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Prostaglandin levels in seminal plasma and sperm extracts of the domestic turkey, and the effects of cyclooxygenase inhibitors on sperm mobility

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Abstract

Background: Turkey reproduction is by artificial insemination using pooled semen so there is interest in storing semen. Fertilizing capacity declines after six hours storage, possibly due to poor sperm mobility. Prostaglandins (PG) affect mammalian sperm motility, but avian sperm has not been widely studied. For this study, levels of PG E1, E2, and F2 alpha in turkey seminal plasma and sperm extract, and effects of cyclooxygenase (COX) inhibitors on sperm mobility were determined.

Methods: Seminal Plasma and sperm extract PG E1, E2, and F2 alpha, from 1.0 mL pooled semen, were measured by ELISA. In Trial 1, PG were determined from 122 wk old toms (n = 4). Trial 2 used 36 wk old toms (n = 7). For Trial 3, PGE2 only was measured from 48 wk (n = 6) and 154 wk old toms (n = 3). The effects of non-specific COX inhibitors indomethacin, diclofenac, tolmetin, or aspirin (n = 10), or specific COX-1 or COX-2 inhibitors (n = 3) on sperm mobility were measured (Accudenz swim-down test).

Results: Seminal plasma PG (pg/mL) in Trials 1 and 2, respectively, were 185.2 ± 88.4 and 187.2 ± 33.7 for PGE1; 141.4 ± 43.1 and 100.4 ± 14.6 for PGF2 alpha; and 431.0 ± 155.1 for PGE2 (Trial 1 only). Sperm extract PG (pg/10 billion cells) in Trials 1 and 2, respectively, were 215.1 ± 38.1 and 208.9 ± 41.5 for PGE1; 133.7 ± 51.7 and 49.8 ± 8.3 for PGF2 alpha; and 52.3 ± 8.6 for PGE2 (Trial 1 only). In Trial 3, seminal plasma PGE2 (pg/mL) in older versus younger males was 1097.9 ± 99.3 versus 853.2 ± 144.6 and sperm extract PGE2 (pg/10 billion cells) was 208.0 ± 56.1 versus 102.4 ± 14.8 . Cyclooxygenase inhibitors (0.001 to 10 mM) decreased sperm mobility: indomethacin 15 to 100%; diclofenac 4 to 100%; tolmetin 27 to 74%; aspirin (tested at 0.01 to 15 mM) 22 to 42%; resveratrol (COX-1) and NS-398 (COX-2), both tested at 0.1 to 10 mM, 38 to 98% and 44 to 85%, respectively.

Conclusion: These results indicate that PG are present in turkey seminal plasma and sperm, and COX inhibitors decrease turkey sperm mobility.

Background

Commercial breeders use artificial insemination (AI) for reproduction of domestic turkeys (*Meleagris gallopavo*).

Semen is collected from a flock of toms, pooled, and diluted prior to insemination of hens. A goal for breeders has been extending the interval between semen collection

and time of insemination. Cryopreservation has not given satisfactory results, for even when suitable extenders and adequate oxygen is supplied, turkey semen cannot be stored for more than six hours without a loss in fertilizing capacity [1–5]. Fertilizing capacity has been correlated with sperm mobility of individual chicken and turkey males [6–12] as well as pooled turkey semen [13] using the Sperm Mobility Test (SMT). The SMT measures the ability of sperm to penetrate an Accudenz® solution. The assay allows objective measurement of the proportion of sperm that move with forward linear movement, i.e., mobility. Researchers attempting to develop successful storage regimens for turkey semen base their investigations on the rationale that by preserving sperm mobility, the fertilizing capacity of the stored semen can be improved.

Prostaglandins are eicosanoid hormones that have been shown to have varied effects on mammalian sperm motility. Schlegel et al. [14] found that $\text{PGF}_{2\alpha}$ was negatively correlated with human sperm motility, but disruption of prostaglandins with 15-hydroxydehydrogenase also caused a dramatic decrease in motility. High levels of $\text{PGF}_{2\alpha}$ were also found to suppress bull sperm motility and induce membrane damage [15]. Studies of human sperm have shown that prostaglandins of the E series promote calcium influx via a receptor-linked mechanism that is capable of inducing the acrosome reaction [16,17]. In general, prostaglandins of the E series stimulate sperm motility, whereas $\text{PGF}_{2\alpha}$ inhibits motility [18,19]. Little is known on the effects of PG on avian sperm.

The formation of the most common seminal prostaglandin series, the series-2 prostaglandins, involves conversion of arachidonic acid to cyclic endoperoxides by cyclooxygenase (COX), followed by conversion of the endoperoxides to prostaglandin by prostaglandin isomerase [20]. Multiple forms of the COX enzyme exist, distinguished by expression patterns in mammalian tissues. The COX-1 enzyme is constitutively expressed and regulates normal physiological processes such as gastrointestinal, renal, and platelet function. In contrast, COX-2 is inducible by such factors as hormones, growth factors, and cytokines and functions to generate prostaglandins involved in the inflammatory process [21]. A variant of the COX-1 enzyme, COX-3, has been found in canine cerebral cortex and human cerebral cortex and heart [22]. While the anti-inflammatory and analgesic effects of non-steroidal anti-inflammatory drugs are generally thought to be due to inhibition of COX, recent evidence suggests that these types of compounds may have additional activities independent of COX inhibition [23].

The purpose of this study was to determine the levels of PG E_1 , E_2 , and $\text{F}_{2\alpha}$ in turkey seminal plasma and sperm

extract, and the effects of cyclooxygenase (COX) inhibitors on sperm mobility.

Methods

Experimental Animals

Small White Turkey toms bred for high egg production [24] were used in these studies. Approval for the use of animals was obtained from the Animal Research Committee at Clemson University. The turkeys were maintained in closed houses with free access to feed and water. Two flocks of turkeys were used and were classified as Group 1 (older toms) and Group 2 (younger toms). Group 1 was 106 wk older than Group 2. Beginning at 29 wk old, both flocks of toms were placed on the following light regimen to initiate semen production: fluorescent light (Cool White Delux, Sylvania F40CW, GTE Products Corp., Versailles, KY) at 189 lx intensity at head height with a 14 hour light/10 hour dark cycle. All toms received a 2,650 kcal ME/kg diet containing 12% protein. Semen was collected by abdominal massage [25].

Determination of Sperm Concentration

Sperm concentration was determined using a modification of the technique of Donoghue et al. [26]. A 10 μL aliquot of semen or semen dilution was added to 2 mL of 3% sodium chloride in a 4.0 mL polystyrene cuvette. The cuvette was inverted 10 times to mix, and the absorbance was read at 550 nm using a Turner SP-830 spectrophotometer (Thermolyne Corp., Dubuque, IA). Sperm concentration was then determined using a standard curve prepared from diluted semen samples of known concentration as determined by counting in a sperm/bacteria counting chamber (Petroff-Hausser Counter, Hausser Scientific, Horsham, PA).

Prostaglandin Concentrations in Turkey Spermatozoa and Seminal Plasma

Semen Collection

Samples of pooled semen were collected from Small White turkey toms at three separate times for PG measurement. Semen PGE_1 , PGE_2 , and $\text{PGF}_{2\alpha}$ were measured for Group 1 males when they were 122 wk old (Trial 1, $n = 4$) and for Group 2 males at 32 wk of age (Trial 2, $n = 7$). An error occurred for the standard curve for PGE_2 in Trial 2, so a third trial was done in which semen was collected from both Group 1 (154 wk old, $n = 3$) and Group 2 birds (48 wk old, $n = 6$) to repeat the measurement of PGE_2 .

For each trial, toms were randomly selected for semen collection from the group or groups being tested, and the semen was alternately added to successive 1.5 mL microcentrifuge tubes to produce the number of pools of semen indicated above. This was repeated until all tubes had 1.0 mL of semen pooled from three or four birds, and each

microcentrifuge tube of pooled semen was considered an independent sample.

Seminal Plasma Preparation

After collection, each pooled sample was centrifuged at $12000 \times g$ for 2 min in a microcentrifuge (Biofuge 13, Heraeus Instruments, Inc., South Plainfield, NJ). The seminal plasma was removed from the sperm pellet, transferred to a new 1.5 mL microcentrifuge tube, and centrifuged at $12000 \times g$ twice for 2 min each. Following the final centrifugation, the seminal plasma was filtered using a 0.2 μm syringe filter and diluted 1/10 with Assay Buffer ED1.

Spermatozoal Extracts

The sperm pellet remaining after removal of seminal plasma was washed by centrifugation two times with tris-buffered saline (TBS, 0.02 M tris/0.5 M sodium chloride, pH 7.5). The pellet was then resuspended to 1.5 mL in TBS and sperm concentration was determined as described above. An aliquot of the sperm suspension containing 5×10^9 sperm was placed in a microcentrifuge tube (1.5 mL) and centrifuged. The supernatant was discarded, 0.5 mL of TBS was added to the pellet, and the pellet was homogenized using a Pellet Pestle (Kontes Glass Company, Vine-land, NJ). The homogenized pellet was sonicated (Vibro Cell, Sonics and Materials Inc., Danbury, CT) at 50% power with an alternating 15 sec. on, 15 sec. off regimen that was repeated sequentially four times. The sample was centrifuged, the sperm extract (supernatant) was collected, and the volume determined. The sperm extract was then diluted 1/10 with Assay Buffer ED1.

Enzyme-linked Immunosorbent Assay (ELISA)

Concentrations of PGE_1 , PGE_2 , and $\text{PGF}_{2\alpha}$ were determined using competitive binding ELISA kits (R & D Systems, Minneapolis, MN) which contained antibodies, standards, and reagents. Cross-reactivities of the antibodies that were greater than 5% were as follows: the PGE_1 antibody had 6.5% and 2.2% cross-reactivity with PGE_2 and PGE_3 , respectively; the PGE_2 antibody had 70% and 16.3% cross-reactivity with PGE_1 and PGE_3 , respectively; and the $\text{PGF}_{2\alpha}$ antibody had 11.8% or 20% cross-reactivity with $\text{PGF}_{1\alpha}$ depending upon the kit used. Three replicate wells were used per sample, and the samples from each trial were assayed separately at the time of collection. The absorbance at 405 nm was inversely proportional to PG concentration, which was determined from a standard curve of absorbance versus prostaglandin concentration generated using a four-parameter logistic curve fit with Softmax software (Molecular Devices Corp., Menlo Park, CA).

Effect of Cyclooxygenase (COX) Inhibitors on Sperm Mobility

Semen Collection/Sperm Concentration

Semen from turkey toms (68 wk old at the beginning of these studies) was used to test the effect of six COX inhibitors on sperm mobility. One inhibitor was tested per week, and all assays for that inhibitor were completed before proceeding to the next inhibitor. Semen was pooled in a 1.5 mL microcentrifuge tube until approximately 1.0 mL was obtained (sample 1). The sperm concentration was determined as described above, and the semen was diluted to 1.0×10^{10} sperm/mL with Mobility Buffer (MB) consisting of 50 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid/128 mM sodium chloride/2 mM calcium chloride, pH 7.4, 280 to 320 mOsm/kg. The sperm mobility, with and without COX inhibitor, was determined for the sample as described below. Then another semen sample was collected, and the procedure was repeated until a total of ten samples had been assayed for each inhibitor, except for the specific COX-1 and COX-2 inhibitors where three samples were assayed.

COX Inhibitor Treatments

Inhibitors used were indomethacin, diclofenac sodium, tolmetin, acetylsalicylic acid, resveratrol, and NS-398 (17378, D6899, A5376, T6779, R5010, and N-194, Sigma Chemical Company, St. Louis, MO). Resveratrol and NS-398 are specific inhibitors of the COX-1 and COX-2 enzymes, respectively. A volume of 100 μL of semen that had been diluted to 1.0×10^{10} sperm/mL was added to 900 μL of MB with or without inhibitors such that the final concentrations of inhibitor was 0 to 15 mM. Due to the limited solubility of diclofenac, indomethacin, resveratrol, and NS-398, these COX inhibitors were first dissolved in dimethyl sulfoxide (DMSO) and then added to MB such that the final concentration of DMSO was 5%. For all treatments, mobility was determined using the Sperm Mobility Test (SMT) as described below. Aliquots of semen diluted with the various treatments were also examined microscopically (CH-2, Olympus Corporation, Lake Success, NY) to assess sperm morphology.

Sperm Mobility Test (SMT)

Sperm mobility was determined using a procedure developed for chicken semen [6] and modified for turkey semen [9]. For each assay, a volume of 0.06 mL of semen diluted as described above was overlaid onto 0.6 mL of prewarmed (41°C) 4% Accudenz[®] (Accurate Chemical and Scientific Corp., Dubuque, IA) solution in a 1.5 mL polystyrene cuvette. The cuvette was incubated at 41°C for 5 minutes in a dry bath (Thermolyne Corp., Dubuque, IA). Following incubation, the cuvette was placed in the Turner SP-830 spectrophotometer. After 1 minute, the

absorbance was recorded at a wavelength of 550 nm. Accudenz[®] solution (4%, 41 °C) was used as the blank.

Statistical Analysis

Prostaglandin Concentrations

The means and SEM of the PG concentrations in turkey seminal plasma and sperm extract were generated using the Descriptive Statistics package in Microsoft Excel 2000 (Microsoft Corp., Redmond, WA). In Trial 3, PGE₂ levels in seminal plasma and sperm extracts of younger versus older toms were analyzed using a Student's *t*-test.

Sperm Mobility Scores

For each COX inhibitor tested, the absorbance values from sample aliquots that contained no drug (Control) were equated to 100% mobility. The absorbance values from sample aliquots containing COX inhibitors were expressed as the percentage of the value from the respective Control sample aliquot. The percentage data were arcsine transformed and analyzed using the General Linear Model procedure and Duncan's Multiple Range test of SAS (SAS Institute Inc., Cary, NC). Significance was determined at $P \leq 0.05$.

Results

Prostaglandin Concentrations in Turkey Seminal Plasma and Sperm Cells

Seminal Plasma

The PG concentrations found in seminal plasma of turkey toms are shown in Table 1 (Trial 1 and 2). Results for PGE₂ concentrations in Trial 2 were discarded due to an error in the standard curve. In Trial 3, PGE₂ concentrations (pg/mL) in seminal plasma were not different ($P \leq 0.05$) between older (1097.9 ± 99.0) and younger (853.2 ± 144.6) males.

Table 1: Prostaglandin concentrations (pg/mL) in seminal plasma¹

Trial Number	PGE ₁	PGE ₂	PGF _{2α}
Trial 1 ^A	185.2 ± 88.4	431.1 ± 155.1	141.4 ± 43.1
Trial 2 ^B	187.2 ± 33.7	No Data	100.4 ± 14.6

¹Mean ± SEM. ^AFor Trial 1, n = 4. Group 1 toms: 122 wk old. ^BFor Trial 2, n = 7. Group 2 toms: 36 wk old.

Table 2: Prostaglandin concentrations (pg/10⁹ cells) in turkey sperm extract¹

Trial Number	PGE ₁	PGE ₂	PGF _{2α}
Trial 1 ^A	215.1 ± 38.1	52.3 ± 8.6	133.7 ± 51.7
Trial 2 ^B	208.9 ± 41.5	No Data	49.8 ± 8.3

¹Mean ± SEM. ^AFor Trial 1, n = 4. Group 1 toms: 122 wk old. ^BFor Trial 2, n = 7. Group 2 toms: 36 wk old

Sperm Extract

The prostaglandin concentrations found in extracts of turkey sperm are shown in Table 2 (Trial 1 and 2). Results for PGE₂ concentrations in Trial 2 were discarded due to an error in the standard curve. In Trial 3, the PGE₂ concentration (pg/10⁹ cells) in sperm extracts from older birds (208.0 ± 56.1) was significantly higher when compared to that from younger birds (102.4 ± 14.8).

Effect of Cyclooxygenase Inhibitors on Sperm Mobility

DMSO Test

As DMSO was required to solubilize some of the COX inhibitors, a preliminary experiment testing the effects of 5% DMSO on sperm was performed. No significant difference was seen in sperm mobility when 5% DMSO was added to the assay (OD = 0.355 ± 0.016) when compared to results using MB alone (OD = 0.353 ± 0.020).

COX Inhibitor Treatments

The effects of general COX inhibitors on sperm mobility are shown in Table 3. Values have been expressed as a percentage of the Control, which contained no drug and was equated to 100% mobility. All compounds tested caused a progressive decline in sperm mobility with increasing dosage: indomethacin (0.001 to 10 mM) 15 to 100%; diclofenac (0.001 to 10 mM) 4 to 100%; tolmetin (0.001 to 10 mM) 27 to 74%, and aspirin (0.01 to 15 mM) 22 to 42% inhibition. Indomethacin significantly depressed sperm mobility above concentrations of 0.1 mM. The results with diclofenac sodium were similar to those seen with indomethacin. Tolmetin also inhibited mobility, but inhibition was only 20 to 25% until the concentration exceeded 1.0 mM. Aspirin resulted in only 15 to 23% inhibition even at concentrations as high as 10 mM.

The effects of specific inhibitors of COX-1 and COX-2 on sperm mobility are shown in Table 4. Addition of 0.1 mM to 10 mM resveratrol (COX-1) or NS-398 (COX-2) resulted in 38 to 98% inhibition for resveratrol and 44 to 85% inhibition for NS-398. Similar to indomethacin and diclofenac, both resveratrol and NS-398 depressed sperm mobility approximately 38% at the 0.1 mM level and 80% or greater at the 1 mM level.

When semen samples diluted in MB containing COX inhibitors were examined microscopically, sperm were generally intact. However, at the 10 mM level of indomethacin and diclofenac, mostly debris was seen with only a few intact, immobile sperm.

Discussion

For mammals, seminal PG are produced mainly in the accessory glands of the reproductive tract, and PG have been shown in vitro to affect sperm motility, capacitation, and the acrosome reaction [16–18,27–29]. In contrast,

Table 3: The effect of cyclooxygenase inhibitors on turkey sperm mobility¹

Dose Level (mM)	Indomethacin	Diclofenac Sodium	Tolmetin	Aspirin
0.0 (Control)	100 ^a	100 ^a	100 ^a	100 ^a
0.001	84.9 ± 2.3 ^b	97.6 ± 2.2 ^b	73.2 ± 4.2 ^b	No Data
0.01	73.4 ± 3.2 ^c	87.8 ± 1.9 ^c	81.0 ± 4.3 ^b	82.2 ± 3.5 ^b
0.1	63.7 ± 1.2 ^d	83.1 ± 2.1 ^c	78.2 ± 6.1 ^b	85.0 ± 4.4 ^b
1.0	11.8 ± 0.8 ^e	35.7 ± 3.0 ^d	75.8 ± 4.7 ^b	83.0 ± 3.8 ^b
10.0	0.8 ± 0.2 ^f	0.2 ± 0.2 ^e	25.3 ± 1.9 ^c	77.4 ± 3.8 ^b
15.0	No Data	No Data	No Data	56.7 ± 3.4 ^c

¹Mean ± SEM. Values are expressed as the percentage of the Control which contained no drug (Control = 100%). ^{a-f}Within columns, values without common superscript differ significantly ($P \leq 0.05$; $n = 10$).

Table 4: Inhibition of sperm mobility by specific COX-1 and COX-2 inhibitors¹

Dose Level (mM)	Resveratrol (COX-1 Inhibitor)	NS-398 (COX-2 Inhibitor)
0.0 (Control)	100 ^a	100 ^a
0.1	61.7 ± 2.3 ^b	55.7 ± 5.4 ^b
1.0	20.0 ± 3.4 ^c	2.1 ± 1.4 ^d
10.0	2.2 ± 1.3 ^d	15.0 ± 2.3 ^c

¹Mean ± SEM. Values are expressed as the percentage of the Control which contained no drug (Control = 100%). ^{a-d}Within columns, values without common superscript differ significantly ($P \leq 0.05$; $n = 3$).

avian species do not have accessory glands, nor do avian spermatozoa undergo capacitation or a mammalian-like acrosome reaction. Given these facts, it is perhaps not surprising that reported PG levels in the seminal plasma of aves [30,31], and those presented here, are lower than those of most mammals, and that a role for avian seminal PG is unknown. A probable mechanism whereby PG could regulate sperm mobility would be through control of calcium, an ion previously shown to be necessary for turkey sperm mobility [32]. In this regard, PGE₁ and PGE₂ have been shown to promote calcium influx in mammalian sperm [17].

Using RIA, Hertelendy [30] reported PGE levels of 300 and 200 pg/mL in seminal plasma of chicken and turkey, respectively, and levels of PGF less than 100 pg/mL in chicken seminal plasma. In the present study, seminal plasma levels of PGE₁ and PGF_{2α} determined using ELISA were comparable to levels reported by Hertelendy [30] for PGE and PGF. No PG levels have been previously reported for turkey sperm extracts. While the PG concentrations detected in each trial were not statistically compared, in general the values for PGE₁ were consistent between Trial 1 and Trial 2. The levels of PGE₂ in Trial 3, however, were two to four times greater than those seen in Trial 1 for

both seminal plasma and sperm extract. The reason for this difference is unknown, but does not appear to be related to the age of the turkeys as levels of PGE₂ were higher in both young (48 wk old) and old toms (154 wk old) in Trial 3 than for the 122 wk old turkeys used in Trial 1. It is also unlikely that this difference was due to the ELISA kits themselves, as the standard curves performed for each PGE₂ assay were comparable, and the same antibody was supplied in each kit. For PGF_{2α} values in Trial 1 were somewhat higher than those found for Trial 2 for both seminal plasma and sperm extract. These differences may have been due to a change in the antibody used in the PGF_{2α} assay kit (with reported lower cross-reactivity), but also may have been related to differences in age of the birds (122 versus 36 wk for Group 1 versus Group 2, respectively).

The accurate measurement of tissue PG concentrations is not without pitfalls. Detected PG levels may not accurately reflect the true endogenous tissue concentrations because PG are easily degraded [33]. Furthermore, cross-reactivity of the PG antibodies, especially the PGE₂ antibody, contributes to variations in measuring specific PG concentrations.

In Trial 3, the measured PGE₂ in sperm of older toms versus that of younger toms was significantly elevated while that in the seminal plasma was not. The reason for this is unknown. While the difference was statistically significant, the difference was not numerically great, and may not be of physiological significance. The presence of PG in these extracts, however, suggests that turkey sperm may possess the enzyme systems necessary for PG synthesis. Both human and bovine sperm have been reported capable of synthesizing PG from arachidonic acid precursor in vitro [28,34], and it would be interesting to determine if this ability is shared by turkey sperm.

The finding that COX inhibitors decreased turkey sperm mobility may indicate that PG regulate mobility. Specific

inhibitors of both COX-1 and COX-2 produced the same degree of inhibition, even at the concentration of 0.1 mM, a level at which the COX-2 inhibitor was reported to have no inhibitory effect on COX-1 activity [35]. However, in vitro experiments involving the effects of drugs on cells must be interpreted with caution. For example, in addition to inhibition of COX enzymes, diclofenac, indomethacin and the specific COX-2 inhibitor SC-236 have been shown to uncouple mitochondrial respiration and inhibit substrate oxidation and ATP turnover in rat mitochondria and thymocytes [36]. Such activity in sperm would likely decrease mobility. Direct toxicity also must be considered. Rocha et al. [37] reported that indomethacin and NS-398 were toxic to renal medullary cell cultures, but only at levels that greatly exceeded those necessary for COX inhibition. Most interest concerning the toxicity of PG inhibitors has focused on the gastrointestinal tract, where it has been shown that they inhibit colorectal carcinogenesis. The mechanism of this actions is independent of COX-inhibition, possibly by decreasing the cell-cycle regulating proteins p21 or inhibition of transcription factors [23,38]. Such effects may not be relevant for sperm, which do not divide.

In the present study, the 10 mM level of indomethacin and diclofenac disrupted turkey sperm membranes, leaving mostly debris with only a few recognizable spermatozoa. This effect might be due to mechanisms unrelated to their action as COX inhibitors. However, sperm appeared intact at lower doses of indomethacin and diclofenac, levels at which there was still significant inhibition of mobility. Tribble et al [39] found that indomethacin causes membrane damage by increasing permeability, along with uncoupling oxidative phosphorylation. Turkey sperm are sensitive to osmotic changes and increased permeability may have been a contributor in the membrane damage observed for indomethacin and diclofenac in this study. Disintegration of sperm was not seen with tolmetin or aspirin at any of the dose levels tested, nor was it observed when the specific COX-1 or COX-2 inhibitors (resveratrol and NS-398) were tested.

Conclusions

These results indicate that PG are present in both seminal plasma and spermatozoa of the domestic turkey, and inhibition of COX may affect mechanisms of avian sperm mobility, although effects of toxicity of the inhibitor or actions via other mechanisms cannot be ruled out.

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