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Alterations in the Erythrocyte Membrane and Ultrastructural Changes in the Liver and Kidney of Albino Mice Exposed to Fipronil

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ABSTRACT

Fipronil is a highly active, broad spectrum systemic pesticide belonging to the phenyl pyrazole group of chemicals. The aim of the present study was to observe the alterations in the surface morphology of erythrocyte membranes of Swiss albino mice exposed to fipronil with the help of scanning electron microscope (SEM) and also its effect on the liver and kidney with the help of transmission electron microscope (TEM). The animals were divided into control group which received distilled water via intraperitoneal injection, and a treated group which received 1/3rd of LD_{50} dose of fipronil intraperitoneally, every 24 hours for 30 days. Various types of alterations in the morphology of the red blood cells of the fipronil treated mice were observed. There was decrease in discocytes and increase in the number of echinocytes, spherocytes and ovalocytes. In case of liver, the degeneration of the nucleus, nucleolus, nuclear membrane and mitochondria were commonly observed in all the treatments compared to the control. The cytoplasm was also found to be vacuolated with the breakdown of the organelles. The kidney cells of the treated mice were also observed with different degrees of degeneration. The mitochondria were damaged with destructed cristae surrounded by basal membrane that is disorganized and the nucleus was shrunken with dense chromatin condensation. Presence of vacuoles in the cytoplasm was seen as well as thickening of the glomerular basement membrane was observed.

INTRODUCTION

Indiscriminate and inappropriate use of pesticides may lead to their residues in the food chain which may exert their harmful effects on the target and non target organisms. Knowingly or unknowingly, farmers are addicted to using agrochemicals indiscriminately and excessively which has made the situation from bad to worse in India and elsewhere in the world (Rajendran 2003). The exposure of pesticides through occupation and environment causes a range of human health problems like immune suppression, hormone disruption, diminished intelligence, reproductive abnormalities and cancer (Abhilash & Singh 2009).

Fipronil is a highly active, broad spectrum systemic pesticide belonging to the phenyl pyrazole or fiprole group of chemicals and is a potent disrupter of the insect's central nervous system via interference with the gamma-aminobutyric acid (GABA-) regulated chloride channel (Tingle et al. 2003). Fipronil may cause slight skin irritation and mild eye irritation in humans. The clinical signs and symptoms reported after ingestion of fipronil by humans include sweating, nausea, vomiting, headache, abdominal pain, dizziness, agitations, weakness and seizures (Fung et al. 2003, Jennings et al. 2002).

Pesticides have been reported to cause changes in the shape of albino mice erythrocytes as maximum number of pesticides are highly lipid soluble, as a result of which it alters the phospholipids of plasma membrane and consequently the shape of the RBC (Baruffaldi & Cucchi 1989). Erythrocytes can occur in many different shapes. The presence of abnormalities in the number, size or alterations in shapes can provide diagnostic clues for the well being of any species (Sastry & Gaurkar 2014).

The liver and kidney are the target organs in toxicological prospects regarding its role in detoxification, biotransformation and excretion of xenobiotics. After enteric uptake of injurious materials, these organs are exposed to hazards via the portal circulation. Kidney is the major organ for detoxification and is frequently susceptible to nephrotoxic effects of the pesticides (Rekha et al. 2013). Histopathological changes have been widely used as significant biological markers for environmental toxicity and can be used as a warning symptom for organisms health. The analysis of toxic properties of drugs and chemicals using *in vivo* mammalian systems (mice or rat) is of enormous value, which reflects indirect toxic effects on the humans because of their high degree of presumptive human relevance (Bagri et al. 2013).

There is scarce data available on the effect of fipronil on mammals. Therefore, the following study was undertaken to study the effect of the pesticide on the erythrocyte membrane and vital organs (liver and kidney) of Swiss albino mice.

MATERIALS AND METHODS

Experimental design: The mice were obtained from the Central Animal House, Regional Institute of Medical Science, Manipur. The animals were kept in the animal house of Department of Biotechnology, Assam University in polypropylene cages (5 animals per cage with sawdust as the bedding material) at 25±5°C temperature on a 12 hour light/dark cycle. Healthy animals of both sex weighing 25-30 g and 10 to 12 weeks old were used. They were acclimatized in the laboratory conditions seven days prior to starting of the experiment after which the experimental mice were divided into fipronil treated group and control group with 5 mice in each. All the mice were fed with standard balanced diet and water *ad libitum.* LD_{50} value of fipronil was standardized and found to be 70 mg/kg. Dose of 1/3rd of LD_{50} i.e. 23.3 mg/kg body weight in distilled water was injected intraperitoneally for 30 days every 24 hours. After 30 days, the mice in all the groups of treatment as well as control were euthanized in a chloroform chamber and blood was collected via cardiac puncture. The liver and kidney were also harvested for electron microscopy study.

The study was approved by the Assam University Ethical Committee (IEC/AUS/C/2014-011 dated 14/8/14).

Electron microscopy: A drop of blood was fixed in 0.1 M 2% glutaraldehyde buffered with sodium cacodylate for 30 minutes. The samples were centrifuged at 1500 r/min for 5 minutes, washed and resuspended in distilled water. The whole process was repeated three times and a thin film was decanted and applied to a coverslip after resuspending in distilled water and air dried. After drying, the samples were studied under a scanning electron microscope (QUANTA 250 FEI) at the Department of Physics, Manipur University, Canchipur, Manipur.

The liver and kidneys of both, the control and treated group, were cut into 1 mm cube and kept in 2.5-3% Kannovsky's fixative for 4 hours in 4°C temperature for fixation. The samples were further prepared according to the methodology followed by Dey (2013). The sections were made ready for observation in the transmission electron microscope (JSM-100CX, Jeol) at Sophisticated Analytical Instrument Facility, North Eastern Hill University, Shillong.

RESULTS AND DISCUSSION

Scanning electron microscope (SEM) studies of erythrocyte membrane: In the present study, SEM images of control mice showed perfect biconcave discocytes (D) which is the shape of a normal red blood cell (Fig. 1 A,B). SEM observations of the mice treated with fipronil indicated several morphological abnormalities in the erythrocyte membranes. Acanthocytes (Fig. 1 C,D) are deformed crenated RBCs characterized by a spherical core and a spiculated appearance, typically has 3 to 12 spikes or knobs which are irregularly distributed (Perrin et al. 2008). They are observed in the treated mice blood samples. These cells are pathogenic and are seen in chorea-acanthocytosis which is a progressive neurodegenerative disorder correlated with a deformation of the red blood cells called acanthocytosis in humans (Foglia 2010). The shape of the acanthocytes is a result of alterations in the cell membrane lipid content that is irreversible.

Echinocyte formation is also seen in the treated mice (Fig. 1 C, D). The transformation of discocytes to echinocytes is characterized by the tendency of membrane externalization. The echinocyte are known to impair blood flow through cellular interaction and increased the whole blood viscosity despite their advantage of easy passage through narrow splenic sinusoids. They undergo phagocytosis and are eliminated from circulation. Leblond (1973) reported that some factors inducing the discocyte-echinocyte transformation lead to measurable changes in the mechanical properties of the surface of the red blood cells. The bilayer couple hypothesis (Sheetz & Singer 1974) states that the changes in shape of erythrocytes by foreign molecule are due to the differential expansion of their two monolayers. Therefore, the spiculated shapes of echinocytes are induced when the added compound is inserted in the outer monolayer.

Stomatocytes are erythrocytes with a loosely folded, mouth like pale area across the cell and are swollen and cup shaped (Fig. 1D). In the treated mice, stomatocyte formation is visible. They are characterized by a decrease in the ratio of surface area-to-volume which may be induced either by reduction in surface area or increase in red cell volume. When viewed under the electron microscope, the biconcave discs are seen as a uni concave cup.

Spherocytes are cells that, rather than being discoform, are spherical or near spherical in shape with reduced surface area-to-volume ratio (Fig. 1C, D). These cells have lost membrane without the equivalent loss of cytosol. They lack the normal central pallor and may appear smaller than a discocyte. Their presence indicates that there is some sort of hemolysis going on and it also indicates a defect in the membrane function (Shashi & Meenakshi 2012). Ovalocyte formation is seen in the treated mice erythrocyte (Fig. 1C). These are erythrocytes that are elongated, elliptical or like

small cigar shape with blunt rounded cells. The oval shape is attributed to a defect in horizontal red cell membrane interactions (Shashi et al. 2012).

Transmission electron microscope (TEM) studies of liver and kidney: Electron micrographs of the liver of the control mice show normal ultrastructure of the hepatocytes with oval nucleus surrounded by numerous mitochondria in association with rough endoplasmic reticulum in the cytoplasm. The mitochondria are round or oval with visible cristae (Fig. 2A, C). The rough endoplasmic reticulum consists of dense, parallel cisternae with numerous ribosomes, which are closely associated with the mitochondria (Fig. 2A). Hepatic sinusoid can be seen which is a type of sinusoidal blood vessel with fenestrated, discontinuous endothelium that serves as a location for the oxygen rich blood from the hepatic artery and portal vein. Peroxisomes, lipid droplets and glycogen particles were also observed (Fig. 2A, E).

Hepatocytes are separated from the sinusoids by the Space of Disse which is occupied by cells, reticular fibres and hepatocytic microvilli. The Space of Disse is separated from the sinusoids by discontinuous epithelial cells (Fig. 2E).

In the fipronil treated mice liver, damage on the nucleus and cytoplasmic organelles are clearly visible. The cytoplasm appears vacuolated with disappearance of most of its components and appearance of vacuoles (Fig. 2B, D). Compared to the control, the mitochondria appear to have lost its cristae and degenerated rough endoplasmic reticulum is also observed (Fig. 2D). Pronounced changes in the blood sinusoid and bile canaliculi are visible (Fig. 2F), where the canaliculi is dilated with reduced number of microvilli. The wall of the sinusoids were obliterated and congested and the endothelial lining was eroded and ruptured. Where, in the control cell, the Space of Disse is clearly distinguished (Fig. 2E), in the fipronil treated liver sinusoid the Space of Disse is disintegrated and not visible (Fig. 2F).

Compared to the control (Fig. 3A, C, E), the kidney cells of mice treated with fipronil display different degrees of degeneration. The mitochondria are damaged (Fig. 3B) with destructed cristae surrounded by basal membrane that is disorganized. Pesticides can produce oxygen free radicals and affect the configuration and active transport of cell membranes. They may impair the mitochondrial membrane and play a role in mitotic dysfunction. The membranes of mitochondria are rich in unsaturated fatty acids which are sensitive to free radicals.

Presence of vacuoles in the cytoplasm is seen as well as thickening of the glomerular basement membrane (Fig. 3D). Thickness of the basement membrane with deposition of electron dense material may be due to the strong affinity for fipronil to the phospholipids, which may lead to the accumulation of free radicals (Ogutcu et al. 2006). The cytoplasm appeared to be electron lucent and reveals lysis of most of the organelles (Fig. 3F).

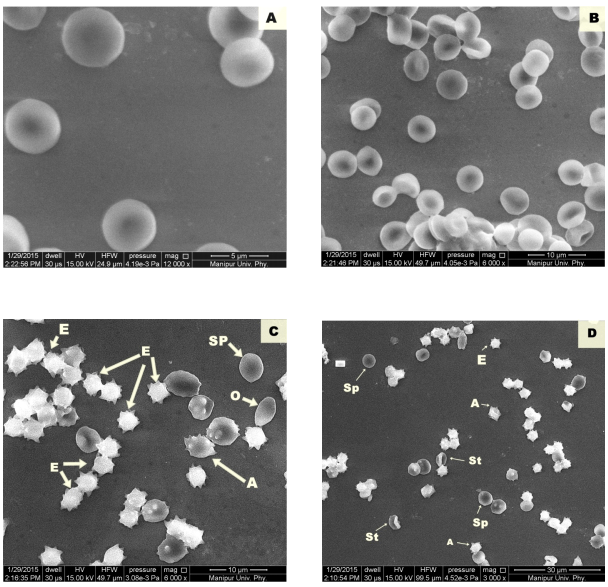

Fig. 1: SEM images of effect of fipronil on morphology of erythrocyte membrane.

A, B. Untreated albino mice erythrocytes showing no abnormalities, 12000x; 6000x.

C. Fipronil treated albino mice erythrocytes showing Spherocytes (SP), Acanthocytes (A), Echinocytes (E), Ovallocytes (O), 6000x.

D. Fipronil treated albino mice erythrocytes showing Spherocytes (Sp), Echinocytes (E), Acanthocytes (A), Stomatocytes (St), 300x

The electron microscopic observation of liver under the treatment of the selected pesticide showed pronounced pathological changes. Degeneration of cytoplasm was also observed in the liver cell, which is irreversible and associated with the destruction of protein lipid structure of intracellular membranes and lysis of the cytoplasm.

Mitochondria are one of the most sensitive indicators of injury to the cell. Swelling of mitochondria, loss of cristae and outer membrane was also observed in the study, as well as the degeneration of the rough endoplasmic reticulum. Membranes of endoplasmic reticulum and mitochondria are rich in unsaturated fatty acids and in a study by Caglar et al. (2013) it was observed that lipophilic metabolites of endosulfan might impair the structure of mitochondrial membrane leading to mitochondrial dysfunction. Increased intracellular reactive oxygen species (ROS) production exceeding the antioxidant capacity of the cell leads to lipid peroxidation and generalized oxidative damage to the mitochondrial components (Franco et al. 2009).

The disintegration of Space of Disse can be seen which

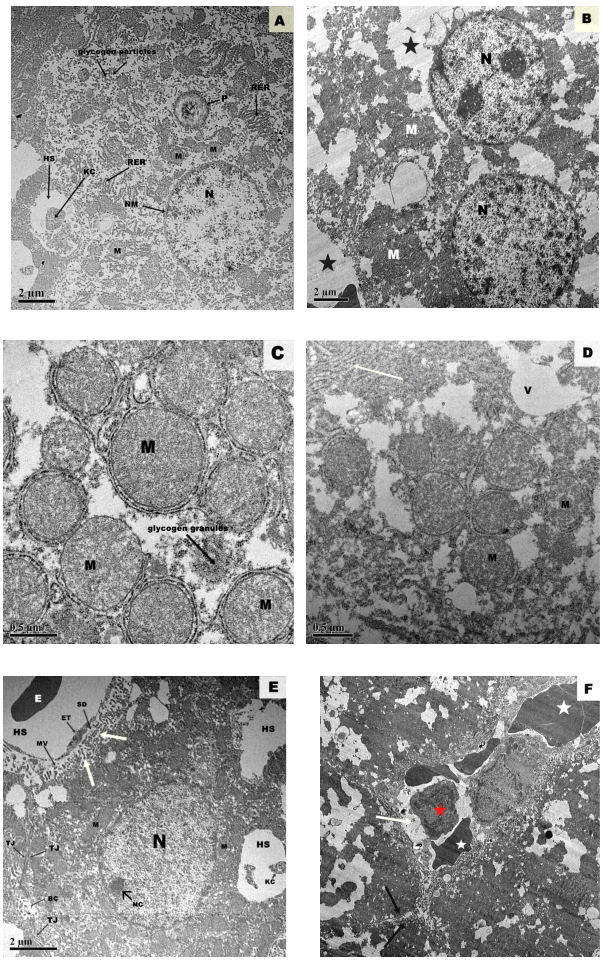

Fig. 2: TEM images on the effect of fipronil on the liver of Swiss albino mice.

A. Hepatocyte of control mice showing an oval nucleus (N) with intact nuclear membrane (NM) in the cytoplasm which contains mitochondria (M), peroxisomes (P), rough endoplasmic reticulum (RER), numerous glycogen particles, a hepatic sinusoid (HS) in which Kupffer cell (KC) can be seen. (1000x).

B. Hepatocyte of fipronil treated mice showing degenerated mitochondria (M), lucent cytoplasm (black star), and nucleus with chromatin condensation (N). (800x).

C. Normal mitochondria (M) dispersed in the cytoplasm of control mice. (6000x).

D. Fipronil treated mice liver showing degenerated mitochondria (M), rough endoplasmic reticulum (white arrow) and vacuoles (V) in the cytoplasm. (5000x).

E. Normal hepatocyte with numerous hepatic sinusoid (HS) is present with erythrocyte (E), microvilli (mv) lining the sinusoid, endothelium (ET), Space of Disse (white arrows). Bile canaliculus (BC) is seen sealed with tight junctions (TJ). (1500x).

F. Fipronil treated mice hepatocyte showing a hepatic sinusoid (white arrow) with Kuppfer cell (red star), erythrocyte (white star), dilated bile canaliculi (black arrows), microvilli indistinguishable (600x).

leads to decrease in uptake by hepatocytes of nutrients and wastes and is commonly seen in the liver diseases. In a study by Selmanoglu et al. (2001), congestion of blood vessels, increase in number of Kupffer cells and cellular infiltration were observed in liver of male rats treated with carbendazim. This may be due to the disrupted microvilli as it acts as a diffusion barrier between membrane and cytoplasm by its actin-based cytoskeletal core structure, which inhibits the entrance of hydrophilic and lipophilic substances into the cytoplasm as suggested by Hashem (2012).

Khagoli et al. (2005) reported that dimethoate induced hepatic pycnosis, vacuolation, blood congestion and high lymphatic infiltration around the central vein in liver and changes in the cortex of glomeruli, swollen cellular lining of the Bowman's capsule in Swiss albino mice. Similarly, in a study by Ksheerasagar & Kaliwal (2010) to study the effect of carbosulfuran in mice, noticed the loss of normal arrangement of cortical tubules and formation of vacuoles in hepatic tissues.

In the present study, degeneration and necrosis of the renal tissues in the form of cytoplasmic vacuolization and destruction of cytoplasmic organelles is visible. According to Rekha et al. (2013), the renal transport, accumulation and metabolism of xenobiotics contribute significantly to the susceptibility of the kidney to toxic injury. It has been reported by Threrien & Blostein (2000) that vacuolised cytoplasm may be caused by disturbed sodium pump as a sequence of impaired oxidative phosphorylation which results in the hydropic degeneration and vacuolization of the cytoplasm of renal tubular cells.

The loss of microvilli and basal infoldings in the PCT and DCT indicate that the pesticides directly damage the integrity of the plasma membranes, which cause the shortening and loss of microvilli and basolateral invagination. Therefore, the reduced reabsorptive surface due to damaged integrity of the brush border membrane might contribute to the reabsorptive and secretory defects (Herak & Sabolic 2001).

Exposure to fipronil was observed to have induced pathological changes in the liver and kidney tissues in a study by Mossa et al. (2015). El-Gerbed (2012) observed nephropathic changes like the thickening of basement membrane, changes in architecture and decrease of podocyte numbers in the study of the effect of deltamethrinin on the mice kidney tissues.

CONCLUSION

Pesticides are known to induce broad spectrum of toxicological effects and biochemical dysfunction constituting serious hazards to health. The results of this study shows

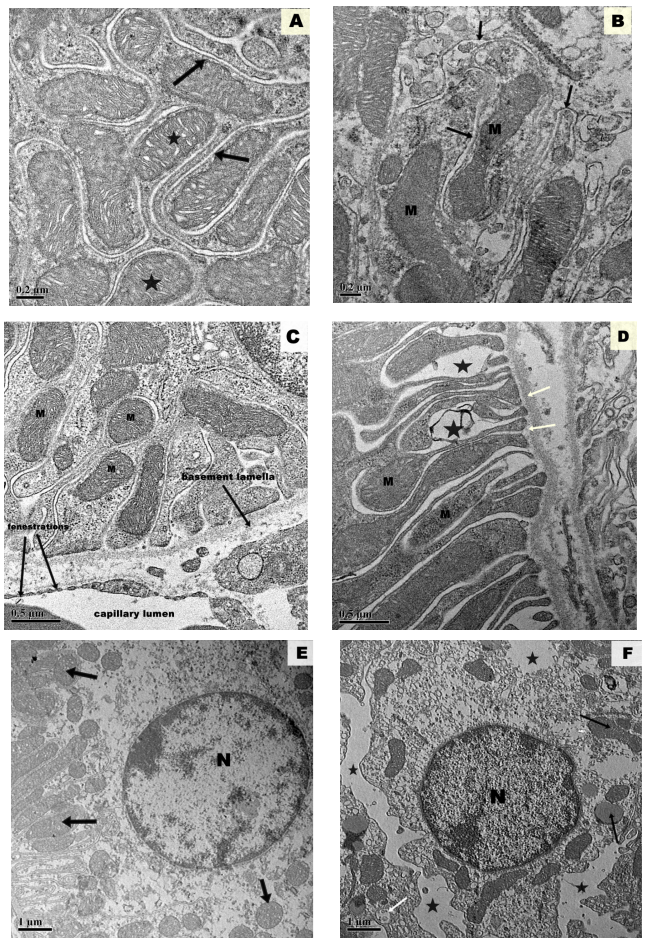

Fig. 3: TEM images on the effect of fipronil on the kidney of Swiss albino mice.

A. Kidney cell of control mice showing numerous mitochondria (black stars) overlaid by deep infoldings (red arrow) of the basal surface of the plasma membrane. (8000x)

B. Fipronil treated mice kidney showing damaged mitochondria (M) surrounded by disorganized basal membrane (black arrows). (5000x)

C. Basal portion of a distal convoluted tubule of kidney of control mice showing basement lamella, mitochondria (M), fenestrations on the outer part of the basement lamella. (6000x)

D. Thickening of basement membrane (white arrows), vacuoles in the cytoplasm (black stars) and damaged mitochondria (M) in the fipronil treated mice. (6000x)

E. Normal nucleus (N) in control mice kidney cell. Mitochondria (black arrows) are seen in their normal shape. (2000x)

F. Fipronil treated mice kidney showing nucleus (N) in an electron lucent cytoplasm (black stars) with reduced organelles and damaged mitochondria (black arrows). Autophagosome is also visible (white arrow). (2000x)

that the pesticides led to extensive degenerative changes in the hepatocyte and kidney cells of the treated albino mice. The changes observed in the study indicate that the pesticides caused damage to the liver and kidney at the cellular and sub cellular level. This study can be used as a potential biomarker of fipronil toxicity in human beings. Prolonged exposure to fipronil may lead to chronic liver and renal failure. The structural changes to the hepatic and renal tissues like haemorrhage, congestion, vacuolation may lead to acute liver or kidney damage. For field or domestic use, the quantities and mode of usage should be strictly monitored to minimize the possibility of its exposure to non target organisms including humans and to the environment.

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