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Effects of supplementation cysteine-coated Fe_3O_4 nanoparticles compared to $FeSO_4$, on reproductive performance in male quail

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Abstract. Iron has a crucial role in growth as part of metalo-proteins like haemoglobin or myoglobin, enzymes; they are also involved in energetic reactions. Iron plays a vital role in fertility. At high doses, Iron has a harmful consequence on the reproductive system, which can be strongly reflected the final stage of spermatogenesis. Nutritional products are claiming to use nanotechnology and it is important to recognize the potential toxicity of nano-sized nutrients. Recently iron nanoparticles were proposed as a food additive for poultry. The objective of this study was to investigate the effects of L-cystein coated iron oxide nanoparticles on reproductive performance in male quails. The results of Fourier Transform Infrared Spectrometer, Alternating Gradient Force Magnetometer and Scaning Electron Microscopy showed that iron oxide nanoparticles was produced and have been coated with L-cycstein (Fe_3O_4-Cys NPs). A total of 100 one-week-old quail chicks were randomly placed to five groups of five replicates. Four quails (two male and two females) were raised in an individual cage for each replicate. The five experimental treatment diets consisted; negative control diet, with no Iron supplementation; positive control diet supplemented with 60 mg/kg of Fe_3O_4; treatment diets supplemented with 0.6, 6 and 60 mg/kg of L-cystein coated iron oxide nanoparticles. The hemoglobin, Red blood cell, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, gonadal somatic index, daily sperm production, total testicular sperm and sperm viability of the male quails that were fed with diet supplemented by 0.6 mg/kg of Fe_3O_4-Cys NPs were improved as compare with negative control. This study showed that not only the use of the Fe_3O_4-Cys nanoparticles had no side effects but also it can be used as a feed additive to improve the reproductive performance in male quails.

Keywords: hemoglobin; red blood cell; gonadal somatic index; daily sperm production; sperm viability; L-cysteine-coated Fe_3O_4 nanoparticles

1. Introduction

Some reports have been shown that nanoparticles (NPs) were able to pass the blood– barriers such as testis and brain barriers (De Jong *et al*. 2008, Lankveld *et al*. 2010, Farzinpour and Karashi 2013, Zanella *et al*. 2017). Nano sized particles can bind to proteins and DNA of tissue cells and may lead to produce the reactive oxygen species (ROS), inflammation induction and cell damage (Asharani *et al*. 2009, Ahamed *et al*. 2010). Coating of nanoparticles surface with organic materials could be used to reduce toxicity of nanoparticles in biological systems (Berry *et al*. 2003). Although there is increasing concern that the NPs can act as reproductive toxicants, only few studies have been performed to reduce the toxicity of nanoparticles. L-cysteine as a powerful antioxidant can bind to dangerous and reactive free molecules and protect cells from the toxic effects of radiation (Lafleur *et al*. 1980). The present study is herefore aimed at evaluating the effects Fe_3O_4 nanoparticles that coated with of Cystein (Fe_3O_4-Cys NPs) on reproductive performance in male quail.

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2. Material and mehods

2.1 Animals

All animal use and procedures were approved by the University of Kurdistan Animal Care Committee. A total of 100 one-week-old quail chicks were weighed and randomly placed to five groups of five replicates. Four quails (two male and two females) were raised in an individual cage (12×15×17 cm) for each replicate. The quails were reared in a light proof environmentally controlled house at 25°C with a photoperiod of 8L: 16D during the growing period and 16L: 8D as a photostimulate program. The ingredient composition of the basal diet is presented in Table 1. The different iron premixes were separately prepared according to its purity and added to each group diet. The purity of $FeSO_4$ and L-cystein coated iron oxide nanoparticles (Fe_3O_4-Cys NPs) were 40% and 95% respectively. The ingredients and composition of diet was shown in Table 1. The corn-soybean meal basal diet was formulated to exceed the nutrients requirement of quail recommended by National Research Council (NRC 1994). Diet and water were provided on ad libitum basis. The five dietary treatments were fed (Table 1): negative control without any Iron supplementation, positive control contained 60 mg/kg of common form of iron (300 mg of $FeSO_4$, 40%) and

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Table 1 Ingredient composition of the diets fed

Ingredients (%)	Diets	
	1-42 day	43-120 day
Corn	48399	47.53
Soybean meal	46.76	46.26
Limestone	0.88	0.88
Soybean oil	2.86	2.86
Dicalcium phosphate	1.41	1.41
Vit premix[1]	0.250	0.25
Min premix[2]	0.251	0.25
Salt	0.335	0.335
DL-methionine	0.112	0.112
L-Lysine	0.122	0.122
Total	100	100
Calculated composition[3]		
Crude Protein	24	20
ME, kcal/kg	3000	3000
Methionine	0.25	0.31
Met and Cys	0.75	0.76
Iron content (mg/kg diet)	Experimental groups	
Negative control	0	0
Positive control*	300	150
0.6**	1.26	0.61
6**	12.6	6.1
60**	126	61

[1]Provided per kilogram of premix: 3600 KUI vitamin A, 800 KUI vitamin D3, 7200 UI vitamin E, 720 mg vitamin B_1, 2640 mg vitamin B_2, 4000 mg pantothenic acid, 12000 mg nicotinic acid, 1200 mg vitamin B_6, 400 mg folic acid, 6 mg vitamin B_{12}, 800 mg vitamin K_3, 40 mg biotin, 100 gr Choline chloride and 40 gr antioxidant.

[2]Provided per kilogram of premix without iron: 40 gr manganese sulfate, 4 gr copper sulfate, 33880 mg zinc, 400 mg iodine, 80 mg selenium.

[3]Crude protein is based on analytical values. Amino acids, MEn, values based on table values from the National Research Council, NRC (1994).

*Positive control, $FeSO_4$; 40%, the levels of 120 and 60 mg Fe/kg dry matter of quail's diet has been recommended by NRC for 1-42 days and 43-120 days respectively.

**Fe_3O_4-Cys NPs, 95%

treatment diets that contained 0.6, 6 and 60 mg/kg of L-cystein coated iron oxide nanoparticles (0.63, 6.3 and 63 mg of Fe_3O_4-Cys NPs 95%) were added to one Kg of the basal diet respectively. Quail sperm were obtained as described by Win *et al*: Quail semen was collected from male quail by squeezing out from the nipple of ductus deferens after decapitation (Win *et al*. 2006). The sperm survival, sperm acrosomal status and Plasma- membrane integrity were determined. Then the Collected semen was suspended at 5.5×106 sperm/ml in Ringer solution.

2.2 Assessment of sperm survival

The parameters of spermatozoa viability included percentages of live, dead and abnormally-shaped sperm. Forty microliters of semen samples of each treatment were added to 150 µl of the staining solution (eosin 16 g/l and nigrosin 60 g/l in BPSE) and put onto slide. Two minutes later, smears were performed for each sample and spermatozoa were observed, using microscope with an oil immersion objective. Live spermatozoa were seen white in color because they were eosin-impermeable. However, dead spermatozoa were pink because they became eosin-permeable. Two hundred spermatozoa per sample were observed and the percentages of live, dead and abnormally-shaped spermatozoa were estimated (El-Gendy *et al*. 2007).

2.3 Assessment of acrosome integrity

The morphological acrosome abnormality was assessed by viewing wet mount of diluted spermatozoa fixed in buffered Formalin-Citrate solution described by Farshad *et al*. (2009). Two drops of solution with one drop of semen on a slide clean and warm (37°C) are mixed. After 30 seconds Put slide under a microscope and with a magnification of 1000 (×100), 20 field (10 sperm in the each field) be counted. Sperms with normal acrosome be counted and the percentage of sperm with intact acrosome considered (Farshad *et al*. 20009).

Assessment of sperm membrane integrity

We used the hypo-osmotic swelling test to examine the integrity of the sperm membrane. To determine plasma membrane integrity, 25 µL of diluted spermatozoa was mixed with 500 µL of a hypo-osmotic solution (100 mOsm/kg) prepared by adding 1 g of sodium citrate to 100 mL of distilled H2O. The percentages of spermatozoa showing coiled mid-pieces and tail segments were determined with examining 300 spermatozoa under a light microscope (magnification 400×).

2.4 Daily sperm production in the testes

The washed testis was homogenized in 10 volumes (wt:vol) of saline-triton-merthiolate (STM) buffer [150 mM NaCl, .05% (vol:vol) Triton X100, and 0.25 mMmerthiolate]. Elongated spermatid nuclei that were resistant to homogenization were counted on a hemocytometer in quadruplicate. Estimates of daily sperm production per gram testis were estimated by dividing homogenization-resistant spermatid nuclei by 2.69. This number corresponds to the time (in days) which developing spermatids spend during spermatogenesis in the quail (Clulow and Jones 1982, Lin *et al*. 1990).

2.5 Preparation of Inner Perivitelline Membrane (IPM)

The IPM was isolated according to a procedure described by Sasanami *et al*. (2002), using freshly laid quails` egg. Eggs of Japanese quail (Coturnix cotumix japonica) were collected within 1 h after lay. Eggs were

broken open. Eggs Yolks were isolated and washed free of albumen in 1% Nacl. Yolk were then immersed in PBS and incubated at 37 for 1 h. After incubation, the PBS was removed and replaced by 1% NaCl. The yolks were placed in a petri dish with the blastoderm positioned on top. The yolk was punctured with forceps. The perivitelline layer obtained was cut around the equator of the egg and the non-germinal disc region was removed. Inner layer of the vitelline membrane was isolated from the outer layer manually by using washing the whole membrane repeatedly in 1% NaCl.

2.6 In vitro sperm penetration into IPM

The in vitro sperm-egg interaction assay accurately reflects the fertilizing ability of spermatozoa and detects spermatozoa damage due to toxicants more sensitively than motility or fertility tests (Kasai *et al*. 2000, Steele *et al*. 1994, Robertson *et al*. 1998, Wishart and Palmer 1986). Firstly, the sperm concentration was determined as the adequate concentration for creating the most countable number of holes in the IPM (Win *et al*. 2006). The sperm penetration assay was determined in laid eggs by fixing and staining the intact IPM section with Schiff's. Briefly, IPM was divided approximately 0.5×0.5 cm, one piece to control and other to treatment (A set of two test tubes were used in this experiment. A control tube contained sperm suspension. The remaining tubes contained sperm and related level of silver nanoparticle). Layers were incubated in micro test tubes with 100 µl of suspension at 5.5×106 sperm/ml in Ringer solution 39°C for 40 min. After incubation, the reaction was terminated by placing the tube at 4°C, and the IPM was washed 3 times with ice-cold PBS. The IPM was positioned onto a glass slide and stained with Schiff's reagent after fixation with 20% (v/v) formaldehyde. The number of holes formed on the IPM in the 40× field was counted under a light microscope. Ten areas were randomly selected for enumeration of perforations. The experiment was repeated six times and the data are represented as the mean ± the standard deviation. A Student's t test was performed and $P < 0.05$ indicated significance.

2.7 Number of epididymal spermatozoa

The left epididymis tissues were removed from each animal, after weighing, using scissors to cut and it shed in a test tube with 10 ml of STM buffer (37°C) and mixed and homogenized for 20 minute until all sperms in the epididymis were issued. Then, 0.2 ml of the homogeneous mixture with 0.8 ml trypan blue. A sub-sample of each homogenate was examined in a Neubauer haemocytometer to determine the total number of spermatozoa. The total number of extragonadal spermatozoa per animal was estimated, using the formula: (number of spermatozoa counted×32000×dilutions) calculated the number of epididymal sperm.

2.8 Blood sample collection and testosterone estimation

Blood samples were taken from wing vein using a needle (13 × 0.45 mm) and syringe (2.0 mL) was suitable for quails. The fresh blood samples were divided into two tubes: one EDTA-coated tube for hematological parameters assay and the second tube without anticoagulant for testosterone estimation (ELISA kits, PishtazTeb, Inc., Tehran, Iran) by ELISA Plate Reader. Hematological parameters: determination of hemoglobin concentration (Cyanomethemoglobin method, BaharAfshan Kit, Tehran, Iran) was performed. The hematocrit was done by centrifuging the blood sample for 5 minute at 11,000 (rpm). Red blood cell (RBC) indices mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated.

2.9 Histopathological assessment

Testes were fixed in 10% neutral buffered formalin for 3 days. Following fixation, tissues were embedded in paraffin, sectioned at approximately 5 µm, and stained with hematoxylin and eosin. They were evaluated under a light microscope to determine structural differences between groups.

2.10 Statistical analysis

The experiments were conducted as a completely randomize design, and statistical analysis of data was performed by the General Linear Model (GLM) procedure of the SAS. A probability level of $p \leq 0.05$ was considered as significant. To optimize the sperm-egg interaction assay was analyzed via student t-test (matched pairs) comparisons of the different results obtained.

$Yij = \mu + Tj + \varepsilon ij$
Yij = Experiment observations;
μ = the overall mean;
Tj = the effect of dietary treatment;
i = T1, ----- T4;
εij = the experimental error.

2.11 Preparation of Fe_3O_4 nanoparticles

Different types of production methods of iron oxide nanoparticles have been developed (Dang *et al*. 2007, Gawande *et al*. 2012). We used a technique that was described by Gawande *et al*. (2012). The Iron (III) chloride hexahydrate (5.4 g) and urea (3.6 g) were dissolved in 200 cm^3 distilled water were with vigorous stirring at 85°C–90°C for two hours, resulting in the solution color gradually turning from red to brown. After cooling the solution to room temperature (30-35°C), Iron (II) sulfate heptahydrate (2.8 g) was mixed to the solution over a period of 30 min under vigorous stirring. Adjust the pH of solution from about < 1 to 10 by adding 1 M NaOH. By elevateing the pH to 10 and the formation of Fe_3O_4 nanoparticles, the solution color will shift to the black color. The produced black materials were treated by ultrasound in the sealed flask for 30 min for dispersing the particles. The black solution flask was placing in a magnetic field in order to sediment the iron oxide nanoparticles. After washing the nanoparticles with ethanol (3 times), it was washed again with distilled water

(3 times). Finally, a black powder was gathered together in a drying chamber after 4 h at 60°C.

2.12 Synthesis of Fe_3O_4 nanoparticles coated by L-cysteine (Fe_3O_4-Cys NPs)

According to Gawande *et al*. (2012), Fe_3O_4-Cys NPs were synthesized. In brief, after dispersing of Fe_3O_4 NPs powder in distilled water (1:20) with ultrasound at 25 C, L-cysteine solution [1 gr of L-cysteine in 40 ml of methanol/water (1:1)] and Fe_3O_4 NPs aqueous solution was then transferred to a flat-bottomed beaker with a 100 mL capacity. The reaction was mixed at room temperature (24 h) in a magnetic stirrer. Finally, after washing the product by absolute methanol and water, produced Fe_3O_4-Cys NPs were dried in a chamber after 4 h at 60°C.

2.13 Fourier Transform Infrared Spectromete (FTIR)

Fourier transform infrared (FTIR) spectroscopy was used to detect the presence of of cysteine on the Fe_3O_4 nanoparticles' surface by using a BRUKER VECTOR 22 Fourier transform infrared spectrometer

2.14 Magnetic properties

The magnetic properties of Fe_3O_4-Cys NPs were confirmed by an alternating gradient force magnetometer (AGFM, model MDKG, Kashan, Iran).

2.15 Scaning Electron Microscopy (SEM)

The morphology of the Cystein-coated Fe_3O_4 nanoparticles was determined by scanning electron microscopy (VEGA\\TES-LMU, Czech Republic).

2.16 Determination of food iron concentration

The iron concentration of experimental diets was determinate by atomic absorption spectroscopy. An amount of 10 g of each experimental diet was dried in an air oven at 100°C for 2-3 hours. The dried production was next charred until it coked to smoke and then ashed in the oven at 450°C until a greyish ash was produced. The greyish ash was treated with concentrated nitric acid, heated to dryness 45 min at 100°C and ashed again for 2 hours at 450°C. The whitish production was dissolved in dilute hydrochloric acid 2%, transferred to a volumetric flask (50 ml) and made up 25 ml. For different experimental diets, at least three readings were recorded and then calculated the average. The Iron amount of the solution was determined by a Varian Atomic Absorption Spectrophotometer model 220 with an air- acetylene flame. A duplicate analysis was conducted for each analytical sample. Based on analytical results, the iron concentration of experimental diets were 71 ± 12.3, 98.6 ± 4.5, 88.6 ± 3.2, 92.6 ± 13.5 and 104.3 ± 7.3 mg/kg of diets for negative control, positive control, 1.2, 12 and 120 mg/kg Fe_3O_4-Cys NPs, respectively.

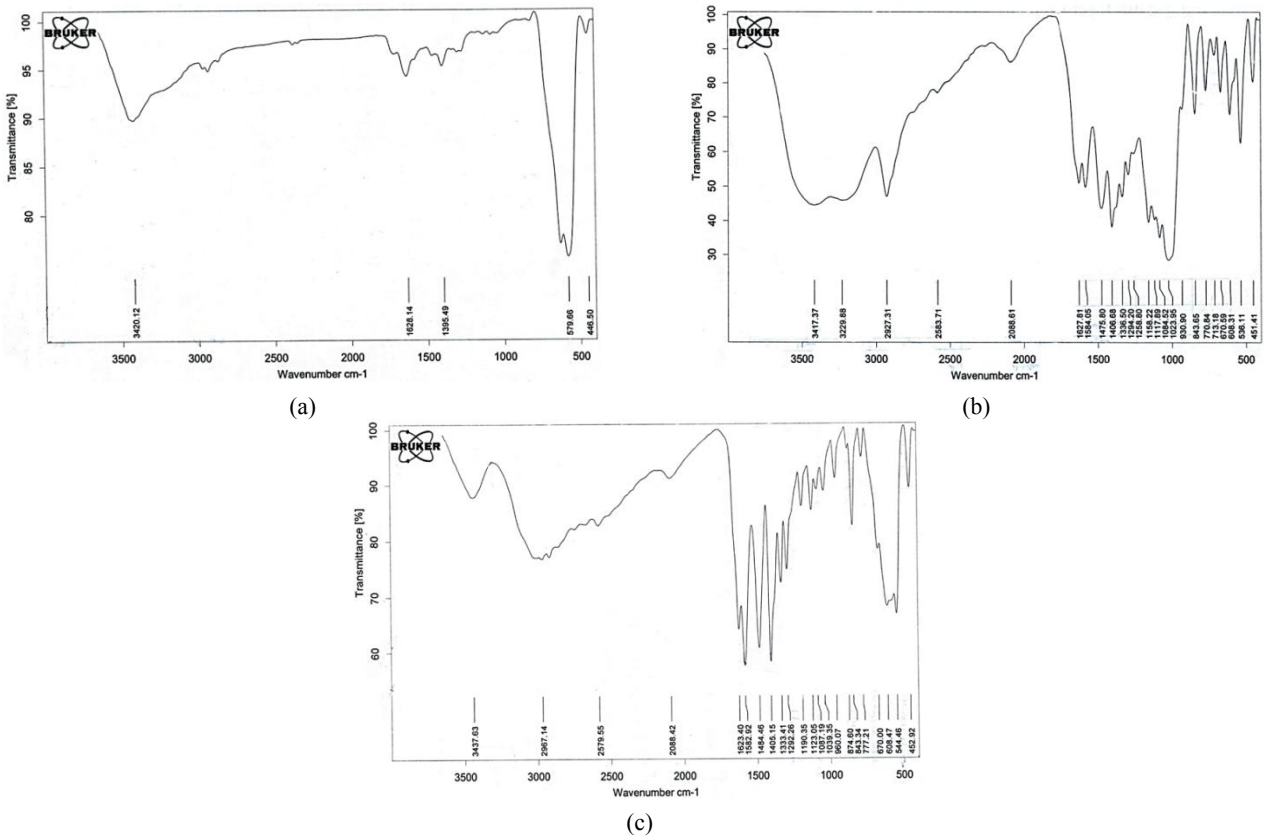

Fig. 1 FTIR absorption spectrum of Fe_3O_4 nanoparticles (a); cysteine (b); and Fe_3O_4 nanoparticles coated by L-cysteine Fe_3O_4-Cys NPs (c)

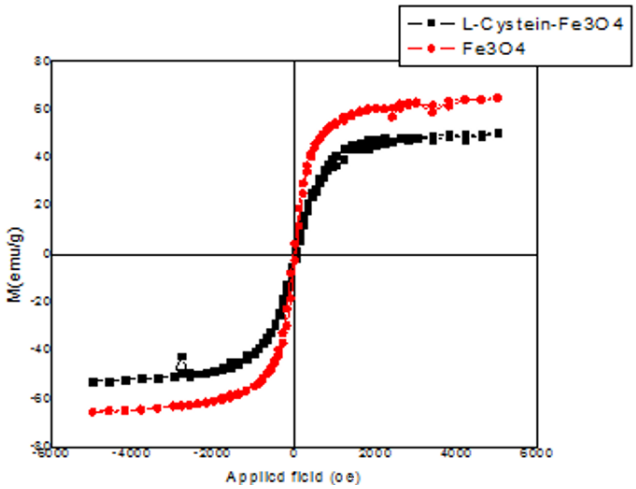

Fig. 2 The alternating gradient force magnetometry (AGFM) results that confirmed the saturation magnetization value for Fe_3O_4 is 66 emu/g, while the MS for Fe_3O_4 nanoparticles coated by L-cysteine (Fe_3O_4-Cys NPs) decreased to 44 emu/g

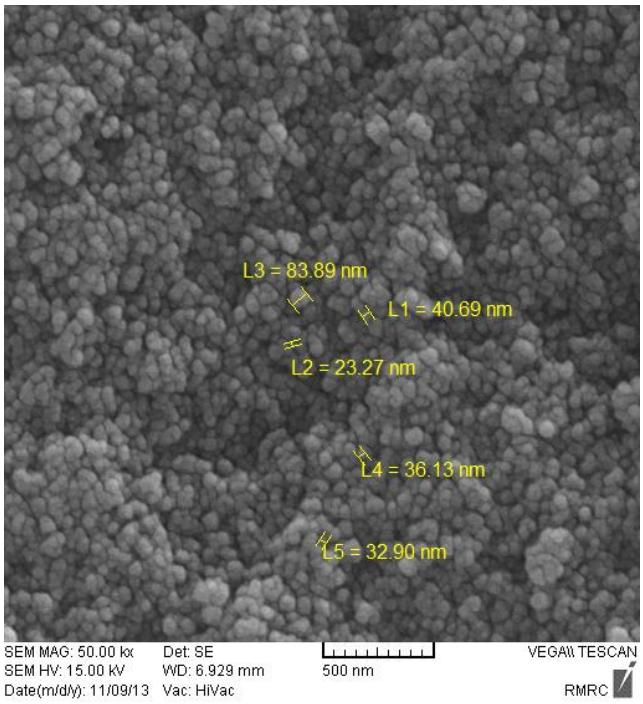

Fig. 3 Scanning Electron Microscopy (SEM) images, the mean particle size of Fe_3O_4-Cys NPs was as 43 nm

3. Results

The proposed structure of the Fe_3O_4 NPs was further confirmed by Fourier transform infrared spectrometer (FTIR) that was coated by L-Cystein (Fig. 1). Magnetization measurements were conducted to establish the magnetic field at room temperature. The hysteresis loops of Fe_3O_4-Cys coated and Fe_3O_4 uncoated samples are shown in Fig. 2. For a given magnetic field variation one can observe that the Fe_3O_4-Cys nanoparticles are less magnetized than the Fe_3O_4 uncoated particles. However, for the Fe_3O_4-Cys coated nanoparticles saturation magnetization occurs. To estimate an average size of nanoparticles, the SEM Image was analyzed. The images obtained by SEM in Fig. 3 indicate that the mean particle size of Fe_3O_4-Cys NPs was determined as 43 nm.

Table 2 Effect of Fe_3O_4-Cys NPs on some carcass parameters in male quails

	Control groups		NPs (mg/kg)			SEM	P value
	0*	60**	0.6***	6***	60***		
Body weight (gr)	203.6	211	209.4	216.2	201.3	3.504	0.27
Carcass characteristics (%)							
Carcass	83.8	80.7	80.6	81.2	85.1	0.016	0.875
Heart	0.73	0.68	0.78	0.69	0.74	0.060	0.09
Liver	2.12	1.88	2.03	1.87	2.04	0.006	0.341
Pancreas	0.25^{a}	0.21^{b}	0.22^{ab}	0.22^{ab}	0.23^{ab}	0.012	0.001
Spleen	0.04	0.11	0.03	0.03	0.05	0.038	0.604
Gizzard	1.52^{b}	1.64^{ab}	1.71^{ab}	1.49^{b}	1.96^{a}	0.007	0.001
Proventriculus	0.31^{ab}	0.33^{ab}	0.36^{a}	0.25^{b}	0.31^{ab}	0.021	< 0.0001
Duodenum	0.69	0.69	0.76	0.66	0.77	0.039	0.547
Jejunum	1.62	1.5	2.31	1.61	1.82	0.014	0.332
Ileum	0.32	0.35	0.41	0.33	0.39	0.023	0.468
Koilin membrane	0.201^{b}	0.226^{ab}	0.212^{ab}	0.17^{b}	0.268^{a}	0.191	< 0.0001
Kidney	0.59^{b}	0.645^{ab}	0.707^{a}	0.634^{ab}	0.59^{b}	0.08	0.01

*Negative control: the basal diet with no supplemental Iron.
**Positive control: 150 mg of $FeSO_4$ (40%) was added to 1 Kg of the basal diet.
***0.61, 6.1 and 61 mg of Fe_3O_4-Cys NPs (95%) were added to 1 Kg of the basal diet respectively.
[a-c] Means within the same row with different superscripts are significantly different ($P < 0.05$).
(%): Given as % of live body weight

Table 3 Effect of Fe_3O_4-Cys NPs on some hematological parameters in male quails, hemoglobin (Hb); Red blood cell (RBC); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC)

	Iron (mg/kg diet)					
	0*	60**	0.6***	6***	60***	value-P
Hb (g/dl)	7.6 ± 0.77^{b}	9.3 ± 0.9^{ab}	8.2 ± 0.5^{ab}	10.5 ± 0.9^{a}	10.6 ± 0.9^{a}	0.02
HCT (%)	38.9 ± 5.3	34.63 ± 1.4	38.00 ± 4.1	39.1 ± 4.6	39.46 ± 1.4	0.16
RBC ($\times 10^6/\mu l$)	3.31 ± 0.04^{c}	3.95 ± 0.13^{b}	2.44 ± 0.23^{d}	3.53 ± 0.22^{bc}	$4.8 \pm 0.0.24^{a}$	0.0001
MCV (fl)	111.1 ± 8.2^{bc}	91.7 ± 3.2^{bc}	153.5 ± 20.1^{a}	119.6 ± 7.8^{b}	82.4 ± 3.8^{c}	0.001
MCH (pg)	24.2 ± 0.04^{b}	30.9 ± 0.86^{ab}	41.4 ± 2.52^{a}	40.3 ± 6.18^{a}	29.5 ± 1.16^{ab}	0.01
MCHC (g/dl)	20.6 ± 0.6^{b}	25.7 ± 1.9^{ab}	23.3 ± 1.1^{ab}	25.1 ± 2.3^{ab}	26.8 ± 1.7^{a}	0.02

*Negative control: the basal diet with no supplemental Iron.

**Positive control: 150 mg of $FeSO_4$ (40%) was added to 1 Kg of the basal diet.

***0.61, 6.1 and 61 mg of Fe_3O_4-Cys NPs (95%) were added to 1 Kg of the basal diet respectively.

[a-c] Means within the same row with different superscripts are significantly different ($P < 0.05$)

Table 4 Effect of Fe_3O_4-Cys NPs on some carcass parameters in male quails

	Iron (mg/kg diet)				
	0*	60**	0.6***	6***	60***
Gonadal Somatic Index (GSI)	3.12 ± 0.25^{ab}	2.58 ± 0.24^{b}	3.62 ± 0.16^{a}	2.56 ± 0.1^{b}	2.89 ± 0.11^{b}
Testosterone (ng/mg)	2.9 ± 0.16	3.04 ± 0.21	3 ± 0.11	3.22 ± 0.24	2.82 ± 0.18
Daily sperm production, DSP$\times(10^6)$	16.5 ± 2.1^{b}	23.6 ± 2.3^{a}	23.1 ± 31.3^{a}	17.4 ± 2.1^{b}	18.7 ± 1.2^{b}
Total testicular sperm$\times(10^6)$	286 ± 37^{b}	342 ± 22^{b}	477 ± 49^{a}	258 ± 29^{b}	296 ± 27^{b}
Epididymal sperm$\times(10^6)$	193 ± 62	350 ± 84	306 ± 50	315 ± 47	232 ± 42
Vaso defferent sperm$\times(10^6)$	196 ± 11^{c}	441 ± 41^{a}	312 ± 40^{b}	368 ± 28^{ab}	295 ± 39^{bc}

*Negative control: the basal diet with no supplemental Iron.

**Positive control: 150 mg of $FeSO_4$ (40%) was added to 1 Kg of the basal diet.

***0.61, 6.1 and 61 mg of Fe_3O_4-Cys NPs (95%) were added to 1 Kg of the basal diet respectively.

[a-c] Means within the same row with different superscripts are significantly different ($P < 0.05$).

The effects of Fe_3O_4-Cys NPs on some carcass parameters are given in Table 2. The relative (organ-to-body weight ratios) weights of the following organs were measured: heart, liver, pancreas, spleen, gizzard, proventriculus, duodenum, jejunum, ileum, koilin membrane and kidney. The body weight was not significantly affected by the Fe_3O_4-Cys NPs. Relative weights of carcass, liver, spleen, duodenum, jejunum and ileum were not influenced by dietary treatments ($P > 0.05$, data shown in Table 1). Dietary treatments affected relative weights of pancreas, gizzard, proventriculus, koilin membrane and kidney (Table 2). A negative control diet without any iron supplementation increased the relative weight of pancreas and there were no overall significant differences between positive control and treatment groups. The highest relative weights of gizzard, proventriculus, koilin membrane and kidney belonged to one of the Fe_3O_4-Cys NPs groups and significantly were greater than negative control.

The hematological parameters were displayed in Table 3. There were statistically differences in hemoglobin (Hb), red blood cell (RBC), mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC) parameters between experimental groups ($P < 0.05$). The quails fed on diets containing higher concentrations of Fe_3O_4-Cys NPs had greater average amount of Hb, RBC, MCH and MCHC than the negative control quails fed diet with no supplemental Iron. The negative control of male quails showed a significant increasing in MCV as compared with other experimental groups. Table 4 presents the results of some reproductive parameters such as gonadal somatic index (GSI), serum testosterone level, daily sperm production (DSP), total testicular sperm, epididymal sperm, vaso defferent sperm in male quails. According to statistical analysis, the highest mean of GSI, DSP and total testicular sperm related to the group that received 0.6 mg/kg Fe_3O_4-Cys NPs ($p < 0.05$). The lowest and highest levels of Vaso defferent sperm count were observed in negative and positive control groups respectively.

The effects of Fe_3O_4-Cys NPs on some sperm cell characteristics containing sperm viability, acrosome integrity, sperm membrane integrity, sperm-egg interaction (number of holes) are given in Table 5. All sperm characteristics decreased in negative control as compared with other experimental groups. The positive control that fed on diets containing Fe_3O_4 significantly improved all sperm cell characteristics ($P < 005$). Sperm viability and

Table 5 Effect of Fe_3O_4-Cys NPs on some sperm cell characteristics in male quails

	Iron (mg/kg diet)				
	0*	60**	0.6***	6***	60***
Sperm viability (%)	49 ± 6.2^{c}	73.2 ± 1.6^{a}	68.8 ± 6.3^{a}	67.8 ± 2.8^{a}	58 ± 3.1^{b}
Acrosome integrity (%)	50.6 ± 1.9^{d}	85 ± 5.6^{a}	80.6 ± 3.3^{a}	71.2 ± 5.1^{c}	65.2 ± 2.7^{c}
Sperm membrane integrity (%)	31.2 ± 1.2^{b}	48.8 ± 4.7^{a}	46.6 ± 4.1^{a}	46.4 ± 2.3^{a}	42.8 ± 5.1^{a}
Sperm-egg interaction (number of holes)	21.1 ± 0.9^{d}	104.2 ± 9^{a}	92.5 ± 8.4^{a}	40.2 ± 3.8^{c}	36.2 ± 1.6^{c}

*Negative control: the basal diet with no supplemental Iron.

**Positive control: 150 mg of $FeSO_4$ (40%) was added to 1 Kg of the basal diet.

***0.61, 6.1 and 61 mg of Fe_3O_4-Cys NPs (95%) were added to 1 Kg of the basal diet respectively.

$^{a\text{-}c}$ Means within the same row with different superscripts are significantly different ($P < 0.05$)

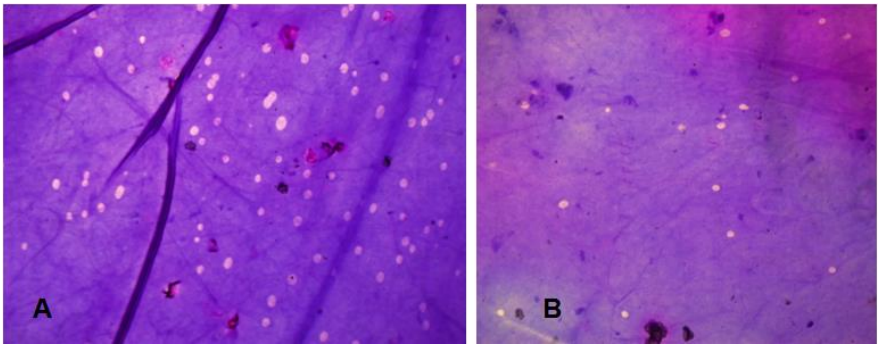

Fig. 4 Light microscopic view of more (A); and less (B) holes of sperm penetration through the inner perivitelline membrane

sperm membrane integrity were increased significantly in group that received 0.6 and 6 mg/kg of Fe_3O_4-Cys NPs same as positive control and improved as compared with negative control($P < 0.05$). The results show that 0.6 mg/kg Fe_3O_4-Cys NPs supplementation significantly same as positive control improved the acrosome integrity and sperm-egg interaction as compared with negative control (Fig. 4).

4. Discussions

The results of FTIR, AGFM and SEM showed that iron oxide nanoparticles was produced and have been coated with L-cycstein (Fe_3O_4-Cys NPs). We reported before, that cysteine-coated Fe_3O_4 nanoparticles could be used as feed additives in quails and had a potential nutritional application for animal (Rahmatollah *et al*. 2017). Also it was published that the fertility of eggs was improved by supplementing Fe_3O_4-Cys NPs (Mohammadi *et al*. 2017). In the present research, we studied the effects of Fe_3O_4-Cys NPs as micronutrient components of feed on male quails. Results of this study extended our previous observation on Fe_3O_4-Cys NPs as food additives in quails.

There was a reduction in some hematological parameters in male quails by feeding an iron deficient purified diet in negative control. These findings agreed well with previous reports (Morck and Austic 1981). A low iron diet caused a rapid decline in hematocrit, total blood hemoglobin (Morck and Austic 1981). The present study provided evidence that of dietary iron significantly affected the hematological parameters in male quails. Fe_3O_4-Cys NPs not only had no side effects but also improved some blood parameters and hematological indices as compared with negative control. Iron (Fe) plays a vital role in normal growth and is essential for fertility (Kňažická *et al*. 2012). A low iron diet induced a rapid reduction in hematocrit, blood hemoglobin in birds (Morck and Austic 1981). According to the blood parameters results, the negative control diet can be considered as a Fe-deficient diet that induced a typical nutritional iron-deficiency anemia (IDA) in male quails same as female quails that we reported it before Rahmatollah *et al*. (2017). Therefore, Fe of negative control diet cannot provide iron needs for quails. In this case, the blood exhibits a reduction in Hb, RBC, MCH and MCHC. Fe concentration in most of cereal grains are 30-60 mg/kg dry matter (DM). Bioavailability of diet minerals is not 100%. The total amount of a mineral in a diet is not a reliable index of its nutritional value (Harland 1989). The major part of iron in diet is present as an organic complex and some bound to phytate (Morris and Ellis 1976). Reduction of mineral bioavailability was related to presence of anti-nutrients such as oxalates, tannins and phytates (Harland 1989). The National Research Council (1994) recommended 60 mg/kg of iron to be supplemented in breeder quail diet (National Research Council – NRC 1994). As the results showed that iron-supplemented diet improved the final weight and prevented IDA in positive control.

The significant statistical difference in means of gonadal somatic index, daily sperm production and total testicular sperm between treatment groups was one of the most

important findings in this study. As the results showed the 0.6 mg/kg diet of Fe_3O_4-Cys NPs had a high performance for DSP, GSI and total testicular sperm as compare with other experimental groups even than positive control. This shows that the iron oxide nanoparticles that coated with cysteine have not negative effect on reproductive parameters in male quails. Between some sperm characteristics, sperm viability is one of important parameters that were influenced by diet iron. And the 0.6 mg/kg diet of Fe_3O_4-Cys NPs can improve it same as positive control that contained 60 mg/kg diet of $FeSO_4$.

High amounts of Fe had harmful results on the reproductive system, which could be strongly reflected in the final step of spermatogenesis associated with pathological disorders (Carriquiriborde *et al*. 2004) Excessive amounts of Fe caused destructive effect on the testicular function and spermatogenesis (Merker *et al*. 1996), but its physiological dose was required for normal spermatozoa production (Kňažická *et al*. 2012). According to Eghbali *et al*. (2010), the Iron content of seminal plasma was important for the preservation of sperm motility and viability after ejaculation, and its presence in the seminal plasma would help spermatozoa to maintain their functions (Eghbali *et al*. 2010). Disproportionate levels of divalent ferrous iron (Fe^{2+}) decreased size of testicular mass (Lucesoli and Fraga 1995). Elevation of Fe^{2+} concentrations might cause smaller testes and reduced sperm production (Merker *et al*. 1996). Iron overdoses increased oxidative stress in testes and epididymal sperm causing infertility (Huang *et al*. 2001). Numerous reports showed that the atrophy and morphological changes of testes, impaired spermatogenesis, epididymal lesions and reduction of reproductive performance might be related to the administration overload of Fe in rat (Crawford 1995).

The insoluble or very poorly soluble iron compounds reduced the absorbability diet iron (Zimmermann and Hilty 2013). Because of this reason, it is a necessity to use the iron supplement in diet. A size-based strategy was designed to increase the absorption of poorly soluble Fe compounds (Zimmermann and Hilty 2013). According to Motzok, reduction of particle size by 50-60% increased iron absorption by 50% in rat (Motzok *et al*. 1975). It was demonstrated that metal nanoparticles might have a nutritional value (Bogunia-Kubik and Sugisaka 2002). Despite the gaining popularity of nanomedicine, the application of nanomaterials has been restricted because of their potential toxicity and long-term secondary adverse effects (Khanna *et al*. 2015). Almost all nanoparticles due to become smaller and increased surface; stimulate toxicological effects on molecular level such the damage to cellular membrane lipids, DNA, and proteins. Some nanoparticles have high toxic effect such as Ag (Farzinpour and Karashi 2013) and zinc oxide (Soleimany *et al*. 2017), while MoO_3 had moderately toxic but Fe_3O_4, Al and MnO2 had less or no toxicity (Rahmatollah *et al*. 2017, Mohammadi *et al*. 2017, Hussain *et al*. 2005). But some reports showed the toxic effects of iron oxide nanoparticles (Di Bona *et al*. 2015, Mohseni Kouchesfehani *et al*. 2013). Coating of nanoparticles surface with albumin, dextran (Berry *et al*. 2003) polyethylene glycol (Gupta and Curtis 2004), Methionine Hydroxy (Saki *et al*. 2014) and cystein (Mohammadi *et al*. 2017) is away to decrease toxicity effects of nanoparticles. The digestibility of the organic minerals was more than inorganic oxide or sulfate (Aoyagi and Baker 1994). The amino acid metal salts provide highly bioavailable of amino acids and metal and it can be utilized better than inorganic sources (Kim *et al*. 2011). Cysteine, methionine and taurine have a protective effect against oxidative stress due to participate in radical scavenging (Atmaca 2004). L-cysteine is a sulphur-containing amino acid and powerful antioxidant due to bind to radical and reactive free molecules to protect the cells (Mukwevho *et al*. 2014, Lafleur *et al*. 1980, Pekas *et al*. 1979). Several investigator have reported iron nanoparticles as a food additive in poultry (Nikonov *et al*. 2011, Saki *et al*. 2014), but there was a little information about nutritional application of cysteine coated iron oxide nanoparticles. Our results demonstrated that Cystein-coated Fe_3O_4 nanoparticles (Fe_3O_4-Cys NPs) improve the performance of quails. A surprising finding in this study was that the lowest supplementary level of Fe_3O_4-Cys NPs (0.6 mg per kg diet) had similar results as compared with positive control. One of the main purposes of the current study was to evaluate the reproductive effects of Fe_3O_4-Cys NPs in male quails. Actual amount of inorganic iron $FeSO_4$ 40% was 150 mg per kg of diet while 0.61 mg of Fe3O -Cys NPs was needed to provide iron nutritional requirements in male quail. Therefore it is a great advantage that we can reduce the diet iron concentration 245 folds by using Fe_3O_4-Cys NPs with purity ≥ 95%.

5. Conclusions

The results confirmed that the supplementation of an Iron source in the corn-soybean meal basal diet is mandatory. The improvements in some hematological parameters, gonadal somatic index, daily sperm production, total testicular sperm and sperm viability of male quails that were fed with the diet supplemented by 0.6 mg/kg of Fe_3O_4-Cys NPs may be due to that Fe_3O_4 nanoparticles that were coated by L-cysteine could improve availability and utilization of diet iron. Finally, this study showed that not only the use of the Fe_3O_4-Cys nanoparticles in diet had no side effects but also it can be used as a feed additive to improve the reproductive performance in male quails.

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