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***In vivo* cytogenotoxicity testing of isotretinoin by the micronucleus assay in the blood of male Sprague-Dawley rats: Isotretinoin is cytotoxic and genotoxic**

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Abstract

Isotretinoin (ISO), one of vitamin A-derived retinoids, is comparatively the most widely prescribed drug in acne vulgaris pathogenesis. Despite its excellent therapeutic success, the systemic use of this drug showed undesirable serious side effects such as teratogenesis, oxidative stress, and genotoxicity. The uses of retinoids in cancer therapy are limited due to severe adverse reactions. This has led the scientific community to ask for further studies qualifying ISO features and comparing their efficacy and safety. The purpose of this study was to evaluate the cytogenotoxicity of multiple oral doses (5, 10, 15, and 20 mg/kg, daily for seven consecutive days) of ISO in male Sprague-Dawley rats. The micronucleus assay was used to investigate genotoxicity biomarkers such as the percentage of micronucleated polychromatic erythrocytes (%MNPCEs) and the percentage of aberrant cells (%Abc). Another goal was to test the cytotoxicity of the drug by measuring the ratio between PCEs and normochromatic erythrocytes (NCEs) (PCEs/NCEs). In comparison with the control, the three cytogenetic endpoints: %MNPCEs, P/N, and %Abc significantly ($P \leq 0.0017$) dose-dependent increase. This suggested genotoxicity and cytotoxicity of the tested ISO doses. Therefore, the therapeutic uses of ISO should be restricted to a very narrow range border. Further studies are needed to shed more light on the safety profile of ISO therapy.

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Introduction

Isotretinoin (ISO) is a synthetic vitamin A derivative (retinoid) that has been the most superior medication in the treatment of moderate and severe acne vulgaris (Douglas Pharmaceuticals Ltd., 2019; Legiawati et al., 2022). The exact mechanism of the therapeutic action of ISO remains unknown. However, several studies have shown that this drug induces apoptosis in sebaceous gland cells (Melnik, 2017; Reigada et al., 2017; Draghici et al., 2021). The use of retinoids in cancer therapy is restricted because of their severe cytotoxic reactions, particularly if administered systemically in aqueous solutions (Diniz et al., 2008). *In vitro* studies on human lymphocytes (Silva et al., 2013) and *in vivo* investigation in female pregnant albino mice (Al-Othman et al., 2019) showed that higher doses of ISO significantly produced severe cytotoxicity via cellular apoptotic and necrotic mechanisms. Isotretinoin has been found to produce oxidative effects (Lemos et al., 2016). The main target of the reactive oxygen species (ROS) is the DNA leading to a detrimental effect on all living cells.

Concerns related to ISO use arise from the selection of the most optimal dosage regimen with the best efficacy and lesser side effect. Many adverse effects have been described for isotretinoin, but by far the most serious and frightening side effect of this drug, at all therapeutic doses, is the induction of congenital birth defects (Gad and Fain, 2014; Al-Othman et al., 2019; Draghici et al., 2021). A review of the National Collections data (Medsafe, 2018) indicated that despite the well-known teratogenic effects of ISO, pregnancy exposures still occur. Both the approved and the off-label oral uses of the drug have never been discontinued and only a few countries have not yet approved it (Kapała et al., 2022; Pile and Sadiq, 2022).

Genotoxicity is one of the major causes of cancer (Mohamed et al., 2017) and is the main cause of fetal abnormalities induced by ISO as suggested by

previous studies (Cinar et al., 2017; Korkmaz et al., 2017). A genotoxic agent may interact with DNA or cellular components such as spindle apparatus or replicating enzymes that control the conformity of the genome (Eker et al., 2019). There have been limited reports about ISO genotoxicity and carcinogenicity (Yilmaz, 2022). The genotoxic effects that result from ISO therapy remain controversial (Layton, 2009; Erturan et al., 2012). The estimation of the frequency of micronuclei (MN) formation is considered a good parameter for the measurement of genetic damage (Sommer et al., 2020). After 3 months and 12 months of ISO therapy, MN counts were higher in the mucosal scrapings of the pre-malignant oral lesion than in the normal-appearing mucosa (Benner et al., 1994). Buccal smear samples taken from women who used ISO for at least 3 months, showed a significantly elevated rate of MN compared to control female patients who did not use ISO (Eker et al., 2019). In contrast, in human lymphocytes, ISO monotherapy (8 or 20 mg/day) was not genotoxic *in vitro* when tested using a cytokinesis-blocked MN assay (Silva et al., 2013). In addition, *in vivo* comet assay test showed no genotoxic effect of the drug when tested in human lymphocytes (Olive and Banáth, 2006; Bordbar et al., 2020).

Both the approved and the off-label oral uses of ISO have never been discontinued and only a few countries have not yet approved it (Kapała et al., 2022). Despite the many years of excellent clinical use of ISO, published data are scarce on the cytotoxicity and genotoxicity of the drug. The present study was carried out for this purpose. The MN test was used to examine the potential of ISO to induce genotoxicity. The genotoxic parameters followed were the percentage of micronucleated young or polychromatic erythrocytes (%MNPCEs) and the percentage of cells carrying at least one micronucleus/cell. To evaluate the cytotoxic action of the drug, a popular and convenient method of monitoring erythropoiesis was employed by measuring the ratio between PCEs and

normochromatic erythrocytes (NCE) PCE/NCE. The results of this study will benefit healthcare centers and dermatology clinics by making them aware of the genotoxic effects of this pharmaceutical product.

Material and methods

Chemicals

Roaccutane soft gelatin capsules (F. Hoffmann-La Roche Ltd, Basel, Switzerland) were used in the current research. Each capsule contains 10 mg of isotretinoin-dissolved soybean oil. Capsules were opened and transferred to class A volumetric flasks and diluted with soybean oil to obtain suspension at the desired concentrations.

Animals

A total of 29 healthy adult male Sprague-Dawley albino rats, approximately weighing 200-250 g and aged 10 weeks, were recruited in this study. The animals were housed in healthy atmospheric conditions, with normal feeding, drinking, and medical care based on the guidelines of experimental animal care, Department of Biological Sciences, Yarmouk University, Irbid, Jordan. The Ethics Committee of the Experimental Animal Care Society at Yarmouk University approved the experimental procedures (Permit Number: IACUC/2021/5).

Dosage and administration

The animals were randomly assigned into six groups (5 rats per group except for the positive group, 4 animals). Four groups were treated, under dim light, with 5, 10, 15, or 20 mg/kg ISO /kg b. w. oral suspension dose for 7 consecutive days. Each rat in the fifth group served as a negative control giving the animal 1.5 ml soybean oil for the same period. The sixth group was injected intraperitoneally with 5 mg/kg mitomycin C (MMC), a known cytotoxic and mutagenic agent.

At these initial doses of the ISO, a cumulative dose of 35-140 mg/kg will be achieved. These dose levels were chosen based on previous investigations in this field (Ferguson et al., 2006; Abali et al., 2013), who orally gavaged rats with 13-cis-retinoic acid either at 7.5 or 15 mg/kg for seven consecutive days (a cumulative dose of 52.5-105). The doses of 7.5 or 15 mg/kg/day were given to produce serum ISO concentrations comparable to those of humans dosed with 1 mg/kg/day based on the previous report (Ferguson et al., 2006). Serum ISO levels were not measured in the present study. The oral administration method was selected for this study since these drugs are administered orally in a clinical setting. The same controlled conditions were applied to each group to eliminate probable variation in the measured parameters. The rats were observed daily for clinical signs as well as physiological and behavioral changes throughout dosing. Toxic manifestations and mortality were also monitored once daily.

Polychromatic Erythrocyte Micronucleus Test Blood smear preparation

About 5 µl of blood was obtained in heparinized capillaries from a small puncture in the eye socket of the rat. The blood was immediately mixed with an equivalent volume of 3 % ethylene diamine tetra-acetic acid (EDTA) solution (1.5 mg/ ml blood). Two blood smears were made for each animal. The smears were allowed to dry at room temperature and fixed in absolute methanol for 1-5 min. After 24 hours they were double stained with hematoxylin (10 min) and 0.1% Giemsa (15 min) according to Khalil et al. (2017). The slides were rinsed thoroughly in tap water, then differentiated in Sorensen's buffer (pH 6.8), and allowed to dry.

Cytogenetic analysis

The protocols followed in the present study complied with the recommendations of the Collaborative Study Group for the Micronucleus

Test (CSGMT, 1995) and guidelines of the Organization for Economic Co-operation and Development (OECD, 1997). Cytotoxicity was followed in a sample of 2000 erythrocytes/animal to determine the ratio of total polychromatic erythrocytes (PCEs) to total normochromatic erythrocytes (PCE / NCE) in blood films from the control and all the treated groups. Because a PCE still contains rRNA it stains blue-grey with Giemsa (Figure 1). This allows the differentiation of PCE from the smaller, mature, hemoglobin-containing erythrocytes (NCE).

Genotoxicity was studied by calculating the percent of micronucleated polychromatic erythrocytes (%MNPCE) (Khalil et al., 2017). All analyses were carried out under blind codes using a light microscope (Nikon). Histograms of cytogenetic data were drawn using GraphPad Software.

Statistical analysis

The results obtained were expressed as mean \pm S.D and analyzed using Statistical Package for the Social Science, version 26 (IBM, SPSS). One-way ANOVA was followed by a post hoc Tukey test to determine significant differences between quantitative variables. The result was considered significant when P is less than 0.05 and highly significant when P is less than 0.0001.

Results and Discussion

Only four animals out of five survived the highest 2 doses, 15mg /kg and 20 mg/kg of the test chemical. In contrast, no death or symptoms of depression, or signs of toxicity were observed among animals that received ISO at 5 mg/kg or 10 mg/kg levels. Control animals showed no signs of illness or unusual behavioral changes.

A single micronucleus was observed in many cells; however, two micronuclei were seen in only some cells (Figure 1). In the present study, 2000 erythrocytes (PCE and NCE) were scored per

animal (5 animals for each group = 10000 PCEs) to show the effects of the four doses of ISO.

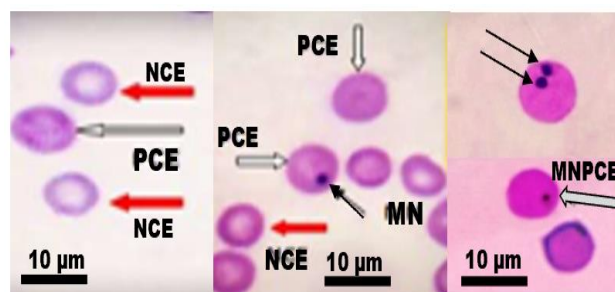


Figure 1. Representative photomicrographs from Blood smears of male albino Sprague-Dawley rats after Isotretinoin treatment. Polychromatic Erythrocytes (PCEs); Normochromatic Erythrocytes (NCEs); Micronucleated PCEs (MNPCEs); Bimicronucleated PCEs (double arrow). Magnification: X1500.

The incidence of micronucleated mature erythrocytes (% MNPCE) and micronucleated young erythrocytes (%MNPCE) as well as the PCEs/NCEs ratios among 2000 erythrocytes (PCE and NCE) from each animal are presented in Table (1). Compared to the negative control, the results show a highly significant increase ($p < 0.0001$) in the mean values of MN nucleated cells calculated for the four ISO-treated groups (Figure 2). It is clear also that the number of NCEs decreases by dose in all the treated groups than the corresponding control group.

Statistical analysis showed that the positive control (MMC) showed significantly increased effects upon %MNPCE. Similarly, ISO at treatment levels caused significant elevations in %MNPCE compared to the negative control. Concerning the PCEs/NCEs, the test chemical induced statistically significant ($P < 0.05$) dose-dependent elevations in these ratios when compared with the matching control group (Figure 3).

In the present study, rats administered with high doses of ISO exhibited extreme tiredness. No signs of hair loss, body weight loss, or nose-rectum bleeds were recorded. Exposure to ISO caused obvious signs of a bad mood in the rats' behavior. However, ISO did not cause any obvious signs of damage to the rat's health although there was a trend to reduce food and water intake. In a previous study (Cisneros et al., 2005), a reduction

in food and water intake was observed and linked to the consumption of ISO.

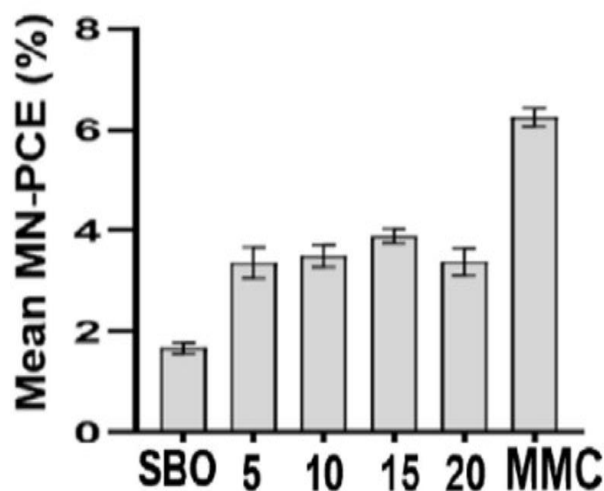


Figure 2. Dose-response for the % Micronucleated Pochromatic Erythrocytes MN-PCE in male albino Sprague-Dawley rat peripheral red blood cells. Isotretinoin doses were delivered orally at 5, 10, 15, and 20 mg/kg for 7 days. Negative control (soybean oil, SBO) was given by the same regimen. Positive control (Mitomycin C, (MMC) was administered intraperitoneally for 2 consecutive days, five animals were sampled except for the MMC. One animal died at 15 and 20 mg/kg. Data represent means \pm SEM. Error bars indicate a standard error at * $p < 0.0001$ and ** $P < 0.0017$.

Peripheral blood was selected as a non-invasive procedure to perform this assay considering the invasive protocol implicated in a bone marrow sample. Additionally, erythrocytes are well-suited to MN assay because, during maturation of erythroblast to PCE (about 6 h following final mitosis), the cell nucleus is expelled, making it easier to detect MN. After all, any MN that has been formed may remain behind in the otherwise anucleated cytoplasm (Jamalpoor and Satheesh, 2014). Moreover, the MN assay measures DNA damage arising from clastogenic or aneugenic insult experienced in progenitor erythroblast (Krishna and Hayashi, 2000). Increased peripheral blood MN-PCE percentages above baseline values (from 0.21 to 0.90 per thousand) are indicative of

MN formed in immature newly formed erythrocytes, during the final cellular divisions, typically from damage that occurred during the last 24 to 48 h from the sampling time (Hedde et al., 1983).

The main finding of the current investigation is that ISO is genotoxic since a significant increase ($p < 0.05$) in the MN number and % MNPCE in all the ISO treatment doses compared with the negative control was observed. The MMC treatment induced a significant MN number and % MNPCE. There are very limited reports about the genotoxicity and carcinogenicity Of ISO (Yilmaz, 2022). The evidence related to its genotoxicity of ISO was and still is controversial (Benner et al., 1994; Bordbar et al., 2020). Although it is difficult to extrapolate the results in one species to those in another species, or from one type of cells to those found in others, our results using rats' peripheral blood cells and the oral route suggest that ISO may be classified as the genotoxic compound. Consistent with our findings are those reported by the Eker group (2019), where there were significant differences between the ISO female patients and female control groups in terms of MN frequency in buccal smear samples. However, contrary to what we report herein regarding the induction of ISO genotoxicity based on MN assay are the findings of Silva et al. (2013). These researchers incubated blood from healthy volunteers for 72 h with ISO (1.2-20 μ M). In *in vivo* studies, they used blood from two patients with acne and three patients with psoriasis vulgaris treated with isotretinoin (8 or 20mg/day). In both protocols, no clear genotoxic effects were reported. No significant MN induction was recorded when the drug was tested in human mucosal scrapings (Benner et al., 1994) and there was a lack of DNA damage using comet assay in lymphocytes (Olive and Banáth, 2006). The absence of genotoxicity data is consistent with *in vivo* testing using the MN test and comet assay (Bordbar et al., 2020) in human mononuclear leucocytes from healthy individuals and patients

with acne on ISO treatment. Shimadaa et al. (2014) reported that erythrocytes with micronuclei were captured and destroyed by the spleen quickly. Another major outcome of the current results of the cytotoxicity test is the erythropoietic cytotoxicity (PCE / NCE ratio) of ISO under the

killing of erythroblasts, removal of damaged cells, or dilution of the existing cell pool with newly formed cells (Vijaylaxmi and Venu, 1999; Boriollo et al., 2018).

Few studies have been found in the literature regarding the effect of ISO on P/N values. Rare

Table 1. The mean and standard error of the mean (SEM) of polychromatic erythrocytes (PCEs), micronucleated polychromatic erythrocytes (MNPCEs), normochromatic erythrocytes (NCEs), and cytotoxicity in peripheral blood cells of adult male Sprague-Dawley albino rats of the control and Isotretinoin treated groups

Treatment		MNPCE (Mean \pm SEM)	P- value**	P/N ratio (Mean \pm SEM)	P- value**	% Abc** (Mean \pm SEM)	P- value*
Negative Control		18.4 \pm 1.18	—	4.2 \pm 0.24	—	2.09 \pm 0.109	—
ISN Dose mg/kg	5	37.0 \pm 3.47	<0.0001	6.5 \pm 0.63	<0.0001	4.11 \pm 0.389	<0.0001
	10	38.4 \pm 2.37	<0.0001	7.82 \pm 0.143	<0.0001	4.25 \pm 0.212	<0.0001
	15*	42.8 \pm 1.62	<0.0001	8.5 \pm 0.092	<0.0001	4.89 \pm 0.109	<0.0001
	20*	37.2 \pm 2.96	0.0003	7.65 \pm 0.329	0.0004	4.36 \pm 0.30	<0.0001
Positive Control		68.8 \pm 1.98	<0.0001	13.3 \pm 0.21	<0.0001	7.31 \pm 0.174	<0.0001

* One animal died ** Significant ($P < 0.05$), highly significant ($P < 0.0001$)*** % Abc: Percent aberrant cells; carrying at least one micronucleus; MN: Micronucleated; NCE: Normochromatic Erythrocytes (N); PCE: Polychromatic Erythrocytes (P).

stated experimental conditions. The test chemical (ISO) and the positive control (MMC) increased the ratio PCEs to NCEs (Table 1 and Figure 3). The blood P/N ratio reveals cellular turnover of PCEs and NCEs next to short-term cellular damage and recovery after exposure by the replacement of erythrocytes with undamaged progenitor cells. To evaluate the cytotoxicity of ISO, a popular and convenient method of monitoring erythropoiesis was employed by measuring the ratio of PCEs to NCEs. Suzuki et al. (1989) reported that assessment of erythropoietic cytotoxicity was a key parameter of safety evaluation in new drug development and PCE counts in peripheral blood were the most popular and convenient method of monitoring erythropoiesis. The ratio of P/N in normal conditions is 1:1. Any deviation from this ratio (e.g., the lower frequency of immature erythrocytes (PCE) relative to mature (NCE) i.e., a decrease in the P/N ratio) indicates cytotoxic activity. A continuous decline in the P/N ratio may occur due to interference in the cell cycle, the

reports of decreased white blood cell count, hemoglobin, and platelets have been encountered in the literature. Our cytotoxicity findings agree with previous cytotoxicity reports from *in vitro* studies on K-562 leukemia cells (Diniz et al., 2008), and human lymphocytes (Silva et al., 2013),

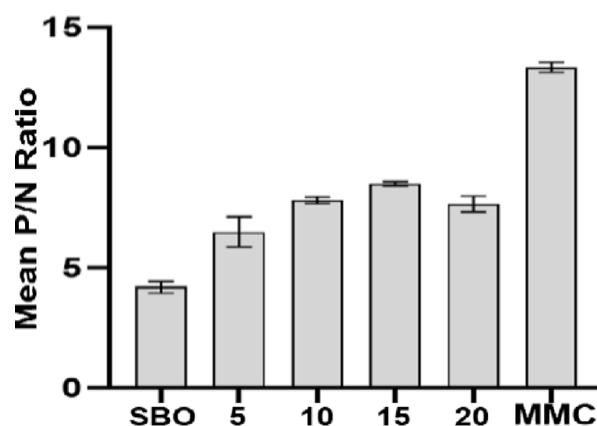


Figure 3. Dose-response for polychromatic/normochromatic erythrocytes ((P/N) ratios in male albino Sprague-Dawley rat peripheral red blood cells. For other details, see the legend of figure (2). Error bars indicate a standard error at * $p < 0.0001$ and ** $P < 0.0004$.

as well as *in vivo* in mice (Al-Othman et al., 2019). Clinically insignificant and reversible complete blood count (CBC) abnormalities were observed with oral ISO (Osofsky and Strauss, 2011). It is supposed that the increase in %PCE value has resulted probably from an increase in PCE in bone marrow due to either direct or indirect stress of ISO on erythropoiesis. One possible explanation for the increase in the P/N ratio is that ISO decreased the numbers of NCE is slow the differentiation and multiplication or denucleation of erythroblasts. Alternatively, the toxicity of ISO caused significant elevations in erythropoiesis so that PCE could be produced but NCE in the circulation remained in the bone marrow because their life span reached its minimum i . e. the effect was on the generated cells populations, but the bulk was mature and not cycled in blood. A similar finding was reported before, where the P/N ratio increased with a decrease in the hemoglobin concentration, hematocrit, and mature erythrocyte count were observed in both mice (El-Alfy et al., 2016) and rats (Kojima et al., 2012), following methotrexate oral dosing of methotrexate. However, this is not consistent with the finding of Suzuki et al. (1989) regarding the gradual decrease in the P/N ratio with time after the administration of MMC into mice. Similarly, decreased frequencies of PCEs in erythrocytes were also observed after exposure of the common carp to chlorpyrifos; an organophosphate pesticide (Mitkovska and Chassovnikarova, 2020). The same conclusion was reached when mice were experimentally treated with doxorubicin (Boriollo et al., 2021). The drastic change in erythropoiesis in bone marrow induced by ISO treatment would affect fluctuations of %PCE or % MNPCE per non-micronucleated erythrocytes in the MN test. It is known that the ISO achieves remarkable pharmacological efficacy in the treatment of severe acne, by influencing the basic cellular processes such as division, growth, survival, differentiation, and apoptosis (Tsukada et al., 2000; Rigopoulos et al., 2010; Miura, 2011;

Nelson et al., 2011; Layton, 2014; Melnik, 2017; Draghici et al., 2021).

Finally, it is worth mentioning that ISO and its analog structures tretinoin, and 4-oxo- isotretinoin are the major systemic metabolites identified in systemic circulation following oral administration of ISO (Colburn et al., 1983), and its plasma concentration is about 4-fold higher than that of the parent drug after multiple dosing (Sadeghzadeh-Bazargan et al., 2021). However, considerable inter-individual variation in the ISO plasma concentrations exists. The differences in anatomy, physiology, and development between the rat and the human (Andreollo et al., 2012) make it difficult to extrapolate the findings and care must be taken into consideration when analyzing the results of any research in rats when age is a crucial factor. Higher doses of acitretin were recommended for use in rats compared to the doses used in humans because the drug metabolism in rats is higher than in humans (Güven et al., 2022). At any rate, the rat remains a major model system in the pharmaceutical industry based on its big size and its inherent physiological features (Jacob, 1999). One shortcoming of our study is that the ISO serum concentration was not measured. Other limitations of our study include the lack of long-term follow-up of the animals. Further studies using other cytogenetic endpoints in different biological systems are required to provide substantial evidence grounds for the clinical safety of ISO. One possible approach for assessing genetic stability is by examining cytotoxicity and genotoxicity using flow cytometry. Currently, rigorous research is being carried out in our laboratory on the ISO histological and histochemical effects as well as the effects of the drug on the rat sperm genome stability.

Conclusion

The data from this research suggest that ISO exhibits cytotoxic and genotoxic activity in rats' erythrocytes. The results of the present and previous studies recommend continuous low-dose treatment as the chosen regimen for acne vulgaris.

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Conflict of interest

Authors declare no conflict of interest.

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