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# Effect of Gibberellic Acid on the Levels of Tumor Necrosis Factor-α, Interleukin-4 and Interleukin-10 in Rats

## Sıçanlarda Gibberellik Asitin Tümör Nekrozis Faktör-α, İnterlökin-4 ve İnterlökin-10 Düzeylerine Etkisi

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## Abstract

**Objective:** Gibberellic acid is a plant growth factor. Several studies have demonstrated the effect of gibberellic acid on tumor development. Tumor necrosis factor-α, interleukin-4 and interleukin-10 are important cytokines involved in the regulation or prevention of cancer. The aim of this study was to investigate whether changes in cytokine balance have any role in the tumor-inducing effect of gibberellic acid.

**Material and Methods:** In order to examine the acute and subchronic effect of gibberellic acid, 50 male Wistar rats were divided into five groups. For the subchronic treatments, the first group received 2 mg/kg gibberellic acid, the second group received 20 mg/kg gibberellic acid, and the third group received only the solvent via intraperitoneal injections given three times a week for a 30 days. In the acute treatment group, a single does of either 20 mg/kg gibberellic acid or the solvent alone was administered via intraperitoneal injection, and the tissues were dissected three hours after the injection.

**Results:** During the study period (30 days), both doses of gibberellic acid resulted in a decrease in Tumor necrosis factor-α levels, and a dose-dependent decrease in interleukin-4 levels was observed. However, no change was observed in interleukin-10 levels.

**Conclusion:** We conclude that gibberellic acid influenced the cytokine balance. The resulting changes may be responsible for the development or advancement of inflammatory diseases and malignancies.

**Keywords:** Gibberellic acid; tumor necrosis factor-α; interleukin-4; interleukin-10; rat

## Özet

**Amaç:** Gibberellik asit, bitki büyüme faktörüdür. GA3'ün tümör oluşumuna etkisini gösteren çalışmalar mevcuttur. Tümör nekrozis faktör-α, interlökin-4, interlökin-10 kanser gelişiminde ya da önlenmesinde önemli sitokinlerdir. Bu çalışmanın ana hedefi, Gibberellik asitin tümör oluşturucu etkisinin sitokin dengesindeki değişikliklerin yer alıp almadığının araştırılmasıdır.

**Gereç ve Yöntemler:** Gibberellik asitin hem akut hem de subkronik etkilerini incelemek için 50 erkek Wistar sıçan beş gruba bölünmüştür. Subkronik tedavi deneylerinde ilk gruba 2 mg/kg, ikinci gruba 20 mg/kg dozunda Gibberellik asit ve üçünce gruba çözücü intraperitoneal olarak haftada üç gün 30 gün süresince enjekte edilmiştir. Akut tedavi grubunda sadece yüksek doz Gibberellik asit tedavisi (20 mg/kg) ya da çözücü intraperitoneal olarak bir kere verilmiş ve enjeksiyondan 3 saat sonra dokular çıkartılmıştır. Ayrılan serumlarda tümör nekrozis faktör-α, interlökin-4 ve interlökin-10 düzeylerine serumda bakılmıştır.

**Bulgular:** Bu çalışmada, Gibberellik asite 4 hafta maruz kalmak her iki dozda tümör nekrozis faktör-α düzeyini, doza bağımlı olarak da interlökin-4 düzeyini azaltmıştır. İnterlökin-10 düzeyinde ise değişiklik gözlenmemiştir.

**Sonuç:** Gibberellik asit sitokin dengesini etkilemektedir. Bu değişiklikler inflamatuar hastalıkların ve malignitelerin gelişimine ya da ilerlemesine katkıda bulunabilmektedir.

**Anahtar kelimeler:** Gibberellik asit; tumör nekrozis faktör-α; interlökin-4; interlökin-10; sıçan

## Introduction

Nowadays, the problem of nutrition is becoming increasingly important worldwide. The distribution of plant-based nutrition is not uniform throughout the world as numerous factors limit crop production depending on the geographical areas. The necessity to meet the nutritional require-

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ments of the ever-growing global population and the obligation to make the most effective use of the agricultural fields have boosted research in this direction. Plant growth factors or plant hormones are substances that are naturally synthesized by plants, which regulate their growth, development, and other related physiological activities, and are effective even at low concentrations (1,2). Plant growth regulators are used commonly and rather uncontrollably in Turkey. The farmers think that higher dosage and more frequent applications of plant hormones will increase the yield. As plant growth substances are used in increasing doses, several adverse reactions such as tumor formation (3-5) and changes in concentration of proteins, vitamin, amino acids and glucose, and alterations in the synthesis of DNA, RNA, and proteins have been observed in plants. This has led investigations on the effect of such substances primarily on plants, animals, and more importantly on human beings.

Gibberellins are plant growth factors that have been known since the 1920s, which play essential roles in the regulation of plant growth and development (6). They are used in agriculture to increase the quantity and quality of the crop (7). Gibberellic acid (GA3) is the most commonly used gibberellin in plants (8).

The experiments on animals have shown that GA3 can cause some carcinogenic effects. Some studies have demonstrated that GA3 can cause hepatocellular carcinoma, profound weight gain in mice, sebaceous adenoma in the axillary region, and adenocarcinomas in the breast and lung (9,10).

Some studies suggest that the use of GA3 can lead to tumor formation by augmenting chronic inflammation. In other words, it is hypothesized that GA3 may induce or facilitate tumor development by altering the cytokine balance and thus, the inflammatory response. The present study is aimed at testing this hypothesis.

Specifically, chronic inflammation is known to facilitate carcinogenesis (11) and metastasis development (12). Tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4) and interleukin-10 (IL-10) are important cytokines involved in the regulation or prevention of cancer. The levels of these cytokines are also investigated in this study. An interesting function of IL-4 is to inhibit the release and expression of proinflammatory cytokines (13). The studies on IL-4, in general, indicate that it prevents tumor formation.

We examined the alterations in TNF-α, IL-4, and IL-10 levels as an indication of imbalance between pro-inflammatory and anti-inflammatory mediators.

## Material and Methods

### Laboratory Animals

The experiments were performed in Akdeniz University Medical Faculty Experimental Animal Laboratory, on male Wistar rats (originally obtained from Harlan, Israel and inbred in our institution). All animal experimentation was conducted in accord with accepted standards of humane animal care, as outlined in the Ethical Guidelines. The animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals. The study was funded by Akdeniz University). The study was approved by Experimental Animal Ethics Committee of Akdeniz University (June 4, 2006, decree no: 1).

Six-week-old (subchronic treatment group) and 15-week-old (acute treatment group) male Wistar rats that were produced in the animal experiment laboratory from pure Wistar rats (Harlan, Israel) were housed individually in a temperature-controlled room (22±2°C), with 50-60% humidity and a 12 hours daylight-12 hours dark in day.

### Experimental Design

Gibberellic acid (GA3; catalog number G7645, Sigma, St. Louis, MO, USA) was used as the plant growth regulator. It was dissolved in methanol and diluted with distilled water in different proportions prior to the administration to animals.

The rats were divided into two treatment groups-subchronic treatment group and the acute treatment group

#### *Treatment Groups*

Subchronic treatment group ($n$=33 rats) was further sub-divided into three groups:

*Control group ($n$=11)*: This group received 20% methanol solution prepared with dis-

tilled water at a dose of 0.1 mL/100 g of per rat body weight.

*Low-dose GA3 group (n=11)*: The rats in this group received a low-dose (i.e., 2 mg/kg) of GA3. About 1 g of GA3 was dissolved in 10 mL methanol, and then diluted to 500 mL with distilled water. The resulting 2 mg/mL GA3 solution was administered to the rats at a dose of 0.1 mL/100 g of per rat body weight.

*High-dose GA3 group (n=11)*: The rats in this group received a high-dose (i.e., 20 mg/kg) GA3. About 1 g of GA3 was dissolved in 10 mL methanol, and then diluted to 50 mL with distilled water. The resulting 20 mg/mL GA3 solution was administered to the rats at a dose of 0.1 mL/100 g of per rat body weight.

In the subchronic treatment group, the calculated doses were administered via intraperitoneal injections performed three times per week for 30 days. At the end of the treatment period, the experiment was terminated, and the serum and tissue samples were obtained.

Acute treatment group ($n$=19 rats) was further subdivided into three groups:

*Control group (n=9)*: This group received 20% methanol solution prepared with distilled water, at a dose of 0.1 mL/100 g of per rat body weight.

*High-dose GA3 group (n=10)*: The rats in this group received a high-dose (i.e., 20 mg/kg) of GA3. About 1 g of GA3 powder was dissolved in 10 mL methanol, and then diluted to 50 mL with distilled water. The resulting 2 mg/mL GA3 solution was administered to the rats, at a dose of 0.1 mL/100 g of per rat body weight.

In the acute treatment group, the calculated dosages of solutions were administered intraperitoneally once, and the experiment was terminated 3 h later; serum and tissue samples were obtained.

### Dissection Procedures and Blood Sampling

Under ether anesthesia, the abdomen was incised and the blood samples were obtained from the abdominal aorta. The rat was then sacrificed and the gallbladder was retracted carefully to avoid any leakage. The liver was removed and stored frozen in liquid nitrogen. The blood samples were drawn into tubes containing EDTA and centrifuged at 3000 rpm for 5 min. The plasma portion was separated within 15 min of sampling, and the blood samples were kept in an ice-box until separation. After centrifugation, the plasma portion was separated and frozen in liquid nitrogen and stored at -80°C.

### Weight of Testes

The rats in the subchronic treatment group were observed to have their testes markedly enlarged during the 30-day experimental period. The testes were removed under ether anesthesia and fixated in formalin solution. Then, they were dried slightly and weighed using a precision scale. A similar procedure was performed for the rats in the acute treatment group.

### Determination of Plasma TNF-α, IL-4, and IL-10 Levels

The separated plasma samples were collected in Eppendorf tubes and stored at -80°C until further use. The plasma concentrations of TNF-α, IL-4, and IL-10 were determined by a commercially available enzyme-linked immunosorbent assay (ELISA) kits (Biosource, France).

### Statistical Analysis

Within-group comparisons were made with paired t-test for the normally distributed data, and Wilcoxon's matched pair test was used for the non-normally distributed data. Comparisons between the groups were made with Student's t-test for normally distributed data and Mann-Whitney *U*-test for non-normally distributed data.

## Results

### Effect of Gibberellic Acid on Testicular Weight

At the end of the experiment, we observed a profound enlargement of the testes in rats exposed to GA3 treatment in the subchronic treatment groups. Therefore, testicular weights were measured. The mean testicular weights were found to be 1725±28.6 mg in the control group, 1765±28.7 mg in the low-dose GA3 group, and 1853±35.5 mg in the high-dose GA3. There was a statistically significant difference between the control group and the group treated with low-dose GA3 ($p$<0.05, unpaired Student's

t-test, Figure 1). No significant difference was observed in testicular weights in the group treated with low-dose GA3.

### Effect of Gibberellic Acid on Plasma TNF-α, IL-4, and IL-10 Levels

The mean TNF-α levels in the subchronic treatment groups were 115.5±100.4 pg/mL in the control group, 45.5±16 pg/mL in the GA3 low-dose group, and 48.4±17 pg/mL in the GA3 high-dose group. Both the groups treated with low and high doses of GA3 showed a statistically significant decrease in TNF-α levels when compared with the control group ($p<0.05$, Mann-Whitney *U*-test, Figure 2).

The mean TNF-α levels in the acute treatment groups were 68.9±34.6 pg/mL for the control group and 38.8±9.9 pg/mL for the GA3 high-dose group. A single dose of GA3 did not cause a statistically significant difference in TNF-α levels.

In the subchronic treatment groups, IL-4 levels showed a statistically significant decrease in the group treated with 20 mg/kg GA3 when compared with the control group ($p<0.05$, Mann-Whitney *U*-test, Figure 3). The mean IL-4 levels were 20.4±5.4 pg/mL in the control group, 12.4±3.4 pg/mL in the GA3 low-dose group, and 9.2±3 pg/mL in the GA3 high-dose group.

The mean IL-4 levels in the acute treatment groups were 9.6±3.1 pg/mL in the control group and 12.8±4 pg/mL in the GA3 high-dose group. No statistically significant difference was observed between the groups.

The mean IL-10 levels in the subchronic treatment groups were 74.1±20.8 pg/mL in the control group, 70.5±6.5 pg/mL in the GA3 low-dose group, and 79.7±20.4 pg/mL in the GA3 high-dose group. The comparison by Mann-Whitney *U*-test did not show a statistically significant change in the IL-10 levels when both GA3 treatment groups were compared against the control group (Figure 4).

The mean IL-10 levels in the acute treatment groups were 27±7.8 pg/mL in the control group and 20.4±2.1 pg/mL in the GA3 high-dose group. The comparison of these groups with Mann-Whitney *U*-test did not show a statistically significant difference.

The results of TNF-α, IL-4 and IL-10 levels in the subchronic treatment group are shown in Table 1 and the results in the acute treatment group are shown in Table 2.

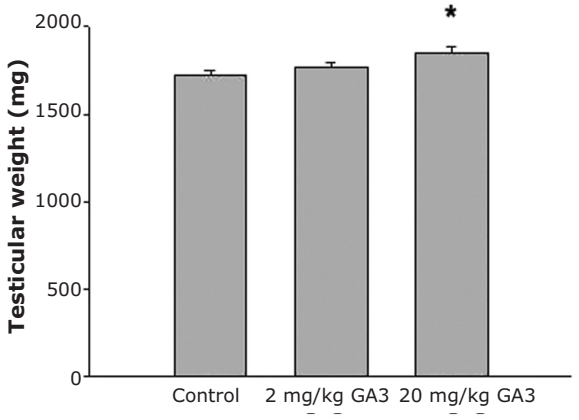


**Figure 1:** The change in testicular weight in the subchronic treatment groups: Control, 2 mg/kg GA3, and 20 mg/kg GA3. Although the subgroups in the subchronic treatment group were similar in terms of the distribution of age and weight, a significant increase was observed in the testicular weights of the rats in treatment groups when compared to the control group. The testis tissues were removed at the end of the experiment and weighed on an analytical scale. The testes of rats treated with 20 mg/kg GA3 showed a significantly higher weight compared to the control group. Although there was some increase in the group treated with 2 mg/kg GA3, the difference was not statistically significant in comparison with the control group *p<0.05 Unpaired Student's t-test.

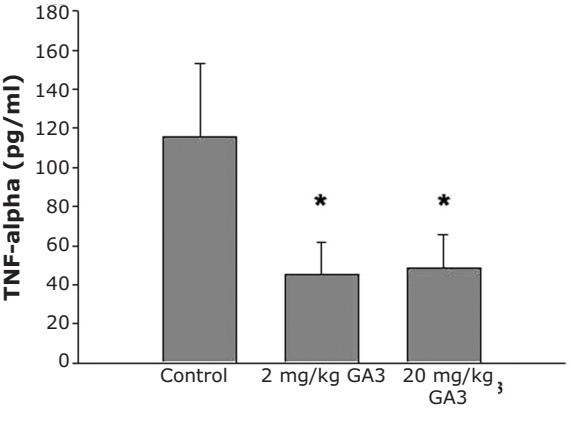


**Figure 2:** The change in TNF-α levels in the subchronic treatment group. After 30 days of GA3 treatment, the TNF-α levels in plasma in the rats that were initially six weeks old were measured with the ELISA method. Statistically significant reduction in TNF-α levels was observed in groups treated with 2 mg/kg GA3 and 20 mg/kg GA3 when compared to the control group. TNF-α (Tumor necrosis factor-α), GA3 (Gibberellic acid), * P<0.05 Mann-Whitney U Test.

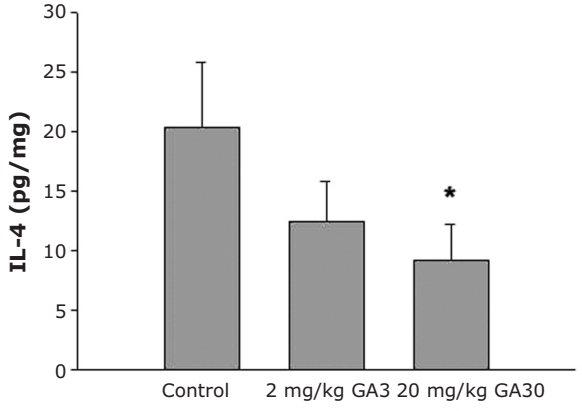


**Figure 3:** The change in IL-4 levels in the subchronic treatment group. After 30 days of GA3 treatment, plasma IL-4 levels of rats that were initially six weeks old were measured with the ELISA method. Statistically significant reduction in the IL-4 levels was observed in the groups treated with 20 mg/kg GA3 when compared to the control group. IL-4 (Interleukin-4), GA3 (Gibberellic Acid), *P<0.05 Mann-Whitney U Test.

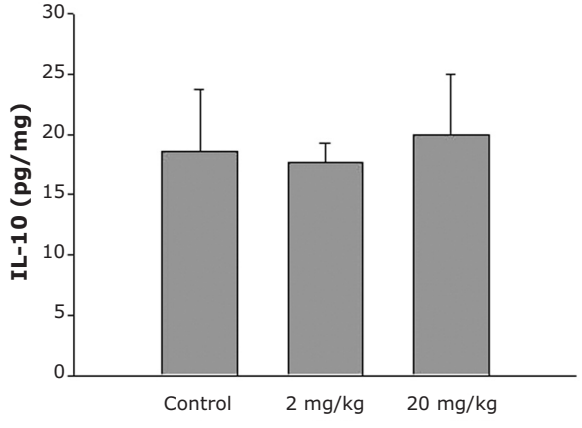


**Figure 4:** The change in IL-10 levels in the subchronic treatment group. After 30 days of GA3 treatment, the plasma IL-10 levels in the rats that were initially six weeks old were measured with the ELISA method. When the Mann-Whitney test was applied to these findings, no statistically significant change in the IL-10 level was found in the two GA3 treated groups was applied compared with the control group.

## Discussion

In this study, the effects of the plant growth factor GA3 on the plasma TNF-α, IL-4, and IL-10 levels were examined. As we observed GA3 treatment caused profound testicular enlargement in rats without any alteration in their feeding patterns, we measured their testicular weights and found that high-dose GA3 treatment resulted in a significant increase in the testicular weight. A study examining the effect of GA3 on weight reported a statistically significant increase in the weights of the liver, heart, adrenal, and thyroid glands of experimental animals that were exposed to GA3 for three weeks (14).

In our study, we observed a statistically significant increase in the testicular weights of rats that received a high dose of GA3 (i.e., 20 mg/kg three times per week for 30 days), which was independent of the age of the animal. Similar findings were reported in a previous study which administered GA3 to rats via intradermal and oral routes during a 45-day study period. The study also demonstrated disruption and reduction in the number of testicular germ cells, a decrease in the size of seminiferous tubules and dystrophy in Leydig cells (15). A long-term high dose administration of GA3 has been shown to cause increased testicular weight and decreased sperm count in male albino rats (16). Another study found markedly reduced aspartate transaminase (AST) and alanine transaminase (ALT) enzyme activities in the testes of rats exposed to GA3, which was explained by the possible toxic effect of GA3 on the testes, resulting in injury to testicular cells (17). A study with neonatal rats

Table 1. The effect of gibberellic acid on plasma TNF-α, IL-4 and IL-10 levels in the subchronic treatment groups.

| | Control | 2 mg/kg GA3 | 20 mg/kg GA3 |
|---|---|---|---|
| TNF-α (pg/mL) | 115.5±100.4 | 45.5±16 | 48.4±17 |
| IL-4 (pg/mL) | 20.4±5.4 | 12.4±3.4 | 9.2±3 |
| IL-10 (pg/mL) | 74.1±20.8 | 70.5±6.5 | 79.7±20.4 |

Tumour necrosis factor-α (TNF-α), interleukin-4 (IL-4) and interleukin-10 (IL-10) GA3: Gibberellic acid.

Table 2. The effect of gibberellic acid on plasma TNF-α, IL-4 and IL-10 levels in the acute treatment groups.

| | Control | 20 mg/kg GA3 |
|---|---|---|
| TNF-α (pg/mL) | 68.9 ±34.6 | 38.8 ±9.9 |
| IL-4 (pg/mL) | 9.6 ±3.1 | 12.8 ±4 |
| IL-10 (pg/mL) | 27 ±7.8 | 20.4 ±2.1 |

Tumour necrosis factor-α (TNF-α), interleukin-4 (IL-4) and interleukin-10 (IL-10) GA3:Gibberellic acid.

found increased weight of the thyroid, ovarian, and adrenal glands but reduced testicular weight in the group exposed to GA3 (18). Despite the contrasting effects of GA3 observed, the common finding of all these studies is that GA3 administration resulted in changes in organs.

A study on Swiss albino mice showed that GA3 accumulated in the hepatic, renal and brain tissues. The most significant damage occurred to the kidneys and GA3 caused lymphocytic interstitial cell infiltration (19). The earlier studies indicate that GA3 affects the vital organs and may lead to cancer and similar pathologies but provide no information on the mechanism responsible. Several mechanisms might play a role in tumor development due to GA3. The association between chronic inflammation and cancer has recently been identified. The main goal of the present study was to investigate whether the alteration in cytokine balance has any role in the tumor-inducing effect of GA3. We found that four weeks of exposure to GA3 caused reductions in both TNF-α and IL-4 levels. GA3 is thought to cause increased cellular infiltration and chronic inflammation. Considering the association between inflammation and cancer, we examined the effects of GA3 on cytokines. The reduction in TNF-α levels might be significant in terms of tumor development. Although many studies suggest that increased TNF-α levels play an important role in tumor advancement, it has been shown that TNF-α can reduce tumor development by preventing angiogenesis for certain tumors (20,21). Recent evidence suggests that the administration of anti-TNF antibodies in Crohn's disease resulted in an increased incidence of multiple myeloma (22). GA3-dependent reduction in the TNF-α might induce tumor development as well. TNF-α is thought to have a dual function. Majority of the related studies have shown that TNF-α causes DNA damage and facilitates or induces cancer development by causing gene mutations, gene amplification, and chromosomal instability (23). In addition to its mutagenic effect, TNF-α has also been shown to have a mitogenic effect and stimulating the development of ovarian epithelial cells (24). On the other hand, TNF-α might as well have an anti-cancer effect. For example, TNF-α infusion treatment to the isolated extremity for soft tissue sarcomas or irresectable melanoma was shown to reduce the tumoral mass (25). Similarly, studies on rats have shown that administration of TNF-α as an adjunct to chemotherapeutic drugs resulted in a synergistic anti-tumor action in large solid organ tumors (20,21).

We observed reduced IL-4 levels with exposure to GA3 in the present study. Several studies have demonstrated the association between reduced IL-4 levels and increased tumor development. IL-4 was shown to inhibit colon and breast cancer cells in a culture medium (26). Recombinant human IL-4 treatment has been shown to result in dose-dependent favorable outcomes in stage IV non-small cell lung cancer (27). Serious effects of IL-4 have been observed, including stabilization of the disease and tumoral cell death. These data suggest that IL-4 can be used as an adjuvant treatment. Therefore, IL-4-reducing effect of GA3 may lead to accelerated cancer progression.

The last cytokine examined in the study was interleukin-10 (IL-10), which is among the anti-inflammatory cytokines similar to IL-4 (28). IL-10 inhibits the proinflammatory cytokines produced by monocytes/macrophages, including TNF-α, IL-1 (interleukin-1), IL-6 (interleukin-6), IL-8 (interleukin-8), IL-12 (interleukin-12) and granulocyte colony-stimulating factor (29). IL-10 deficiency has been found to increase colorectal cancer development (41). Similarly, the overexpression of IL-10 has been shown to be protective against cutaneous malignant melanoma, breast cancer, and ovarian cancer, and prevent tumor development (30-33).

In our previous study, analyzing the association between cancer and inflammation, we investigated the possible effects of GA3 on mast cell degranulation that plays important role in inflammation, and on substance-P levels, which is present in sensory nerve endings (34). Both acute and subchronic effects of GA3 were examined. The changes in substance P levels and mast cell counts were examined in the skin and bladder tissues, which are rich in mast cells and sensory nerve endings. Subchronic treatment resulted in increased mast cell degranulation

in the skin and bladder. While the acute treatment did not cause a change in the bladder mast cell count and degranulation, it resulted in an increase in both degranulated and total mast cell count in the skin. Alterations in substance P levels were found to be similar in both tissues. Unlike subchronic effect, the acute effect of GA3 at 20 mg/kg dose caused increased substance P levels in the skin but did not cause changes in the bladder. The increase in the mast cell degranulation particularly indicates that subchronic exposure to GA3 can trigger inflammatory diseases. Although substance P is regarded as an inflammatory mediator, recent studies have shown that the reduced substance P levels can lead to increased inflammatory damage. Therefore, GA3 exposure in rats can trigger or augment inflammatory events by disturbing the cytokine balance and increasing mast cell degranulation.

Regarding the testicular weights of rats, we found that exposure to 20 mg/kg GA3 for three times a week for a 30-day period resulted in a significant increase in the testicular weight that was independent of the age. Four weeks of GA3 exposure caused a reduction in both TNF-α and IL-4 levels but did not alter IL-10 levels. To sum up, GA3 influences the cytokine balance and causes metabolic changes as well. These changes might contribute to the development or progression of inflammatory or malignant diseases.

In conclusion, the reason behind the tumor-inducing effect of GA3 might be the disturbance of cytokine balance. These cytokines were examined in the present study. The study hypothesis was that GA3 causes increased mutation or prevents the elimination of mutant cells, and accelerates tumor formation by increasing inflammation.

All the evidence in hand, both from the present and previous studies, indicate that subchronic exposure to GA triggers inflammation, and can lead to numerous diseases that are associated with chronic inflammation.

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## Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

## Authorship Contributions

Idea/Concept: Hacer Berna Afacan Öztürk, Mustafa Kemal Balcı; Design: Hacer Berna Afacan Öztürk, Mustafa Kemal Balcı; Control/Supervision: Mustafa Kemal Balcı, Nuray Erin; Data Collection and/or Processing: Hacer Berna Afacan Öztürk, Nuray Erin; Analysis and/or Interpretation: Hacer Berna Afacan Öztürk, Nuray Erin; Literature Review: Hacer Berna Afacan Öztürk, Nuray Erin; Writing the Article: Hacer Berna Afacan Öztürk, Nuray Erin; Critical Review: Hacer Berna Afacan Öztürk, Mustafa Kemal Balcı; References and Fundings: Hacer Berna Afacan Öztürk, Mustafa Kemal Balcı; Materials: Hacer Berna Afacan Öztürk, Mustafa Kemal Balcı.

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