

Pathomorphological Changes in the Organs and Tissues of Animals During Acute Intoxication with Neonicotinoids Containing Nitroso or Cyano Group

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Abstract— This article describes the results of the authors' experimental studies of pathomorphological changes in laboratory animals associated with intoxication with nitroso-containing (clothianidin) and cyano-containing (thiacloprid) compounds from the group of neonicotinoids. Acute intoxication was caused in 20 white outbred rats by single intragastric, via the tube, administration of pesticides Calypso (active ingredient thiacloprid, 480 g/L, BayerCropScience, Germany) and AnoEx (active ingredient clotianidin, 20 g/L, Agravis Raiffeisen AG, Germany) in the doses of 1/10 LD₅₀, respectively. Control group included intact animals (n=10). To assess the overall morphological picture, histological sections were stained with hematoxylin and eosin, and Perls' method was used to indicate hemosiderin. Microphotography of histological preparations was carried out using Altami BIO 1 microscope with USMOSO3100KPA USB digital ocular camera. The research results prove that neonicotinoids, despite differences in their chemical structure (nitroso- and cyano-containing preparations), cause the development of multiple organ pathologies due to vascular disorders. As a result of damaged microvasculature and endothelium of large vessels, the breakdown of histochematic barrier occurs what contributes to the delivery of pesticides and their metabolites into the cells of organs and damage of these organs. In addition, vascular disorders lead to the development of hypoxia which exacerbates the damaging effect on vascular wall and tissues. Dystrophic changes and multiple hemorrhages develop in parenchymal organs, and fibrillogenesis is activated. In some cases, there are alternative changes in the form of focal necrosis of liver. Expanded spleen marginal zone and increased area of periarteriolar lymphoid sheaths reveal immunity stress and potential danger of immunosuppression in the post-intoxication period. Mentioned pathomorphological changes in the animal organism allow planning complex diagnostic and therapeutic measures in the course of the use of neonicotinoids.

Keywords—*neonicotinoids, thiacloprid, clothianidin, Calypso, AnoEx, acute intoxication, pathomorphological changes.*

I. INTRODUCTION

Currently, neonicotinoids are the most popular group of insecticides intended to protect different crops, animals and humans. Due to their multipurpose use and wide coverage, sales of these drugs are allowed in more than 120 countries of the world. Two classes of neonicotinoids are distinguished according to the chemical structure – nitroso-containing compounds (imidacloprid, clotianidin, dinotefuran) and cyano-containing compounds (acetamiprid and thiacloprid).

Thiacloprid - N- [3-(6-chloropyridin-3-methyl)-thiazolidin-2-ylidene]-cyanamide belongs to the group of chlornicotinyls with a systemic effect and intestinal surface activity for insects. According to IUPAC (International Union of Theoretical and Applied Chemistry) classification it belongs to medium toxic compounds with LD₅₀ for rats of 444 mg/kg by oral administration [1]. Clothianidin (N-(2-chlorothiazol-5-ylmethyl)-N'-methyl-N''-nitrosoguanidine) was registered in 2002 and has a non-cyclic structure with a sulfur atom in molecule which significantly influenced the nature of this chemical compound increasing the spectrum of drug effects on target objects. In regards to the irritating effect on the skin of rabbits, acute dermal toxicity for rats, and sensitizing effect on guinea pigs clotianidin is a low-toxic compound, and it is considered to be moderately dangerous referring to acute inhalation toxicity for rats and irritating effect on the mucous membrane of rabbits' eyes [2].

Widespread use of chemical products for protection of plants and animals contributes to the spread of residual pesticides in the environment. Against the background of constantly increasing assortment and the implementation of pesticide use in national economy, information on the consequences of their use for non-target organisms remains extremely insufficient and contradictory.

The literature describes the negative effect of neonicotinoids on non-target objects [3]. Thus, the results of the study performed by scientists from other countries show that neonicotinoid insecticides (imidacloprid, thiamethoxam, clothianidin, acetamiprid, and thiacloprid) can remain in the soil for a long time since their expected half-life is more than 150 days [4]. This indicates the possibility of migration of their residual amounts along the food chains and the potential danger to animals and humans. A proof of the above is the research of Japanese scientists who found that the treatment of forests with neonicotinoids contributes to the appearance of pesticide residues in the human body [5-6]. Other studies revealed that incubation of bovine lymphocytes at various concentrations of thiacloprid led to reduced cell viability and proliferation, apoptosis induction, and DNA damage as evidenced by the formation of micronuclei in cells and breaks of double-stranded DNA [7]. It is worth noting that different drugs of the same group can cause multidirectional effects on body functions. Thus, when assessing the effect of neonicotinoid residues on the locomotor function of bees, it was found that neonicotinoids with a cyano radical caused a

decrease in bee mortality, while these compounds with nitro radical were highly toxic to them. Bees receiving thiacloprid and imidacloprid showed impaired coordination while acetamiprid did not affect their motor activity, and the use of thiamethoxam caused hyperactivity, as evidenced by an increase in average speed by 4.5 times and the distance traveled by 5 times [8].

Massive public outcry about the toxicity of neonicotinoids for the bee population led to the moratorium on the use of these drugs in some countries of the European Union and America [9]. Nevertheless, active advertising and high economic benefits contribute to their further implementation in agricultural production leading to the cases of not only chronic but also acute poisoning. According to Kiev City Toxicology Center, acute poisoning of people with pesticides accounts annually for 0.25-1.5% of all home poisonings; 80% are poisoning with organophosphorus substances, 15% – with carbamates, synthetic pyrethroids and neonicotinoids, and 5% – with unidentified pesticides. Among neonicotinoid poisonings, Confidor and Ratibor (active ingredient imidacloprid), Calypso (active ingredient thiacloprid) insecticides were registered more often, as well as Prestige combined pesticide (active ingredient imidacloprid + pencycuron). The literature describes the symptoms of poisoning by certain classes of pesticides including neonicotinoids. So, clinical picture in the case of neonicotinoid poisoning is characterized mainly by hepatotoxic syndrome, toxic gastroenteritis, general intoxication syndrome, and depends on the chemical structure of the substance. The main symptoms of neonicotinoid poisoning in humans are nausea, sore throat and spastic abdominal pain, urge to vomit, drowsiness, dizziness, disorientation, hemorrhagic gastritis, fever, leukocytosis and hyperglycemia, in severe cases, convulsions were registered; mortality rates in acute cases remain high and tend to increase [3]. In order to get more information on target organs and the pathogenesis of intoxication with thiacloprid and clothianidin, as well as for development of effective poisoning treatment and prevention methods, it is necessary to study pathomorphological changes in experimental animals during intoxication.

Objective: to establish pathomorphological changes in the organs and tissues of animals in the case of poisoning with nitroso- and cyano-containing neonicotinoids.

II. MATERIALS AND METHODS

The experiment was performed on sexually mature outbred white rats in accordance with the international recommendations on the use of animals for biological and medical research (1986). The conditions for keeping and feeding experimental animals were in accordance with the “Sanitary Rules for the Design, Equipment and Maintenance of Experimental Biological Clinics”, approved by the orders of the Ministry of Health of USSR No. 1045 as of 06 April 1973 and No. 1179 as of 10 October 1983. All animals were kept in standard vivarium conditions taking into account the

recommendations set out in the Guide for the care of laboratory animals.

Acute intoxication was caused in 20 white outbred rats (2 groups of 10 animals each) by single intragastric, via the tube, administration of pesticides Calypso (active ingredient thiacloprid, 480 g/L, BayerCropScience, Germany) and AnoEx (active ingredient clotianidin, 20 g/L, Agravis Raiffeisen AG, Germany) in the doses of 1/10 LD₅₀, respectively).

Intact rats (n = 10) received intragastrically purified water in the same volume. Within 7 days after pesticide administration, the clinical status of animals was evaluated. Then, after the end of specified period, animals were euthanized with preliminary sedation with Zoletil drug. Pieces of internal organs were fixed in a 4% neutral formaldehyde solution with phosphate buffer by Biovitrum LLC (St. Petersburg). The material was paraffin-embedded according to the generally accepted technique (D.S. Sarkisov, Yu.L. Perov, 1996; V.V. Semchenko et al., 2006). Frontal sections of organs 4-5 μm thick were prepared using LaboCut 4055 rotary microtome (Slee, Germany) with A35 disposable microtome blades (Feather, Japan). Sections were placed on slides with standard thickness by Menzel-Glaster (Germany) and stained with hematoxylin and eosin to evaluate the overall morphological picture. Perls' method was used to indicate hemosiderin. After staining, the sections were embedded in BioMount medium and covered with standard coverslips. Microphotography of histological preparations was carried out using Altami BIO 1 microscope with USMOSO3100KPA USB digital ocular camera.

III. RESULTS

During a necropsy study 7 days after acute neonicotinoid poisoning, hyperemia of the serous membranes of internal organs was noted. The heart is enlarged, expanded, coronary vessels are overfilled with blood. Transparent yellowish fluid was found in the pericardial cavity of animals undergoing clotianidin intoxication, and coagulated blood was found in the cavities of their hearts. Myocardium was flabby, of gray-red color. Lungs were not collapsed, full-blooded, with single hemorrhages. Liver in rats intoxicated with thiacloprid was dark brown, and in rats intoxicated with clothianidin, gray-clay color and flabby consistency were observed. Kidneys were unevenly colored, their elasticity was reduced. Spleen was slightly enlarged, full-blooded, splenic pulp scrape was without abnormalities.

In the liver of rats intoxicated with Calypso, most hepatocytes had granular cytoplasm, sometimes vacuolated hepatocytes were found. Tubular structure of the organ is broken, most cells with poorly differentiable nuclei. The destruction of hepatocyte plasma membrane was often recorded what is illustrative of necrotic and necrotic processes in this organ. Proliferation of fibroblastic different cells and the appearance of fibrous connective tissue fibers were found around large vessels and bile ducts. Sometimes clusters of mononuclear cells were found near vessels (Fig. 1).

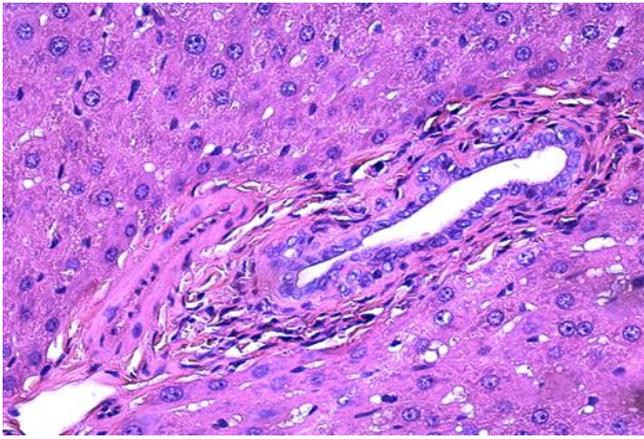


Fig. 1. Granular dystrophy, necrobiosis and necrosis of hepatocytes. The growth of connective tissue around the vessels 7 days after Calypso administration in the dose of 1/10 LD₅₀. Hematoxylin and eosin staining. Magnification × 600.

In the kidneys of rats intoxicated with Calypso, significantly dilated collecting ducts and renal glomerular mesangial swelling were visible. Sludge phenomenon was found in capillaries. In some areas, vascular wall damage was observed with subsequent moving of blood into interstitial space. In the glomeruli, full-blooded capillaries were visible with thickened capsule and basement membrane due to collagen fibers. Vascular extravasation of mononuclear cells was observed in some areas of the cortical substance around glomeruli. Proximal convoluted tubules were in the state of dystrophy, had no lumen, the cytoplasm of epithelial cells contained protein units. In the interstitium of this organ, the growth of connective tissue was observed.

In the heart of animals, 7 days after intoxication, hemodynamic disorders and damaged epithelium of larger arteries characterized by vacuolization and desquamation of endotheliocytes were found. Swelling and red blood cell accumulation were visible in paravasal space. Groups of dystrophically altered muscle fibers were found in subendocardial areas: cardiomyocytes with pale blue cytoplasm had no sharp contours, their nuclei were of lighter color or absent. In some areas, wave-like deformation of myocardial muscle fibers was registered as a sign of possible cardiac rhythm disorder (Fig. 2).

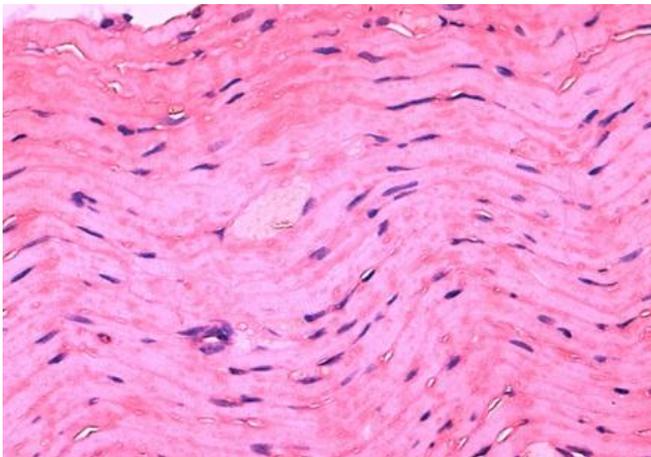


Fig. 2. Areas of wave-like deformation in myocardial muscle fibers (as a sign of possible cardiac rhythm disorder) and dystrophy of cardiomyocytes 7 days after intoxication of rats with Calypso at the dose of 1/10 LD₅₀. Hematoxylin and eosin staining. Magnification × 200.

Large foci of lymphohistiocytic infiltration were registered in the lungs around large vessels and bronchi. The accumulation of alveolar macrophages was observed in alveoli and in thickened interalveolar septa. In several large bronchi, damage and desquamation of epithelial cells was found. Accumulation of mononuclear cells with red blood cells and fibrin was visible in the lumens of alveoli. Several areas with destroyed interalveolar septa were also observed. There were signs of circulatory disorders in the organ in the form of hemorrhages, dilated blood vessels and stasis.

Histological examination of rat adrenal glands 7 days after acute intoxication reveals increased area of glomerular zone which contains endocrinocytes with lightened reticular cytoplasm. In the cytoplasm of bundle zone cells, rounded vacuoles are located. Endocrinocytes of reticular zone without significant changes. The vessels of adrenal medulla are dilated and full-blooded. The growth of fibrous connective tissue in bundle zone and adrenal medulla was registered.

Histological study of rat spleen preparations revealed that the capsule was represented by a dense fibrous connective tissue with trabeculae extending from it and integrating into pulp. White and red pulp represented two compartments of spleen parenchyma with different structure. White pulp formed by accumulation of T and B lymphocytes was located around central arterioles. In the white pulp, different stages of immune response take place, in this regard, it can be divided into several zones. Marginal zone was located on the border with red pulp; it contained a large number of reticular cells which, with the help of long processes, regulate blood flow in spleen. Here, antigens from peripheral blood are absorbed, platelet deposition, capture and phagocytosis of damaged cells occur. In animals with acute intoxication with Calypso, increased marginal zone was observed what was the sign of toxic agent contact with immunocompetent cells. Periarteriolar lymphoid sheaths are the place of T-lymphocytes localization around the eccentrically located central arteriole. The area of this zone in the spleen of experimental animals was increased in comparison with the control group. Primary and secondary lymphoid nodules were densely located along the periphery of the periarteriolar lymphoid sheaths which were a zone of accumulation of B cells located in follicular dendritic cells network (Fig. 3).

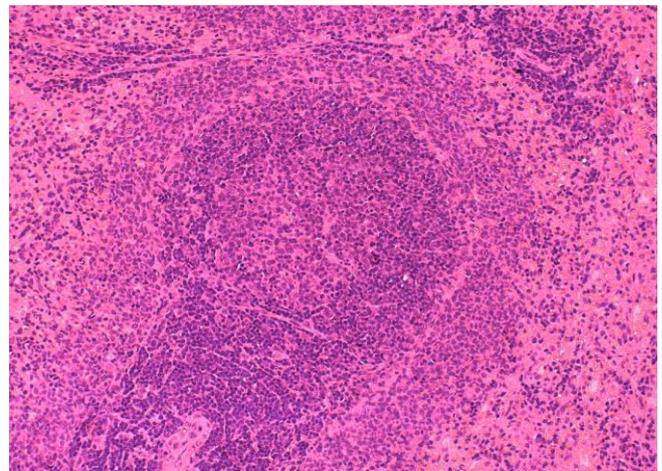


Fig. 3. Increased marginal zone, increased area of PALS of spleen white pulp in rats 7 days after the administration of Calypso® in the dose of 1/10 LD₅₀. Hematoxylin and eosin staining. Magnification × 200.

Red pulp is represented by sinusoidal vessels and pulp cords located between them which consist of reticular tissue and contain accumulations of red blood cells, white blood cells, macrophages.

In the liver of rats intoxicated with AnoEx, in several lobules, dilated and full-blooded central vein and adjacent intralobular capillaries are visible. Hepatic tubules are compressed. In other segments, mainly in their central zones, tubular structure is broken. Hepatocytes are enlarged, their shape is changed, their contours are poorly marked. Cytoplasm of many hepatocytes is vacuolated. Among hepatocytes undergoing hydropic degeneration, there are liver cells in the state of necrosis and necrobiosis. The nuclei of many hepatocytes are in a state of pycnosis and become very compact. The number of unchanged liver cells is sharply reduced; it is especially significant in the center of hepatic lobules. The cells of peripheral zones of liver lobules show the signs of granular dystrophy; their cytoplasm is cloudy, with small grains. In the nuclei of these hepatocytes, pycnosis, rhexis, and less frequently lysis are sometimes found (Fig. 4).

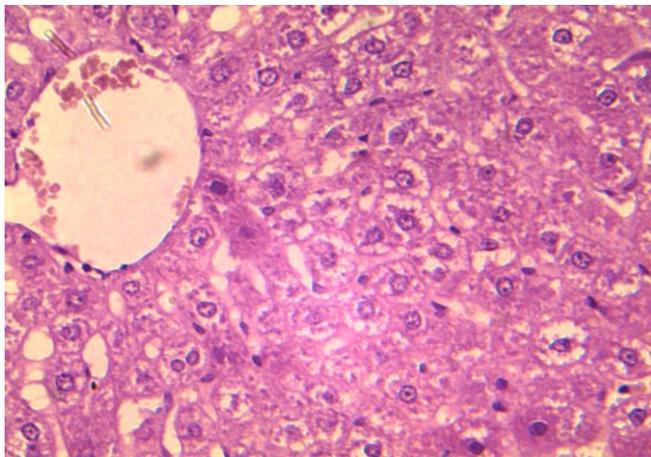


Fig. 4. Rat hepatocytes with large and small vacuoles in their cytoplasm in the case of acute poisoning with AnoEx in the dose of 1/10 LD₅₀. Hematoxylin and eosin staining. Magnification × 600.

Binuclear hepatocytes are more common in the liver of intoxicated animals in comparison with the control group. Some nuclei are enlarged and hyperchromic but most cells are of normal size. During intensifying regeneration processes, activation of fibrillogenesis was registered, especially in the periportal zone of acinus. The nuclei of Kupffer cells were swollen, hyperchromic. Kupffer cells are in some places absent.

Renal blood vessels are full-blooded, there are single hemorrhages which are most pronounced in cortex. Glomeruli are slightly enlarged, hyperemic, single glomeruli are firmly against the capsule. In some areas of cortex and medulla, mononuclear infiltrates were found. In some animals, proliferation of connective tissue in stroma was observed.

The lumen of renal tubules is somewhat narrowed, filled with foamy contents. Many convoluted tubule epithelial cells are enlarged, with granular cytoplasm. The contours of epithelial cells are poorly marked. In some animals, convoluted tubule epithelium was in the state of hydropic dystrophy. There were single epithelial cells with nuclei in the state of pycnosis or lysis.

Microscopic examination of heart revealed full-blooded myocardial vessels. The walls of vessels were in the state of mucoid swelling. Single hemorrhages under epicardium. In myocardium, areas with swollen muscle fibers were visible, with mild cross striation. Contours of cardiomyocytes are poorly marked; many nuclei are in the state of lysis or pycnosis (Fig. 5).

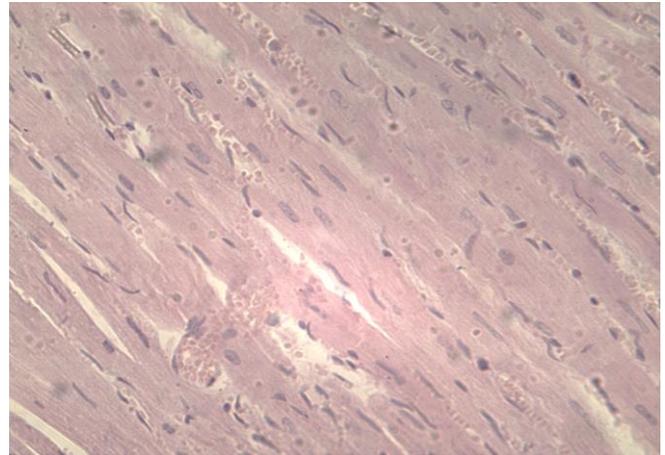


Fig. 5. Myocardial dystrophy in rats associated with acute poisoning with AnoEx in the dose of 1/10 LD₅₀. Hematoxylin and eosin staining. Magnification × 300.

Blood vessels of lungs are dilated, single hemorrhages are visible. In many alveoli, oxyphilic contents are found. Alveolar epithelium is swollen; its cells are in some places desquamated and found in the lumen of alveoli. Red blood cells, lymphocytes and other cells are found in the contents of alveoli. Interlobular connective tissue is thickened, infiltrated with mononuclear cells and neutrophils. Peribronchial lymphoid tissue is increased.

In the adrenal glands, hyperemia was found, more intensive in medulla. The cytoplasm of glomerular zone endocrinocytes is granular, sometimes with vacuoles, many nuclei are reduced in size, pycnotic. The cytoplasm of bundle zone endocrinocytes is cellular, unevenly colored. The nuclei of endocrinocytes are in the state of pycnosis or lysis. Medullary adrenalocytes are swollen, with light cytoplasm, large nuclei.

Histological examination of spleen 7 days after intoxication with clothianidin revealed increased area of white pulp in comparison with control group. In experimental animals, dilated periarteriolar lymphatic sheaths and marginal zones were observed. Lymph nodes in experimental animals, as in control ones, are large and have expanded centers.

Red pulp is filled with red blood cells, white blood cells, macrophages. The parts of red pulp around lymph nodes which are the clusters of macrophages contained large amount of hemosiderin (Fig. 6).

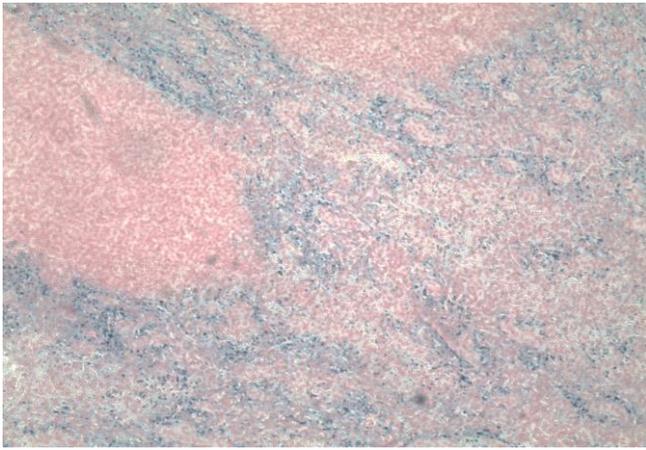


Fig. 6. Hemosiderin in rat spleen during acute poisoning with Anox in the dose of 1/10 LD₅₀. Perls' method. Magnification × 150.

Pigment was mainly found in the cytoplasm of macrophages, reticular cells, and in the endothelium of venous vessels. In experimental animals, more often than in control ones, the extracellular distribution of pigment was found. Large amount of hemosiderin granules was found on the periphery of lymph nodes.

IV. CONCLUSION

Research results convincingly prove that neonicotinoids, despite differences in their chemical structure (nitroso- and cyano-containing agents), cause the development of multiple organ pathologies associated primarily with extensive vascular disorders. As a result of damaged microvasculature and endothelium of large vessels, the breakdown of histochematic barrier occurs what contributes to the delivery of pesticides and their metabolites into the cells of organs and damage of these organs. In addition, vascular disorders lead to the development of hypoxia which exacerbates the damaging effect on vascular wall and tissues. Dystrophic changes and multiple hemorrhages develop in parenchymal organs, and fibrillogenesis is activated. In some cases, there are alternative changes in the form of focal necrosis of liver. Expanded spleen marginal zone and increased area of

periarteriolar lymphoid sheaths reveal immunity stress and potential danger of immunosuppression in the post-intoxication period. Mentioned pathomorphological changes in the animal organism allow planning complex diagnostic and therapeutic measures in the course of the use of neonicotinoids.

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