Modulatory effects of pioglitazone as a ligand for the peroxisome proliferator-activated receptor on semen quality and fertility potential of broiler breeder roosters

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ABSTRACT Fertility potential in roosters is a crucial topic in broiler breeder reproduction which is thought to be associated with age. This study aims to investigate effects of 2 levels of pioglitazone (**PIO**) supplementation on peroxisome proliferator-activated receptor gamma (**PPAR-γ**) expression, semen quality, and fertility parameters of aged broiler breeder roosters. The efficacy of PIO was divided into 2 sections: receptor-dependent and receptor-independent. Expression of PPAR-γ mRNA and protein was assessed in sperm to monitor receptor-dependent actions. Sperm motility, velocity parameters, viability, mitochondrial activity, and apoptosis were assessed for the receptor-independent actions. Broiler breeder roosters were randomly assigned to 3 groups: 1) control received a basal diet (**CTRL**); 2) PIO-5 received a basal diet supplemented with 5 mg PIO/bird/day, and 3) PIO-10 received a basal diet supplemented with 10 mg PIO/bird/day. In addition, semen samples were collected from 24 Ross broiler breeder roosters at 30, 43, and 53 wk of age. Effects of PIO were significant in terms of total motility, straight-line velocity, mitochondrial activity, and apoptosis ($P \leq 0.05$). Total motility, straight-line velocity and mitochondrial activity improved in both PIO groups ($P \leq 0.05$) along with a significant reduction in early and late apoptosis in the PIO groups ($P \leq 0.05$). Pioglitazone addition affected total motility, mitochondrial activity, early apoptosis and late apoptosis in a linearly and quadratically manner ($P < 0.05$). PPAR-γ mRNA and protein expression were not significantly upregulated by the different doses of PIO ($P > 0.05$). Similarly, fertility performance was not significantly changed in the PIO groups ($P > 0.05$). Moreover, PIO improved mitochondrial activity and decreased the apoptosis rate in the sperm of aged broiler breeder roosters. These improvements were associated with the receptor-independent actions of PIO and the mechanism of action of PIO did not appear to be affected by the PPAR-γ receptor in broiler breeder roosters.

Key words: PPAR-γ, synthetic ligand, sperm

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INTRODUCTION

Roosters play an important role in fertility due to the lower ratio in broiler breeder flocks; therefore, it is essential to focus on improvements in semen quality and sperm function to maximize reproductive performance (Abbaspour, Sharifi, Ghazanfari, Mohammadi-Sangcheshmeh, & Honarbakhsh, 2020). In broiler breeder flocks, a reduction in semen quality and fertility potential with increasing age has been reported (Adeldust, Farzinpour, Farshad, Rostamzadeh, & Lopez-Bejar, 2017; Lagares et al., 2017; Shaheen, Mehmood, Mahmud, Riaz, & Ahmad, 2021). One of the physiological pathways responsible for reduced fertility in aged roosters is the hypothalamus-hypophysis-gonadal (**HPG**) axis and the different factors affecting this central endocrine axis. Peroxisome proliferator-activated receptor gamma (**PPAR-γ**) is a member of the PPAR family (Ciavarella, Motta, Valente, & Pasquinelli, 2020) that can modulate the HPG axis (Liu et al., 2015). Expression of PPAR-γ in testicular cells plays a crucial function in spermatogenesis (Hasan, Ibrahim, & El Gendy, 2020). It also has a regulatory role in various conditions, including cell

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proliferation, control of oxidative stress, apoptosis, inflammation, and lipid metabolism (Jalilvand, Hosseini, Beheshti, & Ebrahimzadeh-Bideskan, 2019).

The PPAR-γ activity can be measured by binding to ligands that are divided into 2 types − natural and synthetic. The natural ligands include polyunsaturated fatty acids (**PUFAs**) and eicosanoids, whereas the synthetic ligands include thiazolidinediones (**TZDs**) and nonsteroidal anti-inflammatory medications (**NSAIDs**) (Aquila et al., 2006; Ciavarella et al., 2020; Zhang, Guo, Yang, & Yuan, 2006). Synthetic ligands of PPARs can modify PPAR-mediated transcriptional activation of several key genes involved in energy homeostasis (Liu et al., 2015). Moreover, TZDs exert receptor-dependent and receptor-independent actions (Feinstein et al., 2005). Independent actions include anti-inflammatory roles and cell proliferation, induction of cytotoxicity, energy modification, and mitochondrial function disorder (Feinstein et al., 2005).

Pioglitazone (**PIO**), a member of the TZD family (Sakamoto et al., 2000), is a synthetic ligand of PPAR-γ (Waugh, Keating, Plosker, Easthope, & Robinson, 2006) and may play a critical role in controlling cell metabolism, strengthening the antioxidant defense system (Meneses et al., 2016), and reducing ROS production, which would lead to a decrease in malondialdehyde levels and an increase in glutathione activity in testicular tissue (Jalilvand et al., 2019). An antioxidant role for PIO has been also suggested in the testes of alloxan-induced diabetic rabbits (Gumieniczek, Hopkała, & Ząbek, 2008). The results of a study showed that the 10 mg/kg body weight dose of PIO improved sperm motility and reduced the percentages of deformed and nonviable sperm in treated diabetic rats (Akinola, Dosumu, Sanusi, Ajayi, & Olajide, 2015). Additionally, PIO reduced the numbers of abnormal sperms and increased the sperm concentration in hypothyroid rats (Jalilvand et al., 2019). However, to our knowledge, supplementation of the aged broiler breeder roosters' diet with PIO and its effect on fertility performance through PPAR-γ has not been addressed. Therefore, we designed this study to investigate the synthetic ligand (PIO) receptor-dependent and receptor-independent actions in the aged roosters.

MATERIALS AND METHODS

All chemicals used in this study were obtained from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany). The PIO dietary supplement (Glitoz) was purchased from Zahravi Pharmaceutical Co., Tehran, Iran (http://www.zahravi.com/en/). The Animal Welfare Committee of the Department of Poultry Science, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran approved all of the experimental procedures. The procedures were also conducted in compliance with the standards established by the Royan Institute Ethical Committee, Tehran, Iran (IR.ACECR.ROYAN.REC.1397.187).

Roosters' Management and Study Design

Twenty-four Ross broiler breeder roosters (30 wk old at the beginning of the experiment) were maintained for 24 wk in individual cages in a controlled environment under similar light schedule and temperature conditions. All of the roosters were reared according to the Ross 308 broiler breeder management guide (https://en.aviagen.com/).

Semen sampling and analysis were performed before starting the experiment to ensure that each of the roosters had an active reproductive system. We considered this to be d 0. A search of the literature showed the following previously administered doses for TZD: 1 to 40 mg PIO/bird/day in female broiler breeders (dietary supplement) (Amaleh, Shahneh, & Zaghari, 2019); 50 mg troglitazone/kg/day in male broiler chickens (oral administration) (Sato, Fukao, Seki, & Akiba, 2004); 10 or 30 mg PIO/kg/day in diabetic rats (oral gavage) (Akinola et al., 2015); and 0.6 mg PIO/kg/day in rats with type 2 diabetes mellitus (oral gavage) (Hasan et al., 2020). This enabled us to choose the 5 and 10 mg PIO/bird/day doses as the most appropriate doses that had the least side effects for supplementation in this study.

The basal diet in all experimental groups was a standard commercial mash for Ross broiler breeder roosters (Table 1). The experiment was conducted in three treatments using 8 roosters per treatment. Roosters were allocated into 3 groups: 1) control roosters received a basal diet (**CTRL**); 2) PIO-5 group received the basal diet supplemented with 5 mg PIO/bird/day, and 3) PIO-10 group received the basal diet supplemented with 10 mg

Table 1. Ingredients and composition of the basal diet (g/kg).

Ingredient	Basal diet
Corn	619.00
Soybean meal	80.00
Wheat bran	234.50
Sodium bicarbonate	1.00
Common salt	3.00
Dicalcium phosphate	12.00
DL-methionine	00.50
Bentonite	35.00
CaCO3	10.00
Mineral premix[1]	2.500
Vitamin premix[2]	2.500
Oil	00.00
Calculated composition	
Metabolizable Energy (kcal/kg)	2,708
Crude protein (g/kg)	115.30
Ether extract (g/kg)	35.10
Calcium (g/kg)	7.00
Non phytate phosphorous (g/kg)	3.50

[1]Supplied per kilogram of diet: Fe (FeSO4•H2O), 90 mg; Mn (MnSO4•H2O), 90 mg; Zn (ZnO), 67.3 mg; Cu (CuSO4•5H2O), 10.9 mg; and Se (Na2SeO3), 0.18 mg.

[2]Supplied per kilogram of diet: vitamin A, 15,000 IU; vitamin E, 30 mg; vitamin K3, 4 mg; vitamin D3, 3,000 IU; riboflavin, 7.5 mg; pyridoxine, 5.5 mg; vitamin B12, 25 μg; biotin, 50 μg; niacin, 50 mg; calcium pantothenate, 18 mg; and folic acid, 1.5 mg.

PIO/bird/day. Roosters in the PIO groups received 2 doses of PIO as the daily dietary supplement during experimental period.

Semen Collection

Semen samples were collected at 3 times: baseline (30-wk-old roosters; n = 24 samples), middle (43-wk-old roosters; n = 24 samples), and end (53-wk-old roosters; n = 24 samples) of the experiment. Therefore, 72 semen samples were processed individually. Pioglitazone efficacy was assessed into 2 sections: receptor-dependent (PPAR-γ mRNA and protein expression) and receptor-independent (sperm quality indicators).

Evaluation of PPAR-γ mRNA and Protein Expressions

RNA Extraction and cDNA Synthesis of Sperm Total RNA was isolated from sperm with TRI reagent (Sigma-Aldrich). The amount of RNA was estimated by a spectrophotometer (Thermo Scientific NanoDrop One Microvolume UV-Vis Spectrophotometer). The total RNA was reversed-transcribed into cDNA using a Takara Perfect Real-time cDNA Synthesis kit (Doraville, GA) on a thermocycler apparatus (Master Cycler Gradient Eppendorf, Hamburg, Germany). The cDNA was synthesized at 42°C for 25 min, 85°C for 7 s, and a holding step at 4°C.

Real-Time PCR Analysis The amount of the PPAR-γ transcript was obtained by real-time PCR (RT-PCR; Step One Plus, Applied Biosystems, CA) with *GAPDH* as the housekeeping gene for normalization of the target gene expression. PerlPrimer software (version 1.1.21) and NCBI primer blast were used to design the primers for the PPAR-γ target and housekeeping genes (Table 2). The reaction mixture contained distilled H_2O, either the forward or reverse primers, SYBR Premix Ex Taq II (Takara), and single-strand cDNA. The PCR program consisted of a 5-min activation step at 95°C, followed by 45 cycles of 15 s at 95°C, and 60 s at 60°C. At the end of each PCR, a melting curve analysis was performed at the rate of 0.5°C/s for all genes to check for the specificities of the products. Data analysis was performed by the $2^{-\Delta Ct}$ method.

Western Blot Analysis Total sperm protein was extracted using a lysis buffer (pH 6.8) that consisted of 8 M urea, 1% SDS, 2% CHAPS, and 50 ml Tris-HCl. The protein concentration was determined using a Bradford Assay kit (Thermo Scientific, Rockford, IL) (Bradford, 1976). A total of 10 μg protein from each sample was separated on 10% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (PVDF Western Blotting Membranes, Roche). Then, the membranes were blocked with 1% bovine serum albumin (**BSA**) (Sigma) and nonfat milk at room temperature for 1 h. The membranes were washed three times with TBST (Tris-buffered saline [**TBS**; 100 mM Tris-HCl and 150 mM NaCl] supplemented with 0.05% Tween 20), and incubated overnight at 4°C with the primary antibody to PPAR-γ (1:200 in TBST; NB120-19481, Novusbio LLC, Littleton, CO). After three washes with TBST, bands were detected after a 1.5 h incubation period at room temperature with the secondary antibody, anti-rabbit IgG (1:30,000 in TBST; Sigma). The bands were quantified using Gel Doc (UVITEC Cambridge, Alliance Q9 Advanced). The loading control was α-tubulin (Sigma) for data normalization. The results were quantified with ImageJ software, version 1.50i (National Institutes of Health, Bethesda, MD).

Evaluation of Sperm Parameters

Motility Parameters and Viability Total sperm motility (**TM**), progressive motility (**PM**), average path velocity (**VAP**), straight-line velocity (**VSL**), and curvilinear velocity (**VCL**) were assessed using a sperm class analyzer (**SCA**, Microptic, Spain). Eosin-nigrosin staining was used to assess sperm viability by counting 200 sperm for the presence of unstained sperm heads (live) and stained/partially stained sperm heads (dead) under a phase-contrast microscope at 400 × magnification (Fattah, Sharafi, Masoudi, Shahverdi, & Esmaeili, 2017).

Apoptosis Apoptosis was measured using Annexin V (IQP, Groningen, Netherlands) according to the manufacturer's instructions. The semen was washed with calcium buffer and the concentration of sperm was adjusted to 1×10^6 sperm/mL, after which 10 μL of Annexin V-FITC (0.01 mg/mL) was added to the sperm suspension. The suspension was incubated at room temperature for 15 min. Next, 10 μL of propidium iodide (**PI**) was added to the sperm suspension and the resultant suspension was analyzed by a flow cytometer and the percentage of apoptotic sperm was calculated (Ansari et al., 2017).

Mitochondrial Activity The mitochondrial activity was determined by JC-1 (T4069, Sigma-Aldrich). First, the semen was washed and then the pellet was

Table 2. Sequences of primers used for the quantification of the target and housekeeping genes.

Gene	Sequence	NCBI ID No.	PCR Product (bp)
PPARγ	F: ATACATAAAGTCCTTCCCGCTG R: TTGCTACTTCTTTGTTCTGTTCC	NM_001001460.1	173
GAPDH	F: AGAAACCAGCCAAGTATGATGA R: CTGTCACCATTGAAGTCACAG	NM_204305.1	121

resuspended in phosphate-buffered saline (**PBS**) and the concentration was adjusted to 2 × 10^6 sperm/mL, followed by the addition of 5 μL of JC-1. The mixture was incubated at room temperature in dark for 15 min. Samples were analyzed by a flow cytometer and the percentage of sperm with active mitochondria was recorded (Feyzi, Sharafi, & Rahimi, 2018).

Assessment of Reproductive Performance

Artificial insemination (**AI**) was performed according to the method described by Akhlaghi et al. (2014) with a slight modification (Akhlaghi et al., 2014a) at the end of the experiment (53 wk). Semen from 8 roosters in each treatment group was pooled. Lake extender (potassium citrate [0.25 g/l], D-fructose [0.4 g/l], magnesium acetate [0.035 g/l], polyvinylpyrrolidone [0.15 g/l], glycine [0.187 g/l], and sodium glutamate (0.96 g/l]) was used for dilution. AI was performed at 3:00 pm on specific days by insemination of the pooled semen obtained from each treatment into 20 individual hens per treatment (60 hens total). Hens were inseminated (300 × 10^6 sperm/hen) for approximately 2 wk (2 times per week). Eggs were collected up to 5 d after the last AI, and numbered and stored at 13°C and 75% humidity until they were incubated to assess the fertility and hatchability rates. In each group, 200 settable eggs were placed on turning trays and disinfected with formaldehyde for 15 min according to the recommended concentration of 1.2 mL of formalin added to 0.6 g of potassium permanganate. Next, eggs were placed in a common incubator (Victoria, G. Galilei, 3−22,070, Guanzate, Como, Italy) for 18 d at 37.7°C. After d 18 of the incubation period, eggs were transferred to the hatcher for the remaining 3 d of incubation. On d 7 of the incubation, fertility rate was measured by candling the eggs. Finally, the hatching rate was calculated, after 21 d of incubation according to the numbers of fertilized eggs.

Statistical Analysis

The study was conducted with 3 treatments and eight replications in each treatment. We used Levene's test for equality of variances and the Kolmogorov-Smirnov test to assess data normality. When necessary, the arcsin transformation of data was used for percentage data. The time of the semen sampling was included as a random effect, and repeated-measurements data were analyzed by PROC GLM (SAS, 2002). Means were compared by means and the Tukey's test ($P \leq 0.05$). Fertility and hatchability were analyzed using the chi-square test. The hatching rate was calculated on the 21st day of incubation based on the number of fertilized eggs. Polynomial orthogonal contrasts were also carried out to investigate the linear and quadratic trends.

RESULTS

PPAR-γ Expression

Figure 1 shows the effects of PIO on PPAR-γ mRNA and protein expressions in sperm. PPAR-γ mRNA expression was significantly higher in the CTRL group compared to the PIO groups ($P \leq 0.05$, Figure 1A). PPAR-γ mRNA expressions in both PIO groups was not significantly upregulated by the different doses of PIO ($P >$

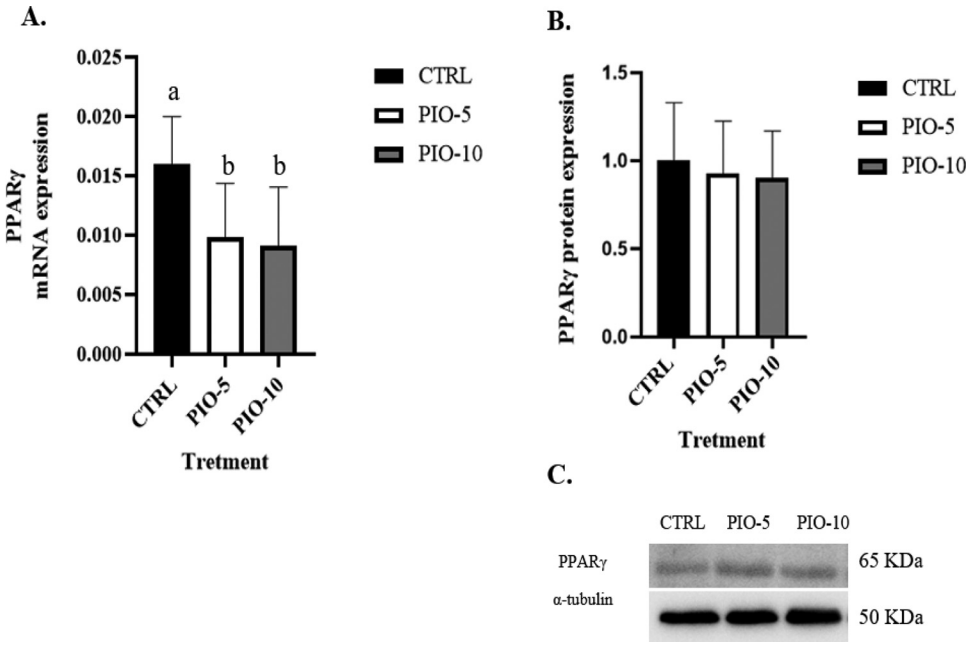

Figure 1. Peroxisome proliferator-activated receptor gamma (PPAR-γ) mRNA expression was determined by real-time reverse-transcription PCR (RT-PCR) in three types of diet (A). The RT-PCR data were analyzed by the $2^{-\Delta Ct}$ method. [a,b] Values with different superscripts are significantly different ($P \leq 0.05$). PPAR-γ protein expression in rooster sperm based on Western blot analysis. PPAR-γ protein content in the three groups (B, C). α-tubulin (Sigma) was used as a loading control for data normalization. The results were quantified by ImageJ software (v 1.50i, National Institutes of Health, Bethesda, MD). The band that corresponds to the PPAR-γ protein mass values of 65 kDa and α-tubulin mass values of 50 kDa.

Table 3. The effects of pioglitazone (PIO) on rooster sperm parameters.

Traits	Treatments			*P*-value				Effect (P)	
	CTRL	PIO-5	PIO-10	SEM	PIO	Time	PIO × time	Linear	Quadratic
Total motility (%)	81.77[b]	87.08[a]	84.86[a,b]	1.65	0.0021	0.17	0.92	0.04	0.001
Progressive motility (%)	13.75	15.35	15.80	1.29	0.14	0.08	0.95	0.11	0.61
VCL (mm/s)	37.28	42.23	38.34	2.33	0.30	0.06	0.30	0.10	0.58
VSL (mm/s)	12.96[b]	16.62[a]	14.49[a,b]	0.80	0.01	0.96	0.10	0.01	0.80
VAP (mm/s)	22.06	25.54	23.27	1.40	0.23	0.33	0.29	0.10	0.76
Viability (%)	80.76	82.25	81.87	1.14	0.37	0.80	0.25	<0.0001	0.20
Mitochondria activity (%)	75.65[b]	85.06[a]	80.70[a,b]	2.63	0.0007	0.07	0.96	0.02	0.002
Early apoptosis (%)	3.05[a]	2.17[b]	2.41[b]	0.21	0.0001	0.01	0.03	0.002	0.002
Late apoptosis (%)	3.82[a]	3.20[b]	3.36[b]	0.20	0.002	0.21	0.01	0.0009	0.0009

The roosters were divided into the following groups: CTRL, PIO-5 (5 mg PIO/bird/day) and PIO-10 (10 mg PIO/bird/day).
[ab]Different letters within the same row show significant differences among the groups ($P \leq 0.05$).

0.05, Figure 1A). Protein expression remained unchanged in all of the groups ($P > 0.05$, Figures 1B and 1C).

Sperm Parameters

Table 3 presents the effects of PIO on PM, TM, VCL, VSL, VAP, viability, mitochondrial activity, and apoptosis at 3 times: baseline (30 wk of age), middle (43 wk of age), and end (53 wk of age) of the experiment. We observed improvements in TM and VSL with 2 doses of PIO compared to control ($P \leq 0.05$, Table 3). The effects of different doses of PIO on PM, VCL, VAP, and viability were not significant ($P > 0.05$, Table 3). The PIO groups had significantly lower early and late apoptosis rates, whereas mitochondrial activity was significantly higher in these groups ($P \leq 0.05$, Table 3). Comparison between PIO groups shows that the low level of PIO had significantly lower apoptosis rates as well as higher mitochondrial activity ($P \leq 0.05$, Table 3). Effects of PIO on TM, mitochondrial activity, early apoptosis and late apoptosis were significant in a linearly and quadratically manner ($P < 0.05$). Also, a linearly effect was showed on viability and VSL ($P < 0.05$). There were no linear and quadratic effects on PM, VCL, and VAP of birds fed treated diets ($P > 0.05$).

Effects of semen sampling time on sperm parameters was not significant in terms of TM, PM, VCL, VSL, VAP, mitochondrial activity, late apoptosis, and viability ($P > 0.05$, Table 3), with the exception of early apoptosis ($P \leq 0.05$, Table 3 and Figure 2B). The interaction effects between PIO doses and semen sampling time were not significant in terms of TM, PM, VCL, VSL, VAP, mitochondrial activity, and viability ($P > 0.05$, Table 3), except during early and late apoptosis ($P \leq 0.05$, Table 3 and Figures 2A and 2C).

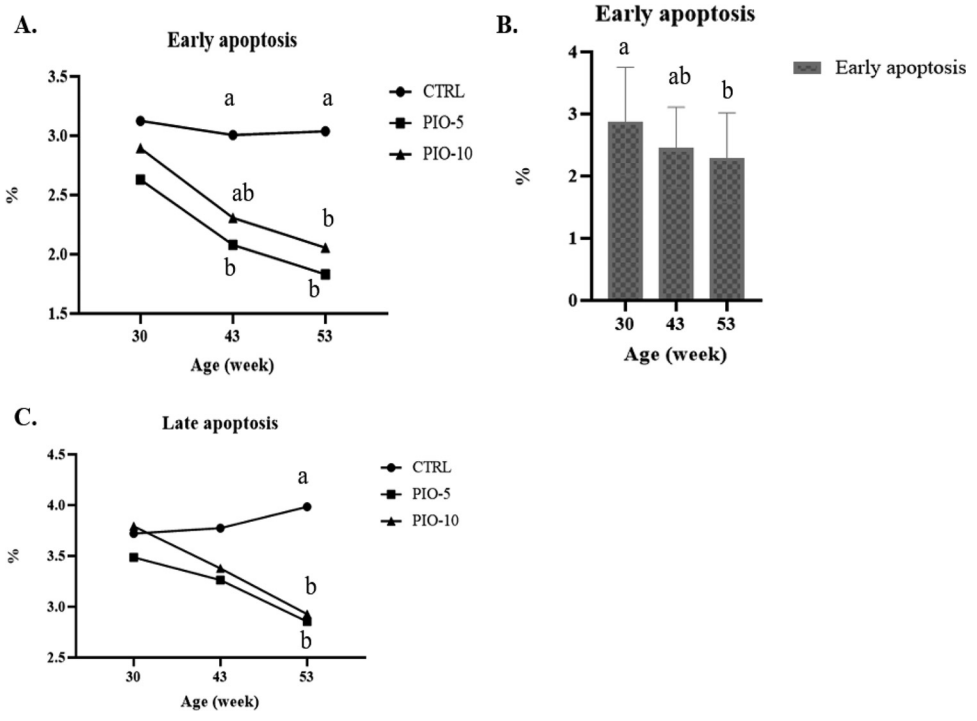

Figure 2. (A) Means of the early apoptosis rate of broiler breeder sperm in roosters in the three treatment groups. Different letters within each day show significant differences among the groups ($P \leq 0.05$). (B) The effect of semen sampling time on early apoptosis rate in broiler breeder sperm. Different letters within each day show significant differences during the weeks ($P \leq 0.05$). (C) Means of late apoptosis rate of broiler breeder sperm in roosters assigned to three treatment groups. Different letters within each day show significant differences among the groups ($P \leq 0.05$).

Table 4. The effects of pioglitazone (PIO) on fertility and hatchability rate.

Traits	Treatments			*P*-value
	CTRL	PIO-5	PIO-10	
Fertility (%)	70	72	71	0.64
Hatchability (%)	82.27	80.43	80.80	0.99

The roosters were divided into the following groups: CTRL, PIO-5 (5 mg PIO/bird/day) and PIO-10 (10 mg PIO/bird/day).

Fertility and hatchability were analyzed according to the chi-square test.

The hatching rate was calculated after 21 d of incubation according to the numbers of fertilized eggs.

Reproductive Performance

Fertility potential in roosters received 2 doses of PIO are presented in Table 4. Both fertility ($P = 0.64$ Table 4) and the hatchability ($P = 0.99$ Table 4) percentages that were based on the numbers of fertilized eggs were not significantly affected by PIO administration (Table 4).

DISCUSSION

Thiazolidinediones are synthetic ligands for the gamma isoform of PPAR, a family of nuclear receptors that control gene transcription. Despite the clinical use of the TZD, exact molecular mechanisms by which TZDs exert their effects remain unknown (Djaouti et al., 2010). Pioglitazone is a member of the TZDs family that has receptor-dependent and independent actions (Feinstein et al., 2005). In this study, PPAR-γ mRNA and protein expression were assessed for the receptor-dependent actions of PIO, whereas semen quality metrics were assessed for the receptor-independent actions. Finally, a fertility trial was performed to validate the sperm parameter results.

Thiazolidinediones receptor-dependent actions can exert cellular effects via activation of PPAR-γ, which lead to changes in gene expression patterns due to binding to PPR elements in the promoter regions of target genes (Feinstein et al., 2005). In contrast, emerging evidence demonstrates that TZDs exert many of their beneficial effects independently of PPAR activation (Feinstein et al., 2005). Our findings have shown that the receptor-dependent action of PIO was not significant and different doses of PIO did not upregulate PPAR-γ mRNA expression. Several researchers have assessed the effects of PIO as a PPAR-γ agonist activator. The results showed cooperation between PPAR-γ expression and its ligands in the diabetic (Akinola et al., 2015; Hasan et al., 2020) and hypothyroid groups (Jalilvand et al., 2019), but not in the normal group. Hasan et al. (2020) reported a significant reduction in PPAR-γ in testicular gene expressions in diabetic rats. They observed a significant elevation in PPAR-γ mRNA in the presence of synthetic and natural PPAR-γ ligands in the diabetic groups and following our result, the CTRL group had the highest PPAR-γ mRNA expression compared to the other groups (Hasan et al., 2020). A lipid metabolism study in mice liver and a comparison of the effects of rosiglitazone (**ROSI**) and PIO showed that PIO cannot modify PPAR-γ expression. It appeared that the effects of PIO on lipid metabolism were independent of the PPAR-γ actions (Djaouti et al., 2010). Upon comparison, PIO and ROSI appear to differ in their actions (Djaouti et al., 2010), and PIO is a less potent PPAR-γ agonist (Jacques et al., 2021).

Our findings showed that PPAR-γ protein expression was the same in all groups. These results agreed with those reported by Mousavi et al. (2019) who observed no significant changes in PPAR-γ protein expressions in human sperm. Their results supported the theory that ejaculated sperm were unable to make new proteins (Miller, Ostermeier, & Krawetz, 2005; Mousavi et al., 2019). Although the function of ejaculated sperm in fertilization is of tremendous interest, their molecular composition and the mechanisms by which their function is regulated are not well-defined (Santoro, De Amicis, Aquila, & Bonofiglio, 2020). Therefore, the results of receptor expression in sperm may not be compared with the studies performed on tissues and further studies are necessary.

The results of studies suggest that the effects of TZDs on mitochondrial function could contribute to the observed PPAR-γ receptor-independent actions (Feinstein et al., 2005). The receptor-independent actions of PIO influence growth, apoptosis, and mitochondrial biogenesis (Feinstein et al., 2005). Although both PIO groups had higher mitochondrial activity compared to the CTRL group, the low dose PIO had the highest mitochondrial activity. There are two possible explanations for this observation. First, it is necessary to choose an effective dose. TZD targets an intracellular organelle, and its accumulation could lead to elevated cytosolic levels. This would appear to be both time- and concentration-dependent and could explain why the in vivo effects take longer (Feinstein et al., 2005). Second, the importance of mitochondrial dysfunction cannot be ignored because it plays an important role in cell death. The mitochondria are key regulators of cellular processes and they act as cellular oxygen sensors. Mitochondria produce adenosine triphosphate (**ATP**) and regulate cell proliferation and death (Hu et al., 2019). Moreover, PIO manages glucose levels, by inhibiting apoptosis of pancreatic β-cells and improving insulin secretion (Ciavarella et al., 2020). In our study, there were significant reductions in both early and late apoptosis in the PIO groups compared to the CTRL group. The mitochondrial dysfunction pathway is initiated by apoptotic signals and causes an increase in intracellular ROS levels, which creates oxidative stress (Amaleh et al., 2019; Sato et al., 2004). Excess ROS generation leads to oxidative damage and cell death (Bilodeau, Chatterjee, Sirard, & Gagnon, 2000). Changes in mitochondrial function elicit numerous responses, some of which have been reported in response to TZDs. In other words, TZDs may exert direct, rapid effects on mitochondrial respiration that lead to changes in glycolytic metabolism

and fuel substrate specificity (Feinstein et al., 2005). In line with our results, the beneficial effects of TZDs improved mitochondrial activity and reduced apoptosis independently of PPAR activation.

Notably, we observed improved sperm motility in both of the PIO groups that had increased mitochondrial activity and decreased apoptosis. The TM had a high presence in the low dose PIO group. Mitochondrial dysfunction decreases ATP, and impacts sperm motility and viability (Feinstein et al., 2005). Mitochondrial damage results in decreased sperm movement (Bazyar, Sharafi, & Shahverdi, 2019). We observed improved mitochondrial activity of sperm in the roosters that received the daily 5 mg PIO dose. The data related to mitochondrial activity showed a logical relationship with motility characteristics, which confirmed the results reported by Appiah et al. (2020) where mitochondrial active potential was mainly responsible for sperm motility (Appiah et al., 2020).

Reproductive performance may be an important assessment to approve sperm parameters. Nevertheless, many factors affect fertility and hatch rate in both males and females, including sperm quality parameters (Zhandi, Ansari, Roknabadi, Shahneh, & Sharafi, 2017), sperm lipid components (Cerolini et al., 1997), total antioxidant capacity level in seminal plasma (Lewis, Boyle, McKinney, Young, & Thompson, 1995), weight of the roosters (Sarabia Fragoso et al., 2013), eggshell quality (Feyzi et al., 2018) and, in general, the critical role of female in terms of egg production and in providing a suitable environment for sperm transport and capacitation (Akhlaghi, Ahangari, Zhandi, & Peebles, 2014b). Researchers have examined effects of dietary supplements on improvement factors that influence on fertility. Natural dietary supplements like flaxseed oil (Zanussi, Shariatmadari, Sharafi, & Ahmadi, 2019), ginger powder (Akhlaghi et al., 2014a), and apple pomace (Akhlaghi et al., 2014b) show a correlation with rooster sperm parameters and reproductive performance. In contrast, synthetic dietary supplements like alpha-lipoic acid showed no significant correlation in broiler breeder rooster fertility and sperm parameters (Behnamifar, Rahimi, Karimi Torshizi, Sharafi, & Grimes, 2021). The results of different studies indicated that the hatch rate was not influenced by various dietary supplements (Behnamifar et al., 2021; Feyzi et al., 2018; Zanussi et al., 2019); rather, it was probably associated with several diverse, individual factors (Sarabia Fragoso et al., 2013). In our study, both fertility and hatching rate based on fertilized eggs did not significantly differ in the PIO groups. Emphasis on factors such as body weight, testicular development, spermatogenesis, and hormone levels can help researchers understand the source for this decline.

Further research is needed to clarify the mechanisms that underlie enhancement of the reproductive performance in creating efficient conditions for the use of dietary sources and the improvement of the fertility and hatch rate in aged broiler breeder roosters (Akhlaghi et al., 2014b).

CONCLUSIONS

We observed that the actions of PIO independent of receptor expression were responsible for increasing total motility, mitochondrial activity and decreasing the apoptosis rate in aged broiler breeder roosters. The relationship between PIO and PPAR-γ expression was not observed in aged roosters.

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DISCLOSURES

The authors have no conflicts of interest to report.

REFERENCES

Abbaspour, B., S. D. Sharifi, S. Ghazanfari, A. Mohammadi-Sangcheshmeh, and S. Honarbakhsh. 2020. Effect of dietary supplementation of whole flaxseed on sperm traits and sperm fatty acid profile in aged broiler breeder roosters. Reprod. Domest. Anim. 55:594–603.

Adeldust, H., A. Farzinpour, A. Farshad, J. Rostamzadeh, and M. Lopez-Bejar. 2017. Increased sperm cell production in ageing roosters by an oral treatment with an aromatase inhibitor and a natural herbal extract designed for improving fertility. Reprod. Domest. Anim. 52:58–60.

Akhlaghi, A., Y. J. Ahangari, B. Navidshad, Z. A. Pirsaraei, M. Zhandi, H. Deldar, M. R. Rezvani, M. Dadpasand, S. R. Hashemi, R. Poureslami, and E. D. Peebles. 2014a. Improvements in semen quality, sperm fatty acids, and reproductive performance in aged Cobb 500 breeder roosters fed diets containing dried ginger rhizomes (Zingiber officinale). Poult. Sci. 93:1236–1243.

Akhlaghi, A., Y. J. Ahangari, M. Zhandi, and E. D. Peebles. 2014b. Reproductive performance, semen quality, and fatty acid profile of spermatozoa in senescent broiler breeder roosters as enhanced by the long-term feeding of dried apple pomace. Poult. Sci. 147:64–73.

Akinola, O. B., O. O. Dosumu, S. A. Sanusi, T. F. Ajayi, and T. H. Olajide. 2015. PPAR-γ agonist pioglitazone improves semen quality and testicular histomorphometrics with partial reversal of hyperglycaemia in alloxan-induced diabetic rats. Middle East Fertil. Soc. J. 20:271–279.

Amaleh, M. H., A. Z. Shahneh, and M. Zaghari. 2019. The effect of pioglitazone on performance and plasma lipids of post-peak female broiler breeders. J. Anim. Sci. Res. 29:141–151.

Ansari, M., M. Zhandi, H. Kohram, M. Zaghari, M. Sadeghi, and M. Sharafi. 2017. Improvement of post-thawed sperm quality and fertility of Arian rooster by oral administration of d-aspartic acid. Theriogenology 1:69–74.

Appiah, M. O., W. Li, J. Zhao, H. Liu, Y. Dong, J. Xiang, J. Wang, and W. Lu. 2020. Quercetin supplemented casein-based extender improves the post-thaw quality of rooster semen. Cryobiology 94:57–65.

Aquila, S., D. Bonofiglio, M. Gentile, E. Middea, S. Gabriele, M. Belmonte, S. Catalano, M. Pellegrino, and S. Ando. 2006. Peroxisome proliferator-activated receptor (PPAR) γ is expressed by human spermatozoa: its potential role on the sperm physiology. J. Cell. Physiol. 209:977–986.

Bazyar, M., M. Sharafi, and A. Shahverdi. 2019. Changes in seminal parameters and hormonal profile with use of aromatase inhibitor in management of aging broiler breeder roosters. Poult. Sci. 98:6100–6107.

Behnamifar, A., S. Rahimi, M. A. Karimi Torshizi, M. Sharafi, and J. L. Grimes. 2021. Effects of dietary alpha-lipoic acid supplementation on the seminal parameters and fertility potential in aging broiler breeder roosters. Poult. Sci. 100:1221–1238.

Bilodeau, J. F., S. Chatterjee, M. A. Sirard, and C. Gagnon. 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol. Reprod. Dev. Inc. Gamete Res. 55:282–288.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

Cerolini, S., R. C. Noble, B. K. Speake, F. Pizzi, L. G. Cavalchini, and U. Kingdom. 1997. Relationship between spermatozoan lipid composition and fertility during aging of chickens. Biol. Reprod. 57:976–980.

Ciavarella, C., I. Motta, S. Valente, and G. Pasquinelli. 2020. Pharmacological (or synthetic) and nutritional agonists of PPAR-γ as candidates for cytokine storm modulation in COVID-19 disease. Molecules 25:2076.

Djaouti, L., T. Jourdan, L. Demizieux, M. Chevrot, J. Gresti, B. Vergès, and P. Degrace. 2010. Different effects of pioglitazone and rosiglitazone on lipid metabolism in mouse cultured liver explants. Diabetes. Metab. Res. Rev. 26:297–305.

Fattah, A., M. Sharafi, R. Masoudi, A. Shahverdi, and V. Esmaeili. 2017. L-carnitine is a survival factor for chilled storage of rooster semen for a long time. Cryobiology 74:13–18.

Feinstein, D. L., A. Spagnolo, C. Akar, G. Weinberg, P. Murphy, V. Gavrilyuk, and C. Dello Russo. 2005. Receptor-independent actions of PPAR thiazolidinedione agonists: is mitochondrial function the key? Biochem. Pharmacol. 70:177–188.

Feyzi, S., M. Sharafi, and S. Rahimi. 2018. Stress preconditioning of rooster semen before cryopreservation improves fertility potential of thawed sperm. Poult. Sci. 97:2582–2590.

Gumieniczek, A., H. Hopkała, and A. Ząbek. 2008. Protective effects of a PPARγ agonist pioglitazone on anti-oxidative system in testis of diabetic rabbits. Die Pharm. Int. J. Pharm. Sci. 63:377–378.

Hasan, M. M., E. M. Ibrahim, and J. El Gendy. 2020. Ameliorative effect of combined low dose of Pioglitazone and omega-3 on spermatogenesis and steroidogenesis in diabetic rats. J. Cell. Biochem. 121:1524–1540.

Hu, Y., L. Huang, M. Shen, Y. Liu, G. Liu, Y. Wu, F. Ding, K. Ma, W. Wang, Y. Zhang, and Z. Shao. 2019. Pioglitazone protects compression-mediated apoptosis in nucleus pulposus mesenchymal stem cells by suppressing oxidative stress. Oxid. Med. Cell. Longev. 22:1–14.

Fouqueray Jacques, V., S. Bolze, S. Hallakou-Bozec, A. W. Czarnik, A. S. Divakaruni, A. N. Murphy, L. H. Van der Ploeg, and S. DeWitt. 2021. Deuterium-Stabilized (R)-Pioglitazone (PXL065) is responsible for pioglitazone efficacy in NASH yet exhibits little to no PPARγ activity. Hepatol. Commun. 1:1–14.

Jalilvand, N., M. Hosseini, F. Beheshti, and A. Ebrahimzadeh-Bideskan. 2019. Protective effect of PPARγ agonist pioglitazone, on testicular tissue and sperm parameters in hypothyroid rats. Toxin Rev 24:1–10.

Lagares, M. A., R. Ecco, N. R. S. Martins, L. J. C. Lara, J. S. R. Rocha, D. A. R. Vilela, V. M. Barbosa, P. F. Mantovani, J. F. V. Braga, I. S. Preis, V. A. Gheller, P. C. Cardeal, and N. C.& Baião. 2017. Detecting reproductive system abnormalities of broiler breeder roosters at different ages. Reprod. Domest. Anim. 52:67–75.

Lewis, S. E. M., P. M. Boyle, K. A. McKinney, I. S. Young, and W. Thompson. 1995. Total antioxidant capacity of seminal plasma is different in fertile and infertile men. Fertil. Steril. 64:868–870.

Liu, L. L., H. Xian, J. C. Cao, C. Zhang, Y. H. Zhang, M. M. Chen, and Y. Qian. 2015. Peroxisome proliferator - activated receptor gamma signaling in human sperm physiology. Asian J. Androl. 2:942–947.

Meneses, M. J., R. L. Bernardino, R. Sá, J. Silva, A. Barros, M. Sousa, B. M. Silva, P. F. Oliveira, and M. G. Alves. 2016. Pioglitazone increases the glycolytic efficiency of human Sertoli cells with possible implications for spermatogenesis. Int. J. Biochem. Cell Biol. 79:52–60.

Miller, D., G. C. Ostermeier, and S. A. Krawetz. 2005. The controversy, potential and roles of spermatozoal RNA. Trends Mol. Med. 11:156–163.

Mousavi, M. S., A. Shahverdi, J. Drevet, V. Esmaeili, F. A. Sayahpour, T. Rezaei, P. Rahimizadeh, and A. Alizadeh. 2019. Peroxisome proliferator-activated receptors (PPARs) levels in spermatozoa of normozoospermic and asthenozoospermic men asthenozoospermic men. Syst. Biol. Reprod. Med. 65:409–419.

Sakamoto, J., H. Kimura, S. Moriyama, H. Odaka, Y. Momose, Y. Sugiyama, and H. Sawada. 2000. Activation of human peroxisome proliferator-activated receptor (PPAR) subtypes by pioglitazone. Biochem. Biophys. Res. Commun. 278:704–711.

Santoro, M., F. De Amicis, S. Aquila, and D. Bonofiglio. 2020. Peroxisome proliferator-activated receptor gamma expression along the male genital system and its role in male fertility. Hum. Reprod. 35:2072–2085.

Sarabia Fragoso, J., M. Pizarro Díaz, J. C. Abad Moreno, P. Casanovas Infesta, A. Rodriguez-Bertos, and K. Barger. 2013. Relationships between fertility and some parameters in male broiler breeders (body and testicular weight, histology and immunohistochemistry of testes, spermatogenesis and hormonal levels). Reprod. Domest. Anim. 48:345–352.

Sato, K., K. Fukao, Y. Seki, and Y. Akiba. 2004. Expression of the chicken peroxisome proliferator-activated receptor-γ gene is influenced by aging, nutrition, and agonist administration. Poult. Sci. 83:1342–1347.

Shaheen, M. S., S. Mehmood, A. Mahmud, A. Riaz, and S. Ahmad. 2021. Interventions in mating strategies of broiler breeder during peak and post peak phase influence hatching traits. Poult. Sci. 100:101095.

Waugh, J., G. M. Keating, G. L. Plosker, S. Easthope, and D. M. Robinson. 2006. Pioglitazone. Drugs 66:85–109.

Zanussi, H. P., F. Shariatmadari, M. Sharafi, and H. Ahmadi. 2019. Dietary supplementation with flaxseed oil as source of omega-3 fatty acids improves seminal quality and reproductive performance in aged broiler breeder roosters. Theriogenology 130:41–48.

Zhandi, M., M. Ansari, P. Roknabadi, A. Zare Shahneh, and M. Sharafi. 2017. Orally administered chrysin improves post-thawed sperm quality and fertility of rooster. Reprod. Domest. Anim. 52:1004–1010.

Zhang, H. J., Y. M. Guo, Y. Yang, and J. M. Yuan. 2006. Dietary conjugated linoleic acid enhances spleen PPAR-γ mRNA expression in broiler chicks. Br. Poult. Sci. 47:726–733.