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IMMUNOTOXIC EFFECTS OF MALLARDS (ANAS PLATYRHYNCHOS, L) EXPERIMENTALLY EXPOSED TO LEAD PELLETS

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ABSTRACT

Lead is one of the most toxic metals and its negative effects range from mild biochemical and physiological disorders to serious pathological processes in which major organs and systems may be affected, with following functional and behavioral changes. The probability for a bird to be poisoned is determined by several factors, such as time of retaining the lead elements, frequency of exposure, nutritional conditions, stress, etc. Our research work focuses on the influence of different total lead content after ingesting lead shots on the mechanisms of non-specific immunity.

Key words: mallards, lead pellets, phagocytosis, NBT-test, non-specific immunity.

Introduction

Lead is one of the most toxic metals and its negative effects range from mild biochemical and physiological disorders to serious pathological processes in which major organs and systems may be affected, with following functional and behavioral changes (Bellrose, 1959). The probability for a bird to be poisoned is determined by several factors such as time of retaining the lead elements, frequency of exposure, nutritional conditions, stress, etc. (Franson and Pain, 2011).

Waterfowl are exposed to the toxic effects of lead due to the ingestion of lead pellets from the bottom of the natural reservoirs in areas with intense hunting activities. The lead pellets used in hunting activities contaminate wetlands worldwide as their density in many places in the upper 20 cm of sediment is over 100 pellets /m^2 (Mateo, 2009). Lead acts at a molecular level and may lead to a number of toxic effects which have been well described in waterfowl (Beyer et al., 1988; Pain et al., 2009).

Many epidemiological studies have demonstrated the immunotoxicity of metals in several species. Increased phagocytosis activity of immune cells has been described in different species of animals in response to metals, such as zinc, cadmium copper, and lead. Studies with reduced phagocytosis activities caused by lead and studies with no connection between phagocytosis activity and metals exist as well (Rainio et al., 2015). Several studies have addressed the immunosuppressive effects of lead exposure in mallards. Mallards dosed with one #4 lead shot exhibited depressed hemagglutination titers to sheep red blood cells, indicating an effect on antibody-mediated immunity by day 7 after treatment, and titers remained low until the end of the 3-week experiment (Trust et al. 1990; Franson and Pain, 2011).

Materials and methods

Sixteen clinically healthy mallards *(Anas platyrhynchos*) aged between 9 and 12 months were evenly divided (n = 4) in four groups and housed in separate aviaries. After a 7-day adaptation period, the birds were treated orally once with lead pellets #3 (medium weight 0.267 g), as follows: I group – 3 lead pellets; II group – 2 lead pellets; III group – 1 lead pellet. The ducks from the IV group were used as a negative control. [1]

Blood samples were obtained by venipuncture from *v. subcutanea ulnaris* before and on the 21st day after lead treatment. The collecting of blood samples was performed using a closed system including needles MN-SV21Q (butterfly type) and 3ml tubes with Li- Heparin for hematology and immunology analysis.

Isolation of heterophils: For isolation of heterophils anticoagulated blood was mixed with 1% methylcellulose and centrifuged at 25 g for 5 min. The supernatant was diluted 1:1 with physiological solution (0.9% NaCl) and then was layered over a *Polysept gradient* (specific gravity 1.077). The sample was then centrifuged at 250 g for 30 min. After centrifugation, the band containing the heterophils was collected and washed three times in RPMI 1640 medium and resuspended in the same RPMI medium. The final concentration of 2 x 10^6 heterophils/cm^3 was prepared and was stored on ice until used.

Phagocytic assay: Phagocytosis of *S. aureus 209 P* by the heterophils was determined in the sterile siliconized glass tubes that contained 2 x 10^6 heterophils and 2 x 10 bacteria in a total volume of 1cm^3. The tubes were centrifuged (450& 15 mm, room temperature) in order to maximize contact [4, 7]. The cells were incubated at 37°C for 30 min. Smears were then prepared from each tube and stained by Romanowsky-Giemsa method, and examined by light microscopy with the oil immersion objective (x100). The results are expressed as a percentage of heterophils containing *S. aureus 209P* and the average number of *S. aureus 209P* per ingesting heterophils.

NBT test: NBT positivity was considered to be characterized by a massive or granular, dark purple or black precipitate, within the leucocytes. Fifty microliters of NBT solution were added to each tube containing 50 µl of zymosan (positive control). Then 100 µl of heterophil suspension was added to each tube. A negative control (to assess the possibility of spontaneous NBT reduction by heterophils) consisted of 50 µl of NBT solution, 50 µl each of RPMI 1640, and 100 µl of heterophil suspension. All tubes were incubated at 37°C in a shaking water bath for 15 min. Then tubes were centrifuged at 60 x g for 5 min. The supernatant was discarded and smears were made from the pellet for microscopic evaluation. The smears were air-dried, fixed with *May-Grünwald-Giemsa* stain, and examined microscopically. The number of NBT-positive cells among 100 heterophils was recorded as a percentage (NBT index).

The analysis and statistical processing of the data were performed by the computer program SPSS 19.0. The data are expressed as mean plus standard error. In this study, the assessment is made with a guaranteed probability of 0.95 (significance level $\alpha = 0{,}05$), where $p < 0.05$ was adopted as the lowest level of statistical reliability.

[1] This study was approved by the Commission for animal welfare at the Faculty of Veterinary Medicine, University of Forestry - Sofia (Permit Number: № 80/2018 for educating students and conducting scientific research in veterinary medicine) and is conducted in compliance with EU and the national legislation.

Results

Morphological changes in red blood cells

The microscopic examination of blood smears of all experimental groups showed destroyed heterophils (Het) with exhausted bactericidal and metabolic potential (Fig. 1). The changes in the phagocytic activity of Het in the different groups showed a statistically significant difference between the mallards from the first experimental group (three lead pellets # 3) compared to the control group.

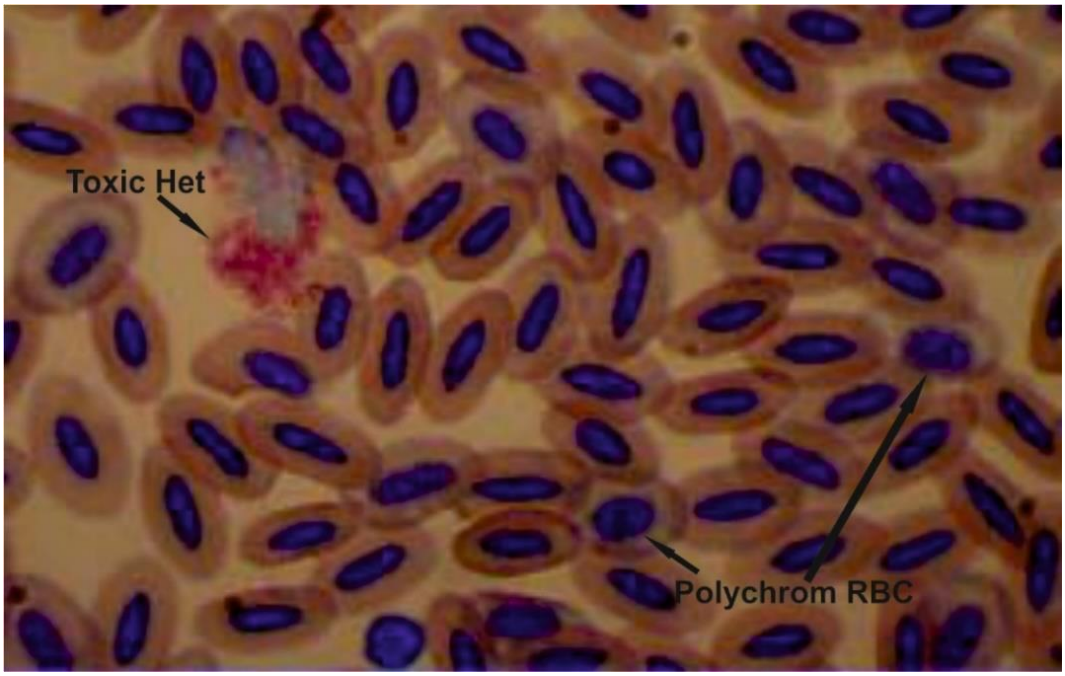

Figure 1: Blood smear from mallard duck experimentally exposed to shot pellets. A toxic heterophils (arrow) is present in the upper left. The arrows in the lower right indicate hypochromic erythrocytes (*May-Grünwald-Giemsa stain*).

The blood smears also showed the presence of hypochromic erythrocytes with bluish stained protoplasm and fine chromatin in the nuclei (Fig. 1).

Phagocytic activity

The changes in the phagocytic activity of heterophils (Het) in the different groups showed a statistically significant difference between the mallards from the first experimental group (three lead pellets # 3) compared to the control group (Table 1).

The obtained results of orally treated mallards with a different number of lead pellets on the percentage of heterophils phagocytizing S. aureus 209 P are shown in Table 1.

Analysis revealed that the phagocytic assay of the first group is decreased significantly ($P < 0.01$). The lowest number of the lead pellets did not depress phagocytosis significantly from control values.

The second measurement of phagocytosis is the mean number of bacteria engulfed per phagocyte. The mean phagocytosis by heterophils was also reduced significantly of the mallards from the first experimental group (three lead pellets # 3) compared to the control group. A similar trend in phagocytic oxidase activity in the NBT test was observed as well.

Table 1: Statistically significant difference compared to the control group

Indicators / Group	Percentage of Het containing S. aureus 209P	Average number of S. aureus 209P per ingesting Het	Spontaneous NBT test	Stimulated NBT test
1group n = 4 3 shot pellets	41,00 ±2,972***	2,8 ± 0,179**	31,75 ± 0,853***	34,50 ±1,936***
2 group n = 4 2 shot pellets	54,25 ± 6,799	3,27 ± 0,246	33,00 ± 2,345*	36, 5 ± 1,554***
3 group n = 4 1 shot pellets	59,75 ± 2,286	3,85 ± 0,417	41,00 ± 3,240	49,50 ± 2,723
4 group n = 4 negative control	59,75 ± 1,108	4,30 ± 0,416	41,25 ± 0,853	57,25 ± 2,780

*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$

The results show a decrease in the percentage of heterophils (Het) that spontaneously regenerate NBT, as well as the percentage of NBT-positive heterophils (Het) as a result of the stimulation.

Discussion

The presence of hypochromic erythrocytes in the peripheral blood of the mallards of the experimental groups treated with higher doses of lead according to other studies is probably due to the accumulation of hemoglobin precursors in the nuclei of red cells. (Roscoe et al., 1979; Fisher et al., 2006).

The study of phagocytic activity is a major indicator of nonspecific immune reactivity. It is known that the process of phagocytosis and the functional activity of phagocytes are sensitive to the action of external and internal environmental factors, including heavy metals.

The idea that the components of the immune system represent a critical target for lead-induced toxicity has been suggested by recent work. In our study, suppression of the phagocytic activity of heterophils was observed, expressed mainly by a decrease in their ability to absorb bacteria. This might be due to the reduced number of opsonizing factors or to the reduction in phagocyte energy sources (Zelikoff, 1993).

In this sense, disorders of fatty acid metabolism, suppression of antioxidant systems, and reduction of TNF-α synthesis are important. Vallverdu-Coll et al. (2015) proved an increased phagocytic activity in female red-legged partridges 21 days after the ingestion of one or three lead pellets. In another study of dietary exposure of lead on oxidative status and phagocytosis activity in Great Tits (Parus major) the effect of phagocytic activity is not observed (Rainio et al., 2015).

The process of phagocytosis is accompanied by an explosion of redox reactions in heterophils. That functional activity of phagocytes determined by the NBT test is an informative indicator of the effect of lead on the immune system

Nitroblue tetrazolium (NBT) is a yellow dye, which penetrates the heterophil cell membrane. If heterophils are stimulated, NADPH oxidase converts the dye in the phagolysosome into blue-black deposits called formazan. Hence, the formation of formazan is indirect evidence to the degree of respiratory burst activity of heterophils (Ghaffari et al., 2008).

Conclusion

The obtained results indicate a dose-dependent suppression of the functional activity of phagocytes. The results show a decrease in the percentage of heterophils that spontaneously recover NBT, as well as in the percentage of NBT-positive heterophils as a result of the stimulation. These data suggest inhibition of oxygen-dependent metabolism in ducks that received more lead pellets.

Many studies about immunotoxicity are available, but the results are contradictory. Although this research demonstrated statistically significant immunosuppression of the phagocytic activity of heterophils of mallards after treatment with lead pellets, further studies to investigate the mechanisms of lead immunotoxicity and its ability to affect the immune response in birds are needed.

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