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The effects of the benzo[a]pyrene on selected blood parameters in the chick embryos *in ovo*

Abstract

Polycyclic aromatic hydrocarbons (PAHs) constitute a large group of persistent environmental pollutants, among which benzo[a]pyrene (BaP) is considered one of the most important compounds from a public health perspective due to its carcinogenic, teratogenic, and cytotoxic properties. Although the biological effects of BaP have been extensively investigated in mammals, its influence on avian embryonic development and haematological processes remains insufficiently characterised. In the present study, the chicken embryo *in ovo* model was used to investigate the effects of BaP exposure on selected embryonic blood parameters. Fertilised eggs were exposed to different doses of BaP administered into the yolk sac during embryonic development, and changes in haematological profiles were evaluated. The following parameters were determined: white blood cell count (WBC), red blood cell count (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), as well as the percentages of heterophils, lymphocytes, monocytes, eosinophils, and basophils. Exposure to BaP resulted in dose-dependent alterations in both erythrocyte and leukocyte parameters, indicating disturbances in embryonic haematopoiesis and immune cell distribution. The observed changes demonstrate the sensitivity of the chicken embryo *in ovo* model to BaP-induced developmental toxicity. The present study provides important insights into the effects of BaP on blood formation and immune system development during vertebrate embryogenesis and supports the use of avian embryos as a valuable model for environmental toxicology studies.

Keywords: animal models, carcinogens, hematopoiesis (blood formation), polycyclic aromatic hydrocarbons

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Introduction

Benzo[a]pyrene (BaP), an example of a polycyclic aromatic hydrocarbon, belongs to the group of persistent organic pollutants (POPs), which are characterised by high environmental persistence and a tendency to bioaccumulation. It originates from various anthropogenic sources, including fossil fuel combustion, smoked food products, and cigarette smoke. Moreover, some natural processes, such as volcanic eruptions and forest fires, may contribute to increased concentrations of this toxic compound in the environment (Gomaa et al., 1993; Sapota, 2002; Wiąckowski, 2010).

The carcinogenic, teratogenic, and cytotoxic effects of BaP have been extensively documented in mammals based on both *in vivo* and *in vitro* studies (Lee, Shim, 2007). Exposure to this hydrophobic molecule may occur through respiratory, gastrointestinal, and dermal routes (Muñoz, Albores, 2011). However, the effects of this important xenobiotic on avian species and embryonic development remain insufficiently investigated.

The chicken embryo *in ovo* model is widely recognised as a valuable experimental system for studying vertebrate development, particularly in modern embryology (Davey, Tickle, 2007). The organogenesis of the avian cardiovascular system shows considerable similarity to that of mammals (Yoshiyama, Kanke, 2005). Furthermore, birds are considered useful bioindicators of urban environmental conditions (Egwumah et al., 2017). Therefore, the chicken embryo *in ovo* model was applied in the present study to evaluate the effects of BaP exposure on selected embryonic blood parameters.

Previous studies investigating the effects of BaP on mice have used doses ranging from 25–100 μg/kg body weight (bw) daily following oral administration (Banks et al., 2016), or 1–6.25 mg/kg bw administered twice weekly via intraperitoneal injection (Zhang et al., 2016).

The chicken embryo *in ovo* model has recently been used to investigate the embryotoxic effects of various compounds, including paracetamol, aflatoxin, homocysteine, and retinoic acid (Osmond et al., 1991; Boot et al., 2004; Lis et al., 2006; Ozaydin, Sur, 2015). These studies demonstrated high sensitivity of this model to toxic agents and highlighted its potential for biomedical research applications (Osmond et al., 1991; Boot et al., 2004; Lis et al., 2006; Davey, Tickle, 2007; Ozaydin, Sur, 2015).

The concentration of carcinogenic polycyclic aromatic hydrocarbons (PAHs) in food products has been estimated to range from 2.6 μg/kg in boiled ham to 43.7 μg/kg in smoked fish, with BaP detected in approximately 92% of analysed products (Gomaa et al., 1993).

Individuals with high nicotine consumption are additionally exposed to approximately 5 µg of carcinogenic PAHs per day through cigarette smoking (Menzie et al., 1992). However, exposure to BaP and other PAHs through inhalation of polluted urban and industrial air is difficult to estimate due to considerable temporal and spatial variability (Gębicki et al., 2015).

The aim of the present study was to determine the effects of BaP exposure on basic morphological parameters of chicken (*Gallus gallus domesticus* L.) embryonic blood following administration of the compound into the yolk sac in an organic oil solution during embryonic development. The proposed methodology represents a simplified model of the cumulative environmental exposure of a vertebrate embryo to BaP.

Materials and methods

Animals, embryos incubation and treatment

The study was conducted using fertilised Ross 308 chicken eggs incubated *in ovo* in a hatchery incubator (HEKA, Germany) at 37.5°C (Fig. 1).

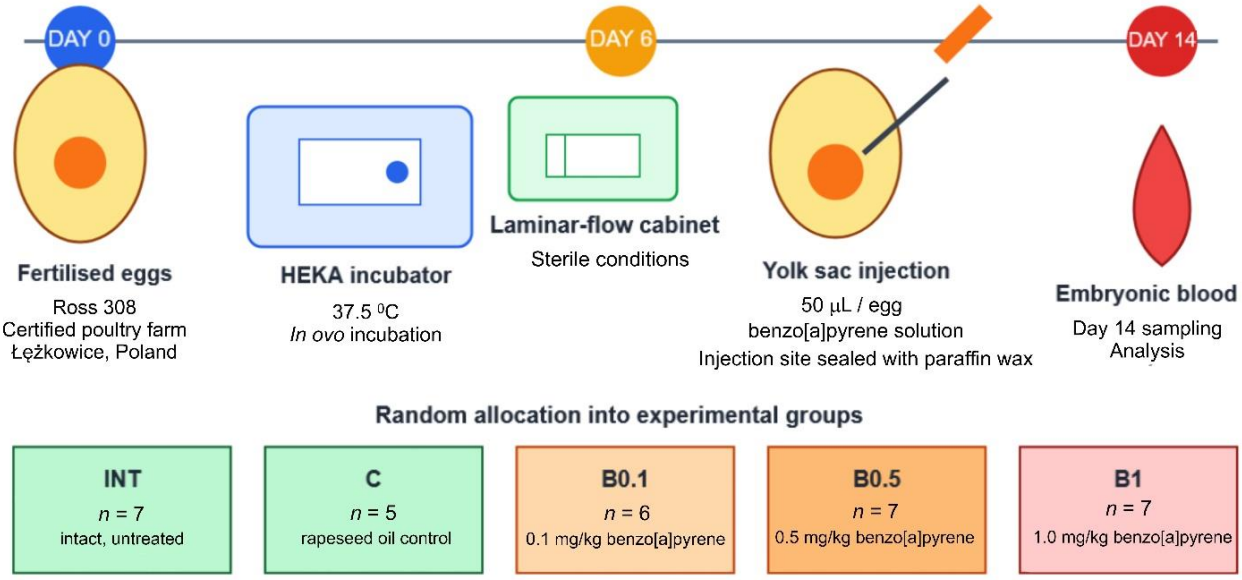


Fig. 1. Experimental design and *in ovo* benzo[a]pyrene exposure protocol; explanation of abbreviations in the text

Fertilised eggs were obtained from a certified poultry farm in Łężkowice, Poland, and randomly assigned to five experimental groups: INT (intact, untreated; n = 7), C (control, injected with rapeseed oil; n = 5), B0.1 (0.1 mg/kg benzo[a]pyrene; n = 6), B0.5 (0.5 mg/kg

benzo[a]pyrene; n = 7), and B1 (1 mg/kg benzo[a]pyrene; n = 7). Benzo[a]pyrene (Sigma-Aldrich, USA) was dissolved in rapeseed oil immediately before administration.

On the 6th day of incubation, eggs from the control and experimental groups were injected into the yolk sac with 50 µL per egg of either rapeseed oil or the appropriate benzo[a]pyrene solution. The injection site was sealed with paraffin wax, and all procedures were performed under sterile conditions in a laminar-flow cabinet (Heal Force HF Safe 1200, Lishen Scientific Equipment, China).

On the 14th day of incubation, the eggs were opened and embryonic blood samples were collected for analysis. All experimental procedures were conducted in accordance with Polish regulations governing the ethical use of animals in research.

The present study represents a part of a larger research project investigating the effects of benzo[a]pyrene exposure on embryonic development. The current manuscript focuses specifically on the evaluation of haematological parameters.

Determination of blood parameters

The following haematological parameters were measured using an automated haematology analyser (Mindray BC-3600, Shenzhen Mindray, China): white blood cell count (WBC), red blood cell count (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), and mean corpuscular haemoglobin (MCH).

Blood smears were prepared manually, stained with Wright's stain (Stamar, Poland), and examined under a light microscope (Jenamed Variant, Carl Zeiss, Germany). Differential leukocyte counts (Schilling's leukograms) were determined by microscopic examination. Statistical analysis was performed using Student's t-test.

Results

The effects of benzo[a]pyrene (BaP) exposure on haematological parameters in embryonic blood are summarised in table 1.

Tab. 1. Changes in haematological parameters in the embryonic blood of chickens (*Gallus gallus domesticus* L.) following exposure to different doses of benzo[a]pyrene (BaP); data are presented for the intact group, the oil-treated control group, and the BaP-treated groups (0.1, 0.5, and 1 mg/kg). Statistical significance is indicated relative to the intact group

Parameter	Intact	Control (oil)	BaP 0.1 mg/kg	BaP 0.5 mg/kg	BaP 1 mg/kg

WBC (×10⁹/L)	70.76	84.85*	17.10***	40.59***	119.67***
RBC (×10¹²/L)	1.46	0.35***	0.36***	1.529 ns	1.56 ns
HGB (g/dL)	6.65	6.90 ns	1.36***	2.57***	7.73***
HCT (%)	27.42	25.50 ns	5.96***	6.34***	24.43**
MCV (fL)	161.96	162.97 ns	162.82 ns	165.14*	160.19 ns
MCH (pg)	53.60	53.77 ns	46.40***	52.99 ns	50.80 ns
Lymphocytes (%)	43.67	50.43**	34.00***	27.83***	68.25***
Monocytes (%)	5.67	7.14 ns	17.60**	7.50 ns	6.00 ns
Eosinophils (%)	5.33	7.28**	7.75*	8.00*	4.00 ns
Basophils (%)	1.50	2.00*	1.83*	1.75 ns	2.00 ns

Note: ns – no statistical significance ($p > 0.05$), * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$

The results obtained for individual parameters of chicken embryonic blood are discussed in detail below.

WBC

Benzo[a]pyrene (BaP) exposure affected the leukocyte count in embryonic blood. The mean WBC value in the intact group was 70.76 × 10^9/L. In the oil-treated control group (without BaP), WBC increased to 84.85 × 10^9/L, representing a significant increase compared with the intact group ($p \leq 0.02$). Exposure to 0.1 mg/kg BaP markedly reduced the leukocyte count to 17.1 × 10^9/L ($p \leq 0.001$). Similarly, treatment with 0.5 mg/kg BaP decreased WBC to 40.59 × 10^9/L ($p \leq 0.001$). In contrast, embryos exposed to 1 mg/kg BaP exhibited an increase in WBC to 119.67 × 10^9/L, which was significantly higher than in the intact group ($p \leq 0.001$).

RBC

BaP exposure also affected the erythrocyte count. The mean RBC value in the intact group was 1.46 × 10^12/L. In the oil-treated control group, RBC decreased to 0.35 × 10^12/L, representing a significant reduction compared with the intact group ($p \leq 0.001$). A similar decrease was observed following exposure to 0.1 mg/kg BaP, with the erythrocyte count reaching 0.36 × 10^12/L ($p \leq 0.001$). In contrast, treatment with 0.5 mg/kg and 1 mg/kg BaP increased RBC to 1.529 × 10^12/L and 1.56 × 10^12/L, respectively; however, these changes were not statistically significant ($p \leq 0.05$).

HGB

BaP exposure altered haemoglobin (HGB) concentration in embryonic blood. The mean HGB concentration in the intact group was 6.65 g/dL. In the oil-treated controls, HGB increased slightly to 6.9 g/dL; however, this difference was not statistically significant ($p \leq 0.05$). Exposure to 0.1 mg/kg BaP significantly reduced haemoglobin concentration to 1.36 g/dL ($p \leq 0.001$). Likewise, embryos treated with 0.5 mg/kg BaP showed a significant decrease in HGB to 2.57 g/dL ($p \leq 0.001$). In contrast, treatment with 1 mg/kg BaP increased HGB to 7.73 g/dL, representing a significant increase compared with the intact group ($p \leq 0.001$).

HCT

BaP exposure affected haematocrit (HCT) values in embryonic blood. The mean HCT in the intact group was 27.42%. In the oil-treated controls, HCT decreased slightly to 25.5%, although this difference was not statistically significant ($p \leq 0.05$). Exposure to 0.1 mg/kg and 0.5 mg/kg BaP markedly reduced HCT to 5.96% and 6.34%, respectively (both $p \leq 0.001$). In embryos treated with 1 mg/kg BaP, HCT decreased to 24.43%, remaining significantly lower than in the intact group ($p \leq 0.01$).

MCV

Mean corpuscular volume (MCV) was only slightly affected by BaP exposure. The mean MCV value in the intact group was 161.96 fL. In the control group, MCV increased to 162.97 fL, although the difference was not statistically significant ($p \leq 0.05$). A similar non-significant increase to 162.82 fL was observed following exposure to 0.1 mg/kg BaP. Treatment with 0.5 mg/kg BaP significantly increased MCV to 165.14 fL ($p \leq 0.05$), whereas exposure to 1 mg/kg BaP resulted in a slight, non-significant decrease to 160.19 fL ($p \leq 0.05$).

MCH

Mean corpuscular haemoglobin (MCH) was also influenced by BaP treatment. The mean MCH value in the intact group was 53.6 pg. In the oil-treated controls, MCH increased slightly to 53.77 pg, although this difference was not statistically significant ($p \leq 0.05$). Exposure to 0.1 mg/kg BaP significantly decreased MCH to 46.4 pg ($p \leq 0.001$). MCH values of 52.99 pg and 50.8 pg were recorded in embryos treated with 0.5 mg/kg and 1 mg/kg BaP, respectively; however, neither differed significantly from the intact group ($p \leq 0.05$).

Lymphocytes

BaP exposure affected the proportion of lymphocytes in embryonic blood. In the intact group, lymphocytes accounted for 43.67% of leukocytes. In the oil-treated control group, the lymphocyte percentage increased to 50.43%, representing a significant increase compared with the intact group ($p \leq 0.01$). Exposure to 0.1 mg/kg and 0.5 mg/kg BaP significantly reduced the proportion of lymphocytes to 34% and 27.83%, respectively (both $p \leq 0.001$). In contrast, embryos treated with 1 mg/kg BaP exhibited a significant increase in lymphocyte percentage to 68.25% ($p \leq 0.001$).

Monocytes

BaP exposure also influenced the proportion of monocytes. In the intact group, monocytes accounted for 5.67% of leukocytes. In the oil-treated controls, the monocyte percentage increased slightly to 7.14%; however, this difference was not statistically significant ($p \leq 0.05$). Exposure to 0.1 mg/kg BaP significantly increased the proportion of monocytes to 17.6% ($p \leq 0.01$). Monocyte percentages of 7.5% and 6% were observed following treatment with 0.5 mg/kg and 1 mg/kg BaP, respectively; however, neither differed significantly from the intact group ($p \leq 0.05$).

Eosinophils

BaP exposure altered the proportion of eosinophils in embryonic blood. In the intact group, eosinophils accounted for 5.33% of leukocytes. In the oil-treated control group, the eosinophil percentage increased significantly to 7.28% ($p \leq 0.01$). Exposure to 0.1 mg/kg and 0.5 mg/kg BaP further increased eosinophils to 7.75% and 8%, respectively, with both changes reaching statistical significance ($p \leq 0.05$). In contrast, embryos treated with 1 mg/kg BaP showed a decrease in eosinophils to 4%; however, this difference was not statistically significant ($p \leq 0.05$).

Basophils

The proportion of basophils was also affected by BaP exposure. In the intact group, basophils accounted for 1.5% of leukocytes. In the oil-treated controls, the basophil percentage increased to 2%, representing a significant increase compared with the intact group ($p \leq 0.05$). A similar significant increase to 1.83% was observed following exposure to 0.1 mg/kg BaP ($p \leq 0.05$). Basophil percentages of 1.75% and 2% were recorded in embryos treated with 0.5 mg/kg and 1 mg/kg BaP, respectively; however, neither differed significantly from the intact group ($p \leq 0.05$).

Discussion

Currently, there are no reports describing the effects of benzo[a]pyrene (BaP) exposure on haematological parameters of chicken embryonic blood. However, it is well established that this hydrocarbon is commonly present in fumes generated during fossil fuel combustion, cigarette smoke, smoked meat products, and various foods processed at high temperatures (Muñoz, Albores, 2011; Kubiak, 2013; Gębicki et al., 2015).

One of the analysed parameters was the white blood cell count (WBC), which is commonly used to evaluate immune status and potential disturbances in haematopoietic function. Increased WBC values are considered indicators of inflammatory responses or neoplastic processes, whereas decreased leukocyte counts may reflect impaired haematopoietic activity or tissue damage (Bukowska, 2015). In the present study, WBC was significantly increased in embryos exposed to 1 mg/kg BaP, showing a 69% increase compared with the intact group (Fig. 2).

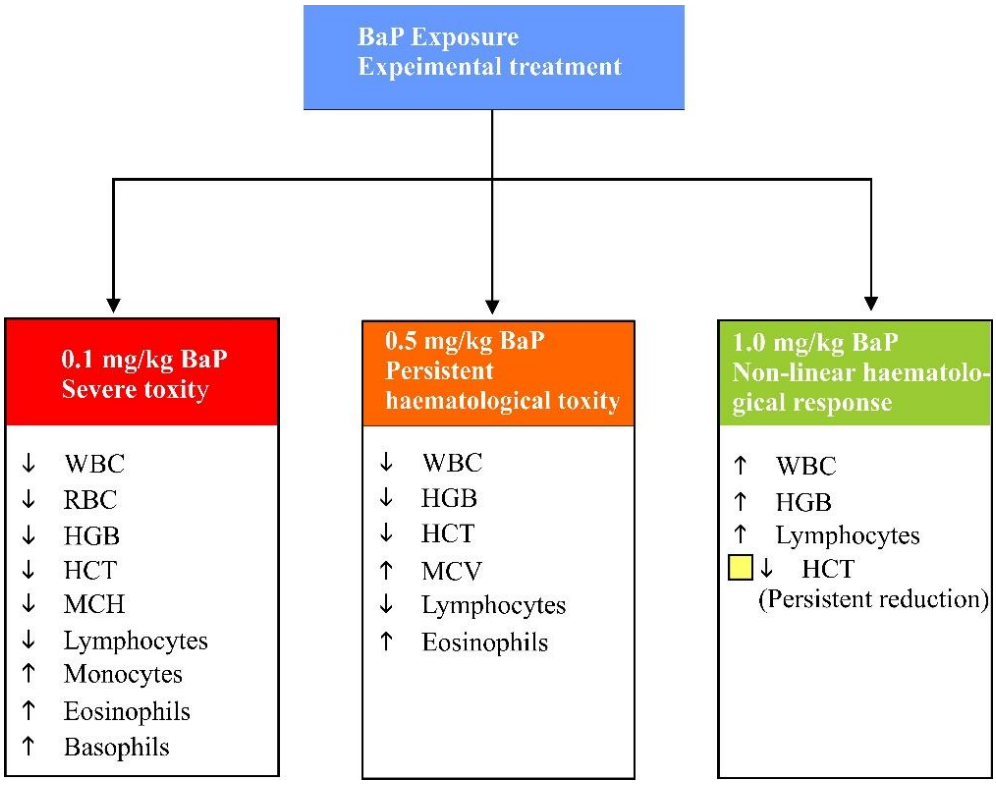


Fig. 2. Dose-dependent effects of benzo[a]pyrene exposure on haematological parameters in chicken embryos (*Gallus gallus domesticus* L.)

In contrast, embryos treated with 0.1 and 0.5 mg/kg BaP exhibited approximately 60% reductions in WBC compared with intact embryos. These findings suggest that BaP may influence leukocyte homeostasis in a dose-dependent manner and that higher concentrations may promote inflammatory responses.

Another analysed parameter, the red blood cell count (RBC), is commonly used to assess erythropoietic activity and iron metabolism. In the present study, a significant decrease in RBC was observed following exposure to 0.1 mg/kg BaP (Fig. 2). Previous studies have demonstrated that polycyclic aromatic hydrocarbons may exert toxic effects on bone marrow cells in mice (Dertinger et al., 2001).

Haemoglobin concentration (HGB) was also evaluated. Alterations in haemoglobin levels may occur as a result of disturbances in electrolyte balance, protein synthesis, or erythrocyte metabolism. In the present study, HGB was significantly reduced in embryos exposed to 0.1 and 0.5 mg/kg BaP (Fig. 2). These changes may indicate an inhibitory effect of BaP on haemoglobin synthesis within erythrocytes (Scherer et al., 2000). Conversely, embryos exposed to 1 mg/kg BaP showed a significant increase in HGB concentration, which may reflect altered regulation of erythropoietic processes.

Haematocrit (HCT) was another parameter analysed in the present study. Reduced HCT values may be associated with anaemia, blood loss, or impaired function of haematopoietic tissues, whereas increased HCT values may occur under conditions related to cardiovascular or respiratory disturbances (Sikorski, Marcinowska-Suchowierska, 2007). In the present experiment, all BaP-exposed groups showed a significant decrease in HCT values compared with the intact group (Fig. 2). This effect may be associated with alterations in erythrocyte metabolism, oxidative stress, or the biotransformation of polycyclic aromatic hydrocarbons resulting in the formation of reactive metabolites that may affect erythrocyte integrity (McNeil, Wills, 1985).

Mean corpuscular volume (MCV) was subsequently analysed as an indicator of erythrocyte size and condition. Increased MCV values may be associated with macrocytic changes, erythrocyte degeneration, or disturbances in haematopoietic processes, whereas decreased MCV values may indicate alterations in iron metabolism or erythrocyte maturation (Sikorski, Marcinowska-Suchowierska, 2007). In the present study, a significant increase in MCV was observed in embryos exposed to 0.5 mg/kg BaP (Fig. 2). This change may indicate alterations in erythrocyte morphology and membrane properties, potentially resulting from interactions between BaP or its metabolites and cellular components (Modica et al., 1983).

Similarly, mean corpuscular haemoglobin (MCH) represents an important parameter reflecting the haemoglobin content of erythrocytes and may provide information about erythrocyte metabolism. A pronounced decrease in MCH was observed in embryos exposed to 0.1 mg/kg BaP, while a decreasing tendency was also noted in embryos treated with other BaP concentrations (Fig. 2). Reduced MCH values may be related to impaired haemoglobin synthesis or structural modifications of haemoglobin molecules caused by the formation of BaP–haemoglobin adducts. Comparable effects have previously been reported in rats exposed to aromatic hydrocarbons (Wolff et al., 1989).

In the differential leukocyte analysis, significant alterations were observed in the proportions of individual leukocyte populations following BaP exposure. The percentage of heterophils was significantly increased in embryos treated with 0.1 mg/kg BaP, whereas a decrease was observed in the control group. The proportion of lymphocytes increased significantly in embryos exposed to 1 mg/kg BaP (Fig. 2). Additionally, an increased percentage of monocytes was detected in embryos treated with 0.1 mg/kg BaP. The levels of eosinophils and basophils were elevated in all BaP-exposed groups, suggesting that exposure to this compound may influence immune cell distribution during embryonic development.

In conclusion, the present study demonstrates that BaP exposure affects haematological parameters in chicken embryos, with the observed responses varying depending on the applied dose. The results indicate that BaP may interfere with erythrocyte development, leukocyte distribution, and overall haematopoietic function during embryogenesis. Further investigations are required to elucidate the molecular mechanisms underlying these effects.

Because BaP is a hydrophobic compound that requires administration in a lipid-based vehicle, the possibility that rapeseed oil itself may influence certain physiological parameters should be considered. The observed changes in the control group may therefore be partially associated with the effects of the vehicle and should be taken into account when interpreting BaP-induced alterations.

Conclusion

The present study demonstrates that benzo[a]pyrene exposure significantly affects haematological parameters in chicken embryos during early development. The chicken embryo *in ovo* model proved to be a useful and sensitive system for evaluating the impact of environmental pollutants on embryonic blood formation.

BaP administration caused alterations in both erythrocyte and leukocyte parameters. The most pronounced changes involved WBC, RBC, HGB, and HCT values, indicating that BaP may interfere with haematopoietic processes and erythrocyte metabolism. Significant changes in leukocyte subpopulations, including lymphocytes, monocytes, eosinophils, basophils, and heterophils, suggest that BaP may also influence immune system development during embryogenesis.

The observed responses varied depending on the applied BaP concentration, indicating a complex dose-related effect of this compound. These findings provide new insights into the effects of BaP on avian embryonic blood development and support the application of the chicken embryo *in ovo* model in environmental toxicology studies. Further research is needed to elucidate the molecular mechanisms underlying BaP-induced haematological alterations.

Conflict of interest

The authors declare no conflict of interest related to this article.

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Wpływ benzo[a]pirenu na wybrane parametry krwi u zarodków kurzych rozwijających się *in ovo*

Streszczenie

Wielopierścieniowe węglowodory aromatyczne (WWA) stanowią dużą grupę trwałych zanieczyszczeń środowiska, wśród których benzo[a]piren (BaP) jest uważany za jeden z najważniejszych związków z perspektywy zdrowia publicznego, ze względu na jego właściwości rakotwórcze, teratogenne i cytotoksyczne. Chociaż biologiczne działanie BaP zostało szeroko zbadane u ssaków, jego wpływ na rozwój zarodka ptasiego i procesy hematologiczne pozostaje niedostatecznie poznany. W niniejszym eksperymencie wykorzystano model zarodka kurzego *in ovo* do zbadania wpływu ekspozycji na BaP na wybrane parametry krwi zarodka. Zapłodnione jaja poddano działaniu różnych dawek BaP podawanych do woreczka żółtkowego w trakcie rozwoju embrionalnego, a następnie oceniono zmiany w profilach hematologicznych. Określono następujące parametry: liczbę białych krwinek (WBC), liczbę czerwonych krwinek (RBC), stężenie hemoglobiny (HGB), hematokryt (HCT), średnią objętość krwinki czerwonej (MCV), średnią hemoglobinę w krwince czerwonej (MCH), a także odsetek heterofili, limfocytów, monocytów, eozynofili i bazofili. Narażenie na BaP spowodowało zależne od dawki zmiany, zarówno parametrów erytrocytów, jak i leukocytów, wskazując na zaburzenia hematopoezy embrionalnej i dystrybucji komórek odpornościowych. Obserwowane zmiany wskazują na wrażliwość zarodka kurzego *in ovo* na toksyczność rozwojową wywołaną przez BaP. Niniejsze badanie dostarcza ważnych spostrzeżeń na temat wpływu BaP na hematopoezę i rozwój układu odpornościowego podczas embriogenezy kręgowców i potwierdza zasadność wykorzystania zarodków ptasich jako cennego modelu w badaniach toksykologicznych środowiska.

Słowa kluczowe: modele zwierzęce, substancje rakotwórcze, hematopoeza (krwiotworzenie), wielopierścieniowe węglowodory aromatyczne

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