Nutrition 57 (2019) 97–108

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Vitamin A supplementation improves the intestinal mucosal barrier and facilitates the expression of tight junction proteins in rats with diarrhea

Lu Xiao M.D. [a], Ting Cui M.D. [a], Shu Liu M.D. [a], Baolin Chen M.D. [a], Yuting Wang Ph.D. [a,b], Ting Yang M.D. [a], Tingyu Li M.D. [a], Jie Chen Ph.D. [a,*]

[a] Children's Nutrition Research Center, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders, China International Science and Technology Cooperation Base of Child Development and Critical Disorders, Chongqing 400014, China
[b] Department of Digestive, Children's Hospital of Chongqing Medical University, Chongqing, China

ARTICLE INFO

Article History:
Received 10 October 2017
Received in revised form 11 June 2018
Accepted 19 June 2018

Keywords:
Diarrhea
Vitamin A deficiency (VAD)
Vitamin A supplementation (VAS)
Intestinal mucosal barrier
Tight junction proteins

ABSTRACT

Objectives: The aim of this study is to investigate the specific effects of vitamin A (VA) on diarrhea in rats and its potential targets to protect the intestinal mucosa.
Methods: Specific pathogen-free Sprague Dawley rats were fed a VA deficient (VAD) or VA normal (VAN) diet for 4 wk. Then, half of the VAN rats were treated with a VAN diet and the other half with a lactose VAN diet. VAD rats were randomly assigned to one of four groups and fed a VAD diet, lactose VAD diet, VAN diet with VA supplementation (VAS) via daily intragastric administration, or a lactose VAN diet with daily VAS. Rat weight and degree of diarrhea were evaluated daily. After 15 d, the serum retinol level was measured by high-performance liquid chromatography, and the serum diamine oxidase (DAO) and zonulin concentrations were analyzed by enzyme-linked immunosorbent assays. The small intestine mucosal pathology was observed by hematoxylin and eosin staining. Western blotting was performed to detect the protein expression levels of occludin and claudin-1 in the intestinal mucosa, and the zonula-occludens 1 expression was assessed using immunohistochemistry.
Results: VAD limited weight gain in rats and increased the degree of diarrhea. The serum retinol levels and the level of tight junction (TJ) proteins claudin-1 and occludin and grip strength were affected by the interaction between lactose-induced diarrhea and the VA diet. Diarrhea, independent of VAD, significantly decreased rat weight, increased serum DAO levels, damaged small intestine villi, and impaired zonula-occludens 1 protein expression. VAD significantly increased the concentration of zonulin independently of diarrhea, but VAS increased the serum retinol level, reduced the severity of diarrhea, increased the expression levels of the TJ proteins, facilitated the restoration of the small intestine villi that were damaged by the diarrhea, and decreased the concentrations of serum DAO and zonulin.
Conclusions: VAD may aggravate the degree of diarrhea and intestinal mucosal damage during the duration of diarrhea, and VAS helps relieve diarrhea and improves intestinal damage likely by regulating the expression of TJ proteins. Therefore, VA plays a pivotal role in the protection of the intestinal mucosa during instances of diarrhea.

Introduction

According to the World Health Organization, approximately 2 billion cases of diarrheal disease occur worldwide, and 1.9 million children aged <5 y die from diarrhea annually, mostly in developing countries [1]. Diarrhea can be classified as acute or persistent based on the course of the disease. Persistent diarrhea (PD), which is also known as chronic diarrhea, refers to diarrhea that persists

Lu Xiao and Ting Cui contributed equally to this report and are regarded as co-first authors.

Sources of support: This work was supported with grants from National Nature Science Foundation of China (81470799, 81770526) and the Science & Technology Project of the Yuzhong District of Chongqing (2012) to Dr. Jie Chen.

Conflicts of interest: The authors have no potential conflicts of interest with regard to the publication of this manuscript. Our team holds a patent (Patent No. ZL201010233032.8) for the formula of the vitamin A normal and vitamin A deficiency animal feed in China.

* Corresponding author. Tel.: +86 23 63630913; fax: +86 23 63622754.
E-mail address: jchen010@foxmail.com (J. Chen).

https://doi.org/10.1016/j.nut.2018.06.007
0899-9007/

for more than 2 wk and leads to deterioration in nutritional status and a substantial risk of death [2,3]. Consequently, diarrhea places a large burden on families and societies. However, according to Das, an increase in the proportion of vitamin A supplementation (VAS) (from 26% to 74%) significantly decreased the ratio of children ages <5 y in Bangladesh who were hospitalized with PD from 8% in 1991 to 1% in 2010 [4].

VA, which is also known as retinol, is an essential fat-soluble vitamin that maintains normal visual function, protects the health of epithelial tissue cells, promotes the synthesis of immunoglobulin, and is conducive to growth and reproduction [5–7]. VA deficiency (VAD) is an important public health problem that affects the health of approximately 190 million preschool-aged children and 19 million pregnant women worldwide [8]. According to a prospective study conducted in Bogotá, Colombia, serum retinol concentrations were inversely related to the rates of gastrointestinal and respiratory morbidity, and VAD was associated with an increased risk of diarrhea with vomiting [9]. Meanwhile, low-dose VAS significantly reduced the incidence of severe diarrhea in severely malnourished children [10]. Although the mechanisms that underlie the effects of VA are unclear, our previous findings have shown that concurrent VAS and zinc supplementation and even a single VAS can significantly boost the therapeutic efficacy of the treatment for PD and decrease disease duration [2]. Zinc supplementation is already included in the guidelines for the clinical treatment of PD in children ages <5 y. However, according to our previous study, VAS alone without zinc supplementation improved the VA nutritional level and anemia in children with PD.

Therefore, the purpose of the present study is to investigate the independent and specific effect of VA on diarrhea in rats and its targets to improve the intestinal mucosa during incidences of diarrhea. First, using VA normal (VAN) and VAD rat models, we established lactose-induced diarrhea. Meanwhile, rats with VAD-related diarrhea were treated with VAS to investigate the VA nutritional levels associated with the severity of the diarrhea. Second, because of the dynamic weight changes in the rats, we focused on the diarrhea index and grip strength in the different groups. Third, we examined the diamine oxidase (DAO) and zonulin levels in the serum to evaluate the intestinal barrier function and the expression level of the tight junction (TJ)-associated proteins claudin-1, zonula-occludens 1 (ZO-1), and occludin in the intestinal mucosa to identify the effective targets of VAS. The present study provides new insight into the use of VA as an adjunctive therapy in children with PD by regulating the TJ-associated proteins in the intestinal mucosa.

Methods

Animals, diets, and diarrhea

Sixty SPF-grade Sprague Dawley (SD) male rats (age: 3 wk) were obtained from the Experimental Animals Centre of the Chongqing Medical University (Chongqing, China). This study was approved by the Animal Experimentation ethical committee of the Chongqing Medical University (Chongqing, China). The rats were housed in the same room with a constant airflow system, controlled temperature (22°C–24°C) and a 12-h light/dark cycle. As shown in Figure 1, 40 randomly chosen rats were fed a VAD-inducing diet that contained 400 IU/kg VA for 4 wk to establish the VAD animal model, and 20 rats were fed a VAN diet that contained 6500 IU/kg VA to establish the VAN animal model (Suppl. Table 1) [7].

After the serum retinol levels in the blood from the tails of the VAD rats decreased to <1.05 μmol/L and that in the VAN rats increased to >1.05 μmol/L, the VAN rats were randomly assigned to two groups and the VAD rats to four groups. For the following 15 d (approximately 2 wk), half of the VAN rats were continuously fed the VAN diet that contained glucose (VANG group) and the other half was fed the VAN diet that contained lactose (VANL group; diarrhea; all glucose in the VANG diet was changed to lactose). Simultaneously, the VAD rats were assigned for the next 15 d to a VAD diet that contained glucose (VADG group), a VAD diet that contained lactose (VADL group; diarrhea; all glucose in the VADG diet was changed to lactose), a VAN diet that contained glucose with VAS via intragastric administration (1 IU/g/d; VASG group), and a VAN diet that contained lactose with VAS via intragastric administration (1 IU/g/d; VASL group; diarrhea).

During the 15-d period, all rats were weighed, and the diarrhea status was observed daily. After 15 d, the rats were sacrificed, their blood was immediately harvested from the femoral artery, the ileum of the small intestine was extracted and stored at −80°C, and part of the ileum was fixed with 4% paraformaldehyde after washing with 0.01 M phosphate-buffered saline for further study.

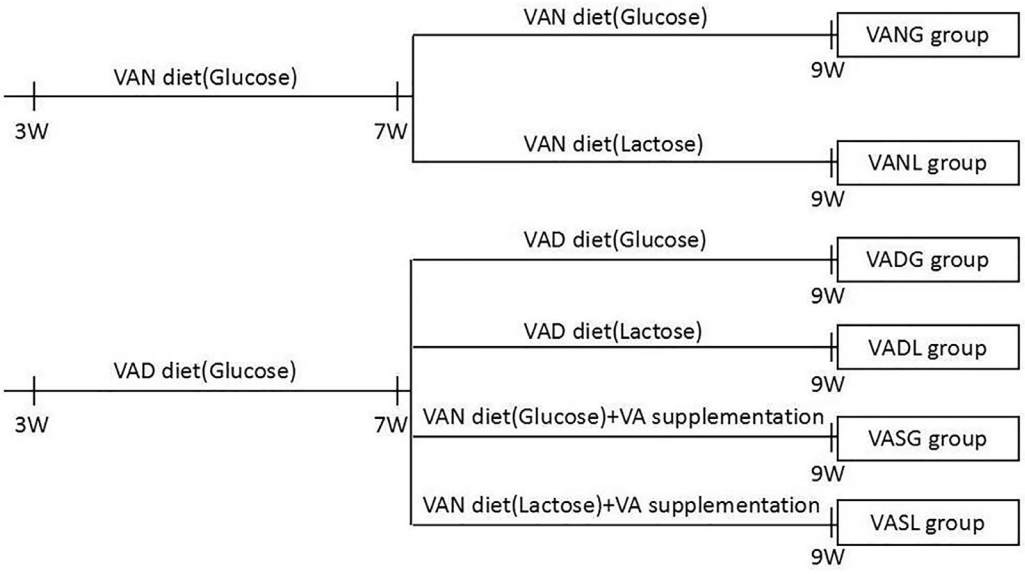

Fig. 1. Schematic diagram of the six experimental groups: Vitamin A normal diet with glucose (VANG), Vitamin A normal diet with lactose (VANL), Vitamin A deficient diet with glucose (VADG), Vitamin A deficient diet with lactose (VADL), Vitamin A supplementation diet with glucose (VASG), and Vitamin A supplementation diet with lactose (VASL). Three-week-old rats were fed a VANG diet until 9 wk of age, of which a subset were fed a VANG diet for 4 wk and then a VANL diet for the following 15 d (approximately 2 wk). Three-week-old rats were fed a VADG diet until 9 wk of age, of which three subsets were fed a VADG diet for 4 wk and then a VADL diet for the following 15 d, a VADG diet for 4 wk and then a VANG diet and treated with VAS via intragastric administration for the following 15 d, or fed a VADG diet for 4 wk and then a VANL diet and treated with VAS via intragastric administration for the following 15 d.

Observation of diarrhea in rats

All rats were reared in single cages, and rat weight and feces were observed daily at the same time. The diameter of each smear was measured using compasses and scales, and a diarrhea index was used as the primary index to estimate the degree of diarrhea [11,12]. The diarrhea index was calculated as the loose stool rate multiplied by the average loose stool degree. The loose stool rate was calculated by the number of loose stools divided by the total stools. The loose stool degree refers to the extent of each animal's watery stools and is divided into four grades according to the diameter of the feces: <1 cm (level 1); 1–1.9 cm (level 2), 2–3 cm (level 3), and >3 cm (level 4). The average stools degree was calculated by the total degree of loose stools divided by the number of loose stools.

Grip strength test

The rats were placed on a grasping force tester with a forelimb grip on the hob. Pulling the rats' tail induced them to seize the hob as a natural instinct. The instrument automatically recorded the maximum grip produced during the process. The operation was repeated five times for each rat with a 5-s resting interval between each test, and the average of five grasping forces was calculated.

Detection of serum retinol

The concentration of serum retinol was measured using high-performance liquid chromatography (HPLC) as previously described [13]. Briefly, 200 μL serum was deproteinized with the same volume of dehydrated alcohol, then 1000 μL hexane was used to extract the retinol from the serum, and the hexane was evaporated using nitrogen gas. The residue of retinol was dissolved in 100 μL of mobile phase mixture (methanol:water = 97:3). Finally, the prepared sample was measured using an HPLC apparatus (DGU-20 As, Shimadzu Corporation, Japan) on a C18 analytical column with a 315 nm ultraviolet photodiode array detector.

Enzyme-linked immunosorbent assay

The levels of DAO and zonulin in the serum of the different treatment groups were measured using a rat DAO enzyme-linked immunosorbent assay kit (Wuhan ColorfulGene Biological Technology) and a rat zonulin enzyme-linked immunosorbent assay kit (Wuhan Cloud-Clone Corp), respectively. All procedures were performed by strictly following the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm, and the optical density values were calculated based on standard curves that were constructed for each assay and performed in triplicate.

Hematoxylin and eosin staining

The fresh ileum were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned using standard methods. Subsequently, the serial sections, which were 4 mm thick, were stained with hematoxylin and eosin. The ileum villi were observed and measured at 100 × magnification. Four discontinuous sections from each experimental group were analyzed, five villi (complete, vertical) in each section were randomly selected, and the average surface area of each villus was calculated (small intestine villus surface area = d*πh, where d is the diameter and h is the height of the villus measured in mm).

Western blotting

The total protein extracted from the ileum was used for Western blotting. The membranes were incubated in primary antibodies, including claudin-1 (1:400, Invitrogen, Carlsbad, CA), occludin (1:1000, Cell Signaling Technology) and GAPDH (1:1000, Santa Cruz, CA), at 4°C overnight, followed by incubation in hydrogen peroxide-conjugated secondary antibodies (Santa Cruz, CA) at room temperature for 1 h. Then, the protein bands were detected using a chemiluminescent hydrogen peroxide substrate (Millipore, Burlington, MA). The images were captured using a Syngene GBox Imaging System (Gene Company, China).

Immunohistochemical staining

The ileum sections (4 μm thick) were deparaffinized, treated with 3% hydrogen peroxide to block the endogenous peroxidase activity, and treated with 0.01 M citric acid (pH 6.0) for 15 min at 100°C for the antigen retrieval. Then, the slides were incubated overnight at 4°C with anti-ZO-1 (1:50 Invitrogen, Carlsbad, CA) and diaminobenzidine as a chromogen and then counterstained with hematoxylin. Positive expression was observed and measured at 1000 × magnification. A quantitative analysis of the positive expression was performed using Image-Pro Plus to calculate the integral optical density values.

Statistics

All data were expressed as mean ± standard deviation. Significant differences were calculated via two-way analysis of variance with the Bonferroni post hoc test and the use of the GraphPad Prism version 5.0 software package. When there was a statistically significant interaction, all experimental groups were compared using the Bonferroni post hoc test. However, when there was not a significant interaction, the Student-Newman-Keuls test was used to analyze the main effect of VA among the three combined VA treatment groups. The Student *t* test was used to analyze the main effect of lactose between the two combined groups with lactose or no-lactose treatment. Only the comparison among the groups were analyzed with one-way analysis of variance. *P* values of <0.05 were accepted as statistically significant.

Results

Vitamin A deficiency decreased serum retinol levels and reduced body weight of rats

To explore the effects of VA on lactose-induced diarrhea, we constructed rat models using VAN and VAD diets. Three-wk-old SD rats were purchased and divided into VAN and VAD groups at random. There was no statistical difference in initial weight between the two groups (Suppl. Fig. 1). After 4 wk of the VAN or VAD diet, the serum retinol levels and body weights of the rats were obtained (Fig. 2). The level of serum retinol in the VAD rats (0.74 ± 0.23 μmol/L) was significantly lower than that in the VAN rats (1.59 ± 0.27 μmol/L; $P < 0.001$; Fig. 2A).

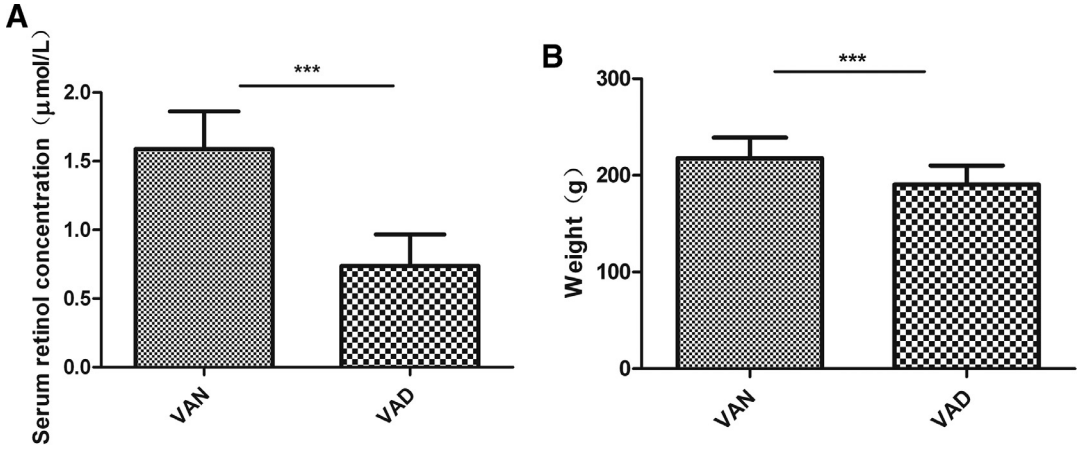

Fig. 2. Changes in (A) serum retinol levels and (B) weight between rats that were fed the vitamin A normal diet (n = 20) and the vitamin A deficient diet (n = 40) after diets with different vitamin A nutrition levels for 4 wk. Mean ± standard deviation, $^{***}P < 0.001$.

According to the international standard of VA levels in humans, both the VAN and VAD rat models were successfully constructed. Meanwhile, the weights of the rats in the VAD group were 190.38 ± 19.63 g and markedly lower than those in the VAN group (217.67 ± 21.48 g; $P < 0.001$; Fig. 2 B). Subsequently, the VAN rats were randomly assigned to the VANG or VANL group, and serum retinol levels and body weights did not significantly differ between the VANG and VANL groups (Figs. 3A and B). The VAD rats were randomly assigned to the VADG, VADL, VASG, or VASL group. No significant differences were observed in the serum retinol levels (Fig. 3C) and body weights (Fig. 3D) among the four groups. Therefore, VAD reduced the serum retinol levels and weights of rats after weaning, which provided a good foundation for the following experiments.

Lactose further reduced serum retinol levels and weight

As shown in Figure 4A, in the absence of lactose, the concentration of serum retinol in VAD rats decreased to 0.329 ± 0.23 μmol/L, which was significantly lower than that in the VAN (1.267 ± 0.40 μmol/L) and VAS (1.267 ± 0.30 μmol/L) rats ($P < 0.001$). No statistical difference was observed between the VAN and VAS groups, which suggests that a VAS can effectively improve serum retinol levels in rats. However, after 15 d of lactose treatment, the serum retinol levels of rats in the VAN and VAS groups were significantly lower than those in rats that did not receive the lactose treatment ($P < 0.001$ and $P < 0.05$, respectively; Fig. 4A).

Although the serum retinol levels in the VAD group after lactose were lower than those in rats that did not receive the lactose treatment, no significant difference was observed. According to the post hoc test, both the VA level ($P < 0.0001$) and lactose treatment ($P < 0.0001$) had significant effects on serum retinol level, and the P value of the interaction between lactose and VA was 0.0186 (Fig. 4A). Therefore, both the VA nutritional level and lactose affected the level of serum retinol.

Furthermore, we observed a dynamic change in daily rat weight among the VAN, VAD, and VAS groups with or without lactose (Fig. 4B). The weights of rats in the VANG, VADG, and VASG groups increased steadily over 15 d of feeding with the diet that contained glucose, even with the weights in the VANG group higher than those in the VADG and VASG groups. However, the weights in the VANL, VADL, and VASL groups did not increase during the 15-d period of the lactose-induced diarrhea. After 15 d, the weights of the VAN, VAD, and VAS rats that were treated with lactose were significantly lower than those of rats not treated with lactose ($P < 0.001$; Fig. 4C). The weights in the VAS group did not significantly differ from those in the VAD group with or without lactose treatment. However, no significant interaction was observed between the VA and lactose effects as determined using two-way analysis of variance (Fig. 4C).

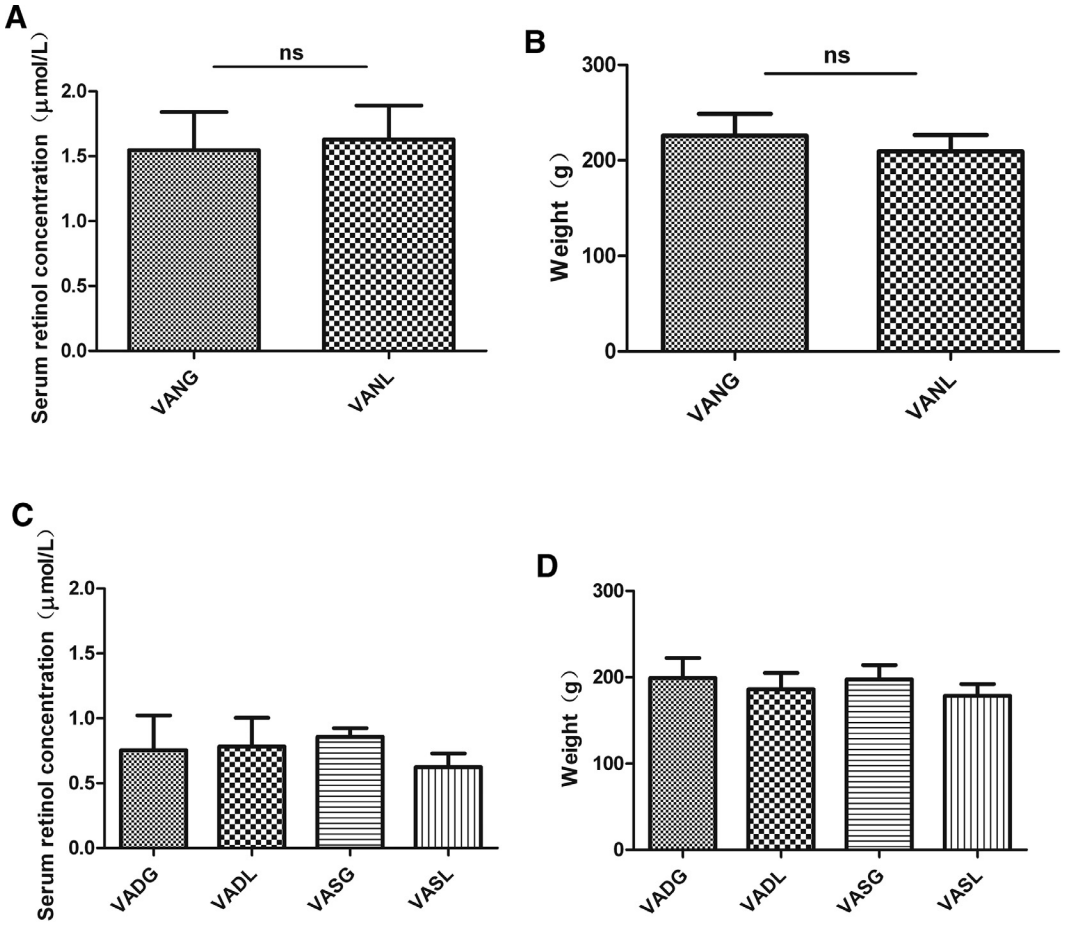

Fig. 3. Comparisons of serum retinol concentrations and weight in rats that were fed the vitamin A normal (VAN) and deficient (VAD) diets and were randomly divided into several groups. (A) Serum retinol levels and (B) weight changes in VAN rats that were fed a diet that contained glucose (VANG; n = 10) or lactose (VANL, n = 10). (C) Serum retinol levels and (D) weight changes in VAD rats fed a diet that contained glucose (VADG, n = 10) or lactose (VADL, n = 10) in the absence or presence of vitamin A supplementation (VASG, n = 10; VASL, n = 10). Mean ± standard deviation. ns, not significant.

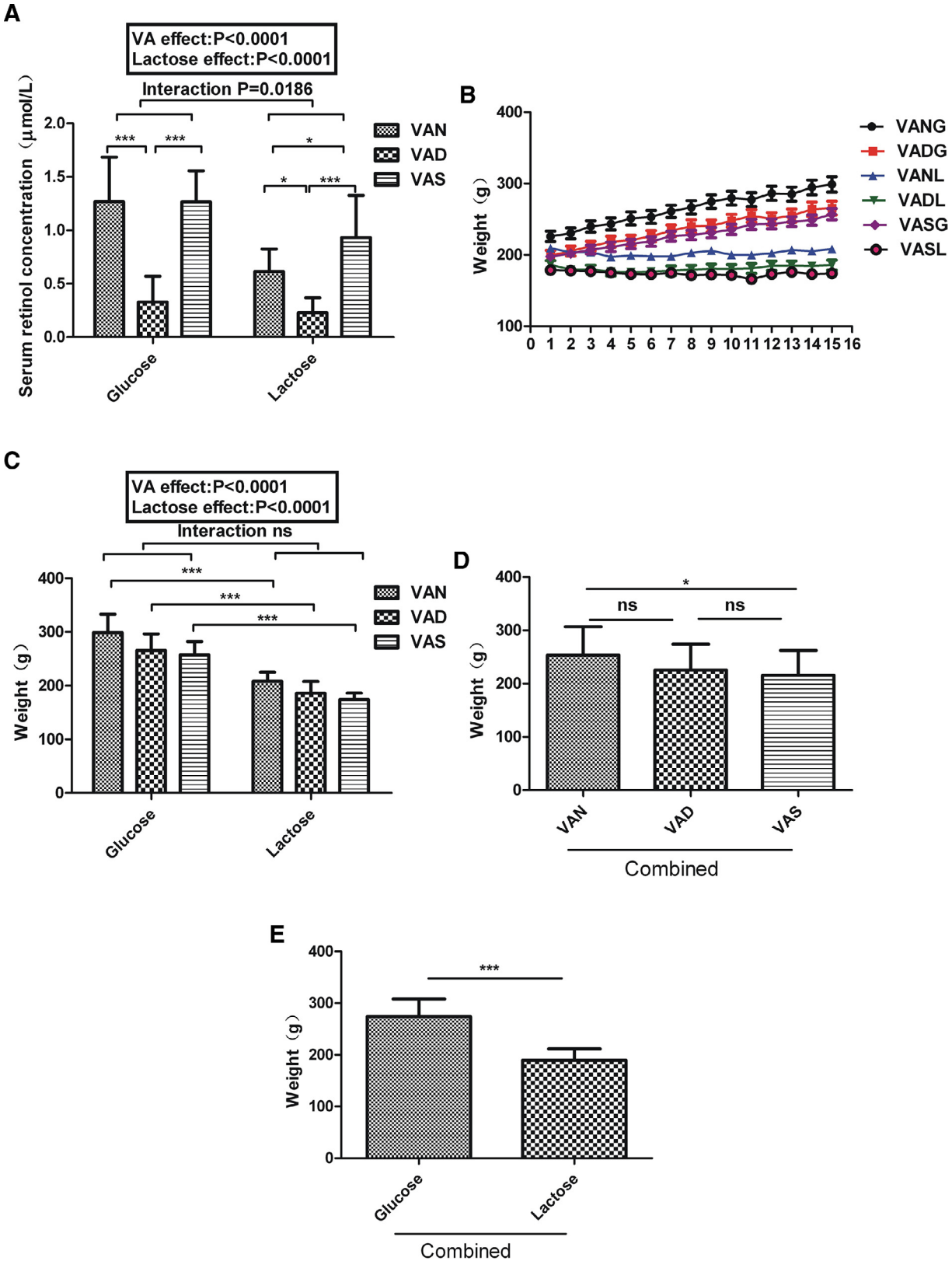

Fig. 4. Effects of different vitamin A nutrition levels and lactose treatment on serum retinol levels and weight changes after 15 d lactose. (A) Changes in serum retinol levels among the vitamin A normal diet (VAN), vitamin A deficient diet (VAD), and vitamin A supplementation (VAS) groups in the presence or absence of lactose (n = 10). (B) Dynamic change trend in daily weight among the VAN, VAD, and VAS groups in the presence or absence of lactose (n = 10). (C) Weight changes among the VAN, VAD, and VAS groups in the presence or absence of lactose (n = 10). (D) The main vitamin A effect, independent of lactose treatment, on weight changes among the combined VAN, VAD, and VAS groups (n = 20). (E) Lactose main effect, independent of vitamin A treatment, by weight between the combined glucose and lactose groups (n = 30). Mean ± standard deviation. Interaction indicates an effect of lactose versus null treatment. $^{*}P < 0.05$; $^{***}P < 0.001$; ns, not significant.

After combining the data of each group with or without the lactose treatment, the weights in the combined VAD group were not significantly different than those in the combined VAN and VAS groups, but the VAN weights were higher than those in the VAS group ($P < 0.05$; Fig. 4D). Simultaneously, the weights of rats in the combined lactose treatment group were significantly lower ($P < 0.001$) than those in the combined glucose group (Fig. 4E). Thus, lactose treatment effectively decreased the weight of rats, but the VAS did not improve the weights in the short term despite increasing serum retinol levels.

Vitamin A deficiency aggravated lactose-induced diarrhea, and vitamin A supplementation improved the severity of diarrhea in rats

The diarrhea index was used as an indicator of diarrhea severity. All diarrhea indices gradually increased over time in the VAN, VAD, and VAS groups after lactose (Fig. 5A), which suggests that the rat lactose-induced diarrhea model was successful. On day 7 of the diarrhea incidence (Fig. 5A), the diarrhea index plateaued, and on day 15, the degree of diarrhea did not decrease. Over the course of the 15 d, the total diarrhea indices in both the VANL and VASL groups were significantly lower than those in the VADL group ($P < 0.01$ and $P < 0.05$, respectively; Fig. 5B), but no significant difference was observed between the VANL and VASL groups. Thus, VAD increased the severity of diarrhea compared with that observed in rats that were fed a VAN diet, but VAS improved the extent of the diarrhea.

Gripping strength is an index of muscle force in rats. As shown in Figure 5C, the grip strengths of rats in the VAD and VAS groups were lower than those in rats of the VAN group in the absence of lactose ($P < 0.001$), but no significant difference was observed between the VAS and VAD groups. However, lactose treatment markedly reduced muscle strength in VAN, VAD, and VAS rats ($P < 0.001$), and no significant difference was observed among the three groups after lactose treatment (Fig. 5C). According to a Bonferroni post hoc analysis of the interaction between the lactose and null treatments, the P value was 0.0012. Therefore, both VAD and diarrhea decrease grip strength in rats.

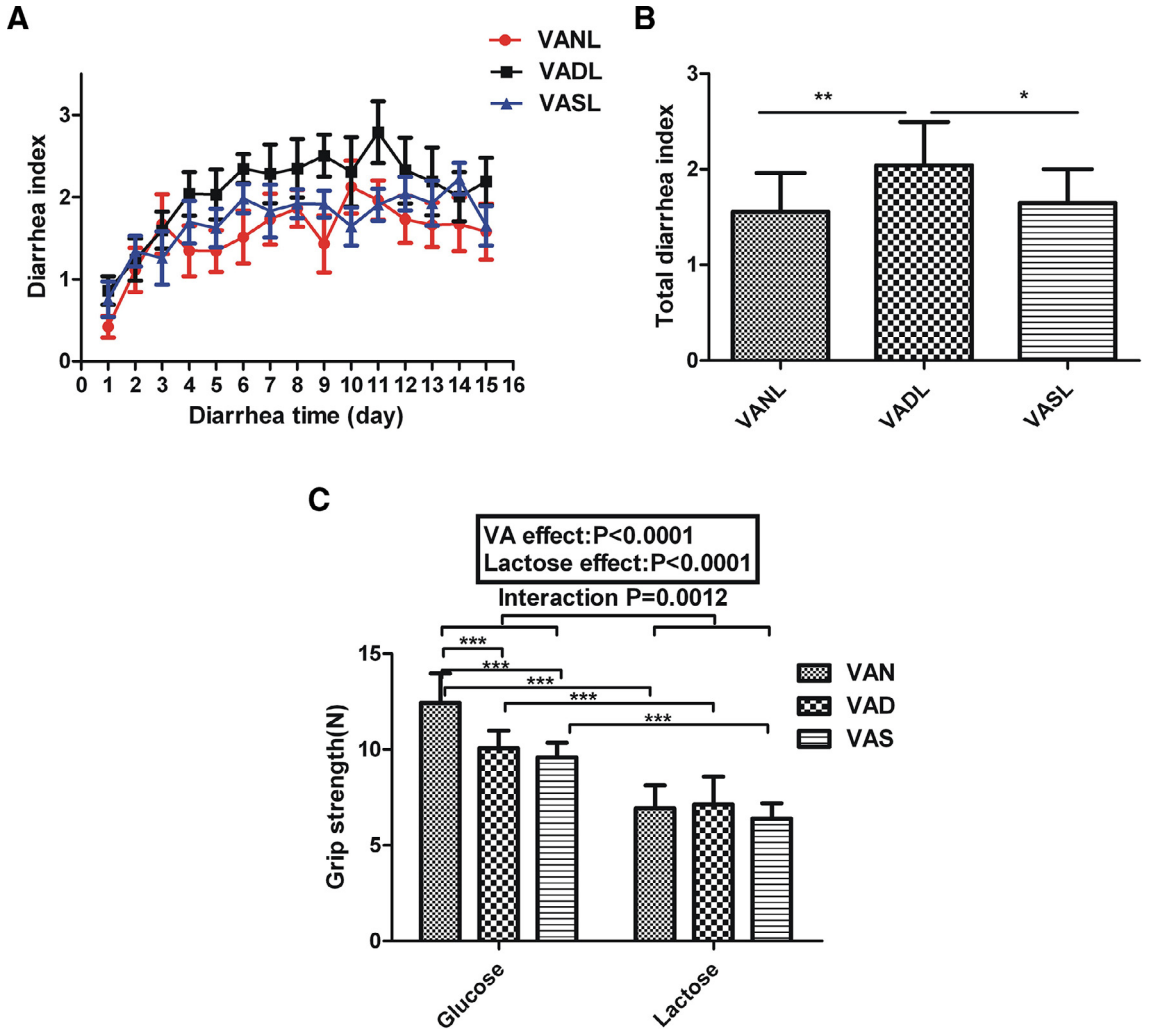

Fig. 5. Comparison of the diarrhea index in the three groups with diarrhea and grip strength among the six groups. (A) Dynamic change trend in the diarrhea index in rats that were fed the vitamin A normal diet with lactose (VANL; n=10), vitamin A deficient diet with lactose (VADL; n=10), and vitamin A supplementation with lactose (VASL; n = 10). (B) Comparison of total diarrhea index over 15 d in the VANL, VADL, and VASL diarrhea groups (n = 10). (C) Comparison of rat grip strength in the VAN, VAD, and VAS groups with or without lactose (n = 10). Mean ± standard deviation. Interaction indicates an effect of lactose versus null treatment. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Vitamin A deficiency-induced intestinal permeability in rats is alleviated by vitamin A supplementation

DAO is a highly active structural enzyme in the intestinal epithelium; thus, the level of serum DAO can reflect the status of intestinal barrier integrity. As shown in Figure 6A, serum DAO levels in VAD rats with or without lactose treatment were significantly higher than those in VAS rats, and lactose-induced diarrhea markedly increased the levels of DAO in both the VAN and VAD groups. According to the post hoc test, lactose and VA treatments had significant effects ($P < 0.0001$ and $P < 0.01$, respectively) on the concentration of serum DAO. However, after combining the lactose and null treatment groups, the concentration of serum DAO in the combined VAD group was higher than that in the combined VAS group ($P < 0.05$; Fig. 6B), and the concentration in the combined lactose treatment group was significantly higher than that in the combined no-lactose group ($P < 0.01$; Fig. 6C).

Zonulin is a master regulator of epithelium TJs and thus modulates the gut barrier. As shown in Figure 6D, the serum zonulin level in VAD rats was higher than that in VAN and VAS rats without lactose treatment ($P < 0.05$), and levels in the VAD group in the presence of lactose was higher than that in the VAS group ($P < 0.01$). The concentration of serum zonulin in the combined VAD group was significantly higher than that in the combined VAS and VAN groups ($P < 0.01$; Fig. 6E), but no significant difference was observed between the combined lactose and no-lactose groups (Fig. 6F). Therefore, the serum DAO level was mainly affected by lactose-induced diarrhea and the concentration of serum zonulin was mainly affected by VAD.

Vitamin A deficiency damaged small intestinal villi, and lactose-induced diarrhea aggravated this injury

To assess the effects of diarrhea and VAD on intestinal mucosa and the role of VAS, a histopathologic analysis of the small intestine (ileum) was performed. The small intestine villi in the VAD group were shorter than those in the VAN and VAS groups in the absence of lactose treatment (Fig. 7A). After 15 d lactose treatment, the villi of the small intestine in the VAN, VAD, and VAS groups were sparse and irregular compared with those in the untreated groups, and the villi in the VAD lactose group appeared to be the most seriously injured among the VAN, VAD, and VAS groups with lactose treatment (Fig. 7A).

Therefore, we quantified the intestinal villus surface area, and the data are shown in Figure 7B. Lactose ($P < 0.0001$) and VA ($P = 0.0001$) treatments had significant effects on the intestinal villus surface area (mm^2). The villus surface areas in the VAD group were significantly smaller than those in the VAN and VAS groups with or without lactose treatment ($P < 0.01$ and $P < 0.05$, respectively), and diarrhea markedly decreased the villus surface area ($P < 0.001$; Fig. 7B). The intestinal villus surface area in the combined lactose treatment group was significantly lower than that in the combined no-lactose group ($P < 0.001$; Fig. 7D), but no significant differences were observed among the combined VAN, VAD, and VAS groups even though the combined VAD group exhibited a decreasing trend (Fig. 7C). Therefore, both VAD and lactose treatment damaged the small intestinal villi, and this damage was rescued by VAS.

Vitamin A deficiency and diarrhea decreased the expression levels of tight junction proteins claudin-1, occludin, and ZO-1 upregulated by vitamin A supplementation

To explore the specific mechanisms by which VA enhances the gut barrier, we examined the expression levels of TJ proteins in the ileum of the small intestine. Claudin-1 and occludin are the major cytoskeletal proteins that form TJs between epithelial cells. The protein expression levels of claudin-1 and occludin in the VAN, VAD, and VAS groups after lactose treatment were notably downregulated compared with those in the untreated groups (Figs. 8A and B). Meanwhile, the expression levels of claudin-1 in the VASL group were significantly upregulated compared with those in the VADL groups, and the expression level of occludin in the VADL group was statistically downregulated compared with that in the VAN and VAS lactose groups (Figs. 8C and D).

According to a Bonferroni post hoc analysis of the interaction between the lactose and no-lactose treatments, the protein levels of claudin-1 and occludin were significantly repressed by lactose exposure in the VAN, VAD, and VAS groups compared with that in the group that did not receive lactose treatment ($P < 0.001$). Notably, VAS significantly upregulated the protein expression level of both claudin-1 and occludin compared with that in the VAD groups with or without lactose treatment, and the levels even exceeded the levels observed in the VAN groups in the presence or absence of lactose (Figs. 8C and D). According to a Bonferroni post hoc analysis of the interaction effect between the lactose and null treatments on the protein expression levels of claudin-1 and occludin, the P values were both <0.0001. Based on these data, we hypothesized that the expression levels of intestinal TJ proteins claudin-1 and occludin were doubly affected by VA nutritional status and lactose-induced diarrhea.

ZO-1 is a TJ protein that is located in cell membrane and cytoplasm. As shown in Figure 9A, the brown granules represent the positive expression of ZO-1 in the ileum of rats. The expression levels of ZO-1 in the VAN, VAD, and VAS groups after lactose treatment were significantly lower than those in the untreated groups ($P < 0.001$; Fig. 9B). Compared with the VAD group with or without lactose treatment, VAS significantly increased the expression of ZO-1 ($P < 0.05$; Fig. 9B). Lactose ($P < 0.0001$) and VA ($P = 0.0039$) treatments had significant effects on the expression level of ZO-1. However, the expression level of ZO-1 in the combined lactose treatment group was significantly lower than that in the combined no-lactose group ($P < 0.001$; Fig. 9D), and no significant differences were observed among the combined VAN, VAD, and VAS group (Fig. 9C), which suggests that lactose-induced diarrhea had a larger effect on the expression of ZO-1 in the small intestinal villi in rats than VA nutritional levels.

Discussion

Retinol is a major metabolite of VA in vivo and has become a diagnostic serum marker in many clinical applications. In our study, we successfully constructed VAD and VAN rat models after weaning, and these models were consistent with the international standard for VA levels in humans [7]. The body weights of VAD rats were significantly lower than those of rats in the VAN group due to VA malnutrition, which is consistent with previous studies that reported that serum retinol levels in children may affect growth likely through the regulation of growth hormone and thyroid-stimulating hormone beta genes [14–16]. In addition, according to our earlier study, daily VA intragastric administration for 7 d could effectively increase the level of serum retinol in VAD rats [17]. The 15-d VAS diet in the present study similarly increased serum retinol levels in VAD rats with or without diarrhea. Intriguingly, the level of serum retinol was affected by the interaction between dietary VA and lactose-induced diarrhea. Diarrhea is known to increase VA excretion and utilization and to decrease its absorption, thus reducing the level of retinol in the serum [18].

A study conducted in Peru investigated 72 children with diarrhea and 65 illness-free control subjects and revealed that serum

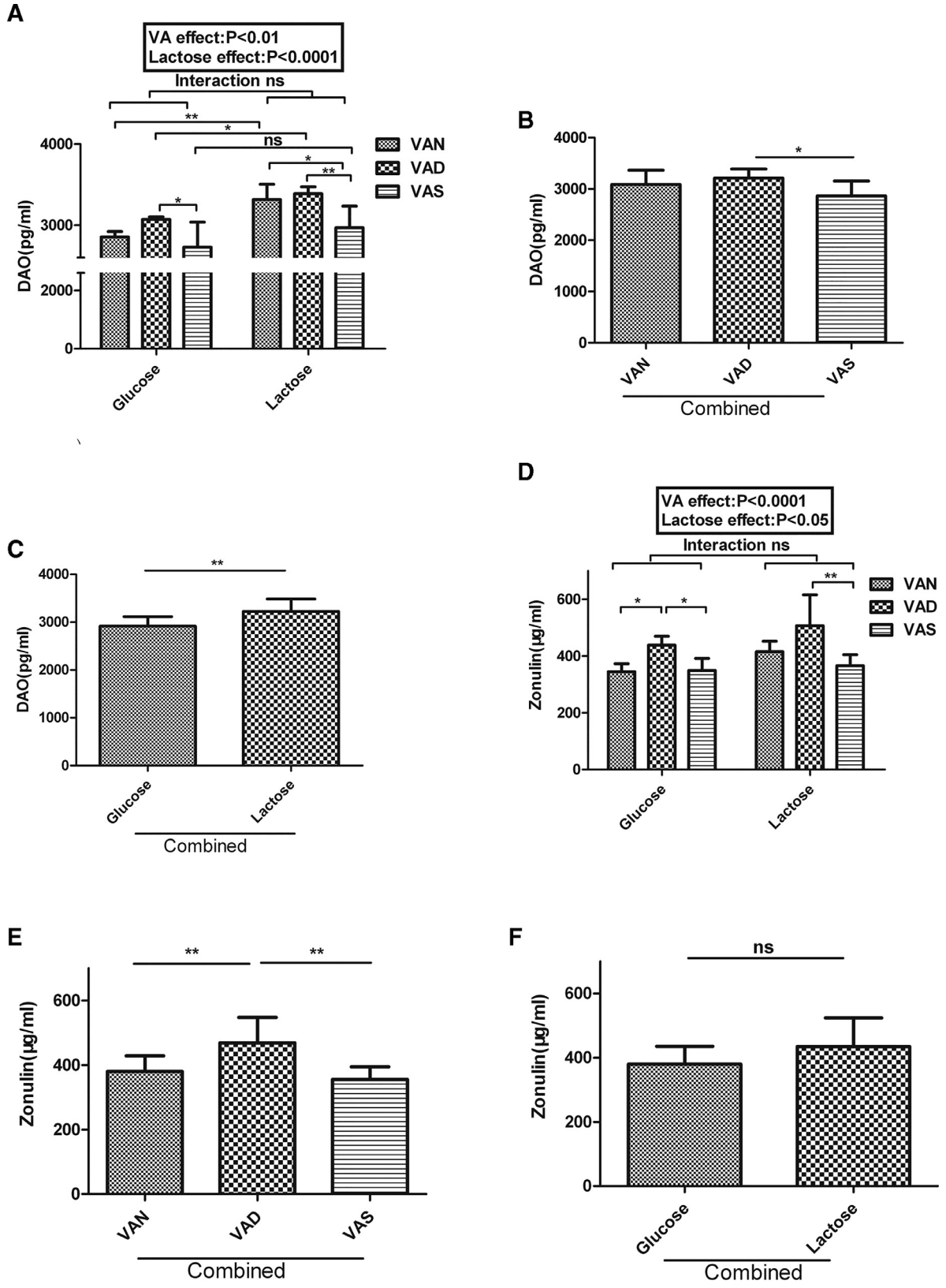

Fig. 6. Changes in (A) serum diamine oxidase (DAO) and (D) zonulin levels in rats that were fed the vitamin A normal (VAN), vitamin A deficient (VAD), and vitamin A supplementation (VAS) diets with or without lactose (n = 10). (B) Vitamin A effect, independent of lactose, on serum DAO concentrations in the combined VAN, VAD, and VAS groups (n = 20). (C) Significant lactose effect, independent of VA treatment, on serum DAO concentrations in the combined glucose and lactose groups (n = 30). (E) Significant vitamin A effect, independent of lactose treatment, on zonulin levels in serum in the three combined VAN, VAD, and VAS groups (n = 20). (F) Zonulin levels in the combined glucose and lactose groups (n = 30). Mean ± standard deviation. Interaction indicates an effect of lactose versus null treatment. ns, not significant in post hoc tests. $^{*}P < 0.05$; $^{**}P < 0.01$.

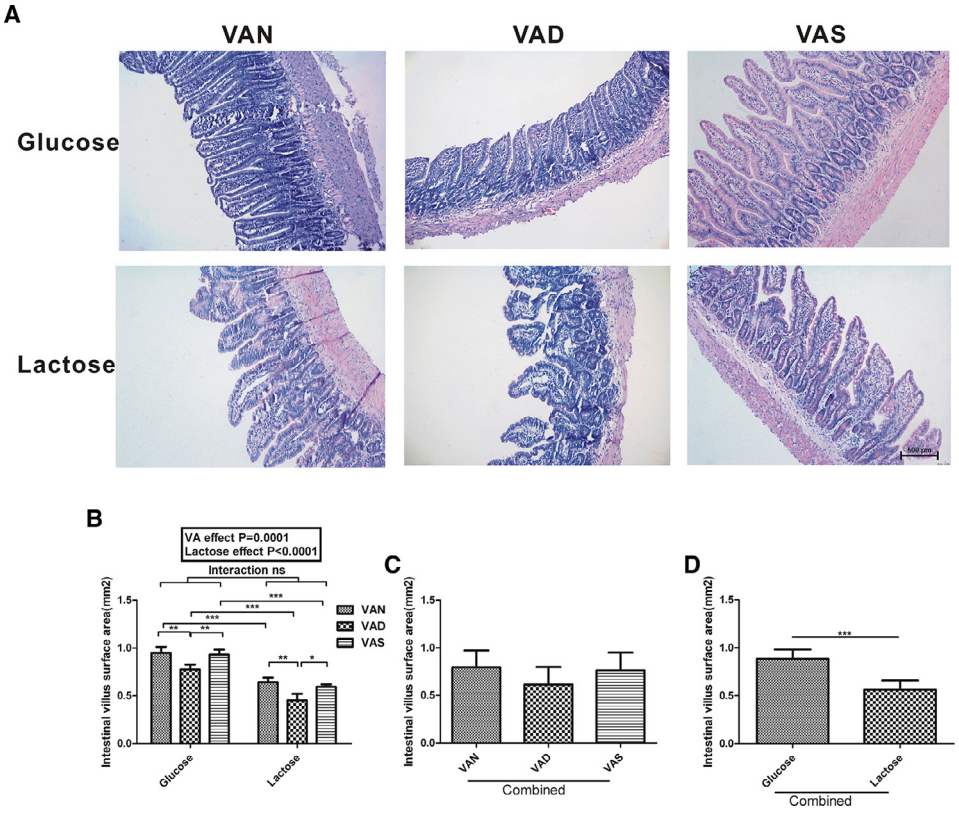

Fig. 7. Pathologic analysis of the small intestine (ileum) in the vitamin A normal diet (VAN), vitamin A deficient diet (VAD), and vitamin A supplementation (VAS) groups with or without lactose. (A) Histologic examination of the small intestine by hematoxylin and eosin staining (× 100). (B) Comparison of intestinal villus surface area in the VAN, VAD, and VAS groups with or without lactose. (C) Intestinal villus surface area in the combined VAN, VAD, and VAS groups. (D) Significant lactose effect, independent of vitamin A treatment, on the intestinal villus surface area in the combined glucose and lactose groups (n = 3). Mean ± standard deviation. Interaction indicates an effect of lactose versus null treatment. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$.

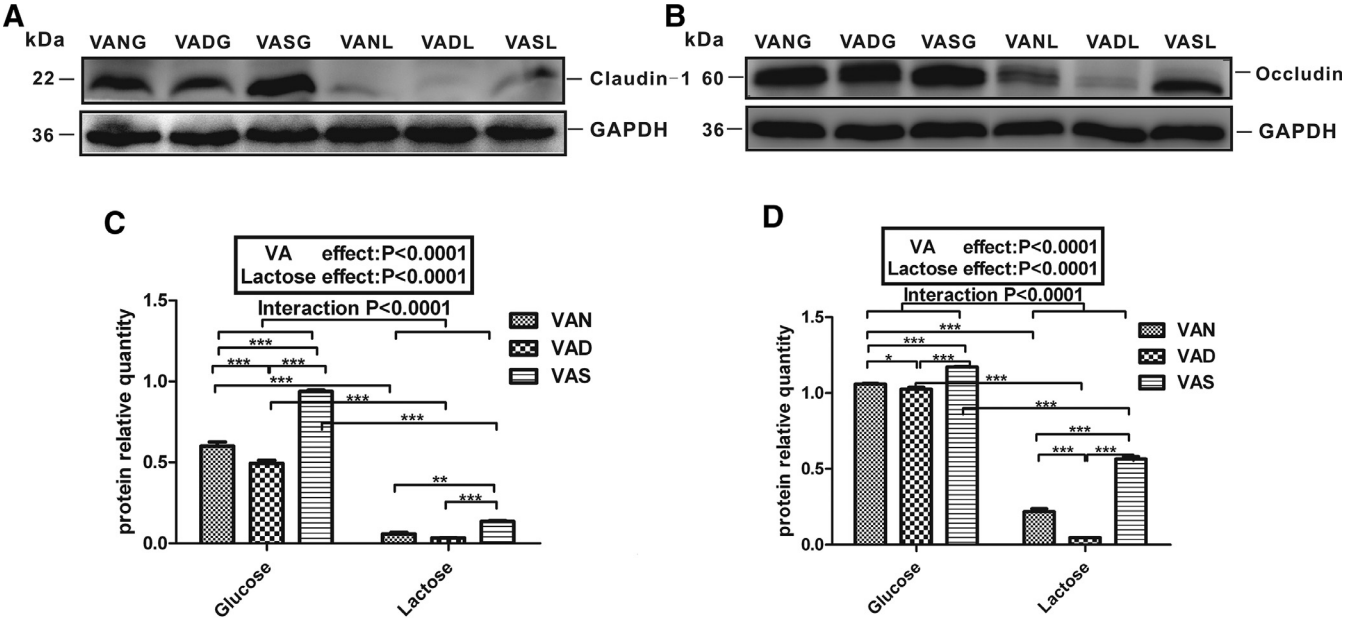

Fig. 8. Protein expression levels of (A) claudin-1 and (B) occludin in the vitamin A normal diet (VAN), vitamin a deficient diet (VAD), and vitamin A supplementation (VAS) groups with or without lactose. (C) Quantification of WB signals shown in (A). (D) Quantification of WB signals shown in (B) (n = 3). Mean ± standard deviation. Interaction indicates an effect of lactose versus null treatment.* $P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

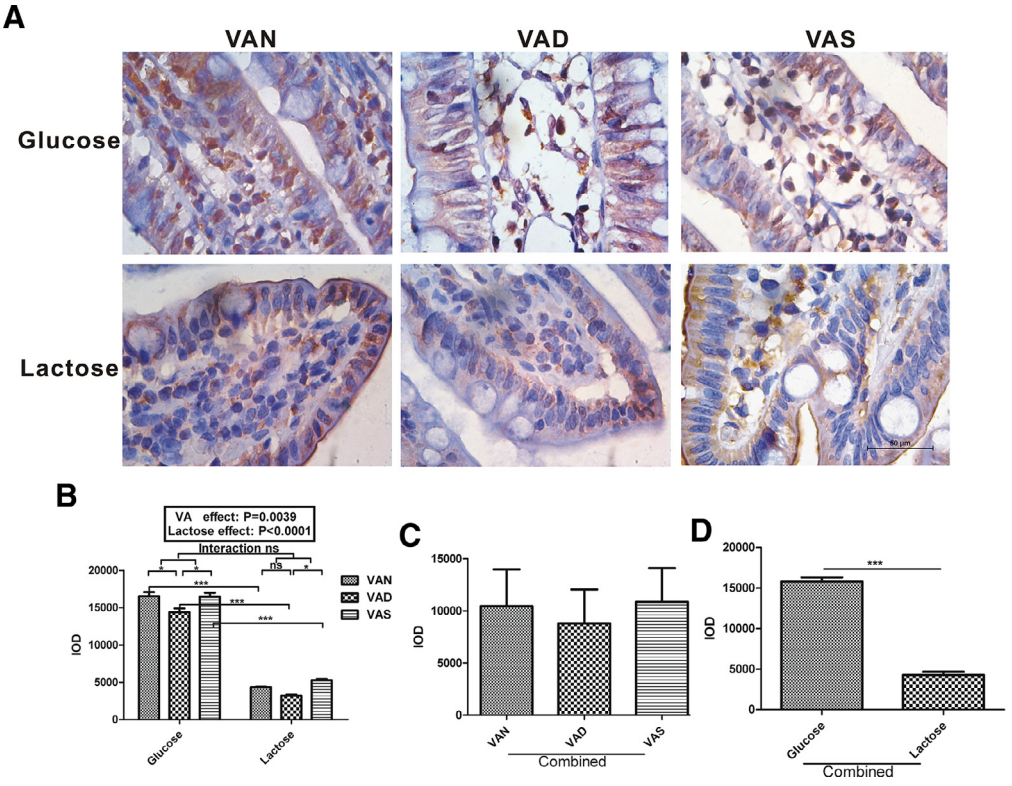

Fig. 9. Expression and distribution of the zonula-occludens 1 protein in the small intestinal (ileum) villi in rats in the vitamin A normal diet (VAN), vitamin a deficient diet (VAD), and vitamin A supplementation (VAS) groups in the presence or absence of lactose by immunohistochemistry staining (× 1000). (A) Immunostaining observation of zonula-occludens 1 protein expression in the small intestinal villi in the six groups. (B) Quantification of positive expression of the integral optical density (IOD) value shown in (A). (C) The levels of IOD in the combined VAN, VAD, and VAS groups. (D) Significant lactose effect, independent of vitamin A treatment, on the IOD in the combined glucose and lactose groups (n = 3). Mean ± standard deviation. Interaction indicates an effect of lactose versus null treatment. $^{*}P < 0.05$; $^{***}P < 0.001$.

retinol levels were significantly lower in children with diarrhea than those in the subjects without diarrhea [19]. Wang also showed that the average serum retinol level in children with PD was lower than that in healthy children ages <5 y in China [2]. Diarrhea can decrease serum retinol levels and slow growth [20,21]. Our results also demonstrated that diarrhea significantly decreased the body weight of VAN, VAD, and VAS rats. According to the post hoc test, no significant interaction exists between VA level and diarrhea with regard to body weight and lactose-induced diarrhea but not VA nutrition level was a primary factor that affects rat body weight. Notably, VAS did not increase body weight in rats with or without diarrhea, but retinol levels were increased by VAS. The following two reasons may explain these results: First, the observation time was only 15 d, which is too short to show the effects of VAS on weight. Second, lactose-induced diarrhea is a major factor that affects weight gain. Thus, short-term VAS has a negligible effect on weight.

The diarrhea index is an indicator of the severity of diarrhea, and the greater the diarrhea index, the more severe the diarrhea. In the present study, both the diarrhea index of dynamic changes during the 15 d and the total diarrhea index were markedly higher in the VADL group than those in the VAN and VAS lactose groups, which suggests that VA nutritional level plays a pivotal role in lactose-induced diarrhea. Our study [2] and another study [22] have shown that concurrent zinc and VAS is the optimal option to treat children ages <5 y with PD and can shorten the duration of PD and markedly improve nutritional status. Zinc supplementation for 10 to 14 d can reduce stool output, prevent weight loss, and promote early recovery [23]. Similar to weight changes in rats, the grip strength of rats in the VAS group with or without diarrhea was not significantly higher than that in VAD rats in the presence or absence of lactose treatment. We speculate that the main reason may be the absence of zinc supplementation. However, our data also suggest that rat grip strength was affected by the interaction between diarrhea and VA level. The World Health Organization suggests that a broader range of vitamins and minerals should be provided, including at least two recommended daily allowances of folate, VA, zinc, magnesium, and copper [1].

DAO is a highly active intracellular enzyme in the cytoplasm of upper chorial cells of the intestinal mucosa. In cases in which intestinal mucosal epithelial cells and barrier function are damaged, the release of DAO is increased in the plasma [23], which is a reliable marker of intestinal mucosal integrity [24–27]. A cross-sectional investigation of children with high rates of subclinical VAD showed that VAD is associated with decreased intestinal barrier function and increased intestinal mucosal permeability [28]. In our study, consistent with the aforementioned results and compared with the VAN group without lactose treatment, VAD slightly increased the level of serum DAO. However, lactose-induced diarrhea had a larger effect on the increase in DAO level.

Furthermore, the surface area of the small intestine villi was significantly decreased in the lactose treatment groups. Based on a study by Zhao et al. [24], the level of serum DAO is related to TJ proteins; thus, we investigated whether VA is a key factor that regulates intestinal permeability via TJ proteins. Zonulin is the only physiological mediator known to regulate intestinal permeability reversibly by intercellular TJs [29,30], and zonulin activates the expression levels of claudin, occludin, JAM, ZOs, and other proteins

by its corresponding receptor to modulate the intestinal mucosal barrier [31]. Our data showed that VAD significantly upregulated zonulin levels in the serum after combining the groups with or without diarrhea, which suggests that VA is a major regulator of serum zonulin. This result is consistent with our previous in vitro study in which all-trans-retinoic acid (ATRA) enhanced the transepithelial resistance of a Caco-2 cell monolayer and decreased zonulin release in the culture medium [32].

TJs consist of the transmembrane protein occludin and claudins and peripheral membrane proteins such as ZO-1, which is linked to the perijunctional cytoskeleton [33,34]. Occludin is a major component of TJs that binds claudin-l, claudin-2, and other peripheral membrane proteins to maintain TJ stability [35]. In a recent study, occludin knockdown was found to induce an increase in the paracellular permeability to macromolecules in intestinal Caco-2 cells and mouse intestines [36]. Our data demonstrated that the protein expression levels of occludin and claudin-1 in the ileum were affected by the interaction between dietary VA and diarrhea treatments. Notably, VAS effectively elevated low protein expression levels of occludin and claudin-1 caused by diarrhea. Simultaneously, we analyzed expression changes in the cytoskeletal protein ZO-1 using immunohistochemistry and found that the protein expression level of ZO-1 was mainly affected by lactose-induced diarrhea even though VAD significantly reduced the protein expression levels of ZO-1 in the presence or absence of lactose treatment.

A growing body of evidence suggests that the regulation of the epithelial barrier requires the mediation of VA. The expression levels of ZO-1 and occludin increase in the retinal pigment epithelium-choroid complex during myopia development, which may be regulated by retinoic acid [37]. Furthermore, the retinoic acid–mediated enhancement of barrier function is potentially associated with the increased expression of TJ-associated genes such as occludin, claudin-1, claudin-4, and ZO-1 [5,38]. Combining the results of the diarrhea index and pathologic observation in the VASL group, we speculate that VAS may regulate TJ proteins to enhance the intestinal barrier function. Therefore, future studies should focus on the mechanism by which VA regulates TJs in the intestinal mucosa.

Conclusions

Our findings demonstrate that VAD limits weight gain in rats and increases the severity of diarrhea. Serum retinol level, grip strength in rats, and the expression levels of TJ proteins claudin-1 and occludin are affected by the interaction effect of VA nutritional levels in diet and lactose-induced diarrhea. VAD aggravates the degree of diarrhea and intestinal mucosal damage during the course of diarrhea, but VAS increases serum retinol levels, decreases the diarrhea index, reduces zonulin and DAO levels in the serum, and enhances expression levels of TJ proteins occludin, claudin-1, and ZO-1. Therefore, VAS helps attenuate the severity of diarrhea and restricts intestinal damage by regulating the expression levels of TJ proteins.

Altogether, VA plays an important role in the protection and restoration of intestinal mucosa during the course of diarrhea. However, the specific clinical applications and identification of the best time point and dosage for VAS in the course of diarrhea need to be further explored.

Acknowledgments

The authors thank Shu Liu, Baolin Chen, Yuting Wang, et al for assisting with the study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nut.2018.06.007.

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