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Administration of silver nanoparticles affects ovarian steroidogenesis and may influence thyroid hormone metabolism in hens (*Gallus domesticus*)

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ABSTRACT

This study aimed to determine the in vivo effect of silver nanoparticles (AgNPs) on the concentration of sex steroids (progesterone – P4, estradiol – E2, testosterone – T) and thyroid hormones (thyroxine – T_4, triiodothyronine – T_3) in the blood plasma as well as the messenger ribonucleic acid (mRNA) and protein expression of HSD3β, CYP17A1 and CYP19A1 enzymes and steroid hormone concentrations in chicken ovarian follicles. AgNPs did not affect serum steroid hormone levels, but increased T_3 levels depending on the size and concentration of AgNPs. At the level of ovarian tissues, AgNPs: (i) affected the levels of E2 and T in prehierachical follicles; (ii) reduced the expression of CYP19A1 mRNA and protein and consequently diminished E2 concentration in small white follicles; and (iii) increased the expression of *CYP17A1* mRNA in large white follicles, without changing its protein expression. The results indicate that AgNPs affect chicken ovarian steroidogenesis. The effects of AgNPs depend on exposure time, the type of follicle and the degree of its development and are associated with the modulation of steroidogenic gene expression and E2 and T synthesis. Prehierachical follicles seem to be more susceptible to AgNPs than preovulatory ones. In conclusion, AgNPs by targeting the chicken ovary may indirectly influence the selection processes of prehierarchical follicles to the pre-ovulatory hierarchy and disturb the ovarian steroidogenesis. Furthermore, AgNPs may affect thyroid hormone metabolism in different ways by size which in turn may influence energy homeostasis of the target cells.

1. Introduction

Nanotechnology is currently a rapidly growing field of science. It is estimated that by 2021 the global nanotechnology market should reach $90.5 billion (BCC, 2016). Silver nanoparticles (AgNPs) are among the most intensively explored nanostructures and, due to their strong antimicrobial properties and promising characteristics, are suitable for various applications. The widespread use of AgNPs oriented towards biomedical and consumer products creates a need to understand the mechanism of their biological interactions and their potentially toxic effects on living organisms and the environment.

Most of the experimental studies reported to date have been focused on the in vitro effects of AgNPs. *In vivo* research has mainly been undertaken on rats (Gaillet and Rouanet, 2015; Hadrup and Lam, 2014) and fish (Bilberg et al., 2012; Khan et al., 2015) and some on birds (Anwar et al., 2019; Saleh and El-Magd, 2018; Vadalasetty et al., 2018). Meanwhile, the utilization of AgNPs in poultry production is gaining more and more applications (Gangadoo et al., 2016). In the 1950s, colloidal silver was used as an additive in poultry feeding to increase their daily growth, but its high price at that time could not compete with that of antibiotics. After the European Union banned the use of antibiotics as feed additives in poultry breeding in 2006, the attention has turned to replace them with AgNPs, whose production is currently generating lower costs (Yaqoob et al., 2020). Compared to silver salts, AgNPs have stronger antibacterial properties, are more resistant to the low pH of gastric juice and can be used at much lower doses, reducing the potential risk of their toxicity (Mahmoud, 2012). In poultry farming, AgNPs have found application not only as feed additives, but also as a disinfectant that reduces bacterial contamination and emissions of odour-forming gases in livestock rooms (Czyż, 2011; Farzinpour and Karashi, 2013). The use of AgNPs as coccidiostats is also being considered (Chauke and Siebrits, 2012).

Research conducted on birds has shown that AgNPs increase the metabolic rate in layer embryos, but do not affect the development, morphology and survival of broiler and layer embryo (Pineda et al., 2012a; Sawosz et al., 2009). Investigations in chicken embryos have

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revealed that AgNPs affect the expression of the myogenic differentiation 1 (*MyoD1*) and ATPase (*ATP1A1*) gene, which is responsible for the differentiation of muscle cells during embryogenesis and the energy metabolism of muscle cells, respectively (Sawosz et al., 2012). According to Sikorska et al. (2010), AgNPs are accumulated in the bones of chicken embryos, but do not affect their mechanical properties. In addition, due to their observed tendency to increase mineral content, the authors suggest that silver nanoparticles can affect bone mineralization. On the other hand, in mature hens, AgNPs upregulate the expression of fibroblast growth factor (*FGF2*) and vascular endothelial growth factor (*VEGFA*) in the pectoral muscles and the heart (Hotowy et al., 2012). Experiments carried out on Japanese quail (*Coturnix japonica*) have shown that AgNPs reduce hen-day egg production and egg yolk weight (Farzinpour and Karashi, 2013). Ahmadi and Kurdestany (2010) have observed increased activity of oxidative stress indicators in the blood of chickens and decreased weight of the bursa of Fabricius under AgNPs treatment. AgNPs have also been found to significantly affect blood parameters such as ALT, AST, ALP, TP, albumin, gamma globulin, triglycerides and cholesterol (Ahmadi, 2012; Saleh and El-Magd, 2018). AgNPs administration have shown a significant decrease in the concentration of immunoglobulins G and immunoglobulins M in the blood of broiler chicks (Pineda et al., 2012a; Vadalasetty et al., 2018) . Song et al. (2017) observed induced intestinal oxidative stress in AgNPs-treated broiler chickens. There has been no evidence in the literature regarding the endocrine aspect of birds' exposure to AgNPs. Meanwhile, there is supporting evidence that they may affect the endocrine system of other vertebrate clusters (Lu et al., 2013). In rainbow trout, a reduction in choriogenin L and vitellogenin levels, which are markers of exposure to estrogen-acting compounds, has been observed (Gagné et al., 2012; Pham et al., 2012). Studies on amphibians have shown that nanosilver affects thyroid receptors' expression (Hinther et al., 2010). In rats, intravenous injection of AgNPs has been found to affect Leydig cell function, manifested by an increase in serum and testis testosterone (T) levels and *CYP11A1* and *HSD3B* mRNA expression (Garcia et al., 2014). Wang et al. (2020) have discovered that AgNPs can accumulate in mouse mammary glands, resulting in a decrease in the percentage of ducts and terminal ducts, reduced estrogen receptor ERα expression and diminished serum levels of estradiol (E2) in adult mice after chronic exposure. In the light of the data presented above, the question arises whether the use of feed additives and formulations containing AgNPs may affect the functions of the reproductive system in domestic birds.

Exposure of chickens to AgNPs can occur in different ways: by inhaling AgNPs from disinfectants used in livestock rooms and through direct contact with the skin as well as taking AgNPs supplemented with feed. Many researchers have carried out oral toxicity experiments in poultry birds (Ahmadi, 2012; Farzinpour and Karashi, 2013; Hotowy et al., 2012; Saleh and El-Magd, 2018; Song et al., 2017; Vadalasetty et al., 2018). Walczak et al. (2013) have observed that during the digestion of nutrients in the human stomach, AgNPs aggregate and form clusters with chlorine, but digestion in the intestine restores its original size and composition. Then, through the intestinal mucosa, they are absorbed into the blood and reach various organs. Nanoparticle silver has been detected in various organs after oral exposure. For instance, in rats after 28 days of repeated oral administration silver distribution has been found to be in the following order: the small intestine, stomach, kidneys, liver, brain and lungs (Loeschner et al., 2011). In an almost identical experiment carried out by Van der Zande et al. (2012), very high levels of AgNPs were also detected in the spleen and testes. In turn, Lee et al. (2013) have observed the presence of nanosilver in the ovary after oral exposure to AgNPs.

Due to the above, the endocrine-disrupting effects of AgNPs on domestic chickens cannot be excluded. Therefore, the objective of this study was to determine the potential effect of the oral administration of AgNPs on steroid hormone concentration in blood plasma and on ovarian steroidogenesis. Given that in the literature there are few studies related to the effects of AgNPs on thyroid hormone action (Carew et al., 2014; Hinther et al., 2010; Sulaiman et al., 2018) and taking into account that thyroid hormones affect chicken ovarian steroidogenesis (Sechman et al., 2013), we additionally decided to determine plasma thyroxine (T_4) and triiodothyronine (T_3) concentrations following exposure to AgNPs.

2. Material and methods

2.1. Animals

The experiment was performed on Hy-Line Brown hens (n = 42) at the age of 25 weeks with an average body weight of 1.67 ± 0.16 kg. The birds were kept in individual cages with free access to water and feed, in a 14L:10D light regime. The chickens were fed a commercial compound feed (DJ). Every day, for 3 weeks before and during the experiment, the laying time was recorded to determine the ovulation time (it was assumed that ovulation occurs 15–30 min after egg laying). The hens were decapitated 22 h before ovulation. The experiments and animal procedures were approved by the First Local Animal Ethics Committee in Krakow, Poland (Permit no. 9/2015).

2.2. Characteristics of silver nanoparticles used in experiments

AgNPs in the form of monodisperse water colloids were produced by the NPIN company (NPIN S.C, Poland) via a chemical method. AgNPs' characteristics were determined using dynamic light scattering (DLS) and scanning transmission electron microscopy (STEM). Nanoparticle sizes were determined at 13 nm and 50 nm. The concentration of both colloids was 100 ppm. The colloids included: silver in the form of nanoparticles 100 ppm; silver in ionic form <5 ppm; sodium cations <354 ppm; citrate anions <970 ppm; other inorganic cations and anions <10 ppm; and other inorganic substances <15 ppm. NPIN also produced a reference solution, whose composition was as follows: sodium cations <354 ppm; citrate anions <970 ppm; other cations and inorganic anions <10 ppm; and other inorganic substances <15 ppm. This solution was used as a control for the experiments.

2.3. Experiment 1

The experiment was carried out on 42 hens, which were randomly divided into 7 equal groups (n = 6). At 8:00 a.m. each day, birds received *per os* 1 ml/kg BW of colloid with the following concentrations of 13 nm or 50 nm AgNPs: 1 ppm, 10 ppm and 100 ppm. The control group received a buffer in which nanoparticles were suspended. During the experiment, feed intake, body weight of chickens and their rate of laying were monitored. Blood was collected on day 14 of the experiment. Blood sampling was undertaken from the wing vein into heparin-containing tubes and centrifuged (3000 rpm, 10 min) to obtain plasma. The plasma was then frozen at −20 °C and stored until hormone concentrations were determined by radioimmunoassay (RIA). Next, an analysis of changes in the concentration of steroid hormones (progesterone (P4), T and E2) and iodothyronines (thyroxine (T_4) and triiodothyronine (T_3)) was performed in order to establish the concentration of AgNPs in the second experiment.

2.4. Experiment 2

The birds were randomly divided into two groups: control (n = 12) and experimental (n = 12). At 8:00 a.m. every day, the hens in the experimental group received 1 ml/kg BW of AgNPs colloidal solution (50 nm, 100 ppm), while the hens in the control group received 1 ml/kg BW of buffer in which nanoparticles were suspended. Blood was collected on days 7 and 14 of the experiment. Six birds from each group were decapitated on day 7 and the rest of them (n = 6 in each group) on day 14 of the experiment. Small (SWF, 1–4 mm) and large (LWF, 4–8 mm) white prehierachical follicles and yellow preovulatory follicles

(F3–F1) were isolated from the ovary. The granulosa layer from the preovulatory follicles was separated by the Gilbert method (Gilbert et al., 1977). Tissues were frozen at −80 °C and stored until RNA isolation, protein and steroid assays.

2.5. Steroid hormones and iodothyronines measurement

The concentration of P4 in the blood plasma of chickens and ovarian tissues (granulosa layer of F3−F1 follicles) was determined by radioimmunoassay using a commercial PROG-RIA-CT kit (DIAsource, Belgium). The Testosterone [125I] RIA KIT kit (IZOTOP, Hungary) was used to determine T in the blood plasma and ovarian tissues (1–4, 4–8 mm follicles, theca layer of F3−F1 follicles). E2 in the blood plasma and ovarian tissues (1–4, 4–8 mm follicles, theca layer of F3−F1 follicles) was determined using a commercial RIA Estradiol kit (Beckman Coulter, USA). The tissue T and E2 concentrations were converted to mg of tissue. P4 concentration was converted to mg of protein determined by the BCA method (Thermo Scientific, Pierce BCA Protein Assay Kit, USA), because the granulosa fragments were very small and weighing them risked distorting the results. The lowest limits of sensitivity for P4, T and E2 were 0.05 ng/ml, 0.087 nmol/L, 9.58 pg/ml, respectively. The intra-assay variability was 5.2% for P4 and T and 14.4% for E2, while the inter-assay variability was 8.6%, 9.6% and 14.5% for P4, T and E2, respectively.

Iodothyronine concentration was determined using T4-RIA-CT and T3-RIA-CT kits (DIAsource, Belgium). The sensitivity of the method for T_4 was 5 nmol/L, with average recovery 103% and the following cross-reactivities: D-T_4 – 48%; L-T_3 – 1.01%; and rT_3 – 7%. The intra-series error was 5.6%, while the inter-series error was 6.5%. For the T_3 kit, the sensitivity of the method was 0.1 nmol/L, with average recovery 109% and cross-binding of anti-T_3 antibodies L-T_4 – 0.17% and D-T4 – 0.04%. Intra-series error was 5.4%, while inter-series error was 8.15%.

2.6. Gene expression analysis

RNA isolation was carried out using the EXTRACTME TOTAL RNA kit (Blirt, DNA Gdańsk, Poland) according to the manufacturer's instructions. The concentration and the purity of the isolated RNA were tested using a NanoDrop Lite spectrophotometer (Thermo Scientific, Madison, USA). All samples had an OD260/OD280 wavelength ratio between 1.9 and 2.0. In addition, electrophoretic separation of isolated RNA was performed to determine its quality. Samples with no signs of degradation were used in the reverse transcription reaction. Two μg of total RNA was transcribed using the RevertAid RT Transcription Kit (ThermoScientific, USA) in a Mastercycler personal thermocycler (Eppendorf, Germany), according to the manufacturer's recommendations. Reverse transcription samples were diluted 10-fold with RNase-free water and in this form used in real-time polymerase chain reaction (PCR).

Real-time PCR was performed as previously described by Katarzyńska-Banasik et al. (2017). TaqMan primer sets and probes (TaqMan Gene Expression Assay) were designed and provided by Applied Biosystems (Table 1). The amplification efficiency was determined for each gene based on a standard curve generated from a series of six 10-fold dilutions of template cDNA (pool of all samples). The reaction efficiency was in the range of 90–110% Reference genes (SDHA and RPL13) were selected as described by Katarzyńska-Banasik et al. (2017). Normalized relative quantitation (NFQ) of tested genes was calculated according to the Pfaffl (2001) modified by Hellemans Mortier et al. (2007).

2.7. Western blot analysis

The tissues were homogenized in liquid nitrogen and suspended in lysis buffer (50 mM 258 Tris, 1 mM EDTA, pH 7.5) containing 10 μL/ml protease inhibitor cocktail and centrifuged at 12,000 rpm for 20 min at 4 °C. The protein was determined by the Bradford method using a Pierce Detergent Compatible Bradford Assay Kit (Thermo Fisher Scientific, USA). Samples containing 20 μg of protein lysate were suspended in 2x Laemmli Sample Buffer (Bio Rad, USA) in a 2:1 ratio and denatured for 4 min at 96 °C. Proteins separated on 12% SDS-PAGE were then transferred to an Immobilon-P membrane (Millipore, Billerica, USA) using a semi-dry Pierce Power Blotter transfer apparatus (Thermo Scientific, USA) (7 min, 25 V). After transfer, the membranes were incubated in blocking buffer (5% skim milk, 0.1% Tween 20, 0.02 M TBS) for 1 h at room temperature, washed and incubated overnight (+4 °C) with primary antibodies (mouse anti-3βHSD monoclonal antibody FDO66Q, Novus Biologicals, Centennial, USA, 1:500; mouse anti-human cytochrome P450 aromatase monoclonal antibody, MCA2077S, Bio-Rad, Hercules, CA, USA, 1:500; mouse anti-CYP17A1 monoclonal antibody, NBP2–01151, Novus Biologicals, Centennial, USA, 1:4000; mouse anti-β-actin antibody, Merck KGaA, Darmstadt, Germany, 1:3000). The membranes were then washed 3 times with TBST buffer (0.1% Tween in 0.02 M TBS) and incubated for 1 h with horseradish peroxidase-conjugated antibodies (goat anti-rabbit or goat anti-mouse antibody; Jackson ImmunoResearch Laboratories, Inc., West Grove, USA, 1:10 000). Signals were detected by chemiluminescence using a Clarity Western ECL Substrate kit (Bio-Rad, USA) and visualized with ChemiDoc-It Imaging System camera (UVP, LLC, Upland, USA). ImageJ software was used for densitometric analysis of the bands (National Institutes of Health, Bethesda, Maryland, USA).

2.8. Statistical analysis

Statistical analysis of the results was carried out using the SAS statistical package (version 9.4). Before proceeding with the analysis, normal distribution was tested using the UNIVARIATE procedure and log transformation was applied for non-normally distributed data. Means and standard errors (SE.) were calculated using the MEANS procedure. Because the first experiment was arranged as a two by three factorial design plus a control, two statistical models were used. First statistical model included all treatments and group effect as the classifying variable. In the case of significant effect of group ($P < 0.05$), the means were separated using the PDIFF option in SAS with Tukey–Kramer adjustment. In the second model, the significance of the Ag nanoparticles' size and concentration was tested. The statistical model included the Ag effect, concentrations and interaction between these effects as classifying variables. As a result, the second model did not include a control group. Polynomial contrasts were used to determine impact of concentration of AgNPs and associated linear and nonlinear changes of investigated parameters (polynomial contrasts did not include control group). In the

Table 1
TaqMan Gene Expression Assays characteristic.

Gene symbol	Gene name	Assay ID	Context sequence	Amplicon size (bp)
CYP11A1	Cholesterol side chain cleavage	Gg03345949_m1	TCTTCCTCATGCACATCCTGGAGAA	65
3βHSD	3-beta-hydroksysteroid dehydrogenase	Gg03372858_s1	CAACCGCCACCTGGTCACTCTGCTG	125
CYP17A1	Steroid 17-alpha-monooxygenase	Gg03346126_g1	GGACGGCCTCGCACCGTGACCACGG	78
CYP19A1	Cytochrome P450, family 19, subfamily A, polipeptyd 1	Gg03346001_m1	ATTGAAACTGTTATGGGTGACAGAG	76
SDHA	Succinate dehydrogenase complex flawoprotein subunit 2	Gg03330765_m1	GCAGAAGACAATGCAAAGCCATGCT	103
RPL13	60S ribosomal protein *L13*	Gg03348054_m1	TTATGCCGATCAGGAACGTTTTCAA	66

second experiment, the statistical model included the group tissue and interaction between these effects as the classifying variable. Once the model had established significant differences ($P < 0.05$), the means were separated using the PDIFF option with Tukey's test. Figures were created using Grapher 13.0 (Golden Software Inc., USA).

3. Results

3.1. Effect of AgNPs on P4, T, E2, T_3 and T_4 concentration

There were no statistically significant changes of steroid hormone concentrations in chicken blood plasma after a 14-day administration of AgNPs. Neither the size nor the concentration of the nanoparticles affected the P4, T and E2 levels (Table 2). Determination of blood steroid hormone levels in the second experiment confirmed the lack of influence of the administration of AgNPs on the P4, T and E2 levels (data not shown).

Regardless of the nanoparticle concentration used, 13 nm nanoparticles did not significantly affect the T_3 blood plasma levels. There was no significant effect on the T_3 level of 50 nm AgNPs used in 1 or 10 ppm concentrations. In turn, the highest concentration of 50 nm AgNPs (i.e. 100 ppm) caused a 3.7-fold increase of T_3 levels in the blood of the examined chickens ($P < 0.05$). T_4 blood plasma level was not affected by either nanoparticle size or nanoparticle concentration (Table 2).

3.2. Effect of AgNPs on HSD3β, CYP17A1 and CYP19A1 gene expression

Administration of AgNPs for 7 consecutive days increased *CYP17A1* mRNA expression in the theca layer of F3 follicles by 140% ($P < 0.05$; Fig. 1C) and diminished the expression of the *HSD3β* gene in LWF follicles by 75% ($P < 0.05$; Fig. 1A).

In turn, after 14 days of AgNPs treatment, *CYP17A1* and *CYP19A1* mRNA expression increased in LWF follicles and in the theca layer of F3 follicles by 130% ($P < 0.05$; Fig. 1A) and 340% ($P < 0,05$; Fig. 1C), respectively. Administration of AgNPs also resulted in *CYP19A1* gene expression decrease in SWF follicles by 46% ($P < 0.05$; Fig. 1A) and *HSD3β* gene expression decrease in the granulosa layer F1 follicles by 60% ($P < 0.05$; Fig. 1B).

3.3. Effect of AgNPs on HSD3β, CYP17A1 and CYP19A1 protein expression

There were no significant changes in expression of the tested proteins on day 7 of AgNPs administration (Fig. 2A; Fig. 3C,E). In turn, after 14 days of the experiment, AgNPs reduced CYP19A1 protein expression in SWF follicles ($P < 0.05$) (Fig. 2B).

3.4. Effect of AgNPs on the concentration of steroid hormones in ovarian tissues

3.4.1. Effect of AgNPs on T and E2 concentration in prehierachical follicles

Under control conditions, there were no significant changes in T concentration between SWF and LWF follicles collected on days 7 and 14 of AgNPs administration (Fig. 4A,B). There were also no significant differences in T concentration between the control group and the experimental group in both classes of follicles on day 7 of the experiment, while on day 14 of the AgNPs administration the T concentration in LWF follicles increased by 42% compared to the control group ($P < 0.05$; Fig. 4B).

The concentration of E2 in control hens was significantly lower in LWF follicles than in SWF ones on both experimental days 7 ($P < 0.05$) and 14 ($P < 0.05$) (Fig. 4C,D). On day 7 of nanoparticle treatment there were no significant changes in E2 concentration in both classes of follicles (Fig. 4C), while on day 14 of AgNPs administration the concentration of E2 in SWF follicles decreased by 27% in comparison to the control group ($P < 0.05$; Fig. 4D).

3.4.2. Effect of AgNPs on P4, T and E2 concentration in preovulatory follicles

In both the control and the AgNPs treated groups, no significant alternations were found in P4 concentration between the granulosa layer of the preovulatory follicles (F3−F1) isolated on days 7 and 14 of the experiment (Fig. 5A,B).

Under control conditions, a gradual decrease in T and E2 concentration was observed in the theca layer of F3 → F1 follicles (Fig. 5C,D and E,F). AgNPs treatment did not significantly affect either T or E2 concentrations in the theca layer of the preovulatory follicles examined (Fig. 5C,D and E,F).

4. Discussion

The rapid development of nanotechnology and the widespread use of nanomaterials in recent years raises concerns over their possible negative impacts on human health and the environment. Studies regarding the effects of nanomaterials on reproductive function suggest that there may be abnormalities in the regulation of sex hormone levels in the blood (Iavicoli et al., 2013; Li et al., 2012). Cell and animal models have been used to study reproductive system dysfunctions under the influence of AgNPs (Asare et al., 2012; Lim et al., 2012; Luaibi and Qassim, 2017; Nair et al., 2011; Philbrook et al., 2011). Although the effect of AgNPs has been tested in numerous studies on broiler chickens (Anwar et al., 2019; Saleh and El-Magd, 2018; Song et al., 2017; Vadalasetty et al., 2018) and chicken embryos (Bhanja et al., 2015; Grodzik and Sawosz,

Table 2
Concentration of progesterone (P4), testosterone (T), estradiol (E2), triiodothyronine (T_3) and thyroxine (T_4) in the chicken blood plasma after 14-day *per os* administration of silver nanoparticles (AgNPs).

Group	Hormone	P4 (ng/ml)	T (ng/ml)	E2 (pg/ml)	T_3 (ng/ml)	T_4 (ng/ml)
Control		2.11 ± 0.25	0.49 ± 0.11	281 ± 20.4	0.77 ± 0.16	38.0 ± 3.80
AgNPs:						
13 nm/1 ppm		1.86 ± 0.26	0.29 ± 0.03	246 ± 12.6	0.61 ± 0.11	33.0 ± 3.94
13 nm/10 ppm		1.78 ± 0.12	0.56 ± 0.03	301 ± 26.4	0.86 ± 0.17	38.9 ± 4.00
13 nm/100 ppm		1.83 ± 0.21	0.52 ± 0.15	239 ± 16.5	0.98 ± 0.16	46.8 ± 2.00
50 nm/1 ppm		1.88 ± 0.20	0.37 ± 0.07	252 ± 10.7	0.79 ± 0.08	38.5 ± 4.92
50 nm/10 ppm		2.04 ± 0.17	0.46 ± 0.07	257 ± 14.9	1.10 ± 0.24	42.5 ± 2.39
50 nm/100 ppm		1.93 ± 0.23	0.62 ± 0.13	253 ± 24.5	2.90 ± 0.21*	45.3 ± 5.07
P-value:						
Group		0.940	0.122	0,271	<0.001	0.486
Ag		0.475	0.709	0.644	<0.001	0.674
Dose		0.983	0.017	0.156	<0.001	0.242
Ag x Dose		0.856	0.329	0.310	<0.001	0.799
Dose effect						
Linear		0.973	0.014	0.751	0.107	0.197
Quadratic		0.803	0.106	0.057	0.737	0.874

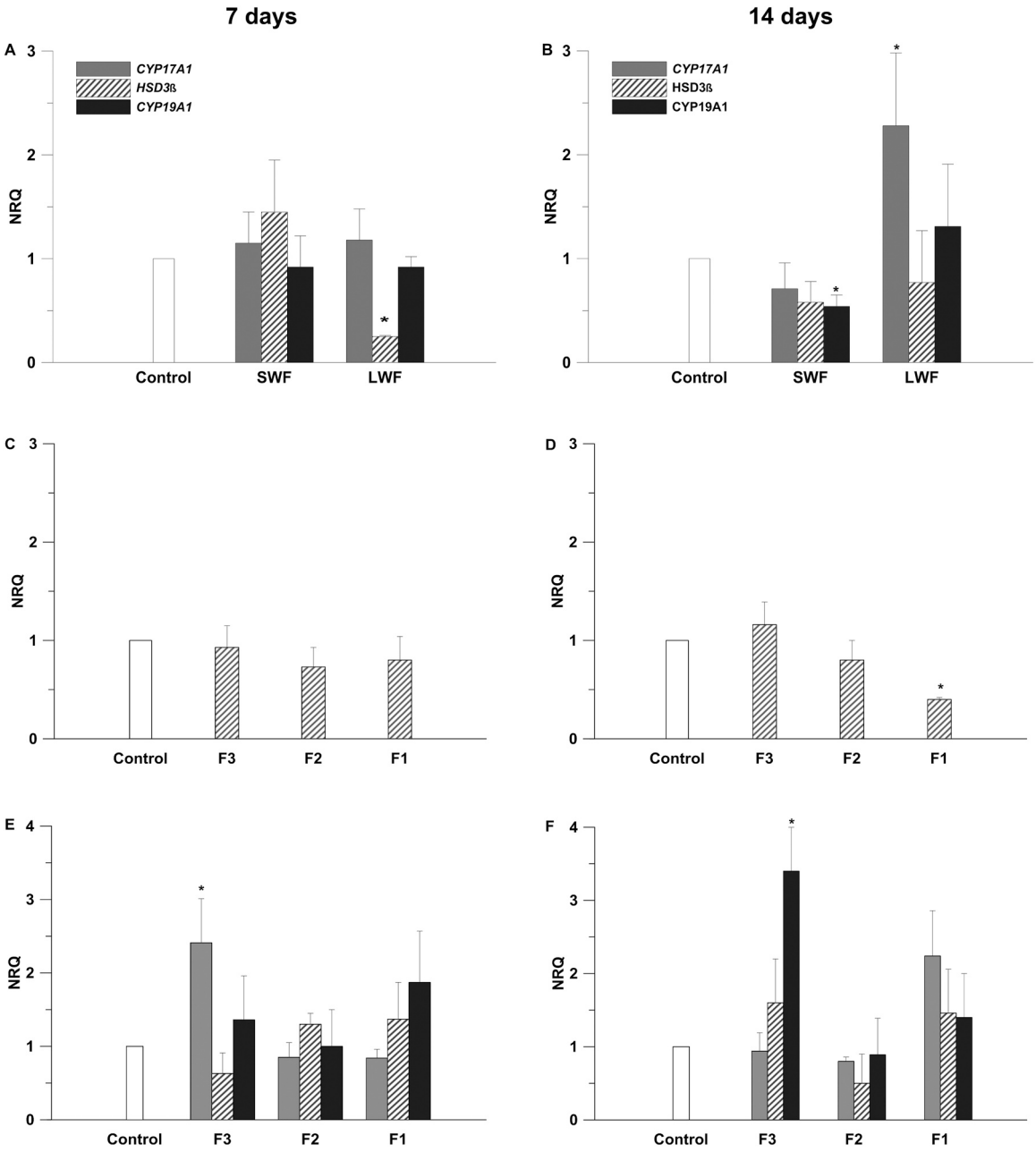

Fig. 1. Effects of silver nanoparticles (AgNPs) administration for 7 (A, C, E) or 14 (B, D, F) days on mRNA expression, evaluated by Real Time PCR, of: CYP17A1, 3βHSD and CYP19A1 genes in small (SWF; 1–4 mm) and large (LWF; 4–8 mm) white ovarian follicles (A, B); *3βHSD* gene in the granulosa layer of preovulatory follicles (F3−F1) (C, D); and *CYP17A1*, *3βHSD* and *CYP19A1* genes in the theca layer of preovulatory follicles (F3−F1) (E, F) of laying chickens. The NRQ values were standardized to the control expression in each follicle taken as 1 (mean ± SE .; n = 6/group). Values marked with an asterisk within a given follicle differ significantly compared to the control group ($P < 0.05$).

2006; Sawosz et al., 2009; Sikorska et al., 2010; Pineda et al., 2012b), there is a lack of data showing the impact of AgNPs on the female reproductive system and its endocrine function.

The present study's analysis of steroid hormone levels in chickens' blood plasma showed no significant changes after the administration of AgNPs. A lack of effect of nanosilver on E2 and T concentration has also been demonstrated by Mathias et al. (2015) and Sleiman et al. (2013) in male rats. In this experiment, we also determined the effect of AgNPs on the concentration of iodothyronines (T_4 and T_3) in chickens' blood plasma. It was shown that the administration of 50 nm AgNPs with a concentration of 100 ppm for 14 days elevated T_3 levels in the blood plasma, while the T_4 levels remained unchanged. In birds, unlike mammals, the thyroid gland synthesizes almost exclusively T_4, while T_3 originates in the extra-thyroidal conversion of T_4 (deiodination). The results obtained indicate that AgNPs may have a positive effect on the deiodination process of T_4 to T_3, which is mainly catalyzed by type I deiodinase in the liver, kidneys and muscles (Orozco et al., 2012). It cannot be excluded that the increase in T3 in the plasma following AgNPs exposure is related to the inhibition of the expression or activity of deiodinase III, which is involved in T_3 to 3,3′-T_2 conversion (Van der Spek et al., 2017). The presence of AgNPs was found in all of the above-mentioned iodothyronine-metabolized tissues, which may further support the hypothesis regarding the extra-thyroidal activity of nanosilver (Ansari et al., 2016; Chakraborty et al., 2016; El Mahdy et al., 2015). The results obtained indicate that only AgNPs with a size of 50 nm increase the concentration of T_3 in the blood of the birds studied here. This may

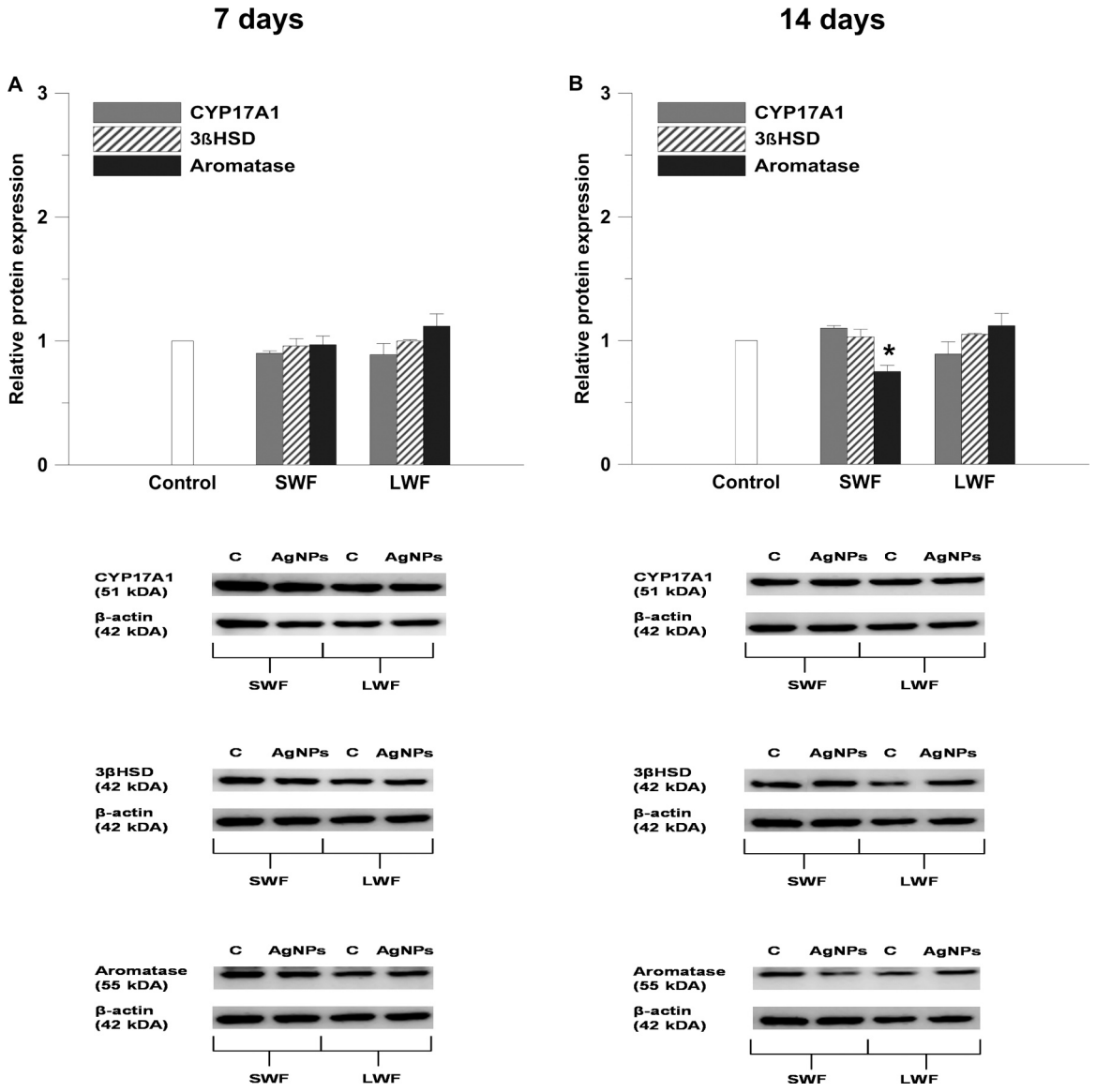

Fig. 2. Effects of silver nanoparticles (AgNPs) administration for 7 (A) or 14 (B) days on protein expression, evaluated by Western blot, of: CYP17A1, 3βHSD and CYP19A1 enzymes in small (SWF; 1–4 mm) and large (LWF; 4–8 mm) white ovarian follicles of laying chickens. The relative quantity values were standardized to the control expression in each follicle taken as 1 (mean ± SE .; n = 4/ group). β-actin served as an endogenous control. Statistically significant values compared to the control group ($P < 0.05$) are marked with an asterisk (*).

be due to the faster and easier endocytosis of nanoparticles of this size into the cells. For small-sized particles, an unfavourable increase in free energy results in a decrease in cellular uptake (Chithrani and Chan, 2007; Gao et al., 2005; Shukla et al., 2005). There are little data available on the effect of AgNPs on the hypothalamic-pituitary-thyroid axis. In line with our results, Saleh and El Magd (2017) showed increased plasma T_3 by AgNPs in broiler chickens. Hinther et al. (2010) have assessed the effect of nanometals on thyroid hormone signaling in tissue obtained from American bullfrog (*Rana catesbeiana*) tadpoles using cultured tail fin biopsy (C-fin) assay, revealing an increase in the basal and T_3-stimulated expression of thyroid receptors β (TRβ) under the influence of AgNPs. The authors suggest that their results are associated with the increased recruitment of TR coactivators to TR-containing transcriptional complexes under the influence of nanosilver. Sulaiman et al. (2018) have shown a significant increase in the weight of the thyroid gland, the disturbance of the normal architecture of the thyroid tissue and decreased thyroxin levels after long-term exposure in female rats. Further studies are needed to clarify the effects of AgNPs on thyroid hormone synthesis, iodothyronine metabolism and/or the molecular mechanisms of their action in the target cells.

The next experiment examined the effect of AgNPs on the mRNA and protein expression of steroidogenic enzymes and steroid hormone concentration in the ovarian tissues. Our results revealed that 7-day administration of AgNPs significantly increased *CYP17A1* mRNA expression in the theca layer of the F3 preovulatory follicle and diminished *HSD3β* expression in LWF follicles. The results obtained after 14-day nanoparticle administration showed significant increases in *CYP17A1* and *CYP19A1* expression in the LWF and theca layers of F3 follicles, respectively. On the other hand, AgNPs inhibited the expression of *CYP19A1* in SWF follicles and *HSD3β* gene in the granulosa layer of the preovulatory follicle F1. There was no noticeable trend regarding changes in the mRNA expression of the examined genes under the influence of AgNPs, except that the longer administration of nanosilver deregulated the expression of more genes and therefore the exposure time was found to be an important factor determining AgNPs action in chickens' ovaries. Modifications of various genes' expression by AgNPs have previously been reported in different species (Griffitt et al., 2012; Hotowy et al., 2012; Sawosz et al., 2012; Song et al., 2017; Wang et al.,

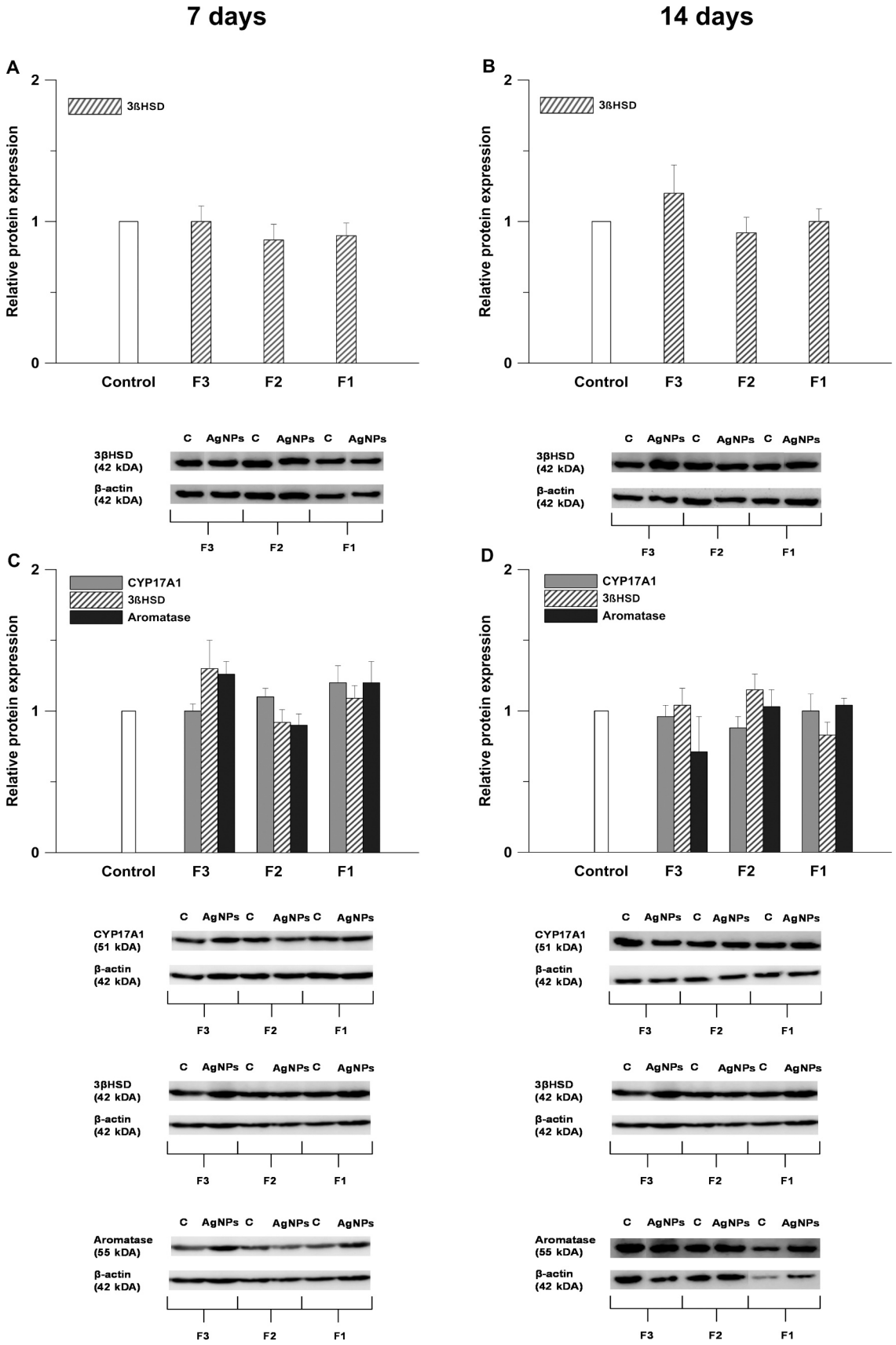

Fig. 3. Effects of silver nanoparticles (AgNPs) administration for 7 (A, C) or 14 (B, D) days on protein expression, evaluated by Western blot, of: 3βHSD enzyme in the granulosa layer of preovulatory follicles (F3−F1) (A, B); and CYP17A1, 3βHSD and CYP19A1 enzymes in the theca layer of preovulatory follicles (F3−F1) (D, E) of laying chickens. The relative quantity values were standardized to the control expression in each follicle taken as 1 (mean ± SE .; n = 4/ group). β-actin served as an endogenous control. Statistically significant values compared to the control group ($P < 0.05$) are marked with an asterisk (*).

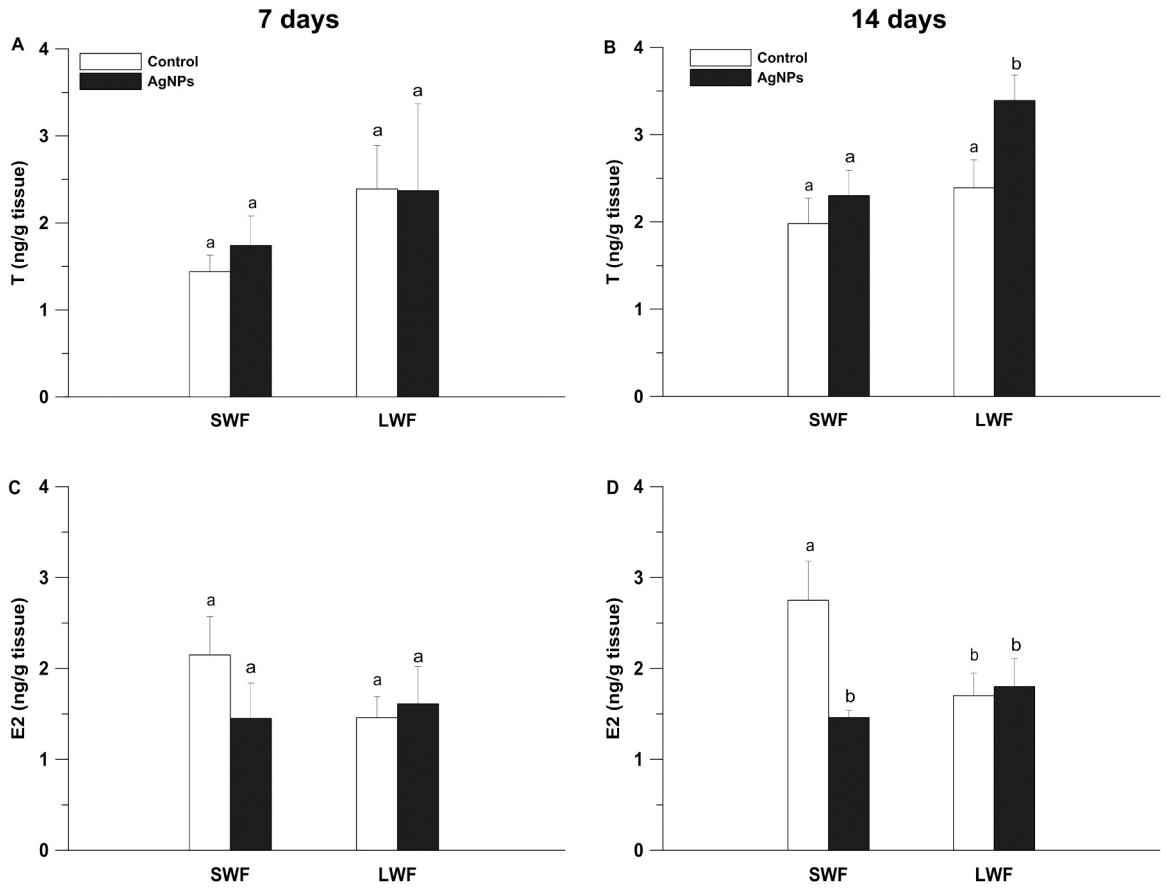

Fig. 4. Effects of silver nanoparticles (AgNPs) administration for 7 (A, C) or 14 (B, D) days on testosterone (T) (A, B) and estradiol (E2) (C, D) concentration in small (SWF; 1–4 mm) and large (LWF; 4–8 mm) white ovarian follicles of laying chicken (mean ± SE; n = 6/group). Hormone concentrations determined by radioimmunoassay (RIA).Values marked with different letters differ significantly ($P < 0.05$).

2020), including genes involved in steroid hormone biosynthesis (Garcia et al., 2014; Han et al., 2016). In our study, the changes in the expression of steroidogenic enzymes at the transcript level were confirmed by protein expression only with respect to the *CYP19A1* enzyme in SWF follicles. Large-scale analyses of mRNA and protein expression conducted by other authors have indicated that the correlation between the amounts of mRNA and protein in the examined tissues is usually low (Sarro et al., 2010; Taquet et al., 2009). Potential reasons for the lack of correlation between changes in mRNA and protein levels are: (i) regulation of translation; (ii) changes in protein half-life; (iii) delay in protein synthesis relative to transcript changes; and (iv) protein transport (Liu et al., 2016; Schwanhäusser et al., 2011). Under constant, defined conditions, the level of protein is largely determined by the concentration of transcripts. On the other hand, during highly dynamic phases such as cell differentiation or stress response, post-transcriptional processes can lead to deviations from the ideal mRNA-protein correlation (Liu et al., 2016). Due to the fact that AgNPs are considered to be oxidative stress-generating factors, we suggest that the lack of correlation between mRNA and protein levels for steroidogenesis enzymes is associated with the post-transcriptional regulation of gene expression and translational processes.

Our data showed that oral exposure to AgNPs significantly affected T and E2 concentration in prehierarchical follicles. The concentration of T in LWF follicles did not change significantly on day 7, while after 14 days of AgNPs administration it increased compared to the control group. These results are consistent with concomitant *CYP17A1* gene expression elevation, which is responsible for the synthesis of androstenedione (A4; substrate for T synthesis) in the theca layer. However, due to the fact that CYP17A1 protein expression did not change significantly, the observed increase in T concentration in LWF follicles was probably the result of the modification of 17β-hydroxysteroid dehydrogenase (*17β-HSD*) mRNA and protein expression. 17β-HSD participates in the conversion of A4 to T. The stimulating effect of AgNPs on T levels has previously been demonstrated in male rats and mice (Cavallin et al., 2017; Garcia et al., 2014). The results presented in this paper revealed a significant reduction in E2 concentration after 14 days of AgNPs administration in SWF follicles, congruent with the observed CYP19A1 mRNA and protein expression decrease. Considering that AgNPs affected the concentration of both hormones in prehierachical follicles and had no effect on their levels in preovulatory follicles (F3−F1), we suggest that prehierachical follicles are more sensitive to AgNPs exposure. It cannot be excluded that by affecting steroids synthesis in this follicular population, AgNPs indirectly affect the processes associated with follicle selection into the preovulatory hierarchy. Furthermore, the effect of nanosilver strongly depends on exposure time, as changes in hormone levels were observed only in tissues from chickens receiving AgNPs for 14 days.

Due to the fact that the synthesis of T and E2 changed only in individual prehierachical follicles, this effect was not large enough to significantly affect the concentration of these hormones in the blood plasma. It also cannot be excluded that AgNPs affect chicken ovarian follicles indirectly by increasing T_3 plasma levels. Nuclear (TRα and TRβ0) and plasma membrane (ITGαVβ3) receptors for thyroid hormones are present in the granulosa and theca layers of chickens' ovarian follicles (Sechman et al., 2009; Sechman, 2013). *In vivo* experiments carried out in laying hens have shown that T_3 injection reduces the level of LH and E2 in the blood and the state of hyperthyroidism caused by multiple T_3 administration reduces the concentration of LH, E2 and P4 (Sechman, 2013). Moreover, using in vitro experiments Sechman et al.

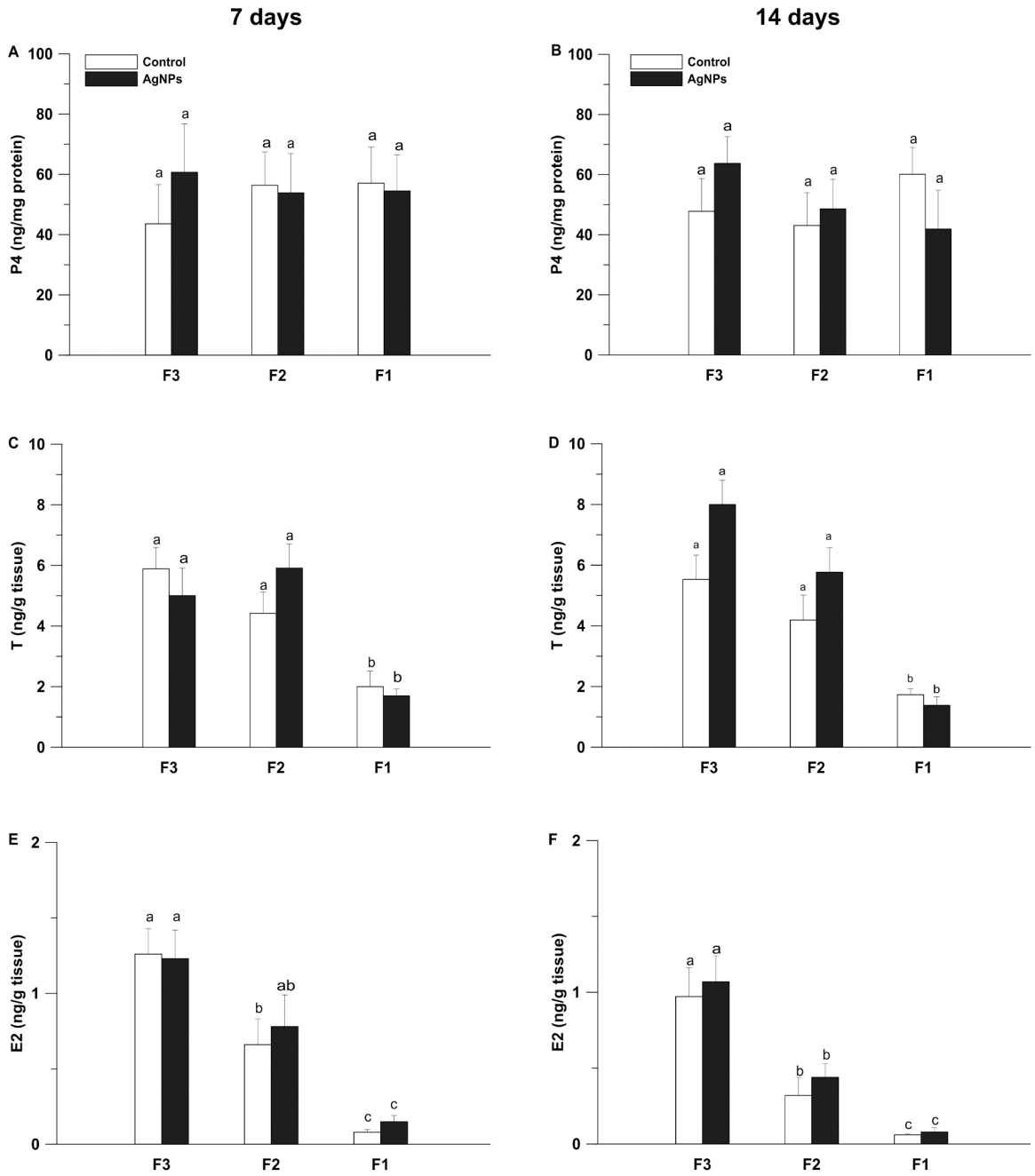

Fig. 5. Effects of silver nanoparticles (AgNPs) administration for 7 (A, C, E) or 14 (B, D, F) days on the concentration of progesterone (P4) (A, B) in the granulosa layer, and testosterone (T) (C, D) and estradiol (E2) (E, F) in the theca layer of laying chicken preovulatory follicles (F3–F1) (mean ± SE; n = 6/group). Hormone concentrations determined by radioimmunoassay (RIA). Values marked with different letters differ significantly ($P < 0.05$).

(2009) showed that the applied dose of 1 ng T_3/ml reduced E2 concentration only in SWF follicles, while the same effect in larger prehierachical follicles and in the theca layer of F3–F1 follicles required a dose of 10 ng T_3/ml. Similar findings were attained in the case of P4 secretion from the granulosa layer of preovulatory follicles (F3–F1), where significant changes were revealed only after the application of 10 ng T_3/ml. In our study, AgNPs increased T_3 concentration up to a maximum of 3 ng/ml, which may be the reason why E2 levels diminished only in SWF follicles, whereas P4 concentration did not change in the preovulatory follicles. The hypothesis regarding the effect of AgNPs on T_3-mediated sex steroid synthesis was also confirmed by the analysis of LH concentration in the blood of chickens receiving AgNPs. Compared to the control group, the administration of AgNPs significantly reduced LH plasma concentration (data not shown). A similar effect has been demonstrated by Sechman (2013) and Sechman et al. (2009) in chickens treated with T_3. The explanation behind the hypothesis presented regarding the iodothyronine-mediated effect of AgNPs on the steroidogenic function of chicken ovarian follicles requires further research.

5. Conclusions

In conclusion, the in vivo effect of AgNPs on the concentration of steroid hormones and the mRNA expression of steroidogenic genes indicates that the chicken ovary is the target site of AgNPs' action and that their effect on ovarian function is associated with the regulation of the steroidogenesis process. Comparing the effects of AgNPs on the E2 and T levels in prehierachical and preovulatory follicles, we suggest that these depend on exposure time, the type of follicle and the degree of its

development, with prehierarchical follicles more susceptible to AgNPs. In addition, observed changes in T_3 plasma levels in chickens receiving AgNPs suggest that AgNPs may affect thyroid hormone metabolism. These results suggest that the exposure of the laying hen to AgNPs disrupt ovarian function as well as may influence energy homeostasis of the target cells.

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CRediT authorship contribution statement

Dorota Katarzyńska-Banasik: Conceptualization, Validation, Formal Analysis, Investigation, Writing - Original Draft, Visualization, Project administration. **Małgorzata Grzesiak**: Investigation, Supervision. **Kinga Kowalik**: Investigation. **Andrzej Sechman**: Supervision, Writing- Reviewing and Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Disclosure statement

The authors report no conflict of interest related to this study.

References

Ahmadi, F., 2012. Impact of different levels of silver nanoparticles (Ag-NPs) on performance, oxidative enzymes and blood parameters in broiler chicks. Pak. Vet. J. 32, 325–328.

Ahmadi, F., Kurdestany, A.H., 2010. The impact of silver nano particles on growth performance, lymphoid organs and oxidative stress indicators in broiler chicks. Glob. Vet. 5, 366–370.

Ansari, M.A., Shukla, A.K., Oves, M., Khan, H.M., 2016. Electron microscopic ultrastructural study on the toxicological effects of AgNPs on the liver, kidney and spleen tissues of albino mice. Environ. Toxicol. Pharmacol. 44, 30–43. https://doi.org/10.1016/j.etap.2016.04.007.

Anwar, M.I., Awais, M.M., Akhtar, M., Navid, M.T., Muhammad, F., 2019. Nutritional and immunological effects of nano-particles in commercial poultry birds. World's. Poult. Sci. J. 75 (2), 261–272. https://doi.org/10.1017/S0043933919000199.

Asare, N., Instanes, C., Sandberg, W.J., 2012. Cytotoxic and genotoxic effects of silver nanoparticles in testicular cells. Toxicology 291, 65–72. https://doi.org/10.1016/j.tox.2011.10.022.

BCC, 2016. BCC Research LLC, ⟨https://www.bccresearch.com/market-research/nanotechnology/nanotechnology-market-products-applications-report.html⟩.

Bilberg, K., Hovgaard, M.B., Besenbacher, F., Baatrup, E., 2012. In vivo toxicity of silver nanoparticles and silver ions in Zebrafish (Danio rerio). J. Toxicol. 2012, 293784–293789. https://doi.org/10.1155/2012/293784.

Bhanja, S.K., Hotowy, A., Mehra, M., Sawosz, E., Pineda, L., Vadalasetty, K.P., Kurantowicz, N., Chwalibog, A., 2015. In ovo administration of silver nanoparticles and/or amino acids influence metabolism and immune gene expression in chicken embryos. Int. J. Mol. Sci. 16 (5), 9484–9503. https://doi.org/10.3390/ijms16059484.

Carew, A.C., Hoque, M.E., Metcalfe, C.D., Peyrot, C., Wilkinson, K.J., Helbing, C.C., 2014. Chronic sublethal exposure to silver nanoparticles disrupts thyroid hormone signaling during Xenopus laevis metamorphosis. Aquat. Toxicol. 159, 99–108. https://doi.org/10.1016/j.aquatox.2014.12.005.

Cavallin, M.D., Wilk, R., Oliveira, I.M., Cardoso, N.C.S., Khalil, N.M., Oliveira, C.A., Romano, R.M., 2017. The hypothalamic–pituitary–testicular axis and the testicular function are modulated after silver nanoparticle exposure. Toxicol. Res. 7, 102–116. https://doi.org/10.1039/c7tx00236j.

Chakraborty, B., Pal, R., Ali, M., Singh, L., Shahidur Rahman, D., Kumar Ghosh, S., Sengupta, M., 2016. Immunomodulatory properties of silver nanoparticles contribute to anticancer strategy for murine fibrosarcoma. Cell. Mol. Immunol. 13, 191–205. https://doi.org/10.1038/cmi.2015.05.

Chauke, N., Siebrits, F., 2012. Evaluation of silver nanoparticles as a possible coccidiostat in broiler production. S. Afr. J. Anim. Sci. 42, 493–497. https://doi.org/10.4314/sajas.v42i5.10.

Chithrani, B.D., Chan, W.C., 2007. Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. Nano Lett. 7, 1542–1550. https://doi.org/10.1021/nl070363y.

Czyż, K., 2011. Praca doktorska: Wpływ nanosrebra na kształtowanie się warunków zoohigienicznych w brojlerni oraz transfer srebra i pierwiastków antagonistycznych (Se, Cu, Zn) do organizmu kurcząt. Uniwersytet Przyrodniczy we Wrocławiu. ⟨http://www.dbc.wroc.pl/Content/10142/Czyz_K_doktor_009_DBC.pdf⟩.

El Mahdy, M.M., Eldin, T.A.S., Aly, H.S., Mohammed, F.F., Shaalan, M.I., 2015. Evaluation of hepatotoxic and genotoxic potential of silver nanoparticles in albino rats. Exp. Toxicol. Pathol. 67, 21–29. https://doi.org/10.1016/j.etp.2014.09.005.

Farzinpour, A., Karashi, N., 2013. The effects of nanosilver on egg quality traits in laying Japanese quail. Appl. Nanosci. 3, 95–99. https://doi.org/10.1007/s13204-012-0097-5.

Gagné, F., André, C., Skirrow, R., Gélinas, M., Auclair, J., van Aggelen, G., Turcotte, P., Gagnon, C., 2012. Toxicity of silver nanoparticles to rainbow trout: a toxicogenomic approach. Chemosphere 89, 615–622. https://doi.org/10.1016/j.chemosphere.2012.05.063.

Gaillet, S., Rouanet, J.M., 2015. Silver nanoparticles: their potential toxic effects after oral exposure and underlying mechanisms – a review. Food Chem. Toxicol. 77, 58–63. https://doi.org/10.1016/j.fct.2014.12.019.

Gangadoo, S., Dragana, S., Hughes, R.J., Moore, R.J., Chapman, J., 2016. Nanoparticles in feed: progress and prospects in poultry research. Trends Food Sci. Technol. 58, 115–126. https://doi.org/10.1016/j.tifs.2016.10.013.

Gao, H., Shi, W., Freund, L.B., 2005. Mechanics of receptor-mediated endocytosis. Proc. Natl. Acad. Sci. U.S.A. 102, 9469–9474. https://doi.org/10.1073/pnas.0503879102.

Garcia, T.X., Costa, G.M.J., Françac, L.R., Hofmann, M.C., 2014. Sub-acute intravenous administration of silver nanoparticles in male mice alters Leydig cell function and testosterone levels. Reprod. Toxicol. 45, 59–70. https://doi.org/10.1016/j.reprotox.2014.01.006.

Gilbert, A.B., Evans, A.J., Perry, M.M., Davidson, M.H., 1977. A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (Gallus domesticus). J. Reprod. Fertil 50 (1), 179–181. https://doi.org/10.1530/jrf.0.0500179.

Griffitt, R.J., Brown-Peterson, N.J., Savin, D.A., Manning, C.S., Boube, I., Ryan, R.A., Brouwer, M., 2012. Effects of chronic nanoparticulate silver exposure to adult and juvenile sheepshead minnows (*Cyprinodon variegatus*). Environ. Toxicol. Chem. 31, 160–167. https://doi.org/10.1002/etc.709.

Grodzik, M., Sawosz, E., 2006. The influence of silver nanoparticles on chicken embryo development and bursa of Fabricius morphology. J. Anim. Feed Sci. 15, 111–114. https://doi.org/10.22358/jafs/70155/2006.

Hadrup, N., Lam, H.R., 2014. Oral toxicity of silver ions, silver nanoparticles and colloidal silver – a review. Regul. Toxicol. Pharmacol. 68, 1–7. https://doi.org/10.1016/j.yrtph.2013.11.002.

Han, J.W., Jeong, J.K., Gurunathan, S., Choi, Y.J., Das, J., Kwon, D.N., Cho, S.G., Park, C., Seo, H.G., Park, J.K., Kim, J.H., 2016. Male- and female-derived somatic and germ cellspecific toxicity of silver nanoparticles in mouse. Nanotoxicology 10, 361–373. https://doi.org/10.3109/17435390.2015.1073396.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, 19. https://doi.org/10.1186/gb-2007-8-2-r19.

Hinther, A., Vawda, S., Skirrow, R.C., Veldhoen, N., Collins, P., Cullen, J.T., van Aggelen, G., Helbing, C.C., 2010. Nanometals induce stress and alter thyroid hormone action in amphibia at or below North American water quality guidelines. Environ. Sci. Technol. 44, 8314–8321. https://doi.org/10.1021/es101902n.

Hotowy, A., Sawosz, E., Pineda, L., Sawosz, F., Grodzik, M., Chwalibog, A., 2012. Silver nanoparticles administered to chicken affect VEGFA and FGF2 gene expression in breast muscle and heart. Nanoscale Res. Lett. 7, 418. https://doi.org/10.1186/1556-276X-7-418.

Iavicoli, I., Fontana, L., Leso, V., Bergamaschi, A., 2013. The effects of nanomaterials as endocrine disruptors. Int. J. Mol. Sci. 14, 16732–16801. https://doi.org/10.3390/ijms140816732.

Katarzyńska-Banasik, D., Grzesiak, M., Sechman, A., 2017. Selection of reference genes for quantitative real-time PCR analysis in chicken ovary following silver nanoparticle treatment. Environ. Toxicol. Pharmacol. 56, 186–190. https://doi.org/10.1016/j.etap.2017.09.011.

Khan, M.S., Jabeen, F., Qureshi, N.A., Asghar, M.S., Shakeel, M., Noureen, A., 2015. Toxicity of silver nanoparticles in fish: a critical review. J. Biodivers. Environ. Sci. 6, 211–217.

Lee, J., Kim, Y., Song, K., Ryu, H., Sung, J., Park, J., Park, H.M., Song, N.W., Shin, B.S., Marshak, D., Ahn, K., Lee, J.E., Yu, I., 2013. Biopersistence of silver nanoparticles in tissues from Sprague–Dawley rats. Part. Fibre Toxicol. 10, 36. https://doi.org/10.1186/1743-8977-10-36.

Li, C., Li, X., Jigami, J., Hasegawa, C., Suzuki, A.K., Zhang, Y., Fujitani, Y., Nagaoka, K., Watanabe, G., Taya, K., 2012. Effect of nanoparticle-rich diesel exhaust on testosterone biosynthesis in adult male mice. Inhal. Toxicol. 24, 599–608. https://doi.org/10.3109/08958378.2012.702140.

Lim, D.H., Jang, J., Kim, S., Kang, T., Lee, K., Choi, I.H., 2012. The effects of sub-lethal concentrations of silver nanoparticles on inflammatory and stress genes in human

macrophages using cDNA microarray analysis. Biomaterials 33, 4690–4699. https://doi.org/10.1016/j.biomaterials.2012.03.006.
Liu, Y., Beyer, A., Aebersold, R., 2016. On the dependency of cellular protein levels on mRNA abundance. Cell 165, 535–550. https://doi.org/10.1016/j.cell.2016.03.014.
Luaibi, N., Qassim, H., 2017. Dose and time dependent effects of silver nanoparticles (AgNPs) on ovarian histology and serum levels of sex hormones in female rats. Pak. J. Biotechnol. 14, 663–672.
Loeschner, K., Hadrup, N., Qvortrup, K., Larsen, A., Gao, X., Vogel, U., Mortensen, A., Lam, H.R., Larsen, E.H., 2011. Distribution of silver in ratsfollowing 28 days of repeated oral exposure to silver nanoparticles or silveracetate. Part. Fibre Toxicol. 8, 18. https://doi.org/10.1186/1743-8977-8-18.
Lu, X., Liu, Y., Kong, X., Lobie, P.E., Chen, C., Zhu, T., 2013. Nanotoxicity: a growing need for study in the endocrine system. Small 9, 1654–1671. https://doi.org/10.1002/smll.201201517.
Mahmoud, U.T., 2012. Silver nanoparticles in poultry production. J. Adv. Vet. Res. 2, 303–306.
Mathias, F.T., Romano, R.M., Kizys, M.M., Kasamatsu, T., Giannocco, G., Chiamolera, M. I., Dias-da-Silva, M.R., Romano, M.A., 2015. Daily exposure to silver nanoparticles during prepubertal development decreases adult sperm and reproductive parameters. Nanotoxicology 9, 64–70. https://doi.org/10.3109/17435390.2014.889237.
Nair, P.M., Park, S.Y., Lee, S.W., Choi, J., 2011. Differential expression of ribosomal protein gene, gonadotrophin releasing hormone gene and Balbiani ring protein gene in silver nanoparticles exposed *Chironomus riparius*. Aquat. Toxicol. 101, 31–37. https://doi.org/10.1016/j.aquatox.2010.08.013.
Orozco, A., Valverde, R.C., Olvera, A., Garcia, G.C., 2012. Iodothyronine deiodinases: a functional and evolutionary perspective. J. Endocrinol. 215, 207–219. https://doi.org/10.1530/JOE-12-0258.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, 45. https://doi.org/10.1093/nar/29.9.e45 7.
Pham, C.H., Yi, J., Gu, M.B., 2012. Biomarker gene response in male medaka (Oryziaslatipes) chronically exposed to silver nanoparticle. Ecotoxicol. Environ. Saf. 78, 239–245. https://doi.org/10.1016/j.ecoenv.2011.11.034.
Philbrook, N.A., Winn, L.M., Afrooz, A.R.M.N., Saleh, N.B., Walker, V.K., 2011. The effect of TiO_2 and Ag nanoparticles on reproduction and development of Drosophila melanogaster and CD-1 mice. Toxicol. Appl. Pharmacol. 257, 429–436. https://doi.org/10.1016/j.taap.2011.09.027.
Pineda, L., Chwalibog, A., Lauridsen, E., Engberg, C., Elnif, R., Hotowy, J., Sawosz, A., Gao, F., Ali, Y., Moghaddam, H.S, A., 2012a. Effect of silver nanoparticles on growth performance, metabolism and microbial profile of broiler chickens. Arch. Anim. Nutr. 66, 416–429. https://doi.org/10.1080/1745039X.2012.710081.
Pineda, L., Sawosz, E., Hotowy, A., Elnif, J., Sawosz, F., Ali, A., Chwalibog, A., 2012b. Effect of nanoparticles of silver and gold on metabolic rate and development of broiler and layer embryos. Comp. Biochem. Physiol. Part A 161, 315–319. https://doi.org/10.1016/j.cbpa.2011.11.013.
Saleh, A.A., El-Magd, M.A., 2018. Beneficial effects of dietary silver nanoparticles and silver nitrate on broiler nutrition. Environ. Sci. Pollut. Res. 25, 27031–27038. https://doi.org/10.1007/s11356-018-2730-7.
Sarro, S.M., Unruh, T.L., Zuccolo, J., Sanyal, R., Luider, J.M., Auer-Grzesiak, I.A., Mansoor, A., Deans, J.P., 2010. Quantification of CD20 mRNA and protein levels in chronic lymphocytic leukemia suggests a post-transcriptional defect. Leuk. Res 34, 1670–1673. https://doi.org/10.1016/j.leukres.2010.06.031.
Sawosz, E., Grodzik, M., Zielińska, M., Niemiec, T., Olszańska, B., Chwalibog, A., 2009. Nanoparticles of silver do not affect growth, development and DNA oxidative damage in chicken embryos. Arch. für Geflügelkd. 73, 208–213.
Sawosz, F., Pineda, L., Hotowy, A., Hyttel, P., Sawosz, E., Szmidt, M., Niemiec, T., Chwalibog, A., 2012. Nano-nutrition of chicken embryos. The effect of silver nanoparticles and glutamine on molecular responses, and the morphology of pectoral muscle. Balt. J. Comp. Clin. Syst. Biol. 2, 29–45. https://doi.org/10.7136/bjccsb.2012.2.0029.
Schwanhäusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., Selbach, M., 2011. Global quantification of mammalian gene expression control. Nature 473, 337–342. https://doi.org/10.1038/nature11848.
Sechman, A., 2013. The role of thyroid hormones in regulation of chicken ovarian steroidogenesis. Gen. Comp. Endocrinol. 190, 68–75. https://doi.org/10.1016/j.ygcen.2013.04.012.
Sechman, A., Pawłowska, K., Rząsa, J., 2009. Influence of triiodothyronine (T3) on secretion of steroids and thyroid hormone receptor expression in chicken ovarian follicles. Domest. Anim. Endocrinol. 37, 61–73. https://doi.org/10.1016/j.domaniend.2009.03.001.
Shukla, R., Bansal, V., Chaudhary, M., 2005. Biocompatibility of gold nanoparticles and their endocytotic fate inside the cellular compartment: a microscopic overview. Langmuir 21, 10644–10654. https://doi.org/10.1021/la0513712.
Sikorska, J., Szmidt, M., Sawosz, E., Niemiec, T., Grodzik, M., Chwalibog, A., 2010. Can silver nanoparticles affect the mineral content, structure and mechanical properties of chicken embryo bones? J. Anim. Feed Sci. 19, 286–291. https://doi.org/10.22358/jafs/66290/2010.
Sleiman, H.K., Romano, R.M., Oliveira, C.A., Romano, M.A., 2013. Effects of prepubertal exposure to silver nanoparticles on reproductive parameters in adult male wistar rats. J. Toxicol. Environ. Health Part A 76, 1023–1032. https://doi.org/10.1080/15287394.2013.831723.
Sulaiman, A.A., Luaibi, N.M., Quassim, A.A., 2018. Effects of silver nanoparticles on thyroid gland structure and function in female rats. Asian J. Pharm. Clin. Res. 11, 509–513.
Song, Z., Lv, J., Sheikhahmadi, A., Uerlings, J., Everaert, N., 2017. Attenuating effect of zinc and vitamin E on the intestinal oxidative stress induced by silver nanoparticles in broiler chickens. Biol. Trace Elem. Res. Dec. 180 (2), 306–313. https://doi.org/10.1007/s12011-017-1016-0.
Taquet, N., Dumont, S., Vonesch, J.L., Hentsch, D., Reimund, J.M., Muller, C.D., 2009. Differential between protein and mRNA expression of CCR7 and SSTR5 receptors in Crohn's disease patients. Mediat. Inflamm. 2009, 1–10. https://doi.org/10.1155/2009/285812.
Vadalasetty, K.P., Lauridsen, C., Engberg, R.M., Vadalasetty, R., Kutwin, M., Chwalibog, A., Sawosz, E., 2018. Influence of silver nanoparticles on growth and health of broiler chickens after infection with Campylobacter jejuni. BMC Vet. Res. 14, 1. https://doi.org/10.1186/s12917-017-1323-x.
Van der Spek, A.H., Fliers, E., Boelen, A., 2017. The classic pathways of thyroid hormone metabolism. Mol. Cell. Endocrinol. 458, 29–38. https://doi.org/10.1016/j.mce.2017.01.025.
Van der Zande, M., Vandebriel, R.J., Van Doren, E., Kramer, E., Herrera Rivera, Z., Serrano-Rojero, C.S., Gremmer, E.R., Mast, J., Peters, R.J., Hollman, P.C., Hendriksen, P.J., Marvin, H.J., Peijnenburg, A.A., Bouwmeester, H., 2012. Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure. ACS Nano 6, 7427–7442. https://doi.org/10.1021/nn302649p.
Walczak, A.P., Fokkink, R., Peters, R., Tromp, P., Rivera, Z.E.H., Rietjens, I.M.C.M., Hendriksen, P.J.M., Bouwmeester, H., 2013. Behaviour of silver nanoparticles and silver ions in an in vitro human gastrointestinal digestion model. Nanotoxicology 7, 1198–1210. https://doi.org/10.3109/17435390.2012.726382.
Wang, Z., Li, Q., Xu, L., Ma, J., Wie, B., An, Z., Wu, W., Liu, S., 2020. Silver nanoparticles compromise the development of mouse pubertal mammary glands through disrupting internal estrogen signaling. Nanotoxicology 13, 1–17. https://doi.org/10.1080/17435390.2020.1755470.
Yaqoob, A.A., Umar, K., Ibrahim, M.N.M., 2020. Silver nanoparticles: various methods of synthesis, size affecting factors and their potential applications – a review. Appl. Nanosci. 10, 1369–1378. https://doi.org/10.1007/s13204-020-01318-w.