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Supplementation of heat treated milk with iron salts in normal or nano form and studying its biological attributes in albino rats

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Abstract

The aim of this research was to study the effect of heat treated milk supplementation with iron salts in normal or nano form on the product properties and biological attributes using Albino rats.

Cow's milk was divided into four portions. The first one was not supplemented by any iron sources, while, the other three portions were supplemented with 39 ppm iron either of ferrous sulfate, ferric chloride or iron nanoparticles. All milks treated by heating at 72°C/15sec and rapidly cooled to 5 °C. Thirty male albino rats were divided into randomly 5 groups, (each group contain six rats). The 1st group was fed on standard diet represent as control (-). While, the 2nd group was fed on standard diet plus dosing 5 ml of heat treated milk without any supplementation served as control (+).The 3rd group was fed on standard diet plus dosing 5 ml of heat treated milk supplemented with ferrous sulfate. For the 4th group, rats were fed on standard diet plus dosing 5 ml of heat treated milk supplemented with ferric chloride. In the 5th group, rats were fed on standard diet plus dosing 5 ml of heat treated milk supplemented with iron nanoparticles. The biological experiments continued 6 weeks.

The results referred to, there were no significant differences in chemical composition among all heat treated milk samples, the ash content increased by adding iron salts. All treatments kept their microbiological quality as pasteurized milk along the cold storage period for 14 days. Addition of iron salts led to a significant decrease in the sensory attributes, but the least severe effect was when the supplementation being with iron nanoparticles whether when fresh or during cold storage period. Biologically, there was a significant increase in body weight in rats when using iron salts, especially in the nano form. There were also significant increases in the weights of the organs (liver and kidney) in the rats fed on milk supplemented with iron salts, in general, and in particular in the nano form. There was significant increase in the percentage of hemoglobin (%Hb) and red blood cells (%RBCs) in the blood samples of the rates group fed on milk supplemented with iron nanoparticles. Histologically, the group iron nanoparticles showed the least significant activity of the kupffer cells.

In conclusion, synthesis of iron complexes in nano size increases iron bioavailability, and problems like unacceptable color, flavor, metallic taste, and rancidity in food vehicles using water-soluble iron salts are solved. Supplementation of milk with iron is considered an important means of producing functional drinking milk especially when it was in the nano form. It had been several benefits for human such as: improved iron absorption which leads to increased iron bioavailability, that was important for preventing iron deficiency anemia which is a common health problem worldwide especially Egypt.

Keywords: Iron deficiency anemia - Ferrous sulfate - Ferric chloride – Nano iron.

1. Introduction

Anemia is a global public health problem. Many types of anemia exist, such as iron-deficiency anemia, pernicious anemia, aplastic anemia, and hemolytic anemia. The different types of anemia are linked to various diseases and conditions (NIH, 2011)[1]. Anemia may be caused by several factors: nutrient deficiencies through inadequate diets or inadequate absorption of nutrients, infection (e.g. malaria, parasitic infections, tuberculosis, HIV), inflammation, chronic diseases, gynecological and obstetric conditions, and inherited red blood cell disorders (Sonja *et al*., 2023)[2]. When mentioning the health problems prevalent in the third world countries, which are based on the deficiency of micronutrients such as mineral elements, the problem of iron deficiency anemia (IDA), which is called IDA must be mentioned (Olson *et al.*, 2021) [3] .When the number of red blood cells or their oxygen-carrying capacity is insufficient to meet physiological requirements of the body this will lead to the Anemia (WHO, 2011)[4]. The most common nutritional cause of anemia is iron deficiency. Iron deficiency anemia is one of the most global health concerns including Egypt. That is affecting all age groups significantly and particularly affects young children, menstruating adolescent girls, pregnant women and postpartum women (De Leeuw *et al.*, 1966)[5]. WHO estimates that 43 % of children months of age, 38% of pregnant women, and 29% of non-pregnant women worldwide are anemic (Stevens *et al.*, 2013)[6].

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Supplementation of foods and dairy products with iron salts conceder is the best and most cost-effective strategy method for targeting large-scale populations to provide nutritional security. The solubility, bioavailability, and chemical characteristics of iron compounds may have a great impact on the quality of iron-fortified food (Blanco and Vaquero, 2019)[7]. Milk is one of the most important food sources rich in many food sources with high nutritional value, it is an excellent source of calcium, protein, vitamins and minerals. However, it was found that milk is poor in iron (Meshref et al., 2014)[8]. The selection of iron compounds for supplementation should be based on the legal and labeling requirements (Hurrell, 2007)[9], the chemical form of iron, the solubility of iron compounds, and the pH of the food, the sensory quality of the supplemented food, and the cost of iron compounds. Dairy products can be fortified with iron in different sources such as iron complexes, ferrous sulfate and ferric chloride, which are highly water-soluble (Unnikrishnan and Bhimasena, 1977)[10], insoluble iron salts (ferric pyrophosphate), while nano-sized iron salts in particular have been highlighted in this research because they are one of the most important alternatives to traditional iron salts commonly used in food fortification, which are either iron salts that have a high solubility in water and are characterized by being of high bioavailability, but it is defective in that it results in the emergence of unwanted flavor and color materials, in addition to the effects they cause for the digestive system or the use of iron salts that are scarce soluble in water, which leaves behind the absence of any unwanted sensory changes in the product, but the lack of solubility of these salts in stomach acids leads to a decrease in bioavailability and thus a lack of nutritional value and highlighting the iron nano salts found that they combine the advantages of traditional iron salts fading their disadvantages mentioned above Hurrell (2002)[11].

Nanotechnology is becoming increasingly important for the food sector. The incorporation of nano-materials into food manufacturing could find applications in fresh fruits and vegetables, bakery products confectionery and dairy products (Sozer and Kokini, 2009)[12]. Among many nanotechnology applications, one is to increase nutrient bioavailability (particle size reduction techniques increase the surface area of iron compound which improves its solubility in the gastric acid leading to higher absorption) and delivery of minerals, nutrients, molecules, and drugs to their target. Researchers found that when some of the materials are prepared in nanometer size, their bioavailability increases (Shafie *et al.,* 2016)[13] and (Foujdar *et al.*, 2020)[14]. In particular nano iron has several health benefits such as (1) improved iron absorption: nano iron particles are small enough to be easily absorbed by the intestines which leads to increased iron bioavailability. This is important for preventing iron deficiency anemia which is a common health problem worldwide.(fragoso *et al.,* 2017)[15]. (2) Enhanced immune function: iron is essential for the proper functioning of immune system. Nano iron can boost immune function and help to protect against infections. (3) Reduced oxidative stress which is a process that can damage cells and tissues because nano iron particles have been shown to have antioxidant properties that can help to protect against oxidative stress (Fernandez *et al.,* 2020)[16]. (4) Improved cognitive function: iron is important for brain development and function. (5) Reduced risk of chronic disease such as heart disease, stroke and cancer (Góral *et al.,*2023)[17].

Therefore, it was aimed to study the effect of heat treated milk supplementation with iron salts in normal or nano form on the product properties and biological attributes using Albino rats.

Materials And Methods

Materials

Fresh cow's milk was purchased from the herd of the dairy cattle at Faculty of Agriculture, Cairo University, Giza, Egypt. The milk contains 3.5% fat, 3.4 protein, and 12.5% T.S. Ferrous sulfate and ferric chloride (food grade salts) were obtained from El- Gomhouria Co. for drugs , Pharmaceuticals and Medical Supplies, Cairo, Egypt.

Commercial kits used for determining alanine aminotransferase (ALT), aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Triglycerides (TG), total cholesterol (TC), High-density lipoprotein (HDL), low-density lipoprotein (LDL), urea and creatinine were obtained from Agricultural Research Center, Giza, Egypt.

Thirty male albino rats weighing about 120±5 g were obtained from Agricultural Research Center, Giza, Egypt. The experimental protocol was approved by the Ethical Committee at Agricultural Research Center. The animal groups were fed on standard diet and placed in an atmosphere of filtered, pathogen-free air, water and maintained at a temperature between 20-25ºC for six weeks.

Experimental Procedures

Preparation of iron solutions

Two iron solutions were prepared using of ferrous sulfate ($FeSo_4$) and ferric chloride ($FeCl_3$) as follow: 0.5 g of ferrous sulfate or ferric chloride was weighed and dissolved in 49.5 ml of sterile distilled water and stirred well until completely dissolved. One ml from each solution was added into 99 ml milk. Thus, milk supplemented with iron salts at rate 0.01% and this concentration equals 39 ppm.

Synthesis of colloidal iron oxide nanoparticles

Colloidal iron magnetite nanospheres 8 ± 2 nm size was synthesized at Nano Fab Technology Co., Giza, Egypt according to (Jakubovics, 1994)[18] and (Bassiony *et a.,* 2014)[19], by co-precipitation method using ascorbic acid reduction of $FeCl_3$. Where, $FeCl_3$ was dissolved in 25 ml distilled water under stirring for five min at the room temperature. In another vessel $NaCO_3$ powder dissolved in 10 ml distilled water, then the solution was added to $FeCl_3$ solution with continued stirring for 10 min. The color of the reaction solution was turned into viscous with brown color. Then, ascorbic acid was instantly added to the previous reaction mixture with vigorous stirring for 15 min, the color of solution turned black, formation of colloidal magnetite nanoparticles capped with ascorbic acid. By examining the crystals formed using a transmission electron microscope (TEM), it is confirmed that the desired nanostate of the molecules has been achieved. When one ml from this solution was added into the 99 ml milk, the final concentration of 39 ppm Fe was done.

Iron supplementation and heat treatment of milk

Milk was divided into four parts. The first part was heat treated at 72 °C /15sec then rapidly cooled to 5 °C without any iron supplementation. The last three parts was supplemented with one % of any iron solution, whether $FeCl_3$, $FeSO_4$ or nano FeO and heat treated as mentioned before. All final milk were filled into 100 ml plastic bottles (previously boiled for 5 min), air tightly closed and cold stored at (5±2°C) for 14 days. Due to the validation time of pasteurized milk was only 14 days at refrigerator temperature (5±2°C); all batches were reproduced again every two weeks to provide milk treatments for the biological studies those continued 6 weeks, taking into account re-melting them well before immediately use.

Analytical Methods

Chemical analysis

Total solids (TS), protein, fat, ash, lactose and titratable acidity contents of milk were determined according to (AOAC, 2012)[20].Iron content was determined according to American Public Health Association (APHA, 2017)[21] using the Agilent 5100 Synchronous Vertical Dual View (SVDV) ICP-OES, with Agilent Vapor Generation Accessory VGA 77.

Physical analysis

The pH value was measured electronically in milk using lab pH-meter with a glass electrode (Hanna model 8417 digital pH meter).

Microbiological examination

Total viable bacterial counts were enumerated using the pour plate method by using Trypticase Soya Agar (TSA), incubation at 37ºC for 48 h according to (APHA, 2017)[21].

Coliform counts were enumerated using Violet Red Bile Agar (VRBA), incubation at 37ºC for 48h according to (APHA, 2017)[21].

Yeasts & Molds were enumerated using the pour plate method by using Malt Extract Agar (MEA) medium, incubation at 25ºC for 3 - 5 days according to (APHA, 2017)[21].

Sensory evaluation

Scoring properties of milk drink were done by ten stuff members at Food Science Department, Faculty of Agriculture, Ain Shams University. Milk drink samples were exposed to a panel judging so that following characteristics were estimated: appearance, mouth feel, taste, flavor and color (9 points for each criterion), a maximum number of points were assigned to each sample according to the scheme of (Nkhata *et al.*, 2015)[22]

Biological evaluation

The experimental animals were divided into randomly 5 groups, (n = 6 per group) as follows: The 1st group, were fed on standard diet represent as control (-). While, the 2nd group, rats were daily fed on standard diet + dosing 5 ml heat treated milk without any iron supplementation served as control (+).The 3rd group, rats were daily fed on standard diet + dosing 5 ml heat treated supplemented milk with 39 ppm $FeSO_4$. The 4th group, rats were daily fed on standard diet + dosing 5 ml heat treated supplemented milk with 39 ppm $FeCl_3$. The 5th group, rats were daily fed on standard diet + dosing 5 ml heat treated supplemented milk with 39 ppm FeO nanoparticles. The body was weighed periodically weekly with a complete blood profile (blood picture, liver function & kidney function). Finally the histopathological examination of anatomical tissue structure of the liver and kidney organs were performed at the end of the experimental period after slaughtering the experimental rats.

Statistical analysis

The obtained data were statistically analyzed using One Way ANOVA. All statistical analysis for the different traits was realized using SAS program (SAS, 1999)[23]. Differences among experimental groups were tested by Duncan's Multiple Range test.

Results And Discussion

Chemical properties

Table (1): Chemical properties of heat treated milk as a function of the supplementation source with iron

Component	Heat treated milk at 72 °C /15 sec			
	Without Fe supplementation	Source of supplementation with 39 ppm Fe		
		$FeSO_4$	$FeCl_3$	Nano FeO
Total solids %	**12.49^A**	**12.32^A**	**12.21^A**	**12.57^A**
Fat %	**3.30^A**	**3.30^A**	**3.40^A**	**3.50^A**
Protein % (Total nitrogen X 6.38)	**3.52^A**	**3.42^A**	**3.44^A**	**3.46^A**
Lactose %	**4.89^A**	**4.84^A**	**4.85^A**	**4.83^A**
Ash %	**0.698^B**	**0.761^A**	**0.752^A**	**0.781^A**
Titratable acidity %	**0.16^A**	**0.16^A**	**0.16^A**	**0.16^A**
Iron (ppm)	**2.6^B**	**33.0^A**	**33.0^A**	**34.0^A**

A, B: Means with same letter among treatments are not significantly differed.

The data in **Table (1)** showed that adding iron salts did not have any significant effect on the chemical composition of milk, whether in the milk's lactose, protein, or total solids content, regardless of the form (ferrous sulfate, ferric chloride and nano iron oxide) in which the milk has been supplemented, but it is causing a slight increase in the ash percentage, as a natural result of adding iron salts. The addition of iron salts did not generally affect the titratable acidity of milk.

These results are in the same line with (Akhter *et al.*, 2014)[24] and (Nkhata, 2015)[22], who found that physico-chemical attributes of raw and pasteurized buffalo milk were not significantly impacted by iron adding.

Microbiological situation

Data of **Table (2)** confirmed the microbiological quality of heat treated milk along the 14 days of cold storage period. These results are in accordance to microbiological limits of the Egyptian Organization for Standardization and Quality (2005)[25], who provided that the total bacterial count of pasteurized milk should not exceed 30X 10^3 cell / ml. This number, when converted logarithmically, equals 4.477.

Table (2): Microbiological quality of heat-treated milk as a function of the supplementation source with iron

Microorganism (Log CFU/ml)	Cold storage period (day)	Heat treated milk at 72 °C /15 sec			
		Without supplementation	Source of supplementation with 39 ppm Fe		
			$FeSO_4$	$FeCl_3$	Nano FeO
Total bacteria	1st	4.04^{Bc}	4.01^{Bc}	4.00^{Bc}	4.02^{Bc}
	7th	4.22^{Bb}	4.20^{Bb}	4.27^{Bb}	4.23^{Bb}
	14th	4.41^{Ba}	4.39^{Ba}	4.36^{Ba}	4.38^{Ba}
Coliform	1st	ND	ND	ND	ND
	7th	ND	ND	ND	ND
	14th	ND	ND	ND	ND
Yeasts and Molds	1st	ND	ND	ND	ND
	7th	ND	ND	ND	ND
	14th	ND	ND	ND	ND

A, B, C: Means with same letter among treatments are not significantly differed
a, b, c : Means with the same letter for the same treatment are not significantly differed
ND: Not detected

Organoleptic quality

Sensory evaluation scores given in **Table (3)** indicated that the addition of iron salts led to a significant decrease in the judging score of appearance, mouth feel, taste, flavor and color criteria compared to the control milk, which was without iron supplementation by any source, but the least severe effect was observed when the supplementation was done using iron nanoparticles, whether when fresh or along the cold storage period (14 days). This is due to the fact that iron nanoparticles is characterized in that it does not produce compounds that affect the taste and flavor *versus* those criticized when normal iron salts, ferrous sulfate or ferric chloride, were used in supplementation of milk. Similar observations were reported by (Kumari and Chauhan, 2022)[26].

Table (3): Organoleptic scores of iron supplemented heat treated milk as affected by the iron source and the cold storage period at 5±1°C for 14 days

Sensory attribute (9 points)	Cold storage period (day)	Heat treated milk at 72 °C /15 sec			
		Without supplementation	Source of supplementation with 39 ppm Fe		
			$FeSO_4$	$FeCl_3$	Nano FeO
Appearance	1st	8.7^{Aa}	8.6^{Aa}	8.6^{Aa}	8.4^{Aa}
	7th	8.7^{Aa}	8.6^{Aa}	8.6^{Aa}	8.4^{Aa}
	14th	7.7^{Ab}	7.5^{Bb}	7.4^{Bb}	7.2^{Bb}
Mouth feel	1st	8.4^{Aa}	4.1^{Da}	6.2^{Ca}	7.3^{Ba}
	7th	8.4^{Aa}	4.1^{Da}	6.2^{Ca}	7.3^{Ba}
	14th	7.4^{Ab}	3.1^{Db}	5.2^{Cb}	6.3^{Bb}
Taste	1st	8.6^{Aa}	4.0^{Da}	5.3^{Ca}	6.3^{Ba}
	7th	8.6^{Aa}	4.0^{Da}	5.3^{Ca}	6.3^{Ba}
	14th	7.6^{Ab}	3.0^{Db}	4.3^{Cb}	5.3^{Bb}
Flavor	1st	8.3^{Aa}	4.3^{Da}	5.2^{Ca}	6.8^{Ba}
	7th	8.3^{Aa}	4.3^{Da}	5.2^{Ca}	6.8^{Ba}
	14th	7.3^{Ab}	3.3^{Db}	4.2^{Cb}	5.8^{Bb}
Color	1st	8.7^{Aa}	7.1^{Ba}	7.1^{Ba}	7.3^{Ba}
	7th	8.7^{Aa}	7.1^{Ba}	7.1^{Ba}	7.3^{Ba}
	14th	7.7^{Ab}	6.1^{Bb}	6.1^{Bb}	6.3^{Bb}
Overall	1st	8.6^{Aa}	4.4^{Da}	6.2^{Ca}	7.9^{Ba}
	7th	8.6^{Aa}	4.4^{Da}	6.2^{Ca}	7.9^{Ba}
	14th	7.6^{Ab}	3.3^{Db}	5.2^{Cb}	6.9^{Bb}

A, B, C: Means with same letter among treatments are not significantly differed.
a, b, c: Means with same letters for same treatment during cold storage periods are not significantly differed

These results are also consistent with those mentioned by (Hurrell *et al.,* 2022)[27], who explained that ferrous sulfate salt often causes completely undesirable sensory changes in food, due to the characteristic metallic taste of dissolved free iron which can form colorful complexes with polyphenol compounds in fruits and vegetables was not preferred to the consumer, as it is possible to oxidize fat in foods containing fat like whole milk or full cream milk powders. These findings may be mainly due to the method used for synthesis of colloidal iron oxide nanoparticles, which was characterized with its composition containing an excellent coating agent, namely ascorbic acid, which gave the product stability throughout the storage period. This is in addition to its effective role in enhancing the absorption of iron within the body and combating the risk of anemia, as indicated by many studies as of (Kumari and Chauhan, 2022)[26] .These results are consistent with (Nile *et al.,* 2020)[28]who found that nano technology is also used to produce foods with better functional properties in taste, appearance and color, and with lower calories and less change in the content and nature of salts and sugars.

This trending is in the same line with (Akhter *et al.,* 2014)[24] who found that sensory attributes of raw and pasteurized buffalo milk supplemented with iron were significantly differed from control.

Biological assessment

Body and organs weights

Regarding the characteristics of the rats that were used in the different conditions of the experimental, including weight and health status, it was noticed that there is a significant increase in body weight when using iron salts, especially iron nanoparticles **(Table, 4)**. This is due to the fact that the bioavailability of iron nanoparticles is greater than that of other iron salts. Therefore, the respiratory rate becomes better, so the digest and metabolize of food were improved, and the rate of infection with microbes, pollution, or any other damage declined. This could be because the immunity of the rats those fed on iron nanoparticles was more powerful than the other rats groups. Such factors will be reflected positively in rats weight increment. The results are in agreement with those of (El-Dreny *et al.,* 2019)[29]. Unlike other iron salts, this is reflected in all the vital processes in which iron participates within the body, including breathing, digestion, absorption, and assimilation of food, as it increases the efficiency of the immune, circulatory, and respiratory systems, and thus the health condition of the body in general, these findings were confirmed by (Kumari and Chauhan, 2022)[26] and in the same line with the results of (El Shemy, 2018)[30].

Table (4): Periodical body weight gain (g) of rats fed on iron supplemented heat-treated milk as affected by the iron source

Periodical Body weight (g)	Feeding diet				
	Standard diet only (Control -)	Standard diet + dosing 5 ml of Heat treated milk at 72 °C /15 sec			
		Without supplementation (Control +)	Source of supplementation with 39 ppm Fe		
			$FeSO_4$	$FeCl_3$	Nano FeO
IBW	162.5	163	161	163	162.8
BW after (1wk)	171.4	173	176	175	178.3
BW after (2wk)	184	186	187	188	190.3
BW after (3wk)	202.5	203	204.5	207	215.5
BW after (4wk)	214	216	217	218.9	223.3
BW after (5wk)	226.8	229.3	230	233.1	237.2
FBW after (6wk)	238.8	239	242	245.4	250.5
%BWG	46.95^{C}	46.63^{C}	50.31^{B}	50.55^{B}	53.87^{A}

IBW, FBW, BWG means initial body weight, final body weight and body weight gain during experimental period, respectively.
wk: week.

A, B, C: Means with same letter among treatments are not significantly differed.

As for the weight of the organs, the indicators showed that there was a significant increase in the weights of the organs (liver and kidney) in rats groups those fed on milk supplemented with iron salts in general and in particular on the iron nanoparticles **(Table, 5)**. This is a logic corollary due to the increase in the bioavailability of the iron nanoparticles, which improved the efficiency of the rats in feeding, and thus its absorption of food was increased, and consequently led to an increase in muscle mass and tissue weight. It is worth noting that the rat's body treated this increase as muscle gain, not fat as will mentioned below in blood lipid profile analysis, which distinguishes this type of iron (nanoparticles) from other iron form. Based on the facts mentioned by (Mohammed *et al.,* 2021)[31] who explained the importance of taking organ weight into account as it is considered a basic part of the determination of drug safety and are used to estimate deep internal organ disease.

Table (5): Mean organs weight gain (g) of rats fed on iron supplemented heat treated milk as affected by the iron source

Organ weight (g)	Feeding material				
	Standard diet only (Control -)	Standard diet + dosing 5 ml of Heat treated milk at 72 °C /15 sec			
		Without supplementation (Control +)	Source of supplementation with 39 ppm Fe		
			$FeSO_4$	$FeCl_3$	Nano FeO
Liver	5.6 ^{B}	5.7 ^{B}	6.5 ^{A}	6.4 ^{A}	6.7 ^{A}
Kidney	1.8 ^{B}	1.8 ^{B}	2.1 ^{A}	2 ^{A}	2.1 ^{A}

A, B, C: Means with same letter among treatments are not significantly differed.

Blood picture

Periodically drawn blood chemical analyses of experimental rats fed on milk supplemented with different sources of iron in **Table (6)** led to observe the significant increase in the percentage of hemoglobin (%Hb) and red blood cells (%RBCs) in the blood of the groups fed on milk supplemented with iron nanoparticles. As for the indicators packed cell volume (%PCV), which expressed the viscosity of the blood, it could be noticed that when comparing the groups with each other, the group fed on milk supplemented with iron nanoparticles had also the best and highest rated, and this is indicative of the increased bioavailability of iron nanoparticles, as the rat's body was able to digest this form of iron better, assimilate and absorb in the body, and thus high concentrations of it were reached to the blood, which appeared in indicators (%PCV).

With regard to the properties related to immunity, it was platelet ratio (%PLT) and white blood cells (WBCs). It was noted that the best treatments in raising and improving the immune efficiency of experimental rats is also this treatment supplemented with iron nanoparticles. These results matched with those of (Elshemy, 2018)[30] who explained that iron oxide nanoparticles administration induced a significant increase in RBCs count, hemoglobin concentration(HB), and packed cell volume (%PCV) when compared to ferrous sulfate group in treatment of anemia. That may be due to its marked small size that increased the bioavailability and absorption of iron oxide nanoparticles.

Table (6): Blood chemical analysis of experimental rats fed on iron supplemented heat treated milk as affected by the iron source

Parameter		Feeding material				
		Standard diet only (Control -)	Standard diet + dosing 5 ml of Heat treated milk at 72 °C /15 sec			
			Without supplementation (Control +)	Source of supplementation with 39 ppm Fe		
				$FeSO_4$	$FeCl_3$	Nano FeO
Blood picture	%Hb	16.9 ^{E}	20.7 ^{D}	38.6 ^{C}	40.6 ^{B}	48.6 ^{A}
	%RBCs	17.2 ^{D}	26.5 ^{C}	30.7 ^{B}	34.8 ^{A}	35.4 ^{A}
	%PCV	16.1 ^{D}	22.1^{C}	26 ^{B}	23.5 ^{C}	31.7 ^{A}
	%WBCs	3.1 ^{E}	4 ^{D}	6.3 ^{C}	13.1 ^{A}	8.8 ^{B}
	%PLT	37.7 ^{D}	36.1 ^{D}	40.6 ^{C}	43.8 ^{D}	47 ^{A}
Liver enzymes (U/dl)	ALT	51.67^{A}	52.66^{A}	52.66^{A}	53.4^{A}	51.2^{A}
	AST	22.33^{C}	23^{C}	29.7^{B}	33.4^{A}	24.33^{C}
	ALP	212.67^{E}	284^{C}	330^{B}	373^{A}	275.57^{D}
Kidney function (mg/dl)	Urea	34.5^{C}	34.83^{C}	39^{B}	42^{A}	35.33^{C}
	Creatinine	0.79^{C}	0.73^{D}	0.80^{B}	0.99^{A}	0.76^{CD}
Lipid profile (mg/dl)	CH	65.4^{C}	68.37^{B}	82.7^{A}	83.63^{A}	70.1^{B}
	TG	117.8^{B}	118.17^{B}	120.8^{B}	132.2^{A}	119.7^{B}
	HDL	62.7^{C}	64.07^{C}	69.5^{B}	73.47^{A}	64.77^{C}
	LDL	61.8^{D}	66.23^{C}	72^{B}	76.18^{A}	67^{C}

Means in a row with different litters are significantly differed ($p<0.05$)
AST, ALT and ALK means aspartate amino transferase, alanine amino transferase and alkaline phosphatase enzyme, respectively.

In studying the effect on liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as shown in **Table (6)**, it was noted that the least effort and the least load on the liver was also in the treatment supplemented with iron nanoparticles. These results were also the closest to the control results, which indicates that they were the best in affecting the liver and not disturbing the proportions and concentrations that secreted by hepatic enzymes.

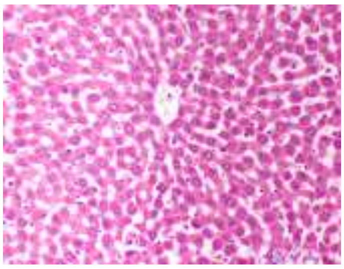
Concerning kidney functions **Table (6)**, milk supplemented with iron nanoparticles gave the least effect and the least effort exerted by the kidneys in the rat of this group, and they were closest to the control samples. This results could lead to suggest that the load did not occur on the liver or kidneys, and this form of iron, which is iron nanoparticles, was not treated as harmful foreign substances that the body needs to purify and filter. Rather, iron nanoparticles was treated as a natural nutritional substance similar to natural food that enters the body naturally, and that was according to unlike other forms of iron salts, which the body treats as a foreign substance and requires greater effort to digest, absorb, and get rid of its impurities, and therefore this is reflected in a change in results and an increase in liver and kidney enzymes. These results are in the same line with those of (Malika *et al.*, 2022)[32], who concluded that the zinc and iron supplementation of wheat flour has very negligible effect on the renal and liver functions.

All indicators of the lipid profile showed in **Table (6)** are considered among the most important results reached by the feeding experiment, as it gave a clear indication that the bioavailability of iron nanoparticles and the rat's body was able to benefit from it as much as possible in the formation of muscle mass, not fat, and this was reflected in the indicators of the lipid profile of the blood samples of the rats of the group that were fed with milk supplemented with iron nanoparticles being closest to the levels of the control samples. The rate of triglycerides (TG) formation was the lowest level in nano-iron feeding rates and most similar to the control samples.

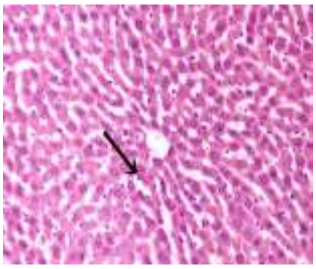
These phenomena led to conclude that, the blood profile improved significantly, and the properties of liver enzymes and kidney functions in the rats group that fed on milk supplemented with iron nanoparticles in comparison with other groups studied. That may be due to the distinctive properties of iron nanoparticles. This finding is in line with (Kumari and Chauhan, 2022) [26]. On the contrary, normal fortificants ($FeSO_4$, $FeCl_3$) caused gastrointestinal problems, black stool, and other health related issues and changed the organoleptic properties of food. While nano sized iron salts have the potential to overcome the problem caused by the use of water-soluble iron complexes.

Histopathological investigation

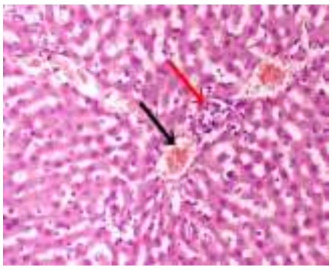
As shown in the **Figure (1)** photomicrograph of liver of rats of the 1st group was showing normal histological architecture of hepatic lobule, while photomicrograph of rats' liver of the 2nd group appeared slight Kupffer cells activation (arrow), photomicrograph of rats' liver of the 3rd group contained congestion of central veins (black arrow) and small focal hepatocellular necrosis associated with inflammatory cells infiltration (red arrow), photomicrograph of rats' liver of the 5th group was showing slight Kupffer cells activation and photomicrograph of rats' liver of the 4th group showed slight Kupffer cells activation.



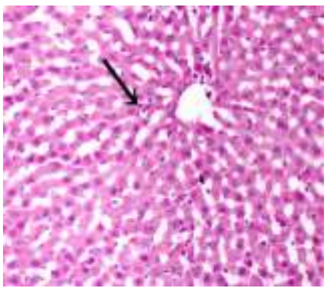
G1
1st group: control (-) fed on basal diet only



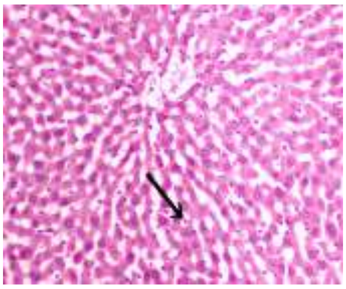
G2
2nd group: control (+) fed on milk without any iron supplementation



G3
3rd group: fed on milk supplemented with ferrous sulfate



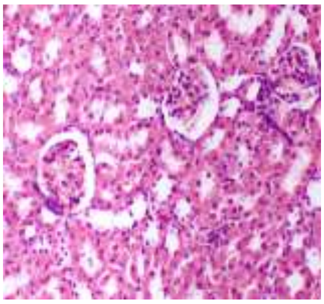
G4
4th group: fed on milk supplemented with ferric chloride



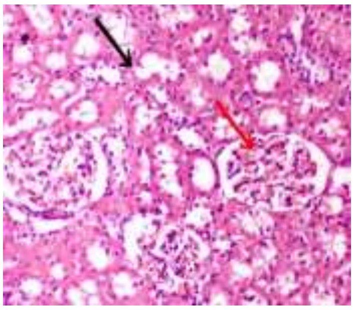
G5
5th group: fed on milk supplemented with iron nanoparticles

Figure (1): Photomicrograph (X400) of liver sections of rats fed on basal diet, iron supplemented heat treated milk as affected by the iron source

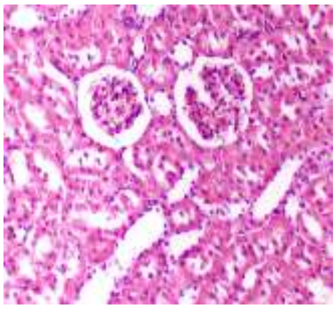
Light microscopic examination of liver sections of rats in different groups revealed that group (1) the control had the normal histological structure of the hepatic lobe, and comparing it with the rest of the groups, the group most similar to it was group (5), which was fed on milk supplemented with iron nanoparticles showing the least significant activity of the kupffer cells. (Lefebvre and Horuzsko, 2015)[33] declared that the activity of Kupffer cells is the main and important indicator when any changes occur in the liver, as they play a critical role in maintaining liver functions. Under physiological conditions, they are the first innate immune cells and protect the liver from bacterial infections. Under pathological conditions, they are activated by different components. These findings are also agree with those of (Malika *et al.*, 2022)[32]. Through which it was indicated that liver samples of rats that were fed on flour fortified with iron salts containing sulfates showed toxicity whereas the ones receiving zinc oxides or iron in Ethylene diamine tetraacetic acid (EDTA) form have been found to be lesser reactive**.** As for the rest of the groups, many changes occurred that appeared upon autopsy of the liver, including congestion in the central veins and necrosis in the hepatic cells, with the occurrence of some inflammation. This was reflected in an increase in the activity of the Kupffer cells, which indicates that an overload had occurred. On the liver cells, they consider these salts as foreign substances that the body needs to get rid of.



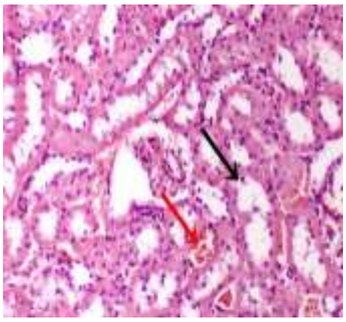
G1
1st group: control (-) fed on basal diet only



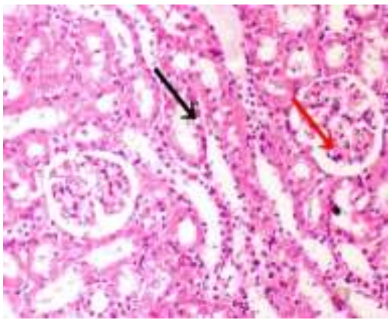
G2
2nd group: control (+) fed on milk without any iron supplementation



G3
3rd group: fed on milk supplemented with ferrous sulfate



G4
4th group: fed on milk supplemented with ferric chloride



G5
5th group: fed on milk supplemented with iron nanoparticles

Figure (2): Photomicrograph (X400) of kidney sections of rats fed on basal diet, iron supplemented heat treated milk as affected by the iron source

As shown in the **Figure (2)** photomicrograph of kidney of rats of 1st group showing normal histological structure of renal parenchyma, photomicrograph of rats' kidney of 2nd group showing vacuolar degeneration of epithelial lining some renal tubules (black arrow) and slight congestion of glomerular tuft (red arrow), photomicrograph of rats' kidney of 3rd group showing apparent normal renal parenchyma, photomicrograph of rats' kidney of 5th group showing vacuolar degeneration of epithelial lining some renal tubules (black arrow) and slight congestion of glomerular tuft (red arrow) and photomicrograph of rats' kidney of 4th group showing vacuolar degeneration of epithelial lining some renal tubules (black arrow) and congestion of intertubular blood capillaries (red arrow).

The results of the feeding experiment concluded that the use of iron nanoparticles to supplement milk achieved greater bioavailability for these products. In addition, the absorption of iron within the digestive system of experimental rats showed that the absorbed iron does not affect the properties of the liver and kidney, and their histological anatomy is very similar to the control samples. In addition to improving the blood profile in general, the absorbed iron added muscle mass and increased the weight and tissues of the rat at the expense of fat. Generally, the obtained results are agree with those found by (Darwish *et al.*, 2021)[34]. They performed histological examinations of the liver, kidney, and spleen and found no significant organ or tissue architectural alterations in the rats groups those ingested nano-encapsulated supplements and reported the safety and efficiency of nutrient delivery *via* organic nanocapsules.

Conclusion

Supplementation of milk with iron is considered an important means of producing a functional milk product especially when added in the form of nanoparticles, as it has the least severe effect on the sensory properties of the product, is most stable during the storage process and is most vital within the body of the organism. Synthesis of iron complexes in nano size increases iron bioavailability, and problems like unacceptable color, flavor, metallic taste, and rancidity in food vehicles using water-soluble iron salts are solved.

References

[1].NIH Publication No. 11-7629A September 2011. IN BRIEF: Your Guide to Anemia

[2].Sonja, Hess, Y,. Aatekah, Owais, Maria Elena D. Jefferds, Melissa F. Young, Andrew Cahill, Lisa M. Rogers, (2023). Accelerating action to reduce anemia: Review of causes and risk factors and related data needs. Ann NY Acad Sci. 1523:11–23.

[3].Olson, R., Gavin-Smith, B., Ferraboschi, C., & Kraemer, K. (2021). Food fortification: The advantages, disadvantages and lessons from sight and life programs. *Nutrients*, *13*(4), 1118.

[4].WHO. Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity. Vitamin and Mineral Nutrition Information System. Geneva, World Health Organization, 2011 (WHO/NMH/NHD/MNM/11.1).

[5].De Leeuw NK, Lowenstein L, Hsieh YS. (1966). Iron deficiency and hydremia in normal pregnancy. Medicine (Baltimore), 45:291–315.

[6].Stevens GA, Finucanen MM, De-Regil LM, Paciorek CJ, Flaxman SR, Branca F. (2013) Nutrition Impact Model Study Group Global, regional, and national trends in haemoglobin concentration and prevalence of total and severe anaemia in children and pregnant and non-pregnant women for 1995–2011: a systematic analysis of population-representative data. Lancet Glob Health 1(1):e16–e25

[7].Blanco-Rojo, R. and Vaquero, M. Pilar. (2019). Iron bioavailability from food fortification to precision nutrition. A review. Innovative Food Science & Emerging Technologies Volume 51, Pages 126-138.

[8].Meshref, A. M., Moselhy, W. A., & Hassan, N. E. H. Y. (2014). Heavy metals and trace elements levels in milk and milk products. *Journal of food measurement and characterization*, *8*, 381-388.

[9].Richard F. Hurrell, R, F,. (2007). Iron fortification: Its efficacy and safety in relation to infections. Food and Nutrition Bulletin, vol. 28, no. 4 (supplement), The United Nations University.

[10].Unnikrishnan V. and BHIMASENA R. M. (1977). Distribution of Added Iron in Milk of Cows and Buffaloes and its Effect on Oxidized Flavor Development. Journal of Food Protection Vol. 40, No.2. Pages 86-89/February.

[11].Hurrell RF .(2002). Fortification: overcoming technical and practical barriers. J Nutr 132:806S-812S.

[12]. Sozer, N. JL Kokini. (2009). Trends in biotechnology, - cell.com

[13].Shafie EH, Keshavarz SA, Kefayati ME, Taheri F, Sarbakhsh P, Vafa MR (2016). The effects of nanoparticles containing iron on blood and inflammatory markers in comparison to ferrous sulfate in anemic rats. Int J Prev Med. 7(1):117

[14].Foujdar R, Chopra H, Bera M, Chauhan A, Mahajan P (2020) Effect of probe ultrasonication, microwave and sunlight on J Food Sci Technol (September 2022) 59(9):3319–3335 3333 123 biosynthesis, bioactivity and structural morphology of punica granatum peel's polyphenols-based silver nanoconjugates. Waste Biomass Valoriz 12(5):2283–2302.

[15].Constante M, Fragoso G, Lupien-Meilleur J, Calve A, Santos MM. (2017). Iron supplements modulate colon microbiota composition and potentiate the protective efects of probiotics in dextran sodium sulfate-induced colitis. Infamm Bowel Dis, 23:753–66.

[16].Garcia-Fernandez J, Turiel D, Bettmer J, Jakubowski N, Panne U, Rivas Garcia L, Llopis J, Sanchez Gonzalez C, Montes-Bayon M. (2020). In vitro and in situ experiments to evaluate the biodistribution and cellular toxicity of ultrasmall iron oxide nanoparticles potentially used as oral iron supplements. Nanotoxicology,14:388–403.

[17].Góral, D., Marczuk, A., Góral-Kowalczyk, M., Koval, I., & Andrejko, D. (2023). Application of iron nanoparticle-based materials in the food industry. *Materials*, *16*(2), 780.

[18].Jakubovics, J. P. (1994). "Magnetism and Magnetic Materials", 2nd Ed., The Institute of Materials, Cambridge.

[19].Bassiony, Heba, Salwa Sabet, Taher A. Salah El-Din, Mona M. Mohamed, and Akmal A. El-Ghor.(2014). "Magnetite nanoparticles inhibit tumor growth and upregulate the expression of P53/P16 in Ehrlich solid carcinoma bearing mice." PloS one 9, no. 11. e111960.

[20].AOAC 2012. Official Methods of Analysis. 18th ed. AOAC International Gaithersburg MD. No. (9685-080), and CH.4, pp. 56-57 and No. (985.01) Ch.3, 6 p.

[21].APHA (American Public Health Association), AWWA (American Water Works Association), and WEF (Water Environment Federation). 2017. Standard Methods for the Examination of Water and Wastewater, 23 rded. (Rice, E. W., Baird, R. B., Eaton, A. D., Clesceri, L. S. eds.) Washington DC.

[22].S Gilliard Nkhata.(2015). Health Food Sci.

[23].SAS 1999. User's Guide Statistics. SAS Institute Inc. Editors, Cary, NC.

[24].Akhter, N. S Iqbal, M Nasir, A Ashraf, U Bacha, MA Khan.(2014). thejaps.org.pk JAPS: Journal of Animal & Plant Sciences.

[25].Egyptian Organization for Standardization and Quality (2005). Egyptian Standards (ES): 1616/2005, Pasteurized milk.

[26].Kumari, A. AK Chauhan. (2022). Journal of Food Science and Technology, Springer.

[27].Hurrell, R. F., Trinidad, T. P., Mallillin, A. C., Sagum, R. S., Foman, J. T., Li, Q., ... & Egli, I. (2022). Iron Bioavailability from Ferrous Ammonium Phosphate, Ferrous Sulfate, and Ferric Pyrophosphate in an Instant Milk Drink—A Stable Isotope Study in Children. *Nutrients*, *14*(8), 1640.

[28]. Nile, S. H. V. Baskar, D. Selvaraj, A. Nile, J. Xiao, and G. Kai. (2020). Nano-Micro Lett., 12, 1-34, doi: 10.1007/s40820-020- 0383-9.

[29].El-Dreny, EG. MA Mahmoud.(2019). Journal of Food & Dairy , search.ebscohost.com
[30].Elshemy, MAE. (2018). Egyptian Journal of Veterinary Sciences, ejvs.journals.ekb.eg
[31].Mohammed, O. N Dyab, E Kheadr, N Dabour.(2021). RSC advances, pubs.rsc.org
[32].Malika, S. A Ullah, AA Anjum, MMK Sattar, T Ali, R Manzoor. (2022). Brazilian Journal of Biology, SciELO Brasil
[33].Nguyen-Lefebvre, AT. A Horuzsko.(2015). Journal of enzymology and metabolism, ncbi.nlm.nih.gov
[34].Darwish, AMG. TN Soliman, HA Elhendy, WM El-Kholy. (2021). Frontiers in Nutrition.frontiersin.org