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Evaluation of Paracetamol Overdose on the Wistar Rat's Liver

Shekhar Kumar Sinoriya* and Kusum Singh
Department of Zoology, Bundelkhand University, Jhansi (Uttar Pradesh) – 284128

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Corresponding Author Email: shekharsinoriya@yahoo.com

Abstract
It has been reported that the liver gets damaged due to overdose of many drugs including paracetamol, several researchers reported a number of hepatoprotective drugs to overcome damage in liver cells. Since a large no. of population uses paracetamol it becomes necessary to evaluate the toxicity and to develop a drug to overcome it. Present study is done to evaluate toxicity of liver due to overdose of paracetamol in Wistar rats. Three doses 1500, 2500 and 3500 mg/kg/ day were given for 3 consecutive days to Wistar rats and various biochemical parameters were performed. To support the study, histopathology of liver was also done simultaneously. It was observed that the Wistar rats were totally resistant to paracetamol except slight gastric ailments. The result of this research is that, the Wistar strain of rat is not suitable for study of paracetamol induce hepatotoxicity.

Keywords
Biochemical parameter, Hepatoprotective, Histopathology, Paracetamol, Wistar rat.

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1.INTRODUCTION
The liver is one of the most important vital organs having a wide range of functions including detoxification, protein synthesis and production of biochemical compounds necessary for digestion. It is involved with almost all the biochemical pathways environmental toxins, drugs and alcohol which can eventually lead to various liver disorders, generally presenting as a distinct pattern of diseases such as hepatocellular, cholestatic (obstructive), or mixed type of liver disorders. Paracetamol is commonly used as antipyretic, anti inflammatory and analgesic drug, worldwide. And we all know that it can induce hepatotoxicity when over dosing occur. So, it becomes one of the challenges for the researchers to develop a potent drug which provides protection against the hepatotoxicity induces by over dose of paracetamol. So, in this situation albino rat is become the first choice of the researchers to induce hepatotoxicity by the paracetamol overdose. And several times they were reported that some kinds of herbal preparations have shown the hepatoprotective effect against paracetamol's over dose in the albino rats. So now it is very important to see the results of this research, Is paracetamol induce the hepatotoxicity in Wistar rats same as human or not?

Hepatocytes are responsible for making many of the proteins (protein synthesis) in the body that are required for different functions, including blood clotting factors, and albumin, required to maintain fluid within the circulation system. The liver is also responsible for manufacturing cholesterol and triglycerides. Carbohydrates are also produced in the liver and the organ is responsible for turning glucose into glycogen that can be stored both in the liver and

in the muscle cells. The liver also makes bile that helps with food digestion.

The liver plays an important role in detoxifying the body by converting ammonia, a byproduct of metabolism in the body, into urea that is excreted in the urine by the kidneys. The liver also breaks down medications and drugs, including alcohol, and is responsible for breaking down insulin and other hormones in the body[https://www.medicineNet.com/Liver_anatomy_and_function/article.htm].

2. Liver Disease Facts

The liver plays an important role in many bodily functions from protein production and blood clotting to cholesterol, glucose (sugar), and iron metabolism. A variety of illnesses can affect the liver, for example: -

- certain drugs like excessive amounts of acetaminophen, and acetaminophen combination medications like Vicodin, Norco, and statins,
- cirrhosis,
- alcohol abuse,
- hepatitis A, B, C, D, and E,
- Epstein Barr virus (infectious mononucleosis),
- non-alcoholic fatty liver disease and
- iron overload (hemochromatosis) etc [https://www.medicinenet.com/liver_disease/article.htm].

3. Liver Disease Symptoms and Signs

Acute and chronic liver diseases can interfere with the functions of the liver and thereby cause symptoms. However, the liver has a hefty reserve capacity. In other words, it usually takes substantial damage to the liver before a disease interferes with the functions of the liver and causes symptoms. Examples of such symptoms are:

Jaundice (yellow skin) that can occur when the liver is unable to properly metabolize or secrete the yellow pigment bilirubin in bile.

Bleeding or easy bruising that can occur when the liver is unable to make enough of the normal blood clotting proteins.

Swelling of the legs with fluid (edema) that can occur when the liver is unable to make enough albumin and the serum albumin gets too low.

Fatigue that is of unknown cause but may be related to some impaired metabolic function of the liver. [https://www.emedicinehealth.com/liver/article_em.htm].

4. Hepatotoxicity induction by Paracetamol's over dose in Human

In cases of paracetamol overdose, the sulfate and glucuronide pathways become saturated, and more paracetamol is shunted to the cytochrome P450 system to produce NAPQI/NAPQBI. As a result, hepatocellular supplies of glutathione become depleted, as the demand for glutathione is higher than its regeneration [1]. NAPQI therefore remains in its toxic form in the liver and reacts with cellular membrane molecules, resulting in widespread hepatocyte damage and death, leading to acute liver necrosis [2]. In animal studies, the liver's stores of glutathione must be depleted to less than 70% of normal levels before liver toxicity occurs [3].

5. MATERIAL

Male albino rats (wt. 150±10gm) were purchased from DRDE, Gwalior (MP) (Ethical clearance no. BU/Pharm/IAEC/A/17/08) and animals were kept under the standard conditions (Temp. 20-25^{o}C, 12 hours light and 12 hours dark. Animals were fed with pelleted diet (Ashirwad animal diet, Mohali) and water ad libitum.

Paracetamol was purchased of GlaxoSmithKline Pharmaceuticals Ltd. Company from medical shop.

6. Method:

24 Wistar male albino rats (wt. 150 + 10 gm) were randomly divided into 4 groups, each group contains 6 rats. The groups are as follows:

Group no.1: These rats were treated with 1500 mg/kg/day paracetamol (Orally) for 3 consecutive days.

Group no.2: These rats were treated with 2500 mg/kg/day paracetamol (Orally) for 3 consecutive days.

Group no.3: These rats were treated with 3500 mg/kg/day paracetamol (Orally) for 3 consecutive days.

Group no.4: These rats were on Vehicle (Distilled water) only (Normal control).

Autopsy was done of all the rats on 4th day or 24 hours after the last dose of paracetamol. Blood sample was collected for the biochemical investigations and Liver sample were collected for the histopathological examination.

6.1. Biochemical Test Procedure:

6.1.A. Billirubin Total, Direct and Indirect [4, 5, 6]:

Diazo method of Pearlman and Lee (Transasia BIO-Medical Ltd.)

Principle: Bilirubin reacts with diazotized suphanilic acid in acidic medium to form pink colored azobilirubin with absorbance directly proportional to bilirubin concentration. Direct Bilirubin being water

soluble directly react in acidic medium. However indirect or conjugated Bilirubin is solubilised using a surfactant and then it reacts similar to direct bilirubin.

Reagents compositions:

Reagent 1: (Table no.1)

Table.1

Surfactant	1.00%
HCl	100 m mol/L
Sulphanilic acid	5 m mol/L

Reagent 2: (Table no.2)

Table.2

Sulphanilic acid	10 m mol/L
HCl	100 m mol/L

Reagent 3: (Table no.3)

Table.3

Sodium Nitrite	144 m mol/L

Reagent preparation for test:

For Total Bilirubin: 10 ml reagent 1 + 200 µ Litre reagent 3.

For Direct Bilirubin: 10 ml reagent 2 + 100 µ Litre reagent 3.

Indirect Bilirubin = Total Bilirubin – Direct Bilirubin.

Method:

Total Bilirubin: 1 ml reagent of total Bilirubin + 50 µ liter sample (Serum)

Direct Bilirubin: 1 ml reagent of direct Bilirubin + 50 µ liter sample (Serum)

- Mix well, Incubate for 5 minutes at 37oC for both total and direct Bilirubin. Read at 546/630 nm against reagent blank.

6.1.B. Serum Glutamate Piruvate Transaminase (SGPT)/ ALT (Alanine Transaminase) [7,8,9,10]:

Modified UV (IFCC), Kinetic Assay: (ARKRAY Healthcare Pvt. Ltd.)

Principle: Alanine Transaminase catalyse the transamination of L-Alanine and α-ketoglutarate and L-glutamine. In subsequent reaction, Lactate dehydrogenase (LD) reduces piruvate to lactate with simuntaneneous oxidation of nicotinamide adenine [reduced] (NADH) to nicotinamide adenine dinucleotide (NAD). The rate of oxidation of NADH is measured kinetically by monitoring the decrease in absorbance at 340 nm.

Reagent Composition: (Table.4)

Table.4

Reagent	Composition	Concentration
Reagent1.	Tris Buffer (pH 7.5)	100 m mol/L
	L-Alanine	500 m mol/L
	Lactate Dehydrogenase	> 1200 U/L
	Stabiliser	Quantity Sufficient
Reagent 2.	A-ketoglutarate	15 m mol/L
	NADH	0.18 m mol/L
	Stabiliser	Quantity Sufficient

Test Procedure:

Reagent 1 (0.8 ml) + Reagent 2 (0.2 ml) + 100 µ lit Serum (sample)

- Mix well and aspirate immediately for measurement
- Read absorbance after 60 seconds. Repeat reading after every 30 seconds i.e. upto 120 seconds at 340 nm wavelength.
- Determine the mean absorbance change / minute.

Calculation:

ALT (IU/L) = Change in absorbance × Kinetic factor (K)

Where, K=1768.

6.1.C. Serum Glutamate Oxaloacetate Transaminase (SGOT)/ Aspartate Transaminase (AST)[11, 12, 13]:

Modified UV (IFCC). Kinetic assay (ARKRAY Healthcare Pvt. Ltd.)

Principle: AST catalyzes the transamination of L-aspartate and α-ketoglutarate to l-glutamate and Oxaloacetate. In subsequent reaction, Malate dehydrogenase (MDH) reduces oxaloacetate to malate with simultaneous oxidation of nicotinamide adenine dinucleotide [reduced] (NADH) to nicotinamide adenine dinucleotide (NAD).The rate of oxidation of NADH is measured kinetically by monitoring the decrease in absorbance at 340 nm and directly proportional to the AST activity in the sample. Lactate dehydrogenase (LD) is added enzyme system to prevent endogenous piruvate interference, which is normally present in serum.

Reagent Composition: (Table.5)

Table.5

Reagent	Composition	Concentration
Reagent1.	Tris Buffer (pH 7.8)	80 m mol/L
	L-Asparted	240 m mol/L
	MDH	≥ 600 U/L
	LD	≥600 U/L
	Stabiliser	Quantity Sufficient
Reagent 2.	α-ketoglutarate	12 m mol/L
	NADH	0.18 m mol/L
	Stabiliser	Quantity Sufficient

Test Procedure:
Reagent 1 (0.8 ml) + Reagent 2 (0.2 ml) + 100 µ lit Serum (sample)

- Mix well and aspirate immediately for measurement
- Read absorbance after 60 seconds. Repeat reading after every 30 seconds i.e. upto 120 seconds at 340 nm wavelength.
- Determine the mean absorbance change / minute.

Calculation:
AST (IU/L) = Change in absorbance × Kinetic factor (K)
Where, K=1768.

6.1.D. Alkaline Phosphatase (ALP)[14, 15, 16, 17]: pNPP-AMP (IFCC), Kinetic Assay: (ARKRAY Healthcare Pvt.Ltd.)

Principle: At pH 10.5 ALP catalyses the hydrolysis of colourless p-nitrophenyl Phosphate (pNPP) to yellow coloured p-Nitrophenol and phosphate.Change in absorbance due to yellow colour formation is measured kinetically at 405 nm and is proportional to ALP activity in sample.

Reagent Composition: (Table.6)

Table.6

Reagent	Composition	Concentration
Reagent1.	AMP	300 mM
AMP (2-amino-2-methyl propanol) buffer	Magnesium acetate	2 mM
	Zinc sulphate	0.8 mM
	Chelator	Quantity Sufficient
Reagent 2.	pNPP	10 mM
pNPP substrate	Stabiliser	Quantity Sufficient

Test Procedure:
Prepare "working reagent" by reconstituting one vial of regent 2 (pNPP substrate) with 1.2 ml Reagent 1 (AMP Buffer) by dissolving properly by gentle swirling.

- 1000 µL Prepared reagent + 20 µL Serum (sample)
- Mix well and aspirate for measurement.
- Blank the analyser with purified water.
- Read absorbance after 30 seconds. Repeat reading after every 30 seconds i.e. upto 120 seconds at 405 nm wavelength.
- Determine the mean absorbance change/minute.

Calculation:
ALP activity (IU/L) =
Change in absorbance/minute × K
Where, K is Kinetic factor = 2712.

6.1.E. Serum Total Protein [18,19]:
Biuret Method (Autozyme kit of Accurex)

Principle: Protein react with cupric ions under alkaline pH to produce colour complex. This colour complex absorb light at 546 nm (530-570 nm). The intensity of colour is directly proportional to the protein concentration in specimen.

Reagent Composition: (Table.7)

Table.7

Composition	Concentration
Cupric sulphate	07 m mol/L
Potassium Iodide	06 m mol/L
Tartarate	20 m mol/L
Surfactant	0.05% w/v
Stabiliser	

Test Procedure:
01 ml reagent + 10 µl Serum (Sample)

- Mix well and incubate for 5 minutes at 37°C
- Measured the absorbance of specimen and standard against the blank.

Calculation:

$$\text{Total Protein in gm\%} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 6$$

6.1.F. Serum Albumin [20, 21]:

BCG Method (Autozyme kit of Accurex)

Principle: Serum albumin in the presence of Bromocresol-green under acidic condition form a green coloured complex. The absorbance of this complex is proportional to the albumin concentration in sample.

Reagent composition: (Table.8)

Table.8

Composition	Concentration
Succinate buffer (pH 3.6)	100 m mol/L
Bromocresol-green	0.15 m mol/L
Stabiliser	

Test Procedure:
01 ml reagent + 10 µl Serum (Sample)

- Mix well and incubate for 01 minutes at 25-30°C
- Measured the absorbance of specimen and standard against the blank at 600 nm.

Calculation:

$$\textbf{Serum Albumin in gm\%} = \frac{\textbf{Absorbance of sample}}{\textbf{Absorbance of standard}} \times \textbf{5}$$

7. Biochemical analysis table and graphs

Effect of paracetamol on various biochemical parameters in Wistar rats at different dose level. (Table.9)

Table.9

Test	1500 mg/kg	2500 mg/kg	3500 mg/kg	Normal Control
Billirubin Total	0.60 ± 0.04	0.62 ± 0.03	0.62 ± 0.06	0.56 ± 0.03
Direct	0.21 ± 0.03	0.25 ± 0.03	0.25 ± 0.04	0.24 ± 0.04
Indirect	0.39 ± 0.04	0.37 ± 0.04	0.37 ± 0.06	0.32 ± 0.02
Serum Protein Total	5.98 ± 0.13	6.09 ± 0.24	6.03 ± 0.11	6.21 ± 0.15
Serum Albumin	2.85 ± 0.36	3.01 ± 0.23	3.00 ± 0.15	2.96 ± 0.16
SGPT	49.46 ± 2.13	51.1 ± 2.34	49.56 ± 3.76	51.0 ± 2.07
SGOT	129.4 ± 10.6	128.9 ± 7.12	124.9 ± 11.8	129.0 ± 4.85
ALP	707.53± 9.82	715.8 ± 17.2	716.7 ± 15.9	724.6 ± 9.54

Values are expressed in mean ± SD, where N=6 and statistical analysis P vs respective control ≥ 0.05.

Figure 1: Comparisons of Billirubin Total, Direct & Indirect.

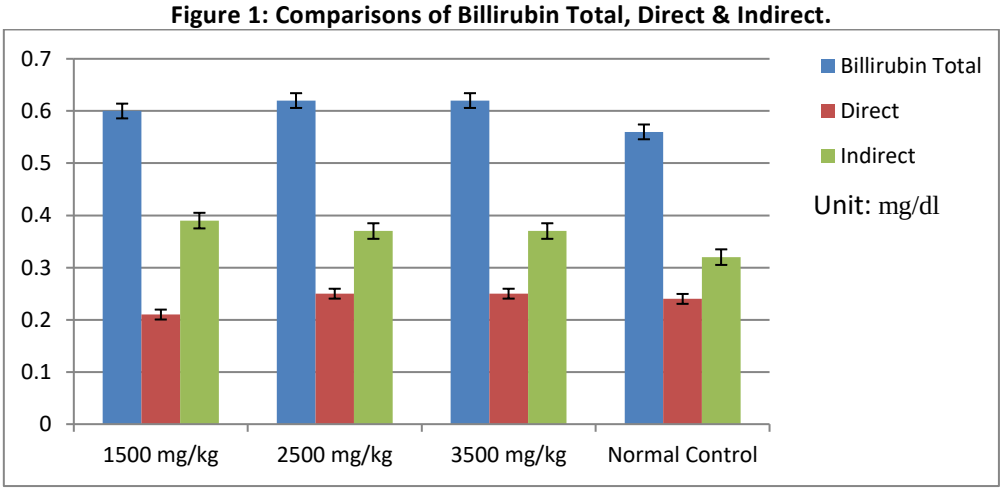


Figure.2 (Comparisons of Total Protein and Albumin.)

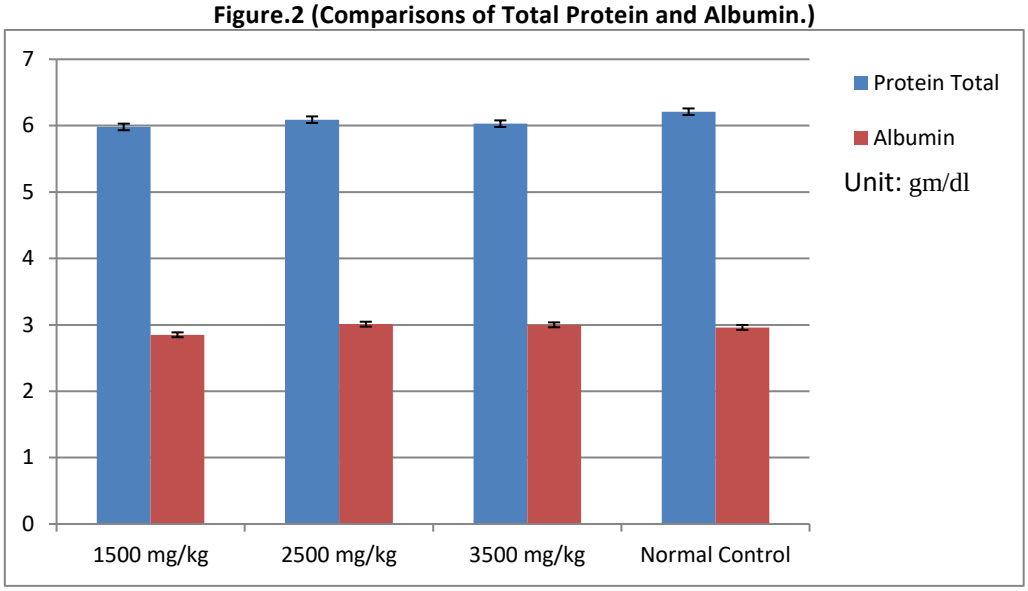
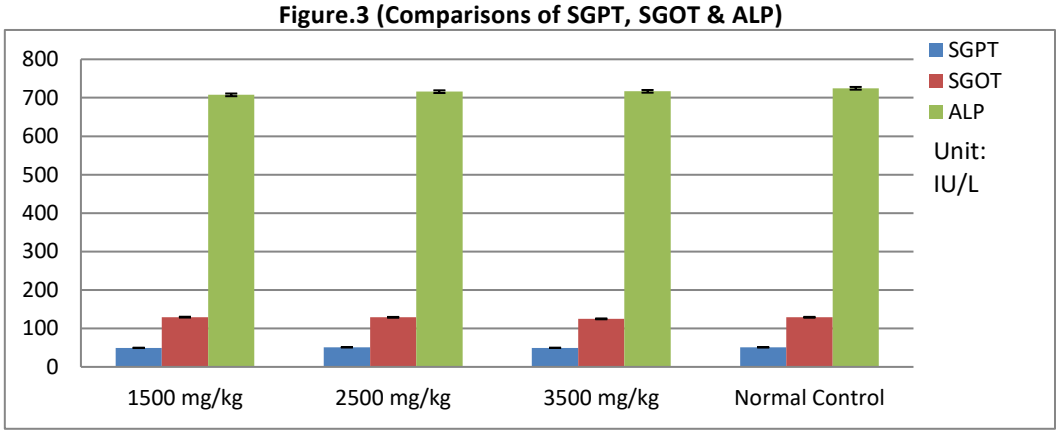


Figure.3 (Comparisons of SGPT, SGOT & ALP)



8. Histopathology of liver

All histopathological images of liver are at 400X Magnification.

Figure 4: (Normal Control) Figure 5: (1500 mg/kg)

Figure 6: (2500 mg/kg) Figure 7: (3500 mg/kg)

H= Hepatocytes, S= Sinosoids, CV= Central vein.

Figure.4 (Normal Control)

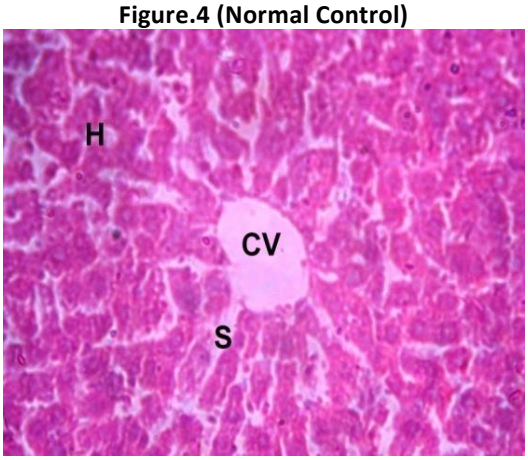


Figure.5 (1500 mg/kg)

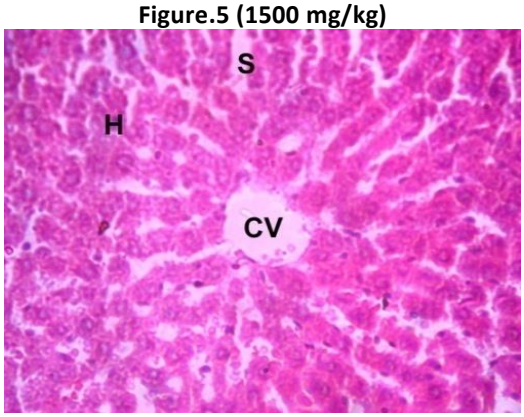


Figure.6 (2500 mg/kg)

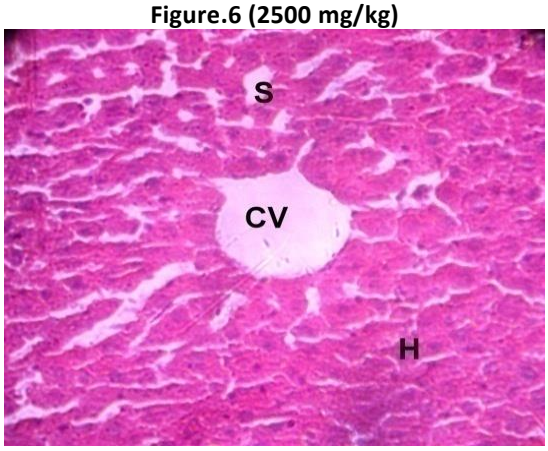
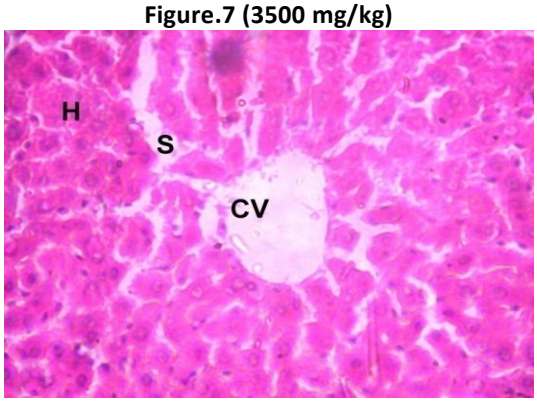


Figure.7 (3500 mg/kg)



Photomicrograph of figure-4 is showing normal structure of liver with central vein surrounded by hepatocytes and sinosoids. Figure-5 is showing the structure of liver after 3 days of treatment with paracetamol at a dose of 1500 mg/kg, but no significant change was observed. Figure-6 is showing the structure of liver after administration of paracetamol at the dose of 2500 mg/kg and figure-7 is showing the structure after administration of the dose of 3500 mg/kg. minor edema in central vein with very slight disturbance in hepatocytes was observed which is not very significant.

9. STATISTICAL ANALYSIS:

Results were expressed as Mean ± Standard Deviation for 6 animals in each group. Hypothesis testing method included one-way analysis of various (ANOVA) by using Dunnette test. P values were more than 0.05 with respect to normal, which considered statistically not significant. All the statistics were carried out in GraphPad InStat Software Inc., v. 3.06, San Digeo, USA.

10. DISCUSSION

After the Overdose of Paracetamol, no hepatotoxicity was observed in Wistar rats, on the

other hand some rats were found under the gastric problems like diarrheal condition at the initial time but at the later stage they undergo the problems of obstruction due to the paracetamol accumulation in the gastrointestinal tract.

11. RESULTS

No hepatotoxicity was induced at the different level doses of paracetamol, as the values of SGPT, SGOT and ALP and other biochemical parameters were not changed significantly with respect to the control. (P vs control $\geq$ 0.05). Histopathogical examination of liver is also supported the biochemical examination.

12. CONCLUSION

The purpose of this research was to make aware about the selection of paracetamol to induce hepatotoxicity in Wistar rats for research work. After performing all the experiments, we reached at the conclusion that the Wistar rats develops resistance against paracetamol over dose. And there is no effect of paracetamol's over dose on Wistar rat liver. Its means that, Paracetamol is not suitable for the research work in which researchers plans to induce hepatotoxicity by paracetamol's over dose in Wistar rats.

13. ABBREVIATIONS

ALP (Alkaline Phosphatase), NAPBQI (*N*-acetyl-*p*-benzoquinone imine), SGOT (Serum Glutamic Oxaloacetic Transaminase), SGPT (Serum Glutamic Pyruvic Transaminase) etc.

14. ACKNOWLEDGEMENTS

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