Original Communication

Vitamin A does not influence mRNA expression of hormone hepcidin but other biomarkers of iron homeostasis in young male Wistar rats

Mauricio Restrepo-Gallego and Luis E. Díaz

Doctorate Program in Bioscience, La Sabana University, Campus Puente del Común, Km. 7, Autopista Norte, Bogotá, Chía, Cundinamarca, Colombia

Abstract: The effects of an adequate supply of vitamin A and iron, in comparison with diets low or absent in vitamin A and low in iron, on the mRNA expression of some biomarkers of iron homeostasis as hepcidin (*Hamp*), transferrin receptor-1 (*Tfrc*), iron regulatory protein-2 (*Ireb2*) and ferritin (*Fth1*) in rats were investigated. 35 male Wistar rats were randomly divided into 5 dietary groups: control, sufficient in iron and insufficient in vitamin A (FesvAi), sufficient in iron and depleted in vitamin A (FesvAd), insufficient in iron and sufficient in vitamin A (FeivAs) and insufficient in both iron and vitamin A (FeivAi). After 6 weeks rats showed no significant effects of variations in vitamin A on the expression of *Hamp* relative to the control group (FesvAi: 1.37-fold; FesvAd: 1.22-fold); however, iron deficiency showed significant reduction on it relative to the control group (FeivAs: 71.4-fold, $P = 0.0004$; FeivAi: 16.1-fold, $P = 0.0008$). Vitamin A deficiency (FesvAd) affects expression of *Fth1* independent of low dietary iron in spleen (0.29-fold, $P = 0.002$) and duodenum (5.15-fold, $P = 0.02$). Variations of dietary iron and vitamin A showed significant effects relative to the control group for expression of *Tfrc* in spleen (FesvAd: 0.18-fold, $P = 0.01$; FeivAs: 0.24-fold, $P < 0.0001$; FeivAi: 0.42-fold, $P = 0.014$), *Ireb2* in spleen (FeivAs: 3.7-fold, $P < 0.0001$; FeivAi: 2.9-fold, $P < 0.0001$) and *Ireb2* in duodenum (FeivAs: 2.68-fold, $P = 0.012$; FeivAi: 2.60-fold, $P = 0.014$). These results show that vitamin A and iron must be supplied together to regulate some of the main biomarkers of iron metabolism as a strategy to reduce prevalence of iron deficiency anemia.

Keywords: retinol, anemia, hepcidin, vitamin A deficiency

Introduction

Micronutrient malnutrition (MNM), or hidden hunger, occurs when the intake of micronutrients and vitamins is below the amount required. It is a common problem in developing and developed countries, and it entails lack or deficiency of iron, vitamin A, iodine, zinc and folate, which are all critical micronutrients related to adequate development [1]. Indeed, MNM is believed to cause a more detrimental impact on cognitive and physical development than calorie restriction. Regarding their importance, iron and vitamin A play a fundamental role in children's development, and health authorities worldwide remain vigilant for iron deficiency anemia (IDA) and vitamin A deficiency (VAD) [2].

According to the World Health Organization (WHO) in its report titled *Global Prevalence of Anemia in 2011*, the most recent official data source, 42.6% of preschool-age children are anemic; among pregnant women, the prevalence is 38.2%. The largest single group in terms of affected individuals is nonpregnant women; based on this group's 29.0% rate of anemia, it includes 496.3 million affected individuals [3]. VAD is the main cause of preventable blindness in children and increases the risk of disease and death from severe infections. In pregnant women, VAD causes night blindness and may increase the risk of maternal mortality [4]. VAD is a public health problem, and it has a major impact on young children and pregnant women in low-income countries [5].

Physiologically, anemia is a condition characterized by low levels of hemoglobin in blood. It is also associated with a deficient quality or quantity of red blood cells. There are a lot of factors that could influence iron absorption as its status, bioavailability, interactions with other minerals, chemical form, enhancers and inhibitors of absorption [6] as plant-derived phytates and tannins [7] and other micronutrient deficiencies. Bleeding, infections and genetic conditions are considered non-nutritional causes

© 2020 Hogrefe

for anemia [8]. Iron deficiency is considered the main nutritional cause [9], it occurs when hemoglobin levels are below the established cutoff points (11 g/dL for children under 5 years old and pregnant women; 12 g/dL for children from 5–12 years old, non-pregnant women and teenage males and 13 g/dL for teenage females) and ferritin levels are below 12 μg/L [10].

At systemic levels and in healthy subjects, iron metabolism is regulated mainly by hepcidin, which is a peptide that the liver produces. It is encoded by the HAMP (hepcidin antimicrobial peptide) gene and considered the master regulator of iron homeostasis. It works by binding to ferroportin and inducing its internalization and lysosomal degradation. Therefore, when an iron overload occurs, hepcidin expression is increased and reduces iron efflux from the enterocyte and macrophages; conversely, in iron deficiency, hepcidin expression is reduced and iron efflux to the blood stream is increased [11–13].

Vitamin A is another nutritional factor that can affect iron use; however, the mechanism of that effect is not well-established. The little evidence available from a mechanistic point of view suggests it affects iron metabolism and not iron absorption or transport [14, 15]. In the case of iron overload and adequate vitamin A levels, retinol stimulates ferritin synthesis, which could be an indirect way to trigger vitamin A's antioxidant activity [16]. However, it is possible that another mechanism exists in which vitamin A regulates excess iron due to nutritional causes [17]. It was demonstrated that in VAD with normal levels of iron, hepcidin expression could be increased [15, 18]. Latter studies employing in vitro (Caco-2) and in vivo (BALB/c male mice and Wistar male rats) models revealed that vitamin A indirectly regulates the presence of ferroportin by regulating hepcidin expression; however, the mechanism is not clear. The same studies revealed that the expression of other proteins related to iron metabolism, such as DMT1 (divalent metal transporter-1), DcytB (duodenal cytochrome B) and TfR (transferrin receptor), was not affected [19].

Based on previous evidence, the present research was conducted to give a better understanding of the role of vitamin A on iron metabolism and go deeper on the different mechanisms related. Our study was an analysis of the mRNA expression of hepcidin and other main biomarkers of iron metabolism under various ratios of iron to vitamin A supply to understand how retinol is related to iron homeostasis in young male Wistar rats.

Materials and methods

Animals, diets and biochemical analysis

The study was conducted at the Bioterium of Antioquia's University in Medellín, Colombia. Thirty-five 21-day-old male Wistar rats (Specific-Pathogen-Free, Neurosciences Laboratory, Antioquia's University, Medellín, Colombia) with a mean body weight of 62.5 ± 6.1 g were housed in polycarbonate cages with stainless steel wire covers and polycarbonate water dispensers. Environmental conditions included a 12-h light cycle, room temperature of 22 ± 2 °C and relative humidity of 55–65%; the air was renewed 15 times per hour. The animals had free access to demineralized water and food and were weighed every two weeks on a digital scale with a resolution of 0.1 g (Precisa BJ2100D, Precisa Gravimetrics AG, Dietikon, Switzerland). The Ethical Committees of Antioquia's University (#108.090217) and La Sabana University (#55.170516) approved the entire procedure.

The animals were randomly assigned five diets, with seven animals in each group. The control group received an AIN-93G growing-rodent diet [20] modified to supply a sufficient amount of iron as ferric citrate (45 mg/kg) and vitamin A as retinol acetate (1200 μg/kg); the experimental groups received modified AIN-93G diets: FesvAd (45 mg/kg iron, no vitamin A), FesvAi (45 mg/kg iron, 120 μg/kg vitamin A), FeivAs (15 mg/kg iron, 1200 μg/kg vitamin A) and FeivAi (15 mg/kg iron, 120 μg/kg vitamin A). Table 1 shows the composition and nutritional profile of the diets. All diets were produced by Research Diets Inc., New Brunswick, NJ.

After six weeks of feeding, animals were anesthetized by inhalation with USP isoflurane (Piramal Critical Care, Inc., Bethlehem, PA), and whole blood was collected by cardiac puncture. 2 mL were collected in serum tubes (Vacutest® gel + cloth activator, Vacutest Kima S.r.l., Arzergrande, Padua, Italy) for iron and retinol analysis. After blood extraction, the animals were euthanized with CO_2 (70%), and death was ensured by cervical dislocation. Their livers, spleens and small bowels were kept at −80 °C for further qRT-PCR analysis. Serum iron concentrations were analyzed by atomic absorption spectrometry on an Analyst 3100 Analyzer (Perkin Elmer Life Sciences, Wellesley, MA). Serum retinol concentrations were measured by HPLC.

Reverse transcription polymerase chain reaction

The extraction of total mRNA from the tissues was performed using Invitrogen TRIzol reagent (Thermo Fisher Scientific Life Sciences Solutions, Carlsbad, CA). mRNA content was assessed in a NanoDrop54 One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific Life Sciences Solutions, Carlsbad, CA). 100 nanograms total mRNA was used for qRT-PCR, using an iTaq54 Universal SYBR® Green One-Step Kit from Bio Rad (Bio Rad

 © 2020 Hogrefe

Table 1. Ingredient composition (g/kg) and nutritional profile of the diets

Ingredient	Control	FesvAi	FesvAd	FeivAs	FeivAi
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
Soybean Oil	70.0	0	0	0	0
Cotton Seed Oil	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-Cystine	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 (500.000 IU/g)	0	0.0008	0	0	0.0008
Nutritional profile					
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 as retinol acetate)	1200	120	0	1200	120

[a] AIN-93G-MX; [b] AIN-93G-MX deficient in iron; [c] AIN-93G-VX; [d] AIN-93G-VX without vitamin A (vA); s: sufficient; d: depleted; i: insufficient.

Laboratories, Hercules, CA, USA). Briefly, 5 μL of SYBR® Green reaction mix was mixed with 0.125 μL of iScript™ reverse transcriptase, 1 μL of forward primer and 1 μL of reverse primer at 300 nM each, 1 μL of mRNA (100 ng/μl) and 1.875 μL of nuclease-free water for a final reaction volume of 10 μL. Hamp (hepcidin antimicrobial peptide) in liver, Ireb2 (iron regulatory protein-2) in duodenum and spleen, Fth1 (ferritin heavy chain) in duodenum and spleen, Il6 (interleukin-6) in liver and Tfrc (transferrin receptor 1) in spleen mRNA expressions were obtained by the reverse transcription polymerase chain reaction method. A CFX96 Touch54 Real-Time PCR Detection System (Bio Rad Laboratories, Hercules, CA, USA) was used. The reverse transcription reaction was carried out at 50 °C for 1 min, followed by polymerase activation and DNA denaturation at 95 °C for 1 min, then denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 60 °C for 30 s, with 40 cycles for all genes. Forward and reverse primers for each gene are summarized in Table 2. The mRNA expression was normalized to the housekeeping gene Actb (β-actin) mRNA expression and is presented by its relative density to β-actin. All the data was processed using the Gene Study module of the Bio-Rad CFX Maestro v.1.1 software (Bio Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Data were presented as mean ± standard deviation. Continuous variables were compared between groups by one-way analysis of variance with a post hoc Tukey's test. The normality of data distribution was tested by the Kolmogorov-Smirnov test; equality of variance was measured using Levene's test. $P < 0.05$ was considered statistically significant. SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) was used. The number of rats was estimated by a power analysis to obtain 80% power at a confidence level of 95% [21].

Results

Biochemical parameters: serum iron and serum retinol

When intergroup comparisons were made both groups insufficient in iron (FeivAs and FeivAi) had significant differences from the control group ($P < 0.05$ for both groups). The group depleted in vitamin A but sufficient in iron

© 2020 Hogrefe

Table 2. qRT-PCR Primers

Gene	Forward primer	Reverse primer	Reference
Hamp	5'-GAAGGCAAGATGGCACTAAGCA-3'	5'-TCTCGTCTGTTGCCGGAGATAG-3'	[35]
Fth1	5'-GCCCTGAAGAACTTTGCCAAAT-3'	5'-TGCAGGAAGATTCGTCCACCT-3'	[36]
Ireb2	5'-CTGCATCCCAGCCTATTGAAAA-3'	5'-GCACTGCTCCTAGCAATGCTTC-5'	[37]
Il6	5'-GTCAACTCCATCTGCCCTTCAG-3'	5'-GGCAGTGGCTGTCAACAACAT-3'	[36]
Tfrc	5'-ATACGTTCCCCGTTGTTGAGG-3'	5'-GGCGGAAACTGAGTATGGTTGA-3'	[37]
Actb	5'-TGTCACCAACTGGGACGATA-3'	5'-AACACAGCCTGGATGGCTAC-3'	[38]

Hamp, hepcidin antimicrobial peptide; *Fth1*, ferritin heavy chain 1; *Ireb2*, iron regulatory protein 2; *Tfrc*, transferrin receptor 1; *Actb*, beta actin.

(FesvAd) showed significant differences when compared when control group ($P < 0.05$) while the group insufficient in vitamin A but sufficient in iron (FesvAi) showed not significant differences when compared with the control group; however, when both grupos with any deficiency of vitamin A (FesvAi and FesvAd) were compared, showed not significant differences between them in terms of serum iron.

In terms of serum retinol levels all experimental groups were significant lower than control grup ($P < 0.05$ for all of them). When both groups sufficient in dietary vitamin A were compared, that one with marginal deficiency of dietary iron (FeivAs) was significant lower than control group. When groups insufficient in dietary vitamin A (FesvAi and FeivAi) were compared, they exhibit the lowest value for serum retinol (0.55 umol.l^{-1}) and no significant differences between them. The results are summarized in Table 3.

Effect of vitamin A status on the mRNA expression of genes related to iron homeostasis in the liver, spleen and duodenum

When compared with the control group, mRNA expression of iron regulatory protein 2 (*Ireb2*) to *Actb* values in the duodenum and spleen showed significant but opposite differences ($P < 0.05$) for groups insufficient in iron regardless of vitamin A supply (FeivAi and FeivAs). Figure 1C shows the results in the duodenum, in which values were higher than those of the control group. The amount of FeivAi was 2.6 times the amount in the control group ($P = 0.0136$), and the amount of FeivAs was 2.7 times the amount in the control group ($P = 0.0118$). In the spleen, as Figure 1D shows, values were lower than in the control group. The amount of FeivAi was 2.9 times the amount in the control group ($P < 0.0001$), and the amount of FeivAs was 3.7 times that in the control group ($P < 0.0001$).

Regarding duodenal ferritin heavy chain 1 (*Fth1*) mRNA relative expression normalized to *Actb* in the duodenum (Figure 1E), the group insufficient in iron but sufficient in vitamin A (FeivAs) showed significant differences when compared with the control group, exhibiting higher values of *Fth1* mRNA relative expression (5.1-fold, $P = 0.0017$). *Fth1* mRNA relative expression in the spleen showed significantly lower values ($P < 0.05$) in comparison with the control group only for the group with sufficient iron and depleted in vitamin A (FesvAd), (3.5-fold, $P = 0.0018$) (Figure 1F).

Transferrin receptor 1 (*Tfrc*) mRNA expression normalized to *Actb* in the spleen showed significant differences ($P < 0.05$) for groups insufficient in dietary iron and for group depleted in vitamin A when compared with the control group (Figure 1G). These groups had lower values than the control group and the group with sufficient iron and insufficient vitamin A: FeivAi, 2.4-fold ($P = 0.0142$); FeivAs, 4.2-fold ($P < 0.0001$) and FesvAd, 5.6-fold ($P = 0.0104$).

Discussion

To explore how vitamin A deficiency alters some of the main biomarkers of iron-homeostasis expression, the present research was focused on the expression of the genes associated with them in young male Wistar rats that received diets with various doses of vitamin A and/or iron. The control group received sufficient amounts of both nutrients according to the reference diets for laboratory animals,

Table 3. Biochemical parameters

Parameter	Control	FesvAi	FesvAd	FeivAs	FeivAi
Serum iron [μg·dl^{-1}]	255.86 ± 43.83 a	219.81 ± 15.58 ab	181.30 ± 14.53 b	131.01 ± 43.83 c	74.74 ± 18.27 d
Serum retinol [umol·l^{-1}]	1.60 ± 0.16 a	0.55 ± 0.10 b	0.24 ± 0.04 c	1.26 ± 0.12 d	0.55 ± 0.08 b

n = 7; different letters indicate significant differences between groups ($P < 0.05$). s: sufficient; d: depleted; i: insufficient.

 © 2020 Hogrefe

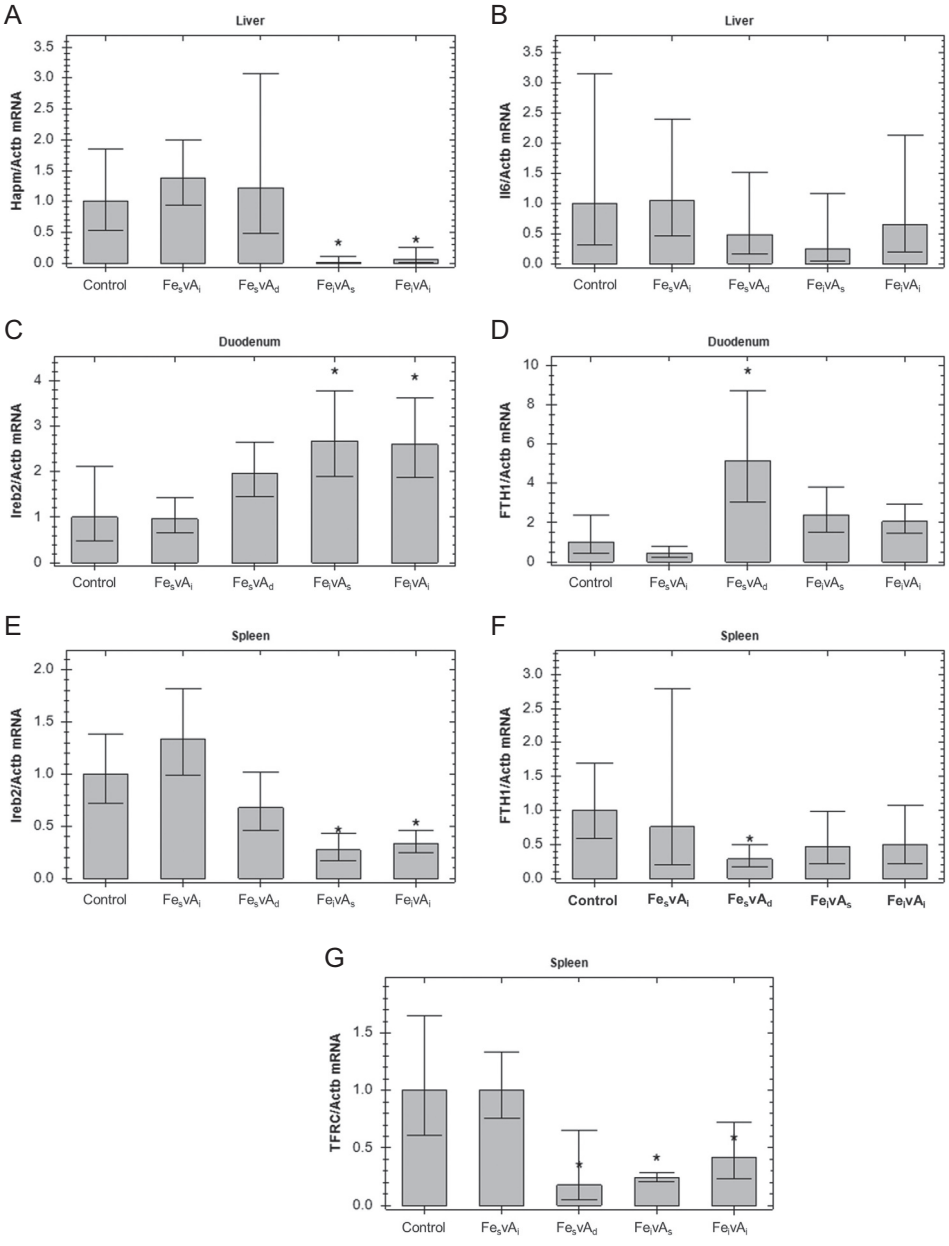

Figure 1. Relative expression of mRNA levels of (A) hepatic *Hamp* (hepcidin antimicrobial peptide); (B) hepatic *Il6* (interleukin 6); (C) duodenal *Ireb2* (iron regulatory protein 2); (D) splenic *Ireb2*; (E) duodenal *Fth1* (ferritin heavy chain 1); (F) splenic *Fth1* and (G) splenic *Tfrc* (transferrin receptor 1). Expression were normalized to *Actb* (beta actin). Data are the means ± S.D. (n = 7). *significant differences (P < 0.05) in comparison with the control group. Error bar = 95% C.I. (A) shows the relative transcript levels of hepatic hepcidin antimicrobial peptide (*Hamp*) in rat livers normalized to beta actin (*Actb*) values. In comparison with the control group, those insufficient and depleted in vitamin A and sufficient dietary iron showed no significant differences (P < 0.001) in *Hamp* mRNA expression (FesvAi: 1.37-fold; FesvAd: 1.22-fold). The groups with low levels of dietary iron showed a significantly lower relative expression than the control group (FeivAi: 16.1-fold, P = 0.0008; FeivAs: 71.4-fold, P = 0.0004). (B) shows that not all the experimental groups displayed significant differences (P < 0.001) in the mRNA expression of hepatic interleukin 6 (*Il6*) normalized to *Actb* values when compared with the control group.

and the four experimental groups received various combinations of iron (Fe) and vitamin A (vA) at sufficient (s), insufficient (i) or depleted (d) levels. No group was fully depleted in iron because it is necessary for development, and a complete lack of it would result in no viable experimental subjects. Given the relationship between vitamin A deficiency and iron homeostasis, biomarkers are important, as they indicate the needed supply of both micronutrients together to prevent iron deficiency anemia in such risk groups as children and women [22].

When results for serum iron in the three groups with sufficient amounts of iron (Control, FesvAi and FesvAd) were

© 2020 Hogrefe

compared, one may expect that no significant differences emerged between them; however, the group without vitamin A (FesvAd) had less iron than the other (Control and FesvAi), which suggests that a small amount of vitamin A is enough to maintain an adequate level of serum iron; that result is consistent with previous studies that revealed that iron deficiency anemia are related to low levels of serum retinol in children between 1 and 5 years of age [15] and low intake of vitamin A in children between 5 and 12 years of age [23]. The eficacy of multinutrient supply has been validated in multiple countries; in South India, researchers found that the supply of a multiple-micronutrient-fortified salt reduced anemia, iron deficiency and serum retinol deficiency and improved the iron status and retinol status [24]. In Benin, researchers found a high prevalence of IDA and VAD among rural women and their children [25], and a strong recommendation was made to further examine stronger strategies of micronutrient supplementation for at-risk populations.

Regarding serum retinol results, this study revealed that dietary iron status has an effect on it even with an adequate supply of vitamin A, but there is no effect when dietary vitamin A levels are low or absent. Although the focus of this research was not to evaluate the effect of iron on vitamin A, previous findings showed a lack of consensus about the unidirectional effect of iron supplementation on the nutritional status of vitamin A as well as differences between results in animal models and humans [26].

About inflammation status, previous studies revealed that vitamin A deficiency and/or iron deficiency enhances the inflammatory state by increasing *Il6* mRNA expression in the liver [14]. Moreover, it has been established that interleukin-6 regulates hepcidin transcription as a way to control the circulation of iron in inflammation [27, 28]. However, the findings of this study showed no evidence of significant differences in *Il6* mRNA expression as an indicator of inflammation in animals, even with vitamin A deficiency, iron deficiency or both. This finding does not mean, however, that no relationship exists between VAD or ID and inflammation, but under the experimental research conditions, not enough evidence supported it. On the other hand, the lack of significant differences in mRNA *Il6* as a biomarker of inflammation allows for comparison with the other biomarkers or iron status without problems due to their acute-phase-protein properties.

As a systemic regulator of iron homeostasis, *Hamp* mRNA expression in the liver was significantly lower in iron-deficient groups, which makes sense given that hepcidin down-regulates ferroportin on the enterocyte's basolateral side [29] as a way to regulate iron exports and thereby prevent oxidative damage. The results showed impact of vitamin A deficiency on Hamp mRNA expression only when iron was deficient too, consistent whit the findings of Xiaolei *et al* [30] when differences between groups

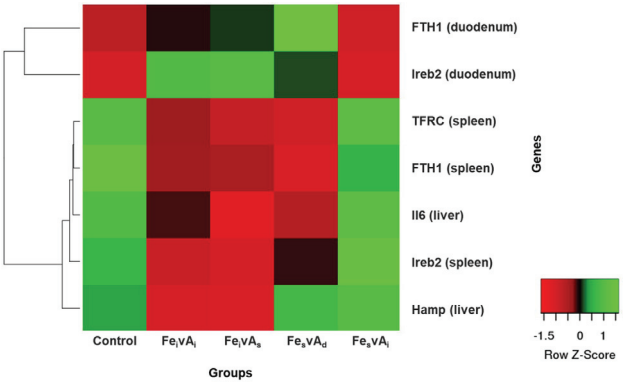

Figure 2. Heat map showing the relative expression of all the genes and experimental groups. Green blocks represent high expression, and red blocks represent low expression. *Fth1*, ferritin heavy chain 1; *Tfrc*, transferrin receptor protein 1; *Ireb2*, iron regulatory protein 2; *Hamp*, hepcidin antimicrobial peptide; *Il6*, interleukin 6.

with fully or partial deficiency of vitamin A and control group were not significant, but when both nutrients were deficient, *Hamp* mRNA expression was significant lower than control group. The work of Arruda *et al* [18] concluded that *Hamp* mRNA increased expression could be a direct effect of vitamin A metabolites or indirect effect mediated by cytokines due to the inflammatory process; as the results of this study showed no inflammation, it is possible that the absence of effect of VAD (at any level) on *Hamp* mRNA expression was due to the absence of cytokines.

Regarding molecular regulation of iron homeostasis, IRP2 is a key regulator of iron metabolism. The results showed that the groups that received a diet low in iron (FeivAs and FeivAi) had significant differences compared to the control group. In the duodenum, the relative mRNA expression of *Ireb2* was higher than that of the control group, but in the spleen, it was lower. Jiang *et al* [31] revealed that vitamin A deficiency could aggravate iron deficiency by upregulating the expression of IRP2; however, the findings of this study suggest that mRNA *Ireb2* expression is not related only to iron or vitamin A supply as independent factors but to both combined.

IRP2 is also related to the regulation of ferritin and transferrin receptor expression. When IRP2 is low, TfR1 expression is reduced and Fn expression is enhanced. Iron storage and mobilization is thus regulated [32]. The results showed that vitamin A was the only factor that impacts ferritin's expression; in the duodenum, only the group that received the diet without vitamin A was significantly higher in ferritin than the others, and in the spleen, the opposite occurred. Those results indicate that vitamin A is necessary to mobilize iron from the enterocyte, reducing its storage in ferritin.

At the duodenal level, these findings were consistent with the study if Citelli *et al* [19] where VAD affected iron mobilization and could lead to oxidative stress. The results

 © 2020 Hogrefe

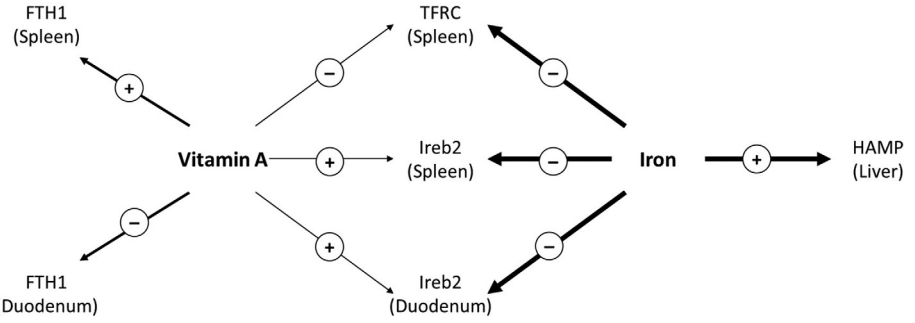

Figure 3. Combined effect of sufficient supply of iron and vitamin A on the mRNA expression of selected biomarkers of iron homeostasis. The arrows' thickness represents the strength of the impact. (+) represents up-regulation. (–) represents down-regulation. Fth1, ferritin heavy chain 1; Tfrc, transferrin receptor protein 1; Ireb2, iron regulatory protein 2; Hamp, hepcidin antimicrobial peptide; Il6, interleukin 6.

regarding the spleen showed a reduction in iron stores represented by relative mRNA ferritin expression for the vitamin A deficient group. That reduction was also demonstrated in findings of Jiang *et al* [31] of the previously discussed study on IRP2 and VAD.

Regarding *Tfrc* mRNA expression in the spleen, the results were consistent with expectations in relation to the results for IRP2 [31]. When comparisons between the dietary-iron sufficient groups were made, only the group without dietary vitamin A showed significant smaller differences, which indicates that, even with an adequate supply of iron, vitamin A is necessary to enhance the *Tfrc* mRNA expression in the spleen. When the low dietary-iron groups were compared to the control group, both iron insufficient groups showed significant smaller differences independent of the vitamin A supply. Those results indicate a combined effect of iron and vitamin A on the *Tfrc* mRNA expression in the spleen.

To give a full picture of the gene expression results, we built a heat map (Figure 2) [33], with which the up-regulation or down-regulation combined effect of iron and vitamin A could be visualized.

When all the results are considered together, the data show a single effect of vitamin A on the mRNA expression of ferritin in the duodenum and spleen, of iron on the mRNA expression of hepcidin in the liver and a combined effect of both on mRNA expression of transferrin receptor 1 and iron regulatory protein 2 in the spleen and duodenum, as seen in Figure 3.

Strengths and limitations

Overall, the main strength of the study is the comparison of five different ratios of iron and vitamin A and the effect of them on the iron homeostasis. Additionally, the study has a strong statistical design adequate to give results of quality without an excessive number of animals according to the main principles of ethics for research with laboratory animals. However, we recognize that the analysis of additional tissues as bone marrow and biomarkers as ferroportin, divalent metal transporter 1 (DMT1) and C-reactive protein (CRP) would give complementary information to support the main findings. A direct measurement of IL6 and CRP as inflammation markers using an ELISA kit would be better to identify the inflammation status. Another limitation, from the methodological approach, was the absence of information of losses of iron and retinol in feces and urine.

Conclusion

The results of the study reinforce the existent relation between vitamin A and iron as demonstrated by different authors as Jiang *et al* [31], da Cunha *et al* [14], Arruda *et al* [18], Mendes *et al* [34] and Xialoei *et al* [30]. This study demonstrated that an adequate supply of dietary vitamin A could enhance the status of serum iron and, as consequence, helps to reduce the prevalence of iron deficiency anemia and make the strategies of nutritional intervention more effective, especially for at-risk populations, such as children and women of reproductive age. In addition, further and deeper research on anemia caused by VAD must be conducted to give a wider explanation of the phenomena related to iron and vitamin A deficiencies.

References

1. Muthayya S, Rah JH, Sugimoto JD, Roos FF, Kraemer K, Black RE. The global hidden hunger indices and maps: An advocacy tool for action. PLoS One. 2013;8(6):1–12.
2. World Health Organization.. Health in 2015: From MDGs, Millennium Development Goals to SDGs, Sustainable Development Goals. Geneva: WHO; 2015.
3. World Health Organization.. The global prevalence of anaemia in 2011. Geneva: WHO; 2015.
4. West KP. Vitamin A deficiency disorders in children and women. Food Nutr Bull. 2003;24(Suppl 4):78–90.

© 2020 Hogrefe

5. World Health Organization.. Global prevalence of vitamin A deficiency in populations at risk 1995–2005: WHO global database on vitamin A deficiency. Geneva: WHO; 2009.
6. Hunt JR. Dietary and physiological factors that affect the absorption and bioavailability of iron. Int J Vitam Nutr Res. 2005;75(6):375–84.
7. Fuqua BK, Vulpe CD, Anderson GJ. Intestinal iron absorption. J Trace Elem Med Biol. 2012;26:115–9.
8. Vieth JT, Lane DR. Anemia. Hematol Oncol Clin North Am. 2017;31(6):1045–60.
9. Wong C. Iron deficiency anaemia. Paediatr Child Health (Oxford). 2017;27(11):527–9.
10. Cascio MJ, DeLoughery TG. Anemia: Evaluation and diagnostic tests. Med Clin North Am. 2017;101(2):263–84.
11. Ganz T. Iron homeostasis: Fitting the puzzle pieces together. Cell Metab. 2008;7(4):288–90.
12. Singh B, Arora S, Agrawal P, Gupta SK. Hepcidin: A novel peptide hormone regulating iron metabolism. Clin Chim Acta. 2011;412:823–30.
13. Theurl I, Schroll A, Nairz M, Seifert M, Theurl M, Sonnweber T, et al. Pathways for the regulation of hepcidin expression in anemia of chronic disease and iron deficiency anemia in vivo. Haematologica. 2011;96(12):1761–9.
14. Da Cunha MSB, Siqueira EMA, Trindade LA, Arruda SF. Vitamin A deficiency modulates iron metabolism via ineffective erythropoiesis. J Nutr Biochem. 2014;25(10):1035–44.
15. Saraiva BC, Soares MC, dos Santos LC, Pereira SCL, Horta PM. Iron deficiency and anemia are associated with low retinol levels in children aged 1 to 5 years. J Pediatr (Rio J). 2014;90(6):593–9.
16. Imam MU, Zhang S, Ma J, Wang H, Wang F. Antioxidants mediate both iron homeostasis and oxidative stress. Nutrients. 2017;9(7):1–19.
17. Da Cunha MSB, Aboudib N, Arruda SF. Effect of vitamin A supplementation on iron status in humans: A systematic review and meta-analysis. Crit Rev Food Sci Nutr. 2019;59(11):1767–81.
18. Arruda SF, Siqueira EMA, De Valência FF. Vitamin A deficiency increases hepcidin expression and oxidative stress in rat. Nutrition. 2009;25(4):472–8.
19. Citelli M, Bittencourt LL, Da Silva SV, Pierucci APT, Pedrosa C. Vitamin a modulates the expression of genes involved in iron bioavailability. Biol Trace Elem Res. 2012;149(1):64–70.
20. Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr. 1997;127:838S–841S.
21. Charan J, Kantharia N. How to calculate sample size in animal studies? J Pharmacol Pharmacother 2013;4(4):303.
22. Pereira RC, Caroso Ferreiira LO, Da Silda Diniz A, Batista Filho M, Natal Figueirôa J. Eficácia da suplementação de ferro associado ou não à vitamina A no controle da anemia em escolares. Cad Saúde Pública. 2007;23(6):1415–21.
23. Visser M, Van Zyl T, Hanekom SM, Baumgartner J, Van der Hoeven M, Taljaard-Krugell C, et al. Nutrient patterns and its relation to anaemia and iron status in 5–12-year-old children in South Africa. Nutrition. 2019;62:194–200.
24. Kumar MV, Nirmalan PK, Erhardt JG, Rahmathullah L, Rajagopalan S. An efficacy study on alleviating micronutrient deficiencies through a multiple micronutrient fortified salt in children in South India. Asia Pac J Clin Nutr. 2014;23(3):413–22.
25. Alaofè H, Burney J, Naylor R, Taren D. Prevalence of anaemia, deficiencies of iron and vitamin A and their determinants in rural women and young children: A cross-sectional study in Kalalé district of northern Benin. Public Health Nutr. 2017;20(7):1203–13.
26. Oliveira JM, Michelazzo FB, Stefanello J, Rondó PHC. Influence of iron on vitamin a nutritional status. Nutr Rev. 2008;66(3):141–7.
27. Lee P, Peng H, Gelbart T, Wang L, Beutler E. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. Proc Natl Acad Sci. 2005;102(6):1906–10.
28. Babitt JL, Lin HY. Molecular mechanisms of hepcidin regulation: Implications for the anemia of CKD. Am J Kidney Dis. 2010;55(4):726–41.
29. Schmidt PJ, Fleming MD. Modulation of hepcidin as therapy for primary and secondary iron overload disorders: Preclinical models and approaches. Hematol Oncol Clin North Am. 2014;28(2):387–401.
30. Xiaolei X, Zhenting Q. Effect of vitamin A deficiency on iron metabolism and hepcidin mRNA expression in rat. In Proceedings 2011 International Conference on Human Health and Biomedical Engineering. 2011;140–2.
31. Jiang S, Wang C, Lan L, Zhao D. Vitamin A deficiency aggravates iron deficiency by upregulating the expression of iron regulatory protein-2. Nutrition. 2012;28(3):281–7.
32. Wilkinson N, Pantopoulos K. The IRP/IRE system in vivo: Insights from mouse models. Front Pharmacol. 2014;5:1–15.
33. Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, et al. Heatmapper: Web-enabled heat mapping for all. Nucleic Acids Res. 2016;44(W1):W147–53.
34. Mendes JFR, De Almeida Siqueira EM, De Brito e Silva JGM, Arruda SF. Vitamin A deficiency modulates iron metabolism independent of hemojuvelin (Hfe2) and bone morphogenetic protein 6 (Bmp6) transcript levels. Genes Nutr. 2016;11(1):1–7. https://doi.org/10.1186/s12263-016-0519-4.
35. Kong WN, Niu QM, Ge L, Zhang N, Yan SF, Bin ChenW, et al. Sex differences in iron status and hepcidin expression in rats. Biol Trace Elem Res. 2014;160(2):258–67.
36. Malik IA, Wilting J, Ramadori G, Naz N. Reabsorption of iron into acutely damaged rat liver: A role for ferritins. World J Gastroenterol. 2017;23(41):7347–58.
37. Malik IA, Naz N, Sheikh N, Khan S, Moriconi F, Blaschke M, et al. Comparison of changes in gene expression of transferrin receptor-1 and other iron-regulatory proteins in rat liver and brain during acute-phase response. Cell Tissue Res. 2011;344(2):299–312.
38. Sheikh N, Batusic DS, Dudas J, Tron K, Neubauer K, Saile B, et al. Hepcidin and hemojuvelin gene expression in rat liver damage: In vivo and in vitro studies. Am J Physiol Liver Physiol. 2006;291(3):G482–90.

History
Received June 28, 2019
Accepted May 29, 2020
Published online July 16, 2020

Acknowledgement
M.R.G. collected and analyzed the data and wrote the manuscript. L.D.B. read and made valuable comments about the text. All authors read and approved the final manuscript. This work was funded by grants from La Sabana University's Grant System (ING-174-2016) and the Colombian Department of Science, Technology and Innovation (Colciencias) through a National Doctoral Grant in call 567-2012.

Conflict of interest
The authors declare that no known conflicts of interest are associated with this publication.

Luis E. Díaz
Campus Puente del Común
Km. 7, Autopista Norte, Bogotá
Chía, Cundinamarca
Colombia
luis.diaz1@unisabana.edu.co

 © 2020 Hogrefe