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Vitamin A Deficiency Causes Ovulation Abnormalities in Mice

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ABSTRACT  Retinoic acid (RA) is an active metabolite of vitamin A (VA) and is involved in tissue organization, patterning, and growth. RA has been shown to regulate male reproduction, however information on its role in ovary development is limited. To investigate the functions of RA in the ovary, we examined its role in ovary development and ovulation using an in vivo dietary VA-deprivation animal model. Our preliminary results have shown that VA deficiency causes a variety of ovarian pathologies, including reduced numbers of total follicles and corpus lutea, formation of hemorrhagic and atretic follicles, and formation of bursa and follicular cysts. To further examine if the reduced numbers of corpus lutea represents a defect in ovulation, in this study, we investigated the effect of VA deficiency on ovulation. We fed CD-1 pregnant mice with either a VA deficient diet (VA-) or a control diet, and at weaning, female pups were maintained on their respective diet. These female pups were then induced super-ovulation at day 19 (D19) or week 7 (Wk7) via subcutaneous injection of pregnant mare’s serum gonadotropins (PMSG) followed by human chorionic gonadotropin (hCG) 48 hours later. Oviducts from these animals were collected 14-16 hours later and ovulated oocytes in the oviducts were counted and scored. Our preliminary data showed that although the total numbers of ovulated oocytes were similar in the control and VA- groups at day 19, VA- mice responded poorly to super-ovulation at week 7. Overall, our study suggests that vitamin A is critical for ovary development and ovulation.

ABBREVIATIONS
RA, retinoic acid; VA, vitamin A; VA-, vitamin A deficient; D19, day 19 mice; Wk7, week 7-old mice; GV, germinal vesicle; GVDB, germinal vesicle breakdown; MII, meiosis II stage; PMSG pregnant mare’s serum gonadotropin; hCG, human chorionic gonadotropin; FSH, follicle stimulating hormone; LH, luteinizing hormone; PVP, polyvinylpyrrolidone.

INTRODUCTION
Normal development of ovarian follicles is essential for proper female reproduction and endocrine function. Activin has been shown to play an important role in the regulation of ovarian follicle development (1). In previous studies done by the Kipp Lab, the gene network related to the signaling of retinoic acid (RA), an active metabolite of vitamin A (VA), was identified as a top activin-regulated pathway (2). RA participates in tissue organization, patterning, and growth in many systems as well as functioning as a sex determination factor during gonadal development (3). However, our understanding of the role of RA in normal ovarian follicle development is quite limited. To investigate further functions of RA in the regulation of ovarian follicle development, we fed CD-1 mice with a VA deficient diet (VA-) and examined the resulting ovarian pathologies.
Our preliminary results revealed several ovarian pathologies in VA deficient animals at day 19 (D19), week 7 (Wk7) and week 15 (Wk15) (manuscript in preparation). Increased follicle atresia, formation of multi-oocytic follicles, and a significant reduction in the number of corpus lutea in these animals was observed, suggesting an important role for RA in the regulation of ovarian follicle development and ovulation. Hormone measurements also showed increased serum testosterone and decreased follicle stimulating hormone (FSH) levels. Testosterone is produced by the ovary while FSH is produced by the pituitary gland. Changes in the latter may potentially cause delay in ovarian development through the hippocampus-pituitary-gonad axis. However, we do not know if the observed decrease in FSH levels represents a primary effect of VA deficiency on the pituitary gland or an effect secondary to the increased testosterone levels via a negative feedback loop. In addition, it is not clear if the observed ovarian pathologies represent a primary intra-ovarian impact of VA deficiency or an effect related to the decreased FSH levels. Therefore, we decided to perform additional experiments that included the super-ovulation of mice to examine if exogenous gonadotropin hormones may rescue the observed ovulation defect. Super-ovulation involves the injection of pregnant mare’s serum gonadotropin (PMSG), followed by human chorionic gonadotropin (hCG) 48 hours later, and the collection of oocytes 14-16 hours post hCG injection (4). In order to examine if exogenous PMSG (which mimics the function of FSH) and hCG (which mimics the function of luteinizing hormone, [LH]) stimulation may rescue the observed ovulation defect in VA deficient mice, pregnant females were fed with either a VA-diet or a control diet and at weaning until D19 or Wk7 after birth, and the female pups were induced super-ovulation at D19 or Wk7. Ovulated oocytes in the oviducts were then counted and compared. To assess the maturation of the oocytes, we classified them based on their developmental stages. The presence of a germinal vesicle (GV) meant that maturation had not yet begun, whereas the presence of germinal vesicle breakdown (GVBD) indicated that the first signs of meiotic maturation had started to occur (5). Oocytes that were captured in the meiosis II (MII) stage with an extruded polar body were considered to be the most mature oocytes and most likely to fertilize (6). Overall, our studies aimed to discover novel functions of RA in the regulation of ovary and oocyte development. The findings from these studies will increase our understanding of ovarian follicle development and have potential applications to human health-related disease prevention and treatment.

**METHODS**

**Animals**

Female CD-1 mice were housed in individual cages and placed on either the VA- or control diet. CD-1 male mice were then added to the cages 24 hours later. All mice were housed in a climate and light-controlled environment with food and water available ad libitum. After litters were born, the mother and her pups were kept on their specific diet while the father was removed. All of the animals were cared for and maintained in accordance to all federal and institutional guidelines.

**Animal Treatments**

Half of the females from the litters began their injection regimen for superovulation at D19 after birth, and the other half at Wk7 after birth. At either the D19 or Wk7 time points, these females were injected subcutaneously with 5 IU PMSG, followed by 5 IU hCG 48 hours later. The mice were then sacrificed via CO₂ gas followed by cervical dislocation 14-16 hours after the hCG injections.

**Tissue Collection**

Oocytes were collected by slashing the swollen part of the ovarian ampullae with a syringe to release the clutch of eggs in a medium containing 30 mL of L15 medium, 90 mg polyvinylpyrrolidone (PVP), and 150
μL penstrep. Each ampullae’s set of oocytes own drop of L15/PVP/PS media containing 3 mg/ml of hyaluronidase to facilitate the digestion of the cumulus mass. After approximately 5 minutes, or once the substrate had cleared itself from the oocytes, the oocytes were washed once into a new drop of clean medium. The oocytes were finally transferred into a final, fresh drop of medium to be counted and the developmental stages were scored as GV (presence of a germinal vesicle), GVBD (absence of a polar body and germinal vesicle breakdown), or MII (presence of polar body and oocyte arrested in meiosis II). Those oocytes in the MII phase were considered to be the most mature, those in the GV were the least mature. The quality of the oocytes was also evaluated and oocytes that were fragmented, had no zona pellucida surrounding the egg, had an empty zona, were degenerative, or appeared blobby were considered unhealthy and they were counted and recorded.

Statistics

Based on our preliminary morphological studies (manuscript in preparation), we hypothesized that VA- mice would have a reduced number of ovulated oocytes. Therefore, the comparisons between two groups were analyzed using the one-tailed student’s t-test. P<0.05 was considered significant. “n” represents the experimental repeats.

RESULTS

The total number of oocytes collected from each ovary from the super-ovulated VA- mice at D19 showed no significant difference when compared to the control difference when compared to the control D19 mice (Figure 1). Although D19 VA- oocytes showed no statistically significant differences in classification types when compared to control, we observed that there is a notable decrease in the number of GV-stage oocytes in the VA- group. We also observed a mild increase in the GVBD and MII stages of oocytes while the fragmented oocyte numbers slightly decreased (Figure 2). At Wk7, a significant decrease in the number of ovulated oocytes was observed in the VA- group as compared to the control group (Figure 3). Classification for oocyte types for this experiment showed a significant decrease in GV, GVBD, and MII oocytes (Figure 4).
Figure 1: The total number of super-ovulated oocytes per ovary at D19. No significant difference in total number of ovulated oocytes was observed between the control and the VA- mice. n=14 for control, 8 for VA-.

Figure 2: Classification of super-ovulated oocytes at D19. The developmental stages for the oocytes were classified as GV (presence of a germinal vesicle), GVBD (breakdown of germinal vesicle), or MII (presence of a polar body and arrested in meiosis II). The quality of the oocytes was evaluated and those that were fragmented, showed no zona pellucida, had an empty zona, were degenerative, or appeared blobby were counted and recorded. n=14 for control, 8 for VA-.

(Frag.: Fragmented; E. Zona: Empty Zona; Deg.: Degenerative.)
Figure 3: Total number of super-ovulated oocytes per ovary at Wk 7. There was a significant decrease observed in the number of ovulated oocytes in VA- mice. *p<0.001, n=12 for control n=6 for VA-.

Figure 4: Classification of super-ovulated oocytes at Wk 7. The total number of VA- oocytes was decreased as well as the numbers of GV (presence of germinal vesicle), GVBD (germinal vesicle breakdown), and MII (arrested meiosis II stage) oocytes when compared to the control. *p<0.05, **p<0.001, ***p<0.01. n= 12 for control, 6 for VA-.
DISCUSSION

The purpose of this study was to examine the ovulation status of VA- mice in response to super-ovulation at D19 and Wk7 of age. Super-ovulation is a treatment method that induces ovulation by injecting animals with the gonadotropin hormones, PMSG and hCG. Using the super-ovulation method, we can quantify ovulation status by counting the numbers of ovulated oocytes and assess the quality of these oocytes by determining the stages of oocyte development and the overall health status of the oocytes. We predicted that mice placed on the VA- diet would not be able to ovulate well as compared to control mice because RA has been shown to be an important factor in ovary development (2). Although there were not statistically significant differences in the D19 group, we observed that the number of oocytes collected from VA- mice at Wk7 was significantly lower than from control mice. These results suggest that prolonged VA- condition has a more severe impact on ovary development and health.

D19 VA- mice show no significant difference in the total number of ovulated oocytes. However, we did observe that there was a decrease in the number of GV stage oocytes and a small increase in the numbers of GVBD and MII stage oocytes, although statistically insignificant. This observation may suggest that the effect of VA- may cause a shift in oocyte maturation. By Wk7, the VA- mice showed a significant decrease in the total number of ovulated oocytes. The oocytes also showed a significant decrease in the numbers of GV, GVBD, and MII stages. These results are consistent with our previous observation that at Wk7, VA- mice had fewer follicles in the ovary (manuscript in preparation). The number of blobby oocytes increased slightly in the VA- group indicating reduced quality, but this increase was not statistically significant. We conclude from these results that the VA- mice at Wk7 have decreased ability to ovulate and that the ovulated oocytes have an increased occurrence of pathologies.

To determine if the duration of time on a VA- diet has a greater effect than the age of the mouse, further studies where mice will be placed on the VA- diet at different time points leading to 7 weeks of age would be plausible. In addition, placing mice on the VA- diet early in development and allowing the mice to recover on a regular feed to week 7 could help to determine if the effect of VA- on early development may program the ovary and lead to ovulation failure in the future. To conclude, mice placed on the VA- diet responded poorly to super-ovulation with the greater effects observed at Wk7 than at D19. This study provides strong evidence that supports the idea that RA plays an important role in ovary development and is the first to demonstrate the impact of vitamin A deficiency on ovulation.

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