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Racemethionine Hepatoprotective Activity against Rifampicin Induced Hepatotoxicity in Albino Rats

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ABSTRACT

The current investigation was aimed to study the minimization of the effect of Rifampicin induced hepatotoxicity by the use of Racemethionine molecule which is an anti dote for paracetamol over dosage in humans. Racemethionine, a drug used for the treatment of toxic effects of paracetamol, was studied for its hepatoprotective activity against anti tubercular drug (Rifampicin) induced hepatotoxicity in wistar albino rats. The serum samples collected were estimated for various biochemical parameters. Racemethionine showed normalization of bio chemical parameters like serum glutamyl oxaloacetate transaminase (ASAT), serum glutamyl pyruvate transaminase (ALAT), alkaline phosphatase (ALP). Total cholesterol (TC) levels were found to be slightly raised in the Racemethionine treated group as compared to the anti tubercular drug treated group. The results indicated that Racemethionine showed to be a more potent hepatoprotective drug against Rifampicin induced hepatic injury in experimental animals. Hence racemethionine could be used for hepatoprotective study models in albino rats indicated by its reduction ability of ASAT, ALAT, ALP and TC in serum.

Keywords: Racemethionine, Rifampicin, Hepatoprotective activity, Total cholesterol.

INTRODUCTION

The liver is the most important organ in which drugs are structurally altered. Some of the resulting metabolites may be biologically inactive, some active and some toxic. The liver is exposed to drugs in higher concentrations than are most organs because most are administrated through the liver to reach the systemic circulation. Because of this liver is vulnerable target for injury from chemicals and drugs, and disorder hepatic function is an important cause of abnormal drug handling and response. The liver is the largest organ in the body which is situated in the upper quadrant of the abdomen. It is respected by the surgeons for its inherent myths as well as because it is one of the most vesicular organ in the abdomen. It is an organ that seldom forgives its violation.

The blood supply to the liver is peculiar in that it is supplied by portal vein, draining the intestines as well as by the hepatic artery, which predominantly nourishes the bile veins. The blood draining away from the liver reaches the heart by means of hepatic multiple veins through the inferior vena cava. The anatomical structure of liver was showed in figure 1.

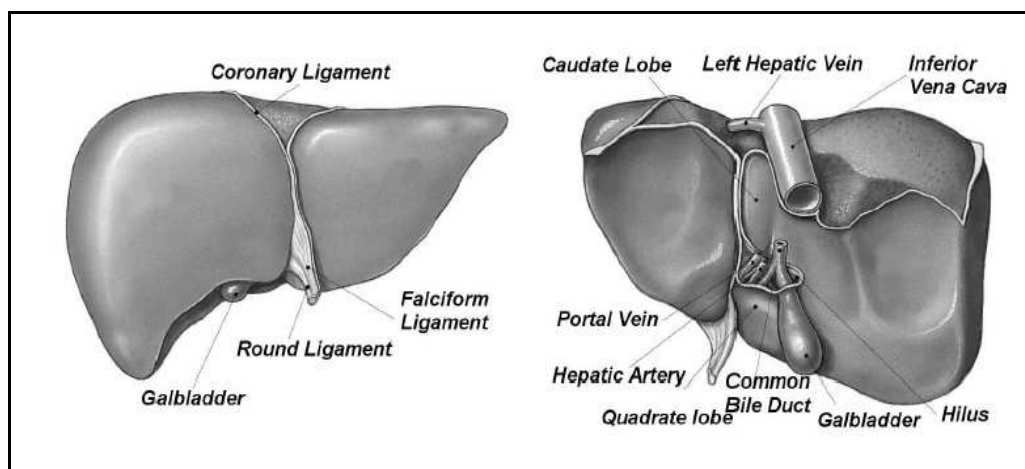


Figure 1: Various anatomical parts of Liver and their organisation

Liver cells called hepatocytes, every second perform several complex biochemical and a number of important functions, including bile production, extraction of bilirubin, cholesterol, hormones and drugs. It is also responsible for metabolism of fats, proteins, carbohydrates, enzymes activation, and storage of glycogen, vitamins, minerals and synthesis of plasma proteins such as albumin, globulin and clotting factors. Toxic liver injury produced by drugs and chemicals are similar to natural liver disease. Continuous use of agents like paracetamol, tetracycline, anti tubercular drugs, oral contraceptives of hormonal origin, chemicals used as food preservatives and agrochemicals are threatening the integrity of liver. Further addiction of alcohol and other drugs aggravated the problem and malnutrition also an important cause of liver damage.

About 20,000 deaths found every year due to liver disorders. Hepatocellular carcinoma is one of the 10 most common tumors in the world with over 2,50,000 new cases each year. In India, about 40 polyherbal commercial formulations are being used for hepatoprotection. It has been reported that 160 phytoconstituents from 101 plants have hepatoprotective activity [1]. Liver protective herbals contain a variety chemical constituents like phenols, coumarins, lignans, essential oils, monoterpenes, carotinoids, glycosides, flavonoids, organic acids, lipids, alkaloids and xanthenes. Plant extracts of many crude drugs are also used for the treatment of liver disorders. Extracts of 25 different plants have been reported to cure liver disorders [2].

Drug Metabolism in Liver:

Biotransformation is the biochemical modification of pharmaceutical substances by living organisms, usually through specialized enzymatic systems, Liver is central to the metabolism of virtually every foreign substance. Most drugs and xenobiotics are lipophilic (lipid soluble), enabling them to cross the membranes of intestinal cells. (more a substance is lipophilic more it is diffused) Drugs are rendered more hydrophilic by biochemical processes in the hepatocyte, yielding water-soluble products that are excreted in urine or bile. This hepatic biotransformation involves oxidative pathways, primarily by way of the cytochrome P-450

enzyme system. After further metabolic steps, which usually include conjugation to a glucuronide or a sulfate or glutathione, the hydrophilic product is exported into plasma or bile by transport proteins located on the hepatocyte membrane, and it is subsequently excreted by the kidney or the gastrointestinal tract.

Drug induced Hepatotoxicity:

Drugs are an important cause of liver injury. More than 900 drugs, toxins, and herbs have been reported to cause liver injury, and drugs account for 20-40% of all instances of fulminant hepatic failure. Approximately 75% of the idiosyncratic drug reactions result in liver transplantation or death. Drug-induced hepatic injury is the most common reason cited for withdrawal of an approved drug. Physicians must be vigilant in identifying drug-related liver injury because early detection can decrease the severity of hepatotoxicity if the drug is discontinued. The manifestations of drug-induced hepatotoxicity are highly variable, ranging from asymptomatic elevation of liver enzymes to fulminant hepatic failure. Knowledge of the commonly implicated agents and a high index of suspicion are essential in diagnosis [3].

Risk factors for drug-induced liver injury

Race: Some drugs appear to have different toxicities based on race. For example, blacks and Hispanics may be more susceptible to isoniazid (INH) toxicity. The rate of metabolism is under the control of P-450 enzymes and can vary from individual to individual. **Age:** Apart from accidental exposure, hepatic drug reactions are rare in children. Elderly persons are at increased risk of hepatic injury because of decreased clearance, drug-to-drug interactions, reduced hepatic blood flow, variation in drug binding, and lower hepatic volume. In addition, poor diet, infections, and multiple hospitalizations are important reasons for drug-induced hepatotoxicity. **Sex:** Although the reasons are unknown, hepatic drug reactions are more common in females. **Alcohol ingestion:** Alcoholic persons are susceptible to drug toxicity because alcohol induces liver injury and cirrhotic changes that alter drug metabolism. Alcohol causes depletion of glutathione (hepatoprotective) stores that make the person more susceptible to toxicity by drugs. **Liver disease:** In general, patients with chronic liver disease are not uniformly at increased risk of hepatic injury. Although the total cytochrome P-450 is reduced, some may be affected more than others. The modification of doses in persons with liver disease should be based on the knowledge of the specific enzyme involved in the metabolism. Patients with HIV infection who are co-infected with hepatitis B or C virus are at increased risk for hepatotoxic effects when treated with antiretroviral therapy. Similarly, patients with cirrhosis are at increased risk of decompensation by toxic drugs. **Genetic factors:** A unique gene encodes each P-450 protein. Genetic differences in the P-450 enzymes can result in abnormal reactions to drugs, including idiosyncratic reactions. Debrisoquine is an antiarrhythmic drug that undergoes poor metabolism because of abnormal expression of P-450-II-D6. This can be identified by polymerase chain reaction amplification of mutant genes. This has led to the possibility of future detection of persons who can have abnormal reactions to a drug.

Host factors that may enhance susceptibility to drugs, possibly inducing liver disease:

- Female - Halothane, nitrofurantoin, sulindac
- Male - Amoxicillin-clavulanic acid (Augmentin)
- Old age - Acetaminophen, halothane, INH, amoxicillin-clavulanic acid
- Young age - Salicylates, valproic acid
- Fasting or malnutrition - Acetaminophen
- Large body mass index/obesity - Halothane

Diabetes mellitus - Methotrexate, niacin
Renal failure - Tetracycline, allopurinol
AIDS - Dapsone, trimethoprim - sulfamethoxazole
Hepatitis C - Ibuprofen, ritonavir, flutamide
Pre existing liver disease - Niacin, tetracycline, methotrexate

Pathophysiology and mechanisms of drug-induced liver injury

Disruption of the hepatocyte: Covalent binding of the drug to intracellular proteins can cause a decrease in ATP levels, leading to actin disruption. Disassembly of actin fibrils at the surface of the hepatocyte causes blebs and rupture of the membrane. Disruption of the transport proteins: Drugs that affect transport proteins at the canalicular membrane can interrupt bile flow. Loss of villous processes and interruption of transport pumps such as multidrug resistance-associated protein 3 prevent the excretion of bilirubin, causing cholestasis. Cytolytic T-cell activation: Covalent binding of a drug to the P-450 enzyme acts as an immunogen, activating T cells and cytokines and stimulating a multifaceted immune response. Apoptosis of hepatocytes: Activation of the apoptotic pathways by the tumor necrosis factor- α receptor of F as may trigger the cascade of intercellular caspases, which results in programmed cell death. Mitochondrial disruption: Certain drugs inhibit mitochondrial function by a dual effect on both beta-oxidation energy productions by inhibiting the synthesis of nicotinamide adenine dinucleotide and flavin adenine dinucleotide, resulting in decreased ATP production. Bile duct injury: Toxic metabolites excreted in bile may cause injury to the bile duct epithelium.

Drug toxicity mechanisms:

The classic division of drug reactions is into at least 2 major groups, 1) drugs that directly affect the liver and 2) drugs that mediate an immune response. Intrinsic or predictable drug reactions: Drugs that fall into this category cause reproducible injuries in animals, and the injury is dose related. The injury can be due to the drug itself or to a metabolite. Acetaminophen is a classic example of a known intrinsic or predictable hepatotoxin at supertherapeutic doses. Another classic example is carbon tetrachloride. Idiosyncratic drug reactions: Idiosyncratic drug reactions can be subdivided into those that are classified as hypersensitivity or immunoallergic and those that are metabolic-idiosyncratic. Hypersensitivity: Phenytoin is a classic, if not common, cause of hypersensitivity reactions. The response is characterized by fever, rash, and eosinophilia and is an immune-related response with a typical short latency period of 1-4 weeks.

Hepatic function tests and their interpretations are as follows:

Bilirubin (total) - To diagnose jaundice and assess severity
Bilirubin (unconjugated) - To assess for hemolysis
Alkaline phosphatase - To diagnose cholestasis and infiltrative disease
AST/serum glutamic oxaloacetic transaminase (SGOT) - To diagnose hepatocellular disease and assess progression of disease
ALT/serum glutamate pyruvate transaminase (SGPT) - ALT relatively lower than AST in persons with alcoholism
Albumin - To assess severity of liver injury (HIV infection and malnutrition may confound this)
Gamma globulin - Large elevations suggestive of autoimmune hepatitis, other typical increase observed in persons with cirrhosis
Prothrombin time after vitamin K - To assess severity of liver disease

Antimitochondrial antibody - To diagnose primary biliary cirrhosis
ASMA - To diagnose primary sclerosing cholangitis

The risk of Hepatotoxicity is apparently greater in persons 70 years old or older occur in liver, by only 2% of all adverse reactions in children of 0-14 years old occur in the liver. Hence there is an increase in interest and need for exploring research frontiers in the area of drug induced Hepatotoxicity, their mechanisms involved and pathways to bring down the risk factor of liver exposure to the toxic effects of drugs. Hence the present investigation is conducted to study the Racemethionine Hepatoprotective activity against Rifampicin induced Hepatotoxicity in albino rats.

MATERIALS AND METHODS

Materials used:

Racemethionine is used as a drug for evaluating its hepato protective activity against Rifampicin induced hepatotoxicity. This drug was obtained as gift sample from Lincon Pharmaceuticals, Ahmedabad. Various kits used for several biochemical parameters estimation were procured from, i.e. ASAT Kit – Merck, Mumbai. ALAT Kit- Merck, Mumbai. Alkaline phosphatase (ALP) kit - Merck, Mumbai. Cholesterol CHOD-PAP Kit – Fortess Diagnostics, Mumbai. Equipments list. Semi auto analyzer – Maysun Technology, Mumbai. Cooling centrifuge of Remi motors, Mumbai. Albino rats were obtained from - sri animals and breeders, madhavaram.

Selection of animals:

Wistar albino rats of either sex of weight ranges between 180-200 g were procured from sri animals and breeders, madhavaram were used for the study. The Animals were housed and acclimatized under standard laboratory conditions in propylene cages in the approved departmental animal house at 30±20C, Light and dark cycles of 10hr and 14hr respectively throughout the experiment. Animals were fed with commercial pelleted rat chow (Ile and Co, Chennai) and water ad libitum

The animals were segregated in to four groups of six animals each and the drug was given for 2 days as follows.

Group I - Control group (Tween 80 1ml/kg b.w.p.o.)

Group II - In toxicated group (Rifampicin 1gm/kg b.w.p.o.)

Group III - Drug treated group (Rifampicin and Racemethionine 1gm/kg and 200 mg/75kg b.w.p.o. respectively)

Group IV - In toxicated Control group (Rifampicin and Silymarin 1gm/kg and 20 mg/kg b.w.p.o. respectively)

Ethical Clearance

The institutional Animal Ethical Committee approval was obtained for carrying out the experimental protocol with Registered no (IAEC 25/2007).

Serum collection [4]

1. The respective drugs were administered to the animal groups for a period of 2 days
2. Blood was collected from all the groups from retro orbital plexus
3. The blood was allowed to clot at room temperature and serum was separated by centrifugation at 2500 rpm for 10 min using cooling centrifuge (Remi motors, Ltd.)

4. The serum was analyzed on semi-auto analyzer for measuring the following biochemical markers, which are: a. Aspartate aminotransferase (ASAT), b. Alanine aminotransferase (ALAT) c. Alkaline phosphatase (ALP) and d. Total cholesterol (TC)

Bio chemical Parameters estimation:

Aspartate aminotransferase (ASAT) estimation [5]:

Principle:

L-Aspartate + 2-oxoglutarate $\xrightarrow{\text{ASAT}}$ Glutamate + Oxalactate

Oxalactate + NADH + H⁺ $\xrightarrow{\text{MDH}}$ L-Malate + NAD⁺

The rate of NADH consumption is measured photometrically and is directly proportional to the ASAT concentration in the sample

Procedure:

1. 100 µl of serum was taken in a clean eppendroff tube.
2. 1000 µl of reagent – 1 (TRIS, L-Aspartate, Malate dehydrogenase (MDH) and Lactate dehydrogenase (LDH) was added to the tube.
3. The tube was mixed well and incubated for 5 min at 37°C
4. 250 µl of reagent – 2 (2-Oxoglutarate and NADH) was added, mixed and incubated for 1 min at 37°C.
5. After 1 min, decrease in absorbance was read every minute
6. Activity of the enzyme was calculated by using the following formula

ASAT activity (U/I) = $\Delta A/\text{min} \times \text{factor}$

Alanine aminotransferase (ALAT) estimation [5]:

L-Alanine + 2-oxoglutarate $\xrightarrow{\text{ALAT}}$ L-Glutamate + Pyruvate

Pyruvate + NADH + H⁺ $\xrightarrow{\text{LDH}}$ L-Lactate + NAD⁺

The rate of NADH consumption is measured photometrically and is indirectly proportional to the ALAT concentration in the sample

Procedure:

1. 100 µl of serum was taken in a clean eppendroff tube.
2. 1000 µl of reagent – 1 (TRIS, L-Alanine and Lactate dehydrogenase (LDH) was added to the tube.
3. The tube was mixed well and incubated for 5 min at 37°C
4. 250 µl of reagent – 2 (2-Oxoglutarate and NADH) was added, mixed and incubated for 1 min at 37°C.
5. After 1 min, decrease in absorbance was read every minute for 3 min at 334 nm, 340 nm and 365 nm.
6. Activity of the enzyme was calculated by using the following formula

ALAT activity (U/I) = $\Delta A/\text{min} \times \text{factor}$

Alkaline phosphatase (ALP) estimation [6]:**Principle:**

p-Nitrophenylphosphate + H₂O $\xrightarrow{\text{ASAT}}$ Phosphate + p-Nitrophenol

The increase in absorbance due to formation of 4-nitrophenolate is measured spectrophotometrically and is directly proportional to ALP activity in sample.

Procedure:

1. 20 µl of serum was taken in a clean eppendroff tube.
2. 1000 µl of reagent – 1 (Diethanolamine and Magnesium chloride) was added to the tube.
3. The tube was mixed well and incubated for 5 min at 37°C
4. 250 µl of reagent – 2 (p-Nitrophenylphosphate) was added, mixed and incubated for 1 min at 37°C.
5. After 1 min, decrease in absorbance was read every minute for 3 min at 405 nm.
6. Activity of the enzyme was calculated by using the following formula

ALP activity (U/I) = $\Delta A/\text{min} \times \text{factor}$

Total Cholesterol estimation [7]:**Principle:**

Cholesterol is present in serum as Cholesterol esters and free Cholesterol. The Cholesterol esters present in serum are hydrolyzed by Cholesterol esterase and the Cholesterol is then measured by oxidizing with Cholesterol oxidase to form hydrogen peroxide. The hydrogen peroxide in turn reacts with phenol and 4-aminoantipyrine present to form the red quinonimine dye. The intensity of dye formed is directly proportional to the level of Cholesterol present in the sample.

Procedure:

1. 10 µl of serum was mixed with 1000 µl reagent (Pipes buffer, Cholesterol oxidase, Cholesterol esterase, 4- aminoantipyrine, Peroxidase and Phenol) in a test tube.
2. For the blank 10 µl of DDH₂O was mixed with 1000 µl of same reagent in another test tube.
3. The tubes were then incubated for 10 min at 20-25°C or for 5 min at 37°C
4. Absorbance was measured at 500 nm against reagent blank. The end point is stable for 60 min at 37°C.
5. Cholesterol level was calculated using the following formula Absorbance of sample

Total Cholesterol = Absorbance of standard x Std. Concentration

RESULTS AND DISCUSSION

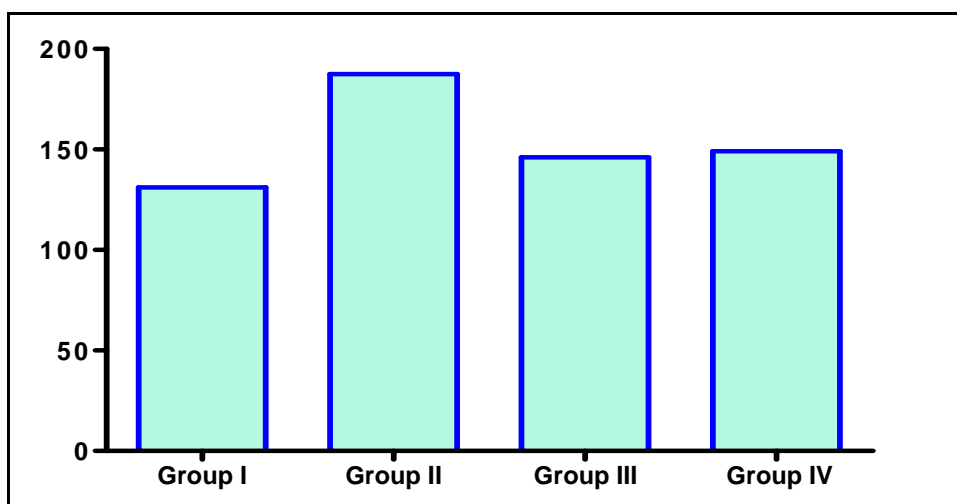
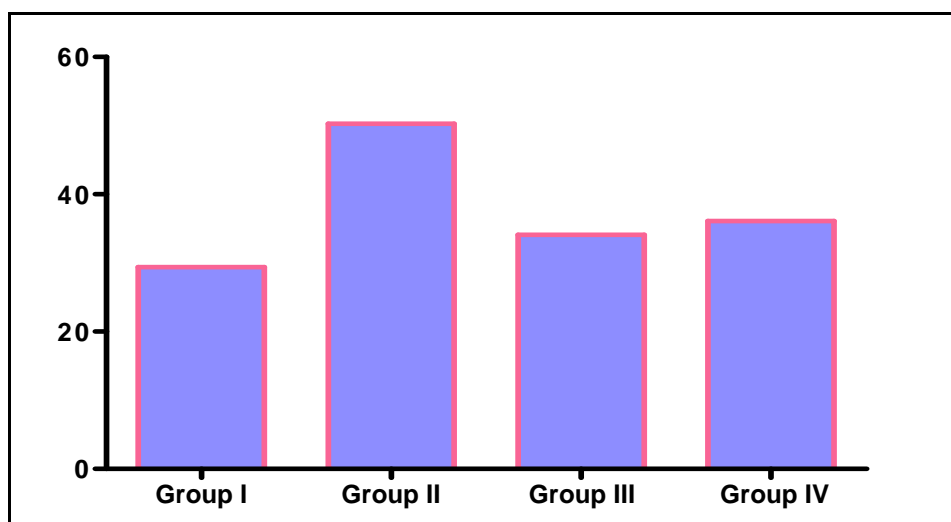
Liver plays a major role in detoxification and excretion of many endogenous and exogenous compounds, any injury to it or any impairment to its functions may leads to many complications on one's health. Management of liver diseases is still a challenge to the modern medicine which has a little to offer for alleviation of hepatic diseases or ailments [8].

Bio chemical parameters: Albino rats were treated with drug and anti tubercular drug (Rifampicin) which leads to the toxicity of the liver cells. The drug regimen was given for 2 consecutive days and the results were tabulated in below table ie. Table 1.

Table 1: Hepatoprotective effect of Racemethionine on Rifampicin induced hepatotoxicity in Albino rats

| S.no. | Parameter | Animal groups | | | |
|-------|-----------------------------------|--------------------------------|--------------------------------|------------------------------------|--------------------------------------------|
| | | Group I | Group II | Group III | Group IV |
| | | Control group (Tween 80) (U/I) | In toxicated group (RMP) (U/I) | Drug treated group (RMP+RMT) (U/I) | In toxicated Control group (RMP+SYM) (U/I) |
| 1 | Aspartate aminotransferase (ASAT) | 133±1.25 | 187.4±1.45** | 146±1.94** | 149.11±1.18** |
| 2 | Alanine aminotransferase (ALAT) | 29.4±1.6 | 50.3±1.03** | 34.1±0.83** | 36.1±0.63** |
| 3 | Alkaline phosphatase (ALP) | 6.2±0.58 | 17.2±0.66** | 10.78±1.1*** | 10.8±0.91** |
| 4 | Total cholesterol (TC) | 49.2±0.8 | 53.6±0.58* | 57.5±0.66* | 52.06±0.51* |

* non significant, ** $P < 0.05$, *** $P < 0.001$ vs Control (Group I), Student *t* test
RMP-Rifampicin, RMT- Racemethionine, SYN- Silimarm

**Figure 2: Effect of Racemethionine on Aspartate aminotransferase (ASAT)****Figure 3: Effect of Racemethionine on Alanine aminotransferase (ALAT)**

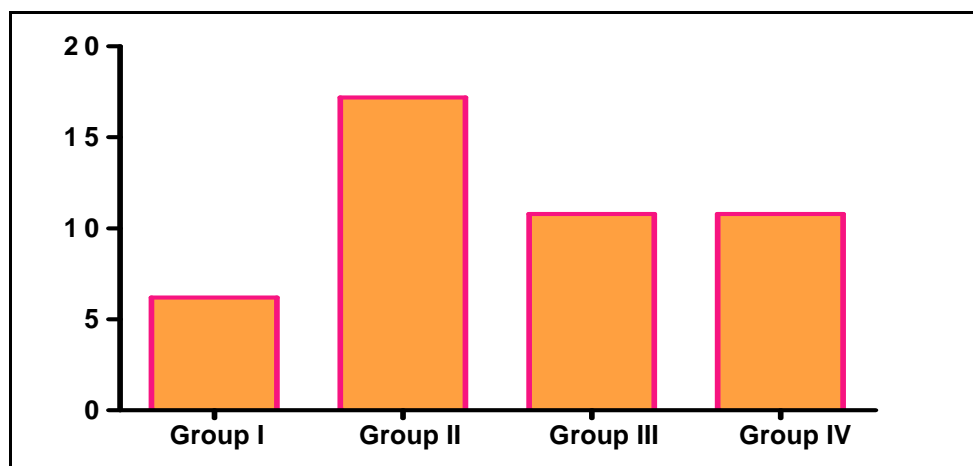


Figure 4: Effect of Racemethionine on Alkaline phosphatase (ALP)

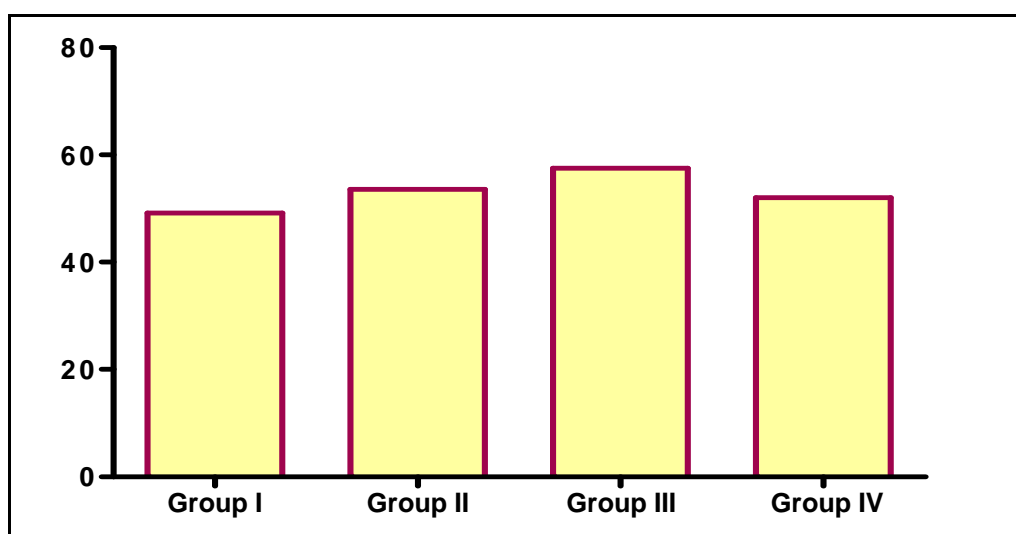


Figure 5: Effect of Racemethionine on Total Cholesterol (TC)

A significant increase in Aspartate aminotransferase (ASAT), Alanine aminotransferase (ALAT), Alkaline phosphatase (ALP) was observed in the intoxicated control group which was treated with. Total cholesterol level in the intoxicated group was also found to increase slightly compared to the control group rats. These biochemical marker enzymes levels were decreased to near normal values in the group of animals treated with racemethionine. Results of racemethionine treated animals were found to decrease significantly in the case of parameters like ASAT, ALAT, ALP when compared with the intoxicated group. Cholesterol levels were found to be not much effected when compared with bio chemical levels of anti tubercular drug treated animals.

Rifampicin is rapidly eliminated in the bile and entero hepatic circulation ensues. During this time the drug is progressively deacetylated, such that after 6 hours nearly all of the antibiotic in the bile will be in the deacetylated form. Intestinal reabsorption is reduced by deacetylation and metabolism thus facilitates elimination of drugs [9].

The effect of Racemethionine on Aspartate aminotransferase (ASAT), Alanine aminotransferase (ALAT), Alkaline phosphatase (ALP) and total cholesterol (TC) was indicated in figures 2, 3, 4 and 5.

Rifampicin is rapidly metabolized to deacetyl rifampicin, which actively and specifically binds to RNA polymerase which inhibits the synthesis of all forms of RNA. Thus inhibiting nucleic acid and protein synthesis it induces fatty liver and finally cirrhosis. This causes fatal liver damage acute hepatic failure, which is accompanied by increase in the activity of some serum enzymes [10].

The enzyme alanine aminotransferase (ALAT) which was estimated in the present study. It is a very specific and sensitive marker of liver injury. The major usefulness of ALAT assay for detecting liver disease is based on the fact that its elevation correlates only with the extent of liver disease and therefore, follows the course of hepatic disease process [11].

Reduction in the levels of ASAT and ALAT towards the normal values and decline in alkaline phosphate levels suggests the stability of the bilayer functions during injury with rifampicin, after administration of racemethionine. This is a sign of regeneration process stimulated by racemethionine against the harmful effects of rifampicin on the hepatic cells. Thus the study indicates that racemethionine has good hepatoprotective activity when toxicated with anti tubercular drug, rifampicin for 2 days.

CONCLUSION

Among the adverse effects of anti tubercular therapy (ATT), hepatotoxicity is a well known complication. The severity ranges from alteration in liver enzymes, chronic active hepatitis and incidence of acute hepatitis, occasionally associated with complicated acute liver failure carrying very high mortality which demands liver transplantation [12].

In the current investigation an attempt was made to minimize the effect of Rifampicin induced hepatotoxicity by the use of Racemethionine which is used as an anti dote for paracetamol overdose [13]

The research study showed that Racemethionine reduced the increasing levels of serum glutamyl oxalo acetate transaminase (ASAT), serum alanine aminotransferase (ALAT) and Alkaline phosphatase (ALP). Total cholesterol (TC) showed an increase in the Racemethionine treated group compared to the anti tubercular drug treated group. The results indicated that Racemethionine showed to be a more potent hepatoprotective drug against Rifampicin induced hepatic injury in experimental animals.

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