doi: 10.17221/8667-CJAS

Preservation effects of melatonin on the quality and fertility of native Fars rooster semen during liquid storage

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ABSTRACT: Liquid or frozen storage of poultry semen negatively affects the sperm motility and has crucial role in reducing fertility. Phospholipids in chicken sperm membrane are composed of high proportions of polyunsaturated fatty acids which are susceptible to lipid peroxidation. One of possible ways to improve semen quality is supplementing the ration with antioxidant compounds such as melatonin. In this study, seventy-two roosters were randomly divided into three equal groups. The first group (control; Group C) was exposed to 14 h light and 10 h darkness. The second group (Group M) was exposed to the same lighting period, but in combination with melatonion supplementation (3 mg/kg body weight daily). The third group (Group L) was exposed to 24 h constant light. Semen was collected using abdominal massage and stored for 6 and 12 h at 5°C. Motility, viability, malondialdehyde (MDA) concentration, fertility, and hatchability were evaluated before and after storage. The results showed that the percentage of viable and motile sperms was significantly ($P < 0.05$) decreased in the control samples after 6 h storage but in Group M these parameters were significantly ($P < 0.05$) decreased after 12 h. The MDA concentration was significantly ($P < 0.05$) decreased in Group M compared with Group C after 6 and 12 h storage. The sperm membrane lipid analysis showed that the percentage of unsaturated fatty acids in Group M was significantly ($P < 0.05$) lower than in Group C. Fertility and hatchability did not change significantly ($P < 0.05$) both in M and L Groups compared with C Group. Melatonin administration improved semen quality and decreased lipid peroxidation during liquid storage. It also reduced the percentage of polyunsaturated fatty acids in the sperm membrane lipid composition but it did not affect fertility and hatchability.

Keywords: chicken sperm; antioxidant; lipid peroxidation; membrane integrity

INTRODUCTION

Several factors may reduce motility and fertilizing ability of chicken spermatozoa during *in vitro* storage. In fact, the *in vitro* storage of sperm is always accompanied by peroxidation of membrane phospholipids which causes irreversible reduction in semen quality. Phospholipids are the basic components of sperm membrane structure and are responsible for the fluidity of membrane bilayer (Zniboni and Cerolini 2009). This characteristic is necessary for capacitation, acrosome reaction, and motility of sperm in human (Nissen et al. 1983), boar (Cerolini et al. 2000), and chicken (Cerolini et al. 2003) sperm.

Supported by the Iran National Science Foundation (grant No. 843171).

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Chicken sperm is characterized by high proportions of polyunsaturated fatty acids (PUFAs) such as arachidonic acid (C20: 4n-6) and docosatetraenoic acid (C22: 4n-6). The high concentration of PUFAs in sperm membrane makes it sensitive to peroxidation during *in vitro* storage (Cerolini et al. 2006).

Sperm membrane is naturally protected by a cellular antioxidant system. The antioxidant defense system in avian spermatozoa includes (1) antioxidant enzymes, e.g. superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), (2) water and lipid soluble antioxidants, e.g. vitamin A, C, and E, and (3) phospholipases, proteases, and transferases which repair or remove damaged molecules especially by cellular oxidative stresses (Breque et al. 2003).

During *in vitro* preservation, reactive oxygen species (ROS) such as hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), and superoxide anion radical (O_2^-) are produced by dead sperms, leukocytes, and atmospheric or molecular oxygen. Oxidative stress is a cellular condition which may occur by unbalancing between the ROS production and the insufficient capacity of the cellular antioxidant system (Reiter et al. 2003).

N-Acetyl-5-methoxytryptamine (melatonin) is a synthetic product of the vertebrate pineal gland. In addition to its effects on circunnal reproductive rhythms (Reiter 1980), immune system stimulation, and anti-inflammatory function (Guerrero and Reiter 2002), it also stimulates glutathione production (Rodriguez et al. 2004) and the activity of antioxidant enzymes such as SOD and GSH-Px (Mayo et al. 2002). Melatonin augments the efficiency of other antioxidants (Reiter et al. 2000) and has powerful and direct effects on scavenging free radicals (Tan et al. 2002). Therefore the purpose of this study was to evaluate the effects of edible melatonin on the semen quality and fertility of roosters after liquid storage at 5°C.

MATERIAL AND METHODS

Animals. For this study, 72 roosters (9 months old with proved fertility) and 270 layer hens (16 months old) kept at Fars native poultry research centre farm (Iran) were used. The roosters were trained to give semen by abdominal massage method (Burrows and Quinns 1936), 14 days before the beginning of the experiment.

Experimental design. Roosters were randomly divided into 3 groups (24 roosters per group) with 3 replicates (8 roosters in each replicate) in the form of a completely randomized design as follows: the first group (control; Group C) was exposed to 14 h light and 10 h darkness. The second group (Group M) was exposed to the same lighting program, but moreover supplemented with 3 mg/kg body weight edible melatonin daily. The content of melatonin capsules (NutriCentury Co., Markham, Canada) dissolved in distilled water was fed to the roosters by a drencher. The third group (Group L) was exposed to 24 h constant light to reduce the blood melatonin concentration to the lowest possible level (Cheung et al. 2003). Constant light was provided by a 100-watt light bulb lamp (Pars Co., Rasht, Iran) situated 1 m above the heads of the roosters which supplied about 150 lux light intensity. The diets for both roosters and hens were prepared following the recommendation of the Fars native poultry research center.

Semen collection, processing, and evaluation. The **s**emen samples were collected four times (on days 0, 20, 40, and 60) during the 60-day experimental period. The semen samples of every 8 roosters in each group were pooled and stored for 6 and 12 h at 5°C. Then, the semen quality, malondialdehyde (MDA) concentration, lipid analysis, fertility, and hatchability were evaluated. For liquid storage the samples were diluted at the ratio of 1 : 2 v/v (one part semen and two parts extender) with Beltsville extender (fructose 27.7mM, potassium citrate 2.1mM, sodium acetate 31.5mM, sodium glutamate 46.3mM, $MgCl_2$ 1.6mM, $K_2HPO_4{\cdot}3H_2O$ 55.7mM, KH_2PO_4 4.7mM, and TES 16.1mM) (Sexton and Giesen 1982). For semen evaluation, the proportion of motile sperm was assessed subjectively (Bakst 1980) under a light microscope DM300 (Leica Microsystems, Wetzlar, Germany) and sperm viability was assessed by an eosin-nigrosin stain (Bajpai 1963). The sperm concentration was measured by a hemocytometer RSS-151 (R.S. Scientific, Delhi, India) and the aliquots of semen containing 1×10^9 and 0.25×10^9 sperm were separated and used for lipid analysis and lipid peroxidation assay, respectively. The samples were frozen at –20°C until further analysis. The hypo-osmotic swelling test (HOST) was used (Blanco et al. 2000) to evaluate functional integrity of the sperm membrane. In brief, 50 µl of hypotonic solution (0.15 ml NaCl in 100 ml of distilled water) was added to 5 µl of semen and preserved at 39°C for 10 min. Under the light microscope

doi: 10.17221/8667-CJAS

(Leica Microsystems) observation the number of curved tail sperms was counted and expressed as the percentage of total spermatozoa.

Lipid peroxidation assay. To measure the rate of sperm lipid peroxidation, MDA concentration of samples was measured by using the thiobarbituric acid reactive substances (TBARS) test (Esterbauer and Cheeseman 1990). In brief, the samples were centrifuged (600 *g*, 15 min, 4°C) and the supernatant discarded. The remains were mixed with 500 µl Na citrate solution (2.9%) and vortexed for 3 min. The Na citrate solution was added again to obtain samples of 1 ml volume. Then, 1 ml EDTA, 1 ml butylated hydroxytoluene (BHT), and 2 ml trichloroacetic acid (TCA) were added and the final sample was centrifuged at 963 *g* for 15 min. The supernatant (1 ml) was mixed with 1 ml of thiobarbituric acid (TBA) and preserved at 95°C for 10 min. The amount of light absorption in 532 nm was assayed using a spectrophotometer Supertonic 70 (Bausch & Lomb, Feldkirchen, Germany) and MDA concentration was expressed as µg/10^9 sperm by a standard curve.

Lipid analysis. Semen samples were thawed and the sperms were separated by a centrifuge (600 *g*, 15 min, 4°C). Total lipids were extracted from sperms by a standard procedure (Christie 1982). In brief, samples were homogenized in a suitable excess of chloroform : methanol (2 : 1, v/v) and extracts of total lipid were prepared. The extracts were separated into phospholipids and free cholesterol by thin layer chromatography. Then the fatty acid composition of phospholipids was determined by a gas chromatograph GC-2010 Plus (Shimadzu, Tokyo, Japan) (Christie et al. 1970).

Assessment of fertility and hatchability. In order to evaluate the fertility and the hatchability of samples, 10 hens were used for each replicates. Each hen was inseminated artificially with 0.2 ml of samples and 2 days after insemination eggs were collected for 2 weeks. The numbers of fertilized and hatched eggs were counted after 21 days of incubation and the results reported as the percentage of total eggs.

Statistical analysis. A completely randomized design with 3 treatments and 3 replicates was used for this experiment. Data were analyzed using the MIXED procedure of SAS (Statistical Analysis System, Version 9.1, 2003) considering the time of storage, day of sampling, treatment, and their interactions as the sources of variation. Differ-

Table 1. Total effects of treatment, storage time, and sampling day on semen characteristics of Fars native roosters (LS means ± SEM)

Characteristics	Treatment			Storage time (h)			Day of sampling			
	control	24 h light	melatonin supplementation[1]	0	6	12	0	20	40	60
Semen pH	7.53 ± 0.01^{a}	7.41 ± 0.01^{b}	7.52 ± 0.01^{b}	7.52 ± 0.01^{a}	7.47 ± 0.01^{b}	7.40 ± 0.01^{c}	7.50 ± 0.01^{a}	7.47 ± 0.01^{ab}	7.43 ± 0.01^{b}	7.46 ± 0.01^{ab}
Motility (%)	58.8 ± 2.1^{a}	57.7 ± 2.1^{a}	62.4 ± 2.5^{b}	75.0 ± 1.9^{a}	59.2 ± 1.9^{b}	44.7 ± 1.9^{c}	67.0 ± 2.6^{a}	51.3 ± 2.2^{b}	59.8 ± 2.5^{a}	60.4 ± 2.2^{a}
Viability (%)	86.5 ± 0.8^{a}	86.5 ± 0.8^{a}	88.5 ± 0.9^{a}	94.2 ± 0.7^{a}	88.5 ± 0.7^{b}	87.7 ± 0.7^{c}	80.7 ± 1.0^{c}	93.5 ± 0.8^{a}	85.7 ± 0.9^{b}	88.7 ± 0.8^{b}
Membrane integrity (%)	12.0 ± 0.6^{a}	12.90 ± 0.6^{a}	13.3 ± 0.8^{a}	18.9 ± 0.6^{a}	11.9 ± 0.6^{b}	7.3 ± 0.6^{c}	14.0 ± 0.8^{ab}	11.70 ± 0.7^{bc}	10.3 ± 0.8^{c}	14.8 ± 0.7^{a}
Malondialdehyde (µg/10^9 sperm)	11.6 ± 0.3^{a}	10.5 ± 0.3^{a}	8.2 ± 0.4^{b}	6.7 ± 0.3^{a}	11.2 ± 0.3^{b}	12.3 ± 0.3^{c}	8.8 ± 0.4^{a}	9.1 ± 0.4^{a}	10.6 ± 0.4^{b}	12.0 ± 0.4^{b}
Fertility (%)	46.7 ± 4.2^{A}	33.8 ± 4.2^{a}	35.1 ± 4.2^{a}	50.5 ± 3.6^{a}	40.2 ± 4.1^{ab}	25.1 ± 3.7^{b}	39.4 ± 4.9^{ab}	54.6 ± 4.9^{a}	35.8 ± 4.9^{b}	24.4 ± 4.9^{b}
Hatching (%)	84.9 ± 3.9^{a}	89.3 ± 3.9^{a}	82.8 ± 3.9^{a}	90.9 ± 2.8^{a}	88.0 ± 3.1^{a}	78.1 ± 4.4^{a}	84.8 ± 4.8^{a}	62.6 ± 4.8^{a}	84.4 ± 4.8^{a}	80.4 ± 4.8^{a}

[1]3 mg/kg body weight edible melatonin daily

means within row in each group with different superscripts significantly differ ($P \leq 0.05$)

doi: 10.17221/8667-CJAS

Table 2. Effect of treatment and storage time on semen pH in Fars native roosters (LS means ± SEM)

Time of *in vitro* storage (h)	Treatment		
	control	24 h light	melatonin supplementation[1]
0	7.62 ± 0.02^{Aa}	7.46 ± 0.02^{Ba}	7.49 ± 0.02^{Ba}
6	7.52 ± 0.02^{Ab}	7.38 ± 0.02^{Ba}	7.51 ± 0.02^{Aa}
12	7.44 ± 0.02^{Ac}	7.39 ± 0.02^{Aa}	7.37 ± 0.02^{Ab}

[1]3 mg/kg body weight edible melatonin daily
means within row (shown in capital letter) and column (shown in small letter) with different superscripts significantly differ ($P \leq 0.05$)

ences between Least Squares means (LS means) were determined by Tukey's test ($P < 0.05$) and the results were presented as LS means ± SEM.

RESULTS

The total effect of treatment, day of sampling, and storage time on semen characteristics (Table 1) showed that pH values increased significantly ($P < 0.05$) in both experimental treatments (Groups L and M) compared with control group (Group C). The lowest concentration of MDA was related to the roosters which received 3 mg/kg body weight melatonin. There were no significant ($P < 0.05$) differences in the percentage of fertility, hatchability, viable and motile sperms in the experimental treatments compared with the control sample. Motility, viability, and plasma membrane integrity of sperm declined by 30.28, 15.48, and 11.16%, respectively, during 12 h of storage.

The highest percentage of sperm viability was observed by the second sampling (Day 20). The percentage of motile sperms was decreased on Day 20, but it increased in the next samplings (Days 40 and 60). The percentage of fertility was decreased since the third sampling (Day 40) till the end of the experiment but the hatchability of fertile eggs did not change during the experiment. There was no significant difference ($P < 0.05$) between the MDA concentration in the first and the second sampling. MDA concentration was significantly ($P < 0.05$) increased by the third and fourth sampling compared with MDA concentration by the first and second sampling.

The effect of storage time and treatment was significant ($P = 0.0001$) for semen pH and the results (Table 2) showed that semen pH in M Group remained unchanged until 6 h but it decreased significantly ($P < 0.05$) after 12 h. In contrast, semen pH of L Group did not change even after 12 h of storage. In control sample, semen pH decreased after 6 h and continued decreasing until 12 h of storage.

The results (Table 3) showed that MDA concentration decreased significantly ($P < 0.05$) in M Group after 6 and 12 h of storage compared with the control group but the interaction between storage time and treatment was not significant ($P > 0.05$) for MDA concentration.

The effect of storage time and treatment was significant ($P = 0.0088$) for sperm motility. The results (Table 4) showed that sperm motility in the roosters who received 3 mg/kg body weight melatonin did not change significantly ($P > 0.05$) after 6 h of storage but in other groups this parameter decreased significantly ($P < 0.05$) during this period.

The effect of storage time and treatment was significant ($P = 0.0077$) for the percentage of viable sperm. The percentage of viable sperm in Groups M

Table 3. Effect of treatment and storage time on malondialdehyde concentration (μg/10^9 sperms) in Fars native roosters (LS means ± SEM)

Time of *in vitro* storage (h)	Treatment		
	control	24 h light	melatonin supplementation[1]
0	8.05 ± 0.57^{Aa}	6.63 ± 0.57^{Aa}	5.51 ± 0.62^{Aa}
6	12.40 ± 0.57^{Ab}	12.01 ± 0.57^{ABb}	9.34 ± 0.62^{Bb}
12	14.24 ± 0.57^{Ab}	12.94 ± 0.57^{Ab}	9.77 ± 0.62^{Bb}

[1]3 mg/kg body weight edible melatonin daily
means within row (shown in capital letter) and column (shown in small letter) with different superscripts significantly differ ($P \leq 0.05$)

Table 4. Effect of treatment and storage time on sperm motility (%) in Fars native roosters (LS means ± SEM)

Time of *in vitro* storage (h)	Treatment		
	control	24 h light	melatonin supplementation[1]
0	76.6 ± 3.4^{Aa}	78.2 ± 3.4^{Aa}	70.1 ± 3.7^{Aa}
6	59.9 ± 3.4^{Ab}	53.2 ± 3.4^{Ab}	64.3 ± 3.7^{Aab}
12	39.9 ± 3.4^{Ac}	41.6 ± 3.4^{Ab}	52.6 ± 3.7^{Ab}

[1]3 mg/kg body weight edible melatonin daily
means within row (shown in capital letter) and column (shown in small letter) with different superscripts significantly differ ($P \leq 0.05$)

doi: 10.17221/8667-CJAS

Table 5. Effect of treatment and storage time on sperm and viability (%) in Fars native roosters (LS means ± SEM)

Time of *in vitro* storage (h)	Treatment		
	control	24 h light	melatonin supplementation[1]
0	93.6 ± 1.3^{Aa}	94.6 ± 1.3^{Aa}	94.5 ± 1.4^{Aa}
6	85.9 ± 1.3^{Ab}	89.8 ± 1.3^{Aa}	89.9 ± 1.3^{Aa}
12	79.9 ± 1.3^{Ac}	75.2 ± 1.3^{Ab}	81.1 ± 1.4^{Ab}

[1]3 mg/kg body weight edible melatonin daily
means within row (shown in capital letter) and column (shown in small letter) with different superscripts significantly differ ($P \leq 0.05$)

Table 6. Effect of treatment and storage time on sperm plasma membrane integrity (%) in Fars native roosters (LS means ± SEM)

Time of *in vitro* storage (h)	Treatment		
	control	24 h light	melatonin supplementation[1]
0	18.0 ± 1.1^{Aa}	20.3 ± 0.02^{Aa}	18.5 ± 1.2^{Aa}
6	11.4 ± 1.1^{Ab}	11.8 ± 0.02^{Ab}	12.5 ± 1.2^{Ab}
12	6.5 ± 1.1^{Ac}	6.7 ± 0.02^{Ac}	8.8 ± 1.2^{Ab}

[1]3 mg/kg body weight edible melatonin daily
means within row (shown in capital letter) and column (shown in small letter) with different superscripts significantly differ ($P \leq 0.05$)

and L remained unchanged and decreased significantly ($P < 0.05$) after 12 h of storage (Table 5).

The percentage of sperm membrane integrity in experimental treatments (Groups L and M) did not significantly differ ($P > 0.05$) from that in the control group (Table 6). The percentage of sperm membrane integrity in M Group did not change significantly during 6 and 12 h of storage but the interaction between storage time and treatment was not significant ($P > 0.05$) for this parameter.

The results of sperm membrane lipid analysis (Table 7) showed that the percentage of saturated fatty acids did not significantly differ ($P > 0.05$) among the groups, but the percentage of unsaturated fatty acids in M Group was significantly ($P < 0.05$) lower than in C Group. The proportion of unsaturated to saturated fatty acids in both experimental treatments was significantly ($P < 0.05$) lesser than in control group. Among the identified fatty acids, although the percentage of docosahexaenoic acid was significantly ($P < 0.05$) decreased in both experimental treatments compared with control group, the percentage of oleic acid, stearic acid, and arachidonic acic did not change significantly ($P > 0.05$).

Table 7. Effect of treatment on fatty acid composition (%) of sperm in Fars native roosters (LS means ± SEM)

Fatty acid	Treatment		
	control	24 h light	melatonin supplementation[1]
9-Hexadecanoic acid	0.40 ± 0.51^{a}	2.67 ± 0.51^{b}	0.39 ± 0.44^{a}
Hexadecanoic acid	10.79 ± 0.57^{a}	13.56 ± 0.57^{b}	13.32 ± 0.49^{b}
Oleic acid	13.55 ± 0.75^{a}	13.88 ± 0.75^{a}	14.22 ± 0.64^{a}
Stearic acid	15.83 ± 0.86^{a}	17.73 ± 0.86^{a}	16.20 ± 0.73^{a}
Arachidonic acid	33.21 ± 2.00^{a}	34.68 ± 2.00^{a}	28.97 ± 1.70^{a}
7,10,13-Eicosatrienoic acid	0.96 ± 0.20^{a}	1.08 ± 0.20^{ab}	1.59 ± 0.17^{b}
11-Eicosanoic acid	3.83 ± 0.27^{a}	4.23 ± 0.27^{ab}	5.07 ± 0.23^{b}
Eicosanoic acid	0.45 ± 0.03^{a}	0.48 ± 0.03^{a}	0.68 ± 0.23^{b}
Docosahexaenoic acid	2.51 ± 0.18^{a}	1.66 ± 0.18^{b}	0.76 ± 0.15^{c}
5,8,11-Eicosatrienoic acid	5.51 ± 0.45^{a}	6.01 ± 0.45^{a}	4.10 ± 0.38^{b}
Saturated fatty acid	26.97 ± 1.47^{a}	31.73 ± 1.47^{a}	30.05 ± 1.25^{a}
Unsaturated fatty acid	64.69 ± 1.77^{a}	64.31 ± 1.77^{a}	55.34 ± 1.51^{b}
Unsaturated to saturated fatty acid ratio	2.40 ± 0.06^{a}	2.02 ± 0.06^{b}	1.85 ± 0.05^{b}
Other lipids	8.34 ± 2.86^{ab}	3.96 ± 2.86^{b}	14.61 ± 2.44^{a}

[1]3 mg/kg body weight edible melatonin daily
means within column with different superscripts significantly differ ($P \leq 0.05$)

doi: 10.17221/8667-CJAS

DISCUSSION

Docosatetraenoic acid (DTA) and arachidonic acid (AA) are the main Omega 6 fatty acids in the phospholipids of the chicken sperm membrane. The Omega 3 family fatty acids, such as docosahexaenoic acid (DHA), are usually present in very low proportions in the sperm membrane phospholipids and account for about 1–5% (Cerolini et al. 1997). The fatty acid composition in the rooster sperm membrane is a species-dependent feature; therefore a few changes can be observed after manipulation of dietary fatty acids (Cerolini et al. 2003). Diets rich in docosahexaenoic acid and docosatetraenoic acid increase the proportion of these fatty acids in membrane lipids of the sperm. Sperm rich in DHA and DTA attain higher fertility compared with the control ones but this result was age-dependent and observed only in young roosters (Cerolini et al. 2006). The results of the present study showed that melatonin administration decreased the percentage of docosahexaenoic acid in sperm membrane phospholipids but it did not affect sperm motility and fertility. The differences in the results of the present and the previous studies are due to differences in the age of roosters (Cerolini et al. 2003), species-specific characteristics of PUFA composition in chicken sperm (Cerolini et al. 2006), and different characteristics of the antioxidant (Melatobin) used in this experiment. In the present study, melatonin administration decreased MDA production due to the reduction of unsaturated fatty acids proportion in sperm membrane phospholipids. Since the high percentage of unsaturated fatty acids leads to increased susceptibility to lipid peroxidation of the sperm membrane and thereby increases production of MDA, this result was not unexpected (Zaniboni and Cerolini 2009). In addition, DHA plays a positive role in chicken sperm motility, but it is also a major target for lipid peroxidation (Ollero and Alvarez 2003). So the reduction of DHA in sperm phospholipids composition is another reason for decreasing MDA production in this study. Addition of antioxidants such as vitamin E to the roosters' diet resulted in increasing of vitamin E concentration in sperm membrane lipids and reducing the susceptibility of phospholipids to peroxidation because of its antioxidant specification (Surai et al. 1997). On the other hand, the protective effects of antioxidants against peroxidation can also be induced by rearranging the fatty acids composition in sperm membrane phospholipids (Maladgian et al. 1998). It is suggested that the effect of melatonin on the reduction of unsaturated fatty acids is similar to that of vitamin E in rearranging the structure of sperm membrane fatty acids and may result in a greater stability of fatty acids to lipid peroxidation (Surai 1999). In addition, melatonin can also induce its antioxidant effects through the strengthening of antioxidant enzymes activities by increasing the gene expression of SOD and GSH-Px in the sperm membrane (Ramadan et al. 2009). So increasing the activity of antioxidant enzymes in rooster spermatozoa might be the second suggestion for decreasing lipid peroxidation during the storage. Melatonin has also a direct antioxidant effect on scavenging the ROS (Tan et al. 2002) and this may be another reason for decreasing MDA production in the present study.

Acknowledgement. The authors wish to thank the employees of the farm of the Fars research centre for native poultry in Kharameh for bird care and technical support.

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Received: 2014–06–06
Accepted after corrections: 2015–03–13

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