Biochemical changes induced by Bioneem (0.03%) formulation in chick embryogenesis (*Gallus domesticus*)

P. V. Gado[1*], S. G. Salokhe[2], S.G. Deshpande[3]

[1,2]PDEA's Prof. Ramkrishna More Arts, Commerce & Science *College* Pune 411044
Email: Purva_gd@yahoo.co.in, shailajasalokhe@yahoo.co.in
[3]National chemical laboratory Pashan Pune 411008
Sg.deshpande@ncl.res.in

Abstract— In ovo studies on the effect of 1,3,5, ppm Bioneem (0.03%) formulation on Biochemical aspect of chick embryo revealed that there was dose dependent total protein reduction in 96 hrs old embryo (treated at 24 hrs) as compared to the control. Also there was reduction in total protein concentration Liver, Brain and Heart of 15 day old chick embryo (treated with Bioneem at 96 hrs. stage) as compared to that of control. Protein carbonyl concentration of 96 hrs old embryo (treated at 24 hrs with Bioneem) and that of Liver, Brain and Heart of 15 day old chick embryo (treated with bioneem at 96 hrs) increased in dose dependent manner. Most affected organ was Liver and least affected organ was Heart. Blood analysis of 15 day old chick embryo (treated with Bioneem at 96 hrs) showed increased level of Blood urea, LDH, SGOT, SGPT, while Serum alkaline phosphatase and serum cholesterol were decreased in dose dependent manner as compared to the control. Thus Bioneem though ecofriendly pesticide can adversely affect vertebrate non target organisms and therefore should be carefully used in pest management programs.

Keywords— Chick embryo, Bioneem, Protein carbonyl, Blood biochemistry.

I. INTRODUCTION

Agrochemicals are beneficial to increase crop yield and efficiency of food production process which ultimately results in decrease in food cost. Plants play important role in crop protection, secondary plant metabolites, such as alkaloids, glycoalkaloids, terpenoids, organic acids or alcohols, are potential sources of pest control substances (Szymon Chowański et al., 2016). Pesticides have been developed to control targeted pests and function with reasonable certainty and minimal risk to human health and environment. Unfortunately, It was reported that less than 0.1% of pesticide applied for pest control reached their target pest while 99.9% finished up in polluting environment and detrimental to human beings (WenJun Zhang et al., 2011). Negative effects of agrochemicals on non-targeted species including humans are conspicuous. Biorational or biopesticides which are referred as 21st century pesticides and considered as less toxic or non-toxic to human and pose least risk to environment. However, toxic effects of such pesticides on non-target species are also documented (Frederick M. Fishel, 2012).

Present study aims to investigate effect of neem based pesticide Bioneem (0.03% Azadirechtin) RAV Products, Punjab. on embryogenesis of chick (*Gallus domesticus*)

Neem and its products are known for their insecticidal properties (Ezekiel et al., 2008, Manish kumar, 2011) However, it was also reported to have toxic effects on vertebrates and non-target organisms. It was observed that leaves of Neem causes toxicity in Sheep, Goats and Gunia pig and water extracts of neem berries showed toxicity to poultry birds also the neem seed oil was found to produce occasional diarrehea and general discomfort. Ingestion of Neem seed oil by infants was found to cause acute poisoning (Abhishekh Raj, 2014) Neem –Azal was found to be lethal to non target organism such as tadpoles, Cyclopes, Daphnia (ei –Shazly, 2000).Neem based insecticide induced oxidative stress in brain and muscles of Zebra fish showed reduction in GSH and CAT causing lipid peroxidation (Dilip kumar Sharma, 2014). There are several reports of non target effect of neem formulation on aquatic organisms (Boonsatien and Vasakorn, 2009). However very few studies are carried out on effect of neem on chick embryogenesis (Kweri J. K, 2006).

In the present study chick embryo was used as vertebrate model to study the effect of Bioneem, because its system gives comprehensive understanding of development of organ system and fundamentals of body formations common to all groups of vertebrates. Advantage of using chick embryo in study of developmental malformation is that during phases of chick development different characters become prominent and hence useful in diagnostic purpose. In order to get information on embryonic lethality , teratogenicity metabolism and systemic toxicity test chick embryo was found to be sensitive , inexpressive and positively correlated with those in other system including mammals (Parisa Sadighara et al., 2011).

II. MATERIALS AND METHODS

2.1 Pretreatment

Freshly laid fertilized eggs (0 hr stage) of *Gallus domesticus* (White Leghorn Strain) were obtained from Dr B V Rao Institute of Poultry Management and Technology Pune. Eggs were washed with distilled water and wiped with 70% ethanol and then incubated for 24 hrs in BOD incubator (Biotechnics India) at 37.5^0 C with a relative humidity of 70 - 80%. For Identification of chick embryonic developmental stage Hamburger V, Hamilton HL., (1951) staging system was used

2.2 Treatment

Four experimental sets of chick embryo each with 6 replicates were prepared. Each set with group I of chick embryos treated with 100 µl of distilled water as a carrier solvent control. Groups II III and IV of chick embryo treated with 100 µl of 1 ppm, 3ppm and 5ppm concentration of Bioneem respectively. Treatment was given in ovo through air sac route at 24 hrs (HH Stage 6-7), 44 -46 hrs (HH stage11-12) and at 96 hrs (HH stage 23-24) of development and transfer to the incubator set at 37.5^0 C The treated and control eggs were manually rotated periodically avoiding yolk and albumin spillage through the incision made for treatment.

2.3 Isolation of chick embryo

Chick embryos and their organs like Brain, Heart and Liver were isolated in 1X chilled PBS.

2.4 Protein Extraction

Proteins were extracted from Isolated chick embryos and tissue samples by homoginizing in cold protein extraction buffer (PEB) 1:10 (w/v) centrifuge at 10000rpm for 10-15 min. supernatant was used for further analysis

2.5 Total soluble Protein Analysis

2.5.1 Quantitative analysis of Protein was done by Lawry's method (1951)

2.5.2 Qualitative analysis of protein was done on 12% SDS PAGE using Coomassie Brilliant Blue **(**CBB) staining method

and gels were photographed and analyzed and band densities were calculated using online software ImageJ 1.50b (http//imageJ.nih.gov/ij) National institute of health science

2.6 Protein carbonyl assay

Protein Carbonyl concentration was measured as marker of oxidative stress according to method of Uchida et al., (1998). It was estimated in chick embryo treated with different concentrations of Bioneem (1, 3, 5 ppm) at 24 hrs (HH 6-7) stage and incubated for 72 hrs post treatment i.e observed at 96 hrs (HH stage 23-24) old stage and in Liver, Brain and Heart of chick embryo treated at 96 hrs old (HH stage 23-24) and incubated for 11 day post treatment (i.e. Embryonic day 15)

2.7 Blood Analysis

Blood was collected in sterile tube from blood vessel and by puncturing heart of 15 days old chick embryo and analyzed using BiOLis 24 I JAPAN fully automated biochemistry analyzer.

III. RESULTS

3.1 Quantitative analysis of protein by Lawry's method

Total soluble protein content of 96 hrs old chick embryo treated with different concentrations of Bioneem (1,3and5ppm) was found to decreased significantly in dose dependent manner as compared to that of control (Figure 1). Further it was estimated that at higher concentration of Bioneem (5ppm) there was 40 % decreased in protein content as compared to that of control although at lower concentration (1ppm) of Bioneem it was not significantly varied than that of control.

Analysis of the total protein of Liver, Brain and Heart of 1ppm,3ppm and 5ppm of Bioneem treated chick embryo 96 hrs old (HH stage 23-24) and incubated for 11 day post treatment (i.e. Embryonic day 15) revealed significant decreased in Liver and Brain protein as compared to control. However, total Heart protein of 1 and 3 ppm treated embryos was not significantly differed than that of control while at 5 ppm concentration of Bioneem total Heart protein significantly reduced than that of control (Figure 2).

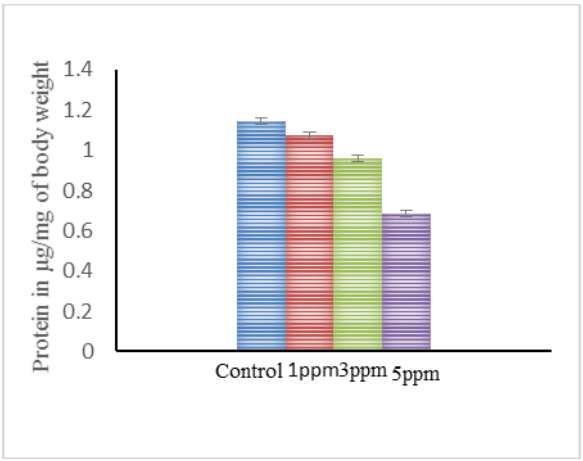


Figure 1: Total protein of 96 hrs old Control and Treated chick embryo.

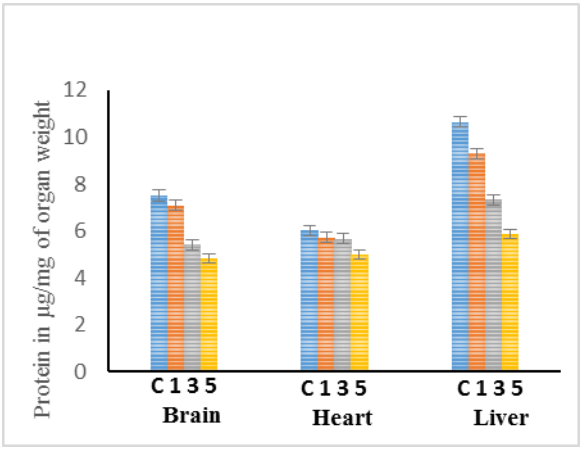


Figure 2: Total protein of 15 day old Control and Treated chick embryonic organs.

3.2 Qualitative analysis of protein

Chick embryo treated at 24 hrs (HH Stage 6-7) with various concentrations of Bioneem (1,3 and 5ppm) when observed on 96 hrs (HH stage 23-24 stage) for expression of protein by SDS PAGE(12%) revealed that there was dose dependent decreased in expression of 56.55kDa and 29.53kDa protein and increased in 50.6 kDa and 42.36 kDa proteins. Analysis of band density revealed decreased 86.4% (0.135 relative density) of 56.55kDa protein at 5 ppm of Bioneem than that of control (Plate1 Band1) while 4.65% (Relative density 0.953) and 39.7% (Relative density 0.602) decreased at 1 and 3 ppm of Bioneem respectively as compared to than that of control (Plate1: Band1, Figure 3).

Protein of 29.53kDa (Plate 1Band 4) was not expressed at 5ppm concentration of Bioneem while decreased by 5 % (Relative density 0.950) and 36.2% (Relative density 0.637) at 1and 3ppm of Bioneem respectively as compared to control (Figure 3).

Protein of 50.6 kDa (Plate 1: Band2) was expressed in Bioneem treated sample in concentration dependent manner. It was found to be 12.12% and 91.27% more in 3 and 5 ppm than 1ppm samples respectively. Similarly expression of 42.36 kDa protein (Plate1: Band 3) increased with increased in concentration of Bioneem. It was 84.9 % (Relative density 1.849) at 1 ppm and 3.1times (Relative density 3.127) and 3.2 times (Relative density 3.244) more in 3 and 5 ppm of Bioneem treated samples respectively, than that of control. (Plate 1: band 4) (Figure 3).

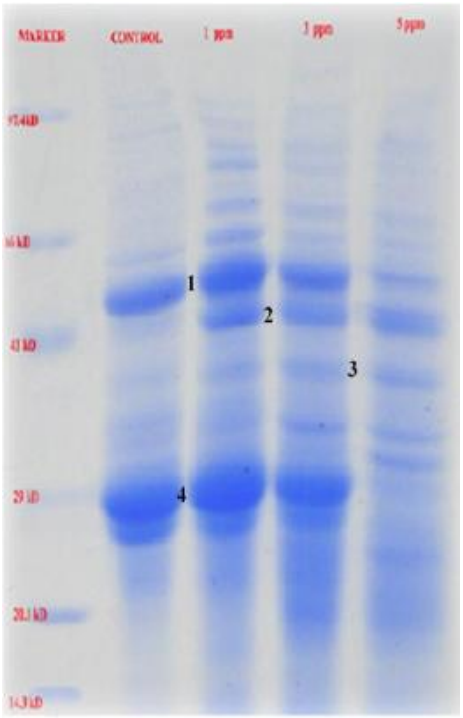
Quantitative analysis of total soluble protein of Brain, Heart and Liver of chick embryo treated with different concentrations of Bioneem (1, 3 and 5ppm) at 96 hrs and incubated till 15 days by SDS PAGE (12%) revealed that expression of Brain proteins of 65.02 kDa, 54.4 KDa and 29. kDa was increased at 1,3 and 5 ppm Bioneem treated samples as compared to control while expression of 39.5kDa protein decreased in all treated samples than that of control (Plate2), (Figure 4). At 5 ppm of Bioneem treatment 34.31kDa Brain protein was failed to express as compared to control while at 1 and 3ppm relatively reduced expression as compared to control was observed.

Analysis of relative density of Protein :Brain protein of 65.02 kDa (Plate2:Band1) showed 23.4% (Relative density 1.234) and 45.5% (Relative Density 1.455) (Figure 4) more expression than that of 1 and 3 ppm treated embryos as compared to control. Further it was 5 times more (Relative density 5.109) (Figure 4) in 5ppm treated embryos than that of control. Similarly brain protein of 54.4 KDa (Plate2: band 2) also increased 2X (Relative density 2.045), 3X (Relative density 3.113) and 6X (Relative density 6.401) (Figure 4) times in 1 ,3 and 5 ppm treated embryos respectively as compared to control. Relative densities of Brain protein 29. kDa (Plate2:band 5) increased in dose dependent manner. It was 52.8% (Relative density 1.528) and 82% (Relative density 1.827) (Figure 4) increased at 1 and 3 ppm while 3 times more (Relative density 2.932) (Figure 4) at 5 ppm than that of control. Brain protein at 39.5kDa (Plate2: Band 3) showed decline in relative densities by 88.5% (Relative density 0.114) 93.8% (Relative density 0.061) and 94.24% (Relative density 0.057) (Figure 4) at 1, 3, 5 ppm of Bioneem respectively as compared with that of control.

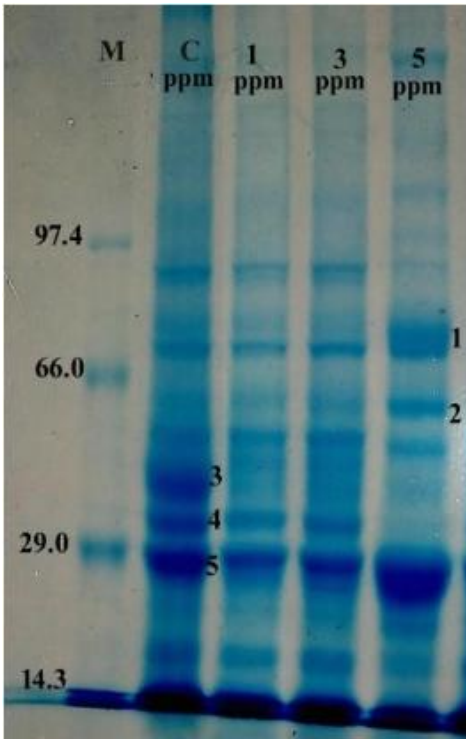
Brain protein at 34.31kDa (Plate2: band 4) was not expressed in 5 ppm Bioneem treated sample. Further it was 92.8% (Relative density 0.725) and 93.85% (Relative density 0.614) (Figure 4) decreased at 1 and 3 ppm of Bioneem treatment respectively as compared with that of control.

Analysis of Heart samples of control and Bioneem treated embryos by SDS PAGE revealed that there was no significant difference in protein expression.

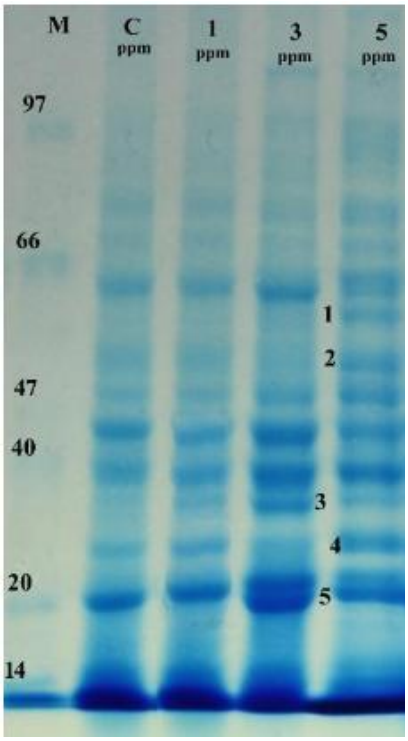
SDS PAGE analysis of Liver proteins revealed that there was variation in the expression of 64.44 kDa, 31.59 kDa, 53.03 kDa, and 26.01 kDa proteins (Plate3) (Figure 5). Liver proteins of 64.44 kDa (Plate3: Band1) was not expressed in control while its expression is highest at 5 ppm of Bioneem treatment while it showed 91.15% and 93.3% decreased in 1 and 3ppm (Figure 5) respectively as compared to5 ppm treated embryos. Expression of 53.03 kDa Liver protein (Plate3: band 2) was not differ from that of control at 1ppm of Bioneem (Relative density 1.001) treated embryo while its density was highest at 5ppm of Bioneem (Relative density 2.12) but showed decreased expression in 3 ppm (0.52 Relative density) and was 52.46% (Figure 5) less than control. 31.59 kDa (Plate 4 band 3) Liver protein was expressed only at 3ppm of Bioneem treated embryos. At 5 ppm of Bioneem treatment 26.01 kDa (Plate3: band 4) Liver protein was expressed with 4.12 Relative density (Figure 5) whereas at 1ppm relative density was 1.406 and at 3 ppm relative density was 0.69 i.e. it was 30% less than control. Low molecular weight (21.47kDa) (Plate3: band 5) protein was expressed at 3 and 5 ppm of Bioneem.



Photograph 1: SDS PAGE of 96 hrs old chick embryo control and treated at 24 hrs development. 1. 56.55kDa; 2. 50.6kDa; 3. 42.36 kDa; 4. 29.53kDa



Photograph 2: SDS PAGE of Brain samples of 15 day old chick embryo Control and treated at 96 hrs development. 1. 65.02kDa; 2. 54.4kDa; 3. 39.5kDa; 4. 34.31kDa; 5. 29.0kDa



Photograph 3: SDS PAGE of Liver samples of 15 day old chick embryo Control and treated at 96 hrs development. 1. 64.44kDa; 2. 53.03kDa; 3. 31.59kDa; 4. 26.01kDa; 5. 21.47kDa

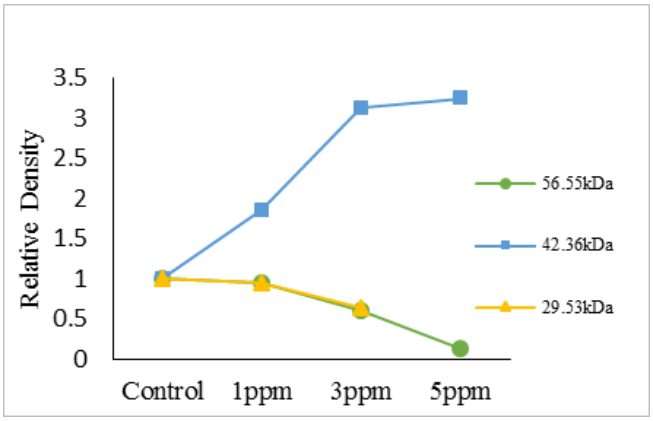


Figure 3: Relative density analysis of gel of 96 hrs old Control and Treated whole embryos.

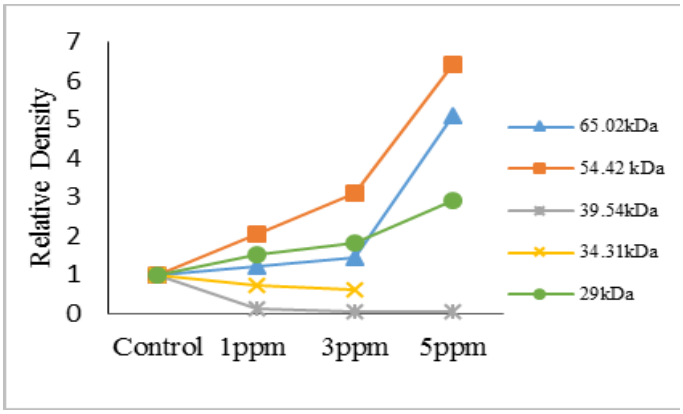


Figure 4: Relative density analysis of gel of 15 day old Control and Treated chick embryonic Brain**.**

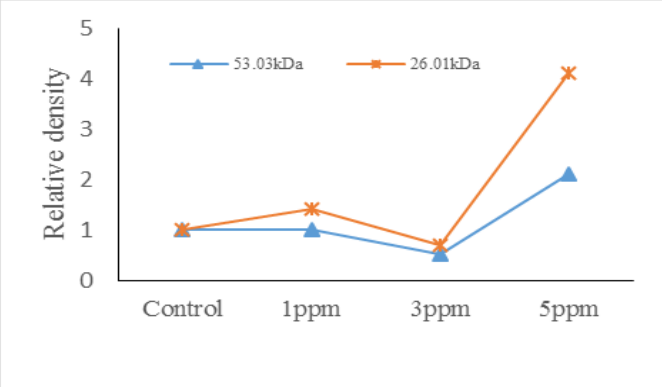


Figure 5: Relative density analysis of gel of 15 day old Control and Treated chick embryonic Liver

3.3 Protein carbonyl assay

Protein Carbonyl concentration of 96 hrs (HH stage23-24) old stage chick embryo showed significant increased in n moles of carbonyl per 500µg protein in treated samples as compared to control in dose dependent manner. Average protein carbonyl per 500µg protein in control sample is 1.07 n moles while it was 1.73 nmoles 2.23nmoles and 4.21nmoles at 1, 3 and 5 ppm Bioneem treated samples respectively (Figure 6).

Protein Carbonyl concentration of Liver, Brain and Heart of 15 day old chick embryo treated 96 hrs old (HH stage 23-24) with at 1ppm,3ppm and 5ppm of Bioneem revealed that there is significant increase in carbonyl level in Liver sample. It was 8.97 n moles in control and increased upto 17.85 n moles in 5 ppm and 12.35 n moles in 3ppm Bioneem treatment although increased at 1 ppm of Bioneem concentration of protein carbonyl (9.95 n moles) was not-significant. (Figure 7).

Brain samples of embryo treated with 1, 3 and 5 ppm of Bioneem showed increased carbonyl concentration 5.21 n moles, 6.118 n moles and 7.047 n moles respectively as compared to that of control (4.976 n moles /500µg of protein) (Figure 7)

Heart sample of embryo treated with different concentrations of Bioneem showed that at 5ppm of Bioneem there was significant increase in protein carbonyl content 4.23 n moles as compared to that of control which is 3.38 n moles. At 1 and 3 ppm Bioneem protein carbonyl content at 1ppm 3.80 n moles and at 3.95 n moles was non-significant as compared to control (Figure 7).

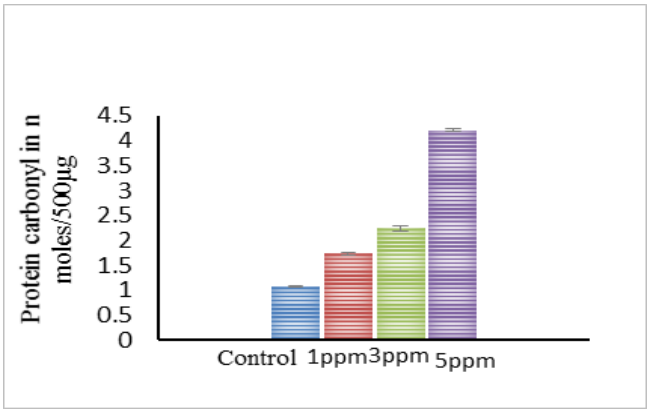


Figure 6: protein carbonyl level in 96 hrs old Control and Treated chick embryo.

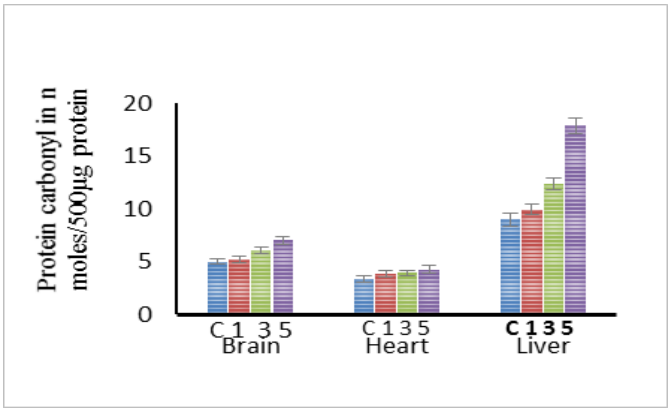


Figure 7: Protein carbonyl level in 15 day old Control and Treated chick embryonic organs.

3.4 Blood analysis of chick embryo treated at 96 hrs old (HH stage 23-24) with at 1ppm,3ppm and 5ppm of Bioneem and incubated for 11 day post treatment (i.e. Embryonic day 15)

Serum urea level of embryonic blood increased with increasing concentration of Bioneem. In control embryos it was 18 mg/dl while at 1, 3 and 5 ppm of Bioneem it was 22 mg/dl, 27mg/dl and 30mg/dl respectively. Further it was estimated that increased in serum urea level was 22.2%, 50.0% and 66% at 1, 3 and 5 ppm of bioneem treatment respectively as compared to that of control (Figure 8).

Serum LDH levels of embryonic blood also showed dose dependent increased. It was found to be increased by 8.2%, 36.1% and 119.1% at 1, 3 and 5 ppm of Bioneem treated embryos respectively as compared to that of control (Figure 9).

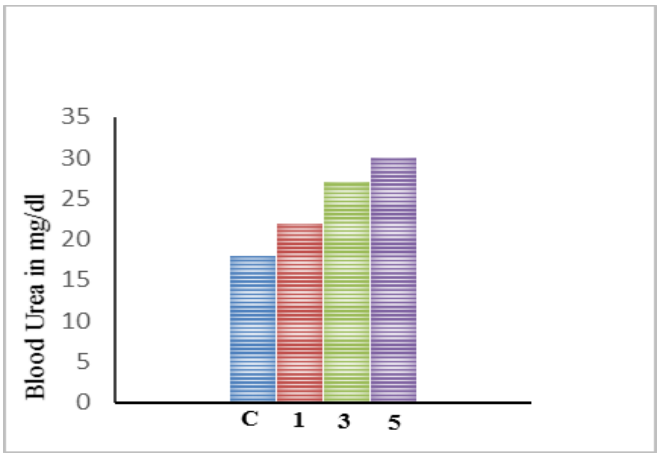


Figure 8: Serum Urea level in 15 day old Control and Treated chick embryo.

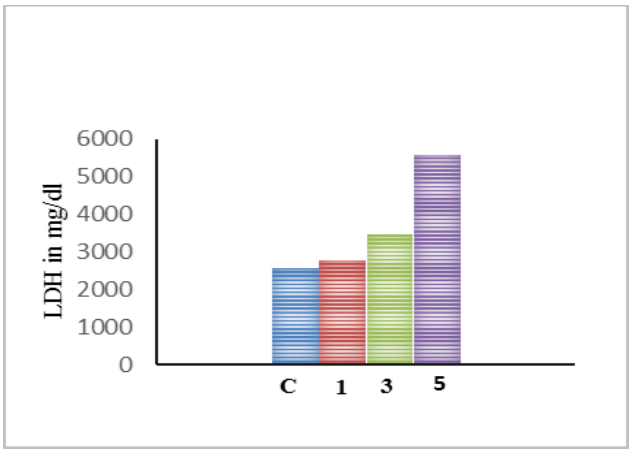


Figure 9: Serum LDH level in 15 day old Control and Treated chick embryo.

SGPT levels of embryonic blood increased with increasing Bioneem concentrations. It was increased by 28.57%, 71.4% at 1 and 3 ppm respectively and 3 times more at 5 ppm of Bioneem treated embryo as compared to that of control (Figure 10).

Serum SGOT of embryonic blood was increased by 9.2%, 30.5% and 116.9% at 1, 3, 5 ppm of Bioneem treated samples respectively as compared to the control (Figure 11).

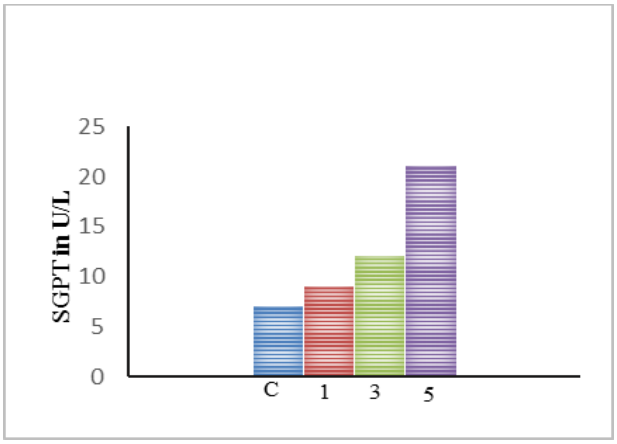


Figure 10: Serum SGPT level in 15 day old Control and Treated chick embryo.

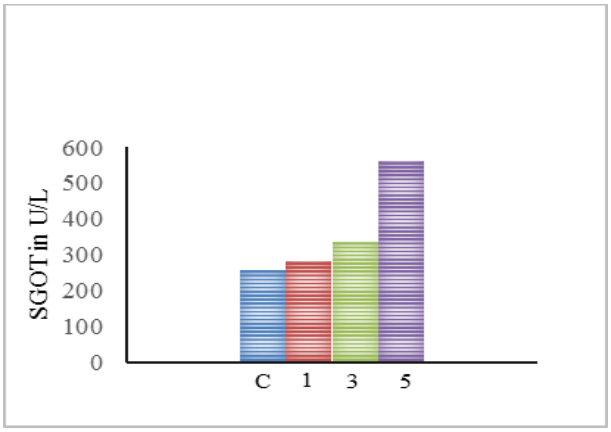


Figure 11: Serum SGOT level in 15 day old Control and Treated chick embryo.

Serum Alkaline phosphatase level of embryonic blood was decreased in all Bioneem treated samples. It was decreased to 12.37 %, 41.88% and 52.0% at 1, 3 and 5 ppm of Bioneem treatment respectively as compared to the control (Figure 12).

Serum cholesterol level of embryonic blood lowered in Bioneem treated samples as compared to that of control. However the values were not much differed in all treated group (1, 3 and 5ppm) It was 8%, 16% and 26% in 1 , 3 and 5ppm respectively of Bioneem treated embryonic blood as compared to that of the control (Figure 13).

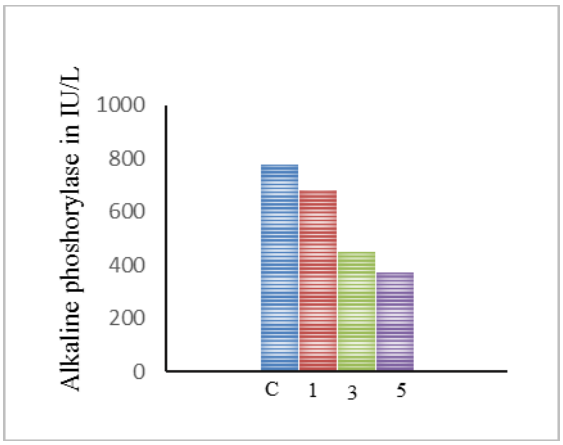


Figure 12: Serum Alkaline phosphatase level in 15 day old Control and Treated chick embryo.

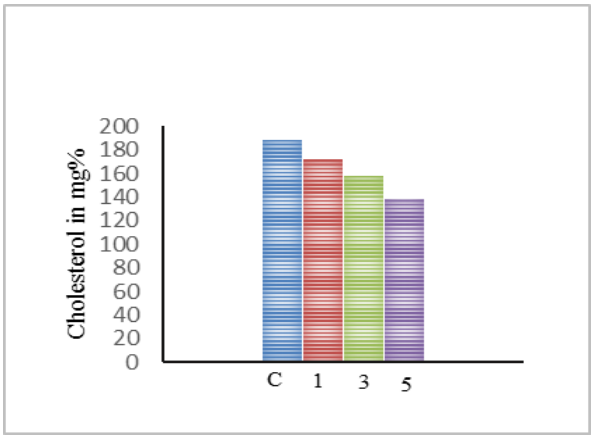


Figure 13: Serum Cholesterol level in 15 day old Control and Treated chick embryo.

IV. DISCUSSION

Bioneem, though considered as potential pesticide to control wide range of pests was shown to affect chick embryogenesis at different biochemical parameters. It was observed that total soluble protein in chick embryo treated with Bioneem at 24 hrs (HH stage 6-7) stage and incubated for 72 hrs post treatment significantly decreased in dose dependent manner. Contradictory observation to our results were reported in Fresh water fish *Prochilodus lineatus* exposed to neem leave extract inducing oxidative stress and not affecting total soluble proteins (Wilkaler et.al 2007). Protein content of Liver, Brain and Heart of 15 day old chick embryo treated with 1, 3 and 5 ppm Bioneem at 96 hrs (HH stage 23-24) decreased as compared to control and the difference in liver protein of treated and control embryo was more profound. Similar observations of decreased in total liver protein was made in Cholrpyrifos treated chick embryo (Lalit Patel 2013), total liver and muscle protein content of biopesticide Neem (*Azadiracta indica*) treated fresh water fish *Heteropneustes fossilis* (Rathod S.H., 2013). In fresh water fish *Claris batrachus* Bioneem treatment reduced the liver protein in dose dependent manner (A. Siddique, 2013). Total protein content of Liver, ovary and muscle of Zebra fish treated with neem based pesticide Achook was found to be reduced with respect to the dose and duration of exposure (Sharma Dilip et al., 2014).

Decreased in total protein concentration in present study may be due to metabolic utilization of protein for different energy producing pathways such as gluconeogenesis to overcome the stress induced by pesticide toxicity as suggested earlier (Murray R.K. et al., 2006).

SDS PAGE analysis of total soluble proteins of chick embryo treated with 1,3,5 ppm of bioneem at 24 hrs (HH stage 6-7) stage and incubated for 72 hrs post treatment, revealed that there was variation in expression of proteins. It was observed that expression of 56.55kDa and 29.33 kDa proteins decreased and that of 50.6kDa and 42.36kDa proteins was increased for all concentrations of Bioneem as compared to that of control. SDS PAGE analysis of total proteins of brain of chick embryo treated with 1,3 and 5 ppm Bioneem at 96 hrs (HH stage 23-24) and incubated for 11 day post treatment revealed that the expression of 65.02 kDa, 54kDa and 29kDa proteins was increased and that of 39.5kDa and 34.1 kDa was decreased in dose dependent manner, while protein of 34.1 kDa was not expressed at 5ppm. Similar observation in seed kernel extracted Azadirachtin treated *Helicoverpa armigera* wherein inhibition of neurosecretory cells and alteration in head polypeptides was reported (N. K. Neoliya et al., 2007). In present studies Bioneem treatment found to decrease embryonic brain proteins in dose dependent manner. It may be due to induction of oxidative stress as observed in brain of *D. rerio* due to treatment with neem based pesticide Achook (Dilip kumar 2014). In the human case of neem oil poisoning neurodeficits due to hypoxic brain damage which was incurable after 2 months of medical care were observed (Ramchandra Dhongde, 2008). Another case of human accidental ingestion of 20ml neem oil reported the toxic encephalopathy (Ajay Mishra 2013). Fresh neem leaves fed to Goat and Pigs for 7 days in 200mg /kg dose caused death of animals at 5^{th} day and postmortem revealed congestion in brain (Abhishekh Raj, 2014). SDS PAGE analysis of heart proteins of the Bioneem treated chick embryo didn't exhibit any significant difference in protein profile as compared to that of control.

Embryonic liver proteins showed significant difference in Bioneem treated sample as compared to the control. Protein of 64.44 kDa was expressed in all treated samples in dose dependent manner. However, at 3 ppm 53.03 kDa protein was not expressed but a 31.59 kDa protein was expressed only at 3ppm. Low molecular weight proteins were expressed at 3 and 5 ppm of Bioneem treated embryo.

This was the first attempt to study SDS PAGE analysis of different organs of chick embryo exposed to pesticides.

Protein carbonyl assay of Bioneem treated (1, 3 and 5ppm) chick embryo increased in concentration dependent manner. Also Protein carbonyl content of Liver, Brain and Heart of 15 day old chick embryo treated with 1,3 and 5 ppm Bioneem at 96 hrs (HH stage 23-24) was increased in dose dependent manner as compared to that of control. Increased in protein carbonyl (PC) was more in embryonic liver as compared to the Heart and Brain. At lower concentration of Bioneem (1ppm) change in PC level was not significant in all the three organs tested. Whereas in embryonic heart sample increased in PC is not significant in all concentration tested. Similar observations were made in liver of mice treated with Diazinon an organophosphate pesticide which showed hepatic injury due to oxidative stress induced to increased protein carbonyl, Lipid peroxidation (LPO) concentration and decreased in oxidative damage defense system such as GSH, CAT, SOD (Nahla s El Shenawy et al., 2010). Increased oxidative stress as conformed by increased PC and LPO was observed in *Gambusia offinis* treated with sub lethal concentrations of Thiocarbencarb (Khaled Y et al., 2014). It was also reported that there was increased protein carbonyl concentration along with overexpression of heat shock protein and other anatomical abnormalities in Black tiger shrimp after treatment with Endosulfan and Deltamethrin (Jennifer Dorts et al., 2009). Increased PC concentration in chick embryonic Liver in present studies indicates increased oxidative stress due to xenobiotics. Bioneem may induced free radicals production in Liver of chick embryo as observed in Chlorpyrifos treated chick embryo (Lalit Patel, 2013).

Observations on biochemical parameters of the blood of 15 day old chick embryo treated with 1, 3 and 5 ppm Bioneem at 96 hrs (HH stage 23-24) and control revealed that Serum urea content and LDH levels of Bioneem treated chick embryo exhibited increasing trends in concentration dependent manner. Similar observations were made in rats after neem leaf extract feeding (Singh et al., 1987) and in rats treated with synthetic pyrethroid insecticide (Muthuviveganandavel Veerappan, 2013). Further, increased blood urea indicated lower clearance suggesting diminished kidney function as observed in poultry birds (Ahrar Khan, 2012). Similar observations of increased LDH level were made in Cypermethrin treated 16 day old chick muscle tissues (Khurshid Anwar et al., 2010), and in neem seed oil treated mice in time dependent manner (Jitendra Kumar et al., 2011).

Enzymes SGOT and SGPT are used as marker of liver damage caused by toxic substances. Our observations revealed that serum level of SGOT and SGPT were increased in Bioneem treated (1,3,5ppm)chick embryo at 96 hrs stage incubated 11 day post treatment (15ED) in dose dependent manner as compared to the control. Similar results were obtained in rat treated with

neem leaf extract (Singh et al., 1987) and Fenvalerate, a pyrethroid pesticide treated chick embryo (Abd-El-Hamid et al., 2004). Also eleveted SGOT concentration was observed in rats exposed to Neem seed smoke (Aliyum Bello, 2014). Increased SGOT and SGPT in kidney, Lungs and Liver of rats exposed to Vepacide neem based pesticide caused in time and dose dependent manner (Rahman et al., 2001), and in rat treated with Pure azadirachtin (Abdel Megeed 2001). Human case of neem oil poisoning in 5 year old child showed raised SGPT level along with other clinical symptoms which were not neutralized even after two months of remedial treatment (Ramchandra Dhonge, 2008). Higher concentration of neem caused elevation in SGPT and SGOT levels in *P. lineatus* (Winkler et al.,2007), and in Argulus-infested goldfish *Carassius auratus* (Saurabh Kumar, 2012). However alkali treated neem kernel cake feeding to Uda lambs showed significant increase in SGOT level although other blood parameters such as SGPT , urea were not affected (A. Aruwayo et al., 2011). Contradictory to our observations were reported in broiler chickens fed with neem leaf powder showing decreased in SGOT and SGPT (Wankar Alok et al., 2009). Increased SGOT and decreased SGPT were observed in Cypermethrin treated chick embryo serum and in muscles of 16 day old chick embryo (Khurshid Anwar, 2010). Increased in concentration of these enzymes in serum of chick embryo treated with Bioneem may be due to change in hepatic function leading to leakage of these enzyme into blood stream as observed in Cypermethrin treated chick embryo (Khurshid Anwar ,2010).

In present study level of alkaline phosphatase (ALP) was decreased in serum of Bioneem treated chick embryo was observed in dose dependent manner. Our observations are in agreement with those in organophosphate pesticide treated mice (Nahla el shenway et al., 2010). Although increased ALP after neem seed smoke treatment in rats were also reported (Aliyum Bello et al., 2014). We also observed decreased blood cholesterol in Bioneem treated chick embryo in dose dependent manner. Similar results were observed in broiler chick treated with aqueous neem leaf extract (Onu Patience et al., 2013). Contradictory to our results were observed in neem treated goats and pigs (Singh et al., 1987).

In present study treatment of Bioneem at 24 hrs stage of chick embryo was responsible for variations in total protein, protein carbonyl and blood biochemistry. Possible cause for this may be to cope the stress induced by Bioneem. Overall observations suggested that Bioneem is comparatively less toxic to Chick embryonic Heart than that of Liver and Brain. It was due to more sensitivity of embryonic Kidney and Liver to the action of teratogen as compared to other embryonic organs as reported earlier in Toxaphene treated rats (Kavlock et al., 1982). It was also reported that aqueous neem leaf extract caused enlarged and congested liver, hepatic vacuolar degeneration with kupffer cell proliferation in adult chick (A.A. Biu et al., 2009). Also water extract of neem berries was found to cause hepatic and nephritic toxicity in poultry birds there were reports of children affected by neem oil along with other clinical signs showing enlargement of liver (Singh et al., 1987). Pure Azadirachtin was also found to cause congestion, hydropic degeneration and necrosis of liver in rat (Abdel Megeed et al.,2011).

The Neem based pesticide used in present study was Bioneem, extracted from neem seed kernel. The adverse effect of Bioneem on Chick embryo, a non-target organism may be because of neem oil based formulation used as reported earlier (Schumutters 1995, Stark, 2001).

V. CONCLUSION

- Bioneem treated chick embryo showed decline in total body and organ weight
- Tenement of chick embryo with Bioneem resulted in decline in total soluble proteins
- Chick embryos treated with Bioneem showed altered expression of developmentally regulated proteins
- Bioneem affects expressions 56.55kDa, 50.6 kDa, 42.36 kDa, 29.53 kDa protein of 24 hrs old whole chick embryo, chick embryonic Brain protein of 65.02, 54.4, 39.5, 34.3,29.0 kDa and Liver protein of 64.4,53.03,31.5,26.01kDa
- Heart proteins of chick embryo did not affected due to Bioneem treatment.
- Bioneem treatment increased oxidative stress biomarker protein carbonyl level of 24 hrs old whole embryo and 15 day old embryonic Liver and Brain in dose dependent manner.
- Increased serum Urea, LDH and decreased ALP level in blood serum of Bioneem treated chick embryo suggested excess energy requirement to cope up the stress induced by Bioneem.
- Bioneem treated chick embryonic blood serum showed Increased SGPT, SGOT and LDH reflected the signs of Hepatic dysfunction

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