Research Article / Araştırma Makalesi

The Effects of Subteratogenic Vitamin A Doses on the Fetal Rat Kidney: A Stereological Study

A Vitamininin Subteratojenik Dozlarının Sıçan Fetüs Böbreği Üzerine Etkileri: Stereolojik Bir Çalışma

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Abstract

Background: Vitamin A (retinol) and its derivatives are essential for maintaining cell differentiation in adult organisms as well as for normal embryonic development in fetuses. On the other hand, high amounts of vitamin A are known to be teratogenic. The formation of urogenital structures depends heavily on retinoic acid receptors. The effects of subteratogenic dosages of retinol on the urinary system have not been adequately studied. The aim of the current study was to investigate the effects of moderate and low doses of vitamin A on the fetal kidney.
Materials and Methods: Pregnant rats were divided into 6 groups. On day 10 to 12 of pregnancy (P10-P12) the first group was administered 10000 IU/kg, the second group 20000 IU/kg, the third group 30000 IU/kg, the fourth group 40000 IU/kg and the fifth group 50000 IU/kg oral vitamin A. The control group only received 1 ml of corn oil on the same days. The fetuses were delivered via cesarean section at P19. The kidneys of the fetuses were removed after cardiac perfusion was used to fixate them. After histological preparation of the kidneys, the slides were stained with hematoxylin and eosin. By using stereological methods, the kidneys' volume (V), glomeruli per unit area (N_Ag), and mean glomeruli diameter (D) were all estimated. The results were statistically analyzed.
Results: The renal volumes of the 20000, 30000 and 40000 IU/kg groups were higher than those of the other groups. It was also found that the N_Ag levels of the group receiving 50000 IU/kg Vitamin A were lower than those of all other groups. Moreover, the N_Ag levels of the groups receiving 20000, 30000 and 40000 IU/kg vitamin A were higher than those of the control group and the group receiving 10000 IU/kg. While the glomeruli diameters of the experimental groups were not different from those of the control group, the glomeruli diameters of the group receiving 20000 and 50000 IU/kg retinol were larger than those of the groups receiving 10000 and 40000 IU/kg vitamin A.
Conclusions: Given the estimated higher V, Na, and D values of the group receiving 20000 IU/kg vitamin A, we can assume that this particular dose has a significant effect on renal morphology and development.

Key Words: Rats, Fetus, Kidney, Vitamin A, Teratogens

Öz

Amaç: A vitamini (retinol) ve türevleri, fetüste normal embriyonik gelişim ve yetişkin organizmada hücre farklılaşmasının sürdürülmesi için gereklidir. Öte yandan aşırı dozda A vitamini alımının teratojenik etkilerinin olduğu bilinmektedir. Retinoik asit reseptörleri, ürogenital yapıların gelişiminde çok önemli bir rol oynar. Subteratojenik doz retinolün üriner sistem üzerindeki etkilerine ilişkin yeterli çalışma yoktur. Çalışmamızın amacı subteratojenik ve düşük doz A vitamininin fetal böbrek üzerine etkilerini araştırmaktır.
Materyal ve Metod: Gebe sıçanlar 6 gruba ayrıldı. Gebeliğin 10 ila 12. gününde (P10-P12). Birinci gruba 10.000 IU/kg, ikinci gruba 20.000 IU/kg, üçüncü gruba 30.000 IU/kg, dördüncü gruba 40.000 IU/kg ve beşinci gruba 50.000 IU/kg oral A vitamini verildi. Kontrol grubuna sadece 1 ml mısır yağı verildi. P19'da fetüsler sezaryen ile çıkarıldı. Fetüsler kalp perfüzyonu ile tespit edildi ve böbrekleri alındı. Histolojik ön aşamalardan sonra, kesitler hematoksilen ve eozin ile boyandı. Böbrek hacmi (V), birim alandaki glomerül sayısı (N_Ag) ve ortalama glomerül çapı (D) stereolojik yöntemlerle hesaplandı. Sonuçlar istatistiksel olarak analiz edildi.
Bulgular: 20.000, 30.000 ve 40.000 IU/kg gruplarının böbrek hacimleri diğer gruplara göre daha yüksekti. Ayrıca 50.000 IU/kg alan grubun N_Ag düzeylerinin diğer tüm gruplara göre daha düşük olduğu saptandı. Ayrıca 20.000, 30.000 ve 40.000 IU/kg A vitamini alan grupların N_Ag düzeyleri kontrol grubu ve 10.000 IU/kg alan gruba göre daha yüksekti. Deney gruplarının glomerül çapları kontrol grubundan farklı değilken, 20.000 ve 50.000 IU/kg retinol alan grubun glomerül çapları 10.000 ve 40.000 IU/kg A vitamini alan gruplardan daha büyüktü.
Sonuç: 20.000 IU/kg A vitamini alan grubun daha yüksek V, N_Ag ve D değerleri göz önüne alındığında, bu dozun böbrek morfolojisi ve gelişimi üzerinde önemli bir etkisi olduğunu düşündürmektedir.

Anahtar Kelimeler: Sıçan, Fetüs, Böbrek, A Vitamini, Teratojenler

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Introduction

Vitamin A (Vit A) is a fat-soluble vitamin that can be obtained from meat and vegetables. It has effects on many systems and organs such as vision, growth and development, and the immune system. Previous studies have shown that vit A deficiency is a serious public problem, especially in developing countries (1-3). Regardless of the cause of vit A deficiency, women of childbearing age are particularly at risk because they have additional needs during pregnancy and lactation (4, 5). Vit A is very important for the fetus, as the vitamin and its metabolites play an important role during prenatal development (6-9). World Health Organization/ Food and Agriculture Organization (2004) stated that a newborn requires about 100 µg retinol/day to meet normal growth requirements and assumed that the fetus has similar requirements in the third trimester of pregnancy. Considering that most pregnant women in the world live in poor conditions, an increase of 200µg retinol activity equivalents/day (RE/day) has been suggested for the entire pregnancy. The reason for this increase is to replenish the mother's stores in the first stage of pregnancy and to meet the needs of the rapidly growing fetus in the last stage of pregnancy (10).

There are many ongoing studies to determine the protective and therapeutic effects of vit A. In addition, many studies focus on the use of vit A in pregnant women and attempt to detect teratogenic and the beneficial dose intervals of vit A. All-trans retinoic acid (RA) is a bioactive vit A metabolite. This metabolite is a crucial signaling molecule for the formation of many organs, including the eyes, heart and kidneys. (11). Insufficient or excessive intake of vit A or RA may cause some malformations in embryos. In fetuses whose mothers do not receive enough vit A, a syndrome with a series of abnormalities called fetal vit A deficiency syndrome (VAD) occurs. The syndrome includes defects of the hindbrain, eyes, ears, heart, lungs, diaphragm, kidneys, testes, limbs and skeleton (12). On the other hand, exposure to high doses of vit A or RA in animal models and humans also results in malformations resembling fetal VAD syndrome (13, 14). Also, subteratogenic doses, doses that do not cause macroscopic malformations, also lead to functional and microscopic abnormalities, particularly in the central nervous system (15). One of the organs most affected by this syndrome is the kidney. Numerous studies on this topic can be found in the literature. In one of these studies, administration of RA to mice in E9 resulted in fulminant apoptosis of the developing metanephroi in E11. As a result, bilateral renal agenesis developed (16). Despite the numerous studies on the effects of vit A and its metabolites on the urogenital system, there are few morphological, morphometric, and stereological studies. In this study, we investigated the effects of various subteratogenic doses of vit A on the fetal kidney using stereological methods.

Materials and Methods

In the current study, 30 female Wistar rats, weighing 250-300 g, were used. Rats were kept in the animal room for 12 h in the dark and 12 h in the light loop. The room temperature was kept constant (21° ± 3° C). Water and food were provided *ad libitum* in cages with a capacity of 5-6 rats. All rats were obtained from Eskişehir Osmangazi University Medical and Surgical Experimental Animal Research Center. Menstrual phases of rats were determined by vaginal swabs. Female rats in estrus were housed in the same cages as males. In vaginal smear examination, sperm-positive females were considered pregnant and on the first day of gestation (P0). The pregnant rats were divided into 6 groups. On the 10th to 12th day of gestation (P10-P12). The first group received 10000 IU/kg, the second group received 20000 IU/kg, the third group received 30000 IU/kg, the fourth group received 40000 IU/kg, and the fifth group received 50000 IU/kg oral vit A by oral gavage. The control group received only 1 ml of corn oil on the same days. At P19, the rats were anesthetized with halothane and the fetuses were removed by cesarean section. Fetuses were removed from the dissected uterus; mothers were sacrificed by exsanguination. After macroscopic examination, fetuses were fixed with cardiac perfusion. The kidneys were removed and postfixed in tissue vessels for histological preparation. After histological preparation, serial sections (5µ) were transferred to slides and stained with hematoxylin and eosin. Kidney volume, number of glomeruli per unit area, and diameter of glomeruli were calculated using stereological methods. The results were statistically analyzed using Jamovi 2.3.

The Cavalieri estimator probe of the Stereo Investigator® (MBF Bioscience) coupled to a Leica DM3000 microscope was used to determine the total volume of the kidneys. The grid of points was randomly placed on a series of sections 50µ slice interval. The points that fell on the related area were counted. The volume (V) was calculated by multiplying the section thickness (T) by the area represented by each point (a/p) and the total number of points counted (∑pi) ($V=T \cdot a/p \cdot \sum pi$). (Fig. 1)

The N_Ag was calculated from 10 photographs selected by systematic random sampling of 10 kidneys from each group. These photographs were taken at 20x magnification, and the glomeruli in the photograph were counted ($\sum pi$). After calculating the area shown in the photographs (A), the number of glomeruli per unit area was calculated using the formula $N_Ag = \sum pi$ /A.

Another measure was the mean diameter (D) of the glomeruli. Glomeruli diameters were calculated by the measuring of length of the line passing through the longest distance between the edges of a glomerulus (a) and a second line crossing the first line at right angles from the center (b). The square roots of these values were taken and multiplied to obtain the mean diameter ($D = \sqrt{a} \cdot \sqrt{b}$). (Fig. 2)

Results

Morphological examinations of the groups showed no difference, there were no major macroscopic or histological differences between groups.

Stereological analyses yielded different results. The V of the 20000 IU/kg group was higher than that of all other

groups (p<0.001). The V of the 30000 IU/kg group was also statistically greater than that of the other groups (p<0,01 compared to 40000 IU/kg, and p<0.001 for the other groups). The V of the 40000 IU/kg group was larger than that of the remaining groups (p<0.001). The mean V of the 50000 IU/kg group was smaller than all other groups (Fig. 3).

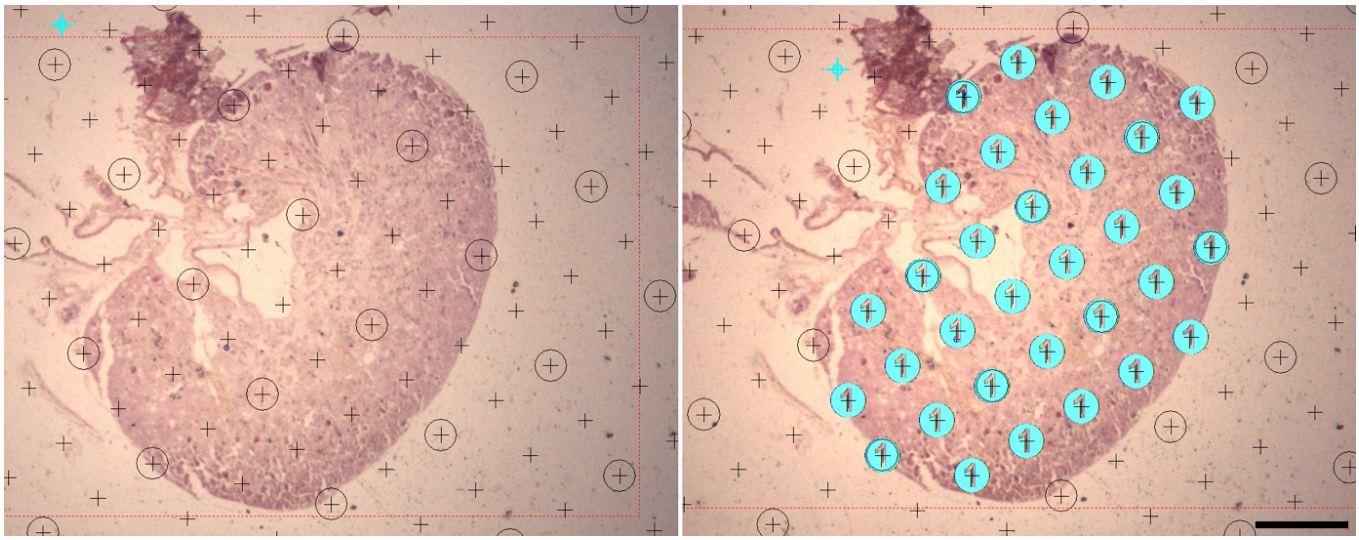


Figure 1. H&E stainings of fetal kidneys. Representative photographs of the Cavalieri method. Point grid and selected dots. Scale 500 µm.

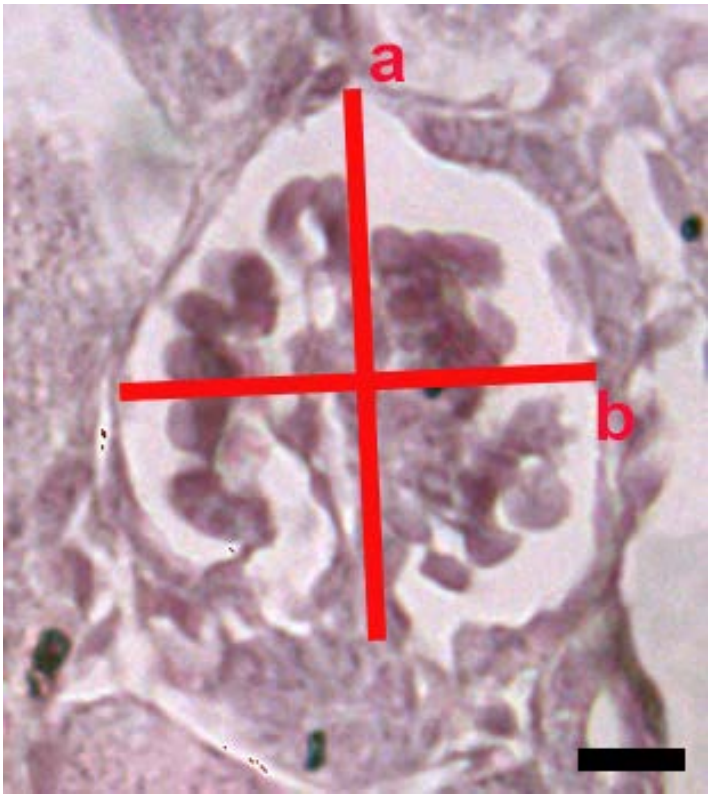
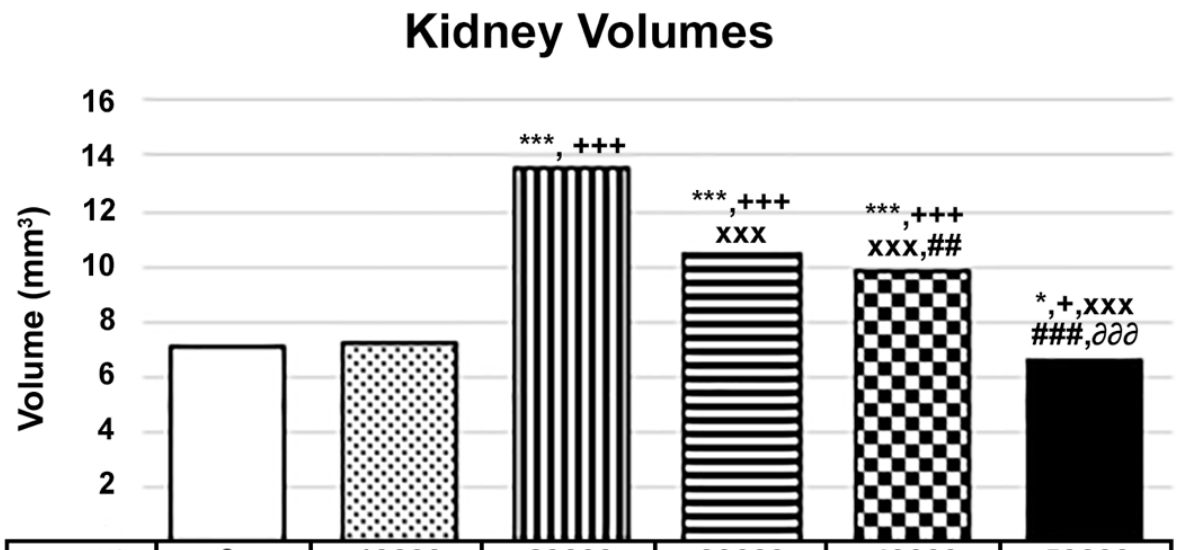


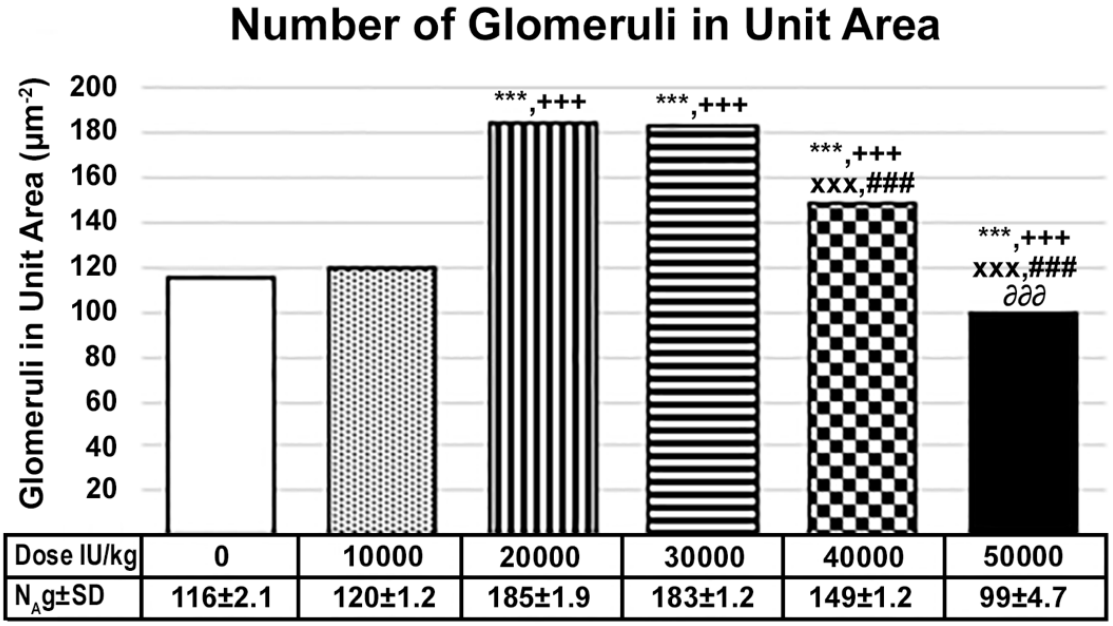
Figure 2. H&E stainings of fetal kidneys. Representative photograph of the measurement of the mean diameter of the glomeruli (D). Glomeruli diameters were calculated by the measuring of length of the line passing through the longest distance between the edges of a glomerulus (a) and a second line crossing the first line at right angles from the center (b). Scale 10 µm.



Dose IU/kg	0	10000	20000	30000	40000	50000
V ± SD	7.1±0.1	7.2±0.2	13.5±0.6	10.5±0.4	9.8±0.5	6.6±0.3

Figure 3. Graph and table representing kidney volumes (V) and statistical significances between groups. Comparison of control group with other groups, *****; comparison of 10000 IU/kg group with higher doses, **+**; comparison of 20000 IU/kg group with higher doses, **x**; comparison of 30000 IU/kg group with higher doses, **#**; comparison of 40000 IU/kg group with higher doses, **ð**. One symbol means p<0.05; two means p<0.01; three means p<0.001.

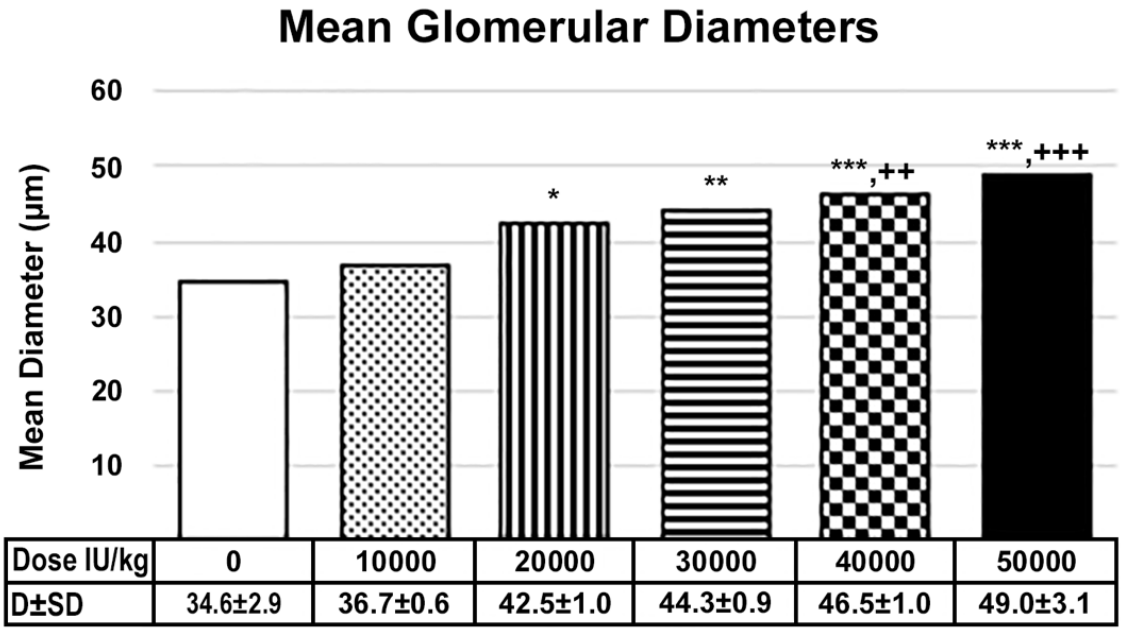
Comparison of the calculated N_Ag area showed that the N_Ag was lower in the group receiving 50000 IU/kg retinol than in all other groups (p<0.001). The highest N_Ag was observed in the groups receiving 20000, and 30000 IU/kg. Although there was no difference between these two groups, the calculated N_Ag values were higher than in the other groups (p<0.001). The second highest N_Ag value was calculated in the group receiving 40000 IU/kg retinol. The statistical difference of this group compared to the other groups was p<0.001. There was no significant difference between the values of the control group and the group receiving 10000 IU/kg retinol (Fig. 4).



Dose IU/kg	0	10000	20000	30000	40000	50000
N_Ag±SD	116±2.1	120±1.2	185±1.9	183±1.2	149±1.2	99±4.7

Figure 4. Graph and table representing number of glomeruli in unit area (N_Ag) and statistical significances between groups. Comparison of control group with other groups, *****; comparison of 10000 IU/kg group with higher doses, **+**; comparison of 20000 IU/kg group with higher doses, **x**; comparison of 30000 IU/kg group with higher doses, **#**; comparison of 40000 IU/kg group with higher doses, **ð**. One symbol means p<0.05; two means p<0.01; three means p<0.001.

When it comes to the D, the values increase with dose. While there was no difference between the values of the control group and the 10000 IU/kg group, the D values of the control group were smaller than those of the other experimental groups (p<0.05 vs 20000 IU/kg, p<0.01 vs 30000IU/kg, p<0.001 vs 40000 and 50000 IU/kg). The D values of the 10000 IU/kg group were also not different from the 20000 IU/kg group, but their measured diameters were smaller than those of the other groups (p<0.01 vs 40000 IU/kg and p<0.001 vs 50000 IU/kg). No difference was observed between the D values of the groups between 20000 IU/kg and 50000 IU/kg (Fig. 5).



Dose IU/kg	0	10000	20000	30000	40000	50000
D±SD	34.6±2.9	36.7±0.6	42.5±1.0	44.3±0.9	46.5±1.0	49.0±3.1

Figure 5. Graph and table representing mean glomerular diameter (D) and statistical significances between groups. Comparison of control group with other groups, *****; comparison of 10000 IU/kg group with higher doses, **+**. One symbol means p<0.05; two means p<0.01; three means p<0.001.

Discussion

We observed that oral retinyl palmitate had a dose-dependent effect on renal V in fetal rats. Retinoids play an important role in the morphogenesis of various organs in mammals. In particular, maternal retinoids can cause syndromic malformations of the urogenital tract like those seen in humans (17). They also affect mesenchymal/epithelial interactions in the developing kidneys, and other organs (18). Nasser and Tahir (2012) found that the offspring of rats administered 60 mg/kg RA between gestational day 7 and 9 exhibited renal hyperplasia, and this growth was particularly due to hyperplasia of the medulla. In addition to these findings, they observed increased endothelial proliferation, and an increase in the number of necrotic cells (19). However, in the present study, we found that the renal volumes of the group receiving 10000 and 50000 IU/kg vit A were not statistically different from the renal volumes of the control group. These results can be clarified when evaluated together with our other results.

Our results showed a gradual increase in renal V with increasing retinol dose, whereas there was a sudden decrease in the 50000 IU/kg group. Lee et al. (2012) reported that RA, when administered at a teratogenic dose, decreased the transcripts of retinal dehydrogenase, which encodes enzymes for the synthesis of RA, and increased the levels of Cyp26a1 and Cyp26b1 mRNAs, which encode enzymes for the degradation of RA. As a result, they observed a significant decrease in retinoic acid levels in whole embryos and kidney rudiments. From these observations, they concluded that an excess of RA would have a similar effect on the developing organism as a deficiency of RA (20).

Our study showed that the N_Ag was higher in the groups with 30000 IU/kg to 40000 IU/kg than in the other groups. The study by Lelié-Pégorier et al (1998) on the effects of mild vit A deficiency on renal and nephron development in newborn rats could support our results, in which they claimed that the number of fetal nephrons was directly related to circulating vit A levels. This study showed that the number of nephrons increased by 21% in the offspring of rats in the control group that received 20 mg/kg RA on day 11 of pregnancy (8). However, as shown by our data, the number of glomeruli decreased with increasing dose. The number of nephrons in the 50000 IU/kg group was computationally lower than in all other groups. The information in the review by Chen et al (2021) may explain this observation. In this review, they found that although RA has a protective effect against renal podocyte injury and glomerular disease at low doses, it is toxic at high doses because it promotes podocyte apoptosis. The authors concluded that the increase in mRNA expression

encoding RARRES1 (Retinoic Acid Receptor Responder Protein 1) was directly related to this effect of RA (21). No difference was observed between the experimental group and the control groups in the measurements we made regarding the D. It is known that glomerular hypertrophy is caused by glomerular hypertension, and hyperfiltration (22, 23). Considering the N_Ag, the increase in glomerular diameters of the 50000 IU/kg group indicates that this result may related to the decreased number of glomeruli.

Angiotensin receptors are thought to increase the expression of transforming growth factor ß1 (TGFß1) and that this causes glomerular and tubular cell hypertrophy. It has been also concluded that these malformations may lead to health problems such as hypertension later in life (24). Although there is conflicting evidence on this, retinoids are thought to increase TGFß expression (25). This information supports our findings. In our study, a dose-dependent increase in glomerular diameter was observed at doses greater than 10000 IU/kg. This increase could also be a compensatory mechanism to protect renal functions due to the increased apoptosis caused by the redundancy of retinoids. Indeed, our previous studies have shown that retinyl palmitate at a dose higher than 10000 IU/kg increases apoptosis in the fetal brain and liver (15, 26).

The presented study shows that oral intake of vit A at doses greater than 10000 IU/kg during pregnancy impairs fetal kidney development and leads to kidney related disorders. We suggest that these changes in the kidneys may be related to the effect of RA on angiotensin receptors and TGFß.

Ethical Approval: Ethical concerns related to the study were reviewed by the Eskişehir Osmangazi University Animal Experimentation Ethics Committee and the study was ethically approved (date:2022, Decree no. 910).

Author Contributions:
Concept: H.A.
Literature Review: H.A., A.O., D.A.
Design : H.A., D.A.
Data acquisition: A.O., D.A.
Analysis and interpretation: H.A., A.O.
Writing manuscript: H.A., A.O.
Critical revision of manuscript: D.A.
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