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RESEARCH ARTICLE

Marginal Vitamin A Deficiency Exacerbates Memory Deficits Following $A\beta_{1-42}$ Injection in Rats

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Abstract: ***Background:*** Although clinical vitamin A deficiency (VAD), which is a public health problem developing throughout the world, has been well controlled, marginal vitamin A deficiency (MVAD) is far more prevalent, especially among pregnant women and preschool children in China. Increasing evidence suggests that VAD is involved in the pathogenesis of Alzheimer's disease (AD). However, whether MVAD, beginning early in life, increases the risk of developing AD has yet to be determined.

Objective: The goal of this study was to investigate the long-term effects of MVAD on the pathogenesis of AD in rats.

Method: An MVAD model was generated from maternal MVAD rats and maintained with an MVAD diet after weaning. The males were bilaterally injected with aggregated amyloid β $(A\beta)_{1-42}$ into the CA3 area of the hippocampus, and the AD-associated cognitive and neuropathological phenotypes were examined.

Results: We found that MVAD feeding significantly aggravated $A\beta_{1-42}$-induced learning and memory deficits in the Morris water maze test. MVAD did not induce the mRNA expression of retinoic acid receptors (RARs), a disintegrin and metalloprotease 10 (ADAM10) or insulin-degrading enzyme (IDE) in $A\beta_{1-42}$-injected rats. Moreover, RARα and RARγ mRNA were positively correlated with ADAM10 mRNA, whereas RARβ mRNA was positively correlated with IDE mRNA.

Conclusion: Our study suggests that MVAD beginning from the embryonic period perturbs the AD-associated genes, resulting in an enhanced risk of developing AD.

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INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder that leads to dementia. Patients with AD typically display progressive memory loss and cognitive impairment. The neuropathology of AD is characterized by extracellular neuritic plaques, intracellular neurofibrillary tangles, synapse loss, glial cell activation and neuronal death. The neuritic plaques are primarily composed of amyloid β (Aβ) protein, which is derived from the sequential cleavage of amyloid β precursor protein (APP) *via* β-secretase and then γ-secretase. The abnormal accumulation of Aβ initiates neuronal dysfunction and plays an important role in AD pathogenesis. Aβ40 and Aβ42 are the major species of Aβ in the brain. Of these two species, Aβ42 is the more toxic form; furthermore, it is hydrophobic, prone to aggregation and predominant in neuritic plaques [1]. Under physiological conditions, the majority of APP is cleaved by α-secretase within its Aβ region to preclude Aβ generation [2-4]. The reduction of a disintegrin and metalloprotease 10 (ADAM10), the central component of α-secretase, promotes AD pathogenesis by shifting α-cleavage to β-cleavage of APP [5]. In addition, the dysfunction of the Aβ clearance mediated by Aβ-degrading enzymes (*e.g.,* insulin degrading enzyme; IDE) is crucial for the accumulation of Aβ in the AD brain [6].

Vitamin A (VA) is one of the most essential micronutrients in humans. In the central nervous system (CNS), VA and its active metabolite retinoic acid (RA) are involved in several essential biological processes including early development of brain structure and function, neuronal patterning,

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proliferation and differentiation, neurite outgrowth and synaptogenesis [7]. RA primarily exerts its biological effects by activating retinoic acid receptors (RARs) and/or retinoid X receptors (RXRs), which act on the retinoic acid responsive elements (RAREs) in the promoter regions of target genes [7]. Both RARs and RXRs have three subtypes: α, β and γ. Over 500 genes might be regulated by RA.

Vitamin A deficiency (VAD) is a serious public health problem throughout the developing world and is primarily caused by chronically low VA intake. VAD affects an estimated 190 million preschool children and 19.1 million pregnant women worldwide [8]. Evidence regarding the involvement of VAD in the pathogenesis of AD has increased. The first evidence was provided by studies that reported decreased serum VA and β-carotene in patients with AD [9-12]. A nutritional analysis of the risk factors for AD also revealed that low dietary VA or β-carotene is associated with an increased risk of AD [13]. Furthermore, the depletion of VA or the dysfunction of RARs results in Aβ deposition, impairment of hippocampal long-term synaptic plasticity and memory deficits in rodents; these effects can be restored *via* VA or RAR agonist replenishment [14-22]. However, the potential mechanisms between VAD and AD remain elusive. Currently, severe VAD has been largely brought under control; however, more widespread marginal states of deficiency have been revealed, designated as mild or marginal vitamin A deficiency (MVAD). MVAD primarily affects pregnant/lactating women and preschool children [23-25]. Our previous study found that MVAD is also prevalent among the elderly. Furthermore, Lahiri first proposed the Latent Early-life Associated Regulation (LEARn) model, suggesting that environmental risk factors (*e.g.,* dietary factors) perturb gene regulation in a long-term fashion, beginning at early developmental stages, which plays a vital role in the etiology of AD [26, 27]. Thus, we hypothesized that long-term MVAD, beginning early in life, might regulate the AD-associated gene and protein expression, thereby increasing the risk for developing AD.

The present study established an MVAD rat model from the embryonic period to the sacrifice. Aggregated $A\beta_{1\text{-}42}$ was injected into the hippocampus of these rats to investigate the effects of MVAD on AD pathogenesis. This study demonstrates aggravated hippocampus-dependent memory deficits in these MVAD rats following $A\beta_{1\text{-}42}$ injection. We subsequently examined the AD-associated gene expression and its correlation with RARs. Our work suggests that MVAD, beginning during the embryonic period, enhances the risk of developing AD.

MATERIAL AND METHODS

Animals and Diets

All Wistar rats used in this study were obtained from the Experimental Animals Center of Beijing, China. The rats were housed in a room with a constant airflow system, controlled temperature (22-24°C) and a 12-hour light/dark cycle. The rats were provided with food and water ad libitum. The female rats were randomly divided into a control group and an MVAD group and fed a VA-normal (VAN) diet (6,500 IU VA per kilogram of the basic diet) and an MVAD diet (400 IU VA per kilogram of the basic diet), respectively, for 4 weeks to generate maternal control and MVAD rats [28]. After confirming that the serum retinol levels matched the recommended standards for MVAD (0.70-1.05 μmol/L) and the VAN control (1.05-2.07 μmol/L) [29], the female rats were mated with the VAN males. After giving birth, the dams continued to be maintained on their respective diets. The male weanlings were subjected to experiment and were maintained throughout the study on a VAN or MVAD diet. This study was conducted in strict accordance with the recommendations of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The Animal Experimentation Ethical Committee of Chongqing Medical University in Chongqing, China, approved the protocol.

Aggregated $A\beta_{1\text{-}42}$ Preparation and Surgery

The preparation of the aggregated $A\beta_{1\text{–}42}$ and the brain stereotaxic surgery were performed as previously described [30]. $A\beta_{1\text{-}42}$ (Sigma-Aldrich, USA) was dissolved in 0.01 M phosphate-buffered saline (PBS), pH 7.4, and then vigorously agitated (200 rpm) at room temperature for 36 hours. The $A\beta_{1\text{-}42}$ peptide was precipitated *via* centrifugation at 15,000×*g* for 10 minutes. Sediments were dissolved with PBS to a 10^{-4} M stock, aliquoted, and stored at -80°C.

After the baseline Morris water maze tests were conducted with 18-week-old pups, the pups in both the control and MVAD groups were randomly divided into the Aβ group (full dose of aggregated $A\beta_{1\text{-}42}$), the 1/2Aβ group (half dose of aggregated $A\beta_{1\text{-}42}$) and the vehicle group. All subjects were anaesthetized, and the injections were made bilaterally into the CA3 area of the dorsal hippocampus with the injectates deposited slowly over 10 minutes. The stereotaxic coordinates were 2.6 mm lateral and 3.5 mm posterior to the bregma, and 3.7 mm ventral. The injections were 5.0 μl of $1x10^{-4}$ M aggregated $A\beta_{1\text{-}42}$ suspension, 5.0 μl of $0.5x10^{-4}$ M aggregated $A\beta_{1\text{-}42}$ suspension and 5.0 μl of PBS for the Aβ group, the 1/2Aβ group and the vehicle group, respectively.

Serum Retinol Assay

The maternal rats at preconception and five to six pups from each group at postnatal 24 hours, 4 weeks, 8 weeks, 12 weeks, 18 weeks, 22 weeks and 26 weeks were randomly selected to detect serum retinol levels. The pups younger than 8 weeks old were decapitated to collect the blood; and the blood from the pups at the age of 8 weeks and older were collected *via* the caudal vein. All pups were decapitated after the last behavioral tests to detect serum retinol levels and confirm the success of the animal models. Serum retinol was measured using high performance liquid chromatography (HPLC) as previously described [31]. Briefly, 200 μl of serum was deproteinized with an equal volume of dehydrated alcohol. The retinol was extracted from the serum by adding 1,000 μl of hexane and evaporated with nitrogen gas. The retinol residue was dissolved in 100 μl of the mobile phase mixture (methanol:water=97:3). Then, 20 μl of the dissolved liquid was tested in the HPLC apparatus (DGU-20As, Shimadzu Corporation, Kyoto, Japan) on a silica column with a 315-nm ultraviolet photodiode array detector. All procedures were performed in a dark room to protect the samples from

light at the Children's Nutrition Research Center in the Children's Hospital of Chongqing Medical University.

Real-time Polymerase Chain Reaction (RT-PCR)

All pups were sacrificed after the last behavioral tests. Total RNA was extracted from the hippocampus of rats using TRI Reagent (Sigma) and reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (TaKaRa) according to the manufacturer's instructions. Real-time PCR was conducted using a SYBR Premix Ex Tag (Perfect Real Time; TaKaRa) and a Bio-Rad Real-Time PCR system. The primer sequences for RARα, RARβ, RARγ, ADAM10, IDE and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are listed in Table **1**. The gene expression levels of all genes were normalized to GAPDH.

Table 1. Primer sequences for the genes of rats.

Gene name	Sequences	PCR products
RARα	F: 5'- CAGGAGGGAGAAGGCAGTGAC -3' R: 5'- ATGGCTTGAGTTCGGAGGACAG -3'	200 bp
RARβ	F: 5'- ACAATGCTGGCTTCGGTCCTC -3' R: 5'- CTCAAGGTCCTGGCGGTCTC -3'	137 bp
RARγ	F: 5'- CTGACCCTGAACCGAACCCA -3' R: 5'- TCCACAGATGAGGCAGATAGCA -3'	144 bp
ADAM10	F: 5'- GCAGTTCAATGGTCGGACTATCAC -3' R: 5'- GAGCCACAATCCACTCAGCAATG -3'	179 bp
IDE	F: 5'- CTGCTTCCGGTCTCATCTTGCC -3' R: 5'- GCATAGCCACGCTTGTTCTTGAAG -3'	200 bp
GAPDH	F: 5'- CAAGTTCAACGGCACAGTCAA -3' R: 5'- TGGTGAAGACGCCAGTAGACTC -3'	149 bp

RARα, retinoic acid receptor α; RARβ, retinoic acid receptor β; RARγ, retinoic acid receptor γ; ADAM10, a disintegrin and metalloprotease 10; IDE, insulin degrading enzyme; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Morris Water Maze Test

Learning and memory at baseline was examined *via* the Morris water maze tests at 18 weeks of age, and the rats were re-tested at 30 days and 80 days after the injection of aggregated $A\beta_{1\text{-}42}$ to evaluate their spatial learning and memory abilities as previously described [32]. The test was performed in a 1.5-m (in diameter) pool with a 10-cm (in diameter) platform. The procedure consisted of 1 day of visible platform tests and 4 days of hidden platform tests, plus a probe trial 24 hours after the last hidden platform test. In the visible platform test performed on the first day, the rats were tested for 5 continuous trials, with an inter-trial interval of 60 minutes. For the hidden platform tests, the platform was placed in the southwest quadrant of the pool. Rats were trained for 5 trials, with an inter-trial interval of 60 minutes. Each rat was allowed 60 seconds to search for the platform. If the rat was unable to find the platform, then it was placed on the platform and allowed to stay for 20 seconds. On the last day of the test, each rat was subjected to the probe trial in which the platform was removed. Each rat was given 60 seconds to locate where the platform was originally placed. Animal movement was tracked and recorded using ANY-maze tracking software (Stoelting).

Statistical Analysis

All results are presented as the means ± SEMs. Serum retinol data between the MVAD and control groups prior to $A\beta_{1\text{-}42}$ injection were analyzed by Student's *t* test. After $A\beta_{1\text{-}42}$ injection, either the serum retinol data or mRNA data were analyzed with the general linear model univariate ANOVA for the main effects of diet (control or MVAD), $A\beta_{1\text{-}42}$ injection (vehicle, 1/2Aβ or Aβ), and their interactions (two-way ANOVA); all significant main effects were further analyzed using Bonferroni post hoc tests. Behavioral data were analyzed by a two-way ANOVA with repeated measures, with diet as the between-subjects factor and learning day as the within-subjects factor. Correlation analyses between the ADAM10/IDE and RARs mRNA levels were conducted by calculating the Pearson's correlation coefficient (r). All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 17.0) software. Differences were considered significant at $P<0.05$. Graphs were built in GraphPad Prism 5.0.

RESULTS

MVAD Exacerbates Memory Deficits in $A\beta_{1\text{-}42}$-injected Rats

To investigate the effects of embryonic-onset MVAD following an $A\beta_{1\text{-}42}$ injection on spatial learning and memory, we first generated a maternal MVAD model by feeding female adult rats an MVAD diet for 4 weeks. Compared with control rats, the serum retinol levels were significantly reduced in the MVAD maternal rat model (0.79±0.07 μmol/L vs. 1.33±0.20 μmol/L, $P<0.05$; Fig. **1A**). After confirming that the maternal model was established, the females were mated with normal males, and the male offspring were used for the subsequent experiments. The serum retinol levels of the pups at 4, 8 and 12 weeks of age were significantly lower in the MVAD group than in the control animals ($P<0.05$; Fig. **1B**). After a half- or full-dose injection of aggregated $A\beta_{1\text{-}42}$ (1/2Aβ or Aβ), only significant main effects of diet ($P<0.05$; Fig. **1C**) without a main effect of Aβ or a significant diet×Aβ interaction were observed at all ages of 18, 22 and 26 weeks ($P>0.05$; Fig. **1C**), indicating that the $A\beta_{1\text{-}42}$ injection did not significantly affect the serum retinol content while the MVAD diet markedly lowered the serum retinol levels. The rats were sacrificed immediately after the last behavioral tests at 80 days post-aggregated $A\beta_{1\text{-}42}$ injection, and the serum retinol levels were measured. A two-way ANOVA revealed a significant main effect of diet in the absence of a main effect of Aβ or a significant diet×Aβ interaction (diet: $F_{(1,59)}=95.47$, $P<0.001$; Aβ: $F_{(1,59)}=2.59$, $P>0.05$; diet×Aβ: $F_{(2,59)}=0.72$, $P>0.05$). Post hoc Bonferroni tests indicated that serum retinol levels were significantly reduced in PBS-, 1/2Aβ- and Aβ-injected MVAD rats compared with those of their respective controls ($P<0.001$; Fig. **1D**). Since there was no significant difference in serum retinol levels among the PBS, 1/2Aβ and Aβ rats, we combined the PBS, 1/2Aβ and Aβ groups together for statistical analysis. The results further confirmed that MVAD treatment decreased the serum retinol levels compared with those of the control group (0.94±0.05 μmol/L *vs.* 1.20±0.053 μmol/L,

P<0.01; Fig. **1E**). These data demonstrate that the MVAD model, which began during the embryonic period, was successfully established. We also detected the $A\beta_{1\text{-}42}$ levels in the hippocampus of both MVAD and control groups after the last behavioral tests at 80 days post-$A\beta_{1\text{-}42}$ injection. Western blot analysis showed that $A\beta_{1\text{-}42}$ expression was significantly elevated after 1/2Aβ or Aβ injection compared with the PBS vehicle injection in either the MVAD or control rats (P<0.01; Supplemental Fig. **S1**), confirming that $A\beta_{1\text{-}42}$ was successfully injected.

To determine whether MVAD affects spatial learning and memory, Morris water maze tests were used for both the MVAD and control pups after an aggregated $A\beta_{1\text{-}42}$ injection. First, we examined learning and memory at baseline (prior to the $A\beta_{1\text{-}42}$ injection at 18 weeks old). On the visible platform tests, all rats exhibited similar escape latencies and path lengths (data not shown), indicating that MVAD did not affect the rats' mobility or vision. On the hidden platform tests, the MVAD pups exhibited similar escape latencies relative to the control group (P>0.05; Fig. **2A**). At 30 days post-injection of vehicle PBS or 1/2Aβ, no significant differences were found between the MVAD and control groups with regard to escape latency (P>0.05; Fig. **2B**, **left** and **middle**), whereas Aβ-injected MVAD rats showed a significantly prolonged escape latency compared with that of their respective controls (P<0.01; Fig. **2B**, **right**). However, 80 days after the injection of vehicle PBS and 1/2Aβ, the MVAD pups showed markedly longer escape latencies than those of controls (P<0.01; Fig. **2C**, **left** and **middle**), whereas the escape latency of MVAD rats was similar to that of controls after the Aβ injection (P>0.05; Fig. **2C**, **right**). These results suggest that MVAD significantly exacerbated the cognitive deficits induced by a half dose of $A\beta_{1\text{-}42}$ during late stages of rat development and that a full dose of $A\beta_{1\text{-}42}$ could accelerate this aggravation.

MVAD Suppresses $A\beta_{1\text{-}42}$-induced ADAM10 Expression *Via* RARα/γ and IDE Expression *Via* RARβ in the Hippocampus of Rats

To further investigate the effect of MVAD on APP processing enzymes, we examined the hippocampal gene expression of RARs, including RARα, RARβ and RARγ, as well as the AD-associated gene expression of ADAM10 and IDE 80 days after the injection of $A\beta_{1\text{-}42}$. A two-way ANOVA of RARα, RARβ, RARγ and ADAM10 revealed significant main effects of diet, Aβ and diet×Aβ interaction (P<0.05; Fig. **3A-D**). Furthermore, significant main effects of Aβ and diet×Aβ interaction (P<0.05) in the absence of a significant main effects of diet were observed for IDE (P>0.05; Fig. **3E**). In addition, post hoc Bonferroni tests showed that a full dose of $A\beta_{1\text{-}42}$ injection appeared to dramatically upregulate the mRNA levels of RARα, RARγ and ADAM10 in the control group (P<0.001); however, upregulation occurred to a much lesser extent in the MVAD group with no significant

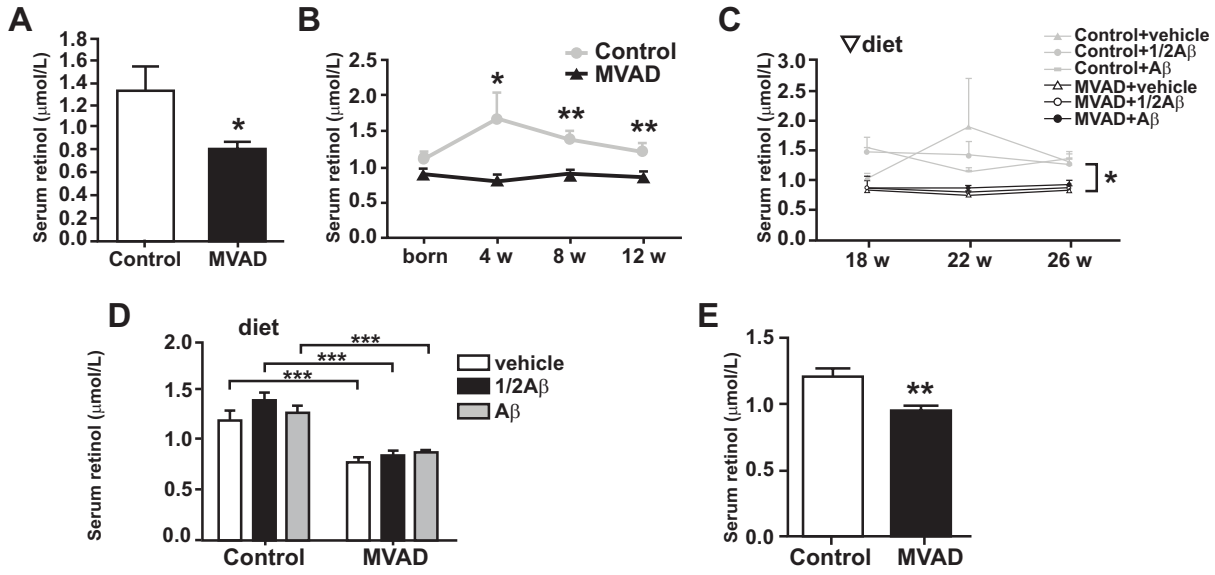

Fig. (1). MVAD reduces serum retinol levels during all life stages and at post-injection of $A\beta_{1\text{-}42}$ in rats. (A) At preconception, the serum retinol levels of the maternal rats in the MVAD group were significantly lower than those in the control group. n=6, $^{*}P$<0.05 by Student's t test. **(B)** At 24 hours, 4 weeks, 8 weeks and 12 weeks postnatal, the serum retinol levels of the pups in the MVAD group were significantly reduced compared with those of the control group. n=5 or 6, $^{*}P$<0.05 and $^{**}P$<0.01 at the same age by Student's t test. **(C)** After vehicle PBS, 1/2Aβ or Aβ injection, the serum retinol levels of the pups in the MVAD group at 18 weeks, 22 weeks and 26 weeks postnatal were significantly reduced compared with those of the control group. n=5 or 6, ∇significant effects (P<0.05 in all cases), $^{*}P$<0.05 at the same age by two-way ANOVA. **(D)** At 80 days post-injection of vehicle PBS, 1/2Aβ or Aβ, the rats were sacrificed immediately after behavioral tests, and the retinol levels in the serum were measured. The serum retinol levels of the MVAD pups after vehicle PBS, 1/2Aβ or Aβ injection were significantly lower than those of the control group. Control group: $n_{vehicle}$=8, $n_{1/2A\beta}$=12, and $n_{A\beta}$=15; MVAD group: $n_{vehicle}$=8, $n_{1/2A\beta}$=12, and $n_{A\beta}$=10; ∇significant effects (P<0.05 in all cases), $^{***}P$<0.001 by two-way ANOVA with Bonferroni post hoc tests. **(E)** The serum retinol levels in the combined groups of MVAD pups were significantly decreased compared with those in the combined groups of control pups. $n_{Control}$=22, n_{MVAD}=17, $^{**}P$<0.01 by Student's t test. All values indicate means ± SEMs. 1/2Aβ, half dose of aggregated $A\beta_{1\text{-}42}$; Aβ, full dose of aggregated $A\beta_{1\text{-}42}$; MVAD, marginal vitamin A deficiency.

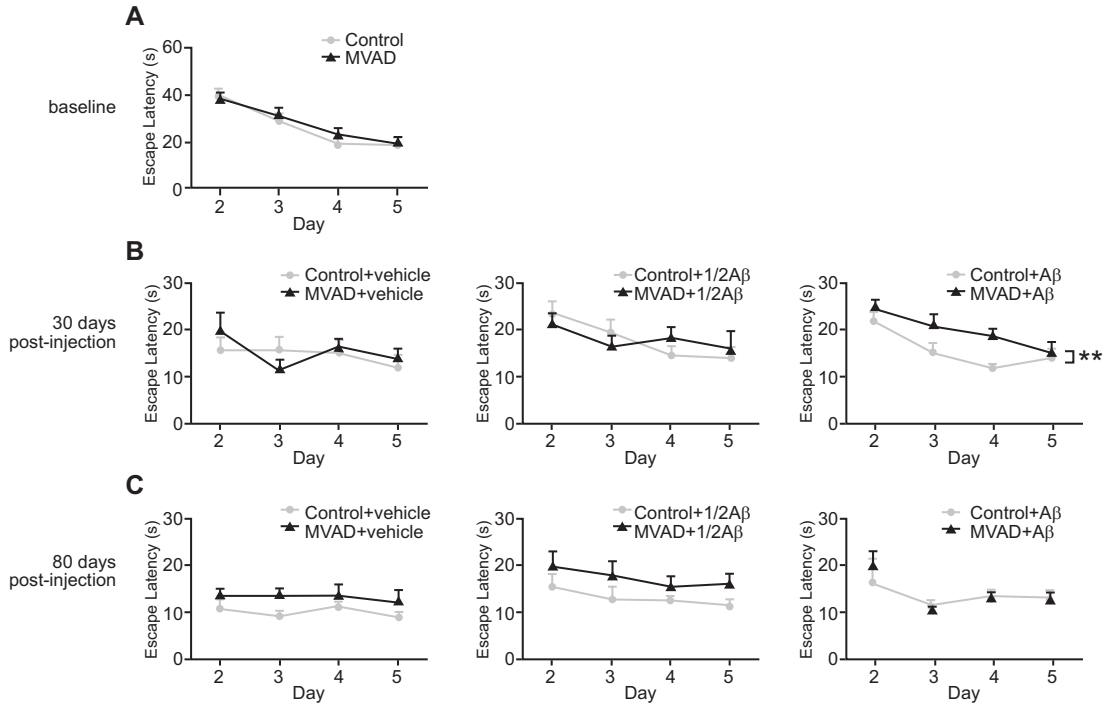

Fig. (2). MVAD with $A\beta_{1\text{-}42}$ injection exacerbates memory deficits in rats. Both the MVAD and control rats were subjected to the Morris water maze test at 18 weeks of age and at 30 and 80 days after vehicle PBS, 1/2Aβ or Aβ injection. **(A)** At 18 weeks of age, prior to $A\beta_{1\text{-}42}$ injectionat baseline, the MVAD pups showed similar escape latencies relative to those of the control group. $n_{Control}$=21, n_{MVAD}=21, P>0.05 by two-way ANOVA with repeated measures. **(B)** At 30 days post-injection of vehicle PBS (left) or 1/2Aβ (middle), no significant differences in escape latencies were observed between the MVAD and control rats; however, the MVAD rats showed a prolonged escape latency compared with the control rats at 30 days post-injection of Aβ (right). **(C)** At 80 days post-injection of vehicle PBS (left) or 1/2Aβ (middle), the MVAD rats showed a longer escape latency than the control rats, whereas similar escape latencies were found between the MVAD and control rats after the Aβ injection (right). Control group: $n_{vehicle}$=5, $n_{1/2A\beta}$=6, and $n_{A\beta}$=10; MVAD group, $n_{vehicle}$=6, $n_{1/2A\beta}$=9, and $n_{A\beta}$=6; $^{*}P$<0.05 and $^{**}P$<0.01 by two-way ANOVA with repeated measures. All values are expressed as the means ± SEMs. 1/2Aβ, half dose of aggregated $A\beta_{1\text{-}42}$; Aβ, full dose of aggregated $A\beta_{1\text{-}42}$; MVAD, marginal vitamin A deficiency.

differences (P>0.05; Fig. **3A-C**). In addition, the mRNA levels of both RARβ and IDE were increased by the half dose of $A\beta_{1\text{-}42}$ but reduced by the full dose of $A\beta_{1\text{-}42}$ in control rats (P<0.01); however, no significant differences were observed in the MVAD rats following $A\beta_{1\text{-}42}$ injection (P>0.05; Fig. **3D** and **E**).

Pearson's correlation analysis showed that ADAM10 mRNA was positively correlated with both RARα and RARγ mRNA ($r_{RAR\alpha*ADAM10}$=0.9946, P<0.0001; $r_{RAR\gamma*ADAM10}$ = 0.9964, P<0.0001; Fig. **4A** and **B**). A positive correlation was also found between RARα and RARγ mRNA ($r_{RAR\alpha*RAR\gamma}$=0.9951, P<0.0001; Fig. **4C**), whereas no significant correlation was found between ADAM10 and RARβ mRNA levels ($r_{RAR\beta*ADAM10}$=-0.2444, P=0.13**)**. Furthermore, IDE mRNA was positively correlated with RARβ mRNA ($r_{RAR\beta*IDE}$=0.6780, P<0.0001; Fig. **4D**) but negatively correlated with both RARα and RARγ mRNA ($r_{RAR\alpha*IDE}$=-0.5118, P<0.001; $r_{RAR\gamma*IDE}$=-0.5152, P<0.001; Fig. **4E** and **F**). These findings suggest that MVAD suppressed Aβ-induced ADAM10 expression through RARα and RARγ and IDE expression through RARβ.

DISCUSSION

VA is an essential micronutrient throughout the life span of mammals. VA and its derivatives, the retinoids, play vital roles in the development, regeneration and maintenance of the CNS in both developing and mature brains. Despite increasing evidence suggests that VA nutrition is related to neurodegenerative diseases including AD, the mechanisms of VAD and MVAD in the pathogenesis of AD remain unclear.

In the developing countries, the dietary pattern is primary an insufficient amount of dominated by grain and vegetables, with meat and VA-rich foods. VAD or MVAD is primarily caused by chronically low VA intake. Furthermore, MVAD is far more prevalent than VAD; however, it has been grossly neglected because its clinical symptoms are obscure. In many countries, including China, postnatal rather than prenatal supplementation is recommended for cases of VAD to avoid the teratogenic effect of excess VA during pregnancy. This convention has resulted in widespread MVAD among pregnant women and preschool children. Our previous work also revealed that twice as many MVAD cases than VAD cases exist in the elderly (25.8% *vs.* 13.3%). Though highly prevalent of MVAD among these people, human studies on MVAD during pregnancy and the child's development are difficult to be conducted. Thus, the present study established the MVAD rat model by partially depriving VA in diets from preconception to the sacrifice of offspring, which was considered to be a long-term status of MVAD.

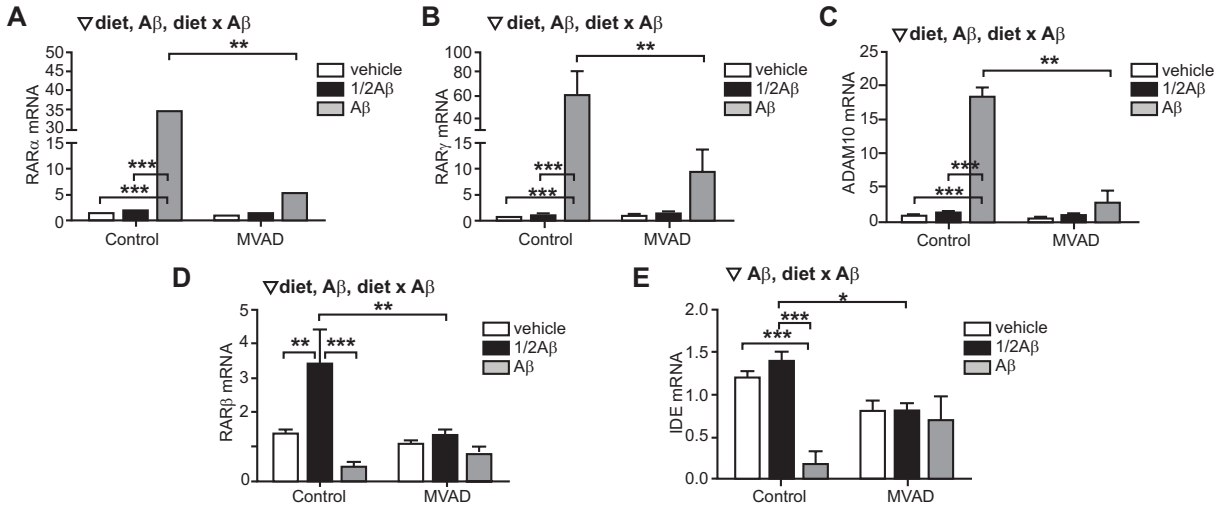

Fig. (3). MVAD suppresses the Aβ-induced mRNA levels of RARα/β/γ, ADAM10 and IDE in the hippocampus of rats. The rats were sacrificed immediately after behavioral tests at 80 days post-injection of vehicle PBS, 1/2Aβ or Aβ, and the mRNA levels of RARα **(A),** RARβ **(D),** RARγ **(B),** ADAM10 **(C)** and IDE **(E)** were detected in the hippocampus of both the MVAD and control groups. **(A-C)** Aβ injection dramatically induced RARα **(A),** RARγ **(B)** and ADAM10 **(C)** mRNA levels in the control group, whereas the increased levels of these mRNAs induced by Aβ were not significant in the MVAD group. **(D and E)** RARβ **(D)** and IDE **(E)** mRNA levels were increased by 1/2Aβ and decreased by Aβ in control rats; however, no significant difference was observed in these mRNA levels among the vehicle PBS, 1/2Aβ and Aβ groups with regard to the MVAD rats. Control group: $n_{vehicle}$=8, $n_{1/2A\beta}$=8, and $n_{A\beta}$=10; MVAD group: $n_{vehicle}$=8, $n_{1/2A\beta}$=9, and $n_{A\beta}$=8; ∇significant effects (P<0.05 in all cases), $^{*}P$<0.05, $^{**}P$<0.01 and $^{***}P$<0.001 by two-way ANOVA with Bonferroni post hoc tests. Values indicate means ± SEMs. 1/2Aβ, half dose of aggregated $A\beta_{1-42}$; Aβ, full dose of aggregated$A\beta_{1-42}$; ADAM10, a disintegrin and metalloprotease 10; IDE, insulin degrading enzyme; RARα, retinoic acid receptor α; RARβ, retinoic acid receptor β; RARγ, retinoic acid receptor γ.

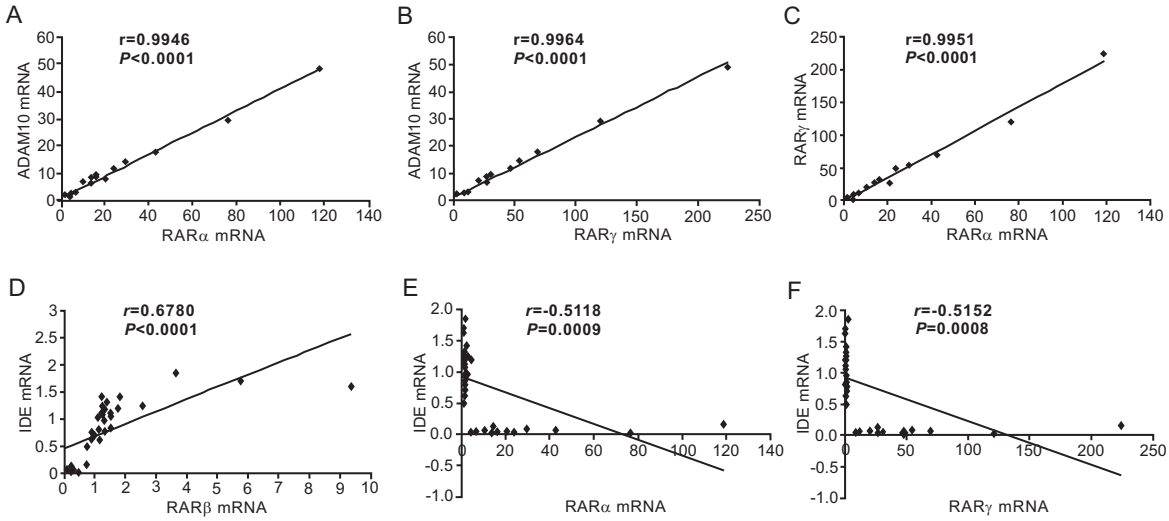

Fig. (4). Pearson's correlations between ADAM10/IDE mRNA and RARα/β/γ mRNA in the hippocampus of rats. Both the MVAD and control rats were sacrificed immediately after behavioral tests at 80 days post-injection of vehicle PBS, a half dose (1/2Aβ) or full dose (Aβ) of aggregated $A\beta_{1-42}$, and the mRNA levels of RARα**,** RARβ**,** RARγ**,** ADAM10 and IDE were detected in their hippocampus. n=39 total: $n_{vehicle}$=4, $n_{1/2A\beta}$=8, and $n_{A\beta}$=10 in Control group; $n_{vehicle}$=3, $n_{1/2A\beta}$=9, and $n_{A\beta}$=5 in MVAD group. Pearson's correlation analysis was performed. **(A and B)** ADAM10 mRNA levels were positively correlated with both RARα **(A)** and RARγ **(B)** mRNA levels. $r_{RAR\alpha*ADAM10}$=0.9946, $r_{RAR\gamma*ADAM10}$=0.9964, P<0.0001. **(C)** RARα mRNA levels were positively correlated with RARγ mRNA levels. $r_{RAR\alpha*RAR\gamma}$=0.9951, P<0.0001. **(D)** A positive correlation was found between IDE and RARβ mRNA levels. $r_{RAR\beta*IDE}$=0.6780, P<0.0001. **(E and F)** IDE mRNA levels were negatively correlated with both RARα **(E)** and RARγ **(F)** mRNA levels. $r_{RAR\alpha*IDE}$=-0.5118, $r_{RAR\gamma*IDE}$=-0.5152, P<0.001. ADAM10, a disintegrin and metalloprotease 10; IDE, insulin degrading enzyme; RARα, retinoic acid receptor α; RARβ, retinoic acid receptor β; RARγ, retinoic acid receptor γ.

The serum retinol content was found to be significantly reduced in this MVAD model in both mother and offspring, confirming that the model was successfully established.

Some previous studies have found that the deprivation of VA or disruption of RARs induces memory impairment in rodents [16, 17, 33]; however, these relationships have not been investigated in an AD mode. In addition, few studies

have investigated the involvement of MVAD in the development of AD. To investigate the long-term effects of MVAD on AD pathogenesis, the current study established the AD model in MVAD rats through the bilateral injection of aggregated $A\beta_{1-42}$ into the CA3 area of the hippocampus in male rats using a single intrahippocampal injection. $A\beta_{1-42}$ levels were significantly increased after this injection (Supplemental Fig. **S1**), confirming that $A\beta_{1-42}$ was successfully injected. This rodent model represents a valid tool for investigating AD because the injection can induce synaptic and memory dysfunction and can facilitate an AD-like pathology [34, 35]. Nevertheless, there have been few studies establishing the AD model on this long-term MVAD model. Behavioral deficits were not apparent during the first 30 days following aggregated Aβ injection; however, significant deficits became apparent approximately 60 to 80 days after injection [30, 36]. Moreover, the current study employed Morris water maze tests at 30 and 80 days post-injection with aggregated $A\beta_{1-42}$. Given that both MVAD and $A\beta_{1-42}$ administration can impair behavioral performance, we injected rats with not only a full dose of $A\beta_{1-42}$ but also a half dose [30]. We found that the half dose of $A\beta_{1-42}$ induced learning and memory deficits compared with the vehicle PBS injection in both the MVAD and control rats at 80 days post-injection but not at 30 days post-injection (Supplemental Fig. **S2**), confirming that the half dose of $A\beta_{1-42}$ induced cognitive impairments during the later stages of development. We also found no significant differences in spatial learning and memory between the MVAD and control rats after 30 days of either vehicle PBS injection or a half dose of $A\beta_{1-42}$ injection. However, at 80 days post-injection of vehicle PBS or a half dose of $A\beta_{1-42}$, the MVAD rats showed significant memory deficits compared with the control animals. The delayed behavioral deficits induced by MVAD might be because of the time required for the neurons to become damaged following a half dose of $A\beta_{1-42}$ injection and the age-related decline in the rats' ability to protect their neurons from Aβ-induced neurotoxicity. However, the memory deficits induced by a full dose of $A\beta_{1-42}$ in the MVAD rats were apparent at 30 days but not 80 days post-injection. Previous studies have demonstrated that intracerebral fibrillar $A\beta_{1-42}$ induces proliferation and activation of microglia [37, 38] and RA suppresses microglial activation in an AD mouse model [39], so it is likely that MVAD accelerates the activation of microglia following $A\beta_{1-42}$ injection. A full dose of $A\beta_{1-42}$ might as well facilitate the damage of neurons. All these results demonstrate that MVAD aggravated $A\beta_{1-42}$-induced cognitive deficits. Furthermore, the age-related decline of anti-neurotoxicity properties might play an important role in the pathogenesis of AD [37].

Numerous in vitro and *in vivo* studies have associated RA and its signaling to APP processing genes or protein expression [40]. Furthermore, RAR/RXR has been long reported significant genetic linkages to AD [41]. Many recent studies have demonstrated that an RA or RAR agonist can reduce Aβ production by activating non-amyloidogenic α-secretase [18, 40, 42-44]. ADAM10, the central component of α-secretase, was identified as a key molecule in RA-mediated anti-AD mechanisms [45]. In addition, RA can increase Aβ clearance by enhancing the expression of the Aβ-degrading enzyme IDE [20, 46]. Moreover, the *IDE* gene contains a RARE in its promoter region [41]. As the current study showed, MVAD did not induce the gene expression of ADAM10 or IDE following $A\beta_{1-42}$ injection, suggesting that MVAD might impair the regulatory feedback of ADAM10 and IDE induced by $A\beta_{1-42}$ injection, resulting in an incompetent protective response. We subsequently investigated the correlations between RARs and each of ADAM10 and IDE. We found that both RARα and RARγ were positively correlated with ADAM10, which somewhat corroborates previous studies reporting that RARα and RARβ increased ADAM10 expression *in vivo* and *in vitro* [43, 44, 47]. In addition, a positive correlation was observed between RARβ and IDE in the current study. This result supports a recent study reporting that RARα and RARβ agonists enhanced the expression of IDE [20]. Moreover, we found that RARα and RARγ were negatively correlated with IDE, which is inconsistent with a previous in vitro study showing that RA-elicited RARα induced IDE expression [46]. Our study of an elderly population also revealed a positive correlation between ADAM10 and RARα and between IDE and RARγ. Together, these data indicated that different AD-associated enzymes were regulated by different RARs. Our findings are of great significance for interpreting the MVAD mechanism concerning AD pathogenesis. Additional studies are needed to gain insight regarding RA signaling and AD-associated gene expression.

Our present study showed that MVAD, beginning from embryonic period, contributed to dysregulation of the Alzheimer-associated gene expression as well as exacerbated memory impairment late in life. Similar viewpoints have been demonstrated in the LEARn model, which was established to integrate environmental risk factors and the developmental basis of AD [26]. This model indicates that accumulated environmental hits, such as dietary factor and toxicological exposure, produce latent epigenetic changes (*e.g.,* DNA methylation) in a long-term fashion, beginning at early developmental stages, until a pathological threshold is reached, resulting in the onset of AD [27, 48, 49]. In addition, deficiency of vitamin B12 or folate has been shown to result in gene promoter methylation with upregulation of AD-associated genes [26]. All these results suggested that environmental risk factors play a vital role in the etiology of late-life disorders, including AD. It was also reported that improving the environment could restore the impaired hippocampal neurogenesis and cognitive function in AD [50]. All these studies supported our hypothesis that VA malnutrition could bring about dysregulation of AD related genes which remain latent for many years until aging, poor mid-life diet or $A\beta_{1-42}$ disturbance, resulting in sustained alterations in these genes to promote AD progression.

However, there are some limitations of our present study. 1) It was reported that the maternal nutritional and metabolic environment is critical in determining long-term health and viability with brain function being most sensitive to influences in the embryonic period [51]. Disturbed maternal nutrition may contribute to deficits in offspring's development and health, resulting in offspring at increased risk of child and adult diseases [51-53]. However, whether maternal MVAD affects the cognitive function of the offspring and increases the risk of AD in the offspring was not investigated in the present study. Additional work is required to address

this issue. 2) Additional research is required to explore the therapeutic potential of VA in MVAD-induced AD pathogenesis and the critical windows of therapy.

CONCLUSION

MVAD beginning during the embryonic period did not induce the AD-associated gene expression of ADAM10 and IDE *via* RARs following $A\beta_{1-42}$ injection in rats. This nutritional gene effect might exacerbate hippocampus-dependent learning and memory deficits. Our results suggest that long-term MVAD results in an increased risk of AD. Long-term nutritional remediation, such as monitoring VA status and correcting low VA levels, during the early stage of life might reduce the risk for AD late in life. Evidently, more research on the therapeutic potential of VA in MVAD-induced AD pathogenesis is of crucial importance and may provide novel targets for the prevention or treatment of AD.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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