (1). Kumar et al. (2022) investigated the effects of rotenone exposure on the reproductive construction and function of female Drosophila melanogaster and third instar larvae and found that exposure to rotenone caused developmental toxicity in Drosophila melanogaster (6). Shen et al. (2018) established a comparative proteome profile of female fetal mouse gonads at certain time points (1.5, 12.5, and 13.5 days post coitum) covering a critical window for the starting of meiosis in female germ cells. They determined that rotenone given in vitro to female gonads 11.5 days post coitum decreased the rate of meiotic germ cells, increased ROS level in gonads

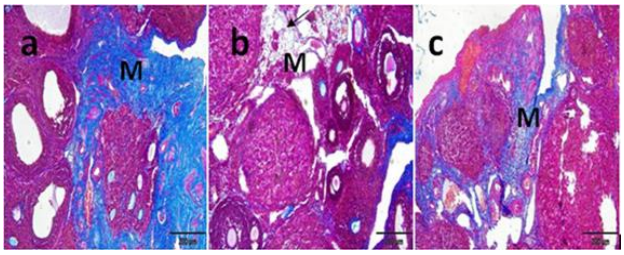

Fig. 3. a: Control group b: Vehicle group c: Rotenone group, M: Medulla, black arrow: fat cells (Masson's trichrome**,** Scale bars: 200 µm)

exposed to rotenone, decreased ATP levels and caused germ cells not to undergo premeiotic DNA replication (15). Pregnant rats fed rotenone 10 mg/kg/day from days 6 to 15 of pregnancy had decreased fertility, increased fetal resorption and lower childbearing weight (16). Experimental studies have revealed that pesticides can interact with reproductive capacity and endocrine system (5).

Rotenone not only reduces ATP synthesis, but also causes lipid peroxidation, both leading to cell death. It has also been shown that rotenone-induced oxidative stress causes cell apoptosis (17). In addition, studies on tubulin linking have reported that rotenone depolymerizes the microtubule assembly to inhibit the linking of colchicine to tubulin heterodimer, caused in mitotic activity arrest and inhibition of cell multiplication (6).

In this study, in which the ovarian structure was evaluated histologically and stereologically after exposure to rotenone, changes in the ovarian structure, decreased ovarian volume, and deterioration in oocyte developmental stages are observed, all in accordance with the literature. Compared to the control, vehicle and rotenone groups sections, which were histologically evaluated selected randomly areas and at different magnifications, it was found that all follicular at different stages especially the secondary follicle and the corpus luteum formed afterwards, remarkably decreased in the rotenone group.

It was determined that in the vehicle group, with the effect of sunflower oil, the fatty tissue in the ovarian tissue increased, ovulation decreased (the decrease in corpus luteum forms showed that), the number of follicles increased, and there was a thickening of the cortex. In the rotenone group, it was found that sunflower oil, which was used as a solvent, did not have this effect.

As a result, it was determined in the study that rotenone, which is widely used in agriculture and has risks for human health, also has harmful effects on the ovary, which is a part of the female reproductive system. The literature is examined, it is seen that studies examining the effects of rotenone on

Table 1: Descriptive Statistics and Comparison Results by Groups

Group		Ovary Total Vol.	Ovary Cortex Vol.	Ovary Medulla Vol.
Control	Mean	1882,67 a	1389,50 ab	493,17 a
	Std. Dev.	416,779	381,695	84,421
	Median	1914,50	1334,00	493,50
Vehicle	Mean	2018,00 a	1607,33 a	410,67 ab
	Std. Dev.	489,672	414,960	136,339
	Median	2263,50	1762,00	418,00
Rotenone	Mean	1299,00 b	975,00 b	324,00 b
	Std. Dev.	371,232	312,242	64,783
	Median	1255,00	948,00	310,00
p		0,025	0,030	0,033

The difference between groups receiving different letters is significant ($p<0.05$)

reproductive organs and functions are limited in number, and we believe that more comprehensive studies are needed to explain the effects of rotenone on reproductive organs and functions in the prenatal and postnatal periods.

Acknowledgments: The authors are grateful for Sıddık Keskin for statistical analysis.

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