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Determining the Effect of Fe3O4 Conjugated with Chitosan Nanoparticles on Labneh Product Characteristics and its Effect on Blood Picture Parameters in Anemic Rats Induced by Phenylhydrazine

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Abstract: ***The research aims to investigate the effect of Fe_3O_4-NPs and it's conjugated with chitosan nanoparticles on the immunological indicators, such as IgA, IgG, and interleukin-6, and the iron indicators, of Ferritin, Transferrin, TIBC, and Iron in the serum of male albino rats induced with anemia by phenyl-hydrazine. The rats were separated into six groups in five replicates, each of which included (T1) the control group, (T2) anemia induced group, T3 group animals given Labneh enrichment with 2.5 mg of Fe_3O_4-NPs ml/day, T4 group animals given Labneh enrichment with 2.5 mg of Ch-NPs ml/day, T5 group animals given Labneh enrichment with 2.5 mg of each Fe_3O_4-NPs conjugated with Ch-NPs ml/day, T6 group animals given Labneh enrichment with 2.5 mg of Hamvir ml/day, and were fed for 30 days. The results showed a change and increase in the values of free fatty acids, as they reached their highest value after 20 days of preservation, where the treatment (T1) recorded a noticeable increase, reaching (2.31). Likewise, in the treatment (T5), we notice a significant increase in it, as it reached (0.75), in contrast to the rest. All coefficients that were (T2), (T3) and (T4) recorded (0.53), (0.58) and (0.63), respectively. the values of RBCs and Hb, which gave a significant decrease and it was at 2.84 (1×10^6/ ml) and 6.4 (g/dl), respectively, compared to the control treatment (T1).***

Keywords: Fe3O4, Anemia, Chitosan, Hb.

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1. INTRODUCTION

The dairy product labneh, which is made by fermenting milk with lactic acid bacteria to give it a distinctive flavour and scent, is very well-liked throughout the world, but especially in Middle Eastern countries reducing lactose intolerance symptoms, serving as a significant calcium source, and boosting the number of good bacteria in the intestines. Labneh is a useful food and a good source of essential nutrients. [1].

On the other hand, many countries adopted nanotechnology in the treatment of many diseases, including incurable ones, as it was noted for its ability to cure some diseases and is used daily in hospitals and health centers to preserve human health through early detection of diseases, which reduces the cost of treatment [2]. Fe3O4-NPs are widely used in numerous significant biological and medicinal applications, and because they are super magnetic, they have various uses in sophisticated technology. [3] It is used in drug delivery and loading systems, as it has the property of carrying more than one treatment material at one time, which includes many diseases, including bacterial and fungal diseases [4]. It also has several applications, including adsorption applications and heavy water treatment applications [5]. Chitosan results from the removal of acetyl groups from chitin, which is considered the second most common compound in nature after cellulose, and its molecular formula is (C6H11O4N)n, which constitutes (20-30)% of crustacean shells such as shrimp and crabs [6] As for nanoscale chitosan (Ch-NP) It has the properties of chitosan and properties of nanoparticles such as the effect of surface area and small size [7]. Chitosan nanoparticles (ChNP) were first described in 1994 by (Ohya) and colleagues. They used ChNP prepared by emulsification and crosslinking to deliver the anti-cancer drug 5-fluorouracil intravenously [8] On the other hand, anemia is viewed as a global public health issue caused by a shortage of red blood cells or a reduction in their ability to carry enough oxygen to meet the needs of the physiological body. The severity of anemia varies with age group., gender and during pregnancy, in 2015, the World Health Organization (WHO) estimates that about 1.62 billion (24.8%) of the world's population suffer from anemia, with a higher rate among pregnant women, as it is The proportion of anemia cases is 38% (32.4 million), 29% (496 million) are non-pregnant, and 43% (273 million) are in children. Anemia may have multiple causes, but according to WHO (2015), 50% of cases of anemia blood caused by an insufficient amount of iron [9]. Due to the lack of studies on the effect of laboratory-made labneh fortified with Fe3O4-NPs, and the complex of chitosan with Fe3O4-NPs Effects on Complete Blood Picture.

2. MATERIALS AND METHODS

Preparation of the Fe_3O_4-NPs with Ch-NPs: 10 mg of Fe_3O_4-NPs was added to 1g of Ch-NPs (NANOSHEL, USA) in 10 ml of deionized aqueous solution and stirred at room temperature for 24 hours. The required amount of the conjugated nanoparticles compound (CNC) was dried using a lyophilized technique and kept at 4 ° C in the refrigerator until use.

Labneh Preparation: the preparation of the support tray template with nanomaterials and the drug Hemafer: local cow's milk was obtained from Salah Al-Alin province (Al-Alam

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district), and the brick was manufactured according to the method mentioned before [10]. Where the milk is heated to 85 °C for half an hour, then the milk is cooled to 42 °C, and the traditional starter culture is added at a rate of 3%. Distributing the salt, then the filtering process is carried out with bags made of cloth type (Japan), which are placed suspended in the refrigerator for 20 hours, after which they were fortified with the aforementioned nanomaterials and the drug at a rate of 10 mg / 100 gm of labneh.

Determination of free Fatty Acids: Free fatty acids were examined according to what was mentioned in [11], by mixing 30 ml of diethyl ether solvent with 30 ml of ethanol alcohol with 1 ml of phenolphthalein indicator. The previous mixture was neutralized using 0.1 sodium hydroxide until the pink color appeared. Then put 10 grams of labneh in the neutralizing solution, mix well, then filter the solution, and the filtrate is calibrated with the previous rule until the neutralization point is reached, which is identified by the appearance of a pink color in the solution. The amount of base consumed after adding the labneh was calculated, through which the amount of free fatty acids was calculated, which is expressed as a percentage of free fatty acids in the labneh and is calculated as oleic acid. According to the following equation:

$$FFA = 2.82 \times T/W$$

pH Measurement: 10 g of labneh was weighed and digested well in a ceramic mortar with 10 ml of distilled water and a pH-meter was used to estimate the pH value of the sample [12].

Initialization of Laboratory Animals

Albino Sprague-Dawley weanling, Male laboratory rats, were obtained from the College of Veterinary Medicine-Tikrit University, at the age of 8-9 weeks. Their weights ranged between 150-152 g, and they were separated randomly into groups of similar weights.

The animals were placed in cages made of plastic, and the floor was covered with sawdust, which was replaced four times a week. The animals were fed regularly using the ready-made diet according to what was mentioned in [13], and the basic food contained (g / kg diet) two cases: 84.95 g, net protein: 158.5 g, oil: 100 g, and a mixture of vitamins: 5 g and 50 g. A mixture of mineral salts, 50 gm of cellulose, 100 gm of glucose, and 536.5 gm of starch. The animals were raised under the supervision of specialized veterinary staff, taking into account the aspect of hygiene.

Experimental animals were distributed into six groups each one including five animals, that included: T1 healthy control group (without treatment, which was given water and food only for the duration of the experiment), T2 induced anemia infection group (given 40 mg by subcutaneous intraperitoneal of phenyl hydrazine/kg of body weight each day for 48 hours) [14], T3 group animals given Labneh enrichment with 2.5 mg of Fe_3O_4-NPs ml/day, T4 group animals given Labneh enrichment with 2.5 mg of Ch-NPs ml/day, T5 group animals given Labneh enrichment with 2.5 mg of each Fe_3O_4-NPs conjugated with Ch-NPs ml/day, T6 group animals given Labneh enrichment with 2.5 mg of Hamvir ml/day.

Diets and water were free of *ad labtum* given, and 0.1 mg of phenyl hydrazine was given for each kg of body weight animal by subcutaneous intraperitoneal throughout the experiment.

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Stages of the Experiment:
The phases of the vital experiment included the following:

- **The first stage** (pre-treatment stage with anemia): Animals in groups (T1-TG6) were fed the usual diet for 5 days, after which blood samples were drawn from each group in two tubes, the first containing EDTA anticoagulant to measure complete blood picture, and the other free of it containing Approximately 2-3 ml of blood was centrifuged using a centrifuge at a speed of 3000 cycles/ min for 15 minutes to obtain serum that was kept at -20 ° C until analyzes were performed, as in [15], to adopt them as control samples before induced anemia.
- **The Second Stage** (treatment with immunosuppression): animals in groups (T2, T3, T4, T5 and T6) were treated with 40 mg by subcutaneous intraperitoneal of phenyl hydrazine/kg of body weight each day for 48 hours until the appearance of symptoms of anemia, which included lethargy, lack of movement, withdrawal and poor appetite. for food that appeared after 3 days. Blood samples were drawn from the groups in two tubes, as in the above paragraph, to ensure that anemia occurred compared to the samples of the first stage.
- **The Third Stage** (treatment with each of Fe_3O_4-NPs or Ch-NPs singly or in conjugated): induced anemia animals in groups (T3, T4, T5 and T6) were treated with each of the Fe_3O_4-NPs or Ch-NPs singly or in conjugated. After the end of the experiment, blood samples were drawn as in the two paragraphs above.

Measured of Biochemicals Parameters: The CBC Mindray device was used according to the manufacturer's instructions, and the blood tests included estimating the hemoglobin concentration (Hb), the red blood cell count (RBCs), the total white blood cell count (WBC), measuring the percentage of packed blood cell volume (P.C.V.) packed cell volume and the average red cell volume. (MCV) and platelet count.

Statistical Analysis: The biological experiment was implemented according to the randomized complete design CRD, and the analysis of variance was carried out using the General Linear Model within the [16] SAS program. When there are significant differences between the means, Duncan's test [17] was used to determine the significance of the differences between the different means at a probability level of 0.05.

3. RESULTS AND DISCUSSION

Determination of Free Fatty Acids (FFA) in Milk Manufactured and Treated with Nanomaterials: Estimating the values of free fatty acids in the labneh manufactured in the laboratory is evidence of its suitability during preservation and experiment. In Table (1) we note from the results that there are no significant differences ($P < 0.05$) at the initial time for all treatments whose rates were between (0.4)- (0.8) However, there are significant differences after 10 days of preservation, especially treatment (T1) the control when compared with other treatments, and the least change was (T2) and (T4) compared with other treatments, and the results showed a change and increase in the values of free fatty acids, as It reached its highest value after 20 days of preservation, as the transaction (T1) recorded a

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significant increase, amounting to (2.31), as well as the transaction (T5), we notice a significant increase in it, as it reached (0.75). In contrast to all the treatments that were (T2), (T3) and (T4), which recorded (0.53), (0.58) and (0.63), respectively, the reason for the decrease in the percentage of free fatty acids in these treatments can be attributed to the role of nanoparticles in inhibiting microorganisms and Gram-negative bacteria that contaminate brick, this is the first study in this field.

Treatment type	**The values of free fatty acids in labneh samples manufactured after a 20-day storage period at 5°C ±2**		
	first hour	**10days**	**20 days**
T1	0.8a± 0.01	0.98a± 0.01	2.31a± 0.01
T2	0.4b± 0.02	0.43c± 0.02	0.53d± 0.02
T3	0.4b± 0.02	0.45bc± 0.01	0.58c± 0.01
T4	0.5b± 0.01	0.43c± 0.01	0.63c± 0.01
T5	0.5b± 0.01	0.51b± 0.02	0.75b± 0.02

Table (1) Values of free fatty acids (FFA) in yoghurt manufactured and treated with nanomaterials after storage for 20 days at a temperature of 5°C ±2.

The different letters in the same column indicate that there are significant differences T1: control, T2: nano-Fe3O4, T3: nano-chitosan, T4: chitosan loaded with nano-Fe3O4, T5: HEMAFER (Ferric hydroxide polymaltose complex).

Estimation of the pH in the Manufactured Brick Treated with Nanomaterials: The pH value indicates the natural and developed acidity, which usually indicates the presence of lactic acid bacteria and the effectiveness of its enzymes in fermenting lactose and converting it into lactic acid, where it participates with other compounds and gives taste and flavor, which gives an indication of its acceptability to the consumer. The results are shown in Table (2) The pH value was similar for all treatments at the beginning of manufacturing.

Treatment type	**The values of** pH **in labneh samples manufactured after a 20-day storage period at 5°C ±2**		
	first hour	**10days**	**20 days**
T1	4.72a± 0.4	4.10b± 0.6	3.05b± 0.5
T2	4.75a± 0.5	4.23a± 0.4	4.15a± 0.3
T3	4.74a± 0.3	4.46a± 0.7	4.24a± 0.6
T4	4.72a± 0.7	4.33a± 0.3	4.29a± 0.7
T5	4.69a± 0.2	4.15b± 0.4	4.01b± 0.5

Table (2) pH values in the manufactured brick treated with nanomaterials after storage for 20 days at 5°C ±2.

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that is, their rates were between 4.69 – 4.75. The pH in the first 10 days of storage for treatment (T1) and (T5) was at 4.10 and 4.15, respectively. As for the end of the experiment, that is, after 20 days of preservation, the decrease in the pH value continued for the treatments (T1) and (T5), as it was at 3.05 and 4.01, while the treatments to which the studied nanomaterials were added (T2), (T3) and (T4) were at 4.15, 4.24, and 4.29. The evolution in the pH value of the treatments (T1) and (T5) is attributed to the activity of the microorganisms present in the labneh product and thus the fermentation of the remaining lactose [18].

Effect of Oral Dosage with Fe_3O_4-NPs Conjugated with Ch-NPs on Physiological Parameters of Anemia-Induced Rats with phenyl-hydrazine.

Effects on Complete Blood Picture: The effect of oral dosage of different treatments on serum iron indicators of anemia-induced rats with phenylhydrazine was noted in Table (3) the impact of doses of nanoparticles, nano complex and hemavir on serum iron levels and indicators of anemia-induced animals.

Treatment type	**symbol**	**RBCs**	**Hb**	**HCT**	**M.C. V**	**Platelet**
		(1×10^6/ml)	**(g/dl)**	%	**µM^3**	**(10^9/L)**
Control	**T1**	6.63a±0.8	12.8a±1.3	39.7b±3.8	59.6d±3.7	295.4d±21.5
Infection with anemia **induced**	**T2**	2.84d±0.2	6.4c±0.9	19.1d±2.4	67.3a±5.2	561.6a±20.8
Treatment	**T3**	4.85c±0.3	12.7a±1.8	40.4b±2.7	60.6d±4.6	319.3c±19.4
	T4	4.86c±0.3	12.3a±1.0	42.9a±3.5	63.6c±5.3	322.6c±15.7
	T5	5.27b±0.4	12.9a±1.2	41.1b±3.3	63.3c±5.1	314.6c±16.6
	T6	5.96b±0.5	11.3b±1.1	35.8c±2.9	65.6b±4.7	530.2b±21.5

Table (3) Effect of oral dosage with Fe_3O_4-NPs conjugated with Ch-NPs on Blood Picture of anemia-induced rats with phenyl-hydrazine.

The Different Letters in the Same Column Indicate that There are Significant Differences. T1: Control Group, T2: Group Orally phenyl-hydrazine Induced Anemia, T3: Group Orally with Fe_3O_4-NPs, T4: Orally with Ch-NPs, T5: Orally with Fe_3O_4-NPs Conjugated with Ch-NPs, T6: Orally with HEMAFER Drug.

The effect of nanoparticles of Fe3O4 and nanoparticles of chitosan, in addition to the complex of chitosan with Fe3O4 nanoparticles, of rats dosed orally for 30 days, can be seen

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in the parameters of the blood picture, which were shown in Table (3). normal levels of their levels in the blood of laboratory rats dosed with the aforementioned substances, where the values of RBCs for treatments T3, T4, T5, and T6 gave 4.85, 4.86, 5.27, and 5.96 (1 x 10^6/ml), respectively, in contrast to treatment T2, which gave a significant decrease when compared T1-treated control.

The results also showed that the value of the hemoglobin Hb concentration decreased significantly in the blood of the group of animals in which anemia was induced experimentally with PHZ and untreated (T2) and was at 6.56 (g / dl) when compared to the groups of treatments T3, T4, T5 and T6 that were treated with nanomaterials and the drug HEMAFER Significant good results, which were 12.7, 12.3, 12.9, and 11.3 (g/dl), respectively, compared with the control group (T1), which was valued at 12.8 (g/dl). We also note that the percentage of agglutinated blood cells (HCT) decreased in the group of animals (T2), when it was at 19.1%, compared with the group of healthy control animals (T1), which was 39.7%. While the results increased significantly after treatment in groups (T3), (T4), ((T5, and (T6), they were at 40.4, 42.9, 41.1, and 35.8%, respectively, compared with the treatment value (T2).

We also notice that the red blood cells (M.C.V) suffered a significant increase in the untreated (T2) group of animals, as it gave a value of 67.3 (µM3), while we notice a significant decrease in its values after treatment in the groups (T3), ((T4), and ((T5). At 60.6, 63.6, and 63.3 (µM3), respectively, compared with (T2), but we find that (T6) remained slightly higher than its predecessors, giving 65.6 (µM3) when compared with the value of the T1 control group, which gave a value of 59.6 (µM3). As well as for platelets PLT, it recorded a significant decrease in the groups of treated animals (T3), (T4), and (T5) and it was at 319, 322 and 314 (103/L) respectively, compared with the two groups (T2) and (T6) where it was at 561 and 530 (103/L) respectively, while in the control group (T1) it was at 295 (103/L).

The results of the study in groups of animals treated with phenylhydrazine and its effects on various blood variables, which include the number of red blood cells, the size of compact cells, and Hb, which is the most important variable indicating anemia and some other variables, agreed with what was mentioned by (Shahenda and (2018, Jehan) [19].The research indicated a decrease in the results of the values of these variables that were studied due to the toxicity of the drug phenylhydrazine resulting from oxidative stress that leads to the generation of free radicals, which in turn attack vital molecules, causing damage to the biological system[20].

These results agreed with the findings of Eman et al., (2022) [21] who indicated an improvement in the levels of RBCs, Hb and PCV concentrations and a decrease in M.C.V in laboratory rats when they were dosed with 1.5 mg of Fe3O4 NPs. [22] also mentioned that nano-chitosan has the ability to enhance the absorption of macro- and micronutrients, including iron. The results of the researcher, Marwa et al. (2021) [23], showed that fortifying the food with nano-chitosan led to an improvement in the levels of Hb, Hct, MCV, and the number of red blood cells in fish fed on it.

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