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Investigation of nitrate and nitrite in commercially available infant formulas and baby foods in Iran and estimation of human health risks

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Levels of harmful nitrogen-containing substances have increased in many foods and drinks around the world. The impacts of dietary nitrate and nitrite on human health have been controversial topics for many years. The present study aimed to quantify the levels of nitrate and nitrite in infant formulas and baby foods sold in Iran and to estimate non-carcinogenic human health risk from exposure to these substances. The samples were gathered randomly and subsequently analyzed to detect nitrate and nitrite via Spectrophotometry. The ranges of nitrate and nitrite in the infant formulas were 0.221–1.347 (mean 0.645) mg/kg and 0.045–0.263 (mean 0.151) mg/kg, respectively. For baby foods, the ranges of nitrate and nitrite were 0.24–1.93 (mean 0.99) mg/kg and 0.04– 1.45 (0.36) mg/kg. Estimated Daily Intake (EDI) values of nitrate and nitrite in all the samples were below the acceptable daily intake (nitrate ADI = 3.7 mg/kg bw/day and nitrite ADI = 0.07 mg/kg bw/day established by FAO/WHO), showing that levels of these contaminants in infant formulas and baby foods may not cause toxicity in the infant and baby population. None of the infant formulas and baby foods in this study showed evidences of a non-cancer risk to the consumers. However, it is suggested that levels of nitrate and nitrite in these products and their related health risks be constantly monitored to prevent significant health hazards in the future.

Keywords Nitrate, Nitrite, Infant formulas, Baby foods, Risk assessment

Nitrate (NO_3^-) and nitrite (NO_2^-) anions are present naturally and ubiquitously in the environment as a part of nitrogen cycle. Air N_2 may also be oxidized to NO_3^- and NO_2^- by microorganisms in soil, plants, and water[1,2]. Nitrate is greatly soluble in water and is an essential nutrient for plants, so it can be distributed in different quantities in plants (particularly in leafy green vegetables and root vegetables) or raw materials for the production of foods[3–7]. Intake of nitrate and nitrite substances occurs through 3 main sources including (vegetable) foods, food additives in some prepared foods and from contaminated water as a result of extensive application of manure and fertilizers[5,7,8]. Microbes can also form nitrite from nitrate during long-term storage of foods in the temperature range from 20 to 25 °C[9]. Nitrate and nitrite in foodstuff have aroused many concerns mainly due to their possible toxicity to human communities, more especially younger children[7,10]. The chemical structure of nitrate and nitrite is shown in Fig. 1. Nowadays, food industry particularly in meat, potassium nitrite (E249), sodium nitrite (E250), sodium nitrate (E251) and potassium nitrate (E252) are commonly and broadly used as food additives and preservatives in almost all countries, in order to fix color, minimize microbial growth (especially Clostridium botulinum), extend the safe shelf life or make a distinguishable flavor[11]. E251 and E252 are legally used as food additives in twenty four foods in the European Union[3]. More than 200 million children

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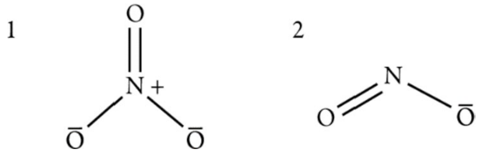

Fig. 1. The chemical structure of, (1) nitrate and, (2) nitrite[45].

globally both in developed and developing countries do not reach their enough developmental potential during first five years due to extreme poverty, poor nutrition, inadequate availability of health care, mental health issues and lack of psychosocial support[12]. The infant and children food industry has expanded dramatically during the past two recent decades[13]. The market for baby foods was reported to be worth above 85.61 billion USD in 2021 and is expected to increase at a yearly growth rate above 6% during 2022–2030 [13]. Generally, infant/baby formulas are milk formulas other than breastmilk produced for children between 0 and 12 months of age; and baby foods are all packaged foods and/or beverages produced for infants and toddlers between 6 and 36 months of age[14].

Although breastfeeding is recommended by the World Health Organization (WHO) during age 0–6 months for achieving an optimal growth, development and health for infants, but approximately 44% of infants 0–6 months of age are exclusively breastfed in the world[15]. UNICEF reported in 2022 that the breastfeeding rate for infants (from 0 to 5 months of age) in some nations located in the Middle East and North Africa in percent as: Iran (53), Iraq (26), Jordan (25), Morocco (35), Algeria (29), Egypt (40), Palestine (39), Syria (29) and Yemen (10)[16]. However, infant formulas and baby foods are being used commonly in many countries as breast milk alternatives mainly because of maternal occupation, study or other engagements, personal preferences, personal beliefs and societal pressure, death and illness [17].

It is reported that 87% of total intake of NO_3^- by an adult is coming from vegetable consumption[1]. High levels of nitrate (above 2500–10000 mg/kg) in some vegetables (particularly in leafy vegetables) have been found in some studies in different nations[18–21]. Concentrations of nitrite in vegetables are comparatively lower. Totally, consumption of some natural fruits and vegetables expose human to higher levels of nitrate and nitrite compared to food additives[22]. Some of the vegetables are used for the manufacture of vegetable-based baby foods[23]. The undesired presence of nitrite in animal feed is also documented[24–27]which may eventually contaminate baby foods[28]. Nitrates and nitrites in baby formulas may originate from several potential sources including the ingestion by dairy cows during grazing and drinking, HNO_3 used during the sanitization of dairy equipment in dairy factory, and the production of N_2O directly through heated spray dryers due to combustion in the presence of excess air[29]. For instance, previous studies have reported nitrate in milk and milk powder samples[27,29–32]. Long-term intake of high levels of nitrates and nitrites through foods and drinks is of great concern for the scientific community and health authorities, as they have been related to detrimental health effects[33,34].

Generally, there are 2 major health concerns associated with the presence of nitrate and nitrite in foods. Nitrate by itself is nontoxic and can be excreted through the urination without adverse effects and thus not of dietary concern but under low pH circumstances or due to the action of commensal bacteria in the mouth and gastrointestinal system of human body it becomes toxic after reducing into nitrite. Generally, nitrate conversion into nitrite in infants occurs in the intestine but in older children and adults in the oral cavity and stomach[23]. After intake, nitrite reacts with haemoglobin (Hb) and forms methaemoglobin (metHb)[5,635]. It may eventually move into the blood and reduce O_2 transport capability by the blood cells, a condition described as cyanosis (blue skin due to low levels of O_2) and asphyxia (low levels of O_2 in blood and tissues) at higher concentrations is dangerous, especially in infants[29,36]. Nitrite, in conjunction with secondary amines from proteins, may also form N-nitroso (NOCs) compounds endogenously in the human body[37]. It is documented that some of these compounds can induce cancer in forty different animal species particularly in higher primates[38]. NOCs are potential human carcinogens and may cause certain types of cancer including gastric, esophageal, nasopharyngeal, and colon cancers[39]. The International Agency for Research on Cancer (IARC) has classified nitrates and nitrites in Group 2 A or probably carcinogens for human because they can form NOCs[40]. For these reasons, regulatory agencies around the world have established upper limits for nitrate and nitrite in infant and baby formulations. France and Germany have set 50 and 250 mg/kg as limits of nitrite and nitrate, respectively, in baby foods. The People's Republic of China has set permissible limits of 2 mg/kg for nitrite and 100 mg/kg for nitrate in baby formula milk and New Zealand declares nitrite < 5 mg/kg as safe for human consumption[29,41]. The European Union has set a permissible limit of 200 mg/kg for nitrate in baby foods for infants and young children[42]. The legal limit for baby foods is also considered 200 mg/kg in Turkey[22]. Brazil has set 250 mg/kg as upper legal limit for nitrate in infant formulations[43]. US FDA doesn't allow use of E250 as an additive for milk or other milk products[44].

Babies are especially more vulnerable from dietary exposure to contaminants though drinking water and foods, because their nervous, reproductive, digestive, respiratory, and immune systems are in a rapid developing stage[46]. Furthermore, dietary exposure of babies to contaminants is usually greater when the exposure is estimated based on mg per kg body weight than that of other people groups, therefore, understanding short-term and long-term exposure to contaminants in baby foods is required to grantee safety and healthy baby growth[47–49]. In the light of the above and also considering the increasing trend of infant formula and baby food consumption, the harmful health effects of nitrate and nitrite as well as limited number of works regarding nitrate and nitrite contents of infant formulas and baby foods in Iran, the objectives of the present study were to gain a better understanding of nitrate and nitrite in these products and to assess the potential human health risks associated with the consumption of the foods containing nitrate and nitrite. Overall, this study offers insights into the levels and the potential health risks of nitrate and nitrite through the consumption of infant formulas and baby foods available in Iran's market.

Materials and methods

Sample collection

In this study, sample selection was done in a way to evaluate a large number of popular brands of infant formulas (milk powder) and baby foods marketed for infants and babies up to the second year of life. The samples in this study were taken from pharmacies in Gonabad, Mashhad, Sabzevar and Tehran in Iran. In total, 47 infant formulas (5 popular brands) and 57 baby foods from 10 different brands (meat-based, fish-based, vegetables-based and fruit-based meals) were randomly collected and analyzed in 2022. Infant formulas and baby foods in this study were coded as IF1-IF47 and BF1-BF57, receptively.

Analysis of contents of nitrite and nitrate

After sampling, they were sent to chemistry laboratory in Gonabad University of Medical Sciences for nitrite and nitrate analysis. The method used for the determination of nitrate and nitrite was according to a previous study[7]. Firstly, nitrate and nitrite stock solutions (1000 mg/L) were prepared using standard sodium nitrate and standard sodium nitrite, respectively. Secondly, color reagent was prepared by dissolving 600 milligrams of 4-aminobenzenesulfonic acid in fifty milliliters of hot water. Then, twenty milligrams of $C_{12}H_{16}C_{l2}N_2$ was dissolved in twenty milliliters of $C_2H_4O_2$ and diluted to 100 milliliters using ultra-pure water. Finally, equal volume of each of the prepared solution was mixed together and used in the experiments. Thirdly, for the sample preparation, 3–10 g of each sample was added into a 200 mL conical flask. Then, for precipitating proteins, 150 milliliters hot water and 10 milliliters of $Na_2B_4O_7{\cdot}10H_2O$ were added and heated for 15 min. The solution finally clarified using $ZnSO_4{\cdot}7H_2O$, diluted with ultra-pure water and filtered through a Whatman paper. Fourthly, nitrate was reduced to nitrite. In this step, 600 mg zinc powder was added into a 50 mL conical flask for each sample. Then, four milliliters $CdSO_4$ was added to each conical flask and left for ten minutes. After that, two milliliters NH_4OH and ten milliliters sample solution were added to one flask. Then, standard nitrate concentrations were prepared by adding ten milliliters of each standard solution to separate volumetric flasks prepared with spongy cadmium. The conical flasks were then shaken for one minute, let stand for 10 min and diluted with distilled water. Finally, 10 mL color reagent was added to each prepared sample and the absorbance was read using a VIS Spectrophotometer (UV-2100).

Statistical data analysis

The data of nitrate and nitrite content in infant formulas and baby foods were statistically analyzed using Excel (Office 2019) and 22th edition of IBM SPSS statistical data.

Health risk assessment from exposure to nitrate and nitrite

Dietary exposure assessment is a useful method to quantify the exposure to contaminated fruits and crops, veterinary residues, nutrients, food additives and other matters from the foods and drinks[50]. Estimated Daily Intake (EDI) was employed to assess the human health risks associated with nitrate and nitrite exposure from infant formulas and baby foods, using the following equation:

$$\mathrm{EDI} = \mathrm{C}_{\text{infant formula/baby food}} \times \frac{\mathrm{IngR}}{\mathrm{BW}} \quad (1)$$

where, $\mathrm{C}_{\text{infant formula/baby food}}$ is nitrate or nitrite concentrations in infant formulas or baby foods expressed in mg/kg dry weight; IngR is average amount of consumed infant formula and baby food which is considered 0.15 kg/day (0–12 months of age) and 0.2 kg/day (12–24 months of age); BW is the average weight body of each age group which was recorded to be 9.7 kg (0–12 months of age) and 11.85 kg (12–24 months of age). Non-cancer risk was calculated using HQ (hazard quotients) described in the following equation:

$$\mathrm{HQ} = \frac{\mathrm{EDI}}{\mathrm{RfD}} \quad (2)$$

where, RfD is the oral reference dose in mg/kg/day which is 1.6 for nitrate and 0.1 for nitrite[51,52]. HQ values greater than or equal to 1 mean that there are potential health risks associated with the consumption of infant formulas and baby foods containing nitrate and nitrite and vice versa.

Toxicological study

For infants and children there is risk of exposure to concentrations of nitrate and nitrite higher than the acceptable daily intake (ADI) because the amounts of food to body weight is higher than that eaten by an adult[7]. In order to assess the exposure levels and to characterize the associated risks, the calculated EDI values were compared to the ADI values recommended by the European Scientific Committee on Food (SCF) and reaffirmed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). According to FAO/WHO, the proposed ADI level for nitrate and nitrite is 3.7 and 0.07 mg/kg bw/day, respectively[53]. The ADI=0.07 mg/kg bw/day for nitrite is obtained from doubling of baseline met-haemoglobin blood levels in male rats investigated in previous studies. Based on the FAO/WHO, an ADI = 3.7 (equivalent to 35.9 mg/kg bw/day for a 9.7 kg infant) and 0.07 (equivalent to 0.68 mg/kg bw/day for a 9.7 kg infant) was considered for infants. For babies, an ADI = 3.7 (equivalent to

Item	Infant formulas		Baby foods	
	Nitrate	Nitrite	Nitrate	Nitrite
Mean	0.645	0.151	0.99	0.36
Standard deviation	0.234	0.063	0.42	0.30
Maximum	1.347	0.263	1.93	1.45
Minimum	0.221	0.045	0.24	0.04

Table 1. Levels of nitrate and nitrite in the infant formulas and baby foods.

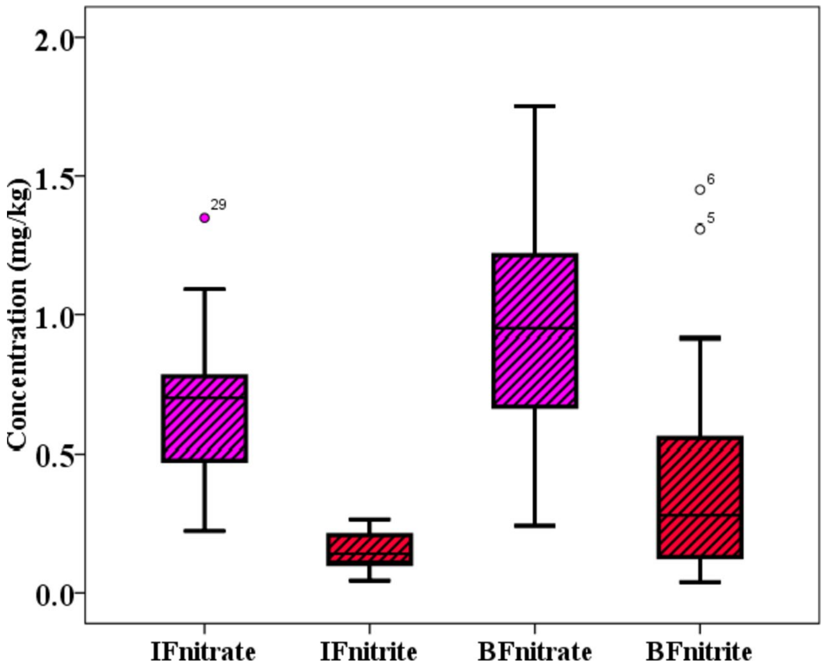

Fig. 2. Box plots of nitrate and nitrite in infant formulas and baby foods.

43.8 mg/kg bw/day for a 11.85 kg baby) and 0.07 (equivalent to 0.83 mg/kg bw/day for a 11.85 kg baby) was used. Percent of ADI was obtained from the following equation:

$$\%\ ADI = \frac{EDI \times 100}{ADI} \tag{3}$$

% ADI values more than 100% signify potential human health risk[54].

Results and discussion

Contents of nitrite and nitrate in infant formulas and baby foods

Since the first year of life is essential for an infant/baby's development, the composition, nutritional value and quality of infant formulas and baby foods must be monitored to decrease the exposure to contaminants[17,28]. Therefore, buyers of infant formulas and baby foods should also be aware of the components and the possible harmful health effects of the food diets they purchase. The contents of nitrate and nitrite obtained by analyzing infant formulas and baby foods are shown in Table 1 and Fig. 2. As given in the figure, the ranges of nitrate and nitrite in the infant formulas were 0.221–1.347 (mean 0.645) mg/kg and 0.045–0.263 (mean 0.151) mg/kg, respectively. For baby foods, the ranges of nitrate and nitrite were 0.24–1.93 (mean 0.99) mg/kg and 0.04–1.45 (0.36) mg/kg.

Nitrate and nitrite was found in all the samples analyzed. In total, nitrate and nitrite concentrations were highly variable within the samples. Concentrations in all of the studied samples were below the admitted limit of 200 mg/kg set for nitrate by the European Commission Directive No 96/5/EC, 100 mg/kg by China and 250 mg/kg by Brazil. Nitrite was also below 2 mg/kg set by China.

The results from the current research were much lower than or some comparable with nitrate and nitrite contents from overseas. In a study, nitrate and nitrite concentrations in infant foods sold in southern Italy was investigated. Concentrations of nitrate and nitrite were in the ranges from 0.35 to 131.68, and from 1.12 to 80.22 mg/kg. The maximum average concentrations were found in foods of plant origin (45.5 mg/kg) for nitrate and in foods of mixed origin (12.48 mg/kg) for nitrite[7]. In another study, the median nitrate concentration in different baby foods (vegetable based, meat based, fish based, and fruit based foods) marketed in Lisbon, Portugal was in the range of 15–61 mg/kg wet weight. Average and median concentrations of nitrate in baby foods were much lower than the legislated value recommended by the European Union (200 mg/kg) and thus these foods were not able to induce harmful effects in the baby population[47]. In a study in Fiji, the commercial baby food brands were investigated. The research showed that nitrate concentrations in the studied baby foods were in the range of 2.10–220.67 mg/kg, whereas, the nitrite concentrations were in the range of 0.44–3.67 mg/kg[1]. In a study in Brazil, 14 baby food samples were taken from local markets and analyzed for nitrate and nitrite. Levels of nitrate and nitrite were in the ranges of 8.44–247.70 mg/kg and < LOD (0.15 mg/kg), respectively[23].

In a previous study, nitrates and nitrites in milk powders in the United States (US) were investigated. The highest nitrite level found in commercial milk powder samples was 17 mg/kg. Nitrite in the examined powders was due to manufacturing or endogenous plant concentrations. The highest nitrate concentration in the retail milk powder samples was 73 mg/kg. The nitrate and nitrite levels in direct heated milk samples were in the ranges of 13–35 and < 0.5–3.2 mg/kg, respectively[29]. McMullen et al. investigated nitrate and nitrite concentrations in baby foods using ion chromatography (IC) and AOAC Official Method (993.03) in the United States of America. Nitrate concentrations were in the range of 95.3–1195.5 mg/kg, values much higher than those detected in the current study. Nitrites were not found in any of the samples analyzed[55].

In a previous study in Taiwan, nitrate and nitrite concentrations in milk powder were investigated. They found nitrate concentrations in the range of 49–417 mg/kg, and nitrite was less than the limit of detection (0.07 mg/kg) in milk powder samples[27]. In a study in Turkey, nitrite levels in infant formulas and baby foods were determined. The mean nitrite level was reported to be 204.07 mg/kg in forty two samples, with 1,073 mg/kg maximum[56]. In another study in Turkey, nitrate concentrations in conventional originated baby food formulas varied from 16.1 to 285 mg/kg with an average amount of 149 mg/kg for all the examined samples. Nitrite concentrations in all the samples were less than the LOQ value (0.021 mg/kg). Briefly, there was no health hazard from the investigated baby foods in respect to nitrate and nitrite[22]. In another research, nitrate and nitrite contents of baby foods in Izmir, the third biggest city in Turkey, were studied. Nitrate levels in baby foods were in the range of 3.32–99.73 (average 37.12) mg/kg. Nitrite levels in the baby foods varied from non-detectable amounts to 30.09 (average 7.90) mg/kg. The findings showed that nitrate contents were all below the upper legislated value (200 mg/kg)[28].

In another work in Turkey, baby purees were investigated, the average nitrite level of all twenty two samples was 31.30 mg/kg. The average nitrite amount in the examined purees was 23.05 and 39.29 mg/kg in purees produced from fruit and vegetable, respectively[57]. A study regarding nitrate in baby food in Estonia, the maximum concentrations of nitrate in baby foods based on carrot and pumpkin were in the range of 62–148 and 124–162 mg/kg, respectively[58]. Mean nitrate concentration found in the baby foods in the current study (0.99 mg/kg) are much lower than the range 59–102 mg/kg reported by the previous studies[58–61]. Moreover, all the samples analyzed in this research had nitrate levels lower than the legal upper limit recommended by the EU (200 mg/kg), the highest amount being 1.93 mg/kg.

Toxicological study

The estimations of nitrate and nitrite exposure through infant formula and baby food consumption were compared with the legal upper limits for ADI of nitrate (3.7 mg/kg bw/day) and nitrite (0.07 mg/kg bw/day), to understand the infant or babies which they may have adverse effects on their health. Fig. 3 shows box plots of ADI values associated with nitrate and nitrite exposure through consumption of infant formulas and baby foods. In the present study, the dietary nitrate exposure or EDI through infant formulas ranged from 0.003 (0.09% of ADI) to 0.021 mg/kg bw/day (0.56% of ADI). For nitrite, exposure levels ranged from 0.001 (0.99% of ADI) to 0.004 mg/kg bw/day (5.8% of ADI). The EDI through baby foods consumption containing nitrate varied from 0.004 (0.11% of ADI) to 0.033 mg/kg bw/day (0.88% of ADI). For nitrite, exposure levels ranged from 0.001 (0.96% of ADI) to 0.024 mg/kg bw/day (34.96% of ADI). EDIs of nitrate and nitrite in all the samples were below the acceptable daily intake (ADI for nitrate = 3.7 mg/kg bw/day and ADI for nitrite = 0.07 mg/kg bw/day), showing that these infant formulas and baby foods may not cause nitrate and nitrite toxicity in the infant and baby population. Generally, the mean EDI of nitrite was higher compared to nitrate. In Portugal study, the vegetable based baby foods for infants of 4 and 8 months of age accounted for 13% and 10% of the ADI, respectively, indicating that the estimated exposures were not of health concern[47].

In Izmir study in turkey, the dietary nitrate exposure through baby foods for the average, maximum and minimum nitrate levels varied from 0.43 (11.60% of ADI) to 1.01 mg/kg bw/day (27.30% of ADI), from 1.15 (31% of ADI) to 2.71 mg/kg bw/day (73.2% of ADI) and from 0.04 (1.08% of ADI) to 0.09 mg/kg bw/day (2.43% of ADI), respectively which all were less than the ADI (3.7 mg/kg bw/day) admitted by the EU. The estimated nitrite exposure through baby food consumption for the average and maximum nitrite levels were in the range from 0.09 to 0.21 mg/kg bw/day and from 0.35 to 0.82 mg/kg bw/day, respectively, all were higher than the ADI level (0.07 mg/kg bw/day) set by the EU[28]. Mean and maximum EDI values of nitrate through baby purees (both fruit and vegetable based) consumption in Turkey were in the ranges of 3.46–5.89 and 12.69–21.88 mg nitrite per 150 gram, respectively which indicated that the intake for one meal is above the ADI established by the FAO/WHO[57]. In Taiwan's study, levels of EDI in all the samples of milk powders were less than the acceptable ADI = 3.7 mg/kg body weight per day recommended by the FAO/WHO[27].

Health risk assessment

Non-carcinogenic risk exposure assessment

The non-carcinogenic risk was calculated using Eq. 2 described in methodology section. Levels of non-cancer risk associated with consumption of infant formulas containing nitrate and nitrite ranged from 0.002 to 0.013 (mean 0.006) and from 0.007 to 0.041 (mean 0.023), respectively. For the baby foods, non-cancer risk associated with nitrate and nitrite varied from 0.003 to 0.02 (mean 0.01) and from 0.007 to 0.245 (mean 0.061), respectively.

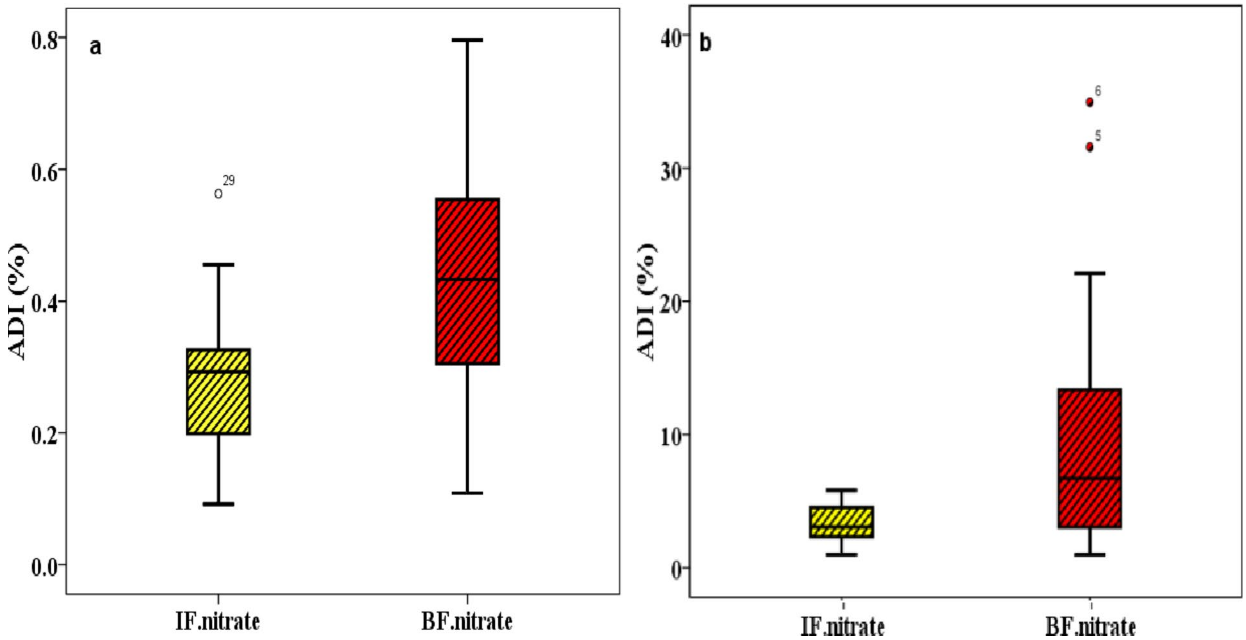

Fig. 3. Box plots of ADI values associated with nitrate (**a**), and nitrite (**b**) exposure through consumption of infant formulas and baby foods.

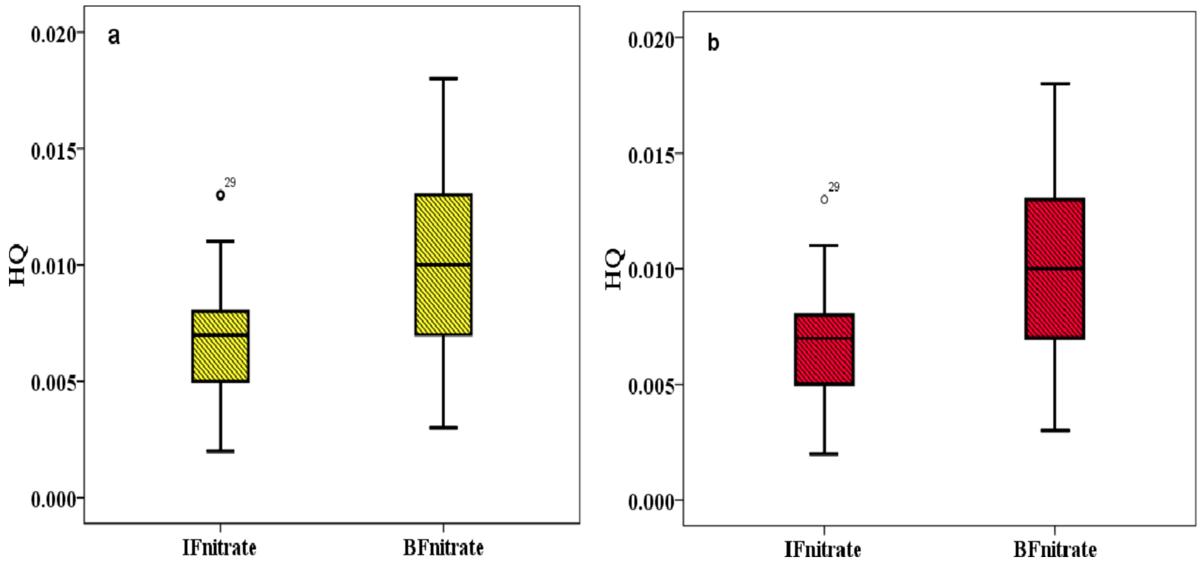

Fig. 4. Box plots of HQ values associated with nitrate (**a**), and nitrite (**b**) through consumption of infant formulas and baby foods.

HQ values of nitrate and nitrite exposure through consumption of infant formulas and baby foods are shown in Fig. 4 (a and b). As seen in the figure, HQ values are all below the upper limit of 1 proposed for non-cancer risk.

There are certainly limitations in this study. Average amount of infant formula and baby food consumption and also average amount of body weight are used in this study. However, some infants and babies may be feed on more/less amounts of these products which affects on the risk they get. Finally, residual concentrations of nitrate and nitrite might be influenced by the storage time and temperature, the primary dosage, pH, components of food, and the presence of microorganisms, resulting in change during storage. These factors should also be considered in the estimation of human health risk, as no relevant data are available in literature. For future risk studies, we therefore suggest to consider a combined dietary intake from all sources for nitrate and nitrite taking into account drinking water and other baby foods in older children.

Conclusions

The main aims of the present study was to determine nitrate and nitrite contamination in infant formulas and baby foods and to assess their possible human health risks. For this purpose, 5 infant formulas and 10 baby foods were randomly collected. Concentrations of all of the studied samples were below the admitted limit of 200 mg/kg set for nitrate by the EU legislation. The estimation of nitrate and nitrite intake from infant formulas and baby foods revealed that infant/baby population were not at risk by considering the ADIs established by the FAO/WHO. None of the infant formulas and baby foods in this study showed evidences of non-cancer risk to the consumers. However, because of the increasing trend of infant formulas and baby foods use and most are specifically sold for their consumption, the evaluation levels of nitrate and nitrite in these products is of great interest and therefore, the frequent monitoring of these foods is important for the safety of the infant/baby foods in regard to nitrate and nitrite.

Data availability

All data generated or analyzed during this study are included in this published article.

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Declarations

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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The ethics committee of Gonabad University of Medical Sciences (IR.GMU.REC.1398.045) approved all the procedures in the study.

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