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#### Original article Effects of High-Dose All-Trans-Retinoic Acid on Tibial Growth of Young Male Albino Rats Nada Khaled Atef Essa , Ibrahim Sayed Ahmed Zamzam , Maha Mohamed Mokhtar , Marwa M. M. Fawzy , Nagah El Sayed Mohamed Ali

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#### Abstract

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**Keywords:** all-transretinoic acid; tibia; growth hormone; bone toxicity Background: All-trans-retinoic acid (ATRA), an active metabolite of vitamin A, plays a significant role in controlling various physiological processes, including embryonic development and epithelial differentiation. ATRA is utilized as a chemotherapeutic agent for treating of acute promyelocytic leukemia (APL) in pediatric patients. Besides its efficacy in APL therapy, ATRA is extensively used in the treatment of various skin diseases such as psoriasis, skin cancer, acne, and ichthyosis. Aim: This study aimed to assess the impact of high-dose ATRA on tibial growth by measuring serum growth hormone "GH," histomorphometric measurements of tibia, and histopathological examination of tibia in young male albino rats. Methods: This experimental study included 54 male albino rats, aged 6 weeks, divided into three groups: group I and group II served as control groups, whereas group III was administered ATRA treatment (40 mg/kg/day of ATRA suspension administered via gavage, dissolved in 1 cc of corn oil at a concentration of 10 mg/ml, with an oral LD50 of 2 g/kg). Each group was subdivided into three subgroups, each comprising six rats. The research was submitted to the Research Ethics Committee of the Faculty of Medicine at Benha University. The study assessed the effects of ATRA treatment on serum growth hormone, weight, tibial length, nose-tail length, outer cortical bone thickness, relative bone resorption eroded surface percentage, osteoclast count, and osteoblast count compared to control groups. Results: There were no notable variations in these parameters among the control groups. In addition, there were no significant differences in osteoblast count among all groups. The ATRA-treated groups demonstrated a significant decrease in serum growth hormone, weight, tibial length, nosetail length, and outer thickness of cortical bone, as well as a highly significant increase in relative bone resorption eroded surface percentage and osteoclast count. Conclusion: High-dose administration of ATRA resulted in a reduction in growth measurements, including weight, tibial length, and nose-tail length with histological alterations and histomorphometric changes.

#### I. Background

Since the human body cannot synthesize vitamin A, it must be obtained through diet. It is an important micronutrient (Gomes et al., 2021). It is a fat-soluble vitamin categorized into two sources: preformed vitamin A or retinol, which is present in animal-based diets, and pro-vitamin carotenoid, which is found in fruit and vegetable sources (Berihun et al., 2023). Enzymes convert vitamin A into retinol, retinaldehyde, and ultimately, irreversibly, into retinoic acid (RA), which is the hormone-active form of vitamin A (Shen et al., 2022). Retinoic acid exists in three isoforms: 9-cis retinoic acid, 13-cis retinoic acid, and ATRA. ATRA represents the predominant and most active isoform (Bahlool et al., 2022). Vitamin A derivatives exist in both synthetic and natural retinoid forms. They play a crucial role in regulating various physiological functions in multiple organ systems, including development, differentiation, and immunological function (Szyma´nski et al., 2020). In physiological microenvironments, ATRA functions as an alcohol and vitamin A metabolite, significantly influencing various physiological processes, such as embryonic development and epithelial differentiation (Wang et al., 2020). ATRA, or all-trans retinoic acid, has been demonstrated to possess both chemotherapeutic and

ATRA is not only effective in APL therapy but is also widely used in the treatment of various skin conditions, such as ichthyosis, psoriasis, acne, and skin cancer, in addition to its role in wrinkle management. The effects on cell differentiation, proliferation, and apoptosis account for this, and it is also highly effective against respiratory syncytial virus (RSV) in neonates (Szyma'nski et al., 2020). ATRA can be associated with life-threatening adverse reactions, such as ulcers, Sweet's syndrome, myocarditis, myositis, and differentiation syndrome (Ferla et al., 2020).

However, it also has short-term adverse effects, such as skin mucosal erosion and mental disorders. Furthermore, it has the potential to result in early epiphysis closure or degeneration in the long term. Additionally, the overconsumption of vitamin A has been associated with a reduction in cortical bone thickness and an increase in trabecular bone content, as well as an increase in osteoclast count and activity. This results in a low stature and the prevention of long bone growth (Shen et al., 2022). Few studies have examined the impact of ATRA on a variety of organs, including the liver and bones. The research that identified the toxic effects of ATRA on bone was insufficient to elucidate the mechanisms of ATRA's bone toxicity and focus on its clinical implications.

Overall, the rationale for this research emphasizes the necessity of comprehending the impact of ATRA on bone remodeling and emphasizes the potential implications for improving bone health and preventing therapeutic harm to the bone. According to our study, ATRA's effect needs more clinical observation for its use as a chemotherapeutic and chemopreventive drug for protection against bone toxicity.

Preclinical studies using animal models, such as albino rats, play a vital part in elucidating the effects of pharmacological agents on bone physiology (Usategui-Martín et al., 2023).

This experimental study aims to assess the effect of a high dose of retinoic acid in its entirety "ATRA" on tibial growth by measurement of serum growth hormone, histomorphometric measurements of the tibia, and chemopreventive effects. The application of this agent as a differentiation inducer, whether used independently or in conjunction with other chemotherapeutic agents, represents the current standard therapeutic strategy for the treatment of pediatric acute promyelocytic leukemia (APL) and neuroblastoma (NB) (Giuli et al.,2020).

histopathological examination of the tibia in young male albino rats.

### II. Materials and Methods

#### II.1 Study design:

This experimental study included fifty-four, 6-week-old male albino rats, which were divided into three groups: group I and group II served as control groups, while group III received ATRA treatment during September 2023.

Group I (negative control group): 18 rats had unrestricted access to food and purified water, and they were all kept unaltered to measure fundamental parameters.

Group Ia: (6 rats) sacrificed after 3 days, group Ib: (6 rats) sacrificed after 7 days, and group Ic: (6 rats) sacrificed after 10 days.

Group II (positive control group): 18 rats received 0.4 ml per 100 grams of corn oil daily.

Group IIa: (6 rats) sacrificed after 3 days, group IIb: (6 rats) sacrificed after 7 days, group IIc: (6 rats) sacrificed after 10 days.

Group III (All-Trans-Retinoic Acid (ATRA) treated group): 18 rats (all were given 40mg/kg per day of ATRA suspension administered via gavage tube (dissolved in corn oil at a concentration of 10 mg/ml).

Group IIIa: (6 rats) were sacrificed after three days, group IIIb: (6 rats) were sacrificed after seven days, and group IIIc: (6 rats) were sacrificed after ten days.

#### **Chemicals:**

ATRA, in the form of powder with a purity of 97%, and corn oil were purchased from Acros Chemical Company, Belgium.

#### **Ethical consideration:**

The study adhered to the guidelines for the use and care of experimental animals. The research was submitted to the Research Ethics Committee of Benha University's Faculty of Medicine and received approval under approval code (MS-17-4-2023).

#### Route of administration:

Both ATRA and corn oil were given by oral gavage using a metallic cannula

**Dosage regimen and vehicle:** The dose of ATRA was 40 mg/kg/day of ATRA suspension administered by gavage (dissolved in 1 cc of corn oil at a concentration of 10

DOI: 10.21608/ZJFM.2024.315762.1199 Corresponding author: Nagah El Sayed Mohamed Ali Email: nageh.ali@fmed.bu.edu.eg mg/ml) with LD50 oral = (2g/kg) (Shen et al., 2022). Corn oil was used to form a suspension with freshly prepared ATRA powder.

**Sample Collection:** In order to separate clear serum for biochemical analysis, blood samples were obtained by puncturing the retro-orbital plexus without the use of an anticoagulant. The tibia was removed from each leg. Removed Tibial bones were cleaned and immersed in 10% formalin to be examined histopathologically and for histomorphometric measurements.

**Studied parameters:** A total of 54 six-week-old male albino rats were subjected to several recordings. The body weights of the rats, nose-tail lengths, and tibial bone lengths were recorded before the experiment and at the end of it for each group.

#### **II.2 Methods**

#### **Biochemical analysis**

#### Preparation of serum

Blood was centrifuged at 2500 rpm for 15 minutes to extract serum after 30 minutes at ambient temperature, then the serum was subsequently stored at -80 °C until further use.

The reagents were equilibrated to room temperature (20– $25^{\circ}$ C) prior to use. Standards A – G and authority 1 and 2 were reconstituted using a dilution buffer. Reconstituted reagents were maintained at room temperature for 15 minutes and subsequently mixed thoroughly and gently using a vortex mixer, ensuring no foam formation. KS1 (control serum 1) and KS2 (control serum 2) were then diluted with the dilution buffer in the same ratio as the sample (Bielohuby et al., 2012).

The serum growth hormone of experimental rats was measured using ELISA kits. Blood samples were collected at night during the peak of GH level (Shen et al., 2022).

#### Histomorphometric measurements of tibial bone

Measurements of specific parameters related to the growth process were conducted for observations and morphological assessments through captured images using an OMAX camera (4x, 10x, 40x). The images were analyzed with Image-Pro Plus (Version 6.3, Media Cybernetics, Nippon Roper Co., Ltd.) image analysis software (Ashraf et al., 2013). The following parameters were measured: (a) Outer cortical bone thickness: the outer cortical bone's mean width. A perpendicular line was drawn from the periosteum to the endosteum in order to quantify this.

(b) Relative bone resorption eroded surface (%): proportion of compact bone surface exhibiting resorption-specific characteristics, such as surfaces containing or lacking osteoclasts. In addition, the length of eroded trabecular surface% and total length of trabecular surface were measured. (c) The count of osteoblasts in an area was equal to 500  $\mu$ m2. The cells were categorized as follows: Osteoblasts were previously characterized as large, multinucleated cells involved in bone resorption, while osteoclasts were considered mononuclear, cuboidal, or columnar cells with a high content of basophilic cytoplasm, serving as bone lining cells.

#### Histopathological examination of tibial bone

Histopathological examinations of the tibia were conducted on all experimental rats, and the slides were analyzed at the Pathology Department of the Animal Health Research Institute, Zagazig Laboratory. All tibial bone tissue sections obtained from experimental rats were prepared using the paraffin technique. Paraffin sections were cut to a thickness of 5  $\mu$ m and subsequently stained with eosin and hematoxylin. Samples were examined under a light microscope (Olympus, Japan) after staining.

#### **II.3 Data analysis:**

Data entry, coding, and analysis were done using international buiseness machines corporation (IBM Corp) (Released 2011, Statistical package for social sciences (SPSS), Statistics for Windows, Version 24.0. Armonk, New york : IBM Corp. Data of this study was of quantitative type. Parametric quantitative data was expressed as means  $\pm$  standard deviation (SD) with median, minimum and maximum range for nonparametric quantitative data. The ANOVA test (F) (analysis of variance) was used to test the significance of numerical information across three groups followed by Fischer's least significant difference (LSD) post hoc test. The repeated ANOVA test (F) was used for the three repeated measures of numeric variables. The area under the curve, sensitivity, specificity, negative predictive value, positive predictive value, and accuracy of the receiver operating characteristic (ROC) curve are analyzed for various cutoffs of a diagnostic test or marker to assess its effectiveness as a diagnostic tool. Our data established a 95% significance threshold, thus a p-value greater than 0.05 was regarded as nonstatistically significant (Peacock, 2020).

#### **III. Results:**

Results of negative and positive control groups showed non-significant statistical difference, so negative control groups' results were used in statistical interpretation in comparison with ATRA treated groups. The serum level of growth hormone is displayed in Table (1) With respect to the control groups, the mean value of serum growth hormone did not exhibit a significant difference between group Ia , Ib, Ic (negative control at 3, 7, 10 days) respectively, group IIa, IIb, IIc (positive control at 3, 7, 10 days) respectively. Group IIIa, IIIb, IIIc (ATRA treated at 3, 7, 10 days) respectively, exhibited a substantial decrease in the mean value of serum growth hormone, but with no significant difference in between (p>0.05). ATRA treated group at 3 days, group IIIa showed significant decrease in serum level of growth hormone compared to control groups group Ia and IIa (p<0.05).The same was found with results at 7 days and 10 days, ATRA treated group at 7 days, group IIIb showed significant decrease in serum level of growth hormone compared to control groups Ib and IIb, while

group IIIc showed significant decrease in serum level of growth hormone compared to groups Ic and IIc (p<0.05). Regarding weight, the control groups: group Ic (negative

control at 10 days) showed significant increase in weight compared to group Ia and group Ib (p < 0.05). Group IIc (positive control at 10 days) exhibited a significant increase in weight compared to group IIa and group IIb (p<0.05). Regarding the ATRA treated groups, group IIIc (ATRA treated at 10 days) exhibited an extremely substantial mean weight decline compared to group IIIa (ATRA treated at 3 days) and group IIIb (ATRA treated at 7 days)(p < 0.05). ATRA treated group at 3 days (group IIIa) showed significant decrease in weight compared to control groups, group Ia and IIa (p < 0.05). The same was found with results at 7 days and 10 days, ATRA treated group at 7 days, group IIIb showed significant decrease in weight compared to control groups, Ib and IIb, while ATRA treated, group IIIc showed significant decrease compared to control groups Ic and IIc (p<0.05).

Table 1: comparison of serum level of growth hormone and weight in control groups and ATRA-treated groups by ANOVA and Fischer's tests

Groups	Gl	Н	(F)/ p (ATRA	treated with
-	Mean ±SD	Range	corresponding	control
			group)	
Group Ia (negative control at 3 days)	5.59±0.136	5.625(5.4-5.77)		
Group Ib (negative control at 7 days)	5.582±0.161	5.615(5.3-5.74)		
Group Ic (negative control at 10 days)	5.442±0.163	5.45(5.2-5.66)		
	p# = 0.152			
Group IIa (positive control at 3 days)	5.522±0.219	5.485(5.21-5.85)		
Group IIb (positive control at 7 days)	$5.43 \pm 0.288$	5.51(5.04-5.740)		
Group IIc (positive control at 10 days)	$5.29 \pm 0.194$	5.28(5.08-5.550)		
	p## = 0.531			
Group IIIa (ATRA treated at 3 days)	$0.98 \pm 0.422$	0.863(0.549-1.760)	F=513.304	p=0.000
Group IIIb (ATRA treated at 7 days)	$0.879 \pm 0.471$	0.710(0.432-1.650)	F=388.816	p=0.000
Group IIIc (ATRA treated at 10 days)	$0.817 \pm 0.228$	0.821(0.495-1.123)	F=1072.913	p=0.000
	p### = 0.643			
	weigl	nt		
Group Ia (negative control at 3 days)	137.0±2.098	137.5(134-139)		
Group Ib (negative control at 7 days)	141.67±1.211	141.5(140-143)		
Group Ic (negative control at 10 days)	$145.33 \pm 1.862$	145.0(143-148)		
	p# = 0.009			
Group IIa (positive control at 3 days)	137.5±1.87	137.5(135-140)		
Group IIb (positive control at 7 days)	141.33±1.966	140.5(140-145)		
Group IIc (positive control at 10 days)	147.50±3.271	148.5(143-151)		
	p## = 0.012			
Group IIIb (ATRA treated at 7 days)	$133.5 \pm 2.168$	133(131-137)	F=38.322	p=0.000
Group IIIc (ATRA treated at 10 days)	129.5±1.871	129.5(127-132)	F=98.387	p=0.000
	p### = 0.014			

Data are expressed as mean±SD, median (Min-Max), F1, F2, F3: ANOVA (analysis of variance test) between groups at 3, 7, 10 days, significant at p<0.05, highly significant (HS) at p<0.01 p#:repeated measure ANOVA test of negativecontrol group, p## :repeated measure ANOVA test of ATRA treated group

Table (2) shows the tibial length results among groups. Group Ia, group Ib and group Ic, group IIa, group IIb and group IIc demonstrated non-significant variation in mean tibial length (p>0.05). In addition, group IIIa (ATRA treated at three days), group IIIb (ATRA treated at seven days), and group IIIc (ATRA treated at ten days) exhibited a substantial non-significant decrease in tibial length (p>0.05). The mean tibial length of group IIIa (ATRA treated at three days) exhibited significant decrease compared to group IIa (positive control at three days) and Ia (negative control at three days) (p<0.05). At the level of seven and ten days, there was non significant difference between ATRA treated group, group IIIb (ATRA treated at seven days) and IIIc (ATRA treated at ten days) and control groups, group Ib (negative control at seven days), group IIb (positive control at seven days), group Ic (negative control at ten days), group IIc (positive control at ten days), group IIc (positive control at ten days (p>0.05) in mean values of tibial length.

Regarding nose-tail length, group Ia (negative control at three days), group Ib (negative control at seven days), group Ic (negative control at ten days), demonstrated non-significant variation in mean nose-tail length (p>0.05). Group IIa (positive control at three days), group IIb (positive control at seven days), and group IIc (positive control at ten days) demonstrated nonsignificant variation in mean nose-tail length (p>0.05). In addition, group IIIa (ATRA treated at three days), group IIIb (ATRA treated at seven days), and group IIIc (ATRA treated at ten days) exhibited a non-significant decrease in nose-tail length, (p>0.05). Regarding results at 3 days, group IIIa (ATRA treated at 3 days) demonstrated a non-significant difference (p>0.05) in the mean value of nose-tail length compared to control groups Ia, IIa. Group IIIb (ATRA treated at seven days) showed a significant reduction (p<0.05) in mean nose-tail length when compared to both the negative and positive control groups, Ib, IIb. Group IIIc (ATRA treated at 10 days) demonstrated a statistically significant reduction (p<0.05) in the mean nose-tail length compared to both the negative and positive control groups, Ib, IIb. Group IIIc (ATRA treated at 10 days) demonstrated a statistically significant reduction (p<0.05) in the mean nose-tail length compared to both the negative and positive control groups.

Table 2: Comparison between control groups and ATRA-treated groups regarding tibial length and nose-tail length by ANOVA and Fischer's tests

Groups	tibial length		F test, p value (ATRA treated	
	Mean±SD	Range	with correspon group)	ding control
Group Ia (negative control at 3 days)	2.415±0.054	2.425(2.33-2.48)		
Group Ib (negative control at 7 days)	$2.495 \pm 0.086$	2.47(2.41-2.63)		
Group Ic (negative control at 10 days)	$2.548 \pm 0.088$ p# = 0.151	2.57(2.40-2.65)		
Group IIa (positive control at 3 days)	2.425±0.0493	2.43(2.35-2.48)		
Group IIb (positive control at 7 days)	$2.4667 \pm 0.067$	2.44(2.40-2.56)		
Group IIc (positive control at 10 days)	$2.533 \pm 0.077$	2.54(2.44-2.63)		
	p## = 0.674			
Group IIIa (ATRA treated at 3 days)	$2.305 \pm 0.0846$	2.315(2.15-2.40)	F=6.389	p=0.010
Group IIIb (ATRA treated at 7 days)	$2.482 \pm 0.058$	2.48(2.42-2.55)	F=0.238	p=0.791
Group IIIc (ATRA treated at 10 days)	2.328±0.275	2.385(1.8-2.61)	F=3.043	p=0.087
	p### = 0.080	· · · · · ·		1
	nose-tail Length			
Group Ia (negative control at 3 days)	21.067±0.242	21.05(20.8-21.5)		
Group Ib (negative control at 7 days)	21.117±0.1722	21.1(20.9-21.4)		
Group Ic (negative control at 10 days)	21.650±0.308	21.75(21.1-21.9)		
	p# = 0.531			
Group IIa (positive control at 3 days)	20.967±0.333	20.95(20.6-21.5)		
Group IIb (positive control at 7 days)	21.20±0.346	21.25(20.7-21.6)		
Group IIc (positive control at 10 days)	21.717±0.214	21.7(21.5-, 22.0)		
	p## = 0.674			
Group IIIa (ATRA treated at 3 days)	21.10±0.261	21.05(20.8-21.5)	F=0.365	p=0.700
Group IIIb (ATRA treated at 7 days)	20.817±0.214	20.75(20.6-21.2)	F=3.746	p=0.048
Group IIIc (ATRA treated at 10 days)	21.25±0.288	21.15(21-21.8)	F=5.127	p=0.020
	p### = 0.140			

Data are expressed as mean±SD, median (Min-Max), F1, F2, F3: ANOVA (analysis of variance test) between study groups at 3 days, 7 days, 10 days, significant at p<0.05, highly significant (HS) at p<0.01 p#: repeated measure ANOVA test of negative control group, p##: repeated measure ANOVA test of ATRA treated group

Table (3) shows the outer cortical bone thickness. In terms of the control groups, group Ic exhibited significant increase in the mean value of outer cortical bone thickness compared to group Ia and group Ib (p<0.05). Group IIc exhibited a significant increase in the mean values of outer cortical bone thickness compared to group IIa and group IIb.

Regarding the ATRA treated groups, group IIIa (ATRA treated at 3 days), group IIIb (ATRA treated at 7 days), and group IIIc (ATRA treated at 10 days) showed a non-significant difference in mean values of outer cortical bone thickness (p>0.05). Group IIIa (ATRA treated at 3 days) showed significant decrease in mean value of outer cortical bone thickness compared to both groups (Ia and

IIa). Group IIIb showed significant decrease in outer cortical bone thickness compared to control groups, Ib and IIb , while group IIIc showed significant decrease in outer cortical bone thickness compared to control groups Ic and IIc, (p<0.05). The mean values of the relative bone resorption eroded surface % did not exhibit any significant difference (p>0.05) among the control groups. The mean values of relative bone resorption eroded surface % demonstrated a significant increase in the group IIIc (ATRA treated at three days) compared to group IIIa (ATRA treated at three days) and group IIIb (ATRA treated at seven days) (p<0.05).

The mean value of the relative bone resorption eroded surface % of group IIIa (ATRA treated at three days) was significantly higher (p<0.05) than that of both control groups IIa and Ia.The same was found with results at 7 days and 10 days. ATRA group at 7 days , group IIIb , showed significant increase in relative bone resorption eroded surface % compared to control groups, Ib and IIb, and ATRA treated group at 10 days, group IIIc, showed significant increase in relative bone resorption eroded surface % compared to control groups, Ic and IIc, (p<0.05).

Table 3: Comparison between the control groups and ATRA-treated groups regarding outer cortical bone thickness and relative bone resorption eroded surface % by ANOVA and Fischer's tests

Groups	outer cortical b	F test, p value (ATRA		
	Mean±SD	Range	treated with co	rresponding
			control g	roup)
Group Ia (negative control at 3 days)	314.447±29.797	137.5(134-139)		
Group Ib (negative control at 7 days)	375.71±39.17	141.5(140-143)		
Group Ic (negative control at 10 days)	422.08±33.04	145.0(143-148)		
	p# = 0.012			
Group IIa (positive control at 3 days)	310.61±37.00	137.5(135-140)		
Group IIb (positive control at 7 days)	364.978±40.70	140.5(140-145)		
Group IIc (positive control at 10 days)	422.73±32.88	148.5(143-151)		
	p## = 0.013			
Group IIIa (ATRA treated at 3 days)	217.513±19.173	218.215 (192.31-	F=20.663	p=0.000
		242.85)		
Group IIIb (ATRA treated at 7 days)	194.56±13.81	198.28(172.33-210.75)	F=54.984	p=0.000
Group IIIc (ATRA treated at 10 days)	$174.99 \pm 19.52$	177.51(141.24 198.67)	F=143.824	p=0.000
	p### = 0.079			
re	lative bone resorption erod	led surface %		
Group Ia (negative control at 3 days)	16.543±0.962	16.135(15.41-18.14)		
Group Ib (negative control at 7 days)	$16.75 \pm 0.899$	16.64(15.61-17.95)		
Group Ic (negative control at 10 days)	$16.52 \pm 0.927$	16.525(15.31-17.65)		
	p# = 0.831			
Group IIa (positive control at 3 days)	16.983±1.163	16.67(15.98-19.11)		
Group IIb (positive control at 7 days)	$16.61 \pm 16.61$	16.35(15.65-18.10)		
Group IIc (positive control at 10 days)	$16.528 \pm 0.884$	16.21(15.54-17.98)		
	p## = 0.674			
Group IIIa (ATRA treated at 3 days)	$32.755 \pm 2.422$	32.39(29.85-36.55)	F=188.448	p=0.000
Group IIIb (ATRA treated at 7 days)	37.512±1.365	37.53(35.89-39.13)	F=755.539	p=0.000
Group IIIc (ATRA treated at 10 days)	41.25±2.399	42.21(37.73-43.47)	F=496.107	p=0.000
	p### = 0.039			

Data are expressed as mean±SD, median (Min-Max), F1, F2, F3: ANOVA (analysis of variance test) between study groups at 3 days, 7 days, 10 days, significant at p<0.05, highly significant (HS) at p<0.01 p#: repeated measure ANOVA test of negative control group, p## :repeated measure ANOVA test of ATRA treated group

Table (4) shows the results of the osteoclast count. Group Ia (negative control at three days), group Ib (negative control at seven days), group Ic (negative control at ten days), group IIa (positive control at three days), group IIb (positive control at seven days), and group IIc (positive control at ten days) exhibited a non-significant difference in the mean osteoclast count (p>0.05). Regarding the ATRA-treated groups, group IIIc (ATRA treated at 10 days) showed significant increase compared to group IIIa (ATRA treated at 3 days) and group IIIb (ATRA treated at 7 days) (p<0.05) in mean values of osteoclast count. At the level of three days, in comparison to positive and negative control groups, Ia and IIa, group IIIa (ATRA treated at 3 days) showed significant increase in the mean values of osteoclast count. The same was found at the levels at 7 days and 10 days, ATRA treated group, IIIb, showed significant increase in osteoclast count compraed to control groups Ib and IIb, (p<0.05). Group IIIc (ATRA treated at 10 days) showed highly significant increase (p=0.000) in

osteoclast count compared to negative and positive control groups, Ic and IIc.

Regarding osteoblast count: there were no significant differences in the mean values of osteoblast count in the control groups, group Ia (negative control at three days), group Ib (negative control at seven days), group Ic (negative control at ten days), group IIa (positive control at three days), group IIb (positive control at seven days), and group IIc (positive control at ten days). As regard ATRA treated groups, group IIIa (ATRA treated at 3 days), group IIIb (ATRA treated at 7 days), group IIIc (ATRA treated at 10 days) showed non-significant differences in the mean values of osteoblast count.

At the level of three days, there was non significant difference (p>0.05) between ATRA treated group IIIa and both control groups Ia and IIa.

The same was at the level of seven days, there was non significant difference (p>0.05) between ATRA treated group IIIb and both control groups Ib and IIb.

Also, a non-significant difference (p>0.05) was observed in the mean osteoblast count of group IIIc (ATRA treated at ten days) when compared to control groups, Ic and IIc.

Table 4: Comparison between the control groups and ATRA-treated groups regarding osteoclast count and osteoblast count by ANOVA and Fischer's tests

Groups	Osteoclast c	F test, p value (ATRA		
	Mean±SD	Range	treated with o	corresponding control group)
Group Ia (negative control at 3 days)	0.333±0.516	0.00(0.00-1.00)		
Group Ib (negative control at 7 days)	$0.333 \pm 0.516$	0.00(0.00-1.00)		
Group Ic (negative control at 10 days)	0.333±0.516	0.00(0.00-1.00)		
	P# = 1.000			
Group iia (positive control at 3 days)	0.333±0.516	0.00(0.00-1.00)		
Group iib (positive control at 7 days)	$0.500 \pm 0.548$	0.50(0.0-1.0)		
Group iic (positive control at 10 days)	0.333±0.516	0.00(0.0-1.0)		
	P## = 0.875			
Group iiia (ATRA treated at 3 days)	$1.167 \pm 0.408$	1.00(1.00-2.00)	F=7.511	p=0.023
Group iiib (ATRA treated at 7 days)	1.33±0.516	1.0(1.0-2.0)	F=7.484	p=0.024
Group iiic (ATRA treated at 10 days)	$2.50\pm0.548$	2.50(2.0-3.0)	F=12.706	p=0.002
	P### = 0.002			
	Osteoblast count			
Group Ia (negative control at 3 days)	$5.0 \pm 1.414$	5.0(3-7)		
Group Ib (negative control at 7 days)	0.333±0.516	0.00(0.00-1.00)		
Group Ic (negative control at 10 days)	$6.5 \pm 1.517$	6.5(4-8)		
	P# = 0.098			
Group iia (positive control at 3 days)	4.83±1.472	4.5(3-7)		
Group iib (positive control at 7 days)	5.33±1.633	5.50(3-7)		
Group iic (positive control at 10 days)	6.50±1.049	6.5(5-8)		
	P## = 0.334			
Group iiia (ATRA treated at 3 days)	4.33±1.211	4.5(3-6)	F=0.385	p=0.687
Group iiib (ATRA treated at 7 days)	4.33±1.033	4.0(3.0-6.0)	F=0.875	p=0.437
Group iiic (ATRA treated at 10 days)	3.83±1.472	3.50(2-6)	F=7.665	_p=0.05
	P### = 0.846			

Data are expressed as mean±SD, median (Min-Max), F1, F2, F3: ANOVA (analysis of variance test) between study groups at 3 days, 7 days, 10 days, significant at p<0.05, highly significant (HS) at p<0.01 p#: repeated measure ANOVA test of negative control group, p## :repeated measure ANOVA test of ATRA treated group

ROC curves were conducted to assess the diagnostic value of the selected markers (independent variables), with the treatment group as the dependent variable. The area under the curves ranged from 0.842 to 1.00, indicating highly significant potent markers. The selection of a cutoff point of 3.102 for growth hormone as a predictive value for the effects of high-dose ATRA on tibial growth in young male albino rats resulted in an area under the curve of 1.00, with 100% sensitivity, 100% specificity, 100% PPV, 100% NPV, and 100% accuracy.

Concerning tibial length, a cutoff point of 2.45 as a predictive value resulted in an area under the curve of 0.842, with 83.33% sensitivity, 83.33% specificity, 71.43% positive predictive value, 90.91% negative predictive value, and 83.33% accuracy. Concerning outer cortical bone thickness, a cutoff point of 299.235 as a predictive value demonstrated an area under the curve of 1.00, with 100% sensitivity, 100% specificity, 100% positive predictive value, 100% negative predictive value, and 100% accuracy. Concerning relative bone resorption eroded surface % a cutoff point

# of 27.855 as a predictive value resulted in an AUC of 1.00, with 100% sensitivity, 100% specificity, 100% PPV, 100% NPV, and 100% accuracy.

Table 5: ROC curves of diagnostic markers between control and treated groups

Markers	Area under the curve	Best cutoff point	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy (%)
			(%/)/ (confidence interval)				
Growth hormone level (ng/ml)	1.000	3.102	100% (54.07-100)	100% (54.07-100)	100% (54.07-100)	100% (54.07-100)	100% (73.54-100)
Tibial length (cm)	0.842	2.450	83.33% (35.88-99.58)	83.33% (35.88-99.58)	83.33% (44.64-96.88)	83.33% (44.64-96.88)	83.33% (51.59-97.91)
Outer cortical bone thickness (mu)	1.000	299.235	100% (54.07-100)	100% (54.07-100)	100% (54.07-100)	100% (54.07-100)	100% (73.54-100)
Relative bone resorption eroded surface %	1.000	27.855	100% (54.07-100)	100% (54.07-100)	100% (54.07-100)	100% (54.07-100)	100% (73.54-100)
Osteoclast count	1.000	1.500	100% (54.07-100)	100% (54.07-100)	100% (54.07-100)	100% (54.07-100)	100% (73.54-100)



Figure 1. ROC curve of serum GH level



Figure 2. ROC curve of tibial length







Figure 4. ROC curve of relative bone resorption eroded surface %



(A) Photomicrograph of H&E stained section of rat tibia of negative control group (after three days) showing a normal outer portion of a dense connective tissue layer as compact bone (CB) from endosteum of the epiphysis with mineralized extracellular bony matrix (CB matrix) consists of collagen fiber and haversian system in a circular pattern including haversian canals surrounded by the osteocytes (Os.c) with characteristic nuclei with the presence of osteoblast cells, osteoclast cells (Os.Cl), vascular supply (Bl.vs) and resorption canals indicating an active process of bone remodeling (scale bar=  $100\mu m$ ).



(C) Photomicrograph of H&E stained section of rat tibia of the positive control group (received corn oil, after three days) showing normal compact bone of diaphysis (CB) with normal regular mineralized bony matrix with collagen fiber and arranged osteocytes (Osc) in a circular pattern forming haversian system, formed from osteons, osteoblast cells, osteoclast cells and vascular supply (Bl.vs). Bone marrow (BM) formed from adipocyte cells in the diaphyseal center is seen (scale bar= 100µm).



(B) Photomicrograph of H&E stained section of rat tibia of the negative control group (after ten days) showing normal spongy or cancellous bone (SB) in the epiphysis with a threedimensional structure consisting of branching anastomosing trabeculae (T), with the same structural organization and components as compact bone. Bone marrow (BM) mainly comprises adipocytes and fills space among trabeculae. Normal compact bone (CB) with vascularity (Bl.vs) is present. (scale bar= 100µm).



(D) Photomicrograph of H&E stained section of rat tibia of the positive control group(received corn oil, after seven days) showing normal cancellous bone of epiphysis with an anastomosing network of trabecular bone (thin arrows) (T) in the form of bone spicules consists of a dense layer of organized, compact bone with arranged osteocytes (Osc) in haversian system forming osteons. Bone marrow (BM) with numerous adipocytes-filled trabecular centers (thick arrows) (scale bar=  $100\mu m$ ).

Figure 6. Control group (negative and positive)



(A) Photomicrograph of H&E stained section of rat tibia of ATRA treated group (3 days post-treatment) showing apparently normal regular growth pattern of trabecular bone (T), which is branching and anastomosing with bony matrix, formed from collagen and osteocytes (OSC). Enclosed bone marrow (BM) formed from adipocytes is seen (scale bar= 100µm).



(C) Photomicrograph of H&E stained section of rat tibia of ATRA treated group (7 days post-treatment), showing regular cartilage replacement by normal regular ossification, vessels formation (Bl.vs) with anastomosing branching trabeculae and compact bone with haversian system with surrounding bone marrow (BM), osteocytes (OSC) and dark basophilic epiphyseal line (EP.L) is present. (black arrow) (scale bar= 100µm).



(E) Photomicrograph of H&E stained section of rat tibia of ATRA treated group (10 days post-treatment), showing compact bone (CB) of lateral epiphysis with an increased number of resorption canals (thin arrows) containing blood vessels (Blvs), osteoclasts as multinucleated cells with collagen deposition of mineralized bony matrix including osteocytes (Osc) (thick arrow) (scalebar= 100  $\mu$ m).



(B) Photomicrograph of H&E stained section of rat tibia of ATRA treated group (3 days post-treatment), showing regular growth pattern through endochondral bone formation type from less wider epiphyseal plate and dark basophilic epiphyseal line (EP.L) with trabeculae (T) enclosed bone marrow (BM) formed from adipocytes and with hyperplasia of osteocytes (Osc) in compact bone. Chondrocytes were seen as regularly arranged columnar cells (scale bar= 100  $\mu$ m).



(D) Photomicrograph of H&E stained section of rat tibia of ATRA treated group (7 days post-treatment), showing apparently normal regular growth pattern towards epiphyseal line (EP.L) with branching anastomosing trabeculae (T) enclosed interrupted bone marrow (BM) with adipocytes. (scale bar=  $100 \mu$ m).



(F) Photomicrograph of H&E stained section of rat tibia of ATRA treated group (10 days post-treatment), showing restricted normal pattern of endo cartilage bone ossification with irregularly arranged chondrocytes(Ch.c) (thin arrows) and osteocytes (OSC) without clear demarcation of the proliferative and hypertrophic zones with wide separated spongy bone (SB) has basophilic patches of bony matrix and enclosed bone marrow (BM) with adipocytes (scale bar= 100  $\mu$ m).

Figure 7. The ATRA-treated group

#### **IV. Discussion**

In 1995, the U.S. Food and Drug Administration (FDA) approved the oral administration of pharmacological ATRA for the treatment of acute promyelocytic leukemia (APL). This resulted in a significant improvement in outcomes, with an average overall survival approaching 95% in contemporary practice. Currently, ATRA remains the recommended treatment for APL (Giuli et al., 2020). The present study aimed to evaluate the impact of ATRA on the growth of the rat tibia Our study demonstrated several effects of ATRA on demonstrated several effects of ATRA on bone growth, including enhanced osteoclastogenesis, decreased growth hormone levels. and aberrant chondrogenesis. Growth hormone (GH) is a peptide secreted by the anterior pituitary gland that is crucial for regulating cell division, growth, and metabolism in various targeted organs (Chang et al., 2023).

Compared to the control groups (positive and negative), the present study revealed a significant reduction in serum growth hormone levels in the ATRA-treated groups. This finding aligns with results indicating that rats administered ATRA demonstrated a substantial decrease in serum GH levels.

ATRA enhances the effects of ghrelin and growth hormone-releasing hormone on GH secretion from the anterior pituitary gland, indicating that differences in the age and duration of treatment of the rats used in their studies may account for the discrepancies observed (Maliza et al., 2016). In the present study, the mean weight of group IIIc (ATRA treated for ten days) was significantly lower than that of groups IIIa (ATRA treated for three days) and IIIb (ATRA treated for seven days). These results align with Shen et al. 2022, who observed decreased body weight in rats that received ATRA. According to Guo et al., 2016, ATRA's ability to reduce weight is attributed to its ability to increase thermogenesis and energy expenditure. ATRA increases the expression of lipogenic genes in adipose tissue while also increasing the expression of lipogenic genes in adipose tissue. These findings suggest a feed-forward loop encompassing lipogenesis, lipolysis, and fatty acid oxidation. Nevertheless, Felipe et al., 2004, showed that the oral administration of ATRA results in weight gain in rats by inhibiting the expression of resistin in adipocytes. There are various potential reasons for this discrepancy, including variations in animal models and doses. In the investigation, male rats aged 12 weeks received a single subcutaneous injection of ATRA daily for four days before euthanasia at doses of 10, 50, or 100 mg/kg body weight, aligning with the current study's findings.

According to Cardoso-Demartini et al., 2023, ATRA may induce developmental damage by facilitating an early

fusion of the growth plates, which accounts for the observed reduction in tibial length and nose-tail length. Shen et al., 2022, gave investigation that clarified the reduction in investigated the reduction in tibial length and nose-tail length. High levels of ATRA induce bone marrow hypoxia, potentially exacerbating bone injury and inhibiting the transduction of the insulin growth factor 1 (IGF1) signal, as ATRA effectively induces p53 in various cell types. The IGF signal axis and p53 signaling pathway exhibit a significant relationship. Numerous studies indicate that p53 can inhibit the promoter of IGF1R, thereby suppressing IGF1 signaling and diminishing survival signals. This interaction may enhance osteoclast activity and lead to insulin growth factor-binding protein 3 (IGFBP3) resistance. In this study, three-week-old male rats were utilized and subjected to a 10-day treatment of high-dose ATRA administered via gavage. Subsequently, the tibial and nasal-tail lengths of the experimental rats were reduced compared to the controls.

Lionikaite et al., 2018, found that short-term ATRA supplementation reduced outer cortical bone thickness because it increased the number of osteoclasts. This finding is consistent with their findings. In a similar vein, Lerner et al., 2024 discovered that ATRA promotes bone growth on endocortical surfaces while decreasing bone resorption. The current investigation demonstrated a significant increase in osteoclast numbers and the relative percentage of degraded bone resorption when comparing the ATRA-treated groups to the control groups.

This result was supported by studies conducted by Lionikaite et al., 2018, who found that the number of osteoclasts on the periosteal surface of the long bones in rats was significantly increased by short-term ATRA supplementation.

Rats treated with high-dose ATRA appear to encourage osteoblast development, but low-dose ATRA therapy appears to have the reverse effect. Alloisio et al., 2021 and Yu et al., 2023 reported that low concentrations of ATRA enhanced the expression of osteoblast-related genes, including osteocalcin (OCN), alkaline phosphatase (ALP), and vascular endothelial growth factor (VEGF). Additionally, ATRA significantly inhibited the expression of osteoclast-related genes, reduced their resorption activities, and facilitated the differentiation of osteoblasts. The histological examination of the tibial bone sample confirmed the growth measurements, histomorphometric analysis, and biochemical assessment of blood growth analysis hormone. The indicated that ATRA administration led to increased resorption canals and a higher count of osteoclasts. The findings revealed a restricted normal endochondral ossification pattern characterized by erratically arranged chondrocytes and separated trabecular widely bone. The resting, proliferative, and hypertrophic zones lacked clear definition, while the enclosed bone marrow, containing

adipocytes, was encircled by basophilic patches of bony matrix. Future researches should focus on examining the various effects of ATRA on bone and provide recommendations for its safer application as a chemotherapy agent for pediatric patients.

#### V. Conclusions:

High-dose administration of ATRA led to a decrease in growth measurements, including weight, tibial length, and nose-tail length. Oral administration of ATRA at high doses for ten days results in tibial bone resorption, as evidenced by various histological alterations and histomorphometric changes. Therefore, using ATRA as a chemotherapy must be carefully adapted to the treatment protocol.

#### VI. Recommendations:

In order to assess the toxic effects of ATRA on bone, it is necessary to conduct precise records of the incidence and prevalence of affected cases as a result of ATRA treatment regimens in oncology hospitals and institutes. Additional experimental studies are required to adjust the exact regimen of ATRA for the treatment of acute promyelocytic leukemia and neuroblastoma in children, as well as clinical studies.

#### **Study limitations**

Limited researches have examined the toxic effects of ATRA on different organs

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