



ORIGINAL ARTICLE

EXPRESSION PATTERNS OF COPPER/ZINC SUPEROXIDE DISMUTASE (SOD1) IN QUAIL EMBRYONIC TISSUES UNDER *IN OVO* AND *EX OVO* CONDITIONSBronislava Pokorná^{1*}, Slavomíra Štefancová¹, Helena Ivanič¹, Eva Petrovová², Lenka Luptáková¹¹Department of Biology and Physiology, University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia; ²Department of Morphological Disciplines, University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Temperature fluctuations can act as stressors affecting embryonic development and cellular homeostasis. This study examined the effects of preincubation at 22°C (P22) or 30°C (P30) on quail embryos under *in ovo* and *ex ovo* conditions, focusing on the expression of the antioxidant enzyme superoxide dismutase (*SOD1*) in selected tissues. Under *in ovo* incubation, *SOD1* expression was significantly upregulated in both liver and heart at P22 and P30, with the highest expression observed in the liver at P30. In contrast, under *ex ovo* conditions, *SOD1* expression was significantly reduced in the liver at P22 but partially restored at P30. In the heart, it was not significantly increased at P22 but slightly decreased at P30. These findings demonstrate tissue- and condition-specific regulation of *SOD1* and suggest that *in ovo* preincubation supports robust antioxidant defence, whereas *ex ovo* culture imposes complex stress responses.

Keywords: *ex ovo*; *in ovo*; preincubation; quail embryo; SOD1

INTRODUCTION

Various factors, such as egg storage period, age, breed, preincubation conditions, lighting, nutritional status, and mating practices, are known to influence fertility in birds [1]. In addition, the process of embryonic development, hatching, and survival of quail (*Coturnix japonica*) can be influenced by multiple environmental and management conditions, including the egg storage conditions, egg rotation, and relative humidity. It has been determined that

temperature has a significant influence on these processes. These environmental and management factors can act as stressors, leading to an increased production of reactive oxygen species (ROS) within the developing embryos [2].

To cope with such oxidative challenges, living organisms have evolved specific antioxidant mechanisms. These natural antioxidant systems are essential for survival in oxygen-rich environments, protecting cells from damage caused by ROS. The first line of defence involves preventing free radical formation by removing their precursors or

deactivating catalytic metal ions, primarily through metal-binding proteins. The second line of defence consists of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), which detoxify ROS once they are formed. SOD, which converts superoxide radicals, is considered a crucial component of this first defence level, as superoxide represents the primary free radical generated in cells [3]. SOD catalyses the disproportionation of the superoxide anion ($O_2^{\cdot-}$), converting it through the reaction $2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$. This process contributes to the reduction of oxidative stress in cells [4].

The antioxidant protection of avian embryos is subject to change over time and between individual tissues during development. The sensitivity of tissues to damage caused by ROS is dependent on the composition of their lipids, resulting in differences between tissues. In avian species, the predominant source of antioxidants consists of substances of maternal origin, which are stored in the yolk. During embryonic development, yolk antioxidants are gradually transported into the embryo, where they are distributed unevenly among different tissues [5].

Embryonic development in birds is highly sensitive to incubation temperature, particularly during early developmental stages. Exposure to continuous or intermittent deviations from optimal incubation temperature has been shown to impair hatchability and alter physiological and hormonal responses [6]. Experimental manipulation during incubation has been used to influence the antioxidant capacity of avian embryos. *In ovo* administration of antioxidant trace elements, such as nano-selenium or nano-zinc oxide, has been shown to enhance antioxidant enzyme activity and reduce oxidative stress under challenging incubation conditions. These findings highlight the importance of controlled experimental approaches for studying embryonic development and redox balance [7].

Most studies utilise the windowing technique (*in ovo*) to observe developmental changes after chemical exposure, as it facilitates long-term experimentation with minimal abnormalities and permits natural hatching [8, 9]. The *ex ovo* method, a widely applied technique in the fields of embryology, genetic manipulation, toxicology [8], and cancer research [10, 11], provides excellent visualisation and unrestricted access to the chorioallantoic membrane (CAM, [12]), thereby enabling direct monitoring of the effects of pathogens or drugs on embryonic vasculature [9]. However, the success of *ex ovo* culture is contingent upon

meticulous regulation of oxygen, moisture, and calcium levels to mitigate the risks of dehydration and infection, which, in the absence of such regulation, can result in elevated mortality rates [13].

In summary, this study focuses on gene expression of *SOD1* as a key component of the antioxidant defence system in quail embryos incubated under different preincubation temperatures and incubation methods. By comparing *in ovo* and *ex ovo* conditions, this work aims to clarify how incubation environment and thermal preconditioning influence antioxidant defence during early embryonic development and to contribute to the optimization of *ex ovo* cultivation strategies.

MATERIALS AND METHODS

Preincubation and Incubation

Fertilized quail eggs (*Coturnix coturnix japonica*, $n = 144$) were purchased from the quail farm (Mala Ida, Kossice, Slovakia), no older than three days after laying. The eggs were disinfected with 70% ethanol and divided into two groups: *in ovo* incubation (65 eggs) and *ex ovo* incubation (79 eggs).

In both the *in ovo* and *ex ovo* incubation groups, the fertilized eggs were divided into three groups according to the incubation conditions (Table 1). During the 12-hour preincubation period, control embryos were stored under standard cold storage conditions at 14 °C in a temperature-controlled wine cooler. Subsequently, this first group of embryos (22 *in ovo*; 27 *ex ovo*) was incubated under standard conditions at 38.2 °C and 60% relative humidity. The second group (21 *in ovo*; 29 *ex ovo*) was preincubated at controlled room temperature (approximately 22 °C; P22), and the third group (22 *in ovo*; 23 *ex ovo*) was preincubated at 30 °C (P30). The embryos were maintained in a closed, climate-controlled, low-disturbance environment during the preincubation period, minimizing potential temperature fluctuations. Preincubation in the P22 and P30 groups lasted 12 hours, after which all eggs were transferred to standard incubation conditions. For the *ex ovo* method, eggs were stored vertically during the preincubation period and placed horizontally during incubation. After 56 hours, the contents of the eggs, including the embryo, were carefully transferred under sterile conditions into a 6-well culture plate. *Ex ovo* incubation required

1. group - standard conditions at $38.2 \pm 0.5^\circ\text{C}$ and 60% relative humidity (22 pcs) without preincubation
2. group - preincubation at a room temperature of 22°C (21 pcs)
3. group - preincubation at 30°C and 60% relative humidity (22 pcs)

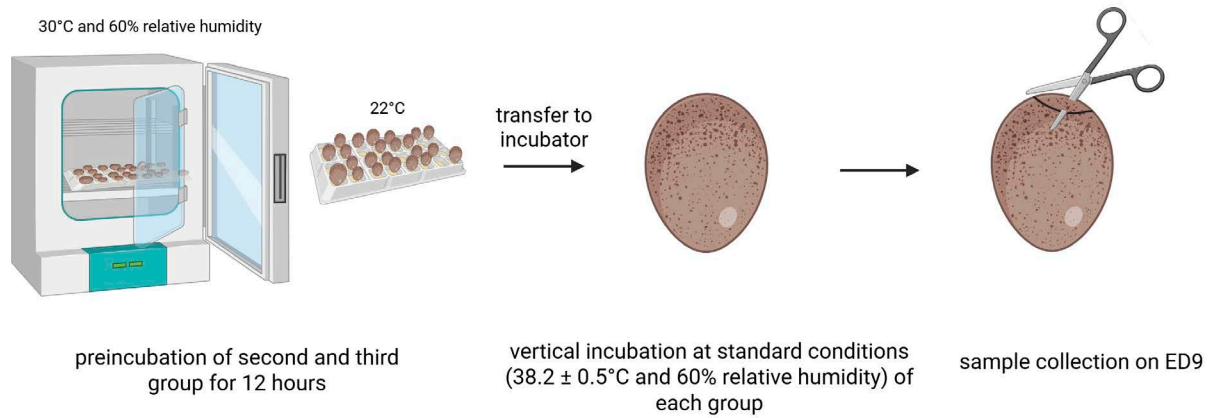


Fig. 1. *In ovo* method

1. group - standard conditions at $38.2 \pm 0.5^\circ\text{C}$ and 60% relative humidity (27 pcs) without preincubation
2. group - preincubation at a room temperature of 22°C (29 pcs)
3. group - preincubation at 30°C and 60% relative humidity (22 pcs)

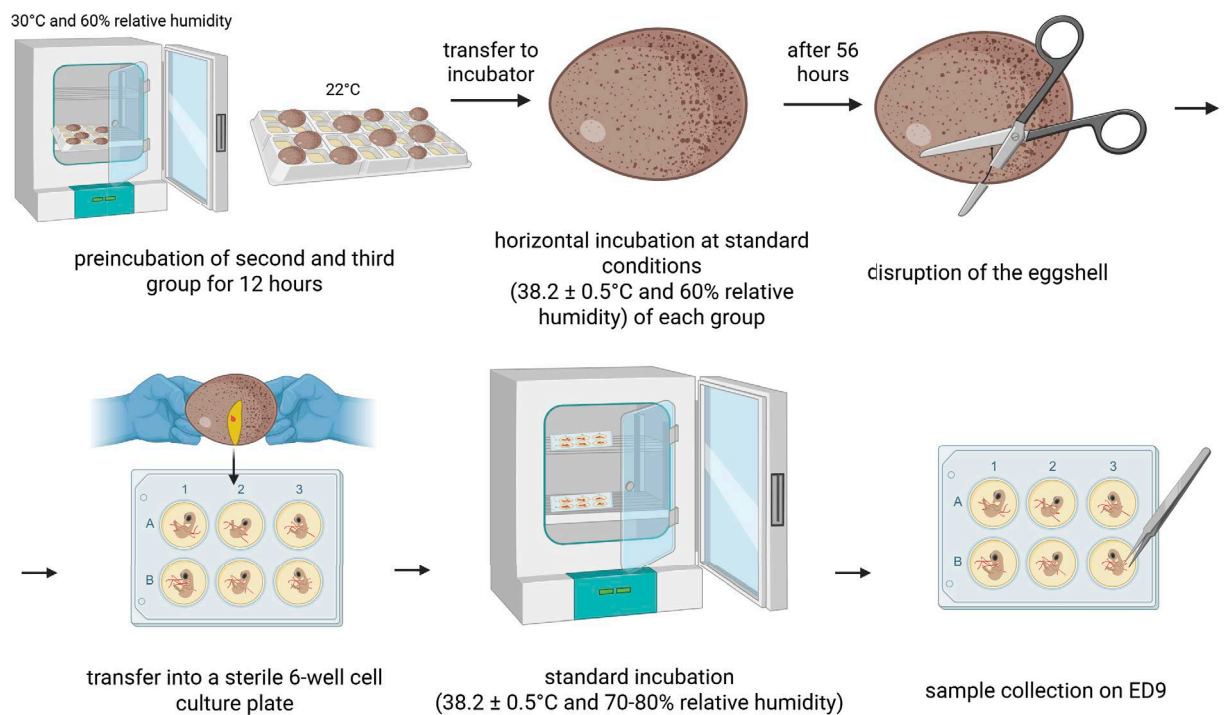


Fig. 2. *Ex ovo* method

higher relative humidity (70–80%), which was applied after transferring embryos to culture plates.

Embryos from both methods were incubated until embryonic day 9 (ED9), when sampling was performed. All incubation was carried out in an automatic incubator

(COVINA ET 49, Italy). For the *in ovo* method, eggs were opened at the blunt end on ED9, and embryos were removed. *Ex ovo* embryos were collected from the culture plates in the same manner. After weighing, samples of the heart and liver were stored at -80°C for RNA extraction.

Figure 1 and Figure 2 show methodological procedures for *in ovo* and *ex ovo* incubation.

Table 1. Number of eggs in experimental groups (*in ovo* and *ex ovo*) at the start of the study

	<i>in ovo</i>	<i>ex ovo</i>
Standard incubation	22	27
Preincubation at 22°C	21	29
Preincubation at 30°C	22	23

Gene Expression Analysis

Total RNA was isolated from liver and heart using the QIAshredder and RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA purity and concentration were assessed with a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Gene expression was analysed using a two-step RT-qPCR protocol. First, cDNA was synthesized from 1 µg of total RNA in a 20 µL reaction using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Waltham, MA, USA) according to the manufacturer's instructions. In the second step, qPCR was performed using SYBR Green Master Mix (Applied Biosystems™, Waltham, MA, USA) and specific primers for *SOD1*. Each 10 µL reaction contained 300 nM of each primer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control, and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method [14]. Amplification conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples were run in triplicate and melt curve analysis confirmed specificity. Gene expression levels were normalized to the control group without preincubation. Melting temperatures ranged from 77.22–78 °C for *SOD1*.

Statistical Analysis

Statistical analysis was performed with the GraphPad Prism program (version 9.3, USA) using a two-way ANOVA test with Tukey's post hoc test. The value of statistical significance was considered as $p < 0.0001$. Data were evaluated as mean ± standard deviation (SD).

RESULTS

Gene Expression Analysis of *In Ovo* and *Ex Ovo* Incubation

Liver

The expression of *SOD1* in the liver was found to be significantly affected by both preincubation temperature and culture system. *In ovo*, *SOD1* expression increased markedly with rising preincubation temperature, showing a significant upregulation ($p < 0.0001$) at P22 compared with the control temperature, and reaching the highest expression level ($p < 0.0001$) at P30.

In contrast, under *ex ovo* conditions, P22 led to a significant decrease ($p < 0.0001$) in *SOD1* expression in comparison with the control group. The P30 demonstrated an increase compared to the control group and P22 group; however, this increase was not statistically significant (Figure 3).

Heart

During the observation of *SOD1* expression in the heart, a significant upregulation ($p < 0.0001$) was observed in both groups with preincubation (P22 or P30) in *in ovo* conditions.

The *ex ovo* conditions demonstrated a range of outcomes. P22 demonstrated a non-significant upregulation in comparison with the control group. P30 demonstrated a marginal yet non-statistically significant reduction in *SOD1* expression when compared to the control group (Figure 3).

To summarize, the *in ovo* incubation approach resulted in a significant upregulation ($p < 0.0001$) of *SOD1* expression in all studied tissues (liver and heart) under both preincubation conditions (P22 and P30). The highest levels of *SOD1* expression were observed in the liver, followed by the heart, particularly when preincubation was conducted at 30°C. This upregulation of *SOD1* may reflect an adaptive increase in antioxidant defence capacity rather than directly indicating elevated oxidative stress levels.

In contrast, the *ex ovo* method exhibited differential effects contingent on tissue and temperature. In the liver, P22 resulted in a significant downregulation of *SOD1* ($p < 0.0001$) in comparison with the control group. In the heart, P30 led to a marginal yet non-significant reduction in *SOD1* expression. The observed patterns may indicate that excessive stress under *ex ovo* conditions is associated with impaired antioxidant responses. The overall expression trends are summarised in Table 2.

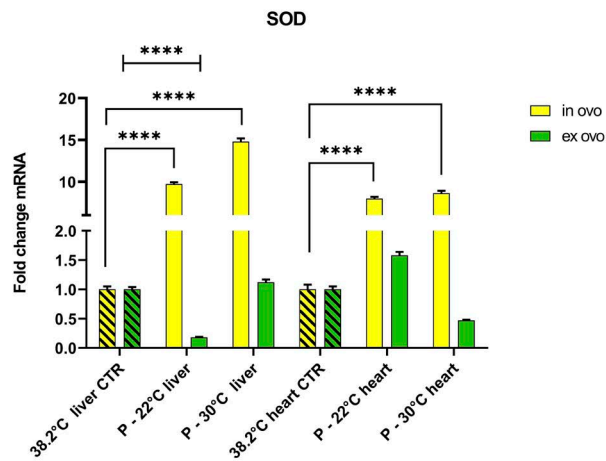


Fig. 3. Gene expression of *SOD1* in *in ovo* and *ex ovo* groups with preincubation (**** indicates statistical significance at $p < 0.0001$)

Table 2. Gene expression of *SOD1* in *in ovo* and *ex ovo* groups with preincubation

		SOD1	
		P22 ^a	P30 ^b
liver	<i>in ovo</i>	+ ^c	+ ^c
	<i>ex ovo</i>	- ^d	+ns ^e
heart	<i>in ovo</i>	+ ^c	+ ^c
	<i>ex ovo</i>	+ns ^e	-ns ^f

P22^a – Preincubation at 22°C; P30^b – Preincubation at 30°C; +^c – Significant upregulation ($p < 0.0001$); -^d – Significant downregulation ($p < 0.0001$); +ns^e – Nonsignificant upregulation; -ns^f – Nonsignificant downregulation

DISCUSSION

The level of oxidative stress in embryos may be influenced by multiple factors, including the interval between laying and storage, the duration of storage, fluctuations in incubation conditions, and the length of the hatch window [15]. During embryogenesis, antioxidants derived from the yolk are distributed unevenly among the developing tissues. Previous studies have demonstrated tissue-specific differences in lipid oxidation and antioxidant enzyme activity. The brain has been reported to exhibit the highest concentrations of malondialdehyde (MDA), a marker of lipid peroxidation, followed by the liver, kidney, and thigh. Antioxidant enzymes such as SOD and CAT have been detected across multiple tissues, including the brain, liver, kidney, and muscle, with their activity varying in a tissue-dependent manner. Among these tissues, SOD activity is tissue-dependent, with the highest levels observed in the heart, followed by muscle, yolk sac membrane, kidney,

lung, and liver [16]. However, these findings are based on enzyme activity measurements and may not directly correspond to gene expression levels.

Our results demonstrated condition-dependent regulation of *SOD1* expression, with tissue-specific differences observed only under *ex ovo* incubation conditions. In the context of *in ovo* incubation, a significant upregulation of *SOD1* was observed in both the liver and heart at both preincubation temperatures (P22 and P30). The highest expression levels were detected in the liver, followed by the heart, particularly at P30. A moderate yet significant increase in *SOD1* was observed at P22 in both organs, suggesting that embryos can mount an adaptive antioxidant response to mild oxidative stress within the stable *in ovo* environment.

In contrast, *ex ovo* conditions elicited more variable tissue-specific responses. The liver showed a significant decrease in *SOD1* expression after P22, while P30 led to a partial restoration of expression, although not to a significant extent. Conversely, the heart exhibited a divergent response: *SOD1* expression was non-significantly increased at P22, suggesting a degree of adaptability. However, a slight, also non-significant decrease in *SOD1* expression was observed after preincubation at P30. These findings suggest that both *ex ovo* culture conditions and preincubation temperature may contribute to the observed tissue-specific differences in *SOD1* expression, likely through combined effects on the embryonic microenvironment.

Several physiological limitations of *ex ovo* incubation, such as the absence of the protective eggshell, increased risk of dehydration, nutrient loss, and impaired calcium availability, may elevate cellular stress and contribute to the reduced antioxidant response [13]. Optimization of humidity, airflow, calcium supplementation, and egg handling has been proposed to improve embryonic viability under *ex ovo* conditions [11, 13].

Consistent with our observations, Amevor et al. reported that extended egg storage periods (7 and 14 days) resulted in increased oxidative stress, reflected by elevated MDA levels and decreased antioxidant indicators, including total SOD (T-SOD), in the liver, heart, and breast muscle [1]. Furthermore, incubation temperature is a critical factor influencing embryonic development, as low temperatures slow development and increase mortality, whereas temperatures within the physiological range accelerate development and improve hatching success [17].

CONCLUSION

Several environmental factors, including temperature fluctuations and modified oxygen supply, can alter avian embryonic development. One of the key cellular responses to such changes is the regulation of genes related to antioxidants, particularly *SOD1*. This gene encodes an enzyme that plays a key role in the body's initial defence against ROS.

Our results indicate that *in ovo* incubation supports a controlled upregulation of *SOD1* gene expression, reflecting a regulated antioxidant response under physiologically stable conditions. In contrast, *ex ovo* incubation exposes embryos to altered environmental conditions, which is associated with reduced *SOD1* gene expression, suggesting differences in the regulation of antioxidant defence-related genes. In addition, preincubation temperature may have contributed to the observed variation in expression patterns, particularly under *ex ovo* conditions.

As this was a pilot study, analysis of other markers of oxidative damage or enzyme activity was not performed. Therefore, the observed changes in *SOD1* expression should be interpreted as an indication of antioxidant capacity.

Data Availability Statement

The data is publicly available.

Ethical Statement

This research study used avian embryos as experimental *ex ovo* and *in ovo* models, which do not fall under the legislation for protecting animals used for scientific purposes (2010/63/EU). No approval of research ethics committees was required to accomplish the goals of this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Generative AI Statement

The authors used AI-based language tools (DeepL Write and Grammarly) solely for improving grammar and style. No generative AI tools were used to create or modify the scientific content of this work.

Authors' Contributions

Bronislava Pokorna: Conceptualization, Formal analysis, Resources, Writing – Original draft. Slavomira Stefancova: Data curation. Helena Ivanic: Conceptualization, Visualization, Investigation, Methodology. Eva Petrovova: Funding acquisition, Project administration, Supervision, Validation, Writing – Reviewing and Editing. Lenka Luptakova: Funding acquisition, Validation, Writing – Reviewing and Editing.

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