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RESTORATIVE EFFECT OF WITHANIA SOMNIFERA AT CELLULAR AND SUBCELLULAR LEVELS IN CARDIOMYOCYTES AGAINST ENDOSULFAN INDUCED TOXICITY IN SWISS ALBINO MICE

Sanjeev kumar jha*[1], Amita kumari[2] and Dilip kumar paul[2]

[1]Department of Zoology, Patna University, Patna, Bihar, India.

[2]MLT College, Saharsa, Bihar, India.

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***Corresponding Author**
Sanjeev kumar jha
MLT College, Saharsa, Bihar, India.

ABSTRACT

India being an agriculture-based country is dependent on cultivation of crops at mass scale. Hence, pesticides are extensively utilized for the better yield of crops. Endosulfan is an organochlorine pesticide, widely used by the farmers of India. In the recent times it has caused serious health hazards in the exposed population. The cardiovascular diseases have also flared up many folds in the exposed population. Hence, the present research work aims to observe the restorative effect of *Withania somnifera* against Endosulfan induced cardiotoxicity at cellular and subcellular levels in Swiss albino mice. Endosulfan at the dose of 3mg/Kg body weight per day was administered orally to Swiss albino mice for 4 weeks. Then after, *Withania somnifera* at the dose of 1000 mg/Kg b.w was orally administered for 4 weeks. Mice were sacrificed after the completion of the entire treatment. After dissection the tissue samples were collected fixed in respective fixatives for light microscopic study as well as Electron microscopic study. The study shows deleterious effect of Endosulfan at cellular and subcellular levels. But, after administration of *Withania somnifera* there was significant normalisation in the cardiomycytes at the cellular as well as cellular levels. Therefore, from the entire study it can be concluded that *Withania somnifera* plays the vital role to control the endosulfan induced cardio-toxicity at cellular as well as sub-cellular levels.

KEYWORDS: Endosulfan, cardiotoxicity, *Withania somnifera*, restorative effect, light microscopy and Electron microscopy.

INTRODUCTION

Agriculture is the major source of revenue for India as crops are cultivated at very large scale by the farmers. Unfortunately, the pests have also damaged the crop yield at large scale in the past. To augment the problem, the farmers are utilizing the pesticides for the better yield of the crops at large scale. Hence, organochlorine pesticides are widely used since their mode of action is precisely more effective than the organophosphate group of pesticides but they have serious health hazards to humans as well (Rajendran, 2003; Pimentel 1995; Jayraj et al., 2016; Akhtar 2009; Gupta 2004)).

Endosulfan (hexachloro-hexahydro-methano-benzodioxathiepinoxide) is an organochlorine (OC) pesticide in group cyclodiene. It is a cyclic sulphurous acid ester with a molecular formula $C_9H_6O_3Cl_6S$ and molecular weight 407. Despite its life-threatening toxic effects, endosulfan continues to be one of the most widely used agricultural pesticides, largely in the developing countries, due to its high efficacy, low cost and environmental stability (Coutselinis et al., 1976; Yadla et al., 2013). It is easily absorbed in the gastro intestinal tract, lungs and skin and exposure through various routes and is very hazardous. Commercially produced endosulfan consists of two isomers α endosulfan and β endosulfan. Both these forms have been proved to be genotoxic to human gonads (ATSDR, 2000 and Helle et al., 2002). The USEPA has rated endosulfan as category Ib – *highly hazardous* compound (EPA 2002) while WHO has classified it as Class II – moderately hazardous to human health (WHO, 2005). Due to its toxic effects and adverse effects on humans the chemical has been banned or severely restricted its use in many countries. Independent of LD_{50} results, these threats warrant the immediate upgrading of endosulfan to WHO Class I b (EPA 2002; Yavuz et al., 2007; Lee et al., 2015; Wilson et al., 2014; Pradhan et al., 1997).

Medicinal plants are widely used as drugs against the vast diseases in India in the Indian medicine system called *Ayurveda*. Such potent medicinal plant which has wide array of medicinal properties is *Withania somnifera*. Ashwagandha (*Withania somnifera*, WS), belongs to the family Solanaceae, and is known to be an Ayurvedic herb worldwide for its numerous beneficial health activities since ancient times. It is widely used for the treatment of various diseases such as epilepsy, depression, arthritis, diabetes, and palliative effects such as analgesic, rejuvenating, regenerating, and growth-promoting effects. It has multifarious effect on vital organs of the body (Pratte et al., 2014; Mirjalili et al., 2009; Rai et al., 2016; Devi et al., 1992; Kumar et al., 2015; Satyavati et al., 1976). Hence, the present research

work aims to observe the restorative effect of *Withania somnifera* against Endosulfan induced cardiotoxicity at cellular and subcellular levels in Swiss albino mice.

MATERIALS AND METHODS

Ethical Approval: Ethical approval was taken from Post Graduate Research Council (PGRC) of Patna University, Patna with no. PGRC No. Acad - / 464, serial No. 7, dated 12/02/2007.

Animals: Twenty four Swiss albino mice (28g to 32 g) were obtained from animal laboratory of Dr. A. Nath, Department of Zoology, Patna University, Patna, India. The research work was approved by the Post Graduate Research Council of the Patna University. Food and water to mice were provided *ad libitum* (prepared mixed formulated feed by the laboratory itself). Animals were maintained in colony rooms with 12 hrs light/dark cycle at 22 ± 2^{o}C.

Chemicals: The commonly used pesticide- endosulfan was obtained (Excel India Pvt. Ltd. Mumbai with EC 35%). The pesticide was prepared to 3 mg/Kg b.w, which was administered orally to mice for 4 weeks.

Plant material: The fresh dried rhizome of *Withania somnifera* (WS) (Ashwagandha) was purchased from the herbal store in Patna and identified by Dr. V Pandit, Department of Botany, Patna Science College, Patna university Patna, India and aqueous extract was made by dissolving it in distilled water using by mortal and pistal. The dose was finally made to 1000 mg/kg body weight for oral administration.

Experimental design: In the present study 24 mice (18 endosulfan treated and 6 as control mice) were taken and divided into groups - control, endosulfan treated and *Withania somnifera* treated. The endosulfan at the rate of 3mg /kg body weight daily were administered orally for 4 weeks. To this endosulfan treated group WS at the rate of 1000mg / kg body weight was administered for 4 weeks. After the completion of the experiment blood samples were collected by orbital sinus puncture method and then serum was extracted.

Histopathological study: For the histopathological study, cardiac tissues were dissected and collected from all the group of sacrificed mice. The tissues were washed in normal saline, grossed into small pieces and finally fixed in 10% neutral formalin for 24 hours. Thereafter, tissue was dehydrated through process of graded series of ethanol and finally embedded into paraffin. Thin sections of 5µm thickness were sliced through digital rotary microtome

(Thermo Scientific Microm HM 340E) and stained with hematoxylin and eosin (H&E) for the histopathological study under light microscope.

Electron microscopic study: For Transmission Electron microscopy it was fixed in 2.5% gluteraldehyde in 0.1M sodium phosphate buffer (pH 7.2) with in 24 h of removal. After rinsing with phosphate buffer, tissues were post fixed with 2% osmium tetraoxide in sodium phosphate buffer. Dehydration was accomplished by gradual ethanol series and tissues were embedded in epoxy resin. Semithin sections were stained with toluidine blue and examined with a light microscope (Olympus, LXi, Tokyo, Japan). Ultrathin sections (800 nm) were stained with uranyl acetate and lead citrate. Sections were then viewed and photographed with Morgagini – 268 D TEM (SEI Co.) at SAIF-EM facility Unit (Sophisticated Analytical Instruments Facility) at All India Institute of Medical Sciences (AIIMS), New Delhi, India. For the light microscopic study the Haemotoxylin- Eosin stained slides were prepared and the sections were viewed under light microscope.

RESULTS

The control cardiomyocytes showed normal architecture with the cytoplasm and the capillaries are well lined with the endothelial cells (Figure 1.1). After, treatment with Endosulfan for 4 weeks cardiomyocytes showed significant degeneration in endothelial cells with ruptured capillaries. Vacuolations in the cytoplasm were also observed and nuclei were scattered in the cytoplasm (Figure 1.2). But, after administration of Ashwagandha for 4 weeks there was significant restoration in the cardiomyocytes. The capillaries were observed to be surrounded with endothelial cells but there were few vacuolations still present in the cytoplasm (Figure 1.3&4).

The Transmission electron microscopic study reveals that the normal cardiomyocytes bears well organized plasma membrane, nucleus with chromatin material of endothelial cells. The M Band are well arranged. The well organized smooth endoplasmic reticulum (SER) and mitochondria shows normal functioning of the myocytes (Figure 2.1). In Endosulfan treated cardiomyocytes shows nucleus with deshaped nuclear membrane of endothelial cells. Cytoplasmic organelles are highly degenerated with as vacuolated spaces. Degeneration in mitochondria with cristae are clearly visible with ruptured secretory vesicles and with degeneration in M-Band region (Figure 2.2). But, after administration of Ashwagandha for 4 weeks it is observed that it plays vital role in normalizing the cellular integrity. The architecture of cell as nucleus is normal with well-defined nuclear membrane, nucleolus of

endothelial cells with abundant mitochondria in normal condition can be observed. Well-arranged M-Band region mitochondria with cristae can be observed shows the smooth functioning of the myocytes (Figure 2.3&4).

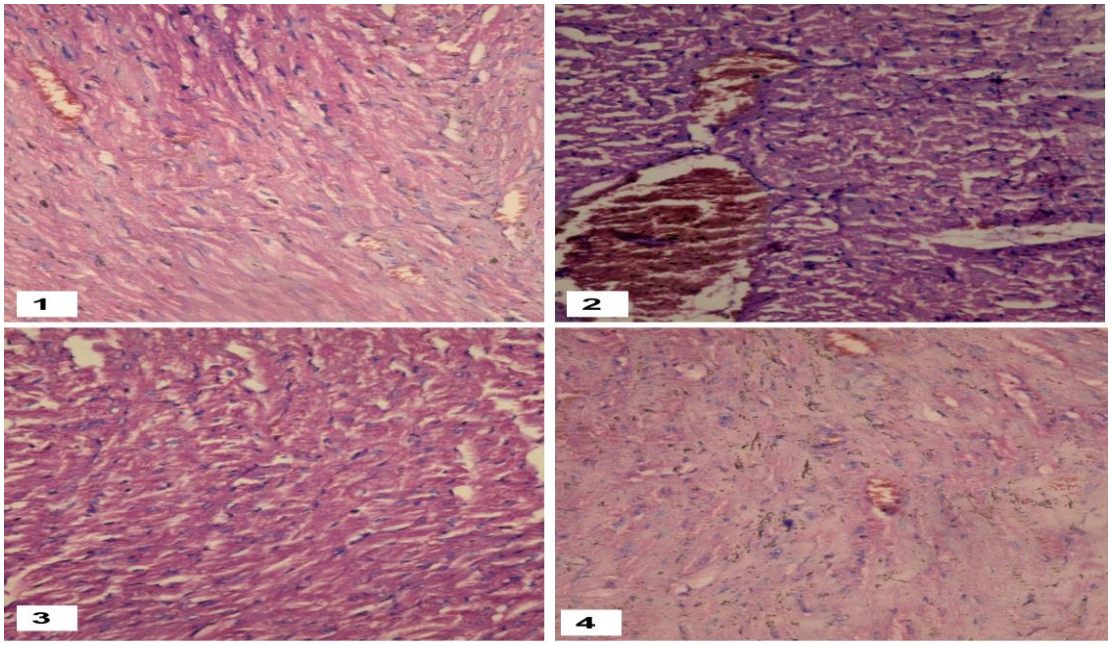

Figure 1: Showing microphotographs sections of cardiomyocytes (H&E) – 1. Control cardiomyocytes, 2. Endosulfan 4 weeks treated cardiomyocytes, 3 & 4 *Withania somnifera* administered cardiomyocytes for 4 weeks X 400.

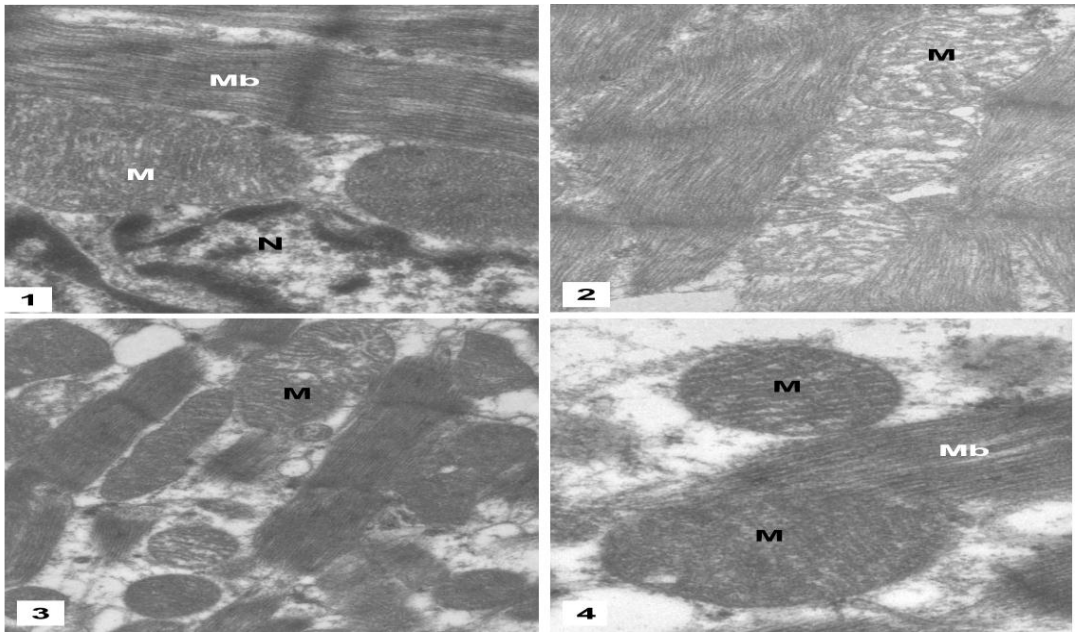

Figure 2: Showing ultra-structure photographs of cardiomyocytes – 1. Control cardiomyocytes, 2. Endosulfan 4 weeks treated cardiomyocytes, 3 & 4 *Withania somnifera* administered cardiomyocytes for 4 weeks X 35000.

DISCUSSION

In the normal physiological functioning of a system, cardiac function has important role, but if it is hampered by any means can cause serious problems in the cardiovascular functions. In the present study, we tried to observe the deleterious effect of pesticide on endosulfan treated 4 weeks treated at cellular level as well as ultrastructure level. The study shows the deleterious effect of endosulfan on the cardio-membrane structure at the cellular and subcellular levels. The membrane lipids are the major barriers which decides the cellular integrity. Hence, they are the important component of the living system maintaining the fluidity and fragility (Shell, 1961; Lehninger, 1975; Suhail et al., 1988). Pesticides cause not only damages to the membrane structure but also the cellular functions of the cardiomyocytes. Moreover, the biochemical parameters such as lipid profile are also hampered due to pesticidal toxicity (Agrahari and Gopal, 2009; Pant and Singh, 1983; Bhushan et *al.*, 2002, Mohamed and Gad, 2008 Jenkins *et al.*, 2003; Rajamanickam and Muthuswamy, 2008; Saravanan *et al.*, 2011 and Yekeen and Fawole, 2011).

In the present study the histopathological changes caused due to endosulfan exposure on cardiomyocytes was significant, as there was changes in the endothelial cells with the ruptured capillaries while vacuolations in the cytoplasm were also observed. Similar studies have been merely studied as there has been significant degenerative changes observed by other authors (Jalili et al., 2007, Anand et al., 1992). However, the biochemical changes caused the serious damage to the cellular cardiovascular functions due to changes in the levels of lipid profile (Jha and Paul, 2020a and 2020b). The study of endosulfan on cardiomyocytes at Transmission Electron microscopy showed serious degenerative changes. The smooth endoplasmic reticulum, the M-Bands, the nucleus etc. were highly damaged (Wei et al., 2018).

In the present study, *Withania somnifera* was used as the medicinal plant to observe the antitoxic effects against endosulfan. There was significant restoration observed at the cellular as well as subcellular levels. The cardiomyocytes were highly restored as the toxicity impact was observed very less. The active ingredients of *Withania somnifera* as Withaferin A and withanolides play the vital role in the restoration due to it antioxidant, immunomodulatory and many other functions. Various studies conducted on *Withania somnifera* on other restorative effects have been reported (Gad and Saad 2008; Vinson et al., 2001; Hincal *et al.*, 1995; Di Rosa et al. 1971; Koblyakov 2001; Sood et al. 2009).

Endosulfan is a known xenoestrogen and due to lipid peroxidation, it causes severe damage to the membranes. The depletion of the lipids from the membrane makes the blood more viscous causing the risk of cardiovascular disease. This causes serious changes in the cytoplasm at the sub-cellular level. The cardiomyocytes are irreparable cells and even a minor damage can be fatal to the system causing cardio-ischemia (Moon and Lee 2013; Avci et al., 2019; Vandenberg 2020; Xu et al., 2017). In the study, the degeneration in the plasma membrane, smooth endoplasmic reticulum and mitochondria was very much significant and denotes impaired cardiovascular functions.

In the present study, there has been remarkable restoration in the cardiomyocytes after *Withania somnifera* administration at the subcellular levels as the transmission electron microscopy denotes the normalisation in the smooth endoplasmic reticulum and the mitochondrial functions, furthermore the nuclear functions also appear to be significantly restored. Similar, significant effect of *Withania somnifera* on other models have been observed (Wang et al., 2010; Reuland et al., 2013; Ravindran et al., 2015; Patel et al., 2016; Yan et al., 2018; Guo et al., 2019).

CONCLUSION

Hence, from the entire study, it can be concluded that endosulfan causes deleterious effect on the cardiovascular functions in the Swiss albino mice, but after the treatment of rhizome extract of WS there was significant restoration at cellular and subcellular levels. But, after administration with *Withania somnifera* there was a significant restoration in the cardiomyocytes. This denotes that *Withania somnifera* possesses antitoxic effects against endosulfan induced cardiotoxicity in Swiss albino mice.

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Conflict of interest

The authors declare that they have no conflict of interests.

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