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Effects of *in ovo* injection of nano-selenium and nano-zinc oxide and high eggshell temperature during late incubation on antioxidant activity, thyroid and glucocorticoid hormones and some blood metabolites in broiler hatchlings

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ABSTRACT. This experiment was conducted to evaluate the effects of *in ovo* injection of nano-selenium (Nano-Se) and nano-zinc oxide (Nano-ZnO) and high eggshell temperature (EST) during late incubation on blood parameters of broiler hatchlings. A total of 750 fertile eggs, were weighed and randomly distributed among 5 treatment groups on each of 5 replicate tray levels. The injection was performed on 17 d of incubation. Treatments included of: 1) Eggs not injected and incubated at normal EST (control); 2) Eggs not injected and incubated at high EST; 3) Eggs injected NaCl solution and incubated at high EST (sham); 4) Eggs injected NaCl solution containing 40 µg Nano-Se and incubated at high EST; 5) Eggs injected NaCl solution containing 500 µg Nano-ZnO and incubated at high EST. EST of 37.8ºC (normal) or 38.9ºC (high) was applied from d 19 to 21 of incubation. *In ovo* injection of Nano-Se and Nano-ZnO significantly increased activity of GSH-Px and SOD and total protein, but decreased the levels of corticosterone, cortisol, T4 and T3 at high EST. Injection of Nano-Se and Nano-ZnO had a significant role in alleviating the negative effects of high temperature incubation and heat stress by increased antioxidant activity and reduced oxidative stress.

Keyword: *in ovo* injection; Nano-selenium; Nano-zinc oxide; high eggshell temperature; blood.

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Introduction

Temperature is a critical factor affecting embryonic development and growth. Numerous studies have investigated the effects of different incubation temperatures on improving thermotolerance and its consequences (Al-Zhgoul et al., 2013; Morita et al., 2016; Piestun, Halevy, & Yahav, 2009). High incubation temperatures can negatively affect hatchability (Sozcu & Ipek, 2015), chick quality (Willemsen et al., 2011), and post-hatch growth performance (Sozcu & Ipek, 2015). High incubation temperature results in changes in hormonal and metabolic regulations of chick embryos. Thyroid hormones and glucocorticoids are implicated in the acclimatory response to heat stress. Thyroid hormones play a major role in the embryonic growth as well as maturation of the organs during late incubation (Christensen et al., 2005). Glucocorticoids are released from the adrenal cortex in response to stress, which affects metabolic pathways, immune function, and endocrine systems. High temperature cause more stress during late incubation; Indeed, with the onset of pulmonary respiration after internal pipping in the chick embryo, the presence of reactive oxygen species (ROS) and oxidative stress is higher (Moran Junior, 2007), while natural antioxidant reserves have not reached an adequate level for innate protection (Yigit, Panda, & Cherian, 2014). Sahin, Sahin, Kucuk, Hayirli, and Prasad (2009) reported that activity of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) is reduced in poultry under heat stress conditions, thus increasing the need for vitamins and minerals.

Both selenium and zinc are essential trace elements in poultry that are important for many normal biologic functions. These elements as the antioxidant can protect the cells from damage caused by oxidative

stress. Selenium is an important and effective part of the selenoenzymes, including GSH-Px, thioredoxin reductase (TrxR), and iodothyronine deiodinase (Duntas, 2010). Likewise, Zinc is an essential component in SOD (Surai, 2016). GSH-Px and SOD are involved in the first line of antioxidant defense (Yigit et al., 2014) and iodothyronine deiodinase plays a major role in the metabolism of thyroid hormones, including the conversion of T4 to T3 (Debonne et al., 2008).

In general, increased temperature during incubation is the main source of stress before hatching, and it affects the development of embryonic organs. Due to the variable and unpredictable antioxidant status of hatching eggs, *in ovo* injection of some nutrients that can potentially affect oxidative stress, alleviating deleterious effects of stress and can beneficially influence on post-hatch growth performance and well-being of broiler chickens (Hajati et al., 2014). *In ovo* administration of nutrients is a possible way to provide more nutrients for the chick embryos (Kucharska-Gaca, Kowalska, & Dębowska, 2017). Recently, numerous studies focused upon the use of nutrients in form of nanoparticles in poultry nutrition. Nanoparticles have new features, including great surface area, high surface activity, high catalytic efficiency, strong adsorbing ability, and low toxicity (Zhao et al., 2014). *In ovo* injection of nanoparticles can be considered as a novel method of nano-nutrition, supplying more nutrients for the chick embryo (Joshua, Valli, & Balakrishnan, 2016).

So far, few studies have been conducted on *in ovo* injection of nanoparticles, particularly selenium and zinc. Furthermore, as far as we know, there are no reports on *in ovo* injection of nanoparticles and their effects on heat stress during incubation. We hypothesized that *in ovo* injection of selenium nanoparticles (Nano-Se) and zinc oxide nanoparticles (Nano-ZnO) could reduce or eliminate the negative effects caused by high eggshell temperature (EST) during late incubation. Therefore, the objective of this study was to evaluate the effect of *in ovo* injection of Nano-Se and Nano-ZnO on antioxidant status, thyroid and glucocorticoid hormones, and some blood metabolites in broiler hatchlings exposed to high EST during late incubation.

Material and methods

Incubation and experimental design

Experiments were carried out at a commercial hatchery complex (Sabzdasht Agro Industrial Co., Abhar, Zanjan, Iran). All eggs were incubated under standard conditions (T = 37.8°C, RH = 55-60%) from 1 to 18 days of incubation (doi) in a Jamesway model PS 500 setter unit (Jamesway Incubator Company Inc., Cambridge, Canada). In this study, the nanoparticle levels were based on other studies conducted on *in ovo* injection on antioxidant effects and low toxicity in fertile eggs (Yair & Uni, 2011). On 16th doi, infertile eggs and eggs containing dead embryos were removed by candling, and then 750 fertile broiler eggs (Cobb 500) from 58-week-old breeders were individually weighed and randomly assigned to experimental treatments. Each treatment consisted of 5 replicates and each replicate containing 30 eggs with average egg weight of 60.2 ± 4. The injection was performed on 17th doi (400h). Treatments including: 1) Eggs not injected and incubated at normal EST (37.8ºC) (control) 2) Eggs not injected and incubated at high EST 3) Eggs injected with 0.5 mL of 0.9% NaCl and incubated at high EST (sham) 4) Eggs injected with 0.5 ml of 0.9% NaCl containing 40 µg Nano-Se and incubated at high EST. 5) Eggs injected with 0.5 ml of 0.9% NaCl containing 500 µg Nano-ZnO and incubated at high EST.

Before the injection, the wide end of the eggs was disinfected with 96% ethanol. The fertile eggs were candled to determine the injection sites and a small hole in the wide end of the egg was made with a 21G needle, and the injections were performed at an approximate depth of 25 mm from the eggshell into amniotic fluid. After injection, the holes were sealed with sterile wax, and then the eggs were returned to the incubator. On 19th doi (444h), the eggs were transferred to two Jamesway model PS 500 hatcher units (Jamesway Incubator Company Inc., Cambridge, Canada). During the last three days of incubation, the relative humidity was maintained at 60-65%. Treatments were exposed to high temperature from 19 to 21 doi except for control treatment. The EST in one hatcher was set to 37.8°C (control group) and in the other to 38.9°C until 21 doi. During the manipulations, the EST was measured every 12 h with an infrared digital thermometer (IRT 6020, Thermoscan, Braun, Germany) and the machine temperature was adjusted to maintain the target EST in each treatment. The physiological requirement in trace mineral is low (Surai, Fisinin, & Karadas, 2016) and the requirement increase under hyperthermic conditions (Rama Rao et al., 2016) and thus, Nano-Se and Nano-ZnO injection was considered only at high EST.

Preparation of nano forms of zinc oxide and selenium and their characterization

The nanoparticles were suspended in distilled water containing 0.9% sodium chloride, stirred with a magnetic stirrer for 5 minutes and then dispersed by ultrasonic vibration for 20 min. The solutions were placed in an autoclave under conventional sterilization conditions (121 C for 20 min).

Sampling, Measurements, and Analysis

Blood samples (10 chicks/treatment) were collected via cardiac puncture into six mL heparinized tubes 12h after hatching. The blood samples were centrifuged (1238×g for 10 min at 4°C) to obtain plasma, and the individual plasma samples were stored at −20°C (Willemsen et al., 2011).

The thyroxine (T4) and triiodothyronine (T3) concentrations in plasma were measured using the T4 ELISA kit (Abnova, KA4013, Taipei, Taiwan) and the T3 ELISA kit (Abnova, KA0925, Taipei, Taiwan). The level of Corticosterone in blood was determined using Enzyme Immunoassay kit (K014-H1) from ARBOR ASSAYS Company (Ann Arbor, USA) and the blood cortisol was measured by ELISA Kit (K7430-100) from Biovision incorporated Company (San Francisco, USA).

The activities of GSH-Px and SOD in blood were quantified using the Glutathione Peroxidase Activity Colorimetric Assay Kit (Biovision K762-100, San Francisco, USA) and the Superoxide Dismutase Activity Assay Kit (Biovision K335-100, San Francisco, USA) following the manufacturer's instructions.

Total protein of plasma samples was determined according to the Biuret method as described by Doumas et al. (1981). Plasma levels of glucose, triglyceride, cholesterol, and total protein were evaluated by Glucose Assay Kit (Abcam, ab65333, Cambridge, UK), Triglyceride Colorimetric Assay Kit (Cayman Chemical, 10010303; Ann Arbor, USA), Total Cholesterol and Cholesteryl Ester Colorimetric/Fluorometric Assay Kit (Biovision K603-100, San Francisco, USA), and (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Statistical analysis

The data were subjected to analysis of variance using the general linear model procedure of SAS software package (Statistical Analysis Software [SAS], 2004). Means were compared using Duncan's test. Statistical significance was determined at $p < 0.05$.

Results

Antioxidant activity

The activities of GSH-Px and SOD in the plasma of broiler hatchlings are shown in Table 1. The high EST during late embryogenesis significantly reduced the activity of GSH-Px ($p = 0.003$) and SOD ($p = 0.006$) compared to the control. The GSH-Px ($p = 0.172$) and SOD ($P = 0.258$) had no significant difference between the high EST without injection and sham groups. Injection of Nano-Se and Nano-ZnO significantly enhanced the activity of GSH-Px ($p = 0.003$) and SOD ($p = 0.006$) under high temperature conditions of incubation.

Table 1. Effect of *in ovo* injection of Nano-Se and Nano-ZnO and high EST during late incubation on antioxidant activity of GSH-Px and SOD in broiler hatchlings.

Experimental Treatments	GSH-Px (Iu mg^{-1}.pr)	SOD (Iu mg^{-1}.pr)
Control	2.670^{c}	3.536^{c}
High EST without injection	1.742^{d}	2.810^{d}
Sham	1.858^{d}	2.716^{d}
Nano-ZnO	3.762^{a}	4.162^{b}
Nano-Se	3.302^{b}	4.402^{a}
SEM	0.058	0.056
P-value	0.003	0.006

^{a,b,c,d} Means in a column with different superscripts are significantly different ($p < 0.01$).

Plasma hormone levels

The effects of *in ovo* injection of nanoparticles and high EST on the plasma levels of hormones of broiler hatchlings are shown in Table 2. Embryos exposed to high EST showed a significant increase in levels of plasma glucocorticoid hormones ($p < 0.01$). Nano-Se and Nano-ZnO significantly reduced concentrations of

corticosterone (p = 0.007) and cortisol (p = 0.009). The lowest level of cortisol was related to Nano-ZnO, which did not have a difference with the control (p = 0.083).

Table 2. Effect of *in ovo* injection of Nano-Se and Nano-ZnO and high EST during late incubation on plasma cortisol, corticosterone, T4, and T3 in broiler hatchlings.

Experimental Treatments	Cortisol (ng mL^{-1})	Corticosterone (ng mL^{-1})	T4 (ng mL^{-1})	T3 (ng mL^{-1})	T3/T4
Control	0.872^{c}	4.724^{d}	1.780^{d}	2.630^{d}	1.48^{a}
High EST without injection	1.252^{a}	11.042^{a}	4.592^{a}	5.120^{a}	1.11^{c}
Sham	1.300^{a}	10.868^{a}	4.684^{a}	5.032^{a}	1.07^{c}
Nano-ZnO	0.786^{c}	7.796^{b}	3.556^{b}	3.410^{c}	0.96^{d}
Nano-Se	0.986^{b}	6.316^{c}	3.050^{c}	3.854^{b}	1.26^{b}
SEM	0.034	0.061	0.063	0.077	0.028
P-value	0.009	0.007	0.006	0.006	0.006

^{a,b,c,d} Means in a column with different superscripts are significantly different (p < 0.01).

Blood metabolites

As shown in Table 3, high incubation temperature significantly increased glucose, triglyceride and cholesterol concentrations and decreased total protein. Administration of nanoparticles significantly reduced the concentration of cholesterol (p = 0.009), triglyceride (p = 0.006) and glucose (p = 0.007) and increased total protein (p = 0.004) in embryos incubated at 38.9° C EST (p < 0.01). In control group, glucose and triglyceride concentration was significantly lower than other groups.

Table 3. Effect of *in ovo* injection of Nano-Se and Nano-ZnO and high EST during late incubation on plasma concentrations of Glucose, Total Protein, Triglyceride, and Cholesterol in broiler hatchlings.

Experimental Treatments	Glucose (mg dL^{-1})	Total Protein (mg dL^{-1})	Triglyceride (mg dL^{-1})	Cholestrol (mg dL^{-1})
Control	169.70^{c}	4.050^{b}	43.25^{d}	111.50^{c}
High EST without injection	248.00^{a}	3.472^{c}	89.90^{a}	148.30^{a}
Sham	245.80^{a}	3.428^{c}	92.00^{a}	150.10^{a}
Nano-ZnO	191.00^{b}	4.388^{a}	56.10^{c}	128.00^{b}
Nano-Se	198.60^{b}	3.904^{b}	69.90^{b}	110.70^{c}
SEM	6.007	0.078	1.528	4.856
P-value	0.007	0.004	0.006	0.009

^{a,b,c,d} Means in a column with different superscripts are significantly different (p < 0.01).

Nano-ZnO had a significant reduction in triglyceride concentration compared to Nano-Se (p = 0.006). The lowest cholesterol concentration was related to Nano-Se, which did not have a significant difference with the control (p ⩾ 0.075). Plasma total protein concentration in Nano-ZnO was significantly higher than other groups (p = 0.004). There was no significant difference in plasma total protein concentration between Nano-Se and control (p = 0.207).

Discussion

High eggshell temperature

The present study showed that antioxidant activity of hatchlings exposed to high EST significantly reduced compared to normal EST. High temperature is one of the factors that leads to free radical generation and subsequently oxidative stress during incubation (Xiao et al., 2016). High incubation temperature is a stressor for embryos of all ages, resulting in decreased development and increased mortality (French, 2000). However, high temperatures lead to more stress during late incubation (Christensen et al., 2005). At this time, embryonic tissues contained relatively high levels of polyunsaturated fatty acids (PUFA), while natural antioxidant reserves have not reached an enough level for innate protection (Yigit et al., 2014). PUFAs are highly susceptible to peroxidation caused by free radicals (Yigit et al., 2014). Therefore, with the onset of pulmonary respiration after internal pipping in the chick embryo, the presence of reactive oxygen species (ROS) and oxidative stress is higher (Moran Junior, 2007). Previous studies have shown that heat stress reduced antioxidant activity and induced oxidative stress in broiler chickens (Azad, Kikusato, Zulkifli, & Toyomizu, 2013). Oxidative stress results in lipid peroxidation and oxidative damages to proteins and DNA (Surai et al., 2016). SOD catalyzes superoxide radical into H_2O_2

and this H_2O_2 is reduced to water by GSH-Px or catalase (Surai et al., 2016), hence the activities of SOD and GSH-Px are beneficial biomarker of resistance to oxidative damage. In the present study, reduced antioxidant activity is attributed to increased ROS generation and oxidative damage during high EST. Piestun et al. (2009) reported that the concentration of thyroid hormones increased sharply during late incubation, reaching a peak during external pipping. T3 plays an important role during the critical time of hatching and is essential in order to supply more energy demands during the hatch (Piestun et al., 2009). Increasing the blood concentration of T4 and T3 seems to be in order to maintain homeostasis and to supply more energy demands during late incubation at high EST. Consistent with current study, Al-Zhgoul et al. (2013) stated that a high temperature (38.8º C) for 6 h during d 10 to 18 of incubation increased T3 and T4 concentrations in 3 days old broiler chickens. Morita et al. (2016) showed the non-significant increase in T4 level at hatch by applying high incubation temperature (38.8º C) from d 13 to hatch. In contrast to these results, various studies have reported that high incubation temperature caused decreased plasma concentrations of thyroid hormones (Piestun et al., 2009; Willemsen et al., 2010). The reason for the conflicting results can be attributed to different duration, amplitude, and period of incubation temperature manipulation (Willemsen et al., 2010).

The present study, similar to other studies (Ayo et al., 2011), found that change in incubation temperature and subsequently hormonal alterations led to changes in some metabolic responses. Consistent with our results, a number of researchers documented that high incubation temperature increased plasma levels of glucose, triglyceride and cholesterol but reduced plasma total protein level in hatchlings (Willemsen et al., 2010; Willemsen et al., 2011). Increased level of glucocorticoids leads to metabolic changes so that focus on mobilizing or producing glucose (Virden & Kidd, 2009) to increase available energy for survival under stressful conditions (Ognik & Sembratowicz, 2012). Glucocorticoids play a critical role in induction of gluconeogenesis (Virden & Kidd, 2009) and greater blood glucose levels in the high EST might suggest a stimulated gluconeogenesis (Willemsen et al., 2011). Indeed, corticosteroids induced by the thermal stress act as an insulin antagonist through reducing glucose utilization in peripheral tissues and increasing blood glucose concentrations (Pechova & Pavlata, 2007). Glucocorticoids quickly mobilize glycogen from the liver to supply energy in form of glucose (Rose, Vegiopoulos, & Herzig, 2010). Additionally, the higher levels of stress hormones stimulate lipolysis (Ognik & Sembratowicz, 2012; Sarıca, Aydın, & Ciftci, 2017), which explains increased concentration of cholesterol and triglyceride under high EST. Reduced plasma concentration of total protein at high EST is also due to the catabolism of proteins to free amino acids for use as gluconeogenic substrates (Ognik & Sembratowicz, 2012). It has been reported that administration of glucocorticoids in chickens reduced protein synthesis in muscle tissue (Klasing, Laurin, Peng, & Fry, 1987).

In *ovo* injection of nano-Se and Nano-ZnO

In order to establish balance between antioxidants and ROS during heat stress, a common practice is to promote antioxidant capability and activity in the bird (Sahin & Kucuk, 2003). It has been shown that antioxidant protection at hatching is an important factor on survival of chicken in the early period after hatching (Surai et al., 2016). Antioxidant status of incubating eggs is variable and unpredictable; hence, *in ovo* injection of some nutrients that potentially attenuate oxidative stress, could alleviate detrimental effects of stress, which might beneficially affect post-hatch growth performance and well-being of broiler chickens (Hajati et al., 2014). It has been shown that the mineral content in the yolk is low in the last days of incubation and therefore, consumption of minerals become very low (Yair & Uni, 2011); hence, high EST during last days of incubation will increase the need for minerals, including selenium and zinc in chick embryos. It has been shown that supplementation of the maternal diet with Se (Surai et al., 2016) and Zn (Zhang et al., 2018) sources can substantially enhance antioxidant capacity and resistance against oxidative stress in developing embryos and hatchlings. Xiao, Yuan, Wang, and Zhan (2016) reported that Se supplementation in the diet of breeders resulted in lower ROS and malondialdehyde (MDA) and higher GSH-Px, SOD and CAT activities in chick embryos exposed to high temperature. Zhu et al. (2017) also indicated that maternal dietary zinc could protect chick embryos against maternal heat stress by elevating antioxidant capacity. Present study demonstrated that antioxidant activities of GSH-Px and SOD in chick embryos under high EST were significantly higher in Nano-Se and Nano-ZnO treatments than other heat treatments. Both Se and Zn are essential trace elements and are involved in several metabolic pathways in both human and

animals (Liu et al., 2015; Zeng & Combs Junior, 2008). About 40 to 50 percent of total body selenium is present in GSH-PX and increases the amount of enzyme activity about 100 to 1,000 times (Ghazanfarpoor, Talebi, & Abedi, 2014). Furthermore, selenium is an essential component of TrxR (Surai et al., 2016). The important role of TrxR in the protection against oxidative damage is also evident (Mustacich & Powis, 2000). Zn is essential for catalytic activity and appropriate spatial conformation of SOD (Yigit et al., 2014). Furthermore, Zn increases the synthesis of metallothionein as a free radical scavenger (Liu et al., 2015; Sahin et al., 2009). Greater efficiency and lower toxicity in the use of Nano-Se (Bagheri, Golchin-Gelehdooni, Mohamadi, & Tabidian, 2015; Boostani, Sadeghi, Mousavi, Chamani, & Kashan, 2015) and Nano-ZnO (Swain, Rao, Rajendran, Dominic, & Selvaraju, 2016) in comparison with other sources of selenium and zinc were reported in poultry feeding, which can be attributed to great surface area, high surface activity, high catalytic efficiency, strong adsorbing ability, and great permeability (Zhao et al., 2014). The small size of nanoparticles allows them to penetrate inside tissues and even makes them able to cross cell membranes (Joshua et al., 2016). Recent studies have shown that the dietary administration of Nano-Se (Bagheri et al., 2015) and Nano-ZnO (Zhao et al., 2014) has promoted antioxidant activity in broiler chicks. Similarly, Lee et al. (2014) found that *in ovo* injection of sodium selenite at 10 and 20 µg of Se/egg improved antioxidant responses in hatched chicks exposed to necrotic enteritis. Boostani et al. (2015) also suggested that the dietary supplementation at 0.3 mg kg^{-1} with different sources of selenium enhanced antioxidant capacity in broiler chickens under oxidative stress. Kucuk, Sahin, and Sahin (2003) reported that supplemental Zn decreased serum MDA concentrations in heat-stressed quails.

In the present study, one of the significant metabolic consequences of *in ovo* injection with Nano-Se or Nano-ZnO was reduced blood concentration of glucocorticoids and thyroid hormones in broiler hatchlings exposed to high EST during late incubation. In fact, due to increased antioxidant activity as a result of *in ovo* administration of Nano-Se or Nano-ZnO, free radicals can be efficiently scavenged, and thus the chick embryo is less exposed to oxidative damage during high EST. Indeed, Alleviation of heat-induced stress leads to less stimulation of the HPA axis, and therefore, less secretion of CRH as well as lower TSH release. Moreover, in alleviated heat-induced stress, the need for energy is reduced (Ognik & Sembratowicz, 2012) and thyroid activity decreases. It is worth mentioning that selenium is an effective part of the iodothyronine deiodinase (Surai et al., 2016); therefore, the higher ratio of T3/T4 when injected Nano-Se compared to Nano-ZnO can be attributed to the effect of selenium on the metabolism of thyroid hormones and the conversion of T4 to T3 (Wang, Wang, & Zhan, 2016). The decreased blood glucocorticoid levels caused by *in ovo* injection of Nano-Se and Nano-ZnO could explain elevated blood total protein level and reduced blood levels of glucose, triglyceride and cholesterol found in hatchlings under high EST. Kucuk et al. (2003) indicated that zinc supplementation resulted in enhanced blood level of total protein and reduced blood concentrations of glucose, triglyceride, and cholesterol in broiler chickens under heat stress. The linear reduction in blood cholesterol by increasing the zinc supplementation from 30 to 60 mg kg^{-1} in the diet of quail under heat stress was reported by Sahin et al. (2005). In addition, Habibian, Ghazi, Moeini, and Abdolmohammadi (2014) also reported that dietary selenium decreased blood levels of total cholesterol, triglyceride, and glucose in broiler chickens under heat stress.

Current study indicated that administration of Nano-Se and Nano-Zno has physiological and biochemical benefits in hatchlings at stressful conditions. In conclusion, high EST significantly impairs antioxidant activity and results in metabolic and hormonal changes, whereas, *in ovo* administration with either Nano-Se or Nano-ZnO improved antioxidant activity and alleviated the negative effects of high EST on metabolic and hormonal alterations of embryos during late incubation.

Concluision

It was concluded that injection of Nano-Se and Nano-ZnO could alleviate the negative effects of high temperature incubation and heat stress by an increase in the antioxidant activity and reduction of oxidative stress.

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