



Administration of retinoic acid to pregnant mice increases the number of fetal mouse glomeruli

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ABSTRACT

The prevalence of chronic kidney disease (CKD) is increasing worldwide, and CKD is a serious global health problem. Low glomerular number is one of the risk factors for CKD; therefore, the glomerular number is associated with the risk of CKD. Increasing the glomerular number above normal levels may reduce the risk of CKD. It has been reported that, *in vitro*, the addition of retinoic acid (RA) to the culture medium increases the glomerular number. However, there is no report of an increase in glomerular number above normal levels with the addition of RA *in vivo*. In this study, RA (20 mg/kg) was administered intraperitoneally to pregnant mice once at embryonic day (E) 10.5, E12.5, E14.5, or E16.5. The fetuses were harvested at E18.5 and fetal mouse kidneys were evaluated. Fetal kidney volume and weight were significantly increased in the E16.5 group compared to the control group. The total glomerular number in the E16.5 group was also approximately 1.46 times higher than that in the control group. In summary, we established a method to increase the glomerular number in the fetal kidney by administration of RA to pregnant mice at E16.5. These results will facilitate the investigation of whether CKD risk is reduced when the glomerular number increases above normal.

1. Introduction

The prevalence of chronic kidney disease (CKD) is increasing worldwide. In 2017, the estimated prevalence was 9.1%, an increase of 29.3% from the prevalence in 1990 [1]. In addition, 2.6 million patients were receiving renal replacement therapy in 2010, and 5.4 million people are expected to receive renal replacement therapy by 2030 [2]. To prevent this, it is necessary to reduce the number of patients with CKD and to control the progression of CKD. However, there are currently no treatments that can improve kidney function, and conservative treatment is the only treatment option. Therefore, it is important to prevent CKD onset.

Infants with low birth weight have a low glomerular number [3,4] and are at a higher risk of CKD [5,6]. Therefore, it is highly likely that the glomerular number and CKD risk are closely related, and higher glomerular numbers at birth are expected to reduce the risk of CKD. In *in vitro* studies, the addition of retinoic acid (RA) to the culture medium increases the glomerular number [7,8]. However, no study has

examined the effect of RA on the glomerular number *in vivo*. Here, we sought to establish a method to increase the glomerular number in the kidney of offspring by intraperitoneal administration of RA to pregnant mice.

2. Materials and methods

2.1. Mouse maintenance and experiments

Mouse experiments were performed according to the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan (2006) and approved by the Institutional Animal Care and Use Committee of the Shimane University School of Medicine (Protocol number IZ3-104). All efforts were made to minimize animal suffering.

Jcl:ICR mice were purchased from CLEA Japan (Tokyo, Japan). Each female mouse (8–20 weeks old) was kept together with a healthy male mouse overnight. If a vaginal plug was observed the next morning, noon of that day was designated embryonic day (E) 0.5.

Abbreviations: CKD, chronic kidney disease; E, embryonic day; GA, glomerular area; GV, glomerular volume; RA, retinoic acid.

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2.2. Reagents

All-trans-RA (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was stored in the dark at -20°C until use. Freshly thawed RA was dissolved in corn oil (2 mg/mL) for administration to pregnant mice.

2.3. All-trans-RA injection

Each pregnant female mouse was injected intraperitoneally with vehicle or 20 mg/kg body weight RA once at E10.5, E12.5, E14.5, or E16.5. The embryos were allowed to develop in situ until E18.5, at which point their tissues were harvested.

2.4. Kidney volume

To determine the kidney volume, the long axis (L) and width (W) of the kidney were recorded using a stereomicroscope. Kidneys were approximated as spheroids, and the kidney volume was calculated using the spheroid calculation method: kidney volume (mL) = $\pi/6 \times L \times W^2$. Changes in kidney volume between the groups were compared using the renal volume divided by the body weight.

2.5. Histology

For histological analysis, fetal kidneys were fixed in 10% neutral-buffered formalin, washed in physiological saline, dehydrated using a graded series of ethanol, and embedded in paraffin. Then, kidney tissue in paraffin blocks was sectioned at a thickness of 5 μm and stained with hematoxylin-eosin.

2.6. Glomerular number per unit area

Glomeruli were counted in the largest cross-sectional slice of hematoxylin-eosin-stained E18.5 fetal mouse kidneys, and the area of the cross-sectioned kidney was determined. Next, the glomerular number was divided by the area of the cross-sectioned kidney to obtain the glomerular number per unit area (pieces/ mm^2). The glomeruli were counted by two different researchers.

2.7. Glomerular number per kidney (Supplemental Fig.)

Glomerular number per kidney was estimated using the stereological estimate. This method provides quantitative information from three-dimensional material based on measurements made on a two-dimensional planar section of the specimens. This well accepted technique is applied for the evaluation of human and animal model kidneys, as described in detail elsewhere [9–11]. Glomeruli were counted in the hematoxylin-eosin-stained E18.5 fetal mouse kidneys. Firstly, we removed a fetal mouse kidney, then it was cut into serial sections (5 μm in thickness). The first and fifth serial sections were used for analysis. Therefore, the distance between the paired section was 20 μm , and the distance between one section pair and the next was 100 μm . Glomeruli were counted under a grid system, and every second square was counted. A glomerulus was counted when present in one section, but not in its paired section, without overlapping the top and right grid line. The number of glomeruli was calculated as $N_{\text{glom}} = 100/20 \times 2/1 \times 1/2 \times Q^-$, where Q^- is the actual number of glomeruli counted per kidney. Fetuses close to the mean weight of the littermate were used in this analysis; that is, three fetuses were examined in each group. The number of glomeruli was counted by two different researchers.

2.8. Glomerular volume

Glomeruli were counted in the hematoxylin-eosin-stained E18.5 fetal mouse kidneys. Glomerular volume (GV) was estimated using the Weibel–Gomez method [12]. The glomerular area (GA) closest to the

maximum diameter was selected and measured as the area described by the outer capillary loop of tuft using Image J (<https://imagej.nih.gov/ij/>) (Bethesda, Maryland, USA). The GV was calculated from the measured GA as follows: $\text{GV} = (\beta/d) (\text{GA})^{3/2}$, where β is a dimensionless shape coefficient ($\beta = 1.38$ for spheres) and d is the size distribution coefficient used to adjust for variations in glomerular size. We used a d of 1.01, as in previous studies [13,14]. Fetuses close to the mean weight of the littermate were used in this analysis. GV was measured in five glomeruli per fetus.

2.9. Statistics

Statistical analyses were performed using GraphPad Prism (version 7.0, GraphPad Software, La Jolla, CA, USA). P-values were determined using unpaired two-tailed Student's *t*-tests. Differences were considered statistically significant at $p < 0.05$.

3. Results

RA (20 mg/kg, 10 mL/kg) was administered intraperitoneally only once at E10.5, E12.5, E14.5, or E16.5. Three pregnant mice were included in each group. The control group received 10 mL/kg of corn oil intraperitoneally at E10.5 ($n = 3$). Fetal weight, kidney volume, and kidney weight were measured in all fetuses. Fetal kidney volumes and weights were divided by the fetal body weight to exclude the effect of fetal weight. Fetal weight was not significantly different between any of the RA groups and the control group (Fig. 1A, Supplemental Table 1). Kidney volume per body weight was increased in the E16.5 group compared to the control group (6.82 ± 0.19 mL/g vs. 8.31 ± 0.18 mL/g, $p < 0.0001$) (Fig. 1B, Supplemental Table 1). Kidney volume was also macroscopically greater in the E16.5 group (Fig. 2). Kidney weight was significantly increased in the E14.5 (8.45 ± 0.12 mg/g vs. 8.91 ± 0.16 mg/g, $p = 0.0364$) and E16.5 (8.45 ± 0.12 mg/g vs. 9.895 ± 0.16 mg/g, $p < 0.0001$) groups compared to the control group (Fig. 1C, Supplemental Table 1).

One fetus close to the average body weight was selected from each littermate, and the number of glomeruli per unit area, GV, and total number of glomeruli per kidney were measured in three fetuses (one from each of the three dams in each group). The number of glomeruli per unit area, GV, and total number of glomeruli per kidney were compared between the control group and the E16.5 group, because both kidney volume and kidney weight increased significantly in the E16.5 group. Five glomeruli were measured for each fetal kidney to determine the GV. The area of the maximum section was larger in the E16.5 group kidneys than in the control group kidneys (Fig. 3A and B). There was no significant difference in the number of glomeruli per unit area ($n = 3$ kidneys in the control group and 3 kidneys in the E16.5 group, 25.98 ± 1.66 pieces/ mm^2 vs. 29.23 ± 1.83 pieces/ mm^2 , $p = 0.2083$, Fig. 3C). There was also no significant difference in GV ($n = 15$ glomeruli in the control group and 15 glomeruli in the E16.5 group, $16.16 \pm 1.42 \times 10^4$ μm^3 vs. $16.25 \pm 1.12 \times 10^4$ μm^3 , $p = 0.9606$, Fig. 3D). However, the total number of glomeruli per kidney was significantly greater in the E16.5 group than in the control group ($n = 3$ kidneys in the control group and 3 kidneys in the E16.5 group, 1557 ± 175.2 vs. 2270 ± 126.6 , $p = 0.0300$, Fig. 4).

All fetuses were alive in the control and all of the RA groups. Exencephaly, micrognathias, ear, eye, hind leg defects, and tail abnormalities were not macroscopically observed.

4. Discussion

We sought to establish a method to increase the glomerular number in the kidney of offspring by intraperitoneal administration of RA to pregnant mice. The glomerular number increases in proportion to the RA concentration in the medium in *in vitro* studies [7,8]. A previous study showed that the fetal glomerular number decreased owing to

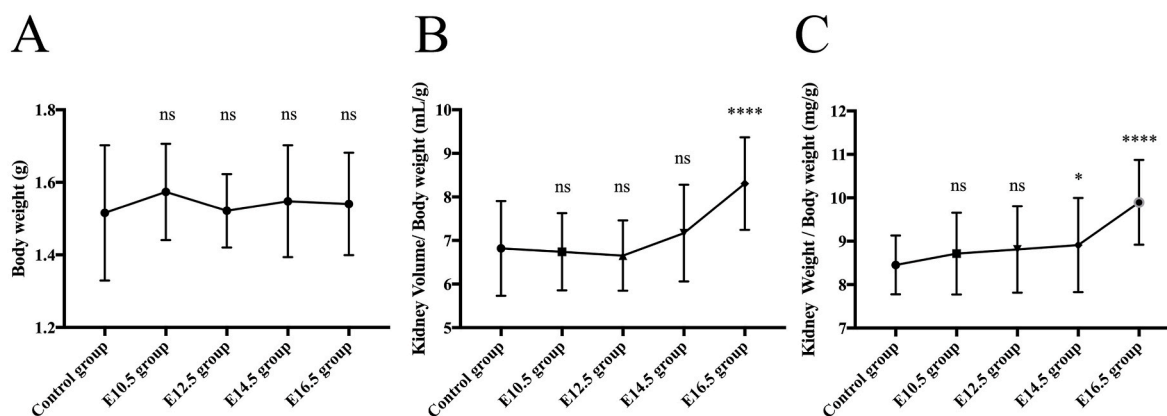


Fig. 1. Body weight, kidney weight, and kidney volume in fetuses treated with retinoic acid. The control group received corn oil intraperitoneally at E10.5. Experimental groups received retinoic acid (RA) intraperitoneally once at E10.5, E12.5, E14.5, or E16.5. Fetuses were harvested at E18.5, and the fetal body weight, kidney volume, and kidney weight were compared. (A) Fetal weight, kidney volume, and kidney weight were measured in all the fetuses ($n = 33$ fetuses in the control group, 53 fetuses in the E10.5 group, 40 fetuses in the E12.5 group, 44 fetuses in the E14.5 group, and 37 fetuses in the E16.5 group). There was no difference in fetal body weight between the control group and the RA groups. (B) Kidney volume was compared after normalizing it to fetal body weight. The E16.5 group had a larger kidney volume than the control group (6.82 ± 0.19 mL/g vs. 8.31 ± 0.18 mL/g, $p < 0.0001$). (C) Kidney weight was compared after normalizing it to fetal body weight. The E14.5 group (8.45 ± 0.12 mg/g vs. 8.91 ± 0.16 mg/g, $p = 0.0364$) and the E16.5 group (8.45 ± 0.12 mg/g vs. 9.895 ± 0.16 mg/g, $p < 0.0001$) had significantly higher kidney weight than the control group. ns: not significant, * $P < 0.05$, **** $P < 0.0001$. The error bars represent standard errors of the mean (SEMs). RA: Retinoic acid, E: Embryonic day.

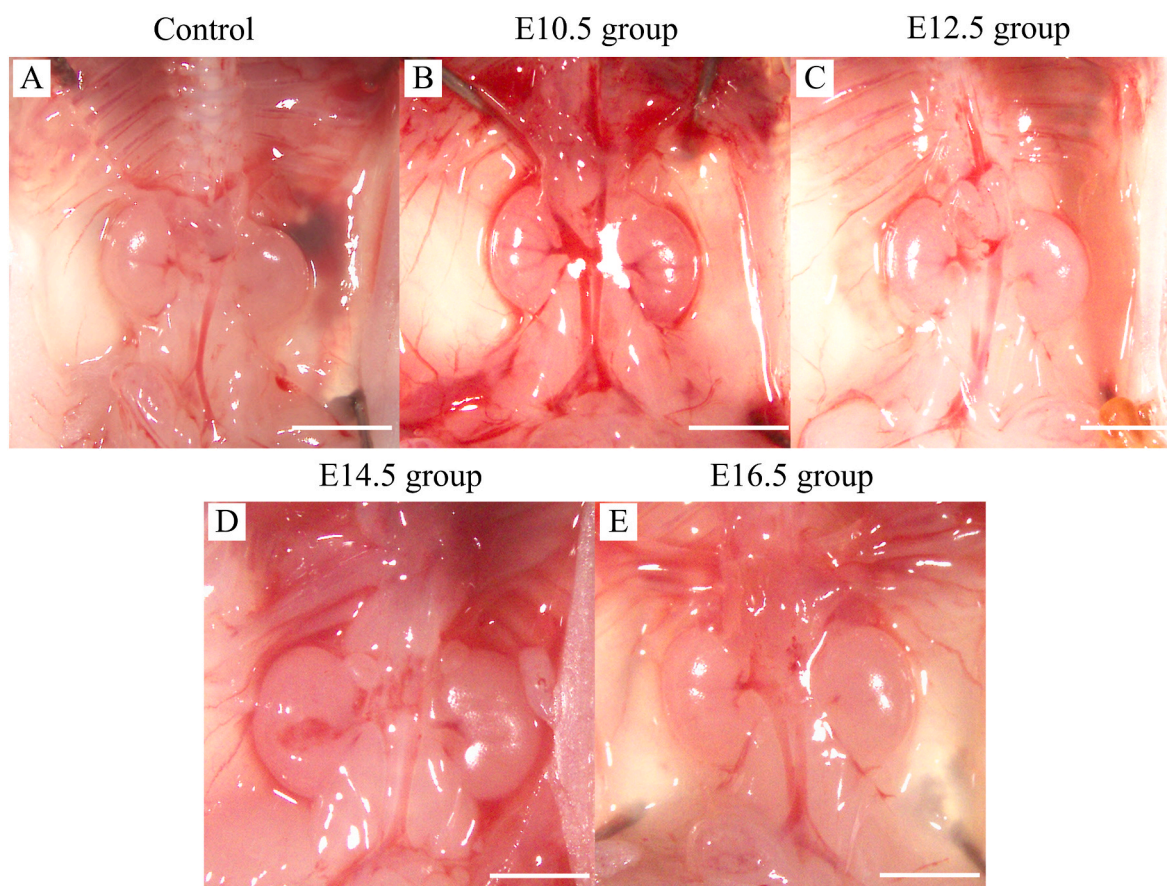


Fig. 2. Fetal kidneys dissected at E18.5 (A–E) Images showing fetal kidneys dissected at E18.5 of the control group (A) and the E10.5 (B), E12.5 (C), E14.5 (D), and E16.5 (E) groups. All images are kidneys taken from fetuses closest to the average fetal body weight (scale bar, 2 mm). The kidneys in the E16.5 group were macroscopically larger than those in the control group. RA: Retinoic acid, E: Embryonic day.

protein restriction in pregnant rats, but the fetal glomerular number was normal after intraperitoneal administration of RA to pregnant rats that had undergone protein restriction [15]. However, no studies have

reported that the glomerular number increases more than normal by RA administration. This study is the first to report that intraperitoneal administration of 20 mg/kg RA to the mother during fetal development

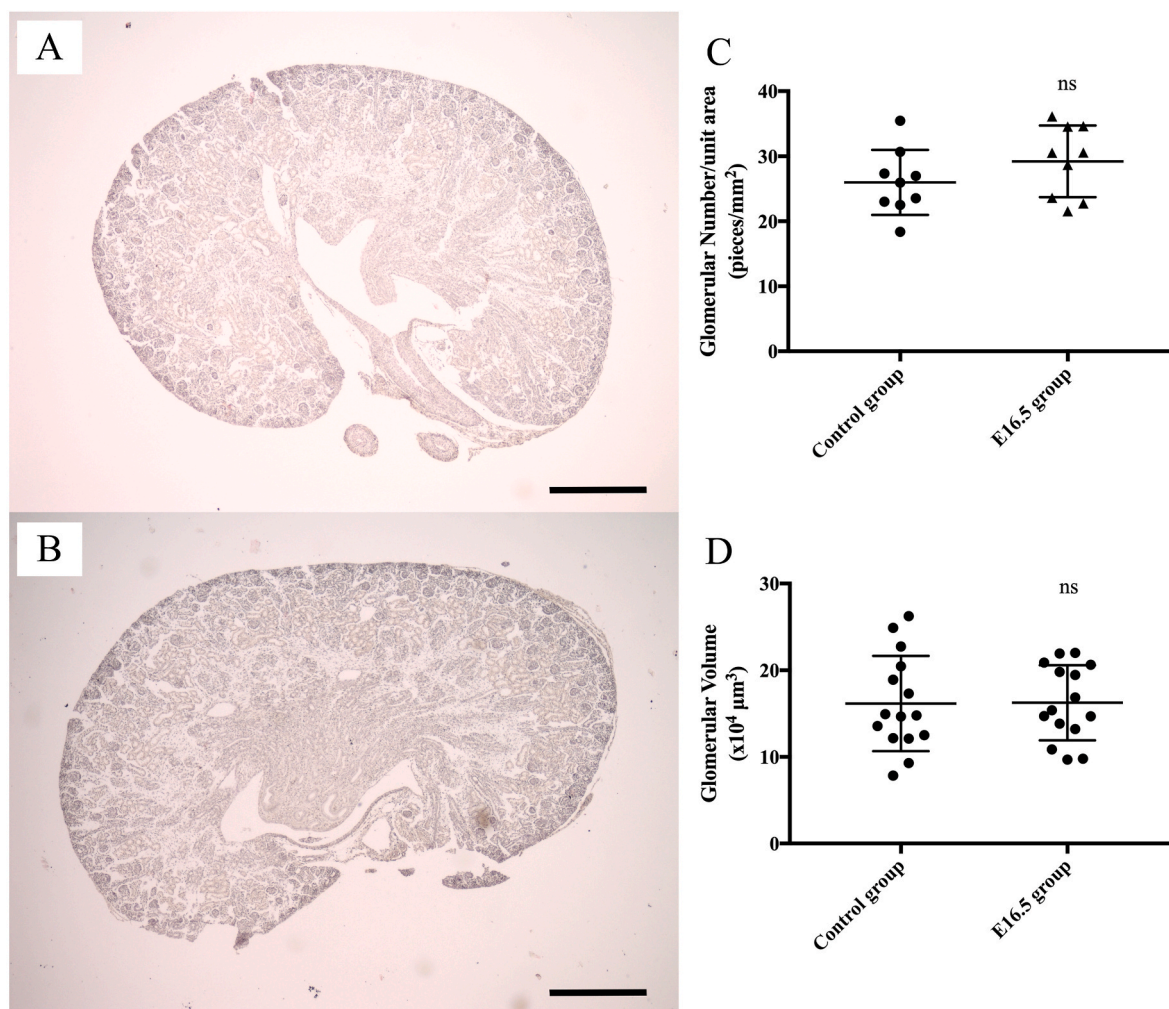


Fig. 3. Histological analysis and glomerular number per unit area

(A, B) Micrographs of fetal kidneys after hematoxylin-eosin staining. The largest cross-sectional slice of kidney in the E16.5 group (B) was larger than that of the control group (A) (scale bar, 0.5 mm). (C) There was no significant difference in the glomerular number per unit area between the control group and the E16.5 group ($n = 3$ kidneys in the control group and 3 kidneys in the E16.5 group, 25.98 ± 1.66 pieces/mm² vs. 29.23 ± 1.83 pieces/mm², $p = 0.2083$). (D) There was no significant difference in the glomerular volume between the control group and the E16.5 group ($n = 15$ glomeruli in the control group and 15 glomeruli in the E16.5 group, $16.16 \pm 1.42 \times 10^4 \mu\text{m}^3$ vs. $16.25 \pm 1.12 \times 10^4 \mu\text{m}^3$, $p = 0.9606$). ns: not significant. The error bars represent standard errors of the mean (SEMs). RA: Retinoic acid, E: Embryonic day.

increases the number of fetal glomeruli.

In this study, the total glomerular number per kidney in the control group was 1557 ± 175.2 , which is similar to the number reported in a previous study (1350 ± 86) [11]. The total glomerular number per kidney in the E16.5 group was 2270 ± 126.6 , 1.46 times higher than that in the control group. However, the GV was not significantly different between the control group and the E16.5 group. In addition, the GV was the same as that observed in a previous report ($14.24 \pm 1.31 \times 10^4 \mu\text{m}^3$) [16]; therefore, the glomeruli presumably developed normally.

The kidney develops through mutual interactions between nephron progenitor cells, ureteric buds, and the stroma. The ureteric bud undergoes branching and segmentation, and the ureteric bud tip interacts with nephron progenitor cells, inducing their conversion into nephrons. RA induces and maintains the expression of Ret at the tip of the ureteric bud and stimulates ureteric bud branching [7,8,17,18]. In this study, the glomerular number per unit area was not different between the control group and the E16.5 group, but the kidney volume, kidney weight, and total glomerular number were higher in the E16.5 group than in the control group. Therefore, the administration of RA to pregnant mice seems to promote ureteric bud branching, and, as a result, nephron

progenitor cells convert into nephrons on the ureteric bud tip, thereby increasing the total glomerular number. In this study, kidney volume and kidney weight did not increase in the E10.5 or E12.5 groups, but they did increase in the E16.5 group, and the glomerular number also increased in the E16.5 group. The ureteric bud begins to branch around E10.5, and the number of ureteric buds increases daily [19]. Because the plasma half-life of all-trans-RA is approximately 30–60 min in the mouse fetus [20], it is likely that the E10.5 and E12.5 groups did not have increased glomerular numbers because the RA was only able to affect the small number of original ureteric buds at E10.5 and E12.5. Because the number of ureteric buds is higher at E16.5, the E16.5 group had a much larger increase in total glomeruli. However, the number of ureteric buds was not measured in this study, and further investigation is needed.

RA may also affect the development of other organs. Excessive RA during fetal development causes exencephaly, micrognathia, cleft lip, cleft lower lip, ear defects, eye defects, hind leg defects, spina bifida, clavicle abnormality, and tail abnormality [21]. In previous reports of malformations caused by RA overdose, the most common RA dose was more than 40 mg/kg, and the most common timing was E8.0–E11.0 [21–25]. Therefore, we administered a single intraperitoneal dose of 20 mg/kg RA in this study. No apparent developmental abnormality was

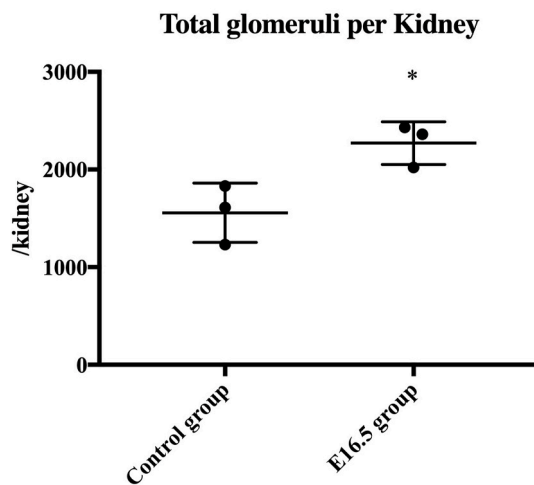


Fig. 4. Glomerular number per kidney

The total glomerular number per kidney in the E16.5 group was significantly higher than that in the control group ($n = 3$ kidneys in the control group and 3 kidneys in the E16.5 group, 1557 ± 175.2 vs. 2270 ± 126.6 , $p = 0.0300$). $*P < 0.05$. The error bars represent standard errors of the mean (SEMs). RA, retinoic acid; E, embryonic day.

observed, and the fetuses all survived; therefore, the RA did not cause any fatal damage to the fetus. However, a previous study showed that the pregnant mice were orally given 20 mg/kg RA at E10.5, E11.5, and E12.5 caused abnormal oral development in some of the fetuses [26]. In addition, a previous study showed that daily oral administration of 10 mg/kg RA to pregnant *Macaca nemestrina* monkeys on day 20–44 resulted in a high frequency of craniofacial and musculoskeletal malformations [27]. The absence of malformations in this study may be attributed to the different route and the frequency of RA administration. It is assumed that there is a difference in the transfer of RA to plasma between oral and intraperitoneal administration routes. The plasma half-life of all-trans-RA is short, approximately 30–60 min in the mouse fetus [20]. Retinoid teratogenicity correlates with the concentration-time curve, or area under the curve (AUC) [28]. The AUCs are possibly different between this previous report and the current study. In our study, plasma RA concentrations were not measured. In addition, malformations were only observed macroscopically, and no histological study was performed. Therefore, it is necessary to confirm in detail whether malformations occur when RA is administered at E16.5 and to determine the optimal RA dosage.

It is still unclear whether increased glomerular number improves kidney function and reduces the risk of CKD. Infants with low birth weight have a low glomerular number [3,4] and a higher risk of CKD [5, 6]; therefore, it is presumed that a low glomerular number increases the risk of CKD. There seems to be a significant relationship between the glomerular number and CKD risk. In addition, Brenner et al. postulated that reduced nephron number contributes to essential hypertension [29]. An increased glomerular number is presumed to reduce the risk of CKD and hypertension, although further investigation is still needed.

In this study, we administered RA during fetal development to increase the glomerular number. We believe that RA administration should be assessed in kidney regeneration using embryonic development programs such as blastocyst complementation [30] and the organogenic niche method [31]. These kidney regeneration methods use the renal developmental environment of the fetus. Kidney regeneration may be more efficient when RA is administered on the renal developmental process.

This study has some limitations. Firstly, only one dose was investigated, and therefore it is necessary to establish the optimal RA dose. Secondly, whether malformations occur owing to RA administration should be investigated. Finally, the effect of increased glomerular

number on postnatal renal function is unclear. Therefore, it is necessary to prepare CKD or acute kidney injury (AKI) mouse models with increased glomerular number by RA administration, and investigate whether the risk of renal function decline can be altered. If it is shown that increased glomerular number by intraperitoneal RA administration during fetal development leads to reduced CKD risk, CKD incidence may decrease.

The final objective of this study is to develop a treatment for humans. RA treatment may contribute to an increase in the number of glomeruli and reduce CKD and AKI risk. It is expected that the number of dialysis patients will decrease, as well disease-related medical expenses, if the risk of CKD and AKI is reduced with the administration of RA. However, RA is teratogenic and should not be administered to pregnant women. The risk of teratogenicity and the optimal dose/route/timing of RA need to be investigated.

In conclusion, the glomerular number in the fetal mouse kidney was increased by intraperitoneal administration of RA at E16.5.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2022.101245>.

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