***Praca oryginalna*** ***Original paper***

# Diverse effect of *in ovo* treatment with metaloestrogen: selenium on chick testis histology and estrogen signaling

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*Received 10.04.2022* *Accepted 24.06.2022*

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Summary

**With the observed in the last years increasing disruption of poultry males' fertility, there is a need to improve knowledge on the morphology and functioning of the avian reproductive system. A number of molecular and endocrine interrelationships indicate that testicular dysfunction may be one of the reasons for the production of poor semen quality. Because selenium (Se) is known to be an essential element for spermatogenesis, thus the aim of the present study was to determine the effect of Se treatment *in ovo* on cellular and molecular status (expression of aromatase, estrogen receptors and estrogen level) of testes of chicks. The study was performed on chick embryons exposed to Se at a dose of 0.6 µg/egg. We revealed diverse changes in testis histology after Se-treatment including developmental changes reflected by cell migration or vacuolization of the marginal stroma. We showed for the first time the expression of GPER in the testes of chicks and changes in the expression of aromatase, GPER, ERα and ERβ after treatment with Se. Simultaneously with these, decreased testicular estradiol concentration indicated an altered receptor protein and/or binding by Se only to GPER, ERα that in turn affects hormone concentration and action. Further studies are needed to evaluate other effects of Se supplementation especially of immature birds or mature ones and eggs laying by them *per se*.**

**Keywords: testis, chick, selenium, estrogen receptors, estrogens**

The essential functions of the male reproductive system include producing and transporting sperm and producing and secreting steroid hormones. In birds, as in mammals, the formation and maturation of male germ cells in spermatogenesis is defined as a tightly regulated process involving multiple cellular interactions (40, 64). However, in recent years in veterinary practice, there have been many reports of decreased reproductive potential not only in mammals but also in birds. Several molecular and endocrine interrelationships indicate that testicular dysfunction may be one of the reasons for poor semen quality production (9, 54). Sun et al. (58) showed that semen from roosters with reduced sperm motility and fertility is characterized by decreased ejaculate volume or a varying degree of tissue disorganization, marked by a shortened diameter

of seminiferous tubules and a reduced thickness of the tubular epithelium. However, the hormonal regulation that lies in the control of molecular mechanisms of these processes in birds remains scarcely known. This may significantly affect the performance of the artificial insemination process, which is common practice in the poultry industry (8, 62).

Androgens are the main male hormones; therefore, any disturbances in concentrations of their metabolites, estrogens, results in severe perturbations of the reproductive function (10, 20). Observations of decreased sperm number and motility in men with genetic deficiency in estrogen synthase-aromatase, together with an animal model data confirm a crucial role for aromatase and estrogens during the development and maintenance of spermatogenesis and final maturation of spermatozoa (6). Importantly, elevated endogenous estrogen levels are often detected in individuals with structural and functional disorders of the reproductive system, such as cryptorchidism or azoospermia (21). The cellular response to estrogen depends not only on the levels of these hormones but also on the presence and localization of specific receptors. The effects of estrogens on cells are mediated by the intracellular estrogen receptors α (ERα, estrogen receptor α) and β (ERβ, estrogen receptor β) (20). Studies investigating the role of sex hormones and androgen receptor expression in turkey testes, and describing the age-dependent distribution of estrogen receptors in rooster testes, demonstrate the importance of steroid hormones in the regulation of the gonadal function in birds and indicate that hormone-receptor interaction may be an important factor in maintaining spermatogenesis and normal male fertility (15, 29). In addition, a study on domestic goose testes showed a reproductive season-dependent expression pattern for ERα and ERβ. This differential distribution of ERs may be related to the regulation of reproduction in sexually mature individuals or the activity of the testes related to the age of the animal. Moreover, it is now known that estrogens may also exhibit a non-classical, extra-genomic mechanism of action that involves activation of the membrane G protein-coupled estrogen receptor (GPER) and a rapid enzymatic cascade involving multiple secondary messengers (13). The presence of GPER has been detected in both spermatogenic cells, including spermatozoa and somatic cells (17, 44), confirming the effect of GPER on the regulation of proliferation and apoptosis of spermatogenic cells and steroidogenesis (7). However, there are still many unknowns about the role of GPER in the testes and other tissues of the male reproductive system.

Diseases related to mineral and element overload or deficiency are commonly and partially described in large animal veterinary practice. One of the essential trace elements for mammals is selenium (Se) (24). Importantly, in many parts of Europe and North America low Se concentrations are applied in locally grown feed (2, 24). A common disorder associated with selenium deficiency in calves, lambs, kids, or foals is white muscle disease or nutritional muscular dystrophy which leads to hyaline degeneration of muscle cells in various skeletal muscles and changes in the frequency and quality of heartbeat (23). Although the impact on animal health is substantial, the clinical evidence of Se excess or deficiency may be complicated by concurrent other vitamins (27). Recently, some reports demonstrated a positive effect of Se on the function of mammalian male reproductive tissues especially through reduction of the level of reactive oxygen species (43, 53). Selenium is named metaloestrogen, as it was shown to interact with estrogen signaling through the estrogen receptor α (ERα) in breast cancer cells (56). During the past decades, the poultry industry has developed in several areas like nutrition, genetics, or engineering, to maximize the efficiency of growth performance and meat yield (57). One of the needs for poultry nutrition supplementation is Se (59). It is an essential trace element of bird reproduction and its application is now commonly practiced in the poultry industry; however, its bio-efficacy depends on its chemical form (54, 60). Importantly, numerous findings indicate that Se can accumulate in high concentrations in reproductive organs and account for an essential element for the spermatogenesis and function of steroidogenic cells (3, 4, 19). Data on the effect of dietary supplementation with organic Se on broiler breeders suggest that Se improves semen quality and production, which correlated with earlier development of secondary sexual characteristics (11). Furthermore, a study by Li et al. (35) on dietary Se-deprived chicks demonstrated poor testicular development, impaired sex hormone synthesis, decreased antioxidant enzyme activity, and increased expression of mRNA and protein of autophagy-related factors. However, the impact of Se on estrogen signalling involved in spermatogenesis, normal sperm maturation, and proper functioning of Avian reproductive tissues is still poorly understood.

In this context, the following experiments were designed to demonstrate the effect of Se treatment *in ovo* on the cellular and molecular status, including the expression of aromatase, estrogen receptors, and the estrogen level of the testes of chicks.

## Material and methods

**Animal, incubation and tissue preparation.** The hatching eggs [57.3 ± 2.9 g (mean ± SD)] of a 29-week-old parental stock of broiler Cobb 500 line (Cobb Germany, Avimex GmbH; Slawomir Domagala's Poultry Farm, Golaczewy, Poland) were incubated in the laboratory incubator (Iglo-Tech, Poland) at temperature (T) 37.8 ± 0.1°C and relative humidity (RH) 50 ± 1% between 1. and 18. – incubation day (E1-E18), and at T 37.2 ± 0.1°C and RH 55-70% during the hatching period (E19-E21). On six days of incubation (E6), the eggs were burned for confirmation of embryo development and the embryonated ones were randomly divided into

two equal groups: control group (C) and selenium group ($Se^{2+}$) (n = 28 eggs per group). The eggshell surface was then disinfected with 70% ethanol, and a hole was made above the air cell using an 18G needle (1.20 mm × 40 mm). The solution (100 µl) of Se (administrated as $Na_2SeO_3 \times 5\ H_2O$, Sigma-Aldrich, USA) at dose 0 or 0.6 µg Se per egg (based on literature data: 28, 49, 52, 53) dissolved in 0.7% saline solution were *in ovo* injected into the albumen using an insulin syringe (1 ml) with a G20 needle (0.9 mm × 40 mm), according to the method described previously (5). After the injections, the holes were sealed with hot paraffin, and incubation was continued.

Testes were obtained immediately post-mortem from one-day-old chicks. For histology, the testes were fixed in Bouin fluid (saturated picric acid, formaldehyde, glacial acetic acid in a 15 : 5 : 1 ratio) for 24 h, dehydrated in an increasing gradient of ethanol, and embedded in paraffin. After mounting on slides, all sections (5 µm) were deparaffinized and rehydrated by decreasing alcoholic solutions cleared in xylene, and rehydrated in a series of grades of ethanol. Other tissue fragments were frozen in liquid nitrogen and stored at –80°C for protein extraction and determination of hormone concentrations. According to Directive 2010/63/EU, the experimental and animal procedures used in this study did not need to be approved by the Local Animal Ethics Committee.

**Morphology.** For histology, hematoxylin-eosin staining (H-E) was performed as described previously described (Slowinska et al. 2021) (55). Fifty cross-sections were examined under a Nikon Eclipse Ni microscope (Nikon).

**Estradiol Concentration Measurement.** The amount of estradiol produced by the chick testes in the control group and selenium group was determined by the direct Estradiol Enzyme Immunoassay Kit (DRG, Marburg, Germany; cat no EIA 2693), according to the manufacturer's instructions. The sensitivity of the assay was 10.60 pq/mL Estradiol concentration was calculated as the mean ± SD (from three separate quantitative determinations) as pg/mL. Three independent experiments were performed, each in triplicate.

**Western blot analysis.** For Western blot analysis, tissue collected from the individual males was pooled and testes samples were homogenized on ice with radio immune precipitation assay buffer (RIPA, Thermo Scientific) with protease and phosphatase inhibitor cocktails (Sigma-Aldrich), sonicated, and centrifuged as described by Kotula-Balak et al. (30). After denaturation, the samples were subjected to electrophoresis on SDS-PAGE gels under reducing conditions and transferred onto a PVDF membrane (Millipore). Nonspecific binding sites were blocked (BSA or non-fat milk) and the membrane was incubated with the respective primary antibodies (Tab. 1). The membranes were then incubated with appropriate secondary antibodies linked to the horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA). Immunoreactivity protein was detected using chemiluminescence and images were captured with transilluminators (GBOX Chemi XRQ Syngene). β-actin (Merck, Invitrogen) was used as a loading control. Molecular masses were estimated by reference to standard proteins (Bio-Rad). For densitometric analyzes, immunoblots were scanned using the public domain ImageJ software (NIH, Bethesda, MD, USA).

**Tab. 1. Primary antibodies used in this study**

| Antibody | Host species | Vendor | Catalog no | Dilution(s) |
|---|---|---|---|---|
| Aromatase | rabbit | BT LAB | DF6884 | WB (1 : 500) |
| GPER | rabbit | Sigma-Aldrich | PA5-67 864 | WB (1 : 500) |
| ERα | rabbit | BT LAB | BT-AP03078 | WB (1 : 500) |
| ERβ | rabbit | BT LAB | BT-AP03082 | WB (1 : 500) |
| β-actin | rabbit | Invitrogen | 71-2700 | WB (1 : 2000) |

**Statistical analysis.** Each variable was tested using the Shapiro-Wilk W-test for normality. The homogeneity of variance was assessed with Levene's test. Statistical differences were determined using the non-parametric Mann-Whitney U-test. The analysis was performed using Statistica 10 (Statsoft, Poland). Data were presented as mean ± SD. Data were considered statistically significant at $P < 0.05$.

## Results and discussion

It is well known that commercial poultry production is associated with several factors that challenge the future growth of the poultry industry like poultry immunity, health, and breeding. Due to the expanding problem of increasing fertility disorders and decreasing semen quality, the protection of farm animal genetic resources is an essential challenge to maintain domestic biodiversity and adaptation of animal species to global changes or breeding circumstances or diseases (39, 47). In this study, the effect of Se impact on *in ovo* development of testes, as well as the function of immature testes in chicks, was evaluated both at the cellular and molecular level.

In testes of control chicks, proper testicular histology was observed: non-active seminiferous tubules lined with a single layer of gonocytes and located in between the tubules with abundant interstitial tissue indicate no terminated development of this tissue in newborn chicks (Fig. 1A). After Se-treatment, occasionally some groups of cells migrating within testicular tissue (open arrows depicted stained in light blue cells; Fig. 1B-D) or vacuolization of the margin testicular stroma (closed arrows; Fig. 1C-D) were seen. Supplementation with Se has been reported to improve the testicular characteristics and semen quality of Saanen bucks (37). Moreover, it was found that the use of nano-Se in the ration of Japanese quail improves several reproductive performance parameters (12). Furthermore, Se caused a significant improvement in sperm count and quality in varicocelized rats (61). Sahu et al. (49) demonstrated that 8 weeks of combined treatment of Se with zinc reduced diabetes-induced germ cell damage in rat testes. However, at the same time, in cauda epididymis of rats alterations in tubule morphology and abnormal spermatozoa were detected (26). Similarly, Se deficiency caused disruption of testes development and autophagy in chick testes (35). In testes of male rats deficient in Se, both morphology (sperm quality and quantity) and spermatogenic function were affected (1, 65). In addition, the mRNA abundance of glutathione peroxidase

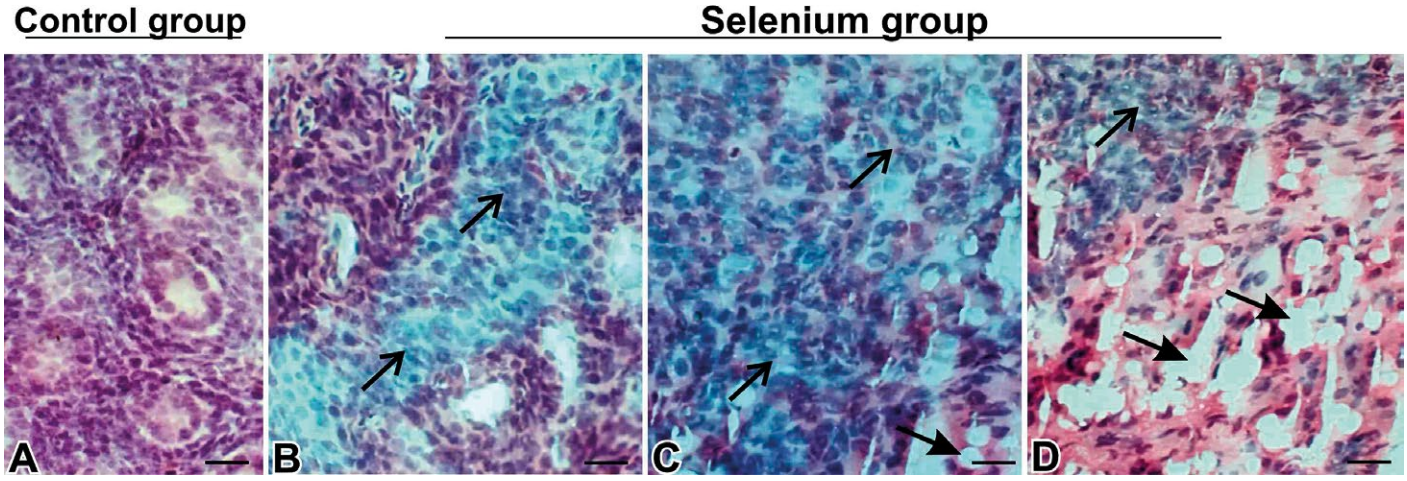


**Fig. 1. Microphotograph of testis (control and selenium groups) cross-sections stained with H-E (A-D). Groups of cells migrating within testicular tissue (open arrows), vacuolization of the margin testicular stroma (closed arrows). Staining was performed at three serial sections from each testis. Bars = 10 µm**

and selenoprotein P was decreased. This indicates possible spermatogenesis perturbations at the cellular and molecular levels in the chicks studied here after Se treatment. Recent studies reported interesting data that Se supplementation influenced on selenoproteins driven by gut microbiota in mouse testes (46). Moreover, numerous reports indicate that the spectrum of non-specific, subacute disorders in farm animals in response to Se concentration alterations besides fertility disorders also includes impaired immune response as well as impaired resilience during parasitic, bacterial and viral diseases (22, 33). It should be emphasized that our results are in line with the earlier mentioned findings that pointed out that Se can exert differential structural functional changes in formatting testis of chicks that may affect the function later in life.

Selenium-treated testes exhibited decreased estradiol concentrations ($P < 0.5$) together with a statistically significant decrease ($P < 0.5$) in aromatase expression compared to the control (Fig. 2). In contrast, a higher expression of either GPER or ERα was revealed ($P < 0.5$) (Fig. 2), but there was no significant change in ERβ expression. The decreased expression of aromatase and estrogen receptors can also act vice versa on endogenous estrogen levels. In addition, these results indicate possible Se effects on GPER and ERα protein and/or on Se binding to these receptors but not ERβ. Studies of mammalian and non-mammalian vertebrate species, including birds, revealed that the metabolism of androgens to estrogens, so-called aromatization, is crucial for male reproduction and fertility and plays a key role in gonadal sex differentiation during development (41, 48). Moreover, any change in the balance of steroid hormones within the seminiferous tubule can result in altered sperm parameters as previously reported earlier by the group of Carreau (50) and our own (31). Kwon et al. (34) demonstrated that germ cells of the male rooster reproductive system contain immunoreactive aromatase. Studies investigating the role of gonadotropins, sex hormones, and androgen receptor expression in the testes of turkeys and describing the age-dependent distribution of estrogen receptors in

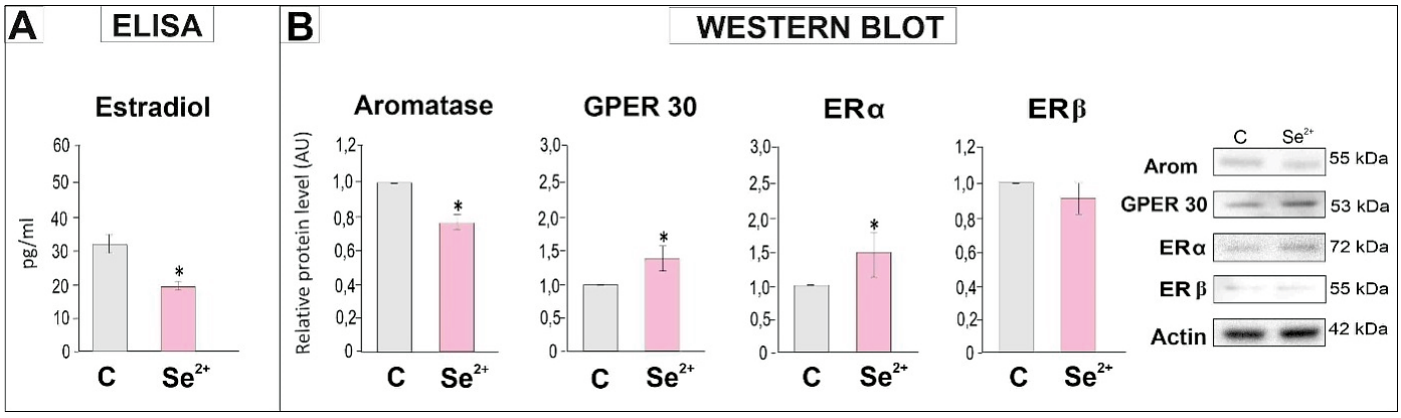


**Fig. 2. Hormonal concentration, aromatase level, as well as estrogen receptor expression in chicks testes. (A) Estradiol concentrations within control (C) and Se-treated testes ($Se^{2+}$). (B) Representative blots of Western analysis and relative expression of aromatase, GPER, ERα and ERβ proteins in testes of control (C) and Se groups ($Se^{2+}$). Densitometric analysis of protein content was normalised against corresponding actin data point. Data obtained from three separate analyses are expressed as means ± SD. Asterisks indicate statistically significant differences (*$P < 0.05$). Each control and Se tissues samples (n = 5 and n = 5, respectively)**

the testes of the rooster demonstrate the importance of steroid hormones in the regulation of gonadal function in birds and indicate that the hormone-receptor interaction may be an important factor in maintaining spermatogenesis and normal male fertility (16, 29). Results of our previous study on turkeys with YSS showed a non-homogeneous distribution of aromatase and its markedly enhanced expression levels in all reproductive tissues and sperm compared to males producing normal semen, which may reflect a higher endogenous synthesis of estrogens in males with abnormal yellow semen (42). In the testes of adult roosters, estrogen is secreted by interstitial Leydig cells and immature germ cells (20). In developing embryonic gonads, the expression of ERα is differentially higher in females, while ERβ is expressed indifferently in the two sexes (51). Interestingly, the early embryonic gonads (bipotential gonads) express ERα in the left but not in the right gonads of both sexes before gonadal differentiation (38). In embryonic day 5, the expression of ERα gradually diminishes in males. In adult testes and epididymides, varying amounts of ERα (highest in the testes) are present, reflecting its importance in the reproductive development of chickens during later stages of life (16, 45). These findings facilitate the argument that the ERα receptor is more sex specific in chickens.

Selenium was demonstrated as a disruptor of ER in breast cancer cells (52, 56). Khalaf et al. (28) have found that Se protects against bisphenol-induced reproductive toxicity in male rats. Moreover, studies describing a positive correlation between plasma Se levels and estrogen fluctuation in pre-menopausal women and higher Se concentration in ovariectomized and estrogen-replaced rats indicate a direct effect of estrogen on Se tissue status (18, 66). Interestingly, in polycystic ovary syndrome, androgens in seminal plasma are elevated, and normal plasma Se levels were observed (64). Moreover, an estrogen-mediated increase in Se was detected, but its mechanisms remains unknown. We for the first time showed here that also GPER is affected by Se in the testes of chicks. Observed histological alterations may be a direct result of perturbation of estrogen signaling *via* GPER and ERα. It was revealed that after blocking of estrogen action in fetal chick gonad development of seminiferous tubules is halted (14). *In ovo* estradiol treatment induced expression of ERα in left chick testis (51). It is also possible that GPER may interact with ER receptors, in various tissues, thus cooperating in the regulation of numerous tissue and cellular processes (25, 32). Therefore, our results may also be the common effect of Se and decreased estradiol level on expression of both estrogen receptors. Furthermore, it has been shown, that in immature rat Sertoli cells of spermatogenic epithelium activation of GPER/EGFR/MAPK signalling cascade causes an increase of anti-apoptotic gene BCL-2 and decreases of proapoptotic gene BAX expression, which indicates that estradiol has an anti-apoptotic effect induced by GPER (36). Thus, we cannot exclude the involvement of ERα or ERβ in the proliferation of testicular cells. On the other hand, vacuolization detected in the marginal stroma of the chick testes after Se may also be a result of a decreased level of estradiol and Se action on GPER and ERα and/or through these receptors but not ERβ.

In conclusion, our data clearly show that Se supplementation during the early days of chick development can have both positive and negative impacts on testicular formation and function in birds that later in life will result in the functioning of either immature or mature testes. As such, these findings open up new perspectives for further studies of the effect of various forms of Se on the reproductive tissues of birds, which may be useful for finding the most effective sources of organic Se for commercial use and, as a result, lead to improved fertility of domestic birds.

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