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Research Article

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EFFECT OF POLY (*N*-ISOPROPYLACRYLAMIDE) "PNIPAM" ON HEPATIC CELLS OF SWISS ALBINO MICE, *MUS MUSCULUS*

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Abstract

The present paper deals with the effect of polymeric compound poly (*N*-isopropyl acrylamide) "PNIPAM" for their toxicity on hepatic cells. The nanoparticle is a xenobiotic compound that accumulates in the liver for their metabolism. Non-metabolizing xenobiotic compounds such as "PNIPAM" produces anomalies in the hepatic cells. Certain enzymes such as ALT and ALP can be assayed for the hepatocytic toxicity. An attempt has been made to know the toxic effect of "PNIPAM" in a concentration of 0.8mg/ml on the hepatic cells of Swiss Albino mice, *Mus musculus*. The ALT and ALP analysis were performed through test kits for their quantitative estimation. The histological result shows that several lesions were produced after the introduction of aqueous solution of PNIPAM for an incubation period of 48 hours. The toxicity was confirmed Spectrophotometrically by the assessment of enzyme ALT and ALP. The increased concentration of ALT (55.0 IU/L) and slight decrease in ALP (40.0 IU/L) concentration was responsible for the metabolic alteration and production of hepatocytic anomalies in the mice.

Keywords: PNIPAM, Polymeric compound, Nanoparticle, ALT, ALP, Hepatocytes, Toxicity.

Introduction

"PNIPAM", poly (*N*-isopropyl acrylamide) is a polymeric nanoparticle. It is also called as "Smart Polymers" (Galaev and Mattiasson, 1999, Aslam *et al.*, 2013). They are considered as an important class of polymeric materials having several important biomedical and industrial applications. These polymers undergo sharp reversible phase transition in response to small changes in relation to environmental stimuli such as pH, temperature; ionic strength etc. Poly (*N*-isopropyl acrylamide) "PNIPAM" is one of the typical examples that have been widely studied for various biotechnological applications. It has a lower critical solution temperature (LCST) of precipitation around 32.58^0C in water and changes reversibly from hydrophilic below this temperature to hydrophobic above it. The reversible phase transition of "PNIPAM" can be used for colloid suspension formation together with an antigen or any other biomolecule as the temperature is increased above the LCST, thus, providing the possibility of their use as an adjuvant in combination with a specific antigen.

The perusal of pertinent literature in this field reveals that considerable information is available on the role of ALT and ALP for knowing the liver and pancreas dysfunction (Hillesheim *et. al.*, 1995, Gebhardt, 1992 and Gebhardt and Gaunitz, 1997, Kikawa, 2006; Wang *et al.*, 2012).

However, practically no information is available on the effect of "PNIPAM" on the hepatic cells, although much work has been done on its application for toxicity assays (Schild, 1992; Galaev and Mattiasson, 1999; Kumar *et al.,* 2007 and Aslam *et al.,* 2013). The objective of the present study is to pin point the effect of "PNIPAM" on the hepatic tissues of the Swiss Albino mice, *Mus musculus* for assessing the extent of hepatotoxicity.

ALT is known as Alanine Transaminase or alanine aminotransferase (ALAT) or serum glutamic pyruvic transaminase (sGPT). It is a homodimeric cytoplasmic pyridoxal phosphate-dependent enzyme. It involved in cellular nitrogen metabolism, amino acid metabolism, and liver gluconeogenesis (Gebhardt, 1992). ALT mediates conversion of major intermediate metabolites, catalyzing reversible transamination between alanine and α-ketoglutarate to form pyruvate and glutamate (Lott *et.al*, 1986). ALT is widely distributed in many tissues but is found in greatest abundance in the liver, and to a much lesser extent in the kidneys, heart, and brain (Gebhardt *et.al*, 1997). The major role of ALT in the liver is the conversion of alanine to glucose which is then exported to the body to be utilized in a multitude of processes. Serum ALT levels are generally low, but may spike during disease states or in the event of tissue injury (Amacher, 1998). As such, ALT levels are routinely used as

indicators of medical issues, particularly liver diseases. An increased level of ALT can be seen in patients with diabetes, cirrhosis, fatty liver disease, and hepatitis. Alanine Transaminase Assay provides ALT activity in serum, plasma, tissue samples, and cell lysates (Wang *et al.,* 2012). Measurement of the ALT activity is carried out by monitoring the rate of NADH oxidation in a coupled reaction system employing lactate dehydrogenase (LDH). The oxidation of NADH to NAD^+ is accompanied by a decrease in absorbance at 340 nm (Dawson, 1985). Under circumstances in which the ALT activity is rate limiting, the rate decrease is directly proportional to the ALT activity in the sample.

Alkaline phosphatase (ALP) catalyzes the hydrolysis of phosphate esters in alkaline buffer and produces an organic radical and inorganic phosphate (Ambler *et al.,* 1970 and Willamson ,1972). Changes in alkaline phosphatase level and activity are associated with various disease states in the liver and bone. The statistical validation through Student's t-test for the data related to ALP and ALT using softwares provided online by different servers using the usual method of calculation (Buda and Jarynowski, 2010). The correlation between ALP and ALT can be made by Pearson correlation using the statistical tool of *Wessa.net*.

Materials and Methods

Model Organism

The model organism Swiss Albino mice, *Mus musculus* balb/C strain is widely used to study toxic effects on various organs such as liver, kidney, pancreas, testis etc. The Swiss Albino mice, *Mus musculus* balb/C strain is easily available, culturable and ethically approved. 4 female Swiss albino mice, *Mus musculus* Balb/C strain with average body weight ranging from 25.0g to 30.0g were obtained from Animal house of the University Department of Zoology, T. M. Bhagalpur University, Bhagalpur, India. Mice were fed with the help of *ad libitum* (prepared mixed formulated feed by the laboratory itself). Animals were housed in colony rooms with 12 hrs light/dark cycle at 30 ± 20^0C in the Post-Graduate Department of Biotechnology, T.M. Bhagalpur University, Bhagalpur. Approval of Institutional Ethical Committee was sought prior to the commencement of experiment.

Chemicals

"PNIPAM", was purchased from Sigma Aldarich. The ALT (Alanine Transaminase Activity) Assay Kit and ALP (Alkaline Phosphatase) Assay Kit were purchased from Cayman Chemicals and Abcam Chemicals respectively.

Treatment Protocol

The four mice were grouped into test and control containing two mice /group. The mice were fed upon normal diet containing carbohydrate and protein sources. After the rearing of fifteen days test group were introduced with 0.8mg/ml of "PNIPAM" nanopolymer. 24 hours starvation was performed before the introduction of "PNIPAM" in test group along with control. The same procedure was repeated after every two days for thirteen days. "PNIPAM" was introduced orally as aqueous solution having a concentration of 0.8 mg/ml.

After thirteen days of dosing the test groups along with control the animals were sacrificed to isolate liver. Liver samples were taken and placed into formalin solution. Small pieces of the liver tissues were removed from the formalin solution and then placed into the alcohol solution with an increasing percentage of solubility (70%, 80%, 90%, and 100% w/w) for the dehydration of samples, then after tissues were kept into destaining xylene solution. Afterwards, the samples were mounted into paraffin wax for microtomy. The samples were cut into pieces of 3–5 nm. The tissue samples were stained with eosin for microscopic examination. The slides were used for microscopic analysis for the morphological anomalies of hepatocytes. For enzymatic assessment the blood samples were taken from supraorbital plexes. The blood sample was mixed with EDTA to prevent clotting and then centrifuged at 5000rpm for 10 minutes to isolate supernatant. Cayman's Alanine Transaminase Assay Kit was used for detecting ALT activity in Supernatant (plasma). Measurement of the ALT activity is carried out by monitoring the rate of NADH oxidation in a coupled reaction system employing lactate dehydrogenase (LDH). The oxidation of NADH to $NAD^+$is accompanied by a decrease in absorbance at 340 nm.

Abcam's Alkaline Phosphatase Assay Kit (Colorimetric) was used to measure ALP activity in plasma. Abcam's Alkaline Phosphatase Assay Kit (Colorimetric) is a highly sensitive, simple, direct and HTS-ready colorimetric assay designed to measure ALP activity in serum and biological samples. It contains 10 substrate tablets providing convenience for multiple usages. The kit uses *p*-nitrophenyl phosphate (*p*NPP) as a phosphatase substrate which turns yellow (λmax= 405 nm) when dephosphorylated by ALP. The Kit can detect 10-250 µU ALP in samples.

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With the help of the alkaline phosphatase (ALP), ALT, aspartate aminotransferase (AST) enzyme recognition kits and the relative spectrometer functions, the relative numbers for the enzymes were studied and analyzed. Student's's *t*-test was used to estimate the differences.

Assessment of hepatotoxicity

Hepatotoxicity was analyzed at both morphological and physiological level. The alteration in tissue organization depicted by various microbiological slides at morphological level.

Statistical Analysis

The data obtained were analyzed for their statistical validity and correlation using *Online Statistical tools* provided by University of Delaware and *Wessa.net* for the calculation of Student's t- test and Pearson correlation. These tools provide the validation parameters and correlation values (-1, 0, +1).

Results

The effect of "PNIPAM" on hepatic cells of Swiss albino mice, *Mus musculus* is depicted through microphotographs showing control and in shots 1.0 - 4.0 respectively (Fig-1). The test slides show necrotic regions in patches as compared with the control. Table-1 shows variation in ALP and ALT concentration on the different dosing days. It was observed that there was a decrease in ALP concentration and increase in ALT concentration was observed on different dosing days. The data was tested through Student's t-test (p=0.004) for statistical validation. The regression analyses of ALT and ALP and students t- test have been depicted in Fig-2 and Fig-3 respectively.

The value of Simple Linear regression is r = 119.157 and P= 0.004. The value of student t- test is 0.5 indicating the significance of data at 5% level.

The ALT and ALP shows antagonistic relationship since the concentration of former is decreasing while the later is increasing. The ALT during the progression of disease produces free radicals in the form of superoxides and having much necrotic effect on hepatic tissues (55.0 IU/L to 96.0 IU/L). ALP is pre-requisite for normal physiology and metabolism of the hepatocytes. During the progression of diseases its concentration decreases resulting in the deformities of the hepatocytes (40.0 IU/L to 17.0 IU/L). Thus, in the present study an inverse relationship was observed between ALT and ALP.

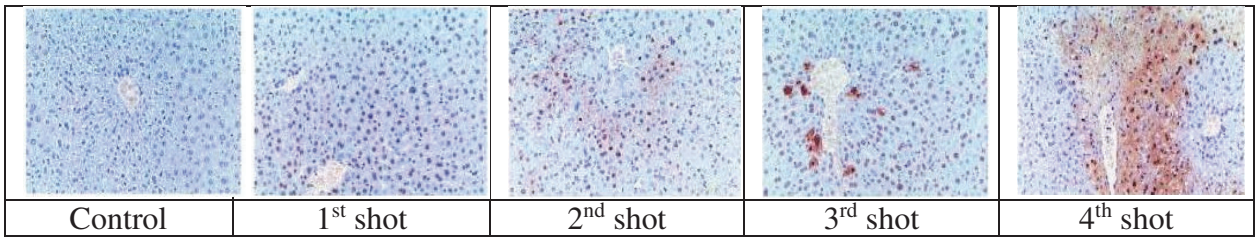

Fig-1: Microphotographs showing control and treated Liver of Swiss Albino mice.

Table-1: Concentrations of ALP and ALT on different days of dosing.

Serial No.	Days of Dosing	ALP		ALT	
		O.D λ= 405nm	(IU/L)	O.D λ= 405nm	(IU/L)
1	01	0.36	40	0.64	55
2	04	0.27	32	0.76	68
3	07	0.21	28	0.83	73
4	10	0.17	20	0.91	82
5	13	0.15	17	0.95	96

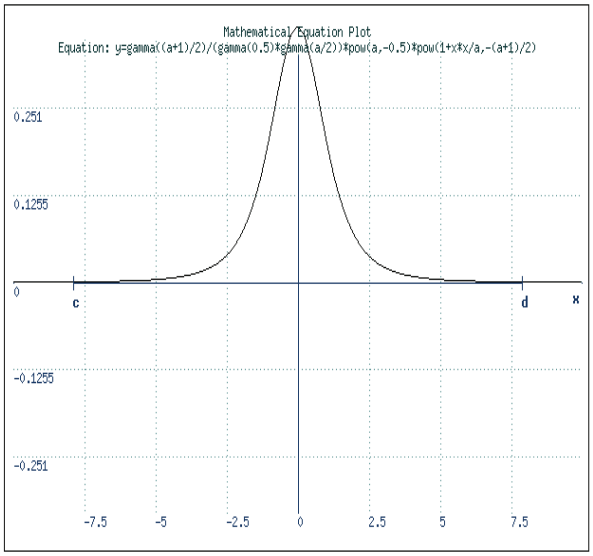

Fig-2: Regression graph of ALT and ALP

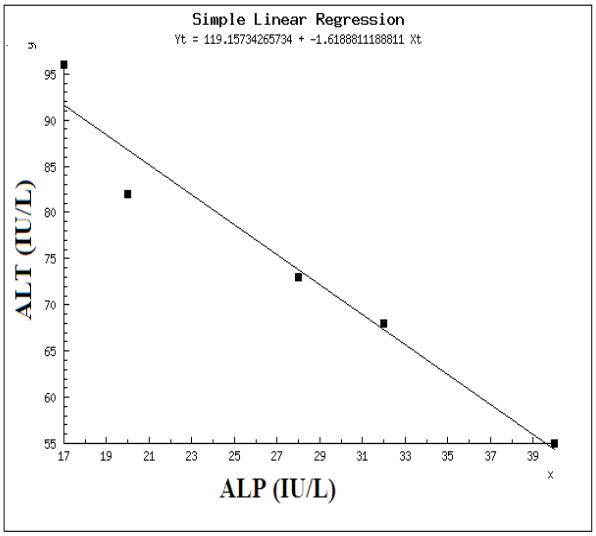

Fig-3: Student's t-test graph

The results were analysed with the help of online statistical tool provided by University of Delaware unpaired t- test to know the statistical significance and

p - value. The confidence interval, degree of freedom, intermediate value, the mean standard deviation and SEM were calculated for ALP &ALT group individually (Table-2).

P value and statistical significance

The two-tailed P value equals 0.0004

By conventional criteria; this difference is considered to be extremely statistically significant.

Confidence interval

The mean of Group One minus Group Two equals -47.40

95% confidence interval of this difference: From -65.89 to -28.91

Intermediate values used in calculations

t = 5.9112

df = 8

Standard error of difference = 8.019

Pearson Correlation

Pearson Correlation of ALP and ALT shows linear correlation for their concentration and activity. The data is significant and the determination value of 0.954020311466217 which is very near to significant value of 1(Fig-4).

Table-2: Showing Statistical Results.

Group	ALP Group	ALT Group
Mean	27.40	74.80
SD	9.26	15.35
SEM	4.14	6.87
N	5	5

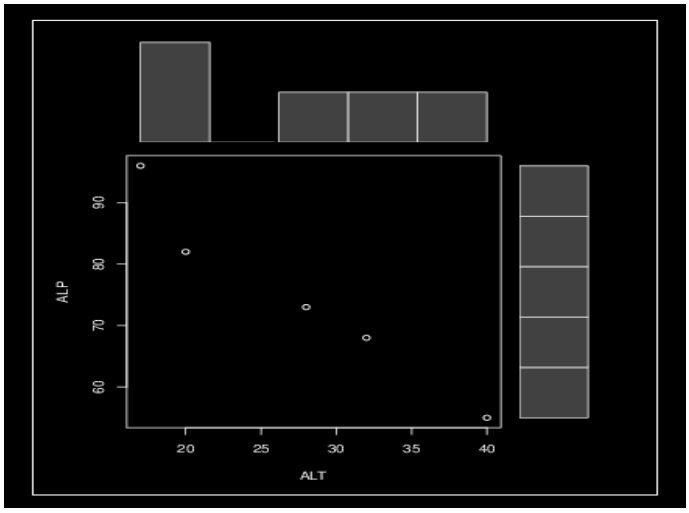

Fig-4: Graph showing Pearson Correlation between ALP and ALT**.**

Discussion

"PNIPAM" as a non-familiar xenobiotic compound for the model organism Swiss Albino mice *Mus musculus* and there is no any obvious mechanism detected to metabolize PNIPAM through liver of the model organism. This condition was detected through ALP and ALT enzymes assay which were taken as a marker for the physiological condition of liver and hepatocytes as a whole (Amacher, 1998). The alteration in the physiology of hepatocytes and organization of liver tissues were depicted through increased concentration of ALT and decreased ALP concentration. The formation of necrotic regions in the liver suggests that

PNIPAM has slightly toxic effect if not metabolized through hepatocytes. As a result disorganization of liver tissues has been occur. The aqueous solution of PNIPAM at the concentration of 0.8mg/ml shows deleterious effect suggesting that the same concentration cannot be tolerable by the liver size of the model organism.

The present result is in the conformity with the work of Cooperstein and Canavan (2010) who observed biological cell detachment from Poly *N*-isopropyl acrylamide.

The statistical analysis through Student's t-test gives the p-value 0.004, confidence interval of 95% with a standard error of difference 8.019 which shows the statistical validity of the data obtained. The correlation between ALP and ALT was made through Pearson correlation which suggests a direct and linear relationship between ALP and ALT activity, antagonizing each other.

The further study in the field of xenobiotic susceptibility in mammals must be practiced due to endless and ever-growing list of newly synthesized compounds. The effect of these xenobiotic compounds must be studied at hematological, physiological and therapeutic level to prevent harmful effects on vital organs of the concerned organism. These xenobiotic compounds have no any immune system yet evolved in animals. They are toxic even in minute quantities. Several information is available on the level of the toxicity of these compounds. Rinaki et al., (2003) have suggested dose/solubility ratio of biopharmaceutics. Acosta et al., (1985) have given an *in vitro* approach to the study of target organ toxicity of drug and chemicals.

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