

Vitamin A deficiency regulates the expression of ferritin in young male Wistar rats

A deficiência de vitamina A regula a expressão de ferritina em ratos Wistar machos jovens

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ABSTRACT

Objective

Iron deficiency and vitamin A deficiency are two of the main micronutrient deficiencies. Both micronutrients are essential for human life and children's development. This study aimed to investigate the effects of vitamin A deficiency on ferritin and transferrin receptors' expression and its relationship with iron deficiency.

Methods

Five diets with different vitamin A-to-iron ratios were given to thirty five 21-day-old male Wistar rats (separated in groups of seven animals each). The animals received the diet for six weeks before being euthanized. Serum iron and

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retinol levels were measured as biochemical parameters. Their duodenum, spleen, and liver were analyzed for the expression of ferritin and transferrin receptors by Western Blotting.

Results

Regarding biochemical parameters, the results show that when both vitamin A and iron are insufficient, the serum iron content (74.74 µg/dL) is significantly lower than the control group (255.86 µg/dL). The results also show that vitamin A deficiency does not influence the expression of the transferrin receptor, but only of the ferritin one.

Conclusion

Vitamin A deficiency regulates the expression of ferritin in young male Wistar rats.

Keywords: Ferritin. Iron deficiency. Receptors, transferrin. Vitamin A deficiency.

RESUMO

Objetivo

A deficiência de ferro e de vitamina A são duas das principais deficiências de micronutrientes, sendo que ambos são essenciais para a vida humana e o desenvolvimento das crianças. O objetivo deste estudo foi investigar o efeito da deficiência de vitamina A na expressão de ferritina e o receptor de transferrina e sua relação com a deficiência de ferro.

Métodos

Cinco dietas com diferentes proporções de vitamina A para ferro foram administradas a 35 ratos Wistar machos de 21 dias de vida (sete animais por grupo). Os animais receberam a dieta por seis semanas antes de serem eutanasiados. Os níveis séricos de ferro e retinol foram medidos como parâmetros bioquímicos. Duodeno, baço e fígado foram analisados quanto à expressão de ferritina e o receptor de transferrina por Western Blotting.

Resultados

Em relação aos parâmetros bioquímicos, os resultados mostram que quando a vitamina A e o ferro são insuficientes, o teor de ferro sérico (74.74 µg/dL) é significativamente menor do que no grupo controle (255.86 µg/dL). Os resultados também mostram que a deficiência de vitamina A não influencia a expressão do receptor da transferrina, mas da ferritina.

Conclusão

A deficiência de vitamina A regula a expressão de ferritina em ratos Wistar machos jovens.

Palavras-chave: Ferritinas. Deficiência de ferro. Receptores da transferrina. Deficiência de vitamina A.

INTRODUCTION

Hidden hunger, also known as Micronutrient Malnutrition (MNM), occurs when the intake of micronutrients is below the recommended quantities. MNM is common in both developing and developed countries and is specifically described for deficiencies of iron, iodine, folate, zinc, and vitamin A, all of which are considered critical nutrients for adequate development [1]. MNM is understood to have a more significant effect on cognitive and physical development than calorie restriction [2]. Both iron and vitamin A are essential for human life and children's development, so authorities around the world are vigilant about Iron Deficiency (ID) and Vitamin A Deficiency (VAD) [3].

Iron sources are divided into heme (from animal sources) and non-heme (from vegetable sources) and their absorption pathways are different – heme iron is absorbed better than non-heme iron [4]. The absorption of iron in the intestine is highly regulated in response to the level of iron body stores and by the amount of iron needed for erythropoiesis [5]. Hepcidin, an antimicrobial peptide discovered in 2000, regulates intestinal iron absorption and affects iron release from hepatic stores and from macrophages involved in the recycling of iron from hemoglobin [6]. Other proteins as ferroportin (strongly regulated by hepcidin), ferritin, transferrin, transferrin receptors, and hemojuvelin are highly related to iron metabolism [7].

The main biomarkers of iron metabolism are acute-phase proteins, which means that they are influenced by infection and inflammation, so markers as Interleukin-6 (IL-6), C-Reactive Protein (CRP) must be considered when iron homeostasis is studied [8-10].

Iron deficiency is diagnosed based on serum ferritin levels ($<12\mu\text{g/L}$) and is considered the main cause of nutritional anemia [11-13]. Anemia could be a consequence of nutritional causes, as micronutrient deficiencies and anti-nutritional factors affect iron absorption. Some studies and reviews explored the relationship between other micronutrient deficiencies and anemia, like vitamin D, vitamin B12 and folate, and vitamin A [14,15].

Vitamin A deficiency is diagnosed based on Serum Retinol Levels (SLR), clinical ($<0.35\text{mmol/L}$), subclinical ($0.36\text{-}0.70\text{mmol/L}$), suboptimal ($0.71\text{-}1.04\text{mmol/L}$), and normal ($>1.05\text{mmol/L}$) [16]. Some of the consequences of VAD are, for example, that it is considered the leading cause of blindness in children and a risk factor of disease and death from severe infections [17]. VAD also causes night blindness in pregnant women and may increase the risk of maternal mortality.

The relationship between iron and vitamin A has been suggested since the early 20th century [18]. Vitamin A is considered a nutrient that could regulate iron use, iron metabolism, and erythropoiesis. VAD should be considered a risk factor for ID, even though it is well known that the precise relationship depends on many factors [19-21]. Still, the mechanism of that effect is not well-established. The little evidence available suggests that VAD affects iron metabolism and not its absorption or transport [22,23]. In situations of iron overload and regular vitamin A levels, retinol has an antioxidant activity stimulating ferritin synthesis, thus reducing the circulating iron to avoid oxidative stress [24].

The studies of Saraiva, Soares, and Santos [23] and of Arruda, Siqueira, and Valencia [25] proposed that the effect of VAD upregulated the expression of hepcidin and reducing the efflux of iron from the enterocyte as a consequence of the internalization of ferroportin induced by hepcidin. That effect of the axis VAD:hepcidin:ferroportin were validated by the work of and Citelli *et al*, [26], that employed in vitro (Caco-2) and in vivo (BALB/c male mice and Wistar male rats) models; however, the mechanism is not clear; the same study revealed that the expression of other proteins related to iron metabolism, such as Divalent Metal Transporter 1 (DMT1), Duodenal Cytochrome B (DcytB), and Transferrin Receptor (TfR), were not affected [26].

The present research hypothesizes that vitamin A somehow regulates iron metabolism in young male Wistar rats, so both must be administered together. Our study analyzed the expression of ferritin and transferrin receptors by Western Blotting under five diets with different vitamin A-to-iron ratios to identify how VAD is related to iron homeostasis in young male Wistar rats. We choose a murine model because research with animal models has been valuable in advancing the knowledge of nutrition, and the murine model has been widely used to study iron metabolism and its disorders [27-29]. The absorption and metabolism of heme iron are known to occur in the rats' mucosa like that in humans, even if the absorption of heme iron is lower in rodents [30].

METHODS

We followed the methods described by Restrepo-Gallego and Díaz for animal housing, diets, and procedures [31]. Briefly, thirty-five 21-day-old male Wistar rats were housed in polycarbonate cages with polycarbonate water dispensers. Five groups of seven animals each were randomly assigned different diets, one group per cage. Table 1 shows the composition and nutritional profile of the diets.

After six weeks of feeding, the animals were anesthetized with isoflurane inhalation (Piramal Critical Care, Mumbai, India) and cardiac punctures were used to collect 2mL of whole blood in serum tubes (Vacutest gel with a cloth activator, Vacutest Kima, Arzegrande, Italy) to analyze the iron and retinol levels.

Table 1 – Ingredient composition and nutritional profile for each diet.

Ingredient	Control	Fes/vAd	Fes/vAi	Fei/vAs	Fei/vAi
Corn starch	397.5	397.5	397.5	397.5	397.5
Casein	200.0	200.0	200.0	200.0	200.0
Maltodextrin 10	132.0	132.0	132.0	132.0	132.0
Sucrose	100.0	100.0	100.0	100.0	100.0
Vegetable oil	70.0	70.0	70.0	70.0	70.0
Cellulose	50.0	50.0	50.0	50.0	50.0
Mineral mix ^a	35.0	35.0	35.0	0	0
Mineral mix ^b	0	0	0	35	35
Vitamin mix ^c	10.0	0	0	10.0	0
Vitamin mix ^d	0	10	10	0	10
L-Cysteine	3.0	3.0	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5	2.5	2.5
Ferric citrate (17.4% Fe)	0.03	0.03	0.03	0.07	0.07
t-Butylhydroquinone	0.014	0.014	0.014	0.014	0.014
Vitamin A palmitate (500.000IU/g)	0	0	0.0008	0	0.0008
Protein (%)	20	20	20	20	20
Fat (%)	7	7	7	7	7
Fiber (%)	5	5	5	5	5
Carbohydrate (%)	64	64	64	64	64
Iron (mg/kg)	45	45	45	15	15
Vitamin A (µg/kg)	1200	0	120	1200	120

Note: ^aAIN-93G-MX; ^bAIN-93G-MX deficient in iron; ^cAIN-93G-VX; ^dAIN-93G-VX without vitamin A; d: deficient; i: insufficient; s: sufficient; Ingredients are listed as g/kg except when noted. Fes/vAd: Sufficient in iron and deficient in vitamin A; Fes/vAi: Sufficient in iron and insufficient in vitamin A; Fei/vAs: Insufficient in iron and sufficient in vitamin A; Fei/vAi: Insufficient in both nutrients.

The fraction of blood was separated after its complete clotting using centrifugation (448 to 700xg/min for 15 to 20min). The supernatant was divided into 2 fractions to determine serum iron and serum retinol levels. Serum iron concentrations were determined using atomic absorption spectrometry (Analyst 3100 Analyzer, Perkin Elmer Life Sciences, Wellesley, MA). Serum retinol concentrations were measured using a HPLC–UV apparatus (model PU-2080 plus chromatography pump, UV-2075 UV detector, and 807-IT integrator; Jasco, Tokyo, Japan).

After extracting the blood, the animals were euthanized with 70% CO₂ and their livers, spleens, and small bowels were kept at -80°C for further Western Blot analysis. The Ethical Committees of Antioquia's University (#108.090217) and La Sabana University (#55.170516) approved the entire procedure following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines [32].

Cell lysates were obtained from the tissues using a lysis buffer (NaCl 150mM, PMSF 1mM, Triton X-100 1%, Tris-HCl 50mM, DTT 200mM, EDTA 1mM) and protease inhibitor (Complete™ ULTRA Tablets, Roche Diagnostics GmbH, Mannheim, Germany). The extraction was carried in a BeadBug D1030 homogenizer (Benchmark Scientific, Edison, NJ, USA) at 4000rpm with 1mM glass beads. The cell extracts' total protein content was quantified in the equipment Qiaxpert 200,630 (Qiagen, Hilden, Germany).

Samples (50µg of total protein) were subjected to 12% SDS-PAGE, transferred to polyvinylidene membranes (PVDF Immobilion-P, Millipore, Burlington, MA, USA), and blocked with skimmed milk powder at 5% in tris-buffered saline and polysorbate 20 (TBST). The primary and secondary antibodies used were obtained from Novus Biologicals (Centennial, CO, USA) and are detailed in Table 2.

The secondary antibodies used were anti-mouse IgG1 [HRP] NB7511 (1:2000), and anti-Rabbit IgG (H+L) [HRP] NB7160 (1:500), both with goat as host and were also obtained from Novus Biologicals. Immunoreactive proteins were visualized by 3,3'-diaminobenzidine staining (Sigma, St. Louis, MO, USA).

Table 2 – Primary antibodies used for Western Blotting tests.

Protein	Primary antibody	Dilution	Host
β -actin	beta-Actin Antibody (AC-15) NB600-501	1:2000	Mouse
Ferritin	Ferritin Antibody NBP1-31944	1:100	Rabbit
Transferrin receptor	TfR (Transferrin R) Antibody NB100-92243	1:100	Mouse
IL6	IL-6 Antibody NB600-1131	1:500	Rabbit

Note: IL6: Interleukin-6.

Data from biochemical parameters were presented as mean \pm standard deviation. Continuous variables were compared between groups with a one-way analysis of variance with a post hoc least-significant-difference t-test ($p < 0.05$). The Kolmogorov-Smirnov test assessed the normality of data distribution; the equality of variance was measured using Levene's test. SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) was used. A power analysis estimated the number of rats to obtain 80% power at a confidence level of 95% [33]. The Western blot procedure was carried out in triplicate for each sample.

Western Blots information was analyzed with the software Prism 7.04 (GraphPad, San Diego, CA, USA). The results were presented as relative expressions of the respective protein to β -actin, giving a value of 1 to the control group, and analyzed with the Tukey method for multiple comparisons. A p -value < 0.05 was considered statistically significant.

RESULTS

The present research analyzed the expression of ferritin and transferrin receptors in five groups of young male Wistar rats fed with diets with different ratios of vitamin A-to-iron. The control group received a standard diet according to the reference diets for laboratory animals, and the four experimental groups received different combinations of iron (Fe) and vitamin A (vA) at sufficient (s), insufficient (i), or deficient (d) levels. None of the diets were deficient in iron because it is necessary for sustaining life; thus, a complete lack of it would result in non-viable experimental subjects [34]. Given the known relationship between VAD and iron homeostasis, biomarkers are essential. They would indicate the needed supply of both micronutrients together to prevent ID in children and women at reproductive age [35].

All the intergroup comparisons indicated significant differences in serum iron content. Both groups with low amounts of iron (Fei/vAs and Fei/vAi) showed significant differences when compared to the control group ($p < 0.001$ for both groups). VAD (Fes/vAi and Fes/vAd) impacted the total serum iron content ($p = 0.024$ and 0.001 , respectively), as the Fes/vAd group exhibited the lowest values of both VAD groups.

In terms of serum retinol levels, the groups with insufficient or deficient dietary vitamin A all had significantly lower serum retinol levels than the control group ($p < 0.001$ for both groups). In the multiple comparisons, only the groups with low dietary vitamin A (Fes/vAi and Fei/vAi) showed no significant differences between them ($p = 0.980$). It is an important remark that those groups insufficient or low in vitamin A were under the normal value of 1.37 ± 0.21 $\mu\text{mol/L}$ for serum retinol and that the group with both in insufficient levels (Fei/vAi) was within the range of iron deficiency ($< 77 \mu\text{g/dL}$) [36,37]. The results are summarized in Table 3.

Table 3 – Biochemical parameters of the different groups.

Parameter	Control	Fes/vAd	Fes/vAi	Fei/vAs	Fei/vAi
Serum iron ($\mu\text{g/dL}$)	255.86 ± 43.83^a	181.30 ± 14.53^b	219.81 ± 15.58^c	131.01 ± 43.83^d	74.74 ± 18.27^e
Serum retinol ($\mu\text{mol/L}$)	1.60 ± 0.16^a	0.24 ± 0.04^b	0.55 ± 0.10^c	1.26 ± 0.12^d	0.55 ± 0.08^e

Note: Data are given as mean \pm 1; n = 7; Different letters indicate significant differences between groups ($p < 0.05$). Fes/vAd: Sufficient in iron and deficient in vitamin A; Fes/vAi: Sufficient in iron and insufficient in vitamin A; Fei/vAs: Insufficient in iron and sufficient in vitamin A; Fei/vAi: Insufficient in both nutrients.

The groups showed no significant differences in food intake ($p=0.126$); the average daily food intake per rat was 15.5g/d, which is consistent with the expected food intake for growing animals of this type [38]. In a previous publication, we showed that iron and vitamin A are essential to improve weight gain and some hematological parameters like reticulocyte count and hemoglobin [31].

Ferritin is an acute-phase protein, and the inflammation status must be considered to avoid misinterpretations and introduce adjustments, if necessary [39-41]. Figure 1A shows that there were no significant differences ($p<0.05$) in the expression of hepatic Interleukin 6 (IL6) values when the experimental groups were compared with the control group, and also when the intergroup comparisons were made; so, inflammation was not a critical factor for the study.

The results for ferritin in the liver (Figure 1B) showed that entirely lacking vitamin A combined with a sufficient amount of iron (Fes/vAd) induces some level of holding or reabsorption of iron as ferritin, reducing its mobilization to other tissues, maybe due to damage at the hepatocyte [42]. Ferritin's significantly higher expression demonstrates this in Fes/vAd group compared to the control group ($p=0.0017$). Those results are consistent with previous findings about the relationship between VAD and oxidative stress by serum iron overload related to an increase of ferritin and IRP2 [43,44].

Regarding ferritin in the duodenum (Figure 1C), we found no significant differences between the experimental and control groups. Ferritin in the spleen (Figure 1D) showed that only the group without vitamin A (Fes/vAd) does not have significant differences in comparison with the control group ($p=0.1076$).

Our study does not show any differences between the experimental groups and the control group for both tissues regarding the effect of VAD on the expression of the transferrin receptor in the liver (Figure 1E) and spleen (Figure 1F).

DISCUSSION

This study's findings showed no significant differences in IL6 expression as an indicator of inflammation in the animals, including with VAD, ID, or both. Previous works showed that VAD or ID enhances the inflammatory state by increasing IL6 expression in the liver [45]. Regarding hepcidin, it has been established that IL6 regulates its transcription, thus controlling the circulation of iron in inflammation [46,47]. So, our findings do not mean that no relationship exists between VAD or ID and inflammation, but that under the experimental research conditions, there is not enough evidence to support it.

When groups with insufficient iron intake (Fei/vAs and Fei/vAi) were compared with one another, there is a subexpression of ferritin in comparison with the control group ($p<0.001$ for both groups) due to the marginal deficiency of iron. However, it is interesting that the group with sufficient iron, but marginal deficiency of vitamin A also showed a subexpression of hepatic ferritin compared to the control group ($p<0.001$). Considering that vitamin A induces the expression of the ferroportin (Fpn1) gene to promote the export of iron into the bloodstream, that means that both nutrients must be in adequate quantities to maintain the appropriate levels of iron storage in the liver [26].

When intergroup comparisons were made, we found that the group with enough iron and a marginal deficiency of vitamin A (Fes/vAi) had significantly higher levels of ferritin in the duodenum than Fei/vAi ($p=0.0351$) and Fei/vAs ($p=0.0487$). That suggests that at duodenal level, only dietary iron seems to influence the expression of ferritin as a mechanism to regulate the circulating iron and prevent oxidative stress, which demonstrated that not only hepcidin but also ferritin are necessary to regulate the iron efflux to the bloodstream [48].

Results of examinations for ferritin in the spleen showed a relationship between the entire absence of vitamin A and some kind of inflammatory disease, which increases the expression of splenic ferritin as a marker of inflammation [49]. In this case, it seems that there is no relationship with the iron status of the body.

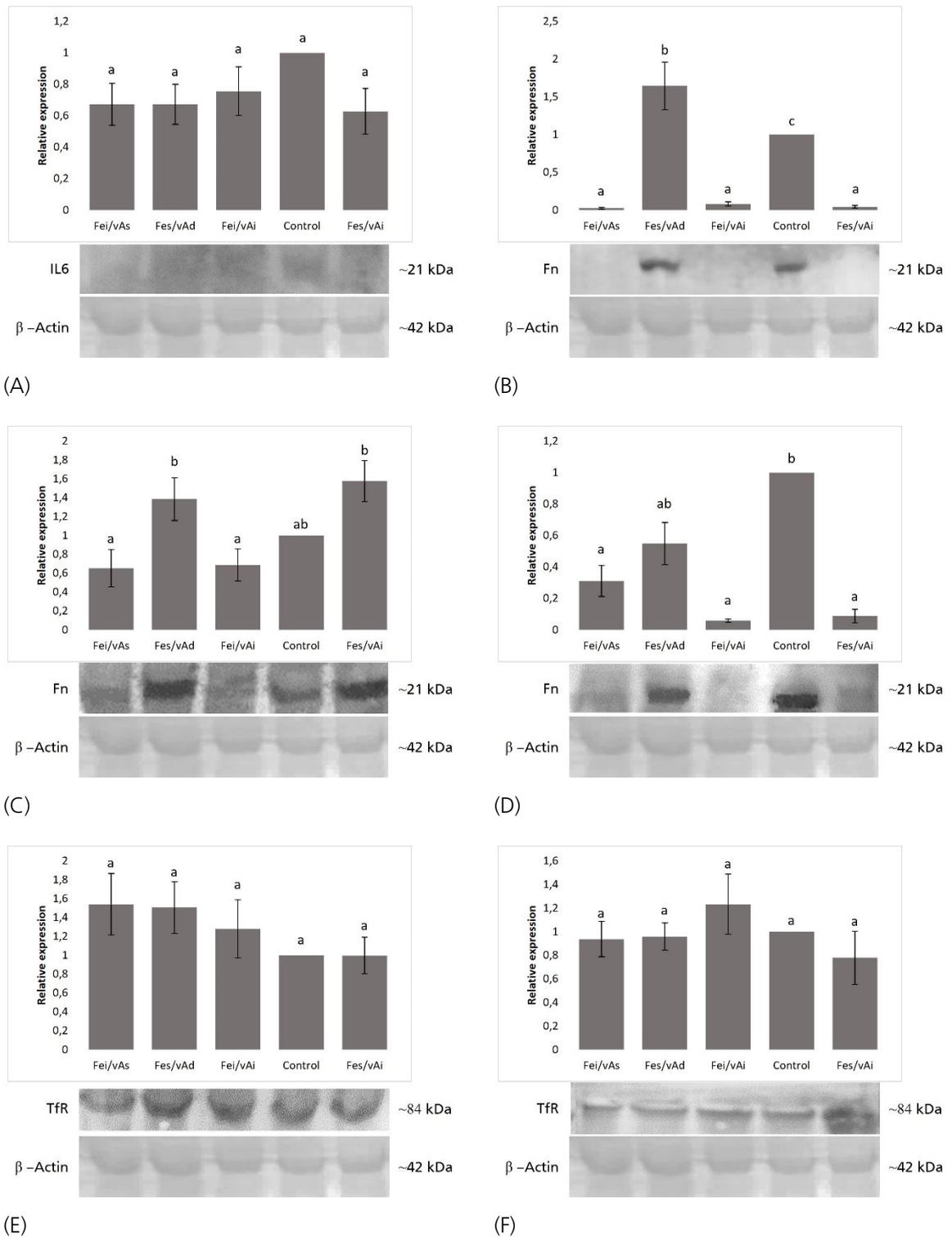


Figure 1 – Relative expression to β -actin of: (A) interleukin-6 in the liver; (B) ferritin in the liver; (C) ferritin in the duodenum; (D) ferritin in the spleen; (E) transferrin receptor in the liver and (F) transferrin receptor in the spleen. Data are given as mean \pm 1 Standard Deviation; groups with different letters differ significantly ($p < 0.05$). IL6: Interleukin-6; Fn: Ferritin; TfR: Transferrin Receptor; Fes/vAd: Sufficient in iron and deficient in vitamin A; Fes/vAi: Sufficient in iron and insufficient in vitamin A; Fei/vAs: Insufficient in iron and sufficient in vitamin A; Fei/vAi: Insufficient in both nutrients.

Our results for the transferrin receptors in the liver and spleen agree with the findings of Citelli, Bittencourt, Silva, Pierucci, and Pedrosa [26], that indicate that VAD impairs iron metabolism and may not affect iron absorption or transport [23,45].

The focus of this research was not to evaluate the effect of iron deficiency on the status of vitamin A. However, it is important to remark that dietary iron changes do not affect serum retinol amounts when dietary vitamin A levels are low or absent. Previous findings showed a lack of consensus about the unidirectional effect of iron supplementation on the nutritional status of vitamin A and differences between the results of animal models and humans [50].

The results of this study give a new approach to the relationship between VAD and ID on the expression of ferritin. Previous findings had been focused on the effects on the expression of hepcidin, iron regulatory protein, and ferroportin [23,25,43,51]. Our findings do not indicate that the previous ones are wrong but give a new approach based on ferritin supported by the review and meta-analysis made by da Cunha, Aboudib, and Arruda [19]. They suggest that supplementation with vitamin A alone may reduce the risk of anemia by improving ferritin levels in individuals with low serum retinol levels. Our study also reinforces the concept that vitamin A acts differently as a regulator of iron homeostasis and metabolism and must be studied more in-depth to understand all the mechanisms related to IDA or anemia by VAD.

Finally, the development of improved technologies for the diagnosis of VAD and ID could lead to a better understanding of the relationship between them and a better interpretation of the results considering variables such as inflammation status, interaction with other micronutrients, and general health status of the individuals [52].

As to considerations regarding the animals' well-being, seven animals per cage were not an issue according to the concepts of the ethical committees that studied the experimental design. Nevertheless, this could be a bias and could influenced the results, being a limitation on the study. However, our previous findings indicated that the food intake and weight gain were consistent with the expected for growing animals of this type [31].

It is important to remark that this study did not measure serum ferritin as a typical parameter for ID. Serum iron was used as an indicator of the iron intake from the diets and, therefore, both ferritin in tissues by Western Blotting and serum iron were used to indicate some level of iron deficiency in the animals. Considering further clinical studies, it should be relevant to include serum ferritin, retinol-binding protein, and immunohistochemical analysis to give more information about the use of iron and vitamin A in the tissues of interest.

CONCLUSION

The study results suggest that VAD influences the expression of the proteins associated with iron storage and could be another mechanism to explain anemia by VAD even with adequate dietary iron levels. Based on our findings, we consider that the supply of iron and vitamin A must be evaluated together in human beings to be considered as a feasible strategy to reduce the ID prevalence, especially for women at reproductive age and children. Further and more in-depth research at the clinical level can give more information about both nutrients' relationship in a more realistic scenario, possibly explaining the mechanism behind that interaction.

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CONTRIBUTORS

Conceptualization, M.R.G. and L.D.; methodology, M.R.G., J.OV., and D.CC.; formal analysis, M.R.G.; investigation, M.R.G.; writing original draft preparation, M.R.G. and J.OV. All authors agree with the manuscript and declare that the content has not been published elsewhere.

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