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Carbofuran Induced Dose and Duration Dependent Alterations in Serum Biomarkers in Male Wistar Albino Rats (*Rattus norvegicus*)

Carbofuran Induced Alterations in Serum Biomarkers

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Abstract: The increase in use of pesticide in agriculture over the recent years have posed serious health concerns in non-target organisms including humans. As the exposure to humans through various means is inevitable by various means, their regulation and evaluation of toxic impact has become the need of the hour. The carbamate pesticide carbofuran, although has a reversible acetylcholine esterase inhibition property, in long-term exposure the effects are more harmful as reported earlier. Our study deals with intragastric exposure of carbofuran to male wistar albino rats (*Rattus norvegicus*) in dose and duration dependent manner emphasizing the role of serum biomarkers for homeostasis disruption in liver. We observed significant alterations ($p<0.05$) in total, direct and indirect bilirubin levels, liver transaminases including alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase levels. The total protein, albumin and globulin levels along with albumin to globulin ratio was also hampered upon long-term exposure. The present study provides an approach for evaluation of serum biomarkers at initial toxicity assessment which not only provides information about homeostasis disruption but also an insight into underlying mechanisms of alteration.

***Keywords*- Carbofuran, Liver function tests, serum proteins.**

I. INTRODUCTION

The carbamic acid derivatives, carbamates represent a vast variety of compounds having applications as herbicides, fungicides and insecticides. Most of these are potent neurotoxicants which usually is accompanied by either intentional, accidental or occupational exposure. Carbofuran (2.3-dihydro-2,-2-dimethyl -7- benzofuranylmethylcarbomate is a carbamate pesticide, used in several farm practices to increase the crop productivity. Owing to its short half-life and broad-spectrum action it is employed as an acaricide, nematicide and insecticide. (Gupta, 1994). The reversible carbamylation of the enzyme acetylcholinesterase allows acetylcholine accumulation (Shalaby *et al*., 2010). It is reported to possess high mammalian toxicity and as it is lipophilic in nature, it accumulates in fat deposits, majorly affecting skeletal muscles, heart, liver and brain (Risher *et al*., 1987; Gupta, 1994; Kaur and Sandhir, 2006; Rai *et al*., 2011). The impact of carbofuran is not just limited to neurotoxicity but also non-specific targets which include liver, testis and so on. It is also reported to induce alterations in serum lipids, protein levels and glucose in rat model (Sadek *et al*., 1989).

As pesticides are metabolized in liver, they are hypothesized to play an important role in inducing alterations in oxidative stress, cell adhesion, immunotoxicity, hormonal levels, tumour promotion and genotoxicity (Dich *et al*., 1997; Gomaa *et al*., 2008; Jin *et al*., 2013; Jin *et al*., 2014). Liver homeostasis disruption is also a result of such exposure wherein pesticide have been regarded as a potential candidate (VoPham *et al*., 2017). Impact of pesticides was evaluated in agricultural farm workers with respect to protein profile. Protein metabolism impairment was reported (Karami-Mohajeri and Abdollahi, 2010). The findings were however contradictory, wherein reports obtained from Araoud *et al* (2012) and Demos *et al* (2013) reveal elevation in serum total protein levels while those from Singh and Singh (2014) and Aroonvilairat *et al* (2015) revealed lower levels of serum albumin and total proteins. the alterations in serum proteins were considered to be due to impact of pesticides on protein synthesis in hepatocytes and altered function of kidney (Arafa *et al*., 2013; Mostafalou and Abdollahi, 2013).

Oxidative stress to erythrocytes causes disintegration of hemoglobin, the globin is re-utilized as a whole or its amino acid constituents whereas the heme in iron form is stored in tissues of the body as hemosiderin and ferritin. This is followed by degradation

of the iron-free porphyrin skeleton causing the formation of biliverdin, a green pigment. This is then reduced to bilirubin, a yellow pigment and is transported to liver by plasma albumin for excretion (Fahimul-Haq *et al*., 2013).

In normal plasma, albumin is responsible for more than 50% of total antioxidant activity (Taverna *et al*., 2013). This is associated with abundance in reduced sulfhydryl group of albumin reported to exhibit oxygen-free radical scavenging activity along with nitric oxide and hypochlorous acid. This property is inherent to albumin. It also binds to unconjugated bilirubin which is also an effective antioxidant. This mechanism is believed to be due to a potent inverse correlation the unconjugated bilirubin of plasma and mortality and morbidity of several disease states (Levitt and Levitt, 2016). Albumin having various multiple physiological functions which include maintaining the colloidal osmotic pressure, corroborating the plasma antioxidant activity along with binding of wide range of compounds. It is also a remarkable and strong indicator in prognosis of health, imbalance in which leads to pathophysiological conditions. Thus, the correlation of serum albumin concentration with health may be possible (Levitt and Levitt, 2016). Therefore, with this as a base, the present study was performed to evaluate impact of carbofuran on serum biomarkers in dose and duration dependent manner.

II. RESEARCH METHODOLOGY

Ethics statement:

The experimentation process employed for the test species used in the present study which includes handling, maintenance and disposal upon experimentation were in accordance with IAEC (Institutional Animal Ethics Committee) as per the guidelines described by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) (Animal House Registration No. 639/02/a/CPCSEA).

Test chemical:

Carbofuran technical grade (95%) purity was obtained from United Chemicals Pvt. Ltd, Vidisha, Madhya Pradesh, India. Corn oil was used as the vehicle for dosing. Initially the stock solution was prepared of 1 mg/ml, the working solutions were freshly prepared while dosing. Other chemicals required for the study were commercially available and of analytical grade.

Test model:

For the present study Wistar albino rats (Rattus norvegicus) weighing 150-180 grams were selected. Rearing and maintenance was carried out at animal house facility, Department of Zoology, Karnatak University Dharwad. They were housed in polypropylene cages and paddy husk was used as bedding material. Room conditions were maintained uniform throughout acclimatization and exposure durations with temperature 24 ± 2◦C and humidity of 64 ± 5%, along with a 12 h of light/dark cycle. Rats had free access to water and food ad libitum, they were feed with commercial feed pellets for laboratory animals. Carbofuran was administered with an oral gavage, the mode of administration being intra-gastric.

Experimental design:

Post acclimatization, animals were segregated into groups ten groups of four each (n=4). Carbofuran sub-lethal concentrations for the present study were calculated by considering available literature on previously determined LD50 values (Dragica *et al*, 2008; Otieno *et al*., 2010). Group A served as control, while Group B, C and D were attributed exposure concentrations, 1.0, 0.5 and 0.3 mg/kg BW respectively. Each of these groups has sub-groups representing the exposure durations B1-30d, B2-60d and B3-90d; C1-30d, C2-60d and C3-90d; D1-30d, D2-60d and D3-90d.

Bilirubin D:

This was estimated by Malloy and Evelyn method (1937) which is based on the Van Den Bergh reaction. The reaction of bilirubin with diazotized sulfanilic acid produces azobilirubin, a purple colour compound. The intensity the colour formed is reflects the bilirubin in serum. This is further calibrated by comparing with standard for determination of unknown concentration. The direct bilirubin is based on the reaction in aqueous medium wherein water-soluble conjugated bilirubin is formed. The reaction is comparatively fast with development of colour. Therefore, direct bilirubin is also termed as conjugated bilirubin and is expressed in mg/dl.

Bilirubin I:

In this reaction, the addition of methyl alcohol solubilizes water-insoluble or unconjugated bilirubin. Therefore, providing an indirect estimation of total bilirubin (both unconjugated and conjugated). As the estimation protocol is indirect, it is called indirect bilirubin and is expressed in mg/dl

Bilirubin T:

The total of both direct and indirect bilirubin gives total bilirubin and is expressed in mg/dl

Total Bilirubin (Bilirubin T) (mg/dl) = Bilirubin Direct (D)+ Bilirubin Indirect (I)

Aspartate aminotransferase (AST):

This was estimated as described previously by Reitman and Frankel (1957). Briefly, to 0.2 ml of serum sample,1.0 ml of the buffer substrate was added and incubated at 37 0C for about 60 minutes. Next to this, 1.0 ml of DNPH (2,4-dinitrophenylhydrazine) was added to arrest the reaction. The tubes were then kept at room temperature for 20 minutes flowed by addition of 10 ml of 0.4N NaOH. Pyruvate was taken as standard to prepare calibration curve which was also treated in a similar way. The color developed was read for absorption at 520 nm.The obtained enzyme activity was expressed as units per liter in serum (U L-1).

Alanine aminotransferase (ALT):

This was estimated as described previously by Reitman and Frankel (1957). To 0.2 ml of serum sample,1.0 ml of substrate (α-ketoglutarate) in phosphate buffer (pH 7.4) with incubation duration of 30 min for ALT. Next to this,1.0 ml of DNPH was added to arrest the reaction and further incubated up to 20 min at 25 0C. This was followed by addition of 1.0 ml of 0.4N NaOH and obtaining the absorbance at 540 nm. The enzyme activity was expressed as units per liter in serum (U L-1).

Alkaline phosphatase (ALP):

This was estimated as described previously by King and Armstrong (1934). Briefly, in a test tube, 4.0 ml of buffer substrate was pipetted out and incubated for 5 minutes at 370C, to this the 0.2 ml of serum was added. The test tubes were further incubated for 15 minutes in water bath. As soon as the tubes were taken out of the water bath, 1.8 ml of diluted phenol reagent was added. The contents of the tubes were mixed thoroughly and centrifuged. To 4.0 ml of supernatant, 2.0 ml of sodium carbonate was added. The absorbance was read at 540 nm and enzyme activities are expressed as units per liter in serum (U L-1).

Total proteins:

For estimation of serum total proteins, the method was described by Lowry *et al* (1951). Briefly, to 1 ml of serum sample 3 ml of 10% of TCA was added. This was centrifuged at 3000 rpm for 10 mins. The pellet was dissolved in 5 ml of 0.1N sodium hydroxide and to 1 ml of this solution 4 ml of reaction mixture was added (mixture of 2% sodium carbonate and 0.5% copper sulphate in the ratio 50:1). The samples were incubated for 10 minutes followed by addition of 0.4 ml of Folin-phenol reagent (diluted prior to use with distilled water in 1:1 ratio). The colour developed was measured for optical density using a Secomam, Anthelie advanced 2 spectrophotometer at 600 nm wavelength.

Serum albumin:

The albumin in serum samples was measured spectrophotometrically as reported previously by Dumas *et al* (1997), based on the specific binding of bromocresol green (BCG) with protein at low pH. Briefly, to 20 µl of the serum sample, 4 ml of BCG was added and incubated at 20-25 0C for 10 minutes. The intensity of colour developed was recorded at 630 nm. For the preparation of standard curve, bovine serum albumin (BSA) was used. Concentration of albumin is thus expressed in gm/dL.

Serum Globulin:

Serum globulin was calculated as described by Busher (1990). This is generally done by subtracting albumin from total proteins. Concentration of globulin is thus expressed in gm/dl.

Total protein (gm/dl) = Albumin + Globulin

Therefore, Globulin = Total protein – Albumin

Serum Albumin to Globulin ratio (AGR):

This was calculated a described by Duran *et al* (2014). The equation being

AGR=albumin/ (Total protein-Albumin)

Statistical tools:

Statistical analysis was performed by comparing the control values with the treated ones obtained from triplicates and were expressed as mean ± standard deviation. Spectroscopic results were further subjected to Kruskal-Wallis ANOVA defining the significant difference at levels $p<0.05$. This was performed in ORIGIN 2022 software.

III. RESULTS AND DISCUSSION

Biomonitoring of pesticides has limitations owing to their duration and concentration of initial exposure as they are bound to vary over time and may necessarily not represent the level of exposure, the source of exposure along with its route, a lack of benchmark in health and challenges faced in evaluation of chemicals which have a relatively short biological half-lives which include carbamates and organophosphates (Angerer *et al*., 2006). The carbofuran exposed groups when compared to control, exhibited significant alterations.

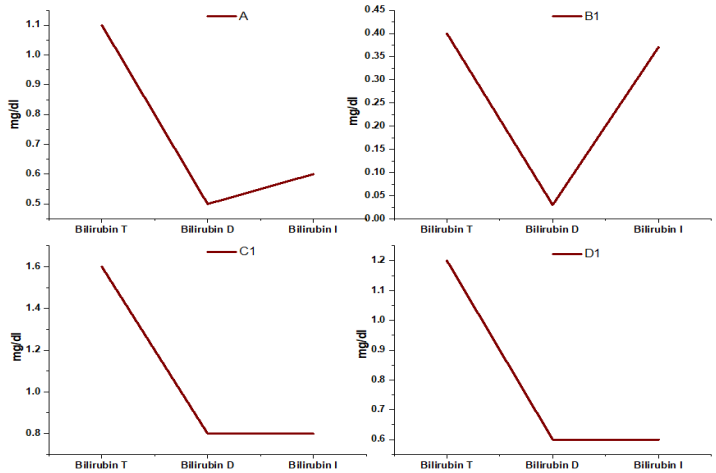

Fig 1a: Depicts the alterations in Bilirubin T, D and I in control and Carbofuran exposed groups for 30 days exposure duration. Values are expressed in mean±SD where $p<0.05$.

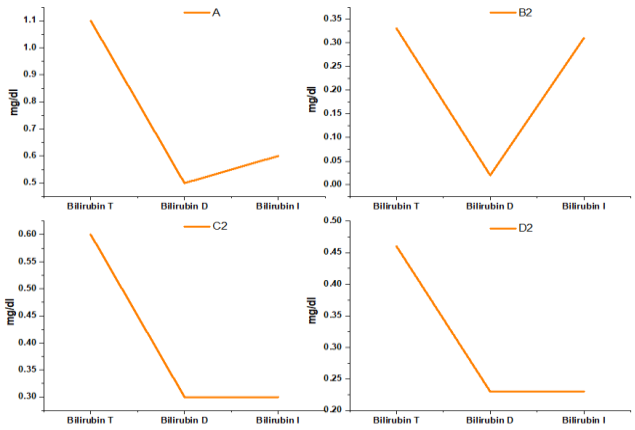

Fig 1b: Depicts the alterations in Bilirubin T, D and I in control and Carbofuran exposed groups for 60 days exposure duration. Values are expressed in mean±SD where $p<0.05$.

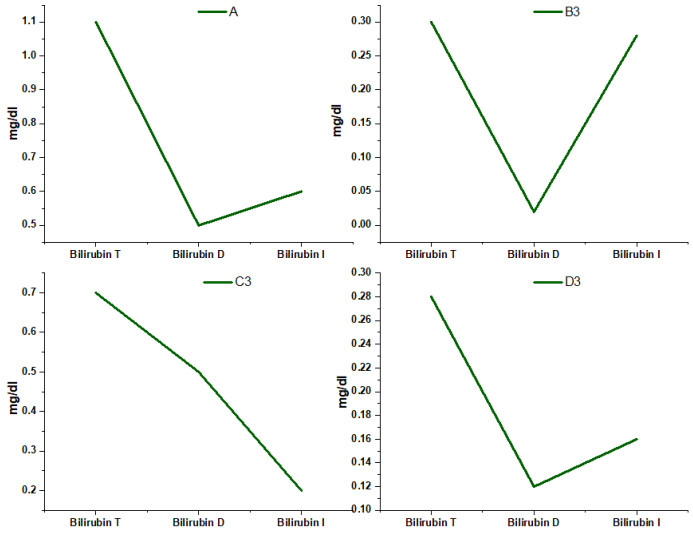

Fig 1c: Depicts the alterations in Bilirubin T, D and I in control and Carbofuran exposed groups for 90 days exposure duration. Values are expressed in mean±SD where $p<0.05$.

Bilirubin is present in bile, reticuloendothelial cells of spleen and in intestines (Cullen, 2005), it is synthesized in the bone marrow and as a product of red blood cells its breakdown occurs in liver. When compared to control group the Bilirubin I was significantly increased in B1 and decreased in C1 and D1 (Fig 1a). The increase in Bilirubin I in Fig 1b followed the similar trend as Fig 1a. In Fig 1c Bilirubin D was significantly increased in C3 and the Bilirubin I was increased in B3 and D3 compared to control. The bilirubin concentration is increased owing to breakdown of red blood cells or reduced levels of transport protein albumin causing decrease in hepatic uptake of bilirubin. The elevation in the concentration of total bilirubin may also be due to the pesticide breakdown in liver causing hepatobiliary damage (Ramaiah, 2007).

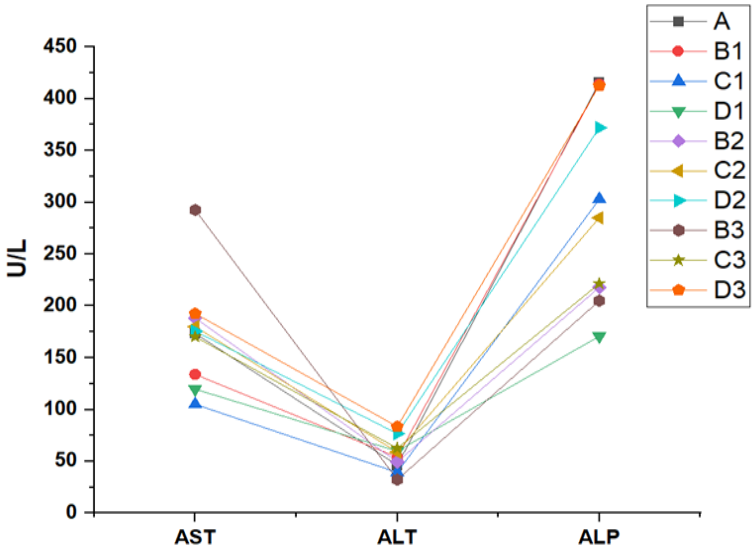

Fig 2: The changes in serum enzymes depict homeostasis disruption in liver in Carbofuran exposed groups when compared to control in dose and duration dependent manner. Values are expressed in mean±SD where $p<0.05$.

Liver transaminases were assessed to evaluate disruption in liver homeostasis. The elevation in their levels can be correlated to oxidative stress, perturbations in the antioxidant defense system, apoptosis, microsomal and mitochondrial metabolism (Karami-Mohajeri *et al.*, 2017). AST comprises of both mitochondrial and cytosolic isoenzymes, found in kidney, skeletal muscle, red blood cells, heart and brain. It is mainly found in liver and is a cytosolic enzyme. Therefore, the increase in their levels can be attributed to liver pathology (Limdi and Hyde, 2003). In our study, the ALT levels were decrease in all exposure groups when compared to that of AST levels. ALP levels however were more pronounced. AST and ALT levels were reported to be increased in organophosphate poisoning cases (Anormallikleri, 2010). Previous studies of carbamate and organophosphate poisoning have described no statistically significant alterations in bilirubin levels (Risal *et al.*, 2019; Anormallikleri, 2010). However, in our study observable alterations have been perceived and the changes were statistically significant in dose and duration dependent manner.

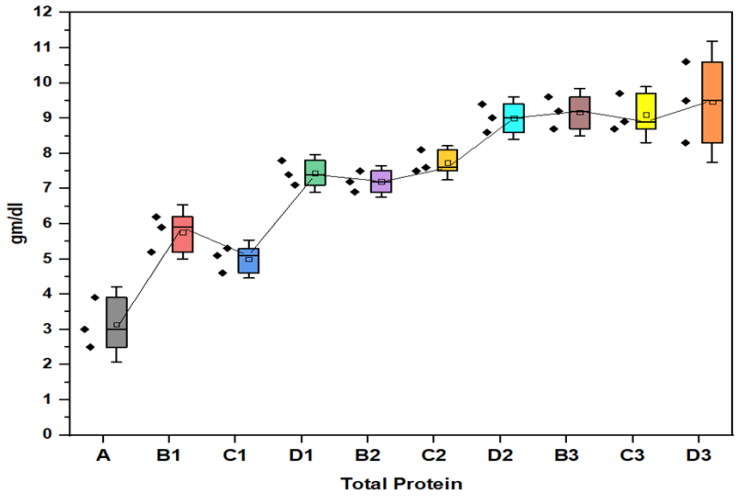

Fig 3: The alterations in serum total protein levels in dose and duration dependent manner in carbofuran exposed male wistar albino rats. the x-axis represents experimental groups while the Y-axis represents grams per decilitre of protein concentration. The median values of the box plots are connected while the outliers depict no. of samples (triplicates). Values are expressed in mean±SD where $p<0.05$.

In plasma, proteins are one amongst the major targets of oxidation (Dean *et al.*, 1997; Stadtman and Oliver, 1991). Plasma protein oxidation has been reported to be involved in chronic hemodialysis which accompanies oxidation of thiol group along with protein carbonyl formation (Himmelfarb *et al.*, 2000). "Carbonyl stress" described by Miyata *et al* (1999) results from formation of excessive carbonyl groups (ketones and aldehydes) which are detectable in carbohydrates, proteins and lipids in chronic renal failure (CRF) patients. Formation of carbonyl may be crucial in developing β2-microglobulin amyloidosis and atherosclerosis in case of renal failure (Miyata *et al.*, 1999, 1998a and b). The level of total proteins was gradually increased in all CF exposed groups when compared to control group A (Fig 3). Increase in total protein levels in the present study explains this phenomenon.

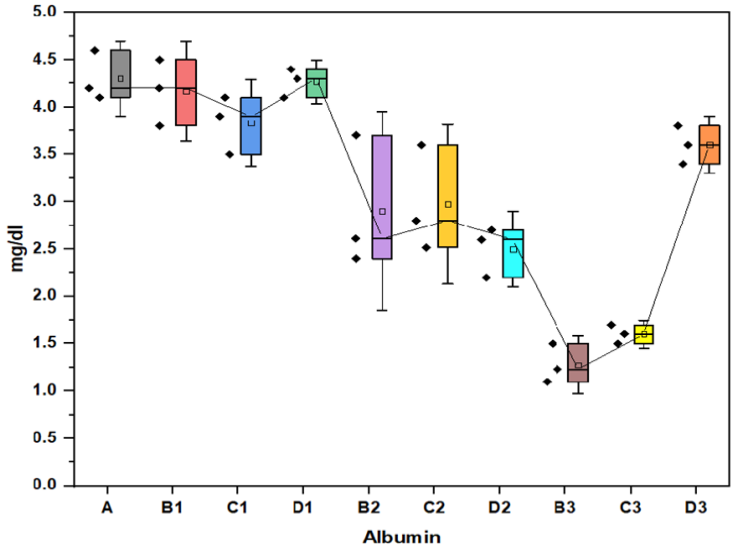

Fig 4: The alterations in serum albumin levels in dose and duration dependent manner in carbofuran exposed male wistar albino rats. The x-axis represents experimental groups while the Y-axis represents milligrams per deciliter of albumin concentration. The median values of the box plots are connected while the outliers depict no. of samples (triplicates). Values are expressed in mean±SD where $p<0.05$.

Albumin is the major protein in serum having a molecular weight of 66.5kDa, which is less than that of gamma globulin being 160kDa, therefore providing an osmotic activity more than gamma globulin. Also, as albumin holds a high negative charge and holds sodium ions but does not bind to them in its filed by Gibbs-Donnan effect, this increases the albumin intrinsic osmotic activity to about 50%. Therefore, about 80% of normal plasma colloidal osmotic activity is provided by albumin, if this declines the elevations in globulin levels are unable in maintaining normal colloidal osmotic pressure (Levitt and Levitt, 2016). Although albumin possesses a negative charge, it has the ability to bind to vast variety of compounds both positively and negatively charged along with hydrophobic organic anions like long-chain fatty acids and bilirubin and divalent cations like magnesium and calcium (Fasano *et al*., 2005). Apart from this it also binds with bile acids, drugs, zinc, copper, thyroxin and vitamin D. Binding of albumin reduces free concentration of compounds, therefore limiting their distribution, biologic activity and rate of clearance (Levitt and Levitt, 2016). There was a contrasting impact observed in albumin levels in CF exposed groups. While in B1 and D1 it was close to group A, in all other groups it was found to be decreased and in D3 the decrease was less significant (Fig 4). Total proteins are composed of albumin whose synthesis take place in liver along with globulin. Albumin is regarded as a potent biomarker in bio monitoring pesticides in relatively low exposure concentrations (Palaniswamy *et al*., 2021; Tarhoni *et al*., 2008). Serum albumin is influenced by several factors that include liver function, nutritional, gastro-intestinal as well as urinary clearance (Aroonvilairat *et al*., 2015; Hassanin *et al*., 2018). Reduced albumin levels have been attributed to increased risk of ischemic heart disease (Ronit *et al*., 2020).

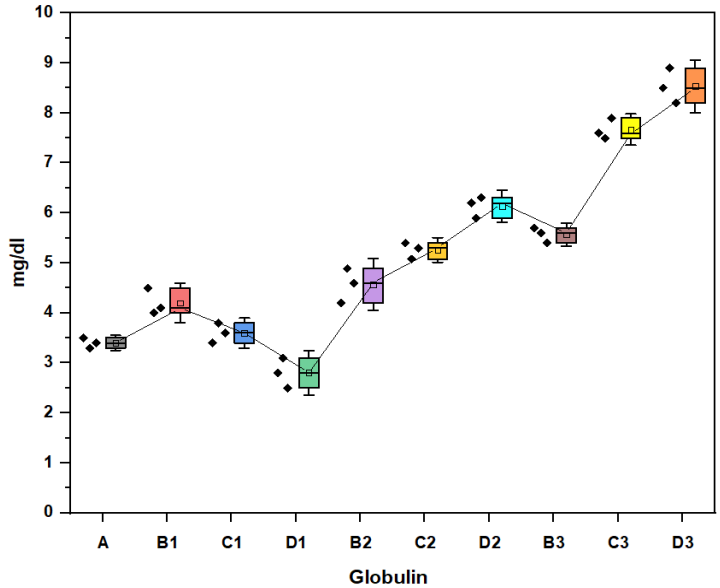

Fig 5: The alterations in serum globulin levels in dose and duration dependent manner in carbofuran exposed male wistar albino rats. The x-axis represents experimental groups while the Y-axis represents milligrams per decilitre of globulin concentration. The median values of the box plots are connected while the outliers depict no. of samples (triplicates). Values are expressed in mean±SD where $p<0.05$.

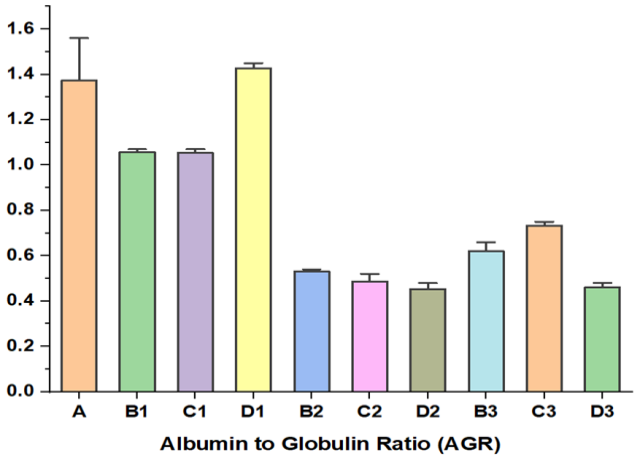

Fig 6: The alterations in albumin to globulin ratio (AGR) upon carbofuran exposure when compared to control group in dose and duration dependent manner

Total protein, globulin and albumin levels were found to be decreased upon pesticide exposure (Hassanin *et al*., 2018; Mostafalou and Abdollahi, 2013). Apart from group D1 in all other CF exposed groups the levels of globulin were gradually found to be significantly increased (Fig 5). The AGR was increased in only D1 while it was significantly reduced in D2 (Fig 6). This decrease is associated with alterations in protein metabolism upon pesticide exposure, they also hamper concentrations of serum proteins by interfering with hepatocytes in weakening protein synthesis and impaired kidney function (Johnson *et al*., 2019; Valcke *et al*., 2017). As the functions of liver and kidney g*et al*ong with each other and elevation in creatinine may additionally impact albumin loss by means of kidney and also reduced albumin production by liver due to liver cell damage (Palaniswamy *et al*., 2021; Johnson *et al*., 2019; Valcke *et al*., 2017). In our study the decrease in albumin and increase in globulin levels in dose and duration manner along with reduction in AGR explains the impact of Carbofuran exposure upon long- term exposure.

IV. ACKNOWLEDGMENT

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