

# RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

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# Assessment of magnetized drinking water on excreta quality, nutrients digestibility, serum components and histomorphology of digestive tract in broiler chickens

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#### **Abstract**

A total number of 150 male Ross 308 day-old broiler chicks were used to assess the effects of magnetizing drinking water on broilers performance. Thus, an experiment was conducted as 3 treatments with 5 replicates of 10 birds each. The control birds consumed an ordinary tap water. One minute and three hours magnetized water by 0.65 Tesla magnetic field was the second and the third experimental treatments. Magnetiziation of drinking water did not have a pronounced effect on the pH of crop and ileum contents, thigh meat, gastrointestinal passage rate and excreta wetness at 21 day of age. In addition, the pH of crop and thigh meat, pH and relative humidity of litter contents and viscosity of jejunal supernatant at 41 day of age were not influenced by experimental treatments. Magnetized water did not have a pronounced impact on apparent fecal digestibility of dry and organic matter, and crude protein and population of lactobacillus and coliforms in jejunal contents at 21 day of age. The activity of aspartate aminotransferase (AST) in serum of broilers that drank magnetized water significantly decreased, but cholesterol, triglycerids, calcium, phosphorus, and alanine aminotransferase (ALT) were not influenced by magnetized water. The relative weights and lenghts of intestinal parts at 21 and 41 day of age were not significantly affected by treatments. Muscularis thickness of small intestine increased significantly by magnetized water both at 21 and 41 day of age. Villus height of jejunum significantly increased, but villus width significantly decreased in birds that drank one-minute magnetized water at 21 day of age. However villus surface area, crypt depth and villus height to crypt depth ratio were not significantly affected by experimental treatments at 21 and 41 day of age. Furthermore, weight, volume, density, length, diameter, and ash of right femur and tibia at 21 and 41 day of age were not influence by megnetized water. In conclusion, magnetized drinking water may influence liver function enzyme and gut physiology in broiler chickens.

**Keywords:** Broilers; histology; litter; magnetized water

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# Introduction

Water is a major component of plants and animals and is the main medium for biochemical reactions. Several reports are available on the application of water magnetization (Coey and Cass, 2000) including broiler production (Gholizadeh et al., 2008; Alhassani and Amin, 2012). Drinking water for successful broiler

production is utmost important. Basically, water characteristics has a close relation to its molecular structure and it can be affected by external processing such as magnetic field (Ozeki and Otsuka, 2006; Pang and Deng, 2008). Lin (1990) reported that diffusion of magnetized water to the cell walls can be better than non-magnetized water. Ma et al. (1992) presented the possibility that magnetized water can prevent aging and

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fatigue by increasing the cell membrane permeability. Also, Buyukuslu et al. (2006) indicated that activity of superoxide dismutase was increased in magnetic field.

Some researches indicated that magnetized water as compared with the ordinary water resulted in better efficiency in agricultural products (Khoshravesh et al., 2011; Mostafazadeh-Fard et al., 2012). In animal husbandry, Lin and Yotvat (1990) reported that magnetized drinking water caused an increased production of milk, mutton, and wool in sheep and more weight gain in geese and egg production and hatchability in turkey. However, Alhassani and Amin (2012) reported that 500 Gauss magnetization with speed of 5, 10, and 15 min for 10 liter drinking water did not significantly affect performance of broiler chickens. The objective of this study was to scrutinize various aspects of magnetized drinking water in broiler chickens.

# **Materials and Methods**

#### Birds housing and treatments

A total number of 150 male Ross 308 day-old broiler chicks were randomly divided into 3 groups. Each group was further divided into 5 replicates of 10 birds each in a completely randomized design. Ordinary drinking tap water of the farm was considered as control treatment. One-minute and three-hours magnetized water were the second and the third experimental treatments respectively. Magnetized water was produced by a commercial magnet namely AQUA CORRECT with 0.65 Tesla (6500 Gauss) magnetic field. Magnetization process for 30 litres of tap water has been done by magnetic apparatus as daily (Fig. 1). Some characteristics of experimental water are given in Table 1. These types of water were offered daily to the birds during 42 days. Each pen (1 m<sup>2</sup>) was equipped with a manual feeder and a manual drinker, and the floor was covered with clean wood shavings.

A corn-soybean meal based diet (Table 2) was formulated to meet or exceed the nutrient requirements of all broiler chickens as recommended by Ross 308 broiler rearing guidelines (Aviagen, 2009). Drinking water and mesh feed were offered *ad libitum* throughout the trial. Lighting was continuous, and the temperature was 32°C in the first week and then gradually decreased to 24°C by the end of the third week. Chicks were vaccinated for Infectious Bronchitis on day 4 and Newcastle Disease on 4, 11, and 20 day of age. The experimental protocol was approved by the Animal Care Committee of the Ferdowsi University of Mashhad, Mashhad, Iran.

# Apparent digestibility of nutrients and analyses of litter, digesta, meat and blood

Gastrointestinal transit time of diets was measured at day 16 by covering the pen floors with clean paper 4 h after feed withdrawal. Then the time between the offering of the diets containing 0.3% chromic oxide and the

appearance of three spotted green excreta in each pens was considered as passage rate of feed (Sedghi et al., 2010). Feeding of these diets was continued until 21 day. A sample of the diet and excreta from each pen was stored at -20°C for further analyses of dry and organic matter. Also, protein concentration of the diets and excreta samples was determined by Kjeldahl standard procedures of AOAC (1996). Chromium in the diets and excreta was determined as described by Fenton and Fenton (1979). Apparent digestibility of nutrients was calculated using the formula of Lazaro et al. (2003). Moreover, freshly voided excreta were scored for wetness on a scale of 1 to 5 with 1 representing normally formed excreta and 5 representing very watery excreta at 21 day of age (Ravindran et al., 2008).

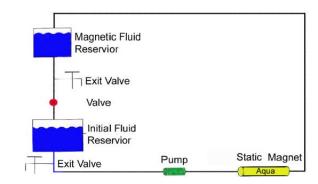


Fig. 1: A schematic diagram of magnetic apparatus. Adapted from Eshaghi and Gholizadeh (2004)

Three male birds with the average weight of each treatment were chosen at 21 and 41 d of age. Following blood sampling from brachial vein, the selected birds were slaughtered. Then, the right femurs and tibiae, small intestine, kidney, and liver were excised, and jejunal digesta were obtained. The pH of crop and ileum contents and litter were measured using a pH meter (Model 691, Metrohm, Switzerland) as described by Esmaeilipour et al. (2011). Furthermore, pH and humidity of litter at 41 day were determined as described by Safaeikatouli et al. (2011). The pH of thigh meat after one month cold storage at -20°C was measured as described by Bernacki et al. (2008). The jejunal digesta was centrifuged at 3000×g for 15 min to obtain the supernatant (0.5 mL) for viscosity. The viscosity (as centipoises [cP]) of the supernatant was measured with a digital viscometer (Brookfield Engineering Laboratories Inc., USA).

Serum was obtained after coagulation of blood samples to measure the chemical components including cholesterol, triglycerides, calcium, phosphorous, aspartate aminotransferase (AST), alanine aminotransferase (ALT) (Tietz, 1995; Young, 1995). These variables were analyzed using an autoanalyzer (Bio Systems S.A. Barcelona, Spain).

Table 1: Some characteristics of ordinary and magnetized drinking water

Type of drinking water	Ordinary tap water	One-minute magnetized water	Three-hours magnetized water
Total dissolved solids (mg/L)	648	654	786
Total hardness (mg/L)	368	368	448
Total count of bacteria (CFU)	2.74	3.86	3.72
Viscosity (cP)	0.78	0.91	0.93

CFU = colony forming unit; Cp = centipoise

Table 2: The composition of basal diets

Item	Starter (0-10 d)	Grower (11-24 d)	Finisher (25-42 d)
Ingredients (%)			
Corn	50.29	53.43	59.28
Soybean meal	40.90	36.32	31.06
Vegetable oil	4.77	6	5.76
Limestone	1.30	1.07	1.04
Dicalcium phosphate	1.95	1.72	1.60
Common salt	0.35	0.35	0.35
L-Lysine HCl	0.30	0.31	0.15
DL-Methionine	0.39	0.30	0.26
Vitamin and mineral premix <sup>1</sup>	0.5	0.5	0.5
Calculated contents (percent unless other	rwise stated)		
ME (kcal/kg)	3025	3150	3200
Crude protein	22.47	21	19
Calcium	1.43	1.34	1.09
Available phosphorus	0.50	0.45	0.42
Lysine	1.43	1.34	1.09
Methionine	0.71	0.61	0.55
Methionine + Cystine	1.07	0.95	0.86
DCAB (mEq/kg)	245	225	204

Tvitamin and mineral premix supplied per kilogram of diet: vitamin A, 10000 IU; vitamin D<sub>3</sub>, 9800 IU; vitamin E, 121 IU; B<sub>12</sub>, 20 μg; riboflavin, 4.4 mg; calcium pantothenate, 40 mg; niacin, 22 mg; choline, 840 mg; biotin, 30 μg; thiamin, 4 mg; zinc sulfate, 60 mg; manganese oxide, 60 mg.

# Microbial study of jejunal contents

Jejunal contents were carefully kept in sterile dishes at -20°C until analyses in the laboratory. The samples were homogenized, and then one gram of each sample was collected and transferred into 10 ml sterile saline solution for dilution. After that, each sample was spread on selective agar plates as follows. The EMB agar (Eosin methylene blue) was utilized for coliforms and MRS agar (Man Rogosa and Sharpes medium) was used for lactobacillus bacteria (APHA, 1993). The number of bacterial colonies was enumerated after incubation at 37°C for 48 hours. Number of microflora was converted to log<sub>10</sub> per ml before statistical analysis.

### Physical traits & histology of bone, gut, liver and kidney

Femurs and tibiae were defleshed, and cartilaginous caps were removed immediately after slaughter and then were frozen. The bones were thawed by leaving them from plastic bags at room temperature for 1 h. The whole length and diaphyseal diameter of femurs and tibiae were measured using a digital caliper. These bones were weighed and the volume of each one was determined in distilled water. The wet densities of bones (g/cc) were calculated as dividing the mass (initial weight) by the volume of that bone (Rath et al., 1999). Then, diaphysial part of the bones were dried at 70°C for 48 h and weighed

after density measurements. These bones were ashed at 600°C overnight in a furnace. The ash percentage was expressed in relative to dry weight of the bone. A segment of jejunum, kidney, liver and proximal part of tibia were kept in 10% neutral buffered formalin. Jejunum was defined as the portion of small intestine extending from the bile duct entrance to Meckel's diverticulum and tissue samples were taken from the midpoint of the aforementioned section. The bones were decalcified with 10% trichloroacetic acid. These tissues were embedded in paraffin and stained with hematoxylin and eosin, and then were processed by microtome for further histomorphology. Moreover, these tissues were observed using microscope for possible changes as described by Liu et al. (2003). The morphometric variables of gut included villus height and width, depth of crypt of Liberkuhn, and muscularis thickness. The mean from 10 measurements per sample was used as the average value for further analysis (Geyra et al., 2001; Akbarian et al., 2013).

# Statistical analyses

All data were analyzed using the General Linear Model procedure of the Statistical Analysis System (SAS, 2004). Tukey's Studentized Range (HSD) test was used to compare the means. All statements of significance were based on probability of P<0.05.

Table 3: Effects of magnetized water on pH of crop and ileum contents and thigh meat, gastrointestinal passage rate and excreta quality in broilers at 21 day of age

		8-			
Treatments of drinking water	Crop	Ileum contents	Thigh pH	Gastrointestinal	Excreta wetness
	contents pH	pН	ringii pri	passage rate (min)	
Ordinary tap water	6.38	6.20	7.45	129.33	3.00
One-minute magnetization	6.47	6.41	7.58	130.33	3.00
Three-hours magnetization	6.67	6.34	7.65	131.66	3.33
±SEM	0.288	0.071	0.057	5.374	0.507
P-Value	0.781	0.183	0.120	0.954	0.869

Table 4: Effects of magnetized water on pH of crop and thigh meat, pH and relative humidity of litter contents and jejunal viscosity in broilers at 41 day of age

Treatments of drinking water	Crop contents pH	Thigh pH	Litter pH	Relative humidity of litter (%)	Viscosity (cP)
Ordinary tap water	5.48	6.68	7.63	47.84	1.59
One-minute magnetization	5.44	6.81	7.54	46.19	1.98
Three-hours magnetization	5.73	6.87	7.62	48.64	1.58
±SEM	0.367	0.135	0.098	3.171	0.223
P-Value	0.605	0.656	0.786	0.858	0.398

Table 5: Effects of magnetized water on apparent nutrients digestibility and jujunal microbiological modifications at 21 day of age

	Ap	parent digestibili	Jujunal microbs		
Treatments of drinking water	DM %	OM %	CP %	Lactobacillus	Coliforms
Ordinary tap water	91.91	87.77	86.00	4.50	4.44
One-minute magnetization	92.68	85.69	86.08	4.24	4.15
Three-hours magnetization	91.72	86.03	86.76	3.94	4.35
±SEM	0.614	0.082	0.616	0.208	0.223
P-Value	0.543	0.064	0.671	0.226	0.671

Table 6: Effects of magnetized water on some serum components at 41 day of age

Treatments of drinking	Cholesterol	Triglycerides	Phosphorus	Calcium	AST (IU/l)	ALT
water	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	AST (10/1)	(IU/l)
Ordinary tap water	125.67	99.00	7.21	14.60	241.67 <sup>a</sup>	22.33
One-minute magnetization	133.33	97.00	8.69	15.04	174.33 <sup>b</sup>	19.66
Three-hours magnetization	124.00	92.00	7.81	13.00	189.33 <sup>b</sup>	18.33
±SEM	7.099	10.614	0.507	0.944	7.187	2.134
P-Value	0.634	0.893	0.199	0.342	0.001	0.451

AST, aspartate aminotransferase; ALT, alanine aminotransferase; <sup>a, b</sup> Means within each column with different superscripts are significantly different (P<0.05).

### **Results and Discussion**

Magnetized drinking water did not affect pH of crop and ileum contents, thigh meat, passage rate and excreta wetness at 21 day of age (Table 3). Also, pH of crop content, thigh meat, pH and relative humidity of litter contents and viscosity of jejunal digesta at 41 day of age were not influenced by magnetized drinking water (Table 4). These results are not in consistent with the results of Xu and Sun (2008) who reported that magnetized water had higher pH as compared with ordinary drinking water. Also, reduction of water viscosity up to 2% through magnetization has been observed by Kronenberg (1985). Moreover, Gilani et al. (2013) indicated that pH of some feed ingredients in magnetized water was significantly more than non-magnetized water.

The effects of magnetized water on apparent fecal digestibility of dry and organic matter, crude protein and

microbial populations in jejunal digesta at 21 day of age are shown in Table 5. These variables were not affected by magnetized drinking water. Furthermore, the evaluation of magnetized drinking water on serum components such as cholesterol, triglycerids, calcium, phosphorus, AST and ALT are presented in Table 6. The activity of AST in serum of broilers drank magnetized water significantly decreased, but the other blood variables were not influenced by magnetized water. The increase in activity of hepatic enzymes is a symptom of liver injury or damage in birds. Also, a reduction in activity of these enzymes may be an indicative of better efficiency of liver (Fudge, 2000). Noteworthy, Ma et al. (1992) revealed that magnetized water increased glutamate decarboxylase activity. More recently, Lee and Kang (2013) indicated that the 8-week intake of magnetized water fortified with 9000-13000 Gauss magnetic field diminished the concentrations of blood

Table 7: Effects of magnetized water on relative weight of small intestine as percentage of live weight at 21 and 41 day of age

Treatments of drinking water	Small intestine	Duodenum	Jejunum	Ileum	Ceca
21 d					
Ordinary tap water	18.55	4.22	7.69	6.60	3.65
One-minute magnetization	18.25	3.23	7.30	7.60	3.10
Three-hours magnetization	21.13	3.85	8.90	8.30	4.03
±SEM	1.739	0.318	0.760	1.040	0.272
P-Value	0.481	0.165	0.371	0.545	0.126
41 d					
Ordinary tap water	6.95	1.26	2.74	2.95	0.64
One-minute magnetization	7.61	1.24	2.94	3.41	0.70
Three-hours magnetization	7.31	1.21	2.98	3.12	0.72
±SEM	0.653	0.090	0.301	0.293	0.069
P-Value	0.780	0.920	0.837	0.555	0.719

Table 8: Effects of magnetized water on relative length of duodenum, jejunum, and ileum to small intestine (%) at 21 and 41 day of age

, <del></del>							
	21d			41d			
Treatments of drinking water	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	
Ordinary tap water	22.06	41.29	36.64	18.14	39.47	42.38	
One-minute magnetization	17.76	40.34	41.89	16.45	38.75	44.79	
Three-hours magnetization	18.82	42.54	38.63	16.67	40.47	42.85	
±SEM	2.382	1.571	2.169	0.960	1.176	0.970	
P-Value	0.320	0.634	0.206	0.225	0.610	0.256	

Table 9: Effects of magnetized water on height, width, and surface area of villus, crypt depth, villus height to crypt depth ratio and muscularis thickness layer of jejunum at 21 and 41 d of age

and muscularis unckness layer of jejunum at 21 and 41 d of age									
Treatments of drinking	Villus height	Villus depth	Villus surface	Crypt depth	Villus height:crypt	Muscularis			
water	(µm)	(µm)	area (µm²)	(µm)	depth	thickness (µm)			
21 d									
Ordinary tap water	1221 <sup>b</sup>	212 <sup>a</sup>	792028	237	5.28	258 <sup>c</sup>			
One-minute magnetization	1317 <sup>a</sup>	157 <sup>b</sup>	672484	228	5.80	368 <sup>a</sup>			
Three-hours magnetization	1302 <sup>ab</sup>	199 <sup>a</sup>	714761	244	5.67	301 <sup>b</sup>			
±SEM	34	10	43432	14	0.330	12			
P-Value	0.039	0.003	0.085	0.749	0.098	< 0.0001			
41 d									
Ordinary tap water	1640 <sup>a</sup>	267	1398009	220	7.96	$208^{b}$			
One-minute magnetization	1510 <sup>b</sup>	298	1281636	213	7.42	231 <sup>ab</sup>			
Three-hours magnetization	1711 <sup>a</sup>	268	1477082	207	8.31	252 <sup>a</sup>			
±SEM	32	13	86740	10	0.365	13			
P-Value	0.0002	0.120	0.249	0.641	0.236	0.041			

a, b Means within each column with different superscripts are significantly different (P<0.05).

glucose and glycated hemoglobin levels, and damaged DNA in diabetic rats. However, the authors found no differences in total cholesterol, HDL-cholesterol, and LDL-cholesterol among different groups. In line with our results, Beata et al. (2010) mentioned that magnetized drinking water did not influence blood constituents such as potasssium and chloride and performance of Guinea fowl. Moreover, conditioned water through 3600 Gauss permanent magnet was used in Saanen lactating goats by Sargolzehi et al. (2009) and found no significant influence on blood components such as urea, Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>++</sup> and P<sup>-</sup>.

The proportional weights and lenghts of intestinal segments at 21 and 41 days of age were not significantly affected by treatments (Tables 7&8). The morphometrical variables of jejunum are presented in Table 9. Muscularis thickness of small intestine was significantly increased in

birds that drank magnetized water both at 21 and 41 day of age. Villus height of jejunum significantly increased, but villus width significantly decreased in birds that drank one-minute magnetized water at 21 day of age. However, villus surface area, crypt depth and villus height to crypt depth ratio were not significanly affected by magnetized drinking water at 21 and 41 days of age. Furthermore, the results of drinking magnetized water on weight, volume, density, length, diameter, and ash of right femur and tibia at 21 and 41 days of age are presented in Tables 10-11. These variables did not change by magnetized drinking water. It is noteworthy that renal and hepatic tissuses along with growth plate of tibia were obsevered for possible pathological alterations, but no changes were found among different treatments. On the other hand, magnetized drinking water did not have any adverse effect on the above mentioned tissues.

Table 10: Effects of magnetized water on right femur characteristics of broilers at 21 and 41 days of age

Treatments of drinking	Weight	Weight	Volume	Density	Length	Diaphysial	Diameter to
water	(g)	(%)	(cc)	(g/cc)	(mm)	diameter (mm)	length ratio (%)
21 d							
Ordinary tap water	5.12	0.70	4.50	1.14	53.47	6.15	11.50
One-minute magnetization	4.99	0.73	4.33	1.15	53.19	6.29	11.80
Three-hours magnetization	5.52	0.75	5.00	1.10	54.30	6.16	11.36
±SEM	0.467	0.055	0.341	0.057	1.515	0.458	0.582
P-Value	0.619	0.774	0.305	0.845	0.814	0.960	0.808
41 d							
Ordinary tap water	16.29	0.60	14.00	1.16	78.20	9.93	12.71
One-minute magnetization	14.76	0.59	13.33	1.10	76.47	9.75	12.81
Three-hours magnetization	15.81	0.64	13.00	1.21	76.03	9.83	12.92
±SEM	1.159	0.040	0.384	0.073	2.591	0.450	0.684
P-Value	0.655	0.706	0.251	0.596	0.827	0.960	0.978

Table 11: Effects of magnetized water on right tibia characteristics of broilers at 41 days of age

Treatments of drinking water	Weight	Weight	Volume	Density	Length	Diaphysial	Diameter to	Ash
	(g)	(%)	(cc)	(g/cc)	(mm)	diameter (mm)	length ratio (%)	(%)
21 d								
Ordinary tap water	6.33	0.86	5.56	1.11	68.04	5.59	8.23	56.17
One-minute magnetization	5.39	0.78	5.33	1.00	66.43	5.66	8.51	52.38
Three-hours magnetization	6.67	0.90	6.33	1.04	68.30	5.63	8.24	55.44
±SEM	0.695	0.066	0.333	0.063	1.593	0.217	0.295	1.912
P-Value	0.453	0.500	0.178	0.525	0.684	0.976	0.757	0.241
41 d								
Ordinary tap water	20.90	0.77	17.66	1.18	104.00	8.65	8.31	50.37
One-minute magnetization	20.23	0.80	16.33	1.23	106.00	8.16	7.71	49.10
Three-hours magnetization	21.62	0.86	18.00	1.20	102.42	8.83	8.62	48.89
±SEM	1.863	0.039	0.860	0.066	2.649	0.347	0.301	1.623
P-Value	0.873	0.311	0.406	0.852	0.653	0.427	0.174	0.792

In agreement with these results, Al-Mufarrej et al. (2005) observed that cosumption of magnetized tab water through 500 Gauss did not significantly influenced the carcass characteristics, immune response and produvtive traits of broilers. Also, Patterson and Chestnutt (1994) concluded that magnetized drinking water containing a relatively high total hardness increased the depth of subcutaneous fat and lipid concentration in carcass of lambs. They did not find any improvement in performance or carcass composition of finishing lambs.

There are controversial results in agriculture about the effectiveness of magnetizing processes. For instance, Vashisth and Nagaraja (2010) exposed seeds of sunflower to different static magnetic fields from 0 to 250 mT in steps of 50 mT for 1-4 h in steps of 1 h. Treatment of sunflower seeds in these magnetic fields improved the germination speed, seedling length and dry weight under laboratory germination conditions. In germinating seeds, enzyme activities of a-amylase, dehydrogenase and protease were significantly higher in treated seeds as compared with controls. The higher enzyme activity in magnetic-field-treated sunflower seeds could lead to fast germination and early vigour of seedlings. However, Maheshwari and Grewal (2009) indicated that magnetically treated irrigation water did not influence the yield of peas. All in all, it seems that plant species responses to magnetic field are unpredictable. Their response depends on magnetic

field intensity, duration of exposure to magnetic field, seed priming methods and species (Dhawi et al., 2009).

Water and water solutions passed through the magnetic field acquire finer and more homogeneous structures (Tkachenko and Semyonova, 1995). This increases the fluidity, dissolving capability for various constituents like minerals and vitamins (Kronenberg, 1985; Mikesell, 1985) and consequently improves the biological activity of solutions positively affecting the performance of human, animal and plants (Lin and Yotvat, 1988). Lin and Yotvat (1990) and Knez and Pohar (2005) reported that magnetizing apparatus, volume, rate and quality of water and even water temperature are determining the magnetizing treatment of water.

#### Conclusion

Magnetized drinking water have no adverse effects on nutrients digestibiliy, litter quality, blood parameters, along with tissues of bone, liver, kidney and gut. Also, micrbiota and some morphometrical variables of small intestine were not influenced by this process; however, magnetized drinking water profoundly improved villus height and muscularis thickness of jejunum in broiler chickens. A reappraisal of magnetizing treatment of water containing more hardness with more powerful magnetic field and longer time on various aspects of poultry production is suggested for future studies.

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