STUDIES ON THE ANTIULCEROGENIC AND ANTIHEPATOTOXIC POTENTIAL OF CHITOSAN AND GLUCOSAMINE IN EXPERIMENTAL RATS

Thesis Submitted to

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

IN BIOCHEMISTRY

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CERTIFICATE

This is to certify that this thesis entitled **"STUDIES ON THE ANTIULCEROGENIC AND ANTIHEPATOTOXIC POTENTIAL OF CHITOSAN AND GLUCOSAMINE IN EXPERIMENTAL RATS"** embodies the original research work conducted by **Mr. Santhosh. S.** under my guidance from 16.10.2002 to 30.07.2006. I further certify that no part of this thesis has previously been formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or in any other university or institution. He has also passed the Ph.D qualifying examination of the COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY, Cochin held in November 2004.

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ABSTRACT

The present study examined the antiulcer effect of glucosamine on mucosal antioxidant defense system in ibuprofen-induced peptic ulcer in male albino rats. The number of lesions in the gastric mucosa, volume of gastric juice, acid output, pepsin activity, lipid peroxides, reduced glutathione content, the activities of glutathione dependent antioxidant enzymes (glutathione peroxidase and glutathione-S-transferase) and antiperoxidative enzymes (catalase and superoxide dismutase), activities of membrane bound ATPases, protein content, glycoprotein components, minerals status, SDS-PAGE electrophoretic pattern of mucosal protein, amino acid composition and histopathology were determined. Prior oral administration of chitosan and glucosamine significantly prevented the ibuprofen-induced increases in the number of lesions in the gastric mucosa, volume of gastric juice and acidity. They also maintained the activity of pepsin at near normal level. Oral pretreatment of chitosan and glucosamine exerted a significant antioxidant effect by preventing ibuprofen-induced lipid peroxidation and by maintaining the level of reduced glutathione and the activities of mucosal antioxidant enzymes at near normalcy. Prior oral administration of glucosamine significantly prevented the ibuprofen-induced depletion of protein and glycoprotein components and maintained the activities of membrane bound ATPases as compared to untreated ulcer induced group of rats. Amino acid composition and mineral status were also brought to the near normal level in the chitosan and glucosamine pretreated groups. The antiulcerogenic activity of glucosamine might be ascribable to its ability to neutralize the hydrochloric acid secreted into the stomach and to its capability to strengthen the mucosal barrier by increasing mucosal glycoprotein synthesis and to its free radical scavenging property. Histopathological investigations and amino acid observations of the mucosal tissue were also supported the antiulcerogenic effect of chitosan and glucosamine. The antioxidant capacity of chitosan and glucosamine to inhibit ibuprofen-induced lipid peroxidation is also supportive to the antiulcer effect.

Tuberculosis is a dangerous disease and its death toll is increasing year by year. Intake of isoniazid and rifampicin, the most common antitubercular drugs, lead to fatal hepatotoxic condition. I have studied the protective effect of chitosan and glucosamine supplementations against the hepatotoxicity induced by antitubercular drugs with respect to the changes in the levels of protein, SDS PAGE-electrophoretic pattern protein, levels of diagnostic marker enzymes, lipid components, lipid peroxidation, albumin, globulin, albumin/globulin ratio, glucose, creatinine and urea in the serum and diagnostic marker enzymes, hepatic thiols, lipid components, lipid peroxidation, reduced glutathione, the activities of glutathione dependent antioxidant enzymes and antiperoxidative enzymes, activities of membrane bound ATPases, protein content, glycoprotein components, minerals status, glycoprotein conjugates, electrophoretic pattern of protein, fatty acid composition and histopathology in the liver tissue of normal and experimental groups of rats. Co-administration of chitosan and glucosamine was found to prevent significantly the antitubercular drugs-induced changes in the levels of diagnostic marker enzymes and albumin/globulin ratio in experimental groups of rats. Isoniazid and rifampicin-induced lipid peroxidation was also found to be prevented by the administration of chitosan and glucosamine. The oral administration of antitubercular drugs caused a significant elevation in the levels of diagnostic marker enzymes and cholesterol, triglycerides, free fatty acids and lipid peroxidation in serum and liver of experimental rats. There was a slight decline in the level of phospholipids in liver tissue also observed. Chitosan and glucosamine exerted a significant antilipidemic effect against isoniazid and rifampicin-induced hepatitis by maintaining the levels cholesterol, triglycerides, free fatty acids and phospholipids in serum and liver at near normalcy. Further, chitosan administration maintained the levels of lipid peroxides, glucose, creatinine, albumin/globulin, urea, hepatic thiols and protein to normalcy in experimental groups compared to hepatotoxicity-induced group of rats. Levels of glycoconjugates were also maintained to near normal level by chitosan and glucosamine co-administration. From the results obtained, it can be concluded

that co-chitosan and glucosamine are beneficial against antitubercualr drugs-induced hepatoxicity. SDS page electrophorectic pattern, fatty acid composition and histopathological observations also support this finding.

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ABBREVIATIONS

ANSA	-	1-amino-2-napthol-4-sulphonic acid
AR	-	Analar
ATP	-	Adenosine triphosphate
BSA	-	Bovine serum albumin
°C	-	Degree celsium
CAT	-	Catalase
CDNB	-	1-chloro-2,4-dinitro benzene
dl	-	Decilitre
DNPH	-	2-4-dinitrophenyl hydrazine
DTNB	-	5,5-dithio-bis-(2-nitro benzoic acid)
EDTA	-	Ethylene diamine tetra acetic acid
Fig.	-	Figure
FFA	-	Free fatty acid
g	-	grams
GPx	-	Glutathione peroxidase
GSH	-	Glutathione
HCl	-	Hydrochloric acid
H_2O_2	-	Hydrogen peroxide
H_2SO_4	-	Sulphuric acid
h	-	Hour
HDL	-	High density lipoprotein
i.p.	-	Intra peritoneal
Kg	-	Kilogram
KOH	-	Potassium hydroxide
KI	-	Potassium iodide.
LDH	-	Lactate dehydrogenase.
LDL	-	Low density lipoprotein
M	-	Molar
mM	-	Millimolar.

mg	-	Milligram
Mm	-	Millil-molar.
mm	-	Millimeter
N	-	Normal
µ moles	-	Micro molesmin
Min	-	Minute
ml	-	Militer
NADH	-	Reduced nicotinamide adenine dicutecldiacid
NADP	-	Nictonamine
Pi	-	Inorganic phohpshris.
OD	-	Optical denstiry
SD	-	Standard division
SOD	-	Superoxide dismutase
TBA	-	Thio barbituric acid.
v/v	-	Volume/volume
VLDL	-	Very low density lipoprotein
w/v	-	Weight/volume
w/w	-	Weight/weight

1. INTRODUCTION

Disease and sickness have been a part of mankind's progress through the ages. It comes as no surprise that a substantial portion of the intellectual efforts of every historical age has been directed at understanding disease and finding cures. It is but natural that each of the formally organized and structured attempts has to depend on the knowledge base and paradigms that were available at the particular period of time when the system evolved and matured. As our view of the world changed, largely due to the efforts of science, newer systems of medicine emerged and older ones fell out of favour or even discarded. Over the last century or less, keeping pace with the advances made by modern science, the Western or allopathic medical system has been the dominant influence on the health care scene. There is no denying its contribution to our well-being. Yet, there remains the lingering reality that no single system of health care delivery is able to provide all the answers to health and disease.

Traditional medicines have been the starting point for the discovery of many important modern drugs (Patwardhan, 2005). This fact has led to chemical and pharmacological investigations and general biological screening programs for natural products all over the world. Ocean, the biggest wonder in the universe, covers almost three quarters of the surface of earth. It is not only a great source of a lot of beautiful living and non-living things, but also a treasure of plenty of substances, which are beneficial in all faces of our lifestyle and in our needs of day-to-day life. Marine organisms produce many bioactive substances, which are having a lot of potential biomedical and pharmaceutical applications.

Marine food has become an inevitable ingredient in our daily menu. Prawn shell, a main waste material of marine food industry, can be efficiently utilized by converting it into various useful marine polysaccharides byproducts such as chitin, chitosan, glucosamine etc. (Synowiecki and Al-Khateeb, 2003). The mucopolysaccharide, chitin is produced by the demineralization and deproteinisation of shrimp shell waste. It is considered as the second most

abundant biopolymer in the world next to cellulose (Sini *et al.*, 2005). Chitin can be converted into two value-added products, chitosan and glucosamine. Chitosan and glucosamine are having immense applications in the fields of biomedical, pharmaceutical and nutraceutical industries.

Chitosan [poly- (ß-1-4)-D-glucosamine] is prepared by the deacetylation of chitin using concentrated alkali (Krisana *et al.*, 2004; Shepherd *et al.*, 1997). It has profound applications in the fields of clarification and purification, chromatography, paper and textiles, photography, food and nutrition, agriculture, pharmaceutical and medical, cosmetics, biodegradable membranes and biotechnology. It has been reported to possess immunological (Nishimura *et al.*, 1984; Mori *et al.*, 1997) antibacterial (Tokura *et al.*, 1997; Tanigawa *et al.*, 1992) and wound healing activities (Okamoto *et al.*, 1993; Kweon *et al.*, 2003; Khnor and Lim, 2003). Chitosan is an effective and adequate haemostatic agent even under the most severe conditions of anticoagulation. Chitosan solution is found beneficial for healing "athletes foot" conditions (Allan *et al.*, 1984). Both hard and soft contact lenses can be made from chitosan. Sapelli *et al.* (1986) studied the application of chitosan in dentistry.

Han *et al.*, (1999) has reported that supplementation of chitosan can reduce high fat diet-induced lipidemia by its antilpidemic property. The free radical quenching property of this marine polysaccharide has also been studied in detail (Xing *et al.*, 2005). Reports by Filipovic-Grcic *et al.*, (2001) indicates the membrane stabilizing property of chitosan. In our laboratory, Anandan *et al.* observed the antiulcerogenic potential of chitosan against HCl-ethanol mixture induced peptic ulcer in rats. Since chitosan can wrap solid particles in liquids to bring them together and agglomerate, it is effectively utilized for the controlled release of drug (Krisana *et al.*, 2004). Dissolution properties and bioavailability of poorly soluble drugs can be improved by grinding them with chitosan. Though the beneficial effects of chitosan have been extensively studied, the antihepatotoxic potential of chitosan on lipid metabolism in antitubercular drugs-induced hepatoxicity has not yet been explored. Muzzarelli (1996) has reported that

supplementation of chitosan can prevent experimentally induced diabetic complications by its antioxidant and membrane stabilizing properties.

Glucosamine is another value added by-product prepared by the hydrolysis of chitin with conc. HCl. This is an amino monosaccharide having a lot of potential biomedical, pharmaceutical and industrial applications. It is an important constituent of cartilage and it has been used for the treatment of osteoarthritis (Timothy *et al.*, 2004). In combination with chondroitin sulfate, it can build blocks for cartilage, up-regulate chondrocyte and reduce the extent of cartilage degradation (Yu Shao *et al.*, 2004). Sal'nikova *et al.* (1990) suggests the antioxidant nature of glucosamine, by virtue of which it can trap and dismute free radicals. Glucosamine is an essential component required for glycoprotein synthesis in living beings (Wu *et al.*, 2004). Mutoh *et al.* (1995) reported the cytoprotectivity of glucosamine rich mucus.

In traditional medicine, the shrimp and cuttlefish exoskeleton powder has been used to cure arthritis, diabetes, stomach disorders, epilepsy and various liver disorders. Yet there is little documentary evidence regarding the exact mechanism of action involved in the cytoprotective effect of these marine polysaccharides. Glucosamine is the basic unit of chitin and chitosan and it is an essential component required for glycoprotein synthesis in living beings (Zhang *et al.*, 2004). The beneficial actions of chitin and chitosan may be ascribable to their basic unit glucosamine. Hence, it thought to be important to study the protective effect chitosan and glucosamine on experimentally induced ulcer and hepatotoxic conditions to derive a conclusion regarding the exact biochemical mechanism involved in the beneficial effects of shrimp and cuttle fish exoskeleton powder in alleviating the ulcerative and liver disorders by virtue of their antilipidemic, antioxidant and membrane stabilizing properties.

2. REVIEW OF LITERATURE

2.1. Chitosan

Chitosan is a natural mucopolysacharide (Van der Lubben *et al.*, 2001) of marine origin. While it has been in existence for millennia, its current form has just recently been prepared. Chitosan is one of the most available polysaccharides with positive charges found in nature (Xing *et al.*, 2005). Technically speaking, Chitosan is a naturally occurring substance that is chemically similar to cellulose (Barbara, 2005), which is a plant fiber. Chitosan is shown to have superior characteristics and especially flexibility in its use.

2.1.1. Source

Chitosan is obtained from chitin (Shepherd *et al.*, 1997; Krisana *et al.*, 2004), which is believed to be the second most abundant biomaterial after cellulose (Sini *et al.*, 2005). Chitin is widely distributed in the nature. Major sources of chitin are shellfish, clams, krill, oysters, squid, fungi and insects. Chitin content in various sources is given in Table 1 (Allan *et al.*, 1978; Kong, 1975). Amongst several sources, the exoskeleton of crustaceans consist of 15-20 % chitin by dry weight. Chitin found in nature is a renewable bio-resource. It is present not only in animals, but also in plants as cell wall material. Chitin resembles cellulose both in chemical structure and in biological function as a structural polymer. The crystalline structure of chitin has been shown to be similar to cellulose in the arrangements of inter- and intra chain hydrogen bonding. The annual biosynthesis of chitin has been estimated to 10^9 to 10^{11} tons. Chitosan is found in nature, to a lesser extent than chitin, in the cell walls of fungi. Zygomycetes contain both chitin and chitosan (Austin *et al.*, 1981; Rudall, 1969).

Chitosan is prepared by alkaline N-deacetylation of chitin (Kittur *et al.*, 2002). Deacetylation of chitin is done by using concentrated NaOH solution (40-50%).

Source	Quantity harvested 10^3 tonnes	Chitinous waste, 10^3 tonnes	Chitin potential, 10^3 tonnes
Shell fish	1700	468	39
Krill (potential landing)	18200	3640	56
Clam/ Oysters	1390	521	22
Squid	660	99	1
Fungi	790	790	32
Total	22,740	5,118	150

Table 2.1. Chitin content in various sources

2.1.2. Properties

Chitosan occurs as odourless substance. It is an amorphous solid and off-white in colour. The properties of chitosan vary considerably depending on the source and production process. Average molecular weight of chitosan is around $1.2X10^5$ Daltons. Most of the commercial polysaccharides like cellulose, dextran, pectin, alginic acid etc. are neutral or acidic. But chitosan is an abundant basic polysaccharide. Its pH comes around 8 and this basic nature makes it unique for different applications. Chitosan has special properties like polyelectolyte behaviour, polyoxysalt formation, film formation and chelation of metal ions (Austin *et al.*, 1981). The main advantage of Chitosan for different biological applications is that it is nontoxic (Arai *et al.*, 1968) in nature. Chitosan is insoluble in water but soluble in dilute acids. Pure chitosan is not hydrolysed by lysozyme

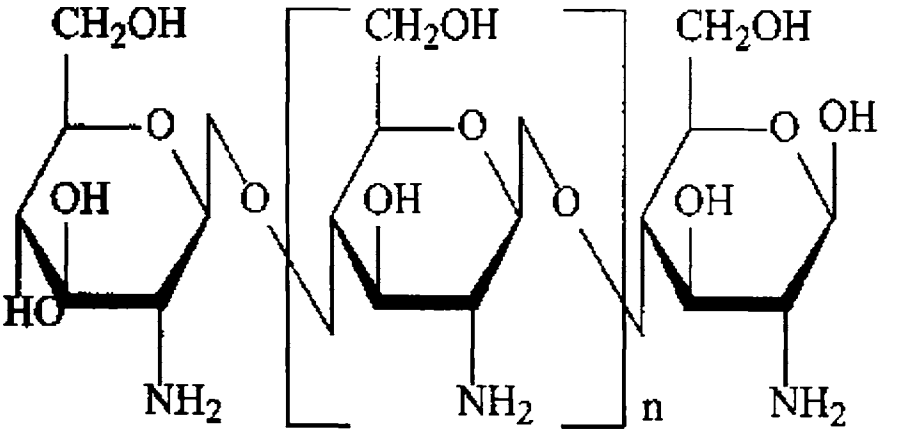
while chitin is hydrolysed. Chitosan undergoes the typical reactions of amines, of which N-acetylation and Schiff reaction are the most important. Although β (1-4) anhydroglucosidic bond of chitosan is also present in cellulose, the characteristic properties of chitosan are not shared by cellulose (Muzarelli, 1978). Chitosan forms aldimines and ketimines respectively with aldehydes and ketones at room temperature. When reacted with ketoacids followed by reduction with sodium borohydride, chitosan produces glucans. N-carboxymethyl chitosan and N-carboxyl benzyl chitosan are examples of glucans derived from chitosan (Muzzarelli, *et al.*, 1982 a,b; Muzarelli and Tanfani, 1982). On hydrogenation with aldehydes, chitosan produces N-alkyl chitosans.In spite of the hydrophobicity of the alkyl chains, N-alkyl chitosans swell in water, but its film forming capacity is retained (Muzarelli *et al.*, 1983). In acidic medium, chitosan develops a violet colour with iodine (Van Wisselingh, 1898; Campbell, 1929; Krishnan and Sundara Rajulu, 1964). This test is used as the detection test for chitosan. An enzymatic method was also put forwarded by Jeuniax (1963 & 1965) for the detection of chitosan. As per the reports of Sundara Rajulu *et al.* (1982), enzymatic test was found more reliable amongst these two tests. Chitosan can be hydrolysed in the presence of chitosanase (Monaghan *et al.*, 1972). According to Fenton and Eveleigh (1981) purified chitosanase from microbes hydrolyses specifically chitosan.

2.1.3. Structure

Chitin, source of chitosan, is a polymer of β (1-4)-N-acetyl-D-glucosamine (Fig. 2.1). Chitin occurs in three polymorphic forms, which differ in the arrangement of molecular chains (Muzarelli, 1977 a). In chitosan, the N-acetyl group of chitin is removed. Chitosan is a polymer of β (1-4)-D-glucosamine (Yasser and Ahmed, 2002)

2.1.4. Applications of chitosan

Since chitosan is nontoxic (Arai *et al.*, 1968), it is suitable for different applications. Chitosan is one of the most abundant natural amino polysaccharides with variety of applications (Majeti and Kumar, 2000) It is having



Chitosan

Fig.2.1. Structure of Chitosan

immunological (Nishimura *et al.*, 1984; Mori *et al.*, 1997) antibacterial (Tokura *et al.*, 1997; Tanigawa *et al.* (1992) and wound healing activities (Okamoto *et al.*, 1993; Kweon *et al.*, 2003; Khnor and Lim, 2003). As chitosan is having antibacterial, haemostatic, fungistatic, antitumoral and anticholesteremic properties (Barbara, 2005), it has wide pharmaceutical applications. Muzarelli *et al.* (1988) studied the application of chitosan in wound dressing since chitosan wound healing property. Chitosan has profound applications in the fields of clarification and purification, chromatography, paper and textiles, photography, food and nutrition, agriculture, cosmetics, biodegradable membranes and biotechnology.

Since chitosan is a long chain polymer, it can wrap solid particles in liquids to bring them together and agglomerate. Thus, chitosan can be used as a coagulant aid. Commercially, chitosan is used for waste water clarification. Free amino group and hydroxyl groups present in chitosan make it suitable for chromatographic applications. Muzarelli (1977) and Allan *et al.* (1972,1975 & 1977) made studies on the application of chitosan on improving the properties of paper. Chitosan can be used for the production of fibers and films (Kunike, 1926). Since chitosan has resistance to abrasion, optical characteristics and behaviour with silver complexes, it has immense applications in photography. Animal feed studies proved that the utilization of whey might be improved if the diet contain small amount of chitinous material. Chitosan feeding studies conducted in chicken, pig, mice, rabbits etc. improved their weights significantly. A thin coating of chitosan in seeds enhances the seed growth. Chitosan coating can be used for the ripening of fruits also. Chitosan can be effectively utilized for the controlled release of drug (Agnihotri and Aminabhavi, 2004). Chitosan is used as a substituent for microcrystalline cellulose (MCC) as a diluent in tablet making by direct compression. Dissolution properties and bioavailability of poorly soluble drugs can be improved by grinding them with chitosan. Enzyme immobilization is another important use of chitosan. Many methods are adopted for enzyme immobilization like entrapment and adaption, fixing by cross-linking etc. There are many advantages when immobilized enzymes are used; enzymic reaction can be stopped at any desired time and small amount of enzyme is

sufficient for large amount of substrate. Since, chitosan is physiologically harmless it can be used in hair cosmetic products instead of synthetic resins (Gross *et al.*, 1982). Chitosan films have good water absorbing property. Chitosan membranes can be used for desalination, ultrafiltration and wastewater treatment (Rutherford and Dunson, 1984). Chitosan is an effective and adequate haemostatic agent even under the most severe conditions of anticoagulation. Chitosan solution is found beneficial for healing "athletes foot" conditions (Allan *et al.*, 1984). Chitosan has good ophthalmologic applications. Both hard and soft contact lenses can be made from chitosan. The effect of chitosan have been investigated in patients with chronic renal failure undergoing long term stable haemodialysis treatment (Jing *et al.*, 1997) Ingestion of chitosan effectively reduced serum cholesterol level and increased serum haemoglobin levels. Significant reduction in urea and creatinine levels in the serum were observed after 4 weeks of chitosan ingestion. The data suggests that chitosan might be effective in the treatment for renal failure patients. Sapelli *et al.* (1986) studied the application of chitosan in dentistry. Knapczyk *et al.* (1989) proved that chitosan is applicable in immunology. Like some plant fibers, chitosan is not digestible; therefore it has no caloric value. No matter how much Chitosan ingest, its caloric count remains at zero. This is a very important property for any weight loss product.

2.2. Glucosamine

Glucosamine is an amino monosaccharide found in most of the tissues in our body. Unlike other forms of sugar in the body, amino sugars are components of carbohydrates that are incorporated into the structure of body tissues, rather than being used as a source of energy. Glucosamine is involved in the formation of the nails, tendons, skin, eyes, bones, ligaments, and heart valves. It also plays a role in the mucous secretions of the digestive, respiratory, and urinary tracts. It is incorporated in the biosynthesis of glycosaminoglycans and proteoglycans, essential for the extracellular matrix of connective tissues. Glucosamine is a nutritional supplement and therefore the authority of the Food and Drug Administration to regulate it is now severely limited (Angell and Kassirer, 1998).

There have been relatively few trials of glucosamine use in humans, and those that have been published are studies that examined the efficacy of glucosamine in the relief of joint pain (Mc Alindon *et al.*, 2000). There have been no reported trials of long-term glucosamine use, and its long-term safety is unknown. Due to favorable, but scientifically inconclusive, reports of glucosamine therapy for arthritis symptoms are less (Loes *et al.*, 1998). Glucosamine is available as an over-the-counter oral supplement, has few adverse side effects, and is relatively inexpensive. Several clinical studies have reported that glucosamine works better in reducing the symptoms of osteoarthritis (Reginster *et al.*, 2001). However, the mechanisms underlying this effect are not yet known.

2.2.1. Source

Exoskeleton of marine organisms are the main source of glucosamine. Chitin is prepared from the shells of crab, shrimp etc. From chitin, glucosamine is prepared by hydrolysis with concentrated acids under drastic conditions (Madhavan, 1992). Glucosamine is found in hyaluronic acid, a compound responsible for the lubricating and shock-absorbing properties of synovial fluid. Glucosamine is synthesized in chondrocytes in the body from glucose and glutamine.

2.2.2. Structure

Glucosamine is an aminomonosacharide. i.e., amino group is attached to a glucose ring (Fig.2.2). Thus comes the name glucose-amine. Chemically glucosamine is 2-amino-2-deoxy-alpha-D-glucose.

2.2.3. Properties

Glucosamine is a colourless and odourless substance with sweet taste. It is crystalline in nature. It is having acidic pH, ranging from 3.5-4.5 and has density of 0.75 g/ml. Specific rotation of glucosamine is 70° and is readily soluble in water. When heated above 80 ^{0}C, it undergoes Maillard reaction and its colour

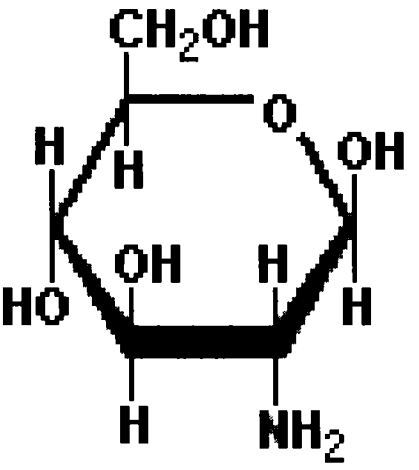


Fig. 2. 2. Structure of β-D-Glucosamine

will be faded. On further heating, glucosamine will be charred. Glucosamine has anti-inflammatory and antioxidant actions. Glucosamine blocks the generation of superoxide free radicals by macrophages. Glucosamine is well tolerated. No severe side effects were reported for glucosamine (Reginster *et al.*, 2001). Glucosamine can increase proteoglycan synthesis (Corinne *et al.*, 1998), which is a fundamental component found in articular cartilage. Proteoglycans synthesized in the presence of glucosamine had smaller glycosaminoglycan chains with a corresponding decrease in lipoprotein retention. This property makes it useful against atherosclerosis (Tannock *et al.*, 2002). Glucosamine inhibits the cartilage-destructive enzyme collagenase. Glucosamine helps in the synthesis of cartilage by increasing key components of cartilage such as glycosaminoglycans. Various reports confirmed that diabetes patients can also consume Glucosamine, which will not increase blood glucose level (Daren *et al.*, 2003). Glucosamine appears to undergo a significant first-pass effect in the liver, which metabolizes a significant proportion of the dose to CO_2, water, and urea (Setnikar *et al.*, 1993)

2.2.4. Applications

Setnikar and colleagues (1984, 1986, 1993) have published several reports on the pharmacokinetics of glucosamine in rats, dogs, and humans. In both humans and rats, oral administration of radio labeled glucosamine results in absorption of more than 90% of the ingested dose (Tannock *et al.*, 2002).

Glucosamine is found largely in cartilage and plays an important role in its health and resiliency. As we age, we lose some of the glucosamine and other substances in cartilage. This can lead to thinning of cartilage and the onset and progression of osteoarthritis. According to Braham (2003), glucosamine supplementation can provide some degree of pain relief and improved function in persons who experience regular knee pain, which may be caused by prior cartilage injury and/or osteoarthritis. Since, glucosamine can increase the synthesis of proteoglycans and glucosaminoglycans, which are necessary ingredients of connective tissue, it is proven to be effective against osteoarthritis

(Timothy *et al.*, 2004; Reginster *et al.*, 2001). Canapp *et al.* (1999) reported that glucosamine was found beneficial against acute synovitis.

Every year about twenty thousand people die from using NSAIDS. The combination of glucosamine and NSAIDs may reduce the doses needed for anti-inflammatory activity as well as the side effects associated with these NSAI drugs (Zupanets *et al.*, 1991). Dettmer (1979) examined the effects of simultaneous administration of glucosamine and chondroitin sulfate on osteoarthritis. This study demonstrated greater effectiveness of this combination therapy. In combination with chondroitin sulfate, it can build blocks for cartilage, up-regulate chondrocyte and reduce the extent of cartilage degradation (Yu Shao *et al.*, 2004).

Fabio *et al.* (2005) synthesized D-glucosaminic acid on a multigram scale by air oxidation of D-glucosamine, catalyzed by the enzyme glucose oxidase. D-Glucosaminic acid is a component of bacterial lipopolysaccharides and a member of the 'chiral pool' and has been used as a starting material for the synthesis of various amino acids.

2.3. Peptic ulcer

Peptic ulcer disease is a very common ailment, affecting one out of eight persons in the United States and commonly seen in adults of India. It is a sore on the lining of the stomach (Fig. 2.3.) or duodenum, which is the beginning of the small intestine. The causes of peptic ulcer have gradually become clear. With this understanding have come new and better ways to treat ulcers and even cure them. These ulcers can occur in the stomach, where they are called gastric ulcers. Or they can occur in the first portion of the intestine. These are called duodenal ulcers. "Peptic Ulcer" is the term used to describe either or both of these two types of ulcers.

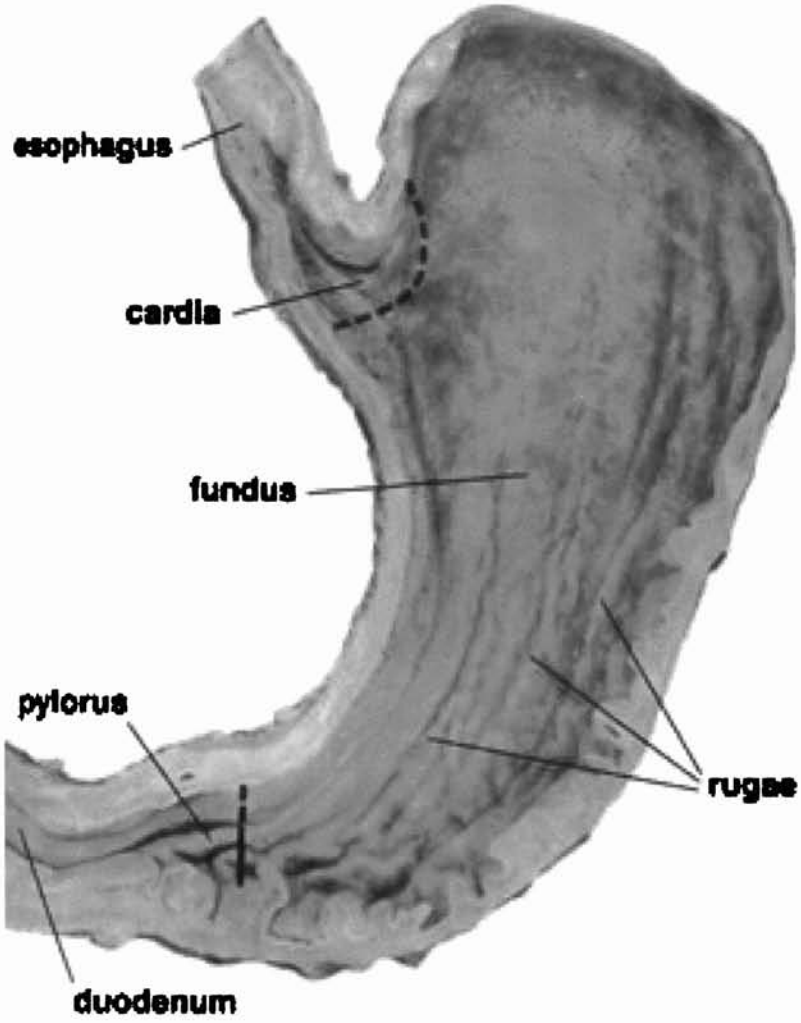


Fig. 2.3. Structure of Stomach

2.3.1. Anatomy and function of the Stomach

The stomach produces a very strong acid. This acid helps to digest and break down food before it enters the small intestine (duodenum). The lining of the stomach is covered by a thick protective mucous layer, which prevents the acid from injuring the wall of the stomach.

2.3.2. Causes of Ulcer

An ulcer is an open wound in the lining of the stomach or intestine, much like mouth or skin ulcers. Peptic ulcers are eventually caused by acid and pepsin, a digestive stomach enzyme.

In the end, it is the production of large amounts of acid in the stomach, passing under the term 'hyperchlorhydria', that causes the injury to the stomach or bowel lining. In a few cases, cancerous tumors in the stomach or pancreas can cause ulcers. Peptic ulcers are not caused by stress or eating spicy food, but these can make ulcers worse. However, a revolutionary and startling recent discovery is that most peptic ulcers result from a stomach infection caused by the bacteria, *Helicobacter pylori* (Fig.2.4). Use of non-steriodal anti-inflammatory drugs (NSAIDs) will also cause ulcers.

2.3.2.1. Helicobacter pylori (*H. pylori*)

Complicating our understanding of stomach ulcers is Barry Marshall and Robin Warren's discovery in 1982 that bacteria are the primary cause of stomach and duodenal ulcers excluding those caused by aspirin or arthritis drugs. This bacterium has a twisted spiral shape and infects the mucous layer lining of the stomach. This infection produces an inflammation in the stomach wall called gastritis. The body even develops a protein antibody in the blood against it. The bacterium is probably acquired from contaminated food or from a drinking glass. It is only after *H. pylori* bacteria injure the protective mucous layer of the stomach, allowing damage by stomach acid, that an ulcer develops. It takes

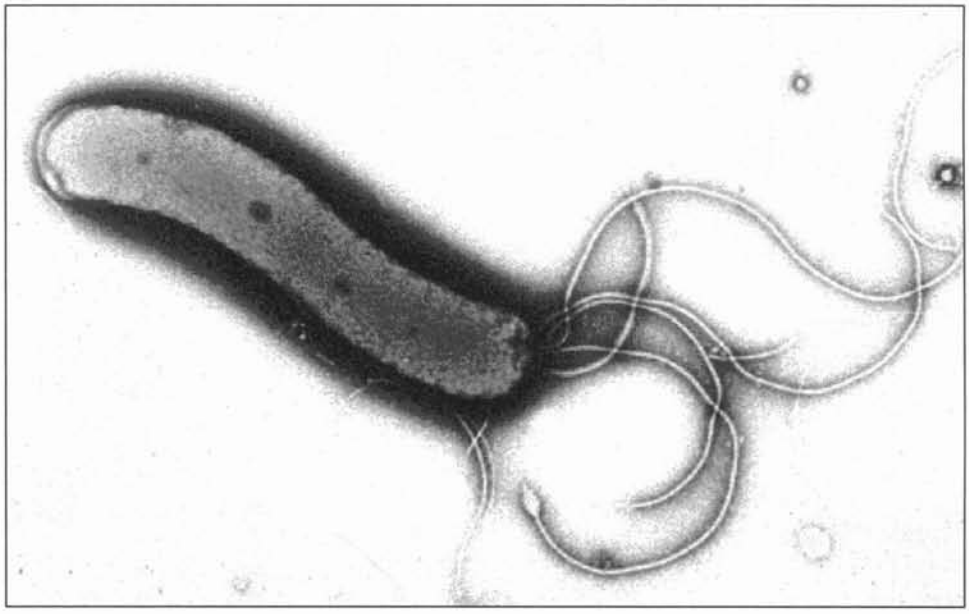


Fig. 2.4. Helicobacter pylori

advantage of the stomach's own mucus for protection. Any acid that does reach the bacteria is converted by *H. pylori*'s urease enzyme in the following reaction

urea + stomach acid + water ⟶ bicarbonate + ammonia

$$C{=}O{\cdot}2NH_2 + H^+ + 2H_2O \longrightarrow HCO_3^- + 2NH_4^+$$

The products of this reaction, bicarbonate and ammonia, are strong bases that further protect the bacteria because of their acid-neutralizing capability. The body's immune system responds to the presence of *H. pylori* and sends infection-fighting cells to the area. However, the neutrophils cannot reach the *Helicobacter pylori* infection because they cannot easily get through the stomach lining. Inflammation in the stomach tissue occurs as the neutrophils die and release superoxide radicals on the stomach wall, damaging tissue. The immune system sends in more nutrients to help the neutrophils, and the *H. pylori* can feed on these nutrients. It may not be the *H. pylori* itself that causes a stomach ulcer, but inflammation in the stomach lining as part of the immune response. Age is also a factor in *H. pylori* infection. In Western countries, children are unlikely to be infected. *H. pylori* infections occur in about 20% of persons below the age of 40 years, and 50% of persons above the age of 60 years.

2.3.2.2. Non Steroidal Anti-inflammatory Drugs (NSAIDs)

NSAIDs include ibuprofen, fenoprofen, aspirin, diclofenac, sulindac, diflusinal, naproxen, tolmetin and many others. They can damage the mucous layer of the stomach, after which the stomach acid causes the final injury.

There are two components to NSAID-induced ulceration. First, there is a local acid effect of the dissolved drug. Most NSAIDs are weakly acidic, lipid-soluble compounds. Since the cell membranes on the stomach wall contain lipids for protection against strong acids, they offer little resistance to the lipid-soluble NSAID. The NSAID acts against the cell membrane, increasing its permeability. This results in cell swelling and death. The local acid effect of NSAIDs has been reduced by enteric-coating the drug, delaying dissolution until later in the

digestive process. However, not all NSAIDs are enteric-coated as it increases cost. In addition, enteric-coating does little more than improving the symptoms of upset stomach. Patients must be informed that enteric-coated NSAIDs are still just as likely to cause stomach ulcers as regular NSAIDs. The second and much more significant component to NSAID-induced ulceration is the systemic effect after being absorbed into the bloodstream. NSAIDs inhibit COX-1, reducing prostaglandin production. Normal COX-1 present in stomach tissue produces prostaglandins which:

- increase mucous and bicarbonate production,
- inhibit stomach acid secretion,
- increase blood flow within the stomach wall.

By acting on COX-1, NSAIDs restrict these self-protection mechanisms, allowing stomach ulcers to develop. It is primarily through this mechanism, not a local acid effect, that NSAIDs cause stomach ulcers.

So, *H. pylori* and certain drugs are the two major factors that cause ulcers. In rare cases, a patient will produce very large amounts of acid and develop ulcers. This condition is called Zollinger -Ellison syndrome. Finally, some people get ulcers for unknown reasons.

2.3.3. Other chemicals inducing Ulcer

Other than NSAIDS, certain chemicals can also cause gastrointestinal damage. In a study conducted by Silvana *et al.* (2003) ulcer was induced with indomethacin. Tan *et al.* (2002) induced ulcer by the administration of HCl-ethanol. Reserpine, an alkaloid can also be used for the induction of ulcer (Salvatore *et al.*, 2003). Jianfeng *et al.* (2002) induced ulcer in rat models by using acetic acid.

2.3.4. Symptoms

Ulcers cause gnawing, burning pain in the upper abdomen. These symptoms frequently occur several hours following a meal, after the food leaves the stomach but while acid production is still high. The burning sensation can occur during the night and be so extreme as to wake the patient. Instead of pain, some patients experience intense hunger or bloating. Antacids and milk usually give temporary relief. Other patients have no pain but have black stools, indicating that the ulcer is bleeding. Bleeding is a very serious complication of ulcers.

2.3.5. Diagnosis

A diagnosis of peptic ulcers can be suspected from the patient's medical history. However, the diagnosis should always be confirmed either by an upper intestinal endoscopy, which allows direct examination of the ulcer or by a barium x-ray of the stomach. Rarely an ulcer can be malignant. With endoscopy, a biopsy specimen can be obtained to determine if this is so.

2.3.6. Treatment

Therapy of peptic ulcer disease has undergone profound changes. There are now available very effective medications to supress and almost eliminate the outpouring of stomach acid. These acid-suppresssing drugs have been dramatically effective in relieving symptoms and allowing ulcers to heal. If an ulcer has been caused by aspirin or an arthritis drug, then no subsequent treatment is usually needed. Avoiding these latter drugs, should prevent ulcer recurrence.

The second major change in peptic ulcer disease treatment has been the discovery of the *H. pylori* infection. When this infection is treated with antibiotics, the infection, and the ulcer, do not come back. Increasingly, physicians are not just suppressing the ulcer with acid-reducing drugs, but they are also curing the underlying ulcer problem by getting rid of the bacterial infection. If this infection is not treated, the ulcers invariably recur.

There are a number of antibiotic programs available to treat *H. pylori* and cure ulcers. Working with the patient, the physician will select the best treatment program available. Antacid, H_2 receptor antagonists (ranitidine, cimetidine, nizatidine, famotidine), gastric acid pump inhibitor (omeprazole), barrier agent (sucralfate) etc. were developed to heal ulcers, but they fail to prevent the occurrence of NSAID-induced ulceration (Agrawal, 1995; Blower, 1996). These treatments are often effective in alleviating ulceration symptoms. They are also used in combination with antibiotic treatment for *H. pylori*-induced ulcers. However, the treatments listed earlier do not prevent NSAID-induced ulcers. Only misoprostol, a synthetic prostaglandin, has been shown to prevent NSAID-induced ulcers.

Some plant extracts were also found effective in the treatment of ulcer. Tan *et al.* (2002) proved the gastric cytoprotective anti-ulcer effects of the leaf methanol extract of *Ocimum suave* (Lamiaceae) in rats. Jianfeng *et al.* (2002) found that sea buckthorn (*Hippophae rhamnoides*) seed and pulp oils have both preventive and curative effects against experimental gastric ulcers in rats. Sea buckthorn (*Hippophae rhamnoides*) is a Euro–Asian wild, newly cultivated, edible berry with exceptionally high contents of nutrients and phytochemicals such as lipids, water and fat soluble vitamins, and flavonoids. Silvana *et al.* (2003) reports that *Tanacetum larvatum* has very good gastroprotective effects. Yeel *et al.* (2003) says that crude extract from *Angelica sinensis* (ASCE), which mainly consisted of polysaccharides, has a direct wound healing effect on gastric mucosa.

2.3.7. Ibuprofen

Ibuprofen is in a class of drugs called Non steroidal Antiinflammatory Drugs (NSAIDs) (Tyagi *et al.*, 2005). Ibuprofen is reported to have analgesic properties also (Polat and Karaman, 2005). Ibuprofen works by reducing hormones that cause inflammation and pain in the body. Ibuprofen inhibits cyclooxygenase (COX); thus inhibits prostaglandin synthesis. Ibuprofen appears to have gastrointestinal adverse drug reactions of all NSAIDs.

2.3.7.1. Structure

Ibuprofen is chemically 2-(p-isobutylphenyl) propionic acid (Fig. 2. 5). It has an empirical formula of $C_{13}H_{18}O_2$.

Ibuprofen like other 2-arylpropionate derivatives (including ketoprofen, flurbiprofen, naproxen etc.) contains a chiral carbon in the β-position of the propionate moiety. As such there are two possible enantiomers of ibuprofen with the potential for different biological effects and metabolism for each enantiomer.

Indeed, it was found that S-ibuprofen (sinisteribuprofen) was the active form both invitro and invivo. Logically then, there was the potential for improving the selectivity and potency of ibuprofen formulations by marketing ibuprofen as a single-enantiomer product (as occurs with naproxen, another NSAID).

Further, invivo testing, however, revealed the existence of an isomerase, which converted R-ibuprofen to the active S-enantiomer. Thus, due to the expense and futility that might be involved in marketing the single enantiomer, all ibuprofen formulations currently marketed are a racemic mixture of both enantiomers.

2.3.7.2. Properties

Ibuprofen has a molecular weight of 206.3 g. It has hepatic metabolism and has renal excretion.

Common adverse effects include nausea, dyspepsia, gastrointestinal ulceration/bleeding, raised liver enzymes, diarrhoea, headache, dizziness, salt and fluid retention, hypertension (Rozzi, 2004). Infrequent adverse effects include oesophaveal ulceration, heart failure, hyperkalaemia, renal impairment, confusion, bronchospasm, rash etc. (Rozzi, 2004).

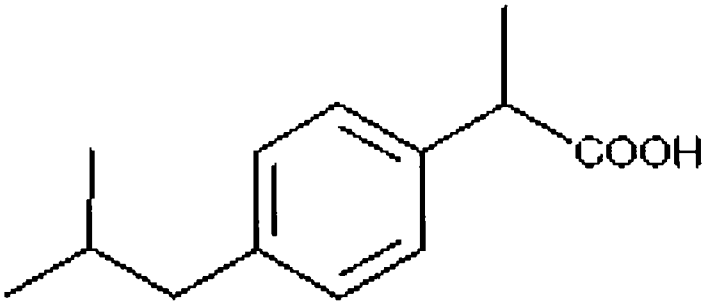


Fig. 2 .5. Structure of Ibuprofen

As with other NSAIDs, ibuprofen has been reported to be a photosensitizing agent (Castell *et al.*, 1987). Ibuprofen however has a very weak absorption spectrum which doesnot reach into the solar spectrum. The molecule contains only a single phenyl moiety and no bond conjugation, resulting in a very weak chromophore system. Ibuprofen, therefore, is only a very weak photosensitizing agent when compared with other members of the 2-arylpropionic acids.

2.3.7.3. Mechanism of action

Nonsteroidal anti-inflammatory drugs work by interfering with the cyclooxygenase pathway (Fig. 2.6). The normal process begins with arachidonic acid a dietary unsaturated fatty acid obtained from animal fats. This acid is converted by the enzyme cyclooxygenase to synthesize different prostaglandin. The prostaglandins go on to stimulate many other regulatory functions and reactionary responses in the body. Earlier research (Tannenbaum, 1996; Vane, 1996; Emery, 1996) have shown that there are two types of cyclooxygenase, denoted COX-1 and COX-2. Each type of cyclooxygenase lends itself to producing different types of prostaglandins.

Different mechanisms stimulate the two types of cyclooxygenase. COX-1 is stimulated continuously by normal body physiology. The COX-1 enzyme is constitutive meaning that its concentration in the body remains stable. It is present in most tissues and converts arachidonic acid into prostaglandins. These prostaglandins in turn stimulate normal body functions, such as stomach mucus production and kidney water excretion, as well as as platelet formation formation. The location of the COX-1 enzyme dictates the function of the prostaglandins it releases (Vane, 1996). For example, COX-1 in the stomach wall produces prostaglandins that stimulate mucous production. In contrast, the COX-2 enzyme is induced. It is not normally present in cells but its expression can be increased dramatically by the action of macrophages the scavenger cells of the immune system (Tannenbaum, 1996) COX-2's most important role is in inflammation. COX-2 is involved in producing prostaglandins for an inflammatory response.

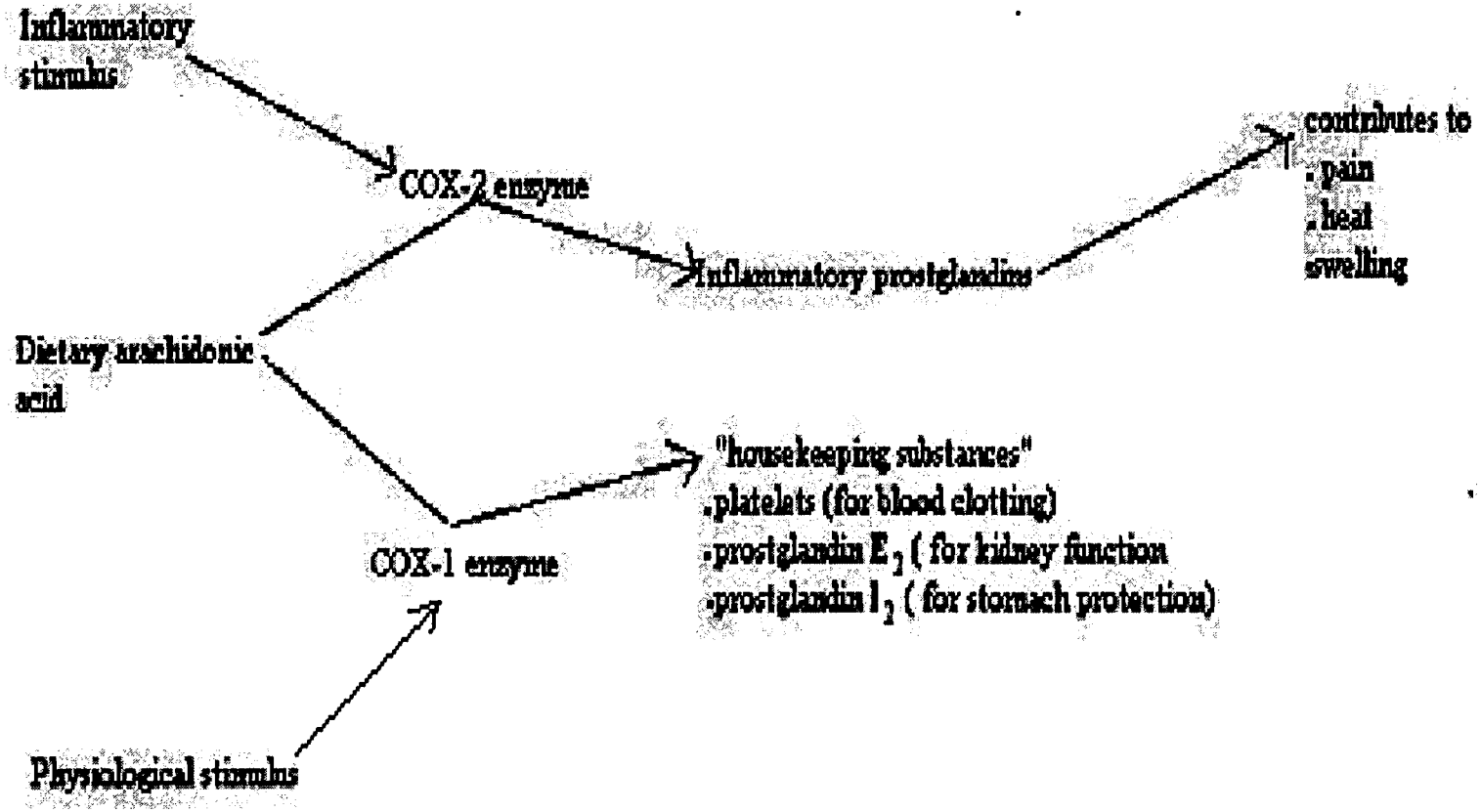


Fig. 2.6. The Cyclooxygenase Pathway

COX-1 is stimulated continually, and COX-2 is stimulated only as a part of an immune response.

2.3.7.4. Biological applications

Ibuprofen is used to reduce the fever, pain, inflammation, and stiffness caused by many conditions, such as osteoarthritis, rheumatoid arthritis, and abdominal cramps associated with menstruation. Ibuprofen is used widely in the community for the relief of headache including migraine. It is also widely marketed as an analgesic agent rather than as an anti-inflammatory and is often used for general pain conditions including those arise from various injuries such as sporting injuries, illness such as influenza, shingles, gout and post operative pain. As with other NSAIDs, ibuprofen inhibits platelet aggregation, but is not used therapeutically for this action since it is a minor and reversible effect. Litkowski *et al.* (2005) studied the analgesic effect of ibuprofen with oxycodone in combination therapy. According to Rostom *et al.* (2005), ibuprofen can cause hepatotoxicity. An earlier study (Hollenz and Labenz, 2004) reported that administration of ibuprofen could lead to gastrointestinal injury like ulcer. Long-term use of ibuprofen will lead to Nephrogenic adenoma, which is an infrequent benign lesion of the urinary system (Scelzi *et al.*, 2004). Dokmeci (2004) proved that Ibuprofen might be a promising new therapeutic avenue for the treatment of neurodegenerative diseases such as Alzheimer's disease (AD).

2.4. Hepatotoxicity

Liver is one of the most important organs in our body. It is the largest gland, which is placed behind the lower right part of ribs. Ribs help in keeping the liver from being injured. Liver is very important to human health and has a lot of functions in the body:

- Stores vitamins, sugars, fats and other nutrients from the food that we eat
- Builds chemicals that the body needs to stay healthy.

- Breaks down harmful substances, like alcohol and other toxic (poisonous) chemicals.
- Removes waste products from the blood.
- Makes sure that the body has just the right amount of other chemicals that it needs.

Many diseases can affect the liver. If anyone has one of these diseases, the liver may not work. Some of the most common diseases that affect the liver are:

1. Viral Hepatitis: Hepatitis is a medical term that means "inflammation (swelling) of the liver." Viruses that attack the liver cause some of the most common forms of hepatitis. Virus attack can be identified by a blood test. Three of the most common viruses that attack the liver are:Hepatitis A virus (HAV), Hepatitis B virus (HBV) and Hepatitis C virus (HCV).

2. Cirrhosis: Cirrhosis is a medical term that means "scarring of the liver." When one has cirrhosis, large parts of the liver are damaged. Because it has been damaged, the liver may not work as well as it should. Cirrhosis of the liver is often the result of drinking too much alcohol. Other common causes of cirrhosis include hepatitis; especially hepatitis C. Cirrhosis can be very dangerous if it is not treated properly.

3. Liver Cancer: Like many other body organs, liver also can get cancer. Liver cancer is a disease in which some of the cells in the liver begin to reproduce faster than they should. These cells form growths called tumors. Having hepatitis B or hepatitis C can increase the chances of getting liver cancer. Liver cancer can be deadly.

4. Hepatotoxicity: Toxicity of liver can be obtained by certain chemicals. Prolonged use of some drugs can also cause hepatoxicity. Millions of people are suffering from hepatic failure. It may lead to death also. Drug induced liver injury was high in patients aged $\geq$35 years (Tasduq *et al.*,

2005). A recent study by Jasmer *et al.* (2002) found that patients >35 years old had a higher risk of grade 3 or 4 hepatotoxicity.

Fulminant hepatitis carries a very high mortality, resulting from acute hepatitis caused by virus infection, alcohol or drugs. Conventional medical therapies can rescue only about 10% of patients with fulminant hepatitis (Hiroaki *et al.*, 2003). Although liver transplantation has improved their mortality, about 40% of these patients die while waiting for liver transplantation (Shakile *et al.*, 2000)

2.4.1. Chemicals inducing hepatotoxicity

Administration of some chemicals can induce hepatotoxicity. Sodium diethyl dithiocarbamate, diclofenac and ketoconazol are three important chemotherapeutic agents that are commonly associated with hepatotoxicity (Amr and Alaa, 2005). One of the most hazardous pollutants called hexachlorobenzene can also cause hepatotoxicity (Billi de Catabbi *et al.*, 2005). Kaufmann (2005) reported that treatment with some drugs causes liver injury. A lot of drugs are devastated from the market due to their induction of hepatotoxicity. Factors affecting susceptibility to drug-induced injury include age, sex, concomitant use of other drugs, and genetic polymorphism in metabolic pathways involved in activation or disposition of therapeutic drugs (Maddrey, 2005). Older persons who are receiving more drugs simultaneously are more susceptible to liver injuries. Guzman *et al.* (2005) studied about the hepatotoxic effect of paroxetine. Galactosamine is capable of inducing wild hepatic failure (Abul *et al.*, 2005). It resembles viral hepatitis. In a study conducted by Senthilkumar and Nalini (2004) hepatotoxicity was induced by the intake of alchohol. Tomiyama *et al.* (2004) studied the effect of DDT in inducing toxicity to liver. Doxorubicin is an anthracycline antibiotic, popularly used in tumour therapy. Administration of doxorubicin was proved to cause hepatotoxicity (Kalender *et al.*, 2005).

Statins are among the most widely prescribed medications in the western world (Eidelman *et al.*, 2002). Their benefit in the primary and secondary prevention of

cardiovascular disease is unequivocal. According to Naga Chalasani (2005), intake of statin will lead to severe liver toxicity. As per the reports of Ishiyama *et al.* (1990) both hepatotoxicity and oxidative stress have been reported in rats following a single dose treatment with diethyl dithiocarbamate.In a study of Crupi *et al.* (2001), liver damage was induced by the use of carbon tetra chloride. Mitra *et al.* (1998) used paracetamol to induce hepatotoxicity in rats.

Herbal drugs have become increasingly popular among patients and physicians (Schuppan *et al.*, 1999) because of the perception that drugs derived from plants are normally safe. However, numerous reports have come forward, which showed hepatic adverse effects induced by plants containing pyrrolizidine alkaloids such as comfrey (Stickel and Seitz, 2000), preparations produced from germander (Larrey *et al.*, 1992), those containing chaparral extracts (Sheikh *et al.*, 1997), and drugs made of Chinese herbs (Yoshida *et al.*, 1996). These plants may contain alkaloids that are biotransformed to toxins. With most of the herbs, hepatotoxic reactions were reversible after withdrawal. Felix *et al.* (2003) studied the hepatotoxic effects of Kava (*Piper methysticum rhizoma*), which is used as a sleeping aid and for the treatment of anxiety disorders and depression.

2.4.2. Mechanism of drug induced liver injury

Mechanisms of drug-induced liver injury are many and varied. With many drugs, intermediary products produced during metabolism are highly reactive and toxic. In these situations, the balance between the rate of production of the metabolite and the effectiveness of the drug may determine whether or not hepatic injury occurs (Maddrey, 2005). Some earlier studies reported that diclofenac induced liver damage through various mechanisms such as mitochondrial permeability transition (Masubuchi *et al.*, 2002), activation of cytochrome P450 (Cantoni *et al.*, 2003) and generation of reactive oxygen species (ROS) (Cantoni *et al.*, 2003: Gomez-Lechon *et al.*, 2003). Ketoconazol has also been reported to mediate hepatotoxicity through chelating GSH with active toxic metabolites (Rodriguez and Buckholz, 2003).

Hepatic DNA fragmentation has been reported in may hepatotoxicity models following treatments with drugs such as acetaminophen (Ray *et al.*, 1992), carbon tetrachloride (Shi *et al.*, 1998) and diquat (Gupta *et al.*, 200). Amr and Alaa (2005) reported that in hepatotoxicity models induced by diethyl dithiocarbamate, diclofenac and ketoconazol, levels of serum ALT and AST has been significantly increased. These three toxic drugs decreased the levels of GSH and SOD. Diethyl dithiocarbamate induced hepatoxicity in rats was associated with reactive oxygen species (ROS) and drug metabolism (Ishiyama *et al.*, 1990).

Other mechanisms of hepatotoxicity are summarized in the following Table 2 (Dominique, 2000)

Liver injuries	Mechanisms
Acute hepatitis	Metabolite-mediated toxicity
	Metabolite-mediated immunoallergy and/or autoimmunity
Acute cholestasis	Inhibition of biliary secretion
Macrovacuolar steatosis	Decreased secretion of lipoproteins
Microvesicular steatosis	Inhibition of fattyacid mitochondrial beta-oxidation
Phospholipidosis	Inhibition of lysosomal phospholipases
Chronic hepatitis	Metabolite-mediated immune reaction
Vanishing bile duct syndrome	Autoimmune destruction of small bile ducts.
	Abnormal multidrug resistance protein system
Sclerosing cholangitis	Biliary ischemia caused by arterial lesions
Veno-occlusive disease	Metabolite-mediated endothelial lesions
Persinusoidal fibrosis	Activation of Ito cells

Table 2. Mechanisms of Hepatotoxicity

2.4.3. Treatment

A lot of treatment methods are found popular in the treatment of hepatoxicity. The stem bark of the Betulaceae plant *Alnus japonica*, which is indigenous to Korea, has been used as a popular folk medicine for hepatitis induced by acetaminophen (Kim *et al.*, 2004). Tazduq *et al.* (2005) reported that Silymarin can reduce hepatic toxicity induced by rifampicin, isoniazid and pyrazinamide. Betaine supplementation blocked or significantly attenuated the induction of hepatotoxicity by alpha-naphthylisothiocyanate (ANIT) (Kim *et al.*, 2005). Jigrine was found to be effective against galactosamine induced hepatitis (Abul, 2005). Jigrine, a polypharmaceutical herbal hepatoprotective formulation containing aqueous extracts of 14 medicinal plants, is used in Indian system of medicine (Unani). Kalender *et.al* (2005) reported that Vitamin E and Catechin could reduce the hepatotoxic effects of antitumour drug doxorubicin. Porchezhian and Ansari (2005) studied the heparoprotective effect of *Abutilon indicum* against carbon tetrachloride and paracetamol-induced hepatoxicity.

2.5.Antitubercular drugs

2.5.1. Tuberculosis

Tuberculosis (TB) is an infection caused by two species of Mycobacteria, "Mycobacterium Tuberculosis and Mycobacterium Bovis". Though it can cause disease involving every organ system in the body, it commonly affects the lungs. The disease was scraping the human kind even in the Neolithic period and till the early 20^{th} century. During the 19th century, up to 25 per cent of deaths in Europe were caused by this disease. The death toll began to fall as living standards improved at the start of the 20th century and from the 1940s, effective medicines were developed. However, there are now more people in the world with TB than there were in 1950, and three million individuals may die this year from this disease - mainly in less developed countries. The disease is more common in areas of the world where poverty, malnutrition, poor general health and social disruption are present. Currently, around 1.7 billion people worldwide, a third of the world's population, are infected by Mycobacterium tuberculosis and 3 million

deaths a year is attributable to tuberculosis. Rapid spread of TB in humans is attributed to crowded living conditions that favour airborne transmission. There was a steady decline in the incidence of tuberculosis, especially in the developed countries till early 1980s; but, since then the trend has reversed and an increasing number of cases have been reported.

2.5.1.2. Causes and Pathogenesis

The term tubercle bacillus refers to two species, "*Mycobacterium Tuberculosis* and *Mycobacterium Bovis*". Other species are classified under a typical Mycobacterial Pathogens. Humans are the only reservoir for *Mycobacterium tuberculosis*. Mycobacterium Bovis was transmitted by contaminated milk once, but is no longer so. Though skin infection by inoculation is seen in pathologists and laboratory personnels, almost all infections are due to airborne transmission by inhalation of droplet nuclei. In general, 3 - 4% of infected individuals will develop active disease during the first year after exposure and a total of 5-15% thereafter. The likelihood of developing active disease varies with the intensity and duration of exposure. Malnutrition, alcoholism, renal failure and uncontrolled diabetes favour the progression of infection to active disease. HIV infection is the strongest risk factor today. The tubercle bacilli, once inhaled, reach the terminal air spaces in the lungs, escaping from the host defense mechanism. They multiply locally but are controlled and retained in the lungs by the body's white blood cells. Sometimes infected white cells carry the bacteria to the lymph nodes and from there to the blood stream. Seeding and unchecked proliferation in other organs can cause disease manifestations elsewhere in the body as well. Pulmonary tuberculosis is usually seen in the upper portions of the lung.

2.5.1.3. Symptoms and Signs

The clinical picture depends on the involved organ. Early tuberculosis of the lung is asymptomatic and may be discovered on a chest x-ray by chance. In case of tuberculosis of the lung, the patient will complain of fever, night sweats, fatigue,

cough, sputum production that is sometimes mixed with blood, weight loss and loss of appetite.

Tuberculosis can also appear as swelling of the glands in the neck with or without fever (lymph node TB), back pain, deformity of the spine and weakness in the lower limbs (TB of the spine), fever, headache, vomiting and drowsiness (TB meningitis), joint pain and swelling (TB arthritis), genitourinary symptoms like flank pain and infertility (genitourinary TB).

2.5.1.4. Treatment and Prognosis

With the advent of 'Combination Chemotherapy', successful treatment of tuberculosis is a reality, but problems of drug resistance, selection of an inappropriate regimen and non-compliance hinder effective therapy. WHO recommends a six-month short course therapy consisting of four drugs - rifampicin, isoniazid, ethambutol and pyrazinamide, for the first two months and two drugs - rifampicin and isoniazid, for the next four months.

2.5.2. Isoniazid

Isoniazid, an antibiotic, is used to treat and prevent tuberculosis since, it prevents Tuberculosis bacteria from multiplying in the body. Isoniazid consumption is associated with a lot of side effects like live difficulty breathing, closing of the throat, swelling of the lips, tongue, or face, unusual weakness or fatigue, abdominal pain, yellow skin or eyes, dark urine etc. Isoniazid toxicity is associated with a high mortality rate (Wason, 1981). If isoniazid is taken acutely, as little as 1.5 g can cause toxicity. Doses larger than 30 mg per kg often produce seizures. Ingestion of the drug in amounts greater than 80 to 150 mg per kg can rapidly lead to death (Shannon *et al.*, 1990). A 1-2 % risk of severe and potentially fatal hepatotoxicity (predominantly hepatic necrosis) associated with the use of isoniazid is problematic in the prophylaxis and treatment of tuberculosis. In addition, daily isoniazid administration is associated with mild elevations of liver enzyme activities in plasma up to 20 % of patients (Troy *et al.*, 1998).

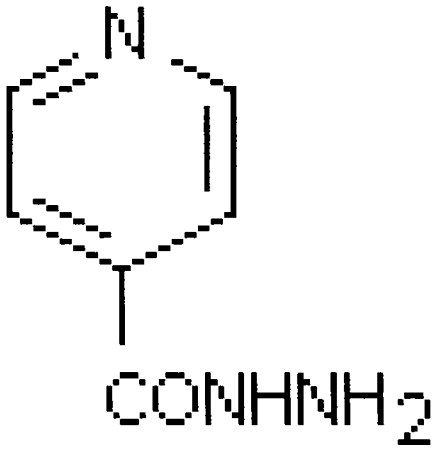


Fig. 2.7. Structure of Isoniazid

2.5.2.1. Structure

Chemically isoniazid is Isonicotinic acid hydrazide. Its molecular formula is $C_6H_7N_3O$. The structure of isoniazid is depicted in Fig. 2. 7.

2.5.2.2. Properties

Isoniazid (INH) has a molecular mass of 137.14g. Isoniazid is a white crystalline powder in occurrence. The substance decomposes on heating and on burning produces toxic fumes including nitrogen oxides. Melting point of Isoniazid comes around 170-173°C. INH is a colourless, odourless, white crystalline powder slowly affected by exposure to air and to light. 1 g isoniazid is soluble in 8 g water. A 10% solution has a pH of 6.0 to 8.0.Isoniazid can induce hepatotoxicity when administered for a long time (Saraswathy and Shyamala Devi, 1999). Hepatitis is due to a toxic metabolite of monoacetyl hydrazine, which binds covalently to liver proteins (Black *et al.*, 1975). In some patients, an allergic mechanism has also been proposed: acetylation of hepatic macromolecules by acetyl hydrazine may lead to the release of antigenic macromolecules, which induce the formation of antibodies directed against the liver (Davies, 1981). Isoniazid interacts with dietary supplements. Isoniazid can interfere with the activity of vitamin B_6 (Goldman and Braman, 1972). Vitamin B_6 supplementation is recommended, especially in people with poor nutritional status, to prevent development of isoniazid-induced peripheral neuritis (inflamed nerves) (Mandell and Petri Jr., 1996). Isoniazid may kill friendly bacteria, in the large intestine, that produce vitamin K. (Holt, 1998). Isoniazid may interfere with the activity of other nutrients, including vitamin B_3 (niacin), vitamin B_{12}, vitamin D, and vitamin E, folic acid, calcium, and magnesium (Werbach, 1997; Holt, 1998).

2.5.2.3. Metabolic pathway of Isoniazid

The major route of isoniazid metabolism is hepatic acetylation by N-acetyl transferase, which produces acetylisoniazed. The rate of acetylation is

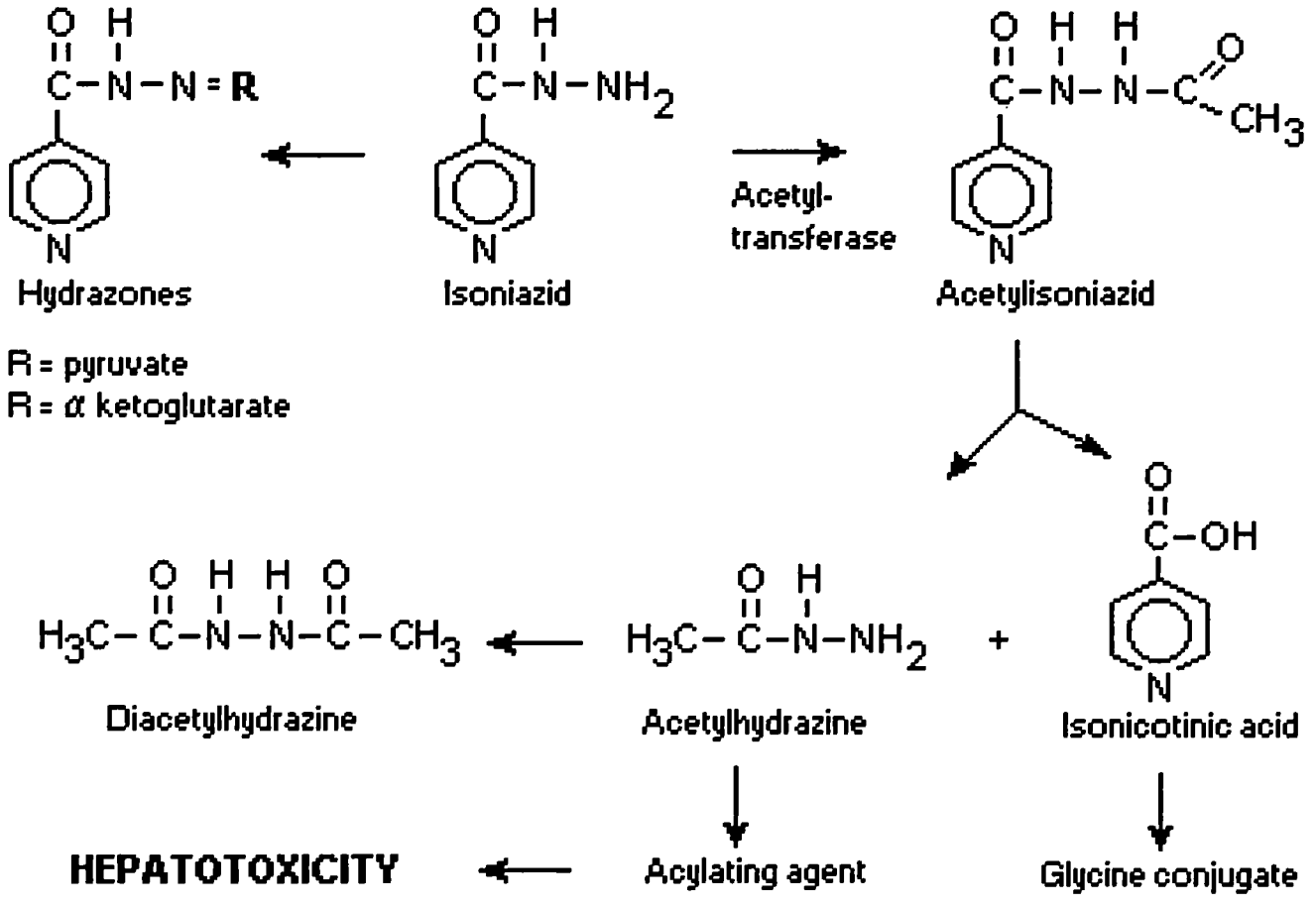


Fig. 2.8. Metabolic pathway of Isoniazid

genetically determined. Acetyisoniazid is further hydrolysed to isonicotinic acid and acetylhydrazine, both of which are excreted in the urine. Isonicotinic acid is conjugated with glycine. Acetylhydrozine is further metabolized to deacetylhydrazine and may be converted by the hepatic microsomal enzymes to the reactive metabolite (presumed to be hydrazine) that are thought responsible for INH-induced hepatotoxicity. Acid labile hydrazones of isoniazid are formed with a-ketoglutarate and pyruvate, but since these do not appear to any extent in the blood, they are thought to be produced in the pointed to cytolytic liver injury in rat studies, as a result of free radical generation, isoniazid bladder (Boxenbaum and Riegelman, 1974). Fig. 2. 8 shows the metabolic pathway of isoniazid.

2.5.2.4. Physiological changes

Some earlier studies have pointed to the cytolytic liver injury in rat studies, as a result free radical generation when isoniazid was used. These cytotoxic materials had been shown to come from lipid peroxidation and the suppression of the antioxidant system (Saraswathy *et al.*, 1998). Studies related to the suppression of the antioxidant system in antituberculosis drugs with rats have also been reported (Skakun and Slivka, 1992). Georgieva *et al.* (2004) reported that lipid peroxidation was found high in isoniazid treated animals.According to Tasduq *et al.* (2005) Alanine aminotrasferase (ALT) and Aspartate aminotransferase (AST) and Alkaline phosphatase (ALP) levels were increased when isoniazid was orally administered in rats for induced hepatotoxicity.

2.5.3. Rifampicin

Rifampicin is an antibiotic drug of the rifamycin group. It is typically used to treat mycobacterium infection, including tuberculosis and leprosy and also has a role in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) in combination with fusidic acid.

Rifampicin is a semisynthetic derivative of rifamycin antibiotics which are produced by the fermentation of a strain of *Streptomyces mediterranei*, a species which was first isolated in Italy in 1957 from a soil sample collected in France. The fermentation produces rifamycin B. Rifamycin B is transformed by a series of reactions into 3- formylrifamycin SV, which in turn is condensed with 1-amino-4-methylpiperazine in peroxide-free tetrahydrofuran to give rifampicin.

Rifampicin inhibits DNA-dependent RNA polymerase in bacterial cells by binding its beta subunit, thus preventing transcription of messenger RNA and subsequent translation to proteins.

2.5.3.1. Structure

Chemically rifampicin is 3-[[(4-methyl-1-piperazinyl)imino]methyl)] rifamycin. Structure of rifampicin is given in Fig. 2. 9.

2.5.3.2. Properties

Molecular formula is $C_{43}H_{58}N_4O_{12}$. Molecular weight is 822.96g. Its colour is red to orange. Powder in nature. Odourless. Melting point is 138 to 188 °C. A 1% suspension in water has pH 4.5 to 6.5. Rifampicin is very soluble in water. Solubility in aqueous solution is increased at acidic pH. Freely soluble in chloroform.

Patients receiving antitubercular drugs frequently develop acute or chronic hepatitis (Hussain, 2003). Prolonged use of Rifampicin may affect gastrointestinal system also (Wada, 1998). When rifampicin is administered the levels of ALT was increased. Bilirubin serum levels were also found increasing (Lenaerts, 2005).

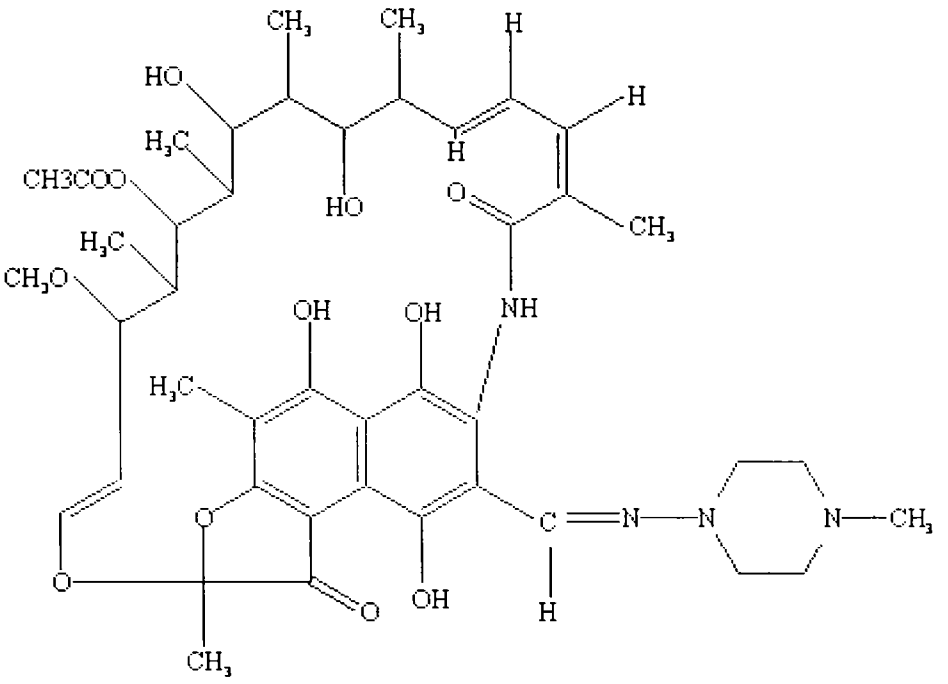


Fig. 2.9. Structure of rifampicin

This is an indication of drug-induced hepatotoxicity. Dawe *et.al* (2004) reported acute renal failure associated with rifampicin administration.

2.5.3.3. Physiological changes

An earlier reported study (Chi *et al.*, 2003) proved that intake of rifampicin increased the level of alanine aminotransferase (ALT). In a study conducted by Prabakan *et al.* (2000) The level of liver mitochondrial protein and the activities of isocitrate dehydrogenase, a-ketoglutarate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, NADH dehydrogenase and cytochrome *c* oxidase were found significantly decreased in rifampicin and isoniazid intoxicated rats.

3. MATERIALS AND METHODS

3.1. Drugs and chemicals

Ibuprofen was obtained from M/s. Cipla Pharmaceuticals Ltd., Mumbai; Bovine serum albumin, tetraethoxy propane, epinephrine and D-galactosamine from M/s Sigma Chemical Company, St. Louis, MO, USA. Glucosamine used in this study was prepared from chitin using a method developed in our laboratory.

Isoniazid and rifampicin were obtained by M/s. Lupin Pharmaceuticals Ltd., Mumbai. Bovine serum albumin, tetraethoxy propane and cholesterol were from M/s Sigma Chemical Company, St. Louis, MO, USA. Chitosan (MW 750000 Da; viscosity 8 cps; deacetylation rate 85-87%; purity 98.6%) used in this study was prepared from chitin in our laboratory (Madhavan, 1992).

All other chemicals used were of analytical grade.

3. 2. Animals

Wistar strain male albino rats, weighing 120-150g, were selected for the study. The animals were housed in polyurethane cages under hygienic conditions and maintained at normal room temperature. The animals were allowed food and water *ad libitum*. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethics Committee (IAEC).

3.2.1. Induction of ulcer (Santhosh *et al.*, 2005)

The ulcer was induced in experimental rats by oral administration of ibuprofen (50mg/kg, p.o.) twice in a day at an interval of 12 h. The animals were deprived of food for 24h prior to ulcer induction.

3.2.1.2. Experimental protocol

The experimental animals were divided into four groups of six rats each.

Group I, normal control rats received only the standard diet.

Group II, ulcer was induced by oral administration of ibuprofen (50mg/kg body weight/each rat, p.o) twice in a day at an interval of 12 h.

Group III, normal rats were treated with chitosan (100 mg/kg body weight/each rat/day, p.o for 20 days).

Group IV, pretreated with chitosan (100mg/kg body weight/each rat/day, p.o for 20 days) before induction of ulcer as described for Group II.

Group V, normal rats were treated with chitosan (100 mg/kg body weight/each rat/day, p.o for 20 days).

Group VI, pretreated with glucosamine (100mg/kg body weight/each rat /day, p.o for 20 days) before induction of ulcer as described for Group II.

At the end of the experiment, all six groups underwent surgery according to Takeuchi *et al.* (1976) and gastric juice was collected for 4 h. Rats were then killed with over dose of chloroform and the stomach was removed after the esophagus had been clamped. The gastric juice was centrifuged and the volume was noted. The stomach was inflated with normal saline and then incised through the greater curvature and examined for the number of lesions. The total acidity was determined by titration with 0.02 N NaOH with phenolphthalein used as indicator. One portion of the stomach tissue was kept in 10% formalin-saline for histopathological observations. The mucosal tissue was scraped from the stomach and the gastric mucosa was homogenized in ice-cold 0.1 M Tris-HCl, pH 7.2 and centrifuged. The supernatant was used for further biochemical analyses.

3.2.2. Induction of Hepatotoxicity (Santhosh *et al.*, 2006)

Hepatotoxicity was induced by the administration of rifampicin and isoniazid (200mg each /kg body weight/each rat/day).

3.2.2.1. Experimental Design

The experimental animals were divided into four groups of six rats each. Rats in **Group I** (normal control) were given only the standard diet. In **Group II**, normal rats were treated with chitosan (100 mg/kg body weight/day, intragastric intubation for 30 days). In **Group III**, normal rats were treated with glucosamine (100 mg/kg body weight/day, intragastric intubation for 30 days). In **Group IV**, hepatotoxicity was induced by oral administration of isoniazid and rifampicin (200mg each /kg body weight/day) for a period of 30 days. **Group V** animals were co-administered with chitosan (100mg/kg body weight/day, intragastric intubation for 30 days) along with antitubercular drugs described for Group IV. **Group VI** animals were co-administered with chitosan (100mg/kg body weight/day, intragastric intubation for 30 days) along with antitubercular drugs described for Group IV.

At the end of the experiment, rats were killed and blood was collected without any anticoagulant. The serum separated was used for different analyses. The liver and kidney tissues were excised immediately and washed with ice-cold saline. One portion of each tissue was fixed in 10% formalin-saline for histopathological observations.

Accurately weighed liver and kidney tissues were homogenized with 0.1M Tris HCl pH 7.4 and the homogenate was used for the estimation of enzyme activities and cellular macromolecules

3.3.1. Assay of alanine aminotransferase (EC 2.6.1.2)

The activity of alanine aminotransferase (ALT) was determined by the method of Mohur and Cook (1957).

1. Buffered substrate solution (0.1 M phosphate buffer, pH 7.4, 0.2 M DL-alanine, 2.0 mM 2-oxoglutarate): 1.5g dipotassium hydrogen phosphate, 0.2g potassium dihydrogen phosphate, 0.03g 2-oxoglutaric acid and 1.78g DL-alanine were dissolved in distilled water. The pH was adjusted to 7.4 with 1N NaOH and made up to 100 ml.

2. 20mg 2,4 dinitrophenyl hydrazine (DNPH) in 100 ml of hot 1N hydrochloric acid.
3. 0.4N Sodium hydroxide.

4. Standard pyruvic acid: 12.5 mg of sodium pyruvate was dissolved in 10 ml of distilled water.10 ml of this was diluted to 100 ml with distilled water and this was prepared freshly for the calibration curve.

Procedure

To 1.0 ml of the buffered substrate, 0.1 ml sample was added and incubated at 37°C for 30 min. The reaction was arrested by adding 1.0 ml of DNPH and left aside for 20 min at room temperature. Color developed by the addition of 10 ml of 0.4N NaOH was read at 540nm in a Shimadzu UV spectrophotometer against the reagent blank.

The enzyme activity was expressed as μ moles of pyruvate liberated /hr/l (serum); μ moles of pyruvate liberated /mg protein (liver)

3.3.2. Assay of aspartate aminotransferase (EC 2.6.1.1)

The aspartate aminotransferase (AST) activity was assayed by the method of Mohur and Cook (1957).

Reagents

1. Phosphate buffer: 0.15M, pH 7.5

2. Substrate: 300mg of L-aspartic acid and 50mg of α-ketoglutaric acid were dissolved in 20-30 ml of the phosphate buffer and added 10% sodium hydroxide to bring the pH to 7.5 and was made up to 100 ml with phosphate buffer.

3. 2,4-dinitro phenyl hydrazine (DNPH) reagent: Dissolved 200mg of DNPH in 85 ml of concentrated hydrochloric acid and made up to a litre with distilled water.

4. 0.4 N sodium hydroxide.

5. Standard pyruvic acid: 12.5mg of sodium pyruvate was dissolved in 10 ml of distilled water.10 ml of this was diluted to 100 ml with distilled water and this was prepared freshly for the calibration curve.

Procedure

To 1.0 ml of the buffered substrate, 0.1 ml of the sample was added and incubated for one hour at 37°C. Then 1.0 ml of DNPH reagent was added and left for 20 min. At the end of the incubation, 10 ml of 0.4 N NaOH was added and the colour developed was estimated by reading OD at 540nm in a Shimadzu UV spectrophotometer after 10 min. The standards were also treated similarly.

The enzyme activity was expressed as μ moles of pyruvate liberated /hr/l (serum); μ moles of pyruvate liberated /mg protein (liver)

3.3.3. Assay of lactate dehydrogenase (EC 1.1.1.27)

The lactate dehydrogenase (LDH) activity was assayed according to the method of King (1965) with slight modification. The amount of pyruvate formed in the forward reaction was measured colorimetrically.

Reagents

1. 0.1M glycine buffer: 7.5 g of glycine and 5.88 g of sodium chloride were dissolved in one liter of distilled water.

2. Buffered substrate: 2.76 g of lithium lactate was dissolved in 125 ml of glycine buffer containing 75 ml of 0.1 N sodium hydroxide to adjust the pH to 10. This was prepared just prior to use.

3. 0.4 N Sodium hydroxide.

4. 5.0 mg of NAD^+ was dissolved in 1.0 ml of distilled water. This was prepared just before use.

5. 2,4-dinitrophenyl hydrazine (DNPH): 200 mg of DNPH was dissolved in one litre of 1 N HCl.

6. Standard pyruvate solution: 12.5 mg of sodium pyruvate was dissolved in 100 ml of buffered substrate.

Procedure

To 1.0 ml of the buffered substrate, 0.2 ml of the sample was added and the tubes were incubated at 37°C for 15 min. After adding 0.2 ml of NAD^+ solution, the incubation was continued for 30 minutes and then 1.0 ml of DNPH reagent was added. And the tubes were incubated at 37°C for 15 min. Then 7.0 ml of 0.4 N NaOH was added and the colour developed was measured at 540 nm in a

Shimadzu UV spectrophotometer against the reagent blank. Suitable aliquots of the standards were also treated in the same manner.

The enzyme activity was expressed as μ moles of pyruvate liberated /hr/l (serum); μ moles of pyruvate liberated /mg protein (liver)

3.3.4. Assay of Alkaline Phosphastase (EC 3.1.3.1)

Alkaline phosphatase was assayed by the method King (1965) using disodium phenyl phosphate as the substrate.

Reagents

1. M carbonate-bicarbonate buffer, pH 10.0

2. Substrate: 0.01M disodium phenyl phosphate solution.

3. Folin's phenol reagent: Into a 1500 ml round-bottomed flask, 100 g of sodium molybdate, 700 ml water, 50 ml of 85% O-phosphoric acid and 100 ml of concentrated hydrochloric acid were added. The mixture was refluxed gently for 10 hours. Then, 150 g of lithium sulfate. 50 ml of water and few drops of bromine were added. The mixture was boiled for 15 minutes to remove excess bromine. The contents were cooled and diluted to one litre and filtered. This was diluted. 1:2 with double distilled water before use.

4. 15% sodium carbonate

5. 0.1 M magnesium chloride

6. Standard phenol solution: A solution of distilled crystalline phenol in water, containing 5.0 μg in 0.1 ml was prepared.

Procedure

The incubation mixture contained the following components in a final wolume of 3.0 ml.1.5 ml of carbonate-bicarbonate buffer, 1.0 ml of substrate and 0.1 ml of magnesium chloride and requisite amount of the enzyme source (0.2 ml serum). The reaction mixture was incubated at 37^0C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of Folin's phenol reagent. If turbidity appeared, the tubes were centrifuged. Controls without enzyme sources were also incubated and the enzyme source was added after the addition of Folin's – phenol reagent. The 1.0 ml of 15% sodium carbonate solution was added and incubated for a further 10 minutes at 37^0C. The blue colour developed was read at 640 nm against a blank. The standards were also treated similarly.

The activity of the enzyme is expressed as μ moles of phenol liberated/hr/l (serum); μ moles of phenol liberated/mg protein (liver)

3.3.5. Assay of Acid Phosphatase(EC 3.1.3.2)

The procedure adopted for the assay of acid phosphatase was the same as described above for alkaline phosphatase (King, 1965), excepting that a citrate buffer (0.1M,pH 4.9) was used and the magnesium ions were omitted from the incubation mixture. The enzyme activity is expressed as μ moles of phenol liberated/hr/l (serum); μ moles of phenol liberated/mg protein (liver)

3.3.6. Estimation of Glucose

Glucose was estimated by the method of Sasaki *et al.* (1972), using o-toluidine reagent.

Reagents

1. Trichloro acetic acid (TCA): 10%

2. O-Toluidine reagent : 12.5 g of thiourea and 12.0 g of boric acid were dissolved in 50 ml of distilled water heating over a mild flame. Exactly 75 ml of redistilled o-toluidine and 375 ml of acetic acid (A.R.) were mixed with thiourea-boric acid mixture and the total volume was made up to 500 ml with distilled water. The reagent was left in a refrigerator overnight and filtered.

3. Standard glucose solution : 10mg of pure glucose was dissolved in 100 ml of 0.2% boric acid in water.

Procedure

Mix 0.1 ml of blood with 1.9 ml of TCA solution to precipitate protein and then centrifuge. 1.0 ml of the supernatant was mixed with 4.0 ml of o-toluidine reagent was kept in a boiling water bath for 15 minutes. The green colour developed was read at 600 nm in a Spectonic Genesys spectrophotometer. A set of standard glucose solutions were also treated similarly. Blood sugar levels were arrived at by comparison with the standard curve.

The values are expressed as mg per dl.

3.3.7. Estimation of protein

The protein content was estimated by the method of Lowry *et al.* (1951)

Reagents

1. Alkaline copper reagent

   Solution A: 2% sodium carbonate in 0.1N sodium hydroxide solution. Solution B: 0.5 copper sulphate in water

   Solution C: 1% sodium potassium tartrate in water. 50ml of solution A was mixed with 0.5ml of solution B and 1ml of solution C just before use.

2. Folin's phenol reagent: Into a 1500ml round bottom flask, 100g sodium molybdate, 700ml water, 50ml 85% O-phosphoric acid and 100ml concentrated hydrochloric acid were added.The mixture was refluxed gently for 10 hours. Then 150g of lithium sulfate. 50ml of water and few drops of bromine were added. The mixture was boiled for 15 minutes to remove excess bromine. The contents were cooled and diluted to one litre and filtered. This was diluted 1:2 with double distilled water before use.

3. Standard bovine serum albumin (BSA): Dissolved 100mg of BSA in 100ml water in a standard flask. Small quantities of alkali could be added to the effect complete dissolution of BSA. 10ml of the stock was diluted to 100ml to get a working standard containing 100µg/ml.

Procedure

Pipetted out 0.01ml of serum, 0.1 ml of tissue homogenate and standard BSA in the range of 20-100µg into test tubes and the total volume was made upto 1.0 ml with water. The blank contained 1.0 ml of water. Exactly 4.5ml of alkaline copper reagent was added o all the tubes and left at room temperature for 10 minutes after which was added 0.5 ml Folin's phenol reagent. The blue colour developed was read after 20 minutes at 640 nm against the reagent blank, in a Schimadzu UV spectrophotometer.

Protein values are expressed as g per dl (serum); mg per g (tissue).

3.3.8. Electrophoretic separation of serum and plasma proteins

Plasma and Serum proteins were separated by SDS-PAGE technique as described by Laemmli (1970).

It is based on the principle that, in the presence of 10% SDS and 2-mercaptoethanol, proteins dissociate into their sub units and bind large quantities of the detergent which mask the charge of the proteins and giving a constant charge to mass ratio. So that the proteins move according to their molecular

weight in an electric field. In this discontinuous buffer system, the separating (resolving gels) and stacking gels are made up in the electrode buffer, Tris-glycine. During electrophoresis, the leading ion is chloride while the trailing ion is glycine. In this experiment, 7.5% gel concentration is used for the effective separation.

Reagents

1. Tris-HCl : 0.5M, pH 6.8
2. Tris HCl : 1.5M, pH 8.8
3. SDS : 10%
4. Acrylamide/Bis : 30%T, 2.67 % C.
5. Sample buffer :

   Distilled water: 3.8 ml

   Tris-HCl 0.5 M, pH 6.8:1 ml

   Glycerol: 0.8 ml

   10% SDS: 1.6 ml

   2-mercapto ethanol: 0.4 ml.

   1% Bromophenol blue: 0.4 ml.

6. Electrode Buffer :

   Tris base : 9 g

   Glycine : 43.2 g

SDS : 3 g

These reagents are dissolved in 600 ml of distilled water.

Working solution: Dilute 100 ml from stock to 500 ml with distilled water.

7. Separating gel (7.5%)

Distilled water: 4.85 ml

Tris-HCl 1.5M: 2.5 ml

10%SDS: 100 μl

Acrylamide: 2.5 ml

APS 10%: 50 μl

TEMED: 5 μl

8. Stacking Gel (4%)

Distilled water: 6.1 ml

Tris-HCl 0.5M: 2.5 ml

10% SDS: 100 μl

Acrylamide: 1.33 ml

APS 10%: 50 μl

TEMED: 10 μl

9. Ammonium per sulphate (APS): 10%

Procedure

Taken 0.1 ml of the suitably diluted plasma into a micro centrifuge tube and added 0.1 ml of the sample buffer, heated in a boiling water bath for 4-min, cooled and kept at 4^0 C in a refrigerator.

The separating gel was prepared without TEMED and APS. Evacuated for 15 min to remove air bubbles. Added TEMED and APS with intermittent shaking after each addition immediately transferred the solution to the apparatus. Added a little water on the top of the gel to level it. Kept for 45 min. Prepared stacking gel in the same way. Kept the comb over the apparatus, tilted it to 45^0, poured the gel slowly, and pressed the comb slowly and evenly. Kept for 45 min. Marked the wells. After removing the comb, the whole apparatus was transferred to the sandwich clamp assembly in to the inner cooling core. Rinsed the apparatus and wells with electrode buffer and filled the inner chamber of the apparatus completely and the outer chamber to the optimum level. Injected 10 μl of the sample into the wells. The electrode lid was placed at proper position and connections were established. The power of 200V was supplied. Electophoresis was carried out for 45 min approximately until the dye reaches the bottom. Subsequently, the gel was removed and is placed in a big petridish containing the stain, Coomassive blue. Kept for 30 min, and transferred the gel into 7% acetic acid for destaining. 7% acetic acid was changed intermittently till the gel got completely destained.

3.3.9. Extraction of Glycoconjugates

500mg of the tissue was weighed and the lipids were extracted using chloroform-methanol mixture by homogenization in a Potter-Elvehjem homogenizer with a Teflon pestle. The extraction was repeated thrice with fresh aliquots of the solvent mixture. The lipid extract was filtered through a fat free Whatmann No.41

filter paper into a separating funnel. The defatted tissue in the filter paper was dried and used for the estimation of hexose, hexosamine and sialic acid.

3.3.10.Estimation of Hexose

Hexose was estimated by the method of Niebes (1972).

Reagents

1. Sulphuric acid: water mixture (3:2,v/v).

2. 800mg of orcinol dissolved in 50 ml of 1 N H_2SO_4.

3. Orcinol-sulphuric acid mixture: 1.0 ml of reagent (2) was mixed with 7.5 ml of reagent (1). This mixture was prepared fresh at the time of assay.

4. Standard hexose: Equal quantities of galactose and mannose were dissolved in water to give a concentration of 100 μg/ml.

Procedure

An aliquot of the delipidised sample was treated with 1.0 ml of 0.1 N NaOH. Blank contained 1.0 ml of 0.1N NaOH .The tubes were cooled by placing in an ice-bath and 8.5 ml of Orcinol-sulphuric acid mixture was added slowly and mixed well. The tubes were stoppered, incubated at 80°C for 15 min in a water bath. Cooled and the color was allowed to develop in the dark for 25 min. The intensity was measured at 540 nm. Standard solutions containing 0.025 to 0.1 mg were treated similarly and hexose concentration was estimated.

The concentration of hexose was expressed as mg/g (tissue).

3.3.11. Estimation of hexosamine

Hexosamine was estimated by the method of Wagner (1979).

Reagents

1. Acetyl acetone reagent: 3.5% acetyl acetone in 1N Trisodium phosphate containing 0.5 N potassium tetra borate (98: 2 V/V).

2. Ehrlich's reagent: 3.2g of P-dimethyl aminobenzaldehyde was dissolved in 30 ml of 1 N HCl and diluted to 210 ml with isopropanol.

3. Standard Hexosamine: Galactosamine hydrochloride solution containing 10 mg/100 ml was prepared.

Procedure

An aliquot of the delipidised sample was hydrolyzed with 3 N HCl in a boiling water bath for 4 hrs and neutralized. 0.8 ml of the neutral hydrolysate was mixed with 0.6 ml of acetyl acetone reagent. The mixture was heated in a boiling water bath for 30 min, cooled and 2.0 ml of Ehrlich's reagent was added. The contents of the tubes were mixed and the absorbance was measured at 535 nm. Standard hexosamine solution containing 20 μg to 80 μg was used for the preparation of standard curve.

Hexosamine was expressed as mg/g (tissue).

3.3.12.Estimation of Sialic Acid

Sialic acid was estimated by the method of Warren (145)

Reagents

1. Sodium meta arsenite: A 10% solution was prepared in 0.5 M sodium sulphate in 0.1N H_2SO_4.

2. Sodium meta periodate : 0.2 M solution in 9 M phosphoric acid.

3. Thiobarbituric acid reagent : 0.6% solution was prepared in 0.5 M sodium sulphate.

4. Acidified butanol: 5 ml of concentration hydrochloric acid in 95 ml of n-butanol.

5. Standard sialic acid : 10mg of N-acetyl neuraminic acid was dissolved in 100 ml of water.

Procedure

An aliquot of the delipidised sample was hydrolysed with 0.1N H_2SO_4. at 80^0C for 1 hour. 0.2 ml of the hydrolysate was mixed with 0.1 ml of metaperiodate and the solution was kept at room temperature for 20 min.1.0 ml of sodium meta arsenite was added and shaken well so that the yellow brown colour disappered. 3.0 ml of thiobarbituric acid reagent was added and heated in a boiling water bath for 15 min. After cooling, 4.3 ml of acidified butanol was added, shaken well and the colour was extracted into butanol phase. The butanol phase was transferred to another set of tubes and the colour intensity was measured at 530 nm. The blank containing 0.2 ml of 0.1N H2SO4 and standard sialic acid solutions were treated similarly.

Sialic acid is expressed as mg/g (tissue)

3.3.13.Lipids

3.3.13.1.Extraction of total lipids

The total lipid content of the tissues was estimated by the method of Folch *et al.* (1957).

Reagents

Chloroform-methanol mixture (2:1 v/v)

Procedure

A weighed amount of the tissue was subjected to lipid extraction using chloroform-methanol mixture (2:1). The extraction was repeated twice with fresh aliquot of chloroform-methanol mixture. The lipid extracts were transferred to a separating funnel and added 20% of water into it and left overnight. Next day the lipid extracts were drained through filter paper containing anhydrous sodium sulphate and was collected in round bottom flask and was evaporated to dryness in a flash evaporator. The lipid in the round bottom flask was made up to 10 ml by using chloroform. From this 1.0 ml was taken into a pre-weighed vial and allowed to dry in warm temperature to constant weight and total lipid content were calculated from the difference in weight. Sample made up to 10 ml was used for the estimation of various lipid components viz., cholesterol (total and free), triglycerides, free fatty acids and phospholipids after evaporating the solvent in air at room temperature.

3.3.13.2. Estimation of total cholesterol

The total cholesterol present in plasma and heart was estimated according to method of Parekh and Jung (1970) with slight modifications.

Reagents

1. Standard cholesterol solution (stock): 1mg /ml in chloroform

2. Working standard: 1.0 ml of the stock was diluted to 10 ml with chloroform.

3. $FeCl_3$ stock solution: 10g $FeCl_3$ in 100 ml acetic acid.

4. $FeCl_3$ - H_2SO_4 reagent: 2.0 ml of $FeCl_3$ stock solution was diluted to 200 ml with conc. H_2SO_4.

5. 33% KOH (w / v): 10g of KOH was dissolved in 20 ml distilled water.

6. Alcoholic KOH solution: 6.0 ml of 33% KOH was made up to 100 ml with distilled ethanol. This solution is prepared fresh before use.

Procedure

1.0 ml of the lipid sample was taken into a 25 ml glass stoppered tube and evaporated off the chloroform. Added 5 ml of freshly prepared alcoholic KOH solution. The tubes were shaken well and incubated in a water bath at 37^0C for 55 min. After cooling to room temperature, added 10 ml of petroleum ether and inverted the tubes once to mix the contents. Then added 5.0 ml of distilled water and shaken the tubes vigorously for 1 min. Took 0.5-2 ml aliquots from the supernatant (petroleum ether) into test tubes. Evaporated the petroleum ether extract under nitrogen. To each of the sample as well as the standard tubes including the blank, added 3.0 ml of glacial acetic acid followed by 0.1ml -distilled water. Mixed the tubes thoroughly and added 2 ml of the $FeCl_3$ - H_2SO_4 reagent to the sides of the test tubes. A brown ring was formed at the interface; tap the bottom of the tubes well to effect mixing and a light colour appeared which changed to an immense purple colour, which was measured in a Shimadzu-UV spectrophotometer at 560 nm.

The amount of total cholesterol was expressed as mg/dl (serum); mg/g (tissue).

3.3.13.3. Estimation of triglycerides

The level of triglycerides in plasma and heart were determined by the method of Rice (1970) with slight modifications.

Reagents

1. Activated silicic acid.

2. Saponification reagent: 5.0g of potassium hydroxide was dissolved in 60 ml distilled water and 4.0 ml isopropanol.

3. Sodium metaperiodate reagent: To 77g of anhydrous ammonium acetate in 700 ml distilled water, added 60 ml glacial acetic acid and 650 mg of sodium metaperiodate and was dissolved and diluted to 1 litre with distilled water.

4. Acetyl acetone reagent: To 0.75 ml of acetyl acetone, 20 ml of isopropanol was added and mixed well.

5. Stock solution: 400mg of triolein was dissolved in 100 ml chloroform.

6. Working standard: 1.0 ml of the stock solution was diluted to 10 ml.

Procedure

0.2 ml of the lipid sample was taken into a test tube and evaporated off the chloroform, added 4.0 ml isopropanol. It was mixed well and added 0.4g of activated silicic acid. It was shaken in a vortex mixer for 15 min and centrifuged at 4000rpm for 5 min. To 2.0 ml of the supernatant and standards ranging from 20-100mg made up 2.0 ml with isopropanol, 0.6 ml of saponifying reagent was added and incubated at 60-70^{0}C for 15 min. After cooling, 1.0 ml sodium metaperiodate solution was added and mixed. To this, 5ml acetyl acetone was added, mixed and incubated at 50°C for 30 min. After cooling, the colour was estimated by measuring OD at 405nm in a Shimadzu-UV spectrophotometer.

The value of triglyceride in plasma was expressed as mg per dl and in tissue as mg per gm.

3.3.13.4. Estimation of free fatty acids

Free fatty acids in plasma and heart were estimated by the modified method of Horn and Menahan (1981) with colour reagent of Itaya (1977).

Reagents

1. Activated silicic acid

2. Chloroform, heptane, methanol (CHM) solvent mixture: It was prepared by mixing chloroform, heptane and methanol in the ratio of 200:150:7(v/v)

3. Copper-triethanolamine solution: 50 ml of 0.1M copper nitrate and 50 ml of 2M triethanolamine were mixed with 33 g of sodium chloride. The pH of the solution was adjusted exactly to 8.1.

4. Diethyldithiocarbomate (DDC) solution: 0.1% DDC in butanol was prepared.

5. Standard Stock: A solution containing 2 mg per ml of palmitic acid was prepared in CHM solvent. For working standard, the stock was diluted 1:10 in CHM to give a concentration of 200μg per ml.

Procedure

To 1.0 ml of the lipid sample, 6.0 ml of CHM solvent and 200mg of silicic acid were added. The mixture was shaken well, centrifuged at 4000 rpm for 5 min and 3.0 ml of the supernatant taken. Standard solution in the range of 25-100 μg were taken and made up to 3.0 ml with CHM solvent. The blank contained 3.0 ml of CHM solvent. To all these samples, 2.0 ml of copper triethanolamine solution was added and then mixed on a mechanical shaker for 10 min. The tubes were centrifuged at 4000 rpm for 5 min. To the 2.0 ml of the supernatant taken, 1.0 ml of DDC solution was added and shaken well. The colour intensity was read immediately at 430 nm in a Shimadzu-UV spectrophotometer.

Values were expressed as mg/dl serum and mg/g wet tissue.

3.3.13.5. Estimation of phospholipids

Phospholipid content of plasma and heart was estimated by the method of Fiske and Subbarow (1925) as inorganic phosphorus liberated after Bartlette's perchloric acid digestion (1959).

Reagents

1. Ammonium molybdate reagent: 2.5 g of ammonium molybdate was dissolved in 100 ml of water.

2. Aminonapthosulfonic acid (ANSA): 0.5 g of 1,2,4 aminonapthosulfonic acid was dissolved in 195 ml of 15% sodium metabisulfite and 50 ml of 20% sodium sulfite was added for complete solubilisation. The solution was filtered and stored in a brown bottle.

3. Stock standard solution: 351 mg of potassium dihydrogen phosphate was accurately weighed, dissolved and made upto 100 ml with double distilled water to give a final concentration of 80 mg phosphorus per ml.

4. Working standard: 1ml of the stock was diluted to 10 ml to give a conc. of 80 μg phosphorus per ml.

Procedure

1 ml of the lipid sample was taken into a test tube and evaporated off chloroform. Added 0.5 ml of perchloric acid, the tubes were made up to 3.0 ml with double distilled water, and 1.0 ml of aliquot was taken. The tubes were made up to 4.0 ml with double distilled water. To all the tubes, 0.5 ml of ammonium molybdate reagent was added. After 10 min, added 0.5 ml of ANSA to all tubes. Aliquots of the standards and blank were carried through the same procedure. The blue colour developed was read after 20 min, at 620 nm in a Shimadzu-UV spectrophotometer.

The phospholipid content of plasma was expressed as mg/dl in serum and mg/g in tissue.

3.3.14.Lipid peroxidation and tissue antioxidant status

3.3.14.1. Estimation of lipid peroxides (LPO)

Lipid peroxides content was determined by thiobarbaturic acid reaction as described by Ohkawa *et al* (1979).

Reagents

1. Acetic acid 20%: 20 ml of glacial acetic acid dissolved in 100 ml distilled water.

2. Thiobarbaturic acid: 8% in 20% acetic acid.

3. Sodium dodecyl sulphate: 8%

4. Standard: 41.66mg of Tetraethoxy propane (TEP) dissolved in 100 ml distilled water. 1.0 ml of above was made up to 10 ml with distilled water.

Procedure

To 0.2 ml of sample, 1.5 ml of 20 % acetic acid, 0.2 ml of sodium dodecyl sulphate and 1.5 ml of TBA were added. The mixture was made upto 4.0 ml with distilled water and heated in a boiling water bath for one hr. After cooling the mixture was centrifuged at 3000 rpm for 10 min. Supernatant was taken and absorbance was read at 532nm in a Shimadzu-UV spectrophotometer.

The lipid peroxides content was expressed as n moles of malondialdehyde per mg protein.

3.3.14.2. Determination of total reduced glutathione (GSH)

The total reduced glutathione was determined by the method of Ellman (1959). The method is based on the reaction of reduced Glutathione with 5,5'-

dithiosbis(2-nitrobenzoic acid) (DTNB) to give a compound that has absorbance at 412 nm.

Reagents

1. DTNB: 0.6mM in 0.2M Phosphate buffer pH 8.0

2. Phosphate buffer: 0.2M, pH 8.0.

3. Trichloroacetic acid: 5%

4. Standard: 61.4mg of reduced glutathione was dissolved in 100 ml .02M EDTA 0.1 ml of this is made up to 10 ml with 0.02M EDTA.

5. Working standard: 2.0 ml of the above was made up to 10 ml.

Procedure

0.5 ml of the homogenate was precipitated with 5% of TCA. The contents were mixed well for complete precipitation of proteins and centrifuged at 4000 rpm for 5 min. To an aliquot of clear supernatant, 2.0 ml of DTNB reagent and 0.2 M Phosphate buffer were added to make a final volume of 4.0 ml. The absorbance was read at 412nm against a blank containing TCA instead of sample, series of standards treated in a similar way were also run to determine the reduced glutathione content.

The amount of glutathione was expressed as n moles/g wet tissue.

3.3.14.3. Estimation of glutathione peroxidase (EC 1.11.1.9)

The method of Paglia and Valentine (1967) was adopted for assay of glutathione peroxidase.

Reagents

1. 0.4 M Phosphate buffer, pH 7.
2. 0.4M Tris buffer, pH 8.9
3. 0.4mM EDTA
4. 2mM GSH
5. 10mM NaN_3
6. 10% TCA
7. DTNB: 99 mg in 25 ml of methanol.
8. H_2O_2: 1mM was prepared freshly from commercial 30% solution.
9. GSH standard: 61.4mg GSH was dissolved in 100 ml distilled water.1.0 ml of this solution was made up to 10 ml with distilled water.
10. Working standard: 2 ml of the stock was made up to 10 ml with distilled water.

Procedure

0.2 ml of tissue homogenate was added to a mixture containing 0.2 ml of buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide, mixed well and added 0.1 ml reduced glutathione and 0.1 ml of hydrogen peroxide. Incubated in a water bath at 37°C for 10min. At the end of incubation period, 0.5ml of 10%TCA was added and centrifuged at 10000rpm for 5 min. 1.0 ml of the supernatant was taken into a separate test tube and added 2.0 ml Tris buffer and 50 µl DTNB. Immediately read the OD at 412nm.

The enzyme activity was expressed as n moles of glutathione oxidized per min per mg protein.

3.3.14.4. Assay of glutathione-s-transferase (EC 2.5.1.18)

Glutathione-S-tranferase activity was determined by the method of Habig *et al.* (1974).

Reagents

1. Phosphate buffer :0. 3 M, pH 6.5
2. 1-chloro-2, 4-dinitrobenzene (CDNB): 30mM
3. Reduced Glutathione (GSH): 30mM.

Procedure

The reaction mixture containing 1.0 ml of buffer, 0.1 ml of CDNB and 0.1 ml of tissue homogenate was made up to 2.5 ml with water. The reaction mixture was pre-incubated at 37°C for 5min. 0.1 ml of GSH was added and the change in the absorbance was measured at 340 nm for 3 min at 30 seconds intervals.

The enzyme activity was expressed as μ moles of CDNB conjugate formed/min./mg protein.

3.3.14.5. Assay of catalase (EC 1.11.1.6)

Catalase was assayed according to the method of Takahara *et al.* (1960).

Reagents

1. Phosphate buffer: 50 mM, pH 7.0
2. Hydrogen peroxide: 30 mM solution in the above buffer.

Procedure

To 2.45 ml of the phosphate buffer, 50 µl of the enzyme source was added and the reaction was started by the addition of 1.0 ml of H_2O_2 solution. The decrease in absorbance was measured at 240 nm at 30 sec intervals for 2 min. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide.

The enzyme activity was expressed as n moles of H_2O_2 decomposed per minute per mg protein.

3.3.14.6. Assay of superoxide dismutase (EC 1.15.1.1)

The superoxide dismutase was assayed according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme.

Reagents

1. 0.1 M Carbonate-bicarbonate buffer: pH 10.2 containing 57mg/dl EDTA.

2. Epinephrine: 3 mM

Procedure

Taken 50 µl of sample into the cuvette and add 1.5 ml buffer and 0.5 ml epinephrine mixed well and immediately read the change in optical density at 480 nm for 2 min in a Shimadzu-UV spectrophotometer.

One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation.

3.3.15. Membrane-bound ATPases

3.3.15.1. Assay of Total ATPase

Total adenonine triphosphatase was assayed according to the method described Hokins *et al.* (1973) which was modified from the method of Evans (1969).

Reagents

1. Imidazole-HCl buffer : 300 mM, pH 7.1
2. Sodium chloride : 1200 mM
3. Potassium chloride : 200 mM
4. Magnesium chloride : 60 mM
5. ATP solution : 40 mM
6. TCA : 4 %

Procedure

For the assay of total ATPase activity the incubation medium in a total volume of 1.0 ml contained the following: 0.1 ml each of buffer, sodium chloride, potassium chloride, magnesium chloride and 0.1 ml of enzyme solution. The mixture was incubated at 37^0C. The reaction was terminated by the addition of 2.0 ml of TCA. The suspension was centrifuged and phosphorous content of the supernatant was estimated.

The enzyme activity is expressed as μ moles of Pi liberated per mg proten

3.3.15.2. Assay of Na^+/K^+ -dependent ATPase

Na^+/K^+ dependent ATPase activity was measured from the amount of Pi released according to the method of Bonting (1970).

Reagents

1. 184 mM Tris buffer, pH 7.

2. 50 mM Magnesium Sulphate

3. 50 mM Potassium Chloride

4. 600 mM Sodium Chloride

5. 1.0 mM EDTA

6. 40 mM ATP

7. 10% TCA

Procedure

1 ml of Tris-buffer and 0.2 ml each of the above assay reagents were mixed together. So the assay medium, in the final volume of 2.0 ml contained, 92 mM Tris-buffer, 5 mM magnesium sulphate, 60 mM sodium chloride, 5 mM potassium chloride, 0.1 mM EDTA and 4.0 mM ATP. After 10 min equilibration at 37°C in an incubator, reaction was started by the addition of 0.2 ml of the enzyme solution. The assay medium was incubated for 30 minutes and at the end of the incubation period. The reaction was stopped by the addition of 2 volumes of ice cold 10% TCA. The phosphorus (Pi) liberated was estimated by the method of Fiske and Subbarow (1925).

The Enzyme activity was expressed as μ moles of Pi liberated per mg protein per hour.

3.3.15.3. Assay of Ca^{2+}-dependent ATPase

Ca^{2+} Dependent ATPase was assayed by the method of Hjerten and Pan (1983).

Reagents

1. 0.125 M, pH 8.0 Tris-HCl buffer.

2. 0.05 M Calcium chloride

3. 0.01 M ATP

4. 10% TCA

Procedure

Tris-HCl buffer 0.1 ml, Calcium 0.1 ml, ATP solution 0.1 ml and distilled water 0.1 ml were taken in test tubes. 0.1 ml of enzyme preparation was added and the tubes were incubated at 37°C for 15 min. The reaction was arrested by the addition of 10% TCA to the incubation mixture. 0.1 ml enzyme source was added to the control tubes. The contents were centrifuged at 4000 rpm for 5 min. The supernatant was used for the estimation of inorganic phosphorous.

The Enzyme activity was expressed as μ moles of Pi liberated per mg. protein per min.

3.3.15.4. Assay of Mg^{2+} -dependent ATPase

Mg^{2+}- ATPase was assayed according to the method described by Ohinishi *et al.* (1982)

Reagents

1. Tris-HCl buffer : 0.375M, pH-7.6
2. Magnesium chloride : 0.205 M
3. ATP : 0.01 M

Procedure

Buffer 0.1 ml, Magnesium chloride 0.1 ml and distilled water 0.1ml were taken in test tubes. 0.1 ml of enzymed preparation was added. The fibre were then incubated at 37^0C for 15 min. The reaction was stopped by the addition of 1.0 ml of 10% TCA. 0.1 ml of enzyme was added to control tubes. The tubes were centrifuged and the phosphorous content of the supernatant was estimated according to the method of fiske and subbarow. The enzyme activity is expressed as μ moles of Pi liberated per mg. protein per min.

3.3.16.Estimation of inorganic phosphorus

Inorganic Phosphorus was estimated by the method of Fiske and Subbarow (1925). The method is based on the formation of phoshomolybdic acid by the reaction between a phosphate and molybdic acid and its subsequent reduction to a dark blue phosphomolybdic acid, the intensity of which is proportional to the phosphate ion concentration.

Reagents

1. Ammonium molybdate reagent: 2.5 g of ammonium molybdate was dissolved in 100 ml of 3 N sulphuric acid.

2. Amino naphthol sulphonic acid (ANSA): 0.5 g of ANSA was dissolved in 195 ml of 15% sodium metabisulphite and 5.0 ml of 20% sodium sulphate was added for complete solubilization. The solution was filtered and stored in a brown bottle.

3. Standard Phosphorus: 35.1 mg of potassium dihydrogen phosphate was accurately weighed, dissolved and made up to 100 ml with distilled water.

Procedure

To suitable aliquots of the supernatant, 1.0 ml of ammonium molybdate reagent was added. 0.4 ml of ANSA was added after 10 min incubation at room temperature, standards and blank were also treated in the above manner. The blue colour developed was read after 20 min at 640 nm in a Shimadzu-UV Spectrophotometer.

The values were expressed as μg per mg. protein.

3.3.17. Pepsin

The activity of pepsin was estimated by the method of Anson (1938)

Reagents

1. Pepsin substrate: Haemoglobin in 0.06 N HCl
2. Sodium carbonate : 0.55 M
3. Trichloro acetic acid : 7%
4. Standard tyrosine : 10 mg of tyrosine dissolved in 100 ml of distilled water
5. Folin's ciocaltaue reagent

Procedure

1.0 ml of the substrate was added to 0.2 ml of the enzyme and incubated at 37^0 C for 10 minutes. The reaction was terminated by adding 1.0 ml of 7% TCA, and the mixture was centrifuged at 1000 mg for 10 min.1.0 ml of supernatant was made alkaline by 5.0 ml of 0.55 M sodium carbonate. Then 0.5 ml of 1N Folin's

reagent was added. The optical density was measured at 660 nm after 30 min. at room temperature in Shimadzu spectrophotometer.

Results were expressed as μ moles of tyrosine liberated/4h.

3.3.18 Estimation of Amino Acids

Total amino acids in the tissue was determined as per the procedure of Rajendra (1987).

Reagents

1. HCl : 6 N

2. HCl : 0.05 M

3. Buffer A: Dissolve tri sodium citrate (58.8 g) in 2 liters of double distilled water, add 210 ml ethanol of 99.5%, adjust the pH to 3.2 by adding 60% perchloric acid and make up to 3 liters using double distilled water.

4. Buffer B: Dissolve tri sodium citrate, 58.8 g and boric acid, 12.4 g in double distilled water, adjust the pH to 10 by adding 4 N NaOH, and make up the volume to 1 liter using double distilled water.

5. Phthaladehyde (OPA) Buffer: Dissolve 122.1 g. of Na_2CO_3, 40.7 g. of H_3BO_3 and 56.4 g. of K_2SO_4 in double distilled water and make up the volume to 3 liters.

6. Phthalaldehyde solution (OPA): Dissolve 400 mg OPA, 7 ml ethanol, 1 ml of 2- Mercaptoethanol and 2ml of 30% w/v Brij-35 in 500 ml OPA buffer.

5. Sodium hypochlorite solution: 4% w/v Sodium hypochlorite in OPA buffer. ie., 0.3 ml Sodium hypochlorite in100 ml OPA buffer.

3.3.18.1 Total amino acids

3.3.18.1.1 Principle

The amount of each amino acid present within a given protein does not vary from molecule and can provide useful information about the nature of the protein molecule. In a typical analysis of the amino acid content of a protein, peptide bonds are broken by acid hydrolysis with 6 N HCl at 110^0 C (24h) so that the released amino acids can be assayed. The amino acid tryptophan is not stable to acid digestion in the presence of even trace amounts of oxygen and is estimated separately by alkali digestion.

3.3.18.1.2 Sample preparation

Take (100 mg tissue) sample in a heat stable test tube. Add 10ml 6NHCl and heat seal the tube after filling with pure nitrogen gas. Carry out the hydrolysis at 110^0C for 24 hours. After the hydrolysis is over, open the test tube. Remove the contents quantitatively and filter into a round bottom flask through Whatmann filter paper No. 42. Wash the filter paper 2-3 times with distilled water. Flash evaporate the contents of the flask to remove all traces of HCl, the process should be repeated for 2-3 times with distilled water. Dissolve the residue and make up to 10 ml with 0.05 M HCl.

3.3.18.1.3 HPLC Analysis

Filter the sample thus prepared again through a membrane filter of 0.45 µm and inject 20 µl of this to an amino acid analyzer (HPLC- LC 10 AS) equipped with cation exchange column packed with a strongly acidic cation exchange resin i.e., styrene di vinyl benzene co polymer with sulphinic group. The column used was Na type i.e., ISC- 07/S 1504 Na having a length of 19 cm and diameter 5mm.

The instrument was equipped with Shimadzu FL 6A fluorescence detector and Shimadzu CR 6A Chrompac recorder. The mobile phase of the system consists of two buffers, Buffer A and buffer B. A gradient system can be followed for the effective separation of amino acids. The oven temperature can be maintained at 60^0 C. The total run was programmed for 62 minutes.The amino acid analysis can be done with non-switching flow method and fluorescence detection after post–column derivatization with o-phthalaldehyde.In the case of proline and hydroxyl proline, imino group is converted to amino group with hypochlorite.

Run an amino acid standard (Sigma chemical Co., St. Lousis, USA) also to calculate the concentration of amino acids in the sample. Calibration of equipment using standards needs to be done before the start of analysis.

3.3.18.1.4 Quantification of amino acids

The standard and the sample were analyzed under identical conditions. The elution time of the amino acids of the sample was compared and identified with those of the standard.Quantification of amino acid was done by comparing the respective peak areas I n the chromatogram of the sample and the standard.

The amount of each amino acid is expressed as nmol/g tissue.

3.3.18.2 Estimation of tryptophan

Tryptophan was estimated as per the method of Sastry and Tummuru (1985) after alkali hydrolysis of the sample using 5% sodium hydroxide at 110^0C for 24 hours.

The 5-hydroxy furfural resulting from sucrose under acidic conditions formed a pale green coloured condensation product with thioglycolic acid, which on treatment with tryptophan formed a pink coloured complex. The colour intensity is measured at 500 nm.

Reagents

1. NaOH : 5%
2. HCl : 6N
3. Sucrose :2.5%
4. Thioglycolic acid : 0.6%
5. H_2SO_4 :50%
6. HCl : 0.1N
7. Tryptophan Standard: 10μg/ ml solution

Procedure

Sample was hydrolyzed with 10 ml of 5% NaOH at 110^0C for 24 hours in a sealed tube with pure nitrogen. The hydrolysate was neutralized to pH 7.0 with 6 N HCl using phenolphthalein indicator. The volume was made up to 25 ml with distilled water. The solution was then filtered through Whatman filter paper no.42 and filtrate was used for estimation.

The test tube containing 4 ml of 50% H_2SO_4, 0.1mlk of 2.5% sucrose and 0.1ml of 0.6% thioglycolic acid were added. These tubes were kept for 5minutes in water bath at 45-50^0C and cooled. The sample was then added to the test tubes. A set of (0.1 to 0.8ml) standard tryptophan (10μg/ ml solution) was run in a similar way. The volume was made up to 5ml with 0.1N HCl and allowed to stand for 5 minutes for the development of colour. The absorbance was measured against a reagent blank at 500 nm in a spectrophotometer.

3.3.19 *Estimation of urea*

Urea was estimated by the method of Natelson *et al.* (1951) using diacetyl monoxime.

Reagents

1. Diacetyl monoxime reagent: 2.0 g of diacetyl monoxime was dissolved in 100 ml of 2.0% acetic acid.

2. Sulfuric acid –phosphoric acid mixture: 25 ml of concentrated sulfuric acid, 75 ml of 85%0- phosphoric acid and 70 ml of distilled water were mixed.

3. Sodium tungstate solution: 10%

4. Sulfuric acid: 0.67 N

5. Standard urea solution: 20 mg urea dissolved in 100 ml of distilled water. 200 μg/ml

6. Working standard: 1.0 ml diluted to 10 ml using distilled water.20μg/ml

Procedure

To 0.1 ml of blood was added 3.3 ml of water and mixed with 0.3 ml of 10% sodium tungstate and 0.3 ml of 0.67 N sulfuric acid reagent. The suspensions were centrifuged and to 1.0ml of the supernatant, 1.0 ml of water, 0.4 ml of diacetylmonoxime and 2.6ml of sulfuric acid-phosphoric acid reagent were added in that order. Aliquots of standard urea were also treated in a similar manner and heated in a boiling water bath for 30 minutes, cooled and the colour developed was measured at 480 nm in a Shimadzu-UV spectrophotometer.

The values were expressed as mg per dl blood.

3.3.20 Estimation of creatinine

Creatinine was estimated by the method of Slot (1965)

Reagents

1. Picric acid : 40mM
2. NaOH : 0.75 M
3. Sodium tungstate solution : 100g/liter of water
4. Sulphuric acid : .33 M
5. Creatinine standard : 10 mM of creatinine in 0.1 M HCl. Just before use
6. Working standard : 2 ml of stock standard was diluted to 100 ml with distilled water.

Procedure

To 0.1ml of serum 1.5 ml of distilled water and 0.5 ml of sodium tungstate were added. 1.0ml sulphuric acid was added with shaking and allowed to stand and centrifuged. To 3.0 ml of supernatant 1.0 ml picric acid and 1.0 ml NaOH were added. The color developed was read at 500 nm after 15 minutes.

The values are expressed as mg/ dl.

3.3.21 Lipoprotein fractionation

Addition of heparin-manganous chloride to serum caused the precipitation of LDL. The supernatant represented the HDL fraction.

3.3.21.1 Estimation of high density lipoprotein fraction

Total HDL was separated by the method of Burstein and Scholnick (1972).

Reagents

1. Heparin-Manganous chloride reagent: 3.167 gm of manganous chloride was added to 1.0 ml of heparin containing 20,000 units/ml. This was made up to 8.0 ml with water.

Procedure

0.1 ml of serum was added to 9μl of heparin-manganous chloride reagent and mixed well. The solution was allowed to stand at 4°C for 30 min. The supernatant represented HDL fraction. Aliquots were taken from HDL fraction for the estimation of cholesterol.

3.3.21.2 Estimation of low density lipoproteins

This differential analysis was made by the method of Brustein and Scholink (1972) using sodium dodecyl sulphate.

Reagent

1. Sodium dodecyl sulphate: 10% in 0.15 M sodium chloride pH 9.0

Procedure

To 0.1 ml of serum, 75 μl of sodium dodecyl sulphate solution was added, which was taken in a polycarbonate centrifuge tube. The contents were swirled briefly and packed for 2 hrs in a water bath at 35°C. The contents were centrifuged in a refrigerated centrifuge at 10,000g for 30 min. VLDL got aggregated as a pellicle at the top. The supernatant was a mixture containing HDL and LDL cholesterol was estimated in 0.05 ml aliquot of the supernatant as described above.

3.3.22 Analysis of fatty acids (FAME)

Fatty acids were analyzed according to the method of AOAC (1975). Lipid content of the tissues was estimated by the method of Folch et al (1957). Methyl esters of fatty acids from animal and vegetable origin having 8-24 atoms are separated and detected by gas chromatography. Method is not applicable to epoxy, oxidised, or polymerized fatty acids. (AOAC-1975 Gas chromatography method.)

Reagents

1. Boron trifluoride reagent.
2. Methanolic sodium hydroxide solution.
3. Petroleum ether.
4. Sodium sulphate.

Procedure

Add sample (lipid of known weight) to flask followed by methanolic NaOH and boiling chip. Attach condenser, and reflux until fat globules disappear (usually 5-10 minutes). Add BF_3 solution from bulb or automatic pipette through condenser and continue boiling for 2 minutes. Remove heat, then condenser, and add 15ml saturated NaCl solution. Stopper flask and shake vigorously 15s while solution is still tepid. Transfer aqueous phase to 250ml separator. Extract with two 30ml portions of petroleum ether (b.p 60-80°C). Wash combined extracts with 20ml portions H_2O, dry over anhydrous Na_2SO_4, filter and evaporate solvent under stream of nitrogen on steam bath.

Methyl esters of the fatty acid thus obtained were separated by gas liquid chromatography (Varian CP 3800. U.S.A) equipped with a capillary column (30m long and 0.54mm diameter) and a flame ionization detector in the presence of

hydrogen and air. The carrier gas was nitrogen and the flow rate was 4ml/min the chromatograph temperature started at150^{0}C and was increased 4 ^{0}C/min until a temperature of 250 ^{0}C was obtained. Fatty acids separated were identified by the comparison of retention times with those obtained by the separation of a mixture of standard fatty acids. Measurement of peak areas and data processing were carried out by electronic integrator. Individual fatty acids were expressed as a percentage of total fatty acids.

3.3.23 Estimation of minerals using Atomic Absorption Spectrophotometer.

Minerals were estimated according to the method of the AOAC (1980).

Reagents

1. Nitric acid
2. Perchloric acid
3. 1& 2 in 9:4
4. Stock solution of sodium, potassium, calcium zinc, copper, magnesium, manganese, selenium and iron were prepared by diluting concentrated solution of 1000 mg/L (Merck).

Procedure

Samples size of 1 g of tissue and 1 ml serum were used for the experiment. To the sample containing flask, 7 ml of nitric acid and perchloric acid (9:4) mixture was added, covered with a watch glass and left at room temperature over night. The sample was then digested using a microwave digester (Milestone ETHOS PLUS lab station Closed Vessel Microwave Digestion System). The completely digested samples were allowed to cool at room temperature, filtered (glass wool) carefully transferred into a clean 50 ml volumetric standard flask and then diluted to the mark with ultra pure water (Milli Q, Millpore). The digested samples were

analyzed using Varian SpectrAA 220 Atomic Absorption Spectrometer equipped with a deuterium back ground corrector, for the determination of minerals viz sodium, potassium, calcium zinc, copper, magnesium, manganese, selenium and iron.

3.4 Statistical analysis

Results are expressed as mean ± SD. One-way analysis of variance (ANOVA) was carried out, and the statistical comparisons among the groups were performed with Tukey's test using a statistical package program (SPSS 10.0 for Windows).

4. RESULTS AND DISCUSSION

4.1. ANTIULCEROGENIC POTENTIAL OF CHITOSAN AND GLUCOSAMINE

4.1.1. Gross pathology

In gross pathology of ulcer-induced stomach, ibuprofen caused ulcerations are apparently visible as dark red lesions **(Plate 4.1.2)**. These are non-malignant wounds on the mucosal membrane by the action of gastric acid. In control animals, the mucosal membrane was lesion free **(Plate 4.1.1)**. Chitosan and glucosamine treated animals showed lesser number of ulcerations on the mucosa **(Plates 4.1.3 and 4.1.4)** showing the antiulcer effect of these compounds.

4.1.2. Histopathology

In the ibuprofen ulcer models, more facial tissue differentiations were observed after administration of the active fraction in bleeding areas and mucosa than those of control group. On the other hand, inflamed cell infiltration and edema in lamina propria were pronounced in the test group when compared with the control group on *per os* administration, while the effects were less on subcutaneous administration. This observation can be interpreted as the co-existence of some compounds in the active fraction, which might affect the gastric mucosa on *per os* application **(Plate 4.1.8)**.

In chitosan and glucosamine pre treated animals, histopathological results of the stomach shows a normal cell architecture **(Plates 4.1.9 and 4.1.10)** as that of control animals **(Plate 4.1.5)**. All the tissue alterations were normalized by chitosan and glucosamine. Histology of the stomach of rats fed with chitosan or glucosamine alone did not show any characteristic change **(Plates 4.1.6 and 4.1.7)**.

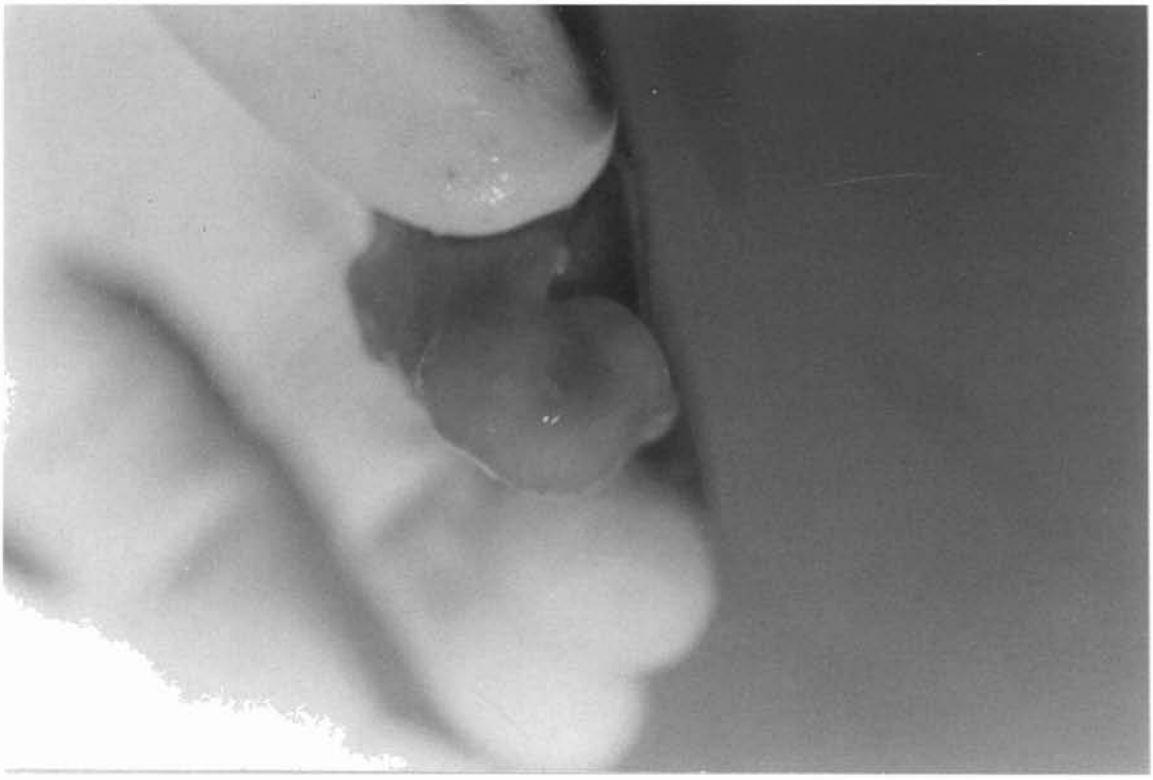


Plate 4.1.1. Gross pathology of the stomach of normal control rat

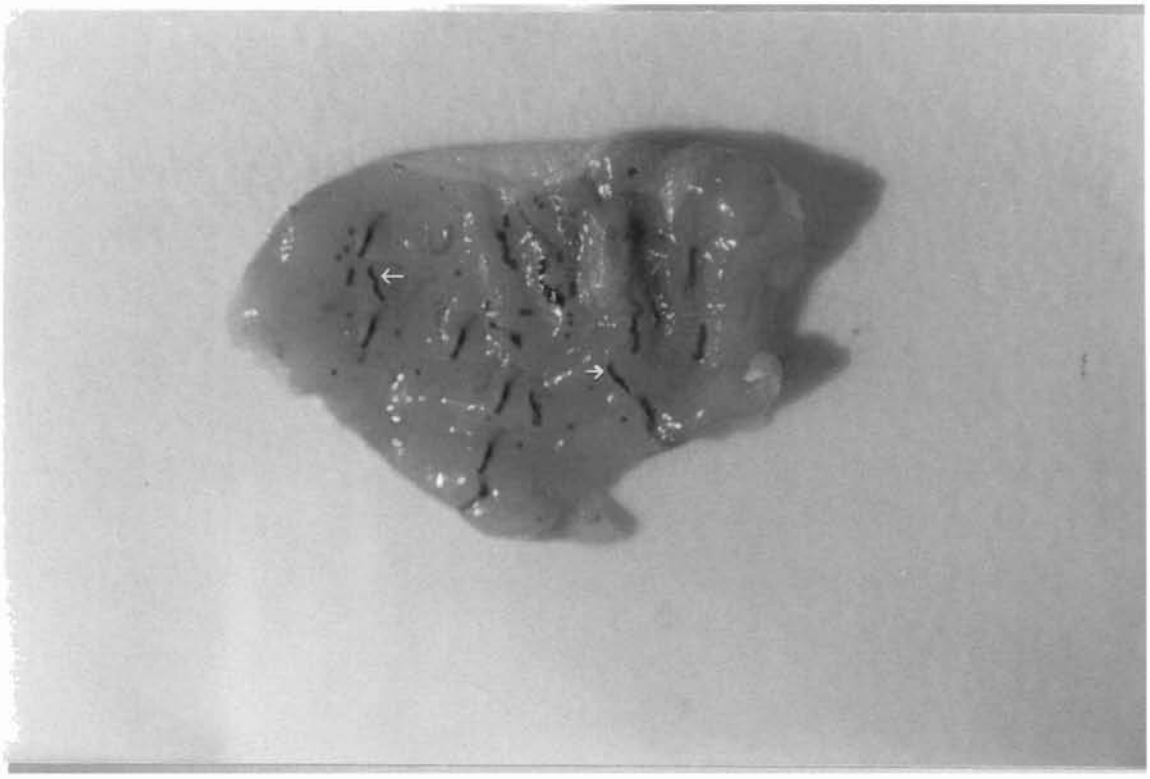


Plate 4.1.2. Ulcer induced stomach of albino rat by the administration of ibuprofen. Mucosal lesion is indicated by arrow

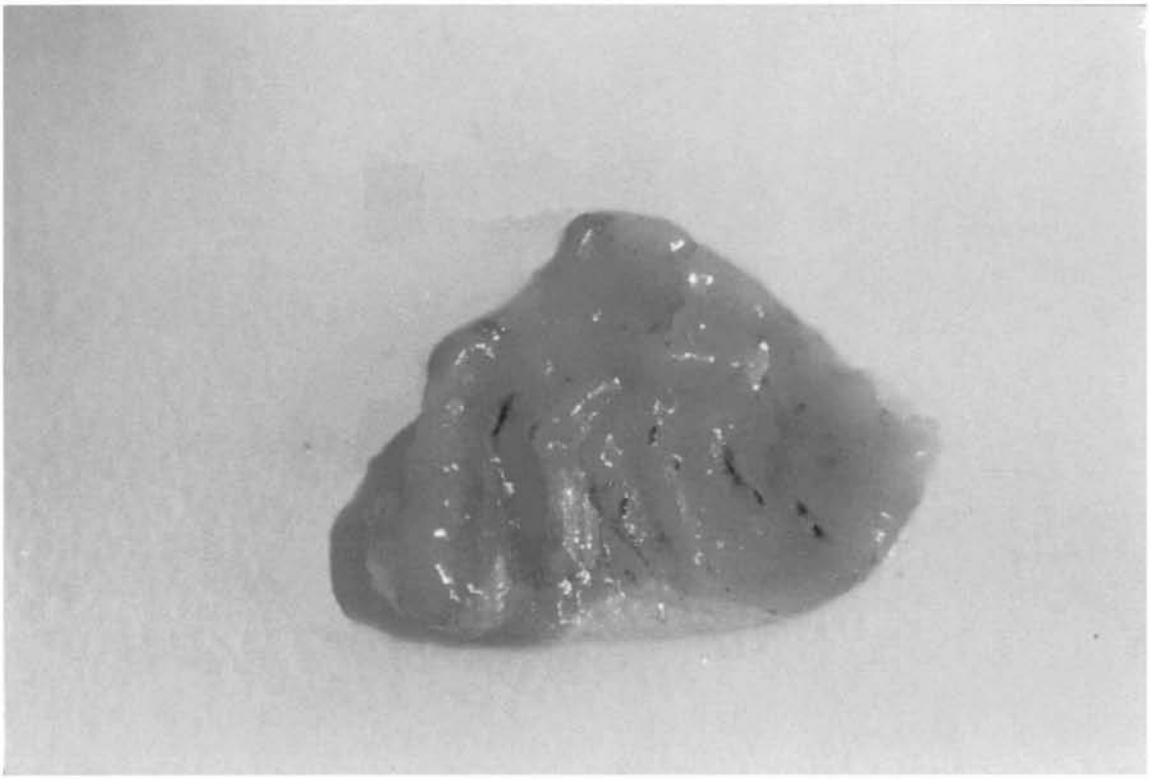


Plate 4.1.3. Gross pathology of the ulcer treated rat stomach with chitosan pre-oral administration, which shows lesser mucosal lesions compared to ulcerated rats.

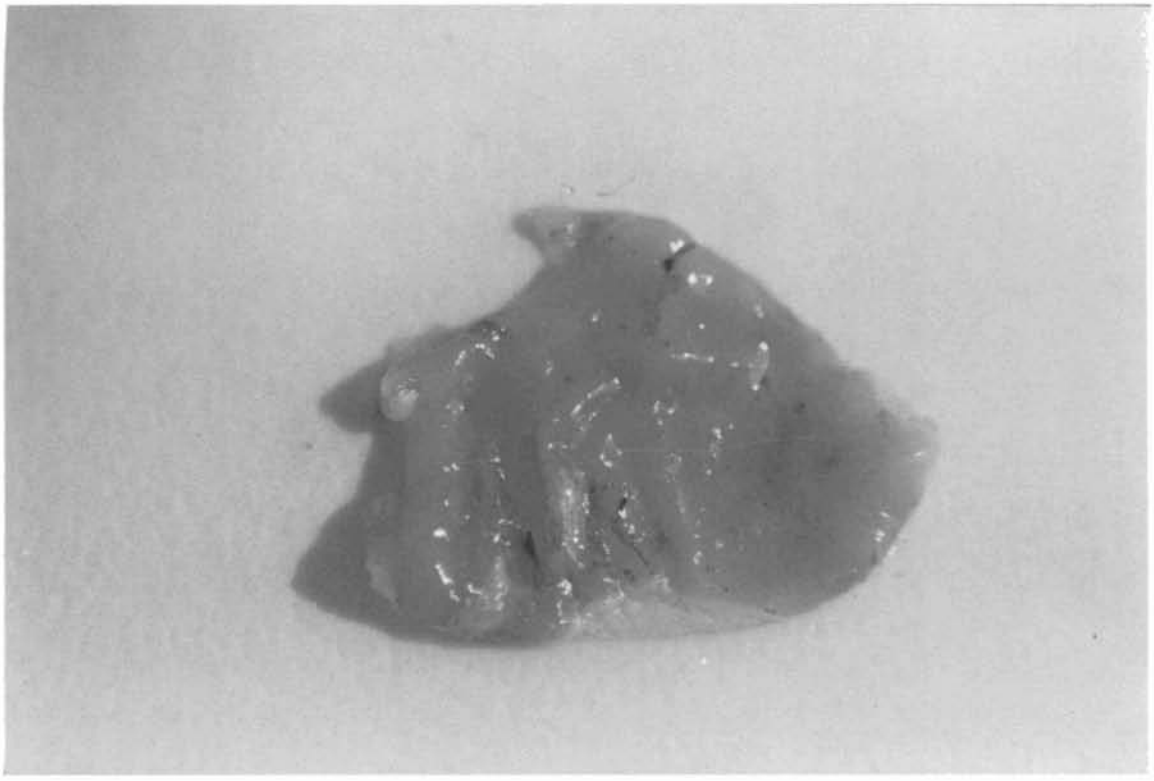


Plate 4.1.4. Stomach of ulcerated rat treated by the pre-oral administration of glucosamine. Most of the ulcerated lesions were vanished as compared to ulcer-induced rats by the treatment with glucosamine.

4.1.3. Gastric mucosal lesions

Table 4.1.1 shows the number of lesions present in the gastric mucosa, of the normal and experimental groups of rats.

Experimental peptic ulcer may be assessed on the basis of number of gastric mucosal lesions. In the present study, a significant ($P<0.001$) increase in the number of lesions in the gastric mucosa was observed in Group III ulcer induced rats as compared to that of Group I control animals. This is in accordance with an earlier report (Lanza *et al.*, 1989), which showed that ibuprofen-induced lesion formation may be multifactorial, with stasis of gastric flow contributing significantly to the hemorrhagic as well as the necrotic aspects of the tissue injury. The products of the 5-lipoxygenase pathway may also play a key role in the development of this ulcer (Rainsford, 2005). The prior oral administration of chitosan and glucosamine resulted in significant ($P<0.001$) reduction in the number of lesions in the gastric mucosa of Group V and Group VI rats as compared to Group IV, ulcer induced rats, indicating the cytoprotective activity of chitosan and glucosamine. Mutoh *et al.* (1995) have postulated that the adoptive cytoprotection in cultured gastric mucus producing cells are mediated by glucosamine rich mucus released in response to a mild irritant. But animals administered with chitosan alone and glucosamine alone did not show adverse effects, which show that chitosan and glucosamine are non-toxic.

4.1.4. Volume of gastric juice, acidity and peptic activity

Volume of gastric juice, acid out put and peptic activity in normal and experimental groups of rats were detailed in **Table 4.1.1**.

Significant ($P<0.001$) increase in acidity and volume of the gastric juice was noticed in Group III ulcer induced rats as compared to that of Group I control animals. Also a significant decline in peptic activity was also observed. These findings concur with an earlier reported study (Welage, 2005). Increased production of hydrochloric acid in ulcerated condition might be a consequence of

Table 4.1.1: Number of lesions, volume of gastric juice, and acid output and pepsin activity of the gastric mucosa of normal and experimental groups of rats.

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Ulcer-Induced C	Group V A+C	Group VI B+C
No of lesions	-	-	-	8.78±1.42[a,b,c]	2.34±0.09[d]	1.25± 0.06[d]
Volume of Gas Juice	1.45±0.05	1.39±0.03	1.37±0.04	2.76±0.09[a,b,c]	1.87±0.06[d]	1.55±0.05[d]
Acid out put	156±12.3	146±11.2	138±14.7	293±18.2[a,b,c]	205±16.6[d]	172±15.5[d]
Pepsin	723±58.1	712±55.3	698±63.5	576±41.9[a,b,c]	668±53.2[d]	682±54.7[d]

Values are expressed as, Mean ± SD for six animals in each group.
Volume of gastric juice- ml/4h; Acid output- µEq/4h; Pepsin- µmol tyrosine liberated/4h.

[a] $P<0.001$- compared with Group-I

[b] $P<0.001$- compared with Group-II

[c] $P<0.001$- compared with Group-III

[d] $P<0.001$- compared with Group-IV

[e] $P<0.01$ - compared with Group-III

[f] $P<0.01$ - compared with Group-II

[g] $P<0.05$ - compared with Group-II

[h] $P<0.05$ - compared with Group-IV

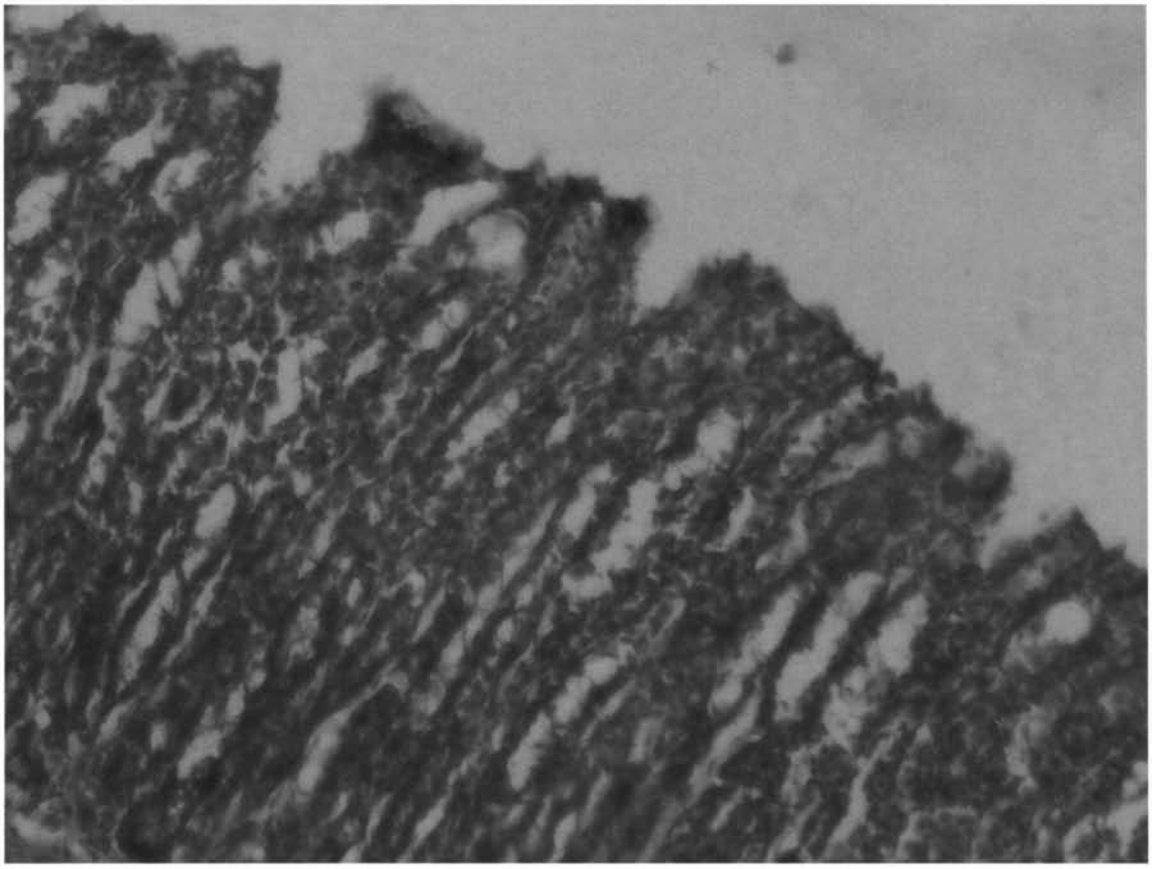


Plate 4.1.5. Histopathology of the gastric mucosa of the normal rat.

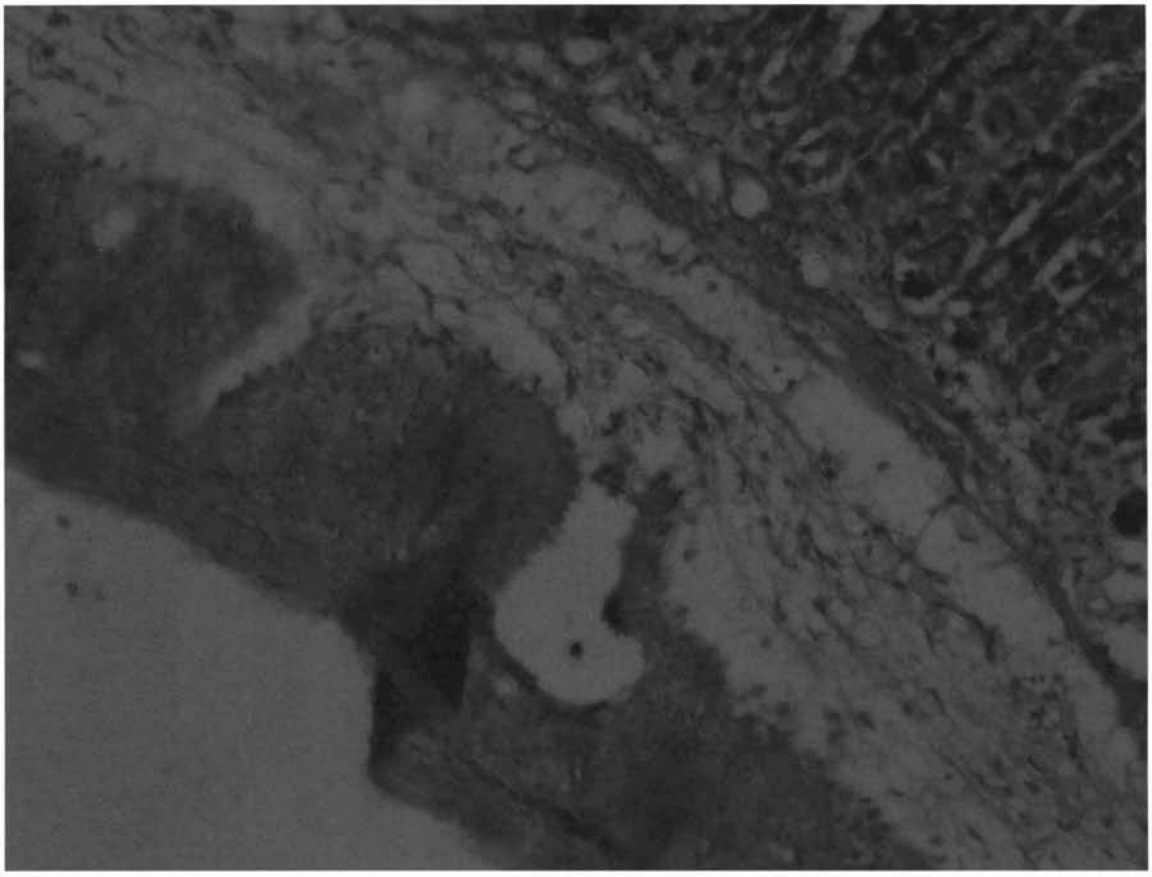


Plate 4.1.6. Histopathology of the stomach of chitosan control rat

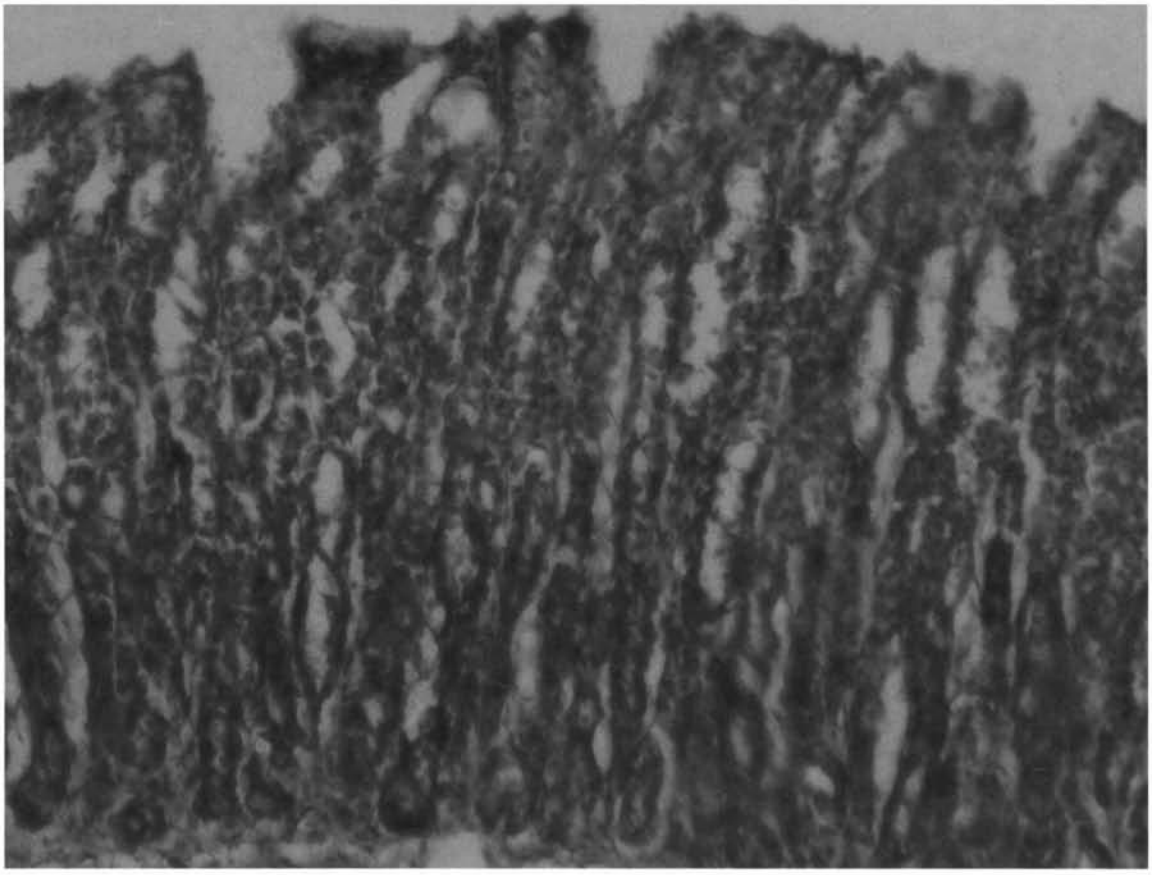


Plate 4.1.7. Histopathology of the stomach of glucosamine control rat

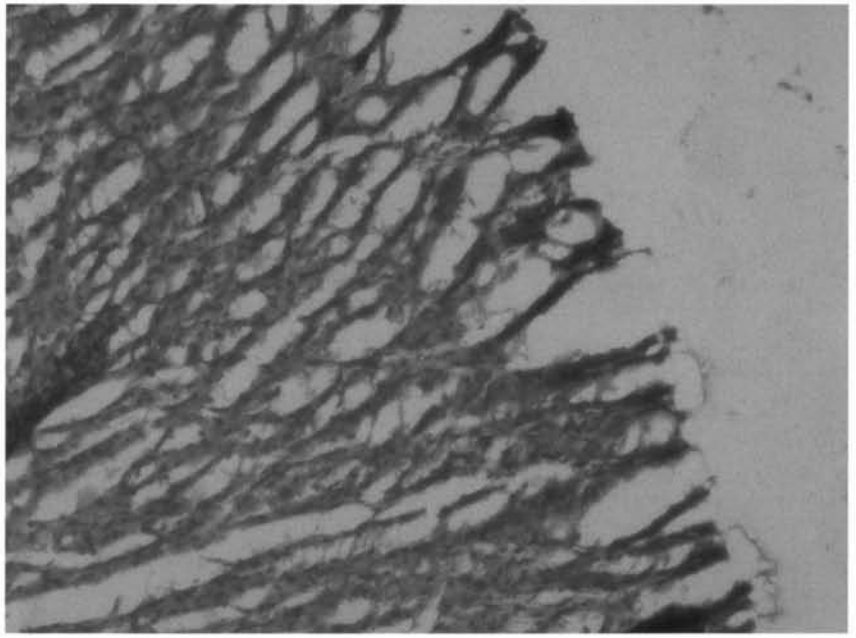


Plate 4.1.8a Illustrates the histopathology of the gastric mucosa of ulcer-induced rat (X100)

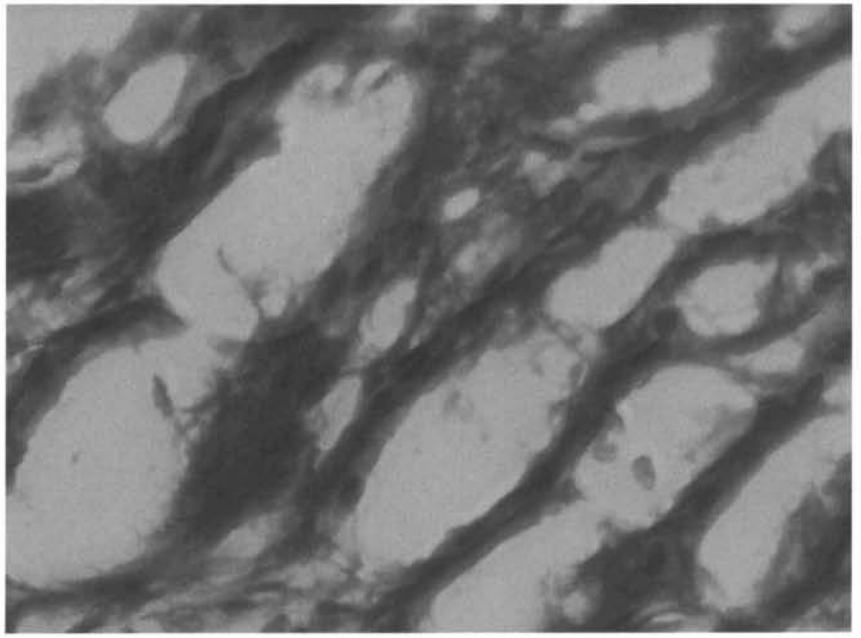


Plate 4.1.8b. Illustrates the histopathology of the gastric mucosa of ulcer-induced rat showing the cell necrosis (X200).

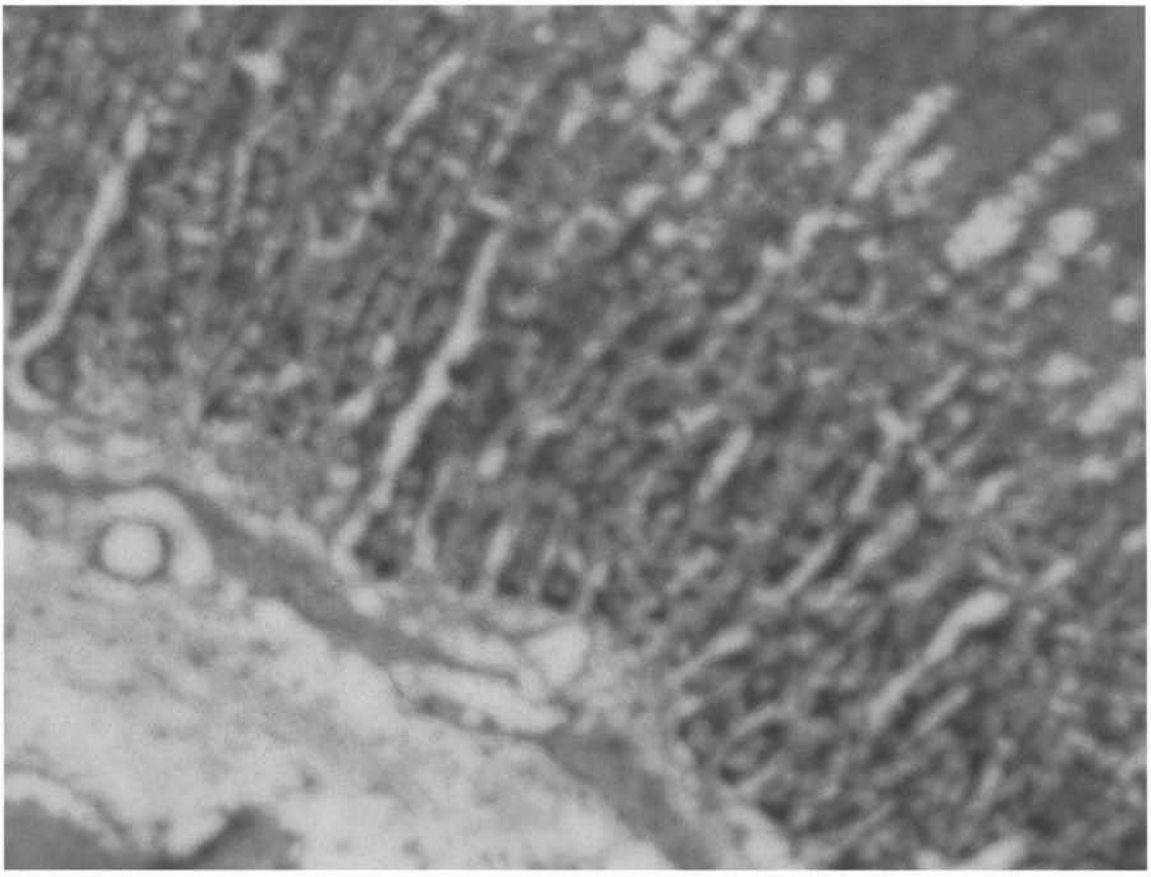


Plate 4.1.9. Histopathology of the stomach of chitosan + ibuprofen administered rat

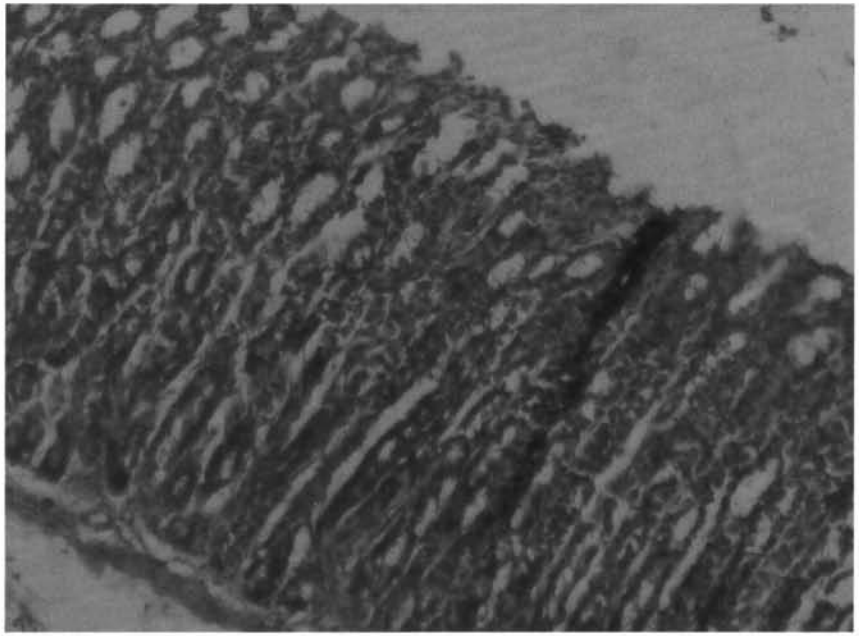


Plate 4.1.10a. Illustrates the histopathology of the gastric mucosa of glucosamine + ibuprofen induced rat (X100)

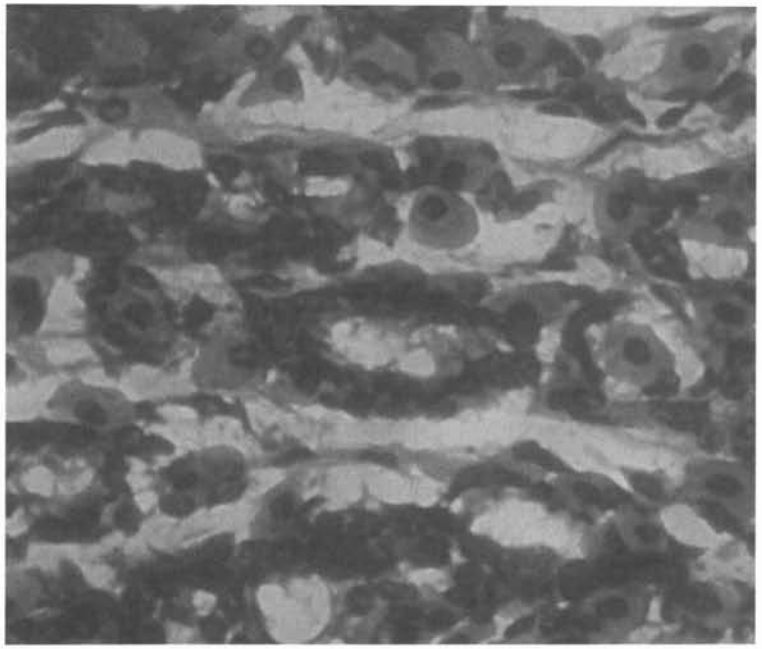


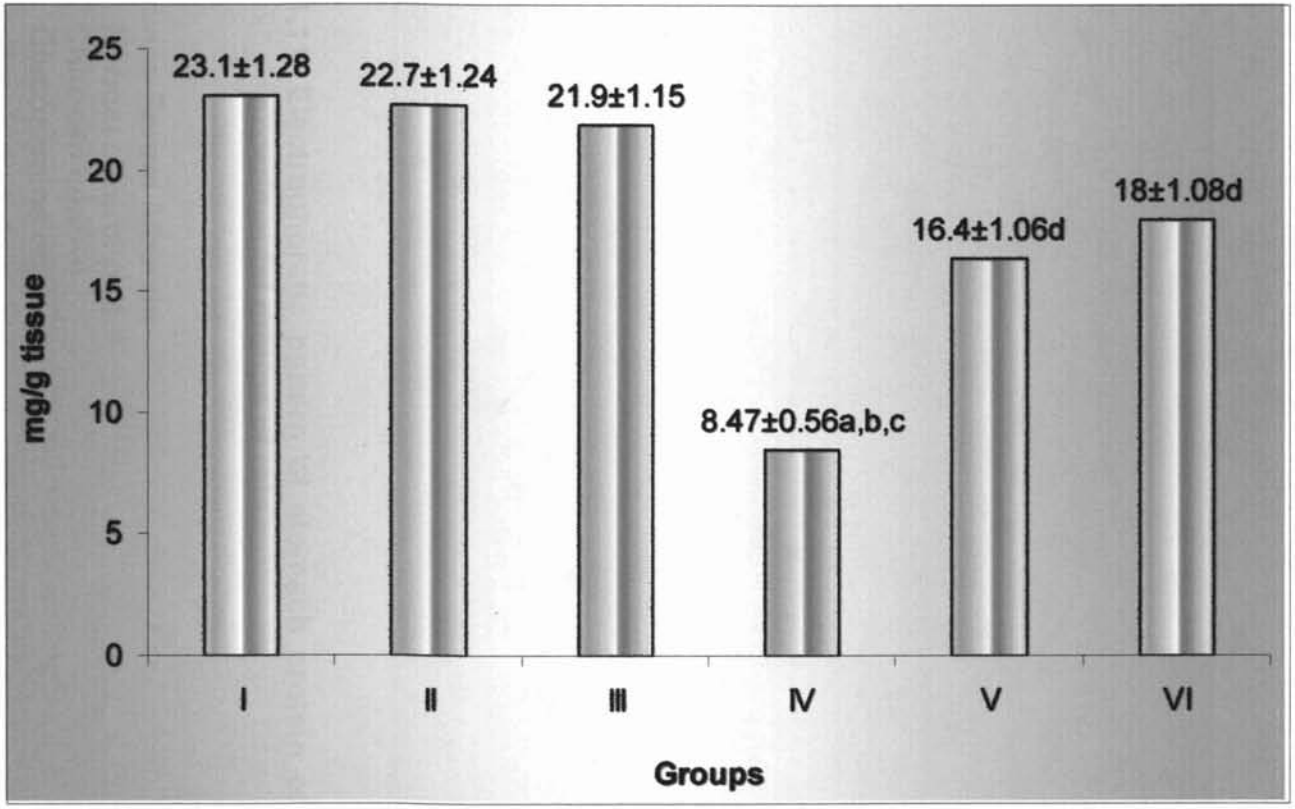
Plate 4.1.10b Illustrates the histopathology of the gastric mucosa of glucosamine + ibuprofen administered rat showing near to normal architecture (X200)

increased permeability of the mucosa, which is an important process in the development of ulcer Bardhan *et al.* (1994). In the present study, the prior oral administration of chitosan and glucosamine resulted in significant (P<0.001) reduction in the volume of gastric juice and acid output in Group V and Group VI animals as compared to Group IV ulcer induced rats. Peptic activity was also maintained at near normal level. It probably did so by the neutralization of hydrochloric acid excessively secreted into the stomach. The neutralization of acid secretion in stomach has already been reported to accelerate ulcer healing (Weberg *et al.*, 1990).

4.1.5. Proteins

The levels of protein are featured in **Fig. 4.1.1**. The level of protein was significantly (P<0.001) decreased in the gastric mucosa of Group IV ulcerated rats as compared to that of Group I normal rats. This is indicated that administration of ibuprofen caused the corrosion of gastric mucosa, resulting in disruption and disintegration of gastric mucosal cells. Similar observations have been reported earlier also (Vanisree and Devaki, 1995). The ulcerative lesions observed in the gastrointestinal tract may also be associated with increased loss protein. According to the Davenport's concept of H^+ release, histamine stimulates cholinergic nerves with a resulting stimulation of acid secretion, an increase in mucosal blood flow, and an increase in capillary permeability (Davenport, 1967). The net effect would be the loss of protein. In the present study, oral pre-treatment with chitosan and glucosamine significantly (P<0.001) prevented the ibuprofen-induced erosion of protein in the gastric mucosa of Group V and Group VI rats, indicating the antiulcer effects of chitosan and glucosamine. The SDS-polyacyrlamide gel electrophoretic separation **(Plate 4.1.11.)** also confirmed the loss of proteins in the ulcerated group as compared to normal rats. The oral chitosan and glucosamine pretreatment nullified the effect of ibuprofen and brought back the level of protein to the near normalcy.

Fig. 4.1.1: Protein levels in the gastric mucosa of normal and experimental groups of rats.



Mean ± SD for six animals in each group.

[a] p<0.001- compared with Group-I, [b] p<0.001- compared with Group-II, [c] p<0.001- compared with Group-III, [d] p<0.001- compared with Group-IV.

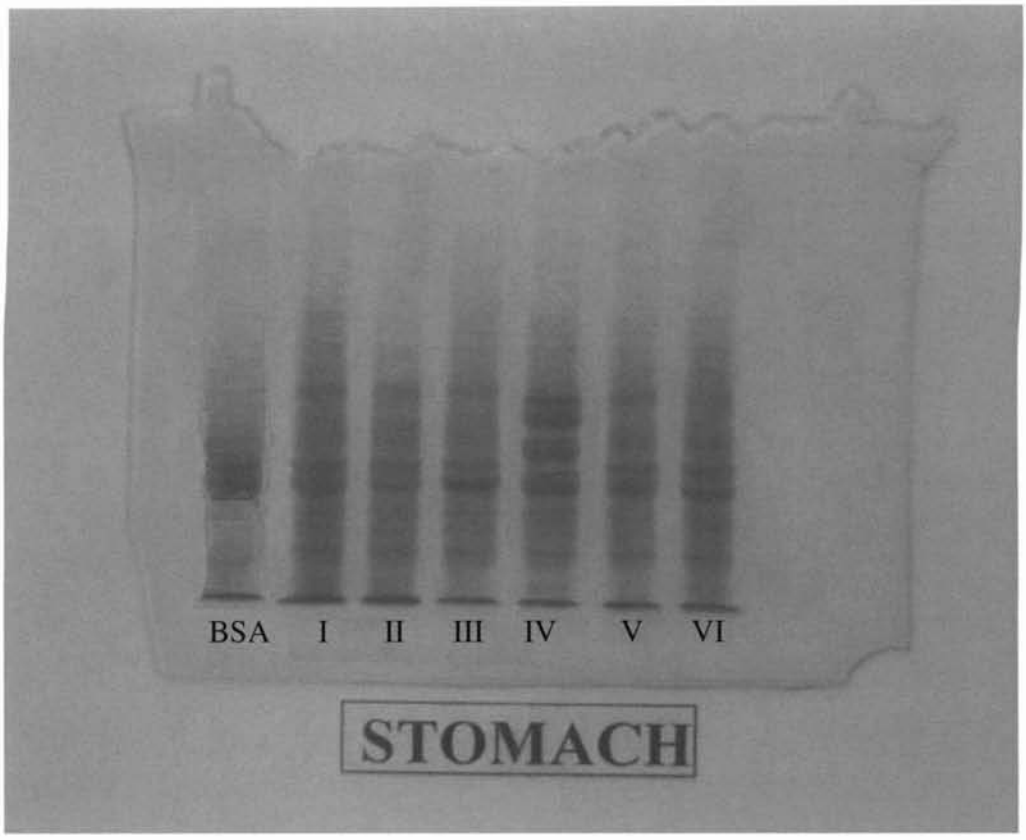


Plate 4.1.11. Electrophoretic pattern of stomach protein of normal and experimental groups of rats.

BSA- Bovine Serum Albumin
I- Normal control rats
II- Chitosan control
III- Glucosamine control
IV- Ulcer induced
V- Chitosan pre treated
VI- Glucosamine pre treated

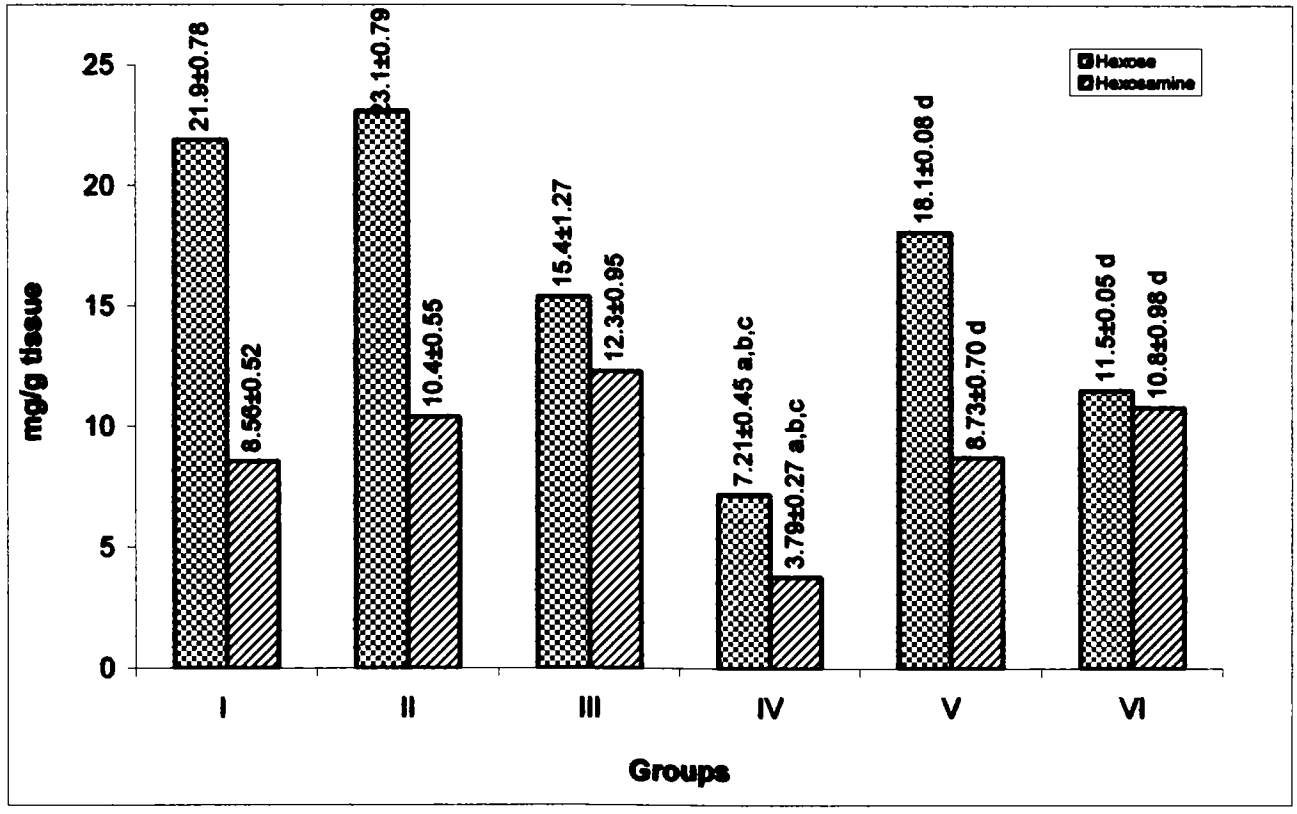
4.1.6. Hexose, hexosamine and sialic acid

Fig. 4.1.2. depicts the levels of hexose and hexosamine and **Fig. 4.1.3.** shows the level of sialic acid in the normal and experimental groups of rats. Intragastric administration of ibuprofen caused significant ($P<0.001$) depletion in the level of gastric mucosal protein as compared to that of Group I control animals. Significant ($P<0.001$) reduction in the levels of mucosal glycoprotein components (hexose, hexosamine and sialic acid) were also observed in the ulcerated mucosa of Group IV animals as compared to Group I normal rats. Prior oral administration of chitosan and glucosamine in Group V and Group VI animals significantly ($P<0.001$) prevented all these alterations and maintained the rats at near normal status. The normal rats receiving chitosan or glucosamine alone did not show any significant change when compared with the normal rats, showing that it does not *per se* have any adverse effects.

Mucus is said to protect the gastrointestinal tract from infective, chemical and physical insults. The glycoprotein component of mucus, which is responsible for the characteristic viscous gel forming property, is believed to be important for the functional role of mucus (Allen *et al.*, 1997). This mucosal barrier lessens the stomach wall friction during contraction, improves the buffering acid and acts as an effective barrier to back diffusion of hydrogen ions (Pfeiffer, 1981). The carbohydrate side chains of mucosal glycoprotein comprise over 80% by weight of the glycoprotein molecule. Each chain consists of sugars such as hexose, amino sugars and sialic acid (Zarzycki *et al.*, 1986). In this study, a significant ($P<0.001$) reduction in the levels of glycoprotein components were observed in the gastric mucosa of Group IV ibuprofen-administered animals as compared to that of Group I rats. Administration of ibuprofen might have affected the cellular processes of exocytosis and apical expulsion or pinching off the apical membrane or exfoliation responsible for the secretion of glycoproteins from the surface epithelial cells by causing the erosion of the epithelial cells.

In the present study, oral pre-treatment with chitosan and glucosamine significantly ($P<0.001$) prevented the ibuprofen-induced depletion of glycoprotein

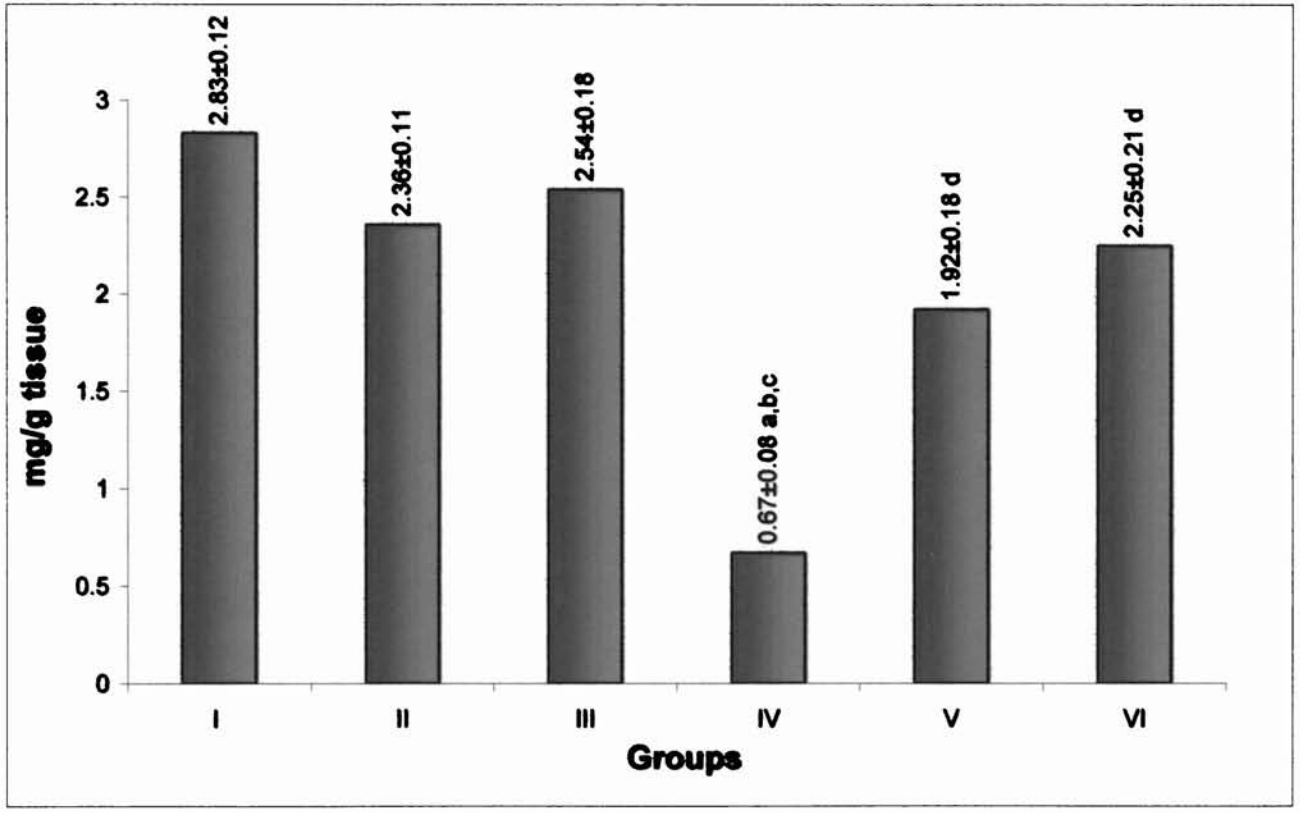
Fig. 4.1.2: Levels of hexose and hexosamine in the gastric mucosa of normal and experimental groups of rats.



Mean ± SD for six animals in each group.

[a] $p<0.001$- compared with Group-I, [b] $p<0.001$- compared with Group-II, [c] $p<0.001$- compared with Group-III, [d] $p<0.001$- compared with Group-IV

Fig. 4.1.3: Level of sialic acid in the gastric mucosa of normal and experimental groups of rats.



Mean ± SD for six animals in each group.

[a] p<0.001- compared with Group-I, [b] p<0.001- compared with Group-II, [c] p<0.001- compared with Group-III

[d] p<0.001- compared with Group-IV

components in the gastric mucosa of Group V and Group VI rats as compared to that of Group IV ulcer induced animals. The incorporation of glucosamine into mucosal glycoprotein might have resulted in strengthening of the mucosal barrier, which is the first line of defense against exogenous and endogenous ulcerogenic agents. When chitosan is administered, it can be converted to oligosaccharides, which can add the strength of mucosal glycoprotein layer and thus can prevent the depletion.

4.1.7. Membrane bound ATPases

Activities of membrane bound ATPases (total ATPase, N^+K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase) were given in **Table 4.1.2**. Significant inhibition in the activities of total ATPase ($P<0.01$), Na^+K^+ ATPase ($P<0.01$), Ca^{2+} ATPase ($P<0.001$) and Mg^{2+} ATPase ($P<0.001$) were observed in the stomach of ulcerated Group as compared to Group I control rats. Oral pretreatment of chitosan and glucosamine significantly maintained the activity levels of total ATPase ($P<0.05$), Na^+K^+ ATPase ($P<0.05$), Ca^{2+} ATPase ($P<0.001$) and Mg^{2+} ATPase ($P<0.001$) near to normal status in Group V and Group VI animals.

Administration of ibuprofen inhibited the activities of membrane bound ATPases and as a result they were lowered. The decreased activities of membrane bound ATPases may be due to the inactivation of phosphates by the free radicals (Mohankumar and Ramasamy, 2006) formed by ibuprofen. Balasubramanian *et al.* (2004) has already reported the formation of free radicals in the gastric mucosa of ibuprofen-induced rats. Pre-treatment of chitosan and glucosamine significantly maintained the activities of membrane bound ATPases by the counteraction of ibuprofen-induced free radicals by their free radical scavenging property (Yan *et al.*, 2006; Sal'nikova *et al.*, 1990)

4.1.8. Lipid peroxidation

Table 4.1.3 shows the lipid peroxidaiton values of normal and experimental rats. Lipid peroxidation reaction, a type of oxidative degeneration of polyunsaturated

Table 4.1.2: Activities of membrane bound ATPases of the gastric mucosa of normal and experimental groups of rats

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Ulcer-Induced C	Group V A+C	Group VI B+C
Total ATPase	6.73±0.58	6.5±0.54	6.52±0.05	5.58±0.47[e,f,g]	6.39±0.53[h]	6.42±0.54[h]
Na-K ATP	4.48±0.38	4.29±0.37	4.33±0.41	3.57±0.31[e,f,g]	4.20±0.42[h]	4.21±0.44[h]
Ca-ATP	3.72±0.35	3.80±0.31	3.86±0.33	2.61±0.28[a,b,c]	3.70±0.32[d]	3.74±0.34[d]
Mg-ATP	4.25±0.37	4.52±0.38	4.48±0.47	3.19±0.32[a,b,c]	4.47±0.38[d]	4.58±0.45[d]

Mean ± SD for six animals in each group. Values are expressed as μ moles of Pi liberated per mg protein per hour.

[a] $P<0.001$ - compared with Group-I

[b] $P<0.001$ - compared with Group-II

[c] $P<0.001$ - compared with Group-III

[d] $P<0.001$- compared with Group-IV

[e] $P<0.01$ - compared with Group-I

[f] $P<0.01$ - compared with Group-III

[g] $P<0.05$ - compared with Group-II

[h] $P<0.05$ - compared with Group-IV

Table 4.1.3: Levels of reduced glutathione (GSH) and lipid peroxides (LPO) in the gastric mucosa of normal and experimental groups of rats.

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Ulcer-Induced C	Group V A+C	Group VI B+C
GSH	5.02±0.36	4.82±0.23	4.89±0.25	2.12±0.12 [a,b,c]	4.21±0.15 [d]	4.73±0.18 [d]
LPO	1.02±0.02	0.98±0.02	0.96±0.01	2.45±0.05 [a,b,c]	1.46±0.03 [d]	1.18±0.01 [d]

Mean ± SD for six animals in each group. Values are expressed as: LPO, nmol MDA/mg protein; GSH, nmol/g wet tissue

[a] $P<0.001$- compared with Group-I

[b] $P<0.001$- compared with Group-II

[c] $P<0.001$- compared with Group-III

[d] $P<0.001$- compared with Group-IV

fatty acids (PUFA), has been linked with altered membrane structure and enzyme inactivation. The level of lipid peroxidation was found to be significantly (P<0.001) higher in the gastric mucosa of Group IV ulcer-induced rats as compared to that of normal controls. Significant increases in the levels of lipid peroxides in the ulcer induced gastric mucosa have already been reported (Yoshikawa *et al.*, 1986, Yoshikawa *et al.*, 1987). Lipid peroxidation worsens the mucosal injury. The exact sources of peroxy radicals in peptic ulcer are not yet thoroughly understood. Xanthine oxidase has been considered as one of the major source of free radicals (Yoshikawa *et al.*, 1990). Another source may be neutrophils, since administration of non-steroidal anti-inflammatory drugs has already been reported to cause neutrophil infiltration into the gastric mucosa (Amagase *et al.*, 2003). Jimenez *et al.* (2004) proposed that the active oxygen species, derived both from xanthine oxidase and activated neutrophils, could play a role in the pathogenesis of gastric injury induced by ibuprofen. The metabolism of arachidonic acid via the lipoxygenase and cyclooxygenase pathways may also result in the formation of reactive oxygen species and other free radicals in the ulcerated gastric mucosa (Argentieri *et al.*, 1994)

Prior administration of chitosan and glucosamine resulted in a significant (P<0.001) reduction in the level of lipid peroxidation towards near normalcy as compared to Group IV ulcer-induced rats, establishing their cytoprotective effect. The unpaired electron present in the ibuprofen-generated free radicals might have been trapped and subsequently dismuted by chitosan and glucosamine by the free radical scavenging capability (Yan *et al.*, 2006; Sal'nikova *et al.*, 1990).

4.1.9. Reduced glutathione (GSH)

Here, GSH level was significantly (P<0.001) decreased in ulcerated group as compared to normal rats **(Table 4.1.3)**. GSH has a direct antioxidant function. It functions by reaction with superoxide radicals, peroxyl radicals and singlet oxygen, followed by the formation of oxidized glutathione and other disulphides (Anandan *et al.*, 1999). Depletion of GSH results in enhanced lipid peroxidation, and excessive lipid peroxidation can cause increased GSH consumption

(Anandan *et al.*, 2004), as observed in the present study This is in accordance with a previously reported study (Lu *et al.*, 1989), which indicated that the depletion of gastric GSH by diethyl maleate produced gastric ulceration. This reduction might have resulted from the oxidation of GSH to ibuprofen-induced generation of free radicals. The depletion of GSH further enhances the susceptibility of the gastric mucosal cells to oxygen metabolites and acid mediated cell damage. Hiraishi *et al.* (1994) has reported that exogenous GSH protects cultured gastric mucosal cells from oxidant-induced damage.

In the present study, the pretreatment with chitosan and glucosamine resulted in significant ($P<0.001$) elevation of the GSH level, which protects against oxidative damage by the way of regulating the redox status of proteins in the cell membrane (Anandan *et al.*, 2004). This increase in intracellular GSH in turn may protect the gastric cells against ibuprofen mediated cellular injury by decreasing their susceptibility to free radicals and acid.

4.1.10. Mucosal antioxidant enzymes

Activities of glutathione-dependent antioxidant enzymes [glutathione peroxidase (GPX) and glutathione s-transferase (GST)] and antiperoxidative enzymes [catalase (CAT) and super oxide dismutase (SOD)] were significantly ($P<0.001$) lower in the gastric mucosa of Group IV ulcer-induced rats as compared to that of Group I control animals **(Table 4.1.4)**. GPX, an antioxidant enzyme, offers protection to the mucosal membrane from peroxidative damage (Prabha *et al.*, 2003). A significant decrease in the activity of GPX makes mucosal membrane susceptible to ibuprofen-induced peroxidative damage, which leads to changes in the gastric mucosal composition and function. GST, another scavenging enzyme, binds to many different lipophilic compounds (Anandan *et al.*, 1999); so it would be expected to bind ibuprofen and act for GSH conjugation reactions. The significant ($P<0.001$) decrease in GST activity noted in this study might have been due to the decreased availability of GSH.

Table 4.1.4: Activities of glutathione-S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) in the gastric mucosa of normal and experimental groups of rats.

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Ulcer-Induced C	Group V A+C	Group VI B+C
GST	5.46±0.38	5.31±0.37	5.34±0.33	3.18±0.25[a,b,c]	4.61±0.32 [d]	4.95±0.34 [d]
GPx	179±14.2	184±14.4	195±16.8	108±7.3 [a,b,c]	153±13.4 [d]	172±15.5 [d]
SOD	5.87±0.27	5.95±0.28	6.02±0.32	2.25±0.18 [a,b,c]	4.46±0.20 [d]	5.18±0.22 [d]
CAT	4.08±0.21	3.90±0.20	3.95±0.26	1.25±0.09 [a,b,c]	3.49±0.16 [d]	3.82±0.18 [d]

Mean ± SD for six animals in each group. Values are expressed as: GST, µmol 1-chloro-2,4 dinitrobenzene conjugate formed /min /mg protein; GPX, nmol GSH oxidized/min/mg protein; SOD, one unit of the superoxide dismutase activity is the amount of protein required to give 50% inhibition of epinephrine autooxidation; CAT, µmol of H_2O_2 consumed/min/mg protein.

[a] $P<0.001$- compared with Group-I

[b] $P<0.001$- compared with Group-II

[c] $P<0.001$- compared with Group-III

[d] $P<0.001$- compared with Group-IV

Significant reduction ($P<0.001$) noticed in the activities of CAT and SOD in experimentally induced ulcerated mucosa is in line with earlier reported studies (Prabha *et al.*, 2003; Raghavendran *et al.*, 2004), indicating that decline in the activities of these antiperoxidative enzymes leads to the formation of peroxyl radicals, which are harmful to the gastric mucosa. In the present study, the prior oral administration of chitosan and glucosamine significantly ($P<0.001$) prevented the ibuprofen-induced alterations in the activities of glutathione-dependent antioxidant enzymes (GPX and GST) and antiperoxidative enzymes (SOD and CAT) in the gastric mucosa of Group IV rats. It probably did so by counteraction of ibuprofen-induced free radicals (Balasubramanian *et al.*, 2004) due to the antioxidant nature.

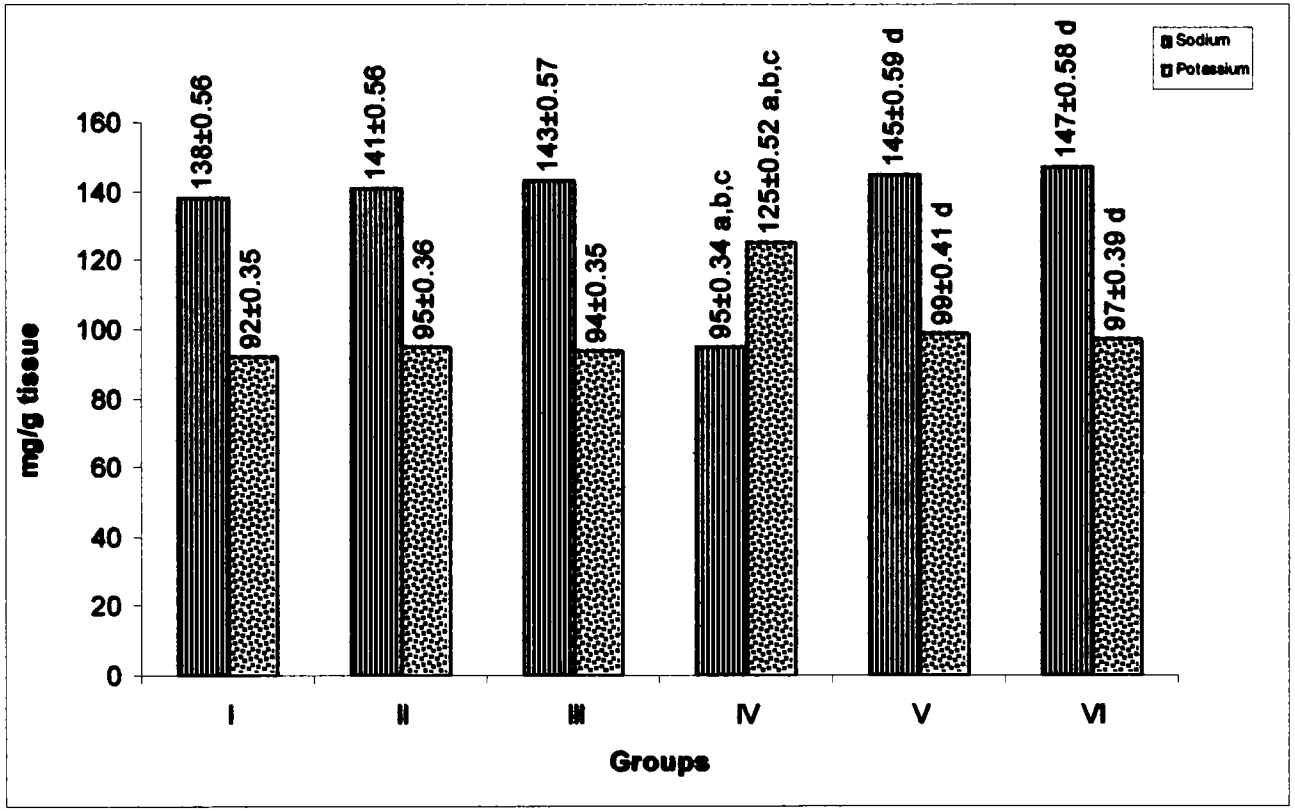
The results of the present study indicates that the overall antiulcerogenic effect of chitosan and glucosamine is probably related to its ability to maintain near to the normal status the activities of the mucosal antioxidant enzymes and the level of GSH which protects mucosa against oxidative damage, by decreasing lipid peroxidation.

4.1.11. Minerals

Mineral status of the stomach tissue of normal and experimental groups of rats were described in **Fig. 4.1.4** and **Table 4.1.5**. Rats treated with ibuprofen showed significant elevation ($P<0.001$) in concentration of calcium in the stomach tissue. The accumulation of intracellular calcium levels also play an important role in the DNA fragmentation and ulcer induced by ibuprofen. An increase in the level of intracellular calcium can simply cause cellular damage via activation of calcium dependent degenerative enzymes including calcium dependent phospholipases, proteases and endonucleases. This could lead to destruction of membranes, critical protein and DNA fragmentation (Timbrell, 2000)

The level of sodium was found to decrease significantly ($P<0.001$) in the ulcerated group. But, levels of potassium, magnesium and zinc were found to

Fig. 4.1.4: Sodium and potassium levels in the stomach of normal and experimental groups of rats.



Mean ± SD for six animals in each group.

[a] p<0.001- compared with Group-I, [b] p<0.001- compared with Group-II, [c] p<0.001- compared with Group-III, [d] p<0.001- compared with Group-IV

increase significantly (P<0.001) in this group. Increased peroxidation may be the cause of increment in the magnesium level. An earlier reported study (Bironaite and Ollinger, 1997) also supports this. Oral pre treatment of chitosan and glucosamine significantly (P<0.001) maintained the levels of these minerals to the normalcy.

4.1.12. Amino acids

There was a significant decrease (P<0.001) in the amino acid levels of ulcer-induced rats as compared to normal control animals **(Table 4.1.6.)**. This may be due to the cytodeterioraton brought about by the ibuprofen created free radicals. In, Groups V and Groups VI animals, due to the administration of chitosan and glucosamine, the levels of these amino acids were maintained to near normalcy. Inhibition of the transsulfuration reactions from homocysteine to cysteine may lead to a decrease in the synthesis of GSH, which would produce an important impact on normal biochemistry and physiology (Sang and Young, 2005). Here, the cytoprotective effect of chitosan and glucosamine plays a good role.

Table 4.1.6: Levels of various amino acids in the stomach of normal and experimental groups of rats

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Ulcer-Induced C	Group V A+C	Group VI B+C
Glycine	1800±81.25	1825±85.34	1831±86.16	1685±76.52[a,b,c]	1790±78.37[d]	1798±78.63[d]
Serine	470±24.32	475±25.51	478±25.82	401±19.34[a,b,c]	465±23.63[d]	468±24.52[d]
Valine	340±16.28	351±19.68	357±20.34	269±12.93[a,b,c]	331±14.57[d]	337±15.69[d]
Histidine	520±25.24	528±26.46	532±26.51	445±19.37[a,b,c]	511±24.91[d]	515±24.87[d]
Tryptophan	89±6.93	95±7.64	97±8.82	45±2.38[a,b,c]	78±5.64[d]	84±5.98[d]
Threonine	290±20.35	301±22.56	305±22.72	180±16.19[a,b,c]	278±18.82[d]	287±20.14[d]
Leucine	155±8.62	160±9.14	163±11.17	91±6.52[a,b,c]	148±7.16[d]	150±7.85[d]

Mean ± SD for six animals in each group. Values are expressed as nmol/g tissue

[a] $P<0.001$- compared with Group-I

[b] $P<0.001$- compared with Group-II

[c] $P<0.001$- compared with Group-III

[d] $P<0.001$- compared with Group-IV

4.2. ANTI HEPATOTOXIC POTENTIAL OF CHITOSAN AND GLUCOSAMINE

4.2.1 Diagnostic marker enzymes

Antibiotic-related liver injuries cover most of the clinical and pathological expressions of hepatic dysfunction, including cytotoxic hepatitis (Westphal *et al.*, 1994). There was a significant ($P<0.001$) elevation observed in the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and acid phosphatase (ACP) in the serum of Group IV antitubercular drugs-administered rats as compared to Group I normal controls **(Table 4.2.1)**, which is in line with an earlier reported study (Imogen *et al.*, 1995). Elevated levels of these enzymes in serum are presumptive markers of drug-induced necrotic lesions in the hepatocytes (Amr and Alaa, 2005). Enhanced susceptibility of hepatocytes cell membrane to the isoniazid and rifampicin-induced peroxidative damage might have resulted in increased release of these diagnostic marker enzymes into the systemic circulation. In the present study, co-administration of chitosan and glucosamine significantly ($P<0.001$) maintained the levels of these diagnostic marker enzymes in the serum of Group V and Group VI animals at near normalcy as compared to Group IV rats, indicating the cytoprotective effect of chitosan and glucosamine (Anandan *et al.*, 2004, Sal'nikova *et al.*, 1990). It probably did so by preventing the antitubercular drugs-induced necrotic damage by the membrane stabilizing action (Filipovic-Grcic *et al.*, 2001). Reports by Anandan *et al.* (2004) showed that chitosan administration prevented experimental ulcer in rats by its cytoprotectivity.

Amino transferases are important class of enzymes linking carbohydrate and amino acid metabolism, thereby clearly establishing the relationship between the intermediates of the citric acid and amino acids. Alanine amino transferase and

Table 4.2.1: Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), acid phosphatase (ACP) and alkaline phosphatase (ALP) levels in the serum of normal and experimental groups of rats

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
ALT	121±7.23	112±6.31	128±7.45	314±29.61 [a,b,c]	133±9.54[d]	153±12.65[d]
AST	102±9.02	94.5±5.69	115±10.23	297±26.74 [a,b,c]	98.4±5.23[d]	127±10.65[d]
LDH	178±14.92	182±16.0	185±16.25	284±25.63 [a,b,c]	193±17.56[d]	210±19.85[d]
ACP	14.3±0.98	12.7±1.02	14.2±1.13	36.8±2.41 [a,b,c]	16.5±1.18[d]	19±1.21[d]
ALP	98.4±7.46	94.1±7.21	97.5±7.35	224±18.0 [a,b,c]	118±9.77[d]	123.6±10.1[d]

ALT, AST and LDH=μmoles of pyruvate liberated/hr/l; ACP and ALP=μmoles of phenol liberated/hr/l

Values are expressed as the mean ± SD for six animals.

[a] $P<0.001$- compared with Group-I

[b] $P<0.001$- compared with Group-II

[c] $P<0.001$- compared with Group-III

[d] $P<0.001$- compared with Group-IV

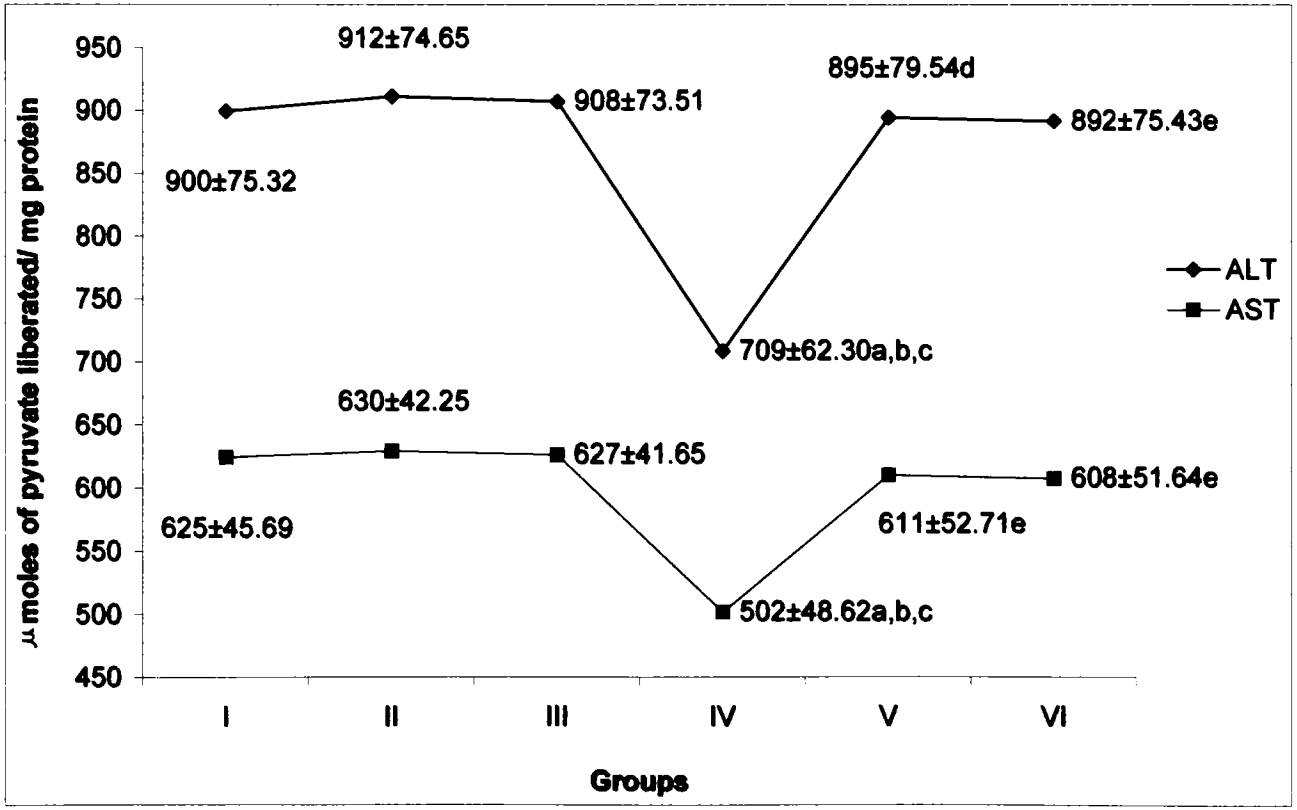
aspartate amino transferase are well known diagnostic indicators of liver disease. The levels of ALT and AST in the liver of normal and experimental groups of rats were shown in **Fig. 4.2.1**. Antitubercular drugs administration in Group IV animals significantly ($P<0.001$) lowered the levels of ALT and AST as compared to Group I normal rats. Co-administration of chitosan and glucosamine brought back the levels of these diagnostic marker enzymes to the normal level. Significant increase in the levels of ALT [($P<0.001$), ($P<0.01$)] and AST ($P<0.001$) was observed in Group V and Group VI animals. In cases of liver damage with hepatocellular lesions and parenchymal cell necrosis, these marker enzymes are released from the damaged tissues into the blood stream. So, in the present study, the activities of these enzymes were significantly lower in the liver of Group IV, antitubercular drugs-administered rats as compared to that of control rats. Observations of significant decrease in the activities these enzymes have already been reported in the liver of isoniazid and rifampicin intoxicated rats (Saraswathy *et al.*, 1998).

The fall in alanine amino transferase activity is almost always due to hepatocellular damage and is usually accompanied by a lowering in the activity of asparate amino transferase. Increased protein catabolism and urea formation that are seen in antitubercular drugs-induced hepatocellular damage may also be responsible for the decline of these amino tranferases activities in liver. In the present study, the activities of alanine amino transferase and aspartate amino transferase were maintained at near normal level in the orally chitosan and glucosamine co-administered rats as compared to that of Group IV antitubercular drugs-induced hepatotoxic rats, showing the normalizing effect of chitosan and glucosamine on protein metabolism.

4.2.2. Histopathology

Liver sections of control rats revealed the normal hepatic architecture and the typical hexagonal hepatic lobules **(Plate 4.2.1)**. Liver tissue of isoniazid and rifampicin treated rats showed severe vacuolation and degeneration of hepatocytes in the three zones of lobules. Membrane disintegration with intense

Fig. 4.2.1: Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the liver of normal and experimental groups of rats.



Values are expressed as the mean ± SD for six animals.

[a] $P<0.001$- compared with Group-I, [b] $P<0.001$- compared with Group-II, [c] $P<0.001$- compared with Group-III, [d] $P<0.001$- compared with Group-IV, [e] $P<0.01$- compared with Group-IV

vacuolation accompanied with cytoplasmic rarefication resulting in loss of polyhedral structure in the liver of rat treated with antitubercular drugs **(Plate 4.2.4)**. There was severe infiltration of inflammatory cells in the portal areas and around the necrotic cell. These are in line with an earlier reported study (Victor and John, 2006). Congested blood vessels and sinusoids were also observed in some cases. Central vein dilation, enlargement of portal areas and bile duct proliferation were seen. However, the other abnormalities like necrosis, macrovesicular steatosis and inflammation were also occasionally present.

Co-administration with chitosan and glucosamine caused reversal of such pathology with no evidence of necrotic areas **(Plates 4.2.5. and 4.2.6.)**, which establishes the cytotoprotectivity of these marine compounds. Liver architecture of rats, fed with chitosan and glucosamine alone did not show any characteristic change **(Plates 4.2.2. and 4.2.3.)**.

4.2.3. Urea, albumin, globulin and albumin/globulin

Drug induced hepatotoxicity is associated with failure to maintain serum albumin level whereas serum globulins, particularly γ-globulin, which is formed in the reticuloendothelial system are increased. Serum albumin level is an index of severity and prognosis in patients suffering from chronic hepatitis. So, in the present study, the level of globulin was elevated to a significantly ($P<0.05$) and levels of albumin and albumin/globulin ratio were found to be decreased significantly ($P<0.001$) in isoniazid and rifampicin induced group as compared to normal rats **(Table 4.2.2)**. Formation of urea, creatine and creatinine are the mode of disposal of nitrogen. Liver is the most important organ in the maintenance of blood ammonia levels through the urea cycle (Stryer, 1995). In hepatotoxic condition, due to the failure of the liver to convert amino acids and ammonia to urea, a significant ($P<0.001$) decrease in urea **(Table 4.2.2)** was observed (Reicher and Paumgartner, 1980). There is an increased catabolism of proteins coupled with the diminished ability of kidneys to excrete the nitrogenous waste. This could be possibly the reason for the lowered level of urea in hepatotoxic rats. This shoulders with a previous report (Eule *et al.*, 1986).

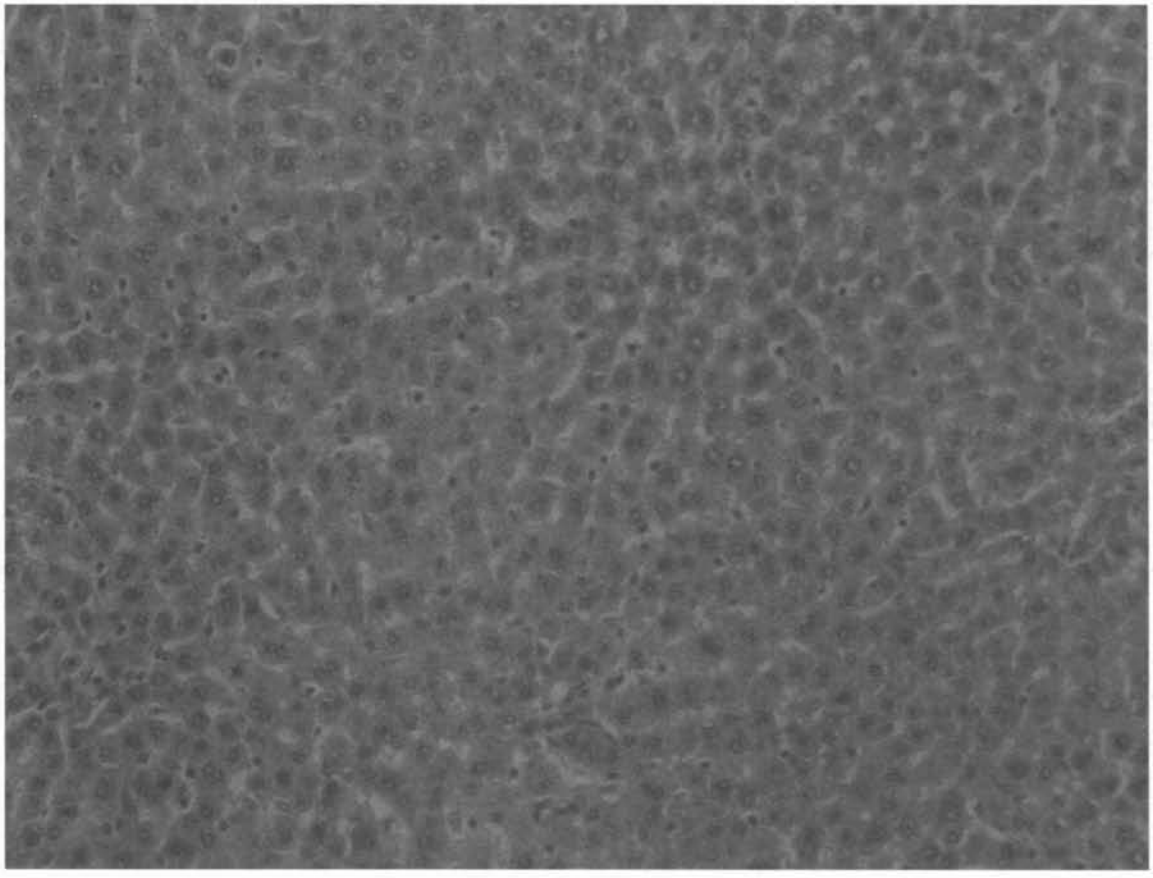


Plate 4.2.1. Histopathological observation of the liver of normal rat.

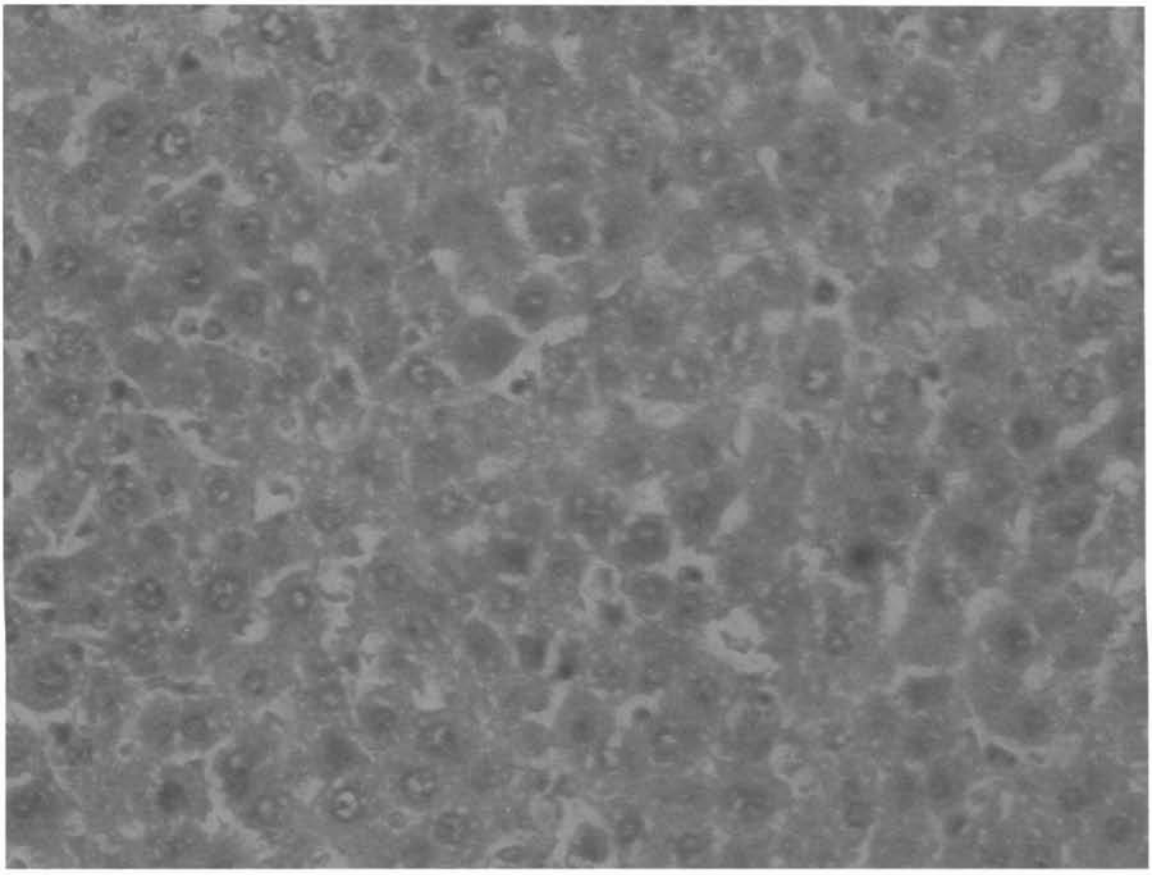


Plate 4.2.2. Histopathological observation of the liver of chitosan control rat.

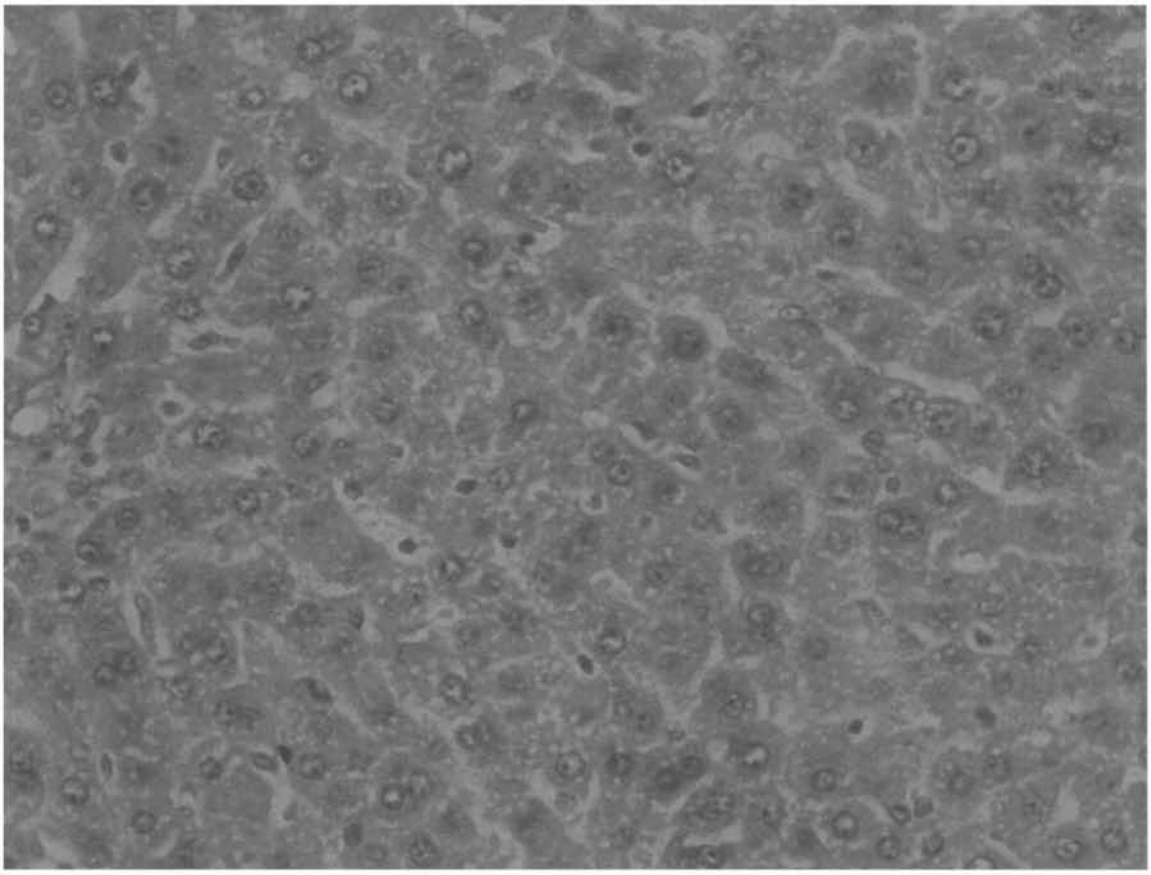


Plate 4.2.3. Histopathological architecture of the liver of glucosamine control rat.

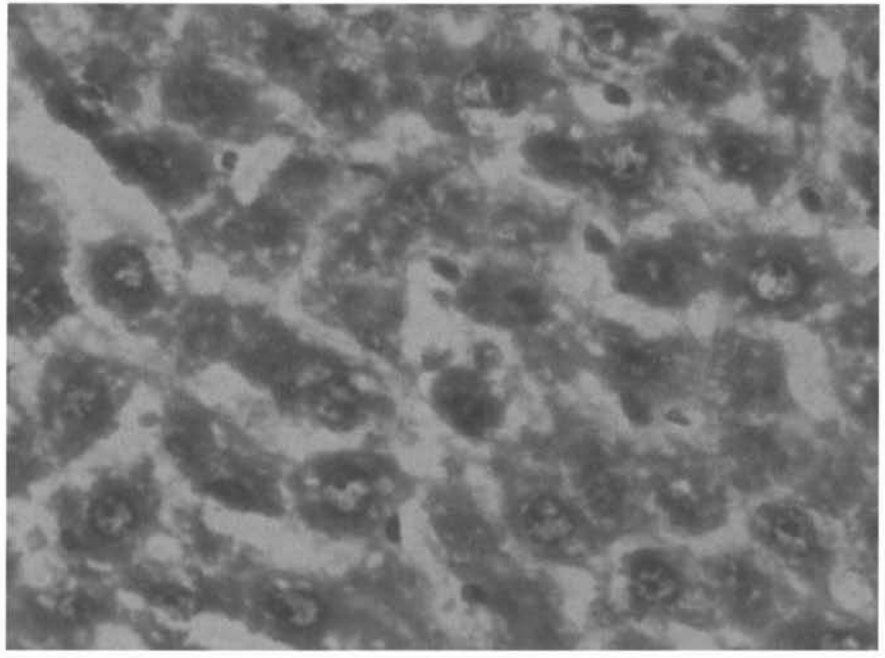


Plate 4.2.4a Illustrates the histopathology of the liver of hepatotoxic rat showing cell necrosis

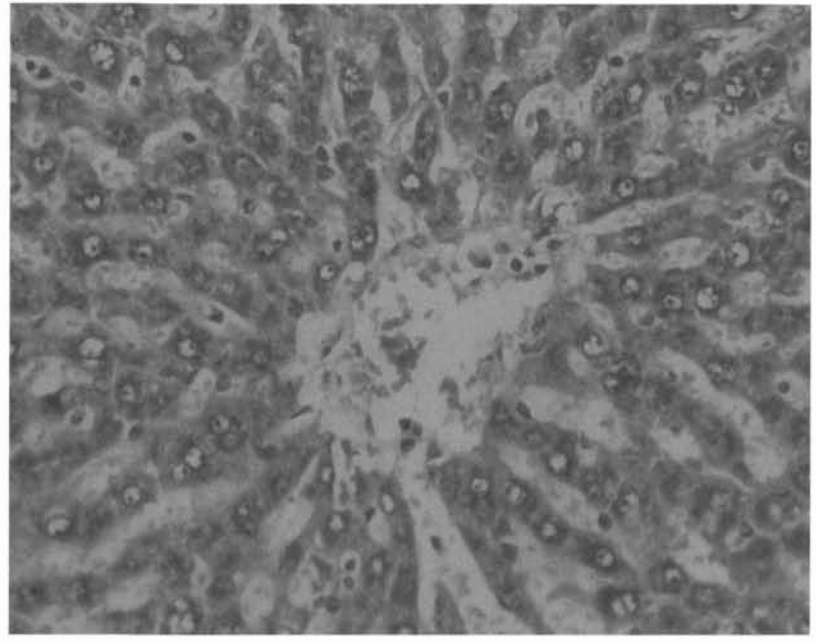


Plate 4.2.4b. Illustrates the histopathology of the liver of hepatotoxicity induced rat. Necrosis is visible.

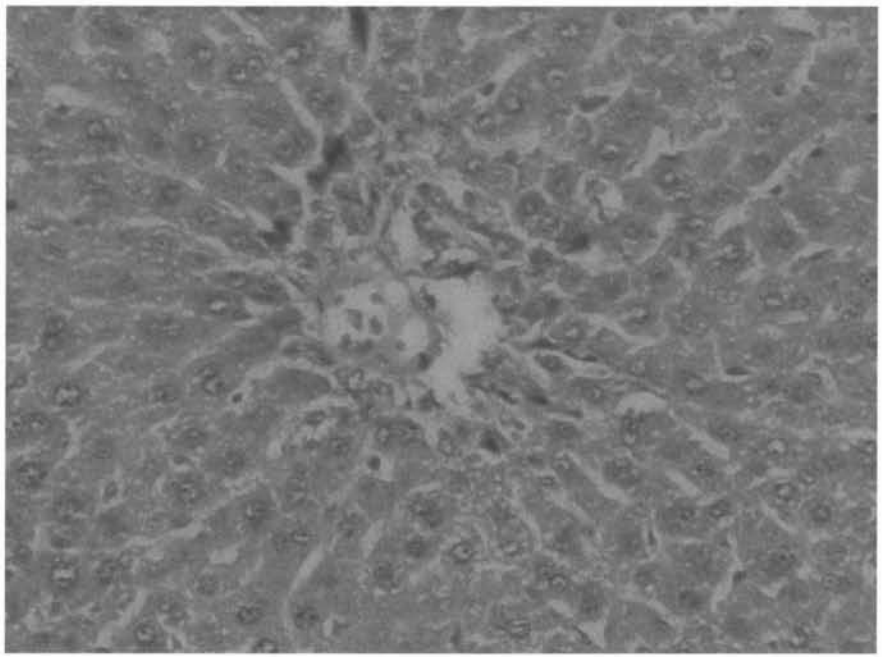


Plate 4.2.5a Illustrates the histopathology of the liver of the rat co-administered with chitosan along with antitubercular drugs.

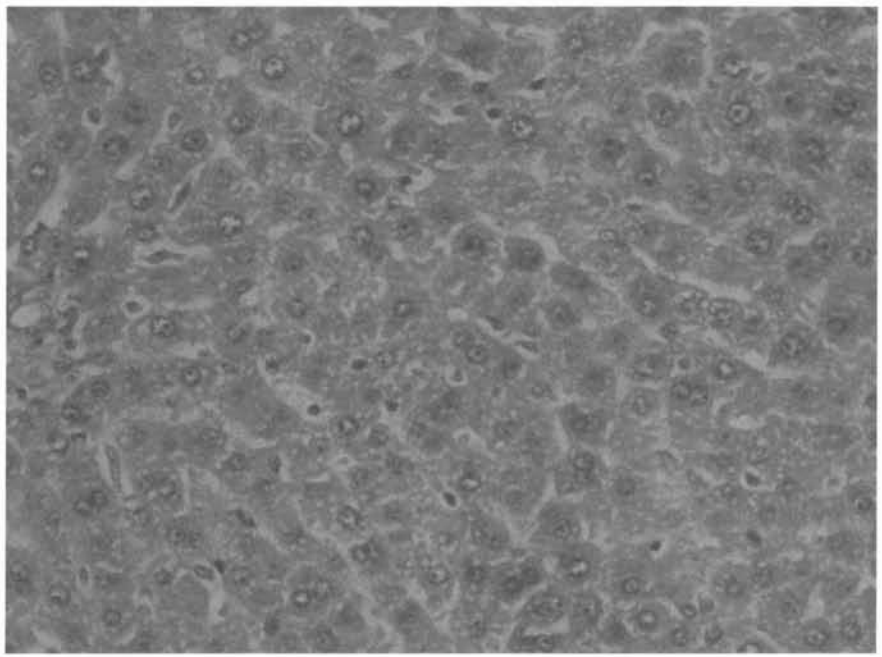


Plate 4.2.5b. Illustrates the histopathology of the liver of chitosan co-administered with isoniazid and rifampicin.

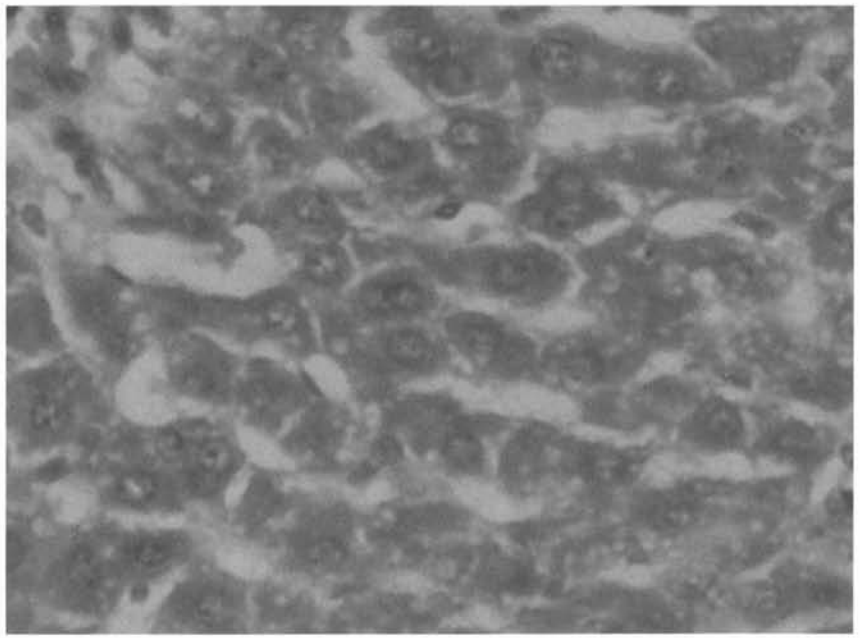


Plate 4.2.6a Illustrates the histopathology of the liver of the liver of the rat, glucosamine co-administered with isoniazid and rifampicin.

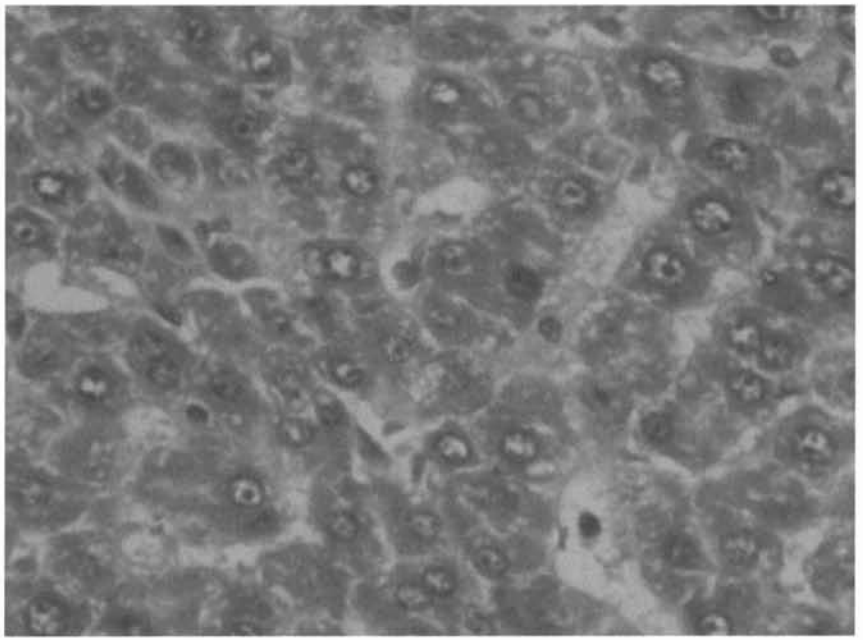


Plate 4.2.6b. Shows the histopathological architecture of rat liver in which glucosamine co-administered with antitubercular drugs.

Table 4.2.2: Levels of urea (mg/dl), albumin(g/dl), globulin(g/dl), albumin/globulin ratio (g/dl) in the serum of normal and experimental groups of rats

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
Urea	22.1±1.90	20.8±2.20	20.7±2.10	11.4±1.30[a,b,c]	18.6±1.55[d]	17.9±1.51[d]
Albumin	4.12±0.32	4.21±0.30	4.24±0.33	2.18±0.23[a,b,c]	3.95±0.28[d]	3.92±0.26[d]
Globulin	3.26±0.35	3.28±0.33	3.30±0.36	3.89±0.37[e,f,g]	3.31±0.29[h]	3.34±0.32[h]
Albumin/globulin	1.25±0.08	1.19±0.09	1.17±0.09	0.53±0.03[a,b,c]	1.04±0.07[d]	1.03±0.08[d]

Values are expressed as the mean ± SD for six animals.

[a] $P<0.001$- compared with Group-I, [b] $p<0.001$- compared with Group-II, [c] $p<0.001$- compared with Group-III,

[d] $P<0.001$- compared with Group-IV, [e] $p<0.05$ - compared with Group-I, [f] $p<0.05$ - compared with Group-II,

[g] $P<0.05$ - compared with Group-III, [h] $p<0.05$ - compared with Group-IV

Creatinine level **(Table 4.2.3)** was also found to be decreased at significant range (P<0.001). Co-administration of chitosan and glucosamine in the present study significantly prevented antitubercular drugs-induced alterations in the levels of creatinine (P<0.001) **(Table 4.2.3)** albumin (P<0.001), globulin (P<0.05), albumin/globulin ratio (P<0.001) and urea (P<0.001) in Group V and Group VI rats as compared to that of Group IV rats **(Table 4.2.2)**. It probably did so by reducing the accumulation of toxic antitubercular drugs derived metabolites, which may contribute to the changes in the rough endoplasmic reticulum and the disturbance of protein metabolism in liver.

4.2.4. Serum glucose

Serum glucose level of experimental and normal groups of rats were described in **Table 4.2.3**. The level of glucose in the serum of hepatotoxic rats were found to be decreased significantly (P<0.001). Administration of isoniazid and rifampicin brought about hypoglycaemia, which may be due to the inactivated pancreatic β cell function and to the inhibition in the activities of the key gluconeogenic enzymes or increase the activities of key glucose utilizing enzymes. The changes in the level of glucose in the serum were normalized significantly by the co-administration of chitosan and glucosamine (P<0.001). Since, chitosan and glucosamine were of saccharide family, they can increase the gluconeogenesis (Krisana *et al.*, 2004). This may be the reason for the hyperglycaemic action of these compounds.

4.2.5. Proteins

The oral administration of antitubercular drugs to rats induces liver cell damage leading to periportal infiltration of inflammatory cells, disruption of lobular architecture, and liver cell necrosis and causes severe disturbance of RNA and protein metabolism. The disaggregation of polyribosomes in isoniazid and rifampicin-induced hepatitis is associated with the inhibition of protein synthesis, which may be partially responsible for the fatty liver, though probably not for the necrosis, although it contributes to the disabling of the cell. Accumulation of

Table 4.2.3: Levels of creatinine and glucose in the serum of normal and experimental

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
Creatinine	1.01±0.08	1.17±0.06	1.19±0.07	0.63±0.03 [a,b,c]	1.14±0.11[d]	1.13±0.13[d]
Glucose	88.4±6.31	86.7±6.34	86.5±6.32	64.58±4.95 [a,b,c]	77.8±5.68[e]	77.7±5.63[e]

Values are expressed as the mean ± SD for six animals. Creatinine and glucose- mg/dl

[a] $P<0.001$- compared with Group-I

[b] $P<0.001$- compared with Group-II

[c] $P<0.001$- compared with Group-III

[d] $P<0.001$- compared with Group-IV

[e] $P<0.01$ - compared with Group-IV

metabolites of antitubercular drugs may also contribute to the changes in rough endoplasmic reticulum and to the disturbance of protein metabolism. My findings confirm the same pattern, and showed a significant decrease ($P<0.001$) in protein content in the serum and liver of Group IV, toxicity induced animals as compared to that of Group I normal rats **(Figs 4.2.2. and 4.2.3.)**. The rats co-administered with chitosan and glucosamine showed a significant increase in liver and serum protein content as compared to hepatotoxic rats.

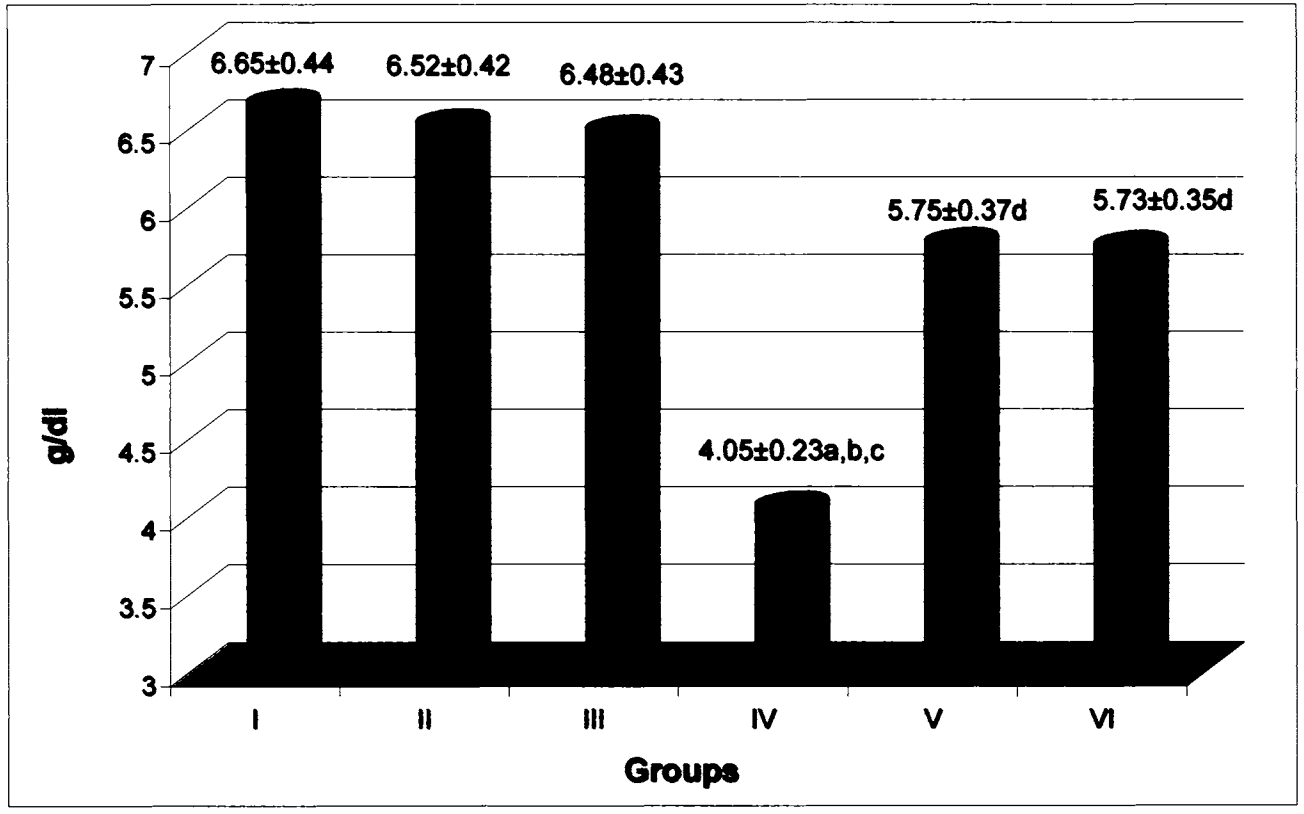
4.2.6. Electrophoretic pattern

In the serum of antitubercular drugs induced rats, the level of albumin was decreased while the level of globulin was slightly increased. The SDS-polyacrylamide gel electrophoretic separation of serum proteins showed the significant reduction in the amount of serum protein especially albumin fraction **(Plate 4.2.7.)**. Serum albumin level is an index of severity and prognosis in patients with chronic hepatic disease. In patients with acute hepatitis, serum albumin level is slightly depressed whereas globulin level is mildly increased (Devaki *et al.*, 1992). In the present study, albumin/globulin ration in antitubercular drugs-induced rats, was less than the normal control rats. When, the SDS-page electrophoretic pattern of liver **(Pate 4.2.8.)** is considered, similar changes were observed. The albumin and globulin levels were maintained to near normalcy in chitosan and glucosamine administered rats.

4.2.7. Gycoprotein components

Levels of glycoprotein conjugates in the liver were showed in **Table 4.2.4**. Group IV, isoniazid and rifampicin administered rats showed a decrease in the levels of hexose, hexosamine and sialic acid. This is in line with an earlier reported study (Leinweber and Mahrt, 1976). The inhibition of protein synthesis in isoniazid and rifampicin-administered rats will disturb the glycoprotein synthesis. Thus, the glycoprotein conjugates (hexose, hexosamine and sialic acid) were found significantly decreased ($P<0.001$) in the Group IV rats. Since, chitosan and glucosamine can induce glycocylation, co-administration of these compounds

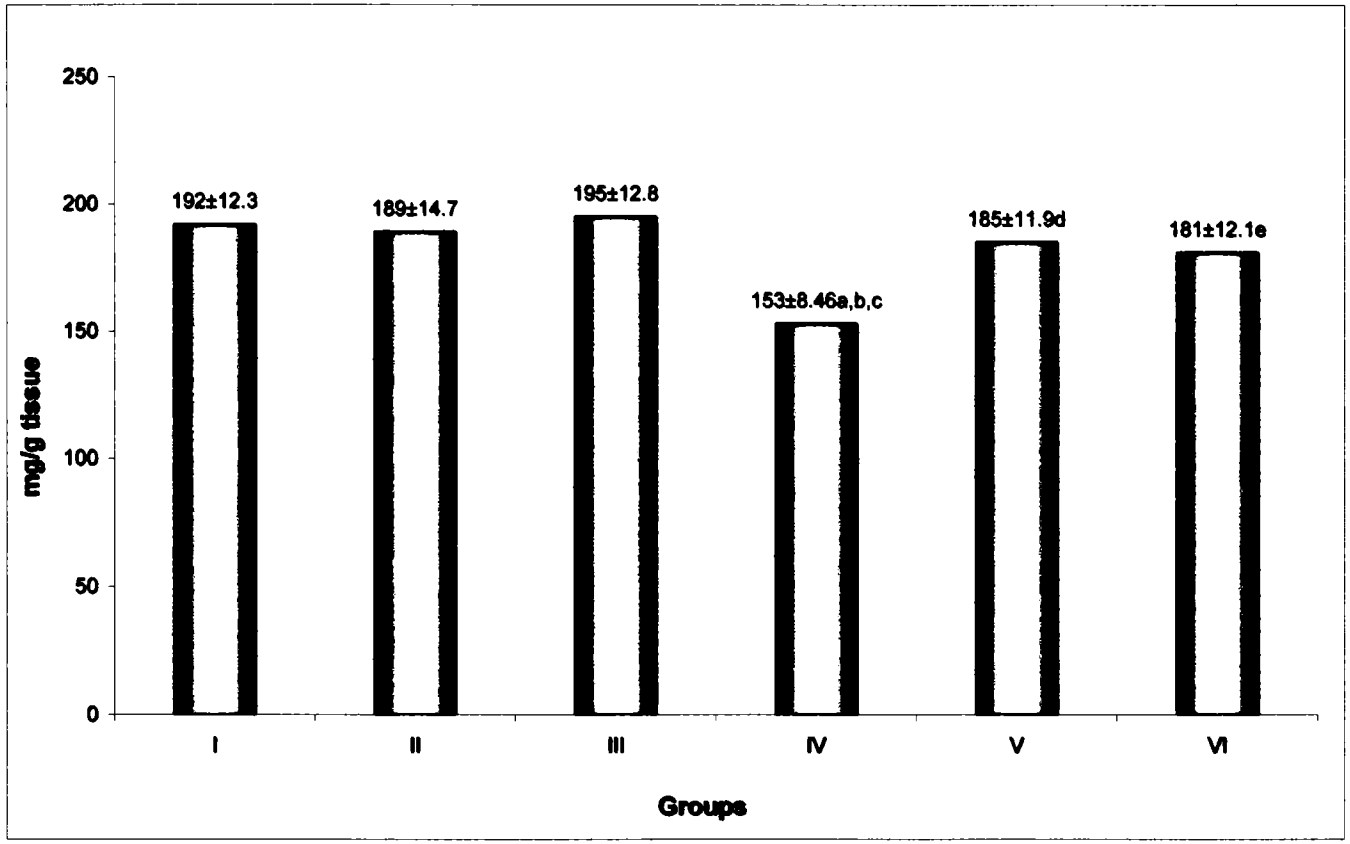
Fig. 4.2.2: Levels of total protein in the serum of normal and experimental groups of rats.



Values are expressed as the mean ± SD for six animals.

[a] $P<0.001$- compared with Group-I, [b] $P<0.001$- compared with Group-II, [c] $P<0.001$- compared with Group-III, [d] $P<0.001$- compared with Group-IV

Fig. 4.2.3: Levels of protein in the liver of normal and experimental groups of rats.



Values are expressed as the mean ± SD for six animals.

[a] $P<0.001$- compared with Group-I, [b] $P<0.001$- compared with Group-II, [c] $P<0.001$- compared with Group-III, [d] $P<0.001$- compared with Group-IV, [e] $P<0.01$- compared with Group-IV

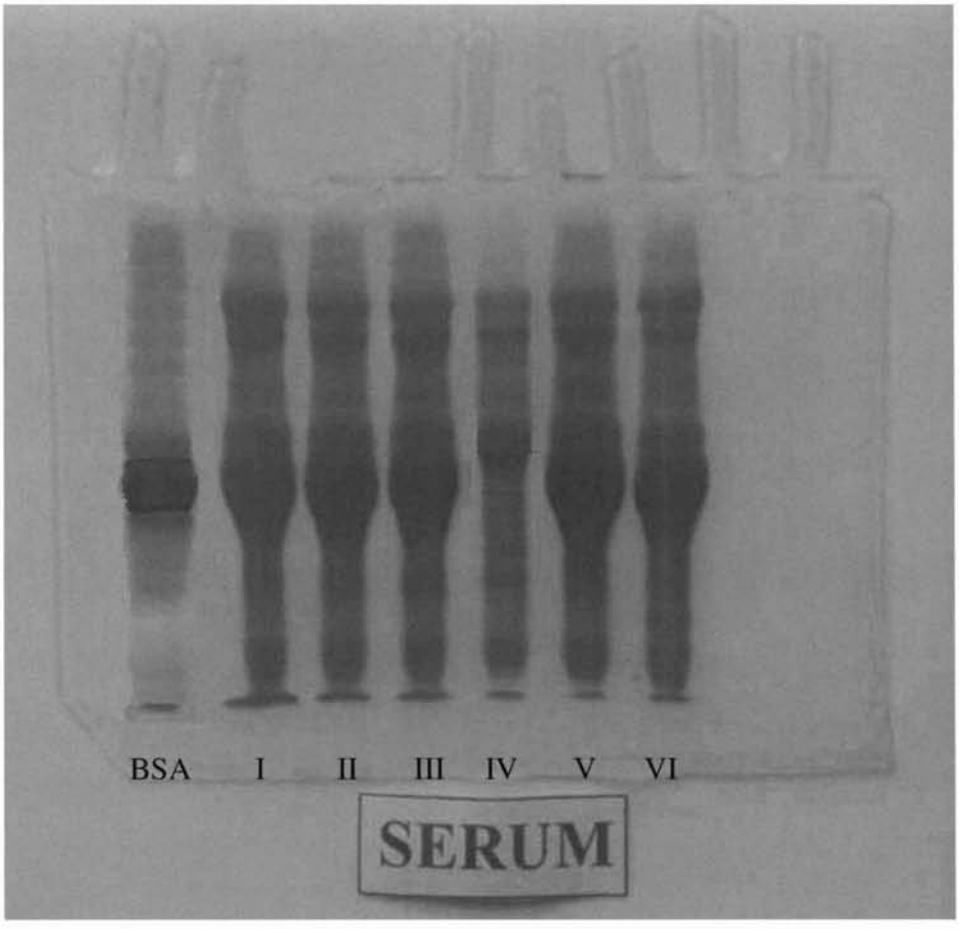


Plate 4.2.7. Electrophoretic pattern of serum protein of normal and experimental groups of rats.

BSA- Bovine Serum Albumin

I- Normal control rats
II- Chitosan control
III- Glucosamine control
IV- Hepatotoxicity induced
V- Chitosan co-administered
VI- Glucosamine co-administered

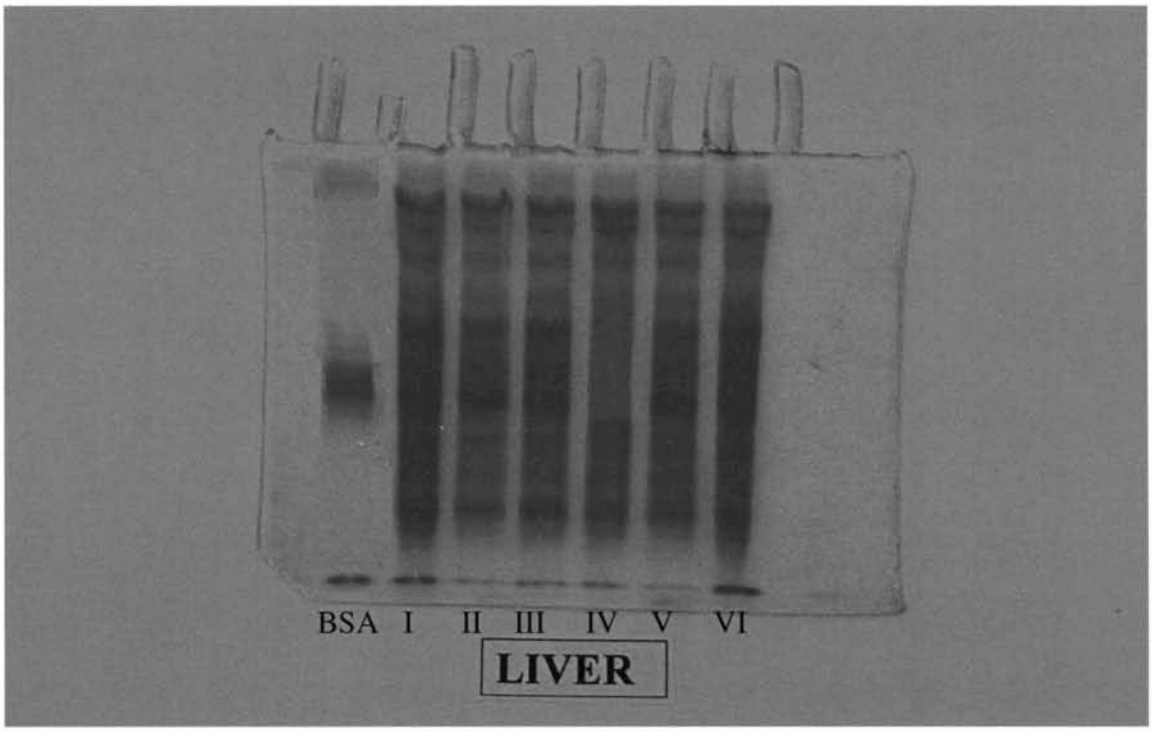


Plate 4.2.8. Electrophoretic pattern of liver protein of normal and experimental groups of rats.

BSA- Bovine Serum Albumin

I- Normal control rats
II- Chitosan control
III- Glucosamine control
IV- Hepatotoxicity induced
V- Chitosan co-administered
VI- Glucosamine co-administered

Table 4.2.4: Levels of hexose, hexosamine and sialic acid in the liver of normal and experimental groups of rats.

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
Hexose	24.7±1.55	28.9±1.68	32.7±2.14	15.6±1.21[a,b,c]	24.3±1.45[d]	27.5±1.99[d]
Hexosamine	8.18±0.49	10.7±0.65	12.8±0.73	5.11±0.44[a,b,c]	7.87±0.56[d]	9.24±0.57[d]
Sialic acid	0.32±0.02	0.36±0.03	0.34±0.0.3	0.21±0.01[a,b,c]	0.28±0.02[d]	0.27±0.03[e]

Values are expressed as the mean ± SD for six animals. Values are expressed in mg/g tissue.

[a] $P<0.001$- compared with Group-I

[b] $P<0.001$- compared with Group-II

[c] $P<0.001$- compared with Group-III

[d] $P<0.001$- compared with Group-IV

[e] $P<0.01$ - compared with Group-IV

may accelerate the synthesis of hexose, amino sugars and sialic acid. As the result, tissue glycoprotein conjugate levels were brought back to normal level in Group V and Group VI animals.

4.2.8. Lipid peroxidation

Lipid peroxidation of membranes is regulated by the availability of substrate in the form of polyunsaturated fatty acids (PUFA), the availability of inducers such as free radicals and the exited state molecules to initiate propagation, the antioxidant defense status of environment and the physical status of membrane lipids (Pepicelli *et al.*, 2005). Antibiotic therapy can favor free radical production in excess of basal rates. Many antibiotics that depend on bound metals for their activity are able to generate free radicals and cause cellular damage (Doroshow & Hochstein, 1982). In the present study, a significant ($P<0.001$) increase in the level of lipid peroxides in the serum and liver tissue of antitubercular drugs-treated rats was observed **(Table 4.2.5)**. This is in corroboration with an earlier investigation (Saraswathy and Shyamala Devi, 2001), which suggested that the high vulnerability of liver to peroxidative damage is mainly due to a decline in the level of free radicals for scavengers. Free radicals initiate lipid peroxidation of biological membranes. Isoniazid and rifampicin induced hepatitis is due to their biotransformation to reactive metabolites that are capable of binding to cellular macromolecules (Georgeiva *et al.*, 2004). As an alternative to inducing cellular damage by covalent binding, there is evidence that these antitubercular drugs causes cellular damage through the induction of oxidative stress, a consequence of dysfunction of hepatic antioxidant defense system. The role of oxidative stress in the mechanism of isoniazid and rifampicin-induced hepatitis has been reported by Attri *et al.* (2000). My findings confirm the same pattern and show significant increase in the level of lipid peroxidation in the serum and liver tissue of Group IV antitubercular drugs administered rats as compared to that of Group I control rats. The combination of isoniazid and rifampicin treatment in experimental animals enhanced lipid peroxidation, indicating increased oxidative stress in liver (Skakun and Yun, 1992).

Table 4.2.5: Levels of lipid peroxides (LPO) in the serum and liver of normal and experimental groups of rats.

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
Serum-LPO	1.43±0.11	1.36±0.09	1.45±0.11	3.42±0.22[a,b,c]	1.57±0.10[d]	1.86±0.15[d]
Liver- LPO	0.96±0.07	0.87±0.04	1.02±0.06	1.91±0.165[a,b,c]	0.98±0.05[d]	1.21±0.06[d]

Values are expressed as the mean ± SD for six animals. Serum LPO- nmoles of MDA/ml; Liver LPO- nmole MDA/mg protein.

[a] $P<0.001$- compared with Group-I

[b] $P<0.001$- compared with Group-II

[c] $P<0.001$- compared with Group-III

[d] $P<0.001$- compared with Group-IV

Antioxidants are necessary for preventing the formation of free radicals and they inhibit some of the deleterious actions of reactive oxygen species that damage lipids, DNA and proteins (Eidelman *et al.*, 2002). Co-treatment with chitosan and glucosamine resulted in significant ($P<0.001$) reduction in the level of lipid peroxidation (Shevtsova, 2000) towards near normalcy, as compared with the levels in the Group IV antitubercular drugs-administered rats, establishing the antioxidant nature (Mohur and Cook, 1957) by blocking isoniazid and rifampicin-induced lipid peroxidation.

4.2.9. Glutathione-dependent antioxidant system

Activities of glutathione-dependent antioxidant enzymes [glutathione peroxidase (GPx) and glutathione *S*-transferase (GST)] and the level of reduced glutathione were significantly ($P<0.001$) lower in the liver tissue of Group IV rats as compared to Group I control animals **(Tables 4.2.6 and 4.2.8)**. Glutathione antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species. Excessive lipid peroxidation can cause increased glutathione consumption (Onyema *et al.*, 2006), as observed in the present study. The cellular tripeptide, GSH (γ -glutamyl cysteinyl glycine) thwarts peroxidative damage by neutralizing the free radicals. Reduction noticed in the activities of GPx and GST in antitubercular drugs -induced hepatotoxicity condition might be due to decreased availability of their substrate, reduced glutathione. This in turn may lead to increase the accumulation of oxidative free radicals and also enhance the susceptibility of liver cell membrane to peroxidative damage.

In the present study, co-treatment with chitosan and glucosamine significantly prevented the antitubercular drugs-induced aberrations in the level of GSH ($p<0.001$) and the activities of GPx ($P<0.001$) and GST ($P<0.001$, $P<0.05$) in the liver tissue of Group V and Group VI rats as compared to that of Group IV rats. Benassi *et al.* (2006) had earlier reported that chitosan administration could increase the level of GSH in hepatotoxic condition.

4.2.10. Antiperoxidative enzymes

Activities of antiperoxidative enzymes [super oxide dismutase (SOD) and catalase (CAT)] were significantly (P<0.001) lower in the liver of antitubercular drugs-administered rats as compared to Group I control rats **(Table 4.2.6)**. SOD and CAT, responsible for the destruction of peroxides have a specific role in protecting tissues against oxidative damage. Reduction in the activities of these enzymes in antitubercular drugs-induced hepatotoxicity may lead to the formation of O^{2-} and H_2O_2, which in turn can form hydroxyl radical ($OH^{\bullet}$) and bring about a number of reactions harmful to the cellular and subcellular membranes. The generation of free radical scavenging enzymes in antitubercular drugs-induced hepatotoxicity might have exceeded the ability of these free radical scavenging enzymes to dismute the radicals, resulting in peroxidative damage to liver cell membranes.

In this study, co-treatment with chitosan and glucosamine significantly (P<0.001) maintained the activities of these antiperoxidative enzymes at near normalcy. They probably did so by their antioxidant action (Xing *et al.*, 2005; Sal'nikova *et al.*, 1990) against isoniazid and rifampicin-induced lipid peroxidation. The unpaired electron present in the hydroxyl free radical, which is mainly responsible for the antitubercular drugs-induced necrotic damage to the hepatocytes, might have been trapped and subsequently dismuted by the antioxidant property of chitosan and glucosamine. Earlier Yan *et al.* (2006) reported that chitosan had a potent reducing effect on the production of free radicals in rats exposed to cytotoxic substance.

4.2.11. Membrane-bound ATPases

There was a significant (P<0.001) reduction noticed in the activities of the membrane bound ATPases in the liver of Group IV, antitubercular drugs administered rats as compared to that of Group I control animals **(Table 4.2.7)**. This is in accordance with an earlier reported study (Saraswathy *et al.*, 1998), which indicated that isoniazid and rifampicin induced hepatitis is characterized by

Table-4.2.6: Levels of catalase (CAT), glutathione s-transferase (GST), glutathione peroxidase (GPx) and super oxide dismutase (SOD) in the liver of normal and experimental groups of rats.

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
CAT	7.33±0.42	7.25±0.39	7.28±0.46	3.05±0.24[a,b,c]	6.84±0.37[d]	5.46±0.27[d]
GST	9.63±0.58	10.4±0.61	9.42±0.55	6.29±0.31[a,b,c]	8.77±0.59[d]	7.23±0.47[e]
GPx	7.59±0.47	7.76±0.45	7.43±0.52	4.83±0.24[a,b,c]	6.99±0.36[d]	6.02±0.32[d]
SOD	6.21±0.55	6.44±0.47	6.18±0.42	3.04±0.18[a,b,c]	5.97±0.46[d]	4.83±0.39[d]

Values are expressed as the mean ± SD for six animals.

CAT, µmol of H_2O_2 consumed/min/mg protein; GST, µmol 1-chloro-2,4 dinitrobenzene conjugate formed /min /mg protein; GPX, nmol GSH oxidized/min/mg protein; SOD, one unit of the superoxide dismutase activity is the amount of protein required to give 50% inhibition of epinephrine autooxidation.

[a] $P<0.001$- compared with Group-I, [b] $P<0.001$- compared with Group-II, [c] $P<0.001$- compared with Group-III,

[d] $P<0.001$- compared with Group-IV, [e] $P<0.05$- compared with Group-IV

Table 4.2.7: Activities of membrane bound ATPases in the liver of normal and experimental groups of rats.

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
Total ATPase	3.34±0.18	3.45±0.15	3.23±0.22	2.08±0.11 [a,b,c]	3.08±0.19[d]	2.67±0.14 [d]
Ca^{2+}-ATP	0.74±0.05	0.69±0.05	0.78±0.04	0.51±0.02 [a,b,c]	0.67±0.04[d]	0.63±0.05 [d]
Mg^{2+}-ATP	1.42±0.11	1.49±0.14	1.35±0.12	1.10±0.07[a,b,e]	1.31±0.11[f]	1.28±0.10[f]
Na^{+}-K^{+} ATP	1.18±0.08	1.27±0.09	1.1±0.07	0.47±0.03[a,b,c]	1.12±0.07[d]	0.86±0.07[d]

Mean ± SD for six animals in each group. Values are expressed as μ moles of Pi liberated per mg protein per hour.

[a] $P<0.001$- compared with Group-I

[b] $P<0.001$- compared with Group-II

[c] $P<0.001$- compared with Group-III

[d] $P<0.001$- compared with Group-IV

[e] $P<0.01$- compared with Group-III

[f] $P<0.05$- compared with Group-IV

Table 4.2.8: Levels of total thiols and free thiol (GSH) in the liver of normal and experimental groups of rats.

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
Total thiols	398±27.4	412±24.5	385±28.2	237±17.5[a,b,c]	353±23.7[d]	319±25.7[d]
Free thiol (GSH)	4.84±0.32	5.18±0.34	4.97±0.31	3.38±0.28[a,b,c]	4.95±0.33[d]	4.65±0.32[d]

Mean ± SD for six animals in each group. Values are expressed as nmoles/g wet tissue.

[a] $P<0.001$- compared with Group-I

[b] $P<0.001$- compared with Group-II

[c] $P<0.001$- compared with Group-III

[d] $P<0.001$- compared with Group-IV

a severe derangement of subcellular metabolism and structural alterations in liver cell membrane. Since membrane-bound ATPases are lipid dependent as well as –SH dependent enzymes, alterations in membrane lipid may lead to changes in membrane fluidity, which in turn affects concentration of intracellular ions and conservation of transmembrane electrochemical gradient. The functional state of liver depends on its active Na^+, K^+ sequestering property. Earlier Tasduq *et al.* (2005) have reported that the appearance of antitubercular drugs induced hepatic injury is dependent on intracellular Ca^{2+} concentrations. Isoniazid and rifampicin intoxication has been previously reported to be associated with alteration in membrane-bound ATPases (Skakun and Tabachuk, 1992). Co-administration of chitosan and glucosamine restored the activities of membrane bound ATPases at near normal level. This could be due to the ability of chitosan and glucosamine to protect the 'SH' groups from oxidative damage though inhibition of peroxidation of membrane lipids. The membrane stabilizing property of chitosan is already reported by Filipovic-Grcic *et al.* (2001).

4.2.12. Hepatic thiols

The levels of membrane protective thiols were found to decrease significantly ($P<0.001$) in antitubercular drugs-administered rats **(Table 4.2.8)**. The remarkable loss in total, Ca^{2+} and Na^+K^+ ATPase activities found in Groups IV animals may also be due to the deteriorations of SH because of increased peroxidative damage caused by isoniazid and rifampicin administration. Co-administration of chitosan and glucosamine was found to restore the levels of thiols in Group V and Group VI animals. This is due to the free radical scavenging property of these compounds

4.2.13. Lipid components

Tables 4.2.9 and **4.2.10** show the levels of lipid components in serum and liver of normal and experimental groups of rats respectively. Serum HDL and LDL cholesterol levels were shown in **Fig. 4.2.4.** Significant ($P<0.001$) increases in

Table 4.2.9: Levels of lipid components in the serum or normal and experimental groups of rats

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
Cholesterol	54.5±0.48	48.1±0.24	58.7±0.31	78.9±0.62[a,b,c]	56.1±0.27[d]	59±0.29[d]
Triglycerides	47.4±0.36	40.6±0.26	48.1±0.28	95.8±0.76[a,b,c]	54.9±0.34[d]	55.3±0.35[d]
Free fatty acids	10.2±0.09	8.34±0.51	9.87±0.58	18.1±0.12[a,b,c]	10.5±0.98[d]	11.4±1.2[d]
Phospholipids	108±9.8	94.2±8.72	98.3±8.91	128±10.40[b,c,e]	102±9.16[d]	107±9.54[f]

Values are expressed as Mean ± SD for six animals in each group. Cholesterol, triglycerides, free fatty acids and phospholipids = mg/dl

[a] $P<0.001$- compared with Group-I, [b] $P<0.001$- compared with Group-II, [c] $P<0.001$- compared with Group-III,
[d] $P<0.001$- compared with Group-IV, [e] $P<0.01$- compared with Group-I, [f] $P<0.01$- compared with Group-IV

Table 4.2.10: Levels of lipid components in the liver normal and experimental groups of rats

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
Cholesterol	9.18±0.81	8.05±0.54	8.71±0.58	15.4±0.98[a,b,c]	10.4±0.87[d]	10.5±0.88[d]
Triglycerides	18.5±1.25	15.3±0.95	18.8±1.23	29.9±1.75[a,b,c]	18.2±1.29[d]	20.3±1.58[d]
Free fatty acids	0.94±0.05	0.83±0.04	1.02±0.05	1.73±0.11[a,b,c]	0.88±0.04[d]	1.1±0.06[d]
Phospholipids	27.3±1.02	28.5±2.15	27.2±1.03	24.2±1.84[e,f,g]	27.6±2.03[h]	27.4±2.01[h]

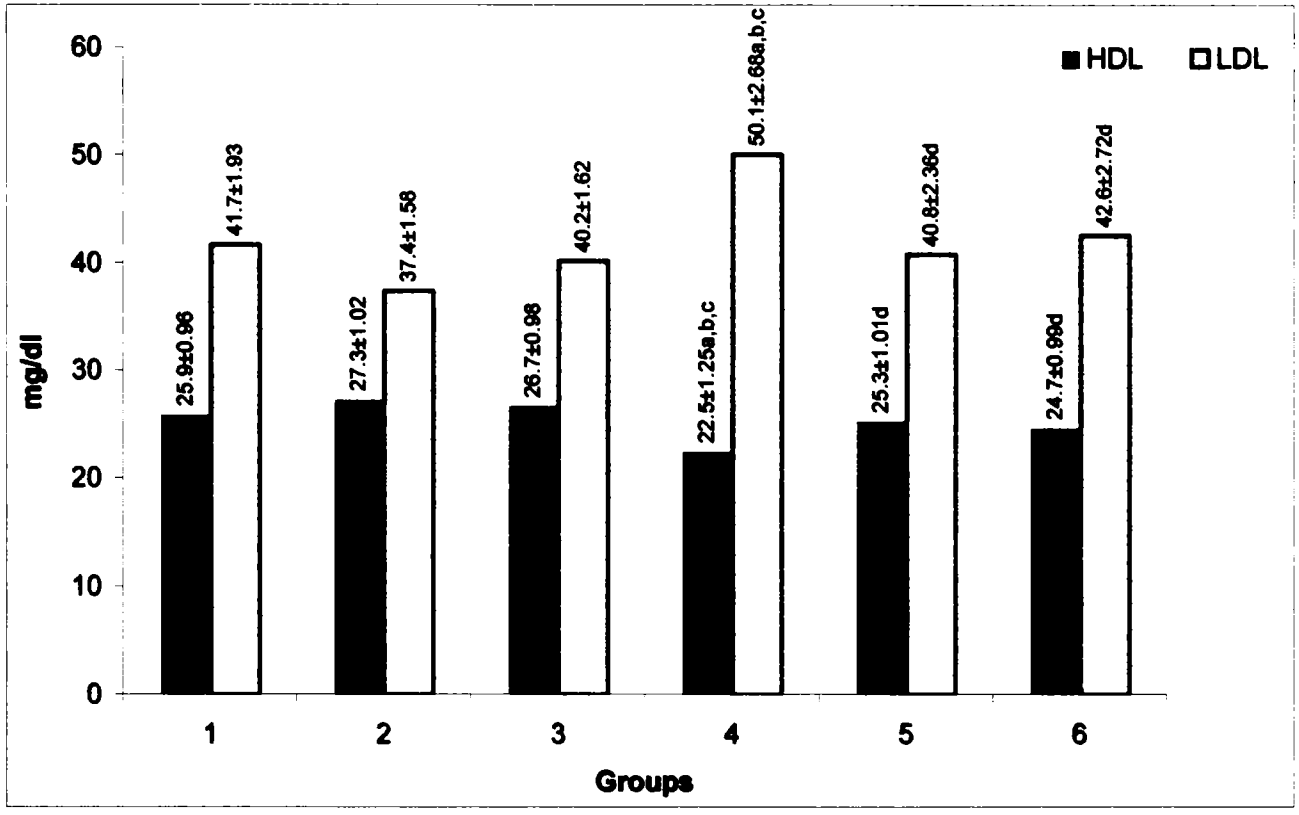
Values are expressed as Mean ± SD for six animals in each group. Cholesterol, triglycerides, free fatty acids and phospholipids = mg/g tissue

[a] $P<0.001$- compared with Group-I, [b] $P<0.001$- compared with Group-II, [c] $P<0.001$- compared with Group-III, [d] $P<0.001$- compared with Group-IV, [e] $P<0.01$- compared with Group-II, [f] $P<0.05$- compared with Group-I, [g] $P<0.05$- compared with Group-III, [h] $P<0.05$- compared with Group-IV

the levels of cholesterol, triglycerides and free fatty acids were observed in the serum and liver of Group IV, isoniazid and rifampicin-administered rats as compared to that of Group I control rats. A slight decline in the level of HDL-cholesterol with concomitant rise in LDL-cholesterol level was also noted in Group IV antitubercular drugs-administered rats **(Fig. 4.2.4.)**. The rats co-administered with chitosan and glucosamine showed significantly near normal levels of lipid components in serum and liver of Group V and Group VI rats as compared to that of Group IV hepatotoxicity induced rats, indicating the antilipidemic effect of chitosan. A significant rise in the level of phospholipids was noted in serum ($P<0.01$) of Group IV antitubercular drugs-administered rats. This was paralleled by a slight reduction ($P<0.05$) in the level of phospholipids in liver tissue of Group IV rats. Chitosan co-administration resulted in the prevention of antitubercular drugs-induced phospholipids depletion in the liver of experimental groups of rats. It also exerted a significant ant ilipid peroxidative effect by blocking the induction of isoniazid and rifampicin-mediated lipid peroxidation in serum and liver of Group IV rats. The rats administered with chitosan alone did not show any adverse effects, indicating that chitosan is non-toxic.

The major disorder encountered in antitubercular drugs-induced hepatitis is fatty accumulation in the liver, which develops either due to excessive supply of lipids to the liver or interference with lipid deposition. The pathogenesis is multifactorial, reflecting complex biosynthetic, enzymatic and catabolic derangement in lipoprotein metabolism. In the present study, the levels of total cholesterol and LDL-cholesterol were significantly ($P<0.001$) higher in Group III isoniazid and rifampicin-administered rats as compared to that of normal control animals, indicating the antitubercular drugs-induced hypercholesterolemic condition. Also the level of HDL-cholesterol was slightly reduced in Group III rats. This is in line with an earlier study (Gibson *et al.*, 1995). Increased cholesterol levels in the liver might due to increased uptake of LDL from the blood by the tissues (Kissler *et al.*, 2005). The abnormal cholesterol deposition is favored by the dangerous tendency of cholesterol to passive exchange between the plasma lipoproteins and the cell membranes (Brown and Goldstein, 1986).

Fig. 4.2.4: Levels of HDL-cholesterol and LDL-cholesterol in the serum of normal and experimental groups of rats.



Values are expressed as the mean ± SD for six animals.

[a] $P<0.001$- compared with Group-I, [b] $P<0.001$- compared with Group-II, [c] $P<0.001$- compared with Group-III, [d] $P<0.001$- compared with Group-IV

In the present study, co-administration with chitosan significantly ($P<0.001$) reduced the antitubercular drugs-induced elevation in the levels of total cholesterol and LDL-cholesterol in Group IV rats as compared to that of Group III rats. Also the level of HDL-cholesterol maintained at near normalcy. Earlier Yao and Chiang (2002) have shown that chitosan intake is effective in lowering the plasma total cholesterol, VLDL cholesterol and LDL cholesterol levels in experimental animals. In the present study, a slight reduction in the level of total cholesterol and LDL-cholesterol were also observed in Group II chitosan and glucosamine fed normal animals, establishing the hypocholesterolemic property of them. The hepatoprotective effect of chitosan and glucosamine is probably related to its ability to inhibit the lipid accumulation in the liver tissue by the antilipidemic property (Xing *et al.*, 2005).

The levels of triglycerides were significantly ($P<0.001$) higher in serum and liver tissue of Group III rats as compared to Group I control animals **(Tables 4.2.9** and **4.2.10).** Increased lipolysis of depot triglycerides liberates free fatty acids from adipose tissue stores (Kruger *et al.*, 1967: Stenberg, 1976) and the free fatty acids liberated by the adipose tissue are also taken up by the liver tissue, leading to the hypertriglyceridemic condition. In the present study, a significant ($P<0.001$) reduction was noticed in the levels of triglycerides and free fatty acids in serum and liver of Group IV chitosan and glucosamine administered rats as compared to that of Group III isoniazid and rifampicin-induced hepatotoxic rats, indicating the hypolipidemic nature of chitosan and glucosamine. The hypolipidemic property of chitosan has already been reported in high fat diet fed experimental animals (Xing *et al.*, 2005).

The levels of phospholipids were significantly ($P<0.01$) decreased in the liver tissue of Group III isoniazid and rifampicin-administered rats as compared to that of normal control animals. This is in accordance with earlier study (Karthikeyan, 2005), which indicates that hepatic injury related alterations in lipid composition of liver tissue appears to occur due to the destruction of hepatocytes. The intracellular calcium concentration has been reported to rise in antitubercular drugs-induced hepatitis (Tasduq *et al.*, 2005). This intracellular Ca^{2+} is an

inducer of phospholipase A_2, which degrades membrane phospholipids. The increased peroxidation of membrane phospholipids releases free fatty acid by the action of phospholipase A_2. Hence, the significant ($P<0.001$) elevation noticed in free fatty acid content in isoniazid and rifampicin-administered rats might be due to enhanced breakdown of membrane phospholipids. Recent studies (Karthikeyan, 2005) indicate that accelerated degradation of membrane phospholipids is very likely the biochemical basis for the antitubercular induced hepatocellular injury.

In the present study, the co-administration of chitosan significantly ($P<0.05$) reduced the antitubercular drugs-induced degradation of membrane phospholipids in liver, establishing its membrane stabilizing effect. It probably did so by preventing changes in fatty acid composition and inhibiting the peroxidative deterioration of hepatocellular membranes. It is possible that stabilization of hepatocelluar membranes might prolong the viability of hepatocytes.

4.2.14. Fatty acid composition

Table 4.2.11 shows the fatty acid composition of the liver of normal and experimental groups of rats. Fatty Administration of isoniazid and rifampicin leads to their biotransformation to toxic metabolites like ROS (reactive oxygen species) and the prime target of ROS is PUFA (Georgieva *et al.*, 2004). Depletion of PUFA alters the fatty acid composition of tissues in isoniazid and rifampicin intoxicated rats. The levels of all fatty acids were found to increase except arachidonic acid in Group IV, hepatotoxic animals. Arachidonic acid is released from cell membrane phospholipids by the effect of calcium dependent phosphatases (PL) A_2. Cyclooxygenase products of arachidonic acid are present in much higher concentrations in inflamed tissues than in healthy tissues (Emken *et al.*, 1997). Administration of isoniazid and rifampicin may increase the membrane PLA_2 activity, liberating arachidonic acid from phospholipids.Thus the arachidonic acid levels in the antitubercular drugs induced rats are lowered than the control animals. This decrease may also be due to the channeling of arachidonic acid for the production of prostaglandins. Arachidonic acid

Table 4.2.11: Fatty acid composition of the liver of normal and experimental groups of rats

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
Palmitic acid	12.52±1.24	10.58±1.21	11.63±1.39	29.24±2.89[a,b,c]	17.91±1.26[d]	19.26±1.98[e]
Palmitoleic acid	16.34±1.58	18.62±1.71	17.46±1.82	9.53±1.06[a,b,c]	14.53±1.21[d]	11.39±1.23[e]
Stearic acid	11.23±1.13	12.65±1.19	11.84±1.16	19.57±1.62[a,b,c]	12.36±1.15[d]	13.54±1.31[e]
Oleic acid	11.16±1.25	9.87±1.08	10.08±1.10	18.68±1.38[a,b,c]	12.62±1.20[d]	13.81±1.19[e]
Arachidonic acid	2.87±0.05	1.98±0.03	2.11±0.04	4.89±0.07[a,b,c]	3.12±0.03[d]	3.56±0.04[f]
Eicosapentaenoic acid	22.86±1.98	23.75±2.02	23.02±2.03	15.64±1.52[a,b,c]	20.86±1.19[d]	19.46±1.12[f]
Docosapentaenoic acid	15.49±1.38	14.72±1.36	13.88±1.41	26.85±2.56[a,b,c]	16.54±1.53[d]	17.62±1.28[e]

Values are expressed as Mean ± SD for six animals in each group. Values are expressed in wt %

[a] $P<0.001$- compared with Group-I, [b] $P<0.001$- compared with Group-II, [c] $P<0.001$- compared with Group-III,

[d] $P<0.001$- compared with Group-IV, [e] $P<0.001$- compared with Group-IV, [f] $P<0.05$- compared with Group-IV

hydrolysed from phospholipids is used for the production of eicosanoids (Holtzman *et al.*, 1991). Chitosan and glucosamine administration to antitubercular drugs induced rats inhibit the release of arachidonic acid in the liver, which may be because chitosan and glucosamine inhibit the activity of PLA_2.

4.2.15. Mineral composition

Mineral composition of the liver of normal and experimental groups of rats were given in **Fig. 4.2.5.** and **Table 4.2.12.** The intracellular concentration of sodium and potassium were regulated by a transport system in the cell membrane. This converts chemical energy by the hydrolysis of ATP to drive the vectorial movement of sodium out of the cell and potassium into the cell against an electrochemical gradient (Skou, 1971). Active calcium transport and resultant low calcium concentration are the necessary conditions for active sodium/potassium ionic transport. Since, sodium and calcium are thought to be competitive at a number of membrane sites, it seems likely that a high concentration of Ca^{2+} in liver cells of ischemic rats would compete with sodium specific sites at the inner surface of the membrane (Vincenzi, 1971) and this may lead to decrease in sodium content. Also, failure of sodium pump results in a depletion of liver sodium and rise in potassium concentration was observed in the present study. This leads to hyponatremia and hyperkalemia, which are the most common electrolyte abnormalities in hepatotoxic condition.

Isoniazid and rifampicin administration increased the level of calcium in the liver tissue. Elevation in Ca^{2+} is known to be involved in the initiation and progression of hepatocellular damage (Bellemo and Orrenius, 1985). Calcium is a messenger of great importance to cells. Antitubercular drugs administration could maximally increase Ca^{2+} influx in whole liver tissue. Since plasma membrane Ca^{2+}-ATPase extrudes Ca^{2+} from the cytoplasm of all the cells (Horward *et al.*, 1994) blocking of intracellular ca2+-ATPase under the condition of isoniazid and rifampicin induced hepatotoxicity results in fast cellular ca^{2+} accumulation. Thus influx of calcium into the cell is uncontrolled under antitubercular drugs induced toxicity,

Table 4.2.12: Levels of calcium, magnesium and zinc in the liver of normal and experimental groups of rats

Parameters	Group I Normal Control	Group II Chitosan-Control A	Group III Glucosamine-Control B	Group IV Hepatotoxicity C	Group V A+C	Group VI B+C
Calcium	302±6.32	299±5.84	301±5.68	355±10.36[a,b,c]	312±9.63[d]	314±9.81[e]
Magnesium	291±5.12	288±5.03	290±5.11	338±9.25[a,b,c]	301±7.94[d]	304±7.82[e]
Zinc	92±3.56	90±3.49	91±3.51	144±5.32[a,b,c]	98±4.68[d]	100±4.85[e]

Mean ± SD for six animals in each group. Values are expressed as mg/g tissue

[a] $P<0.001$- compared with Group-I

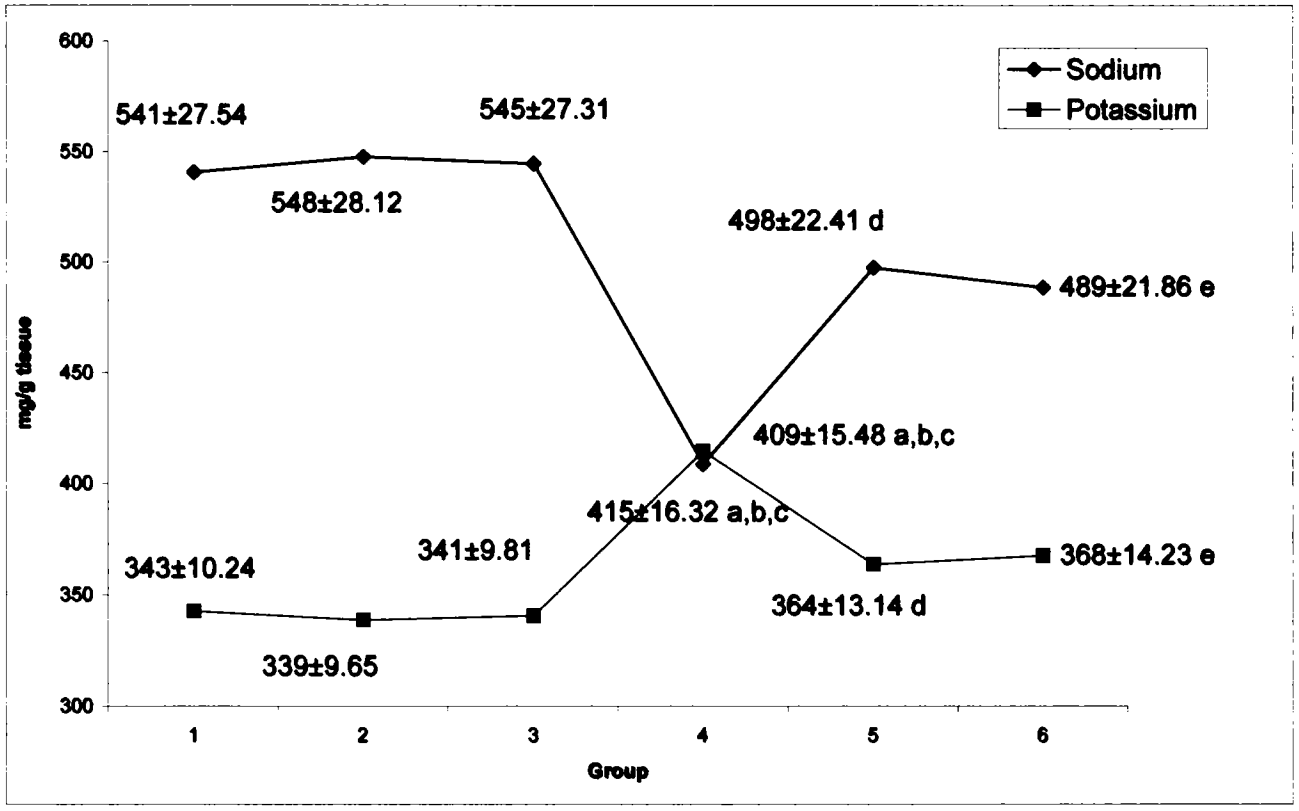
[b] $P<0.001$- compared with Group-II

[c] $P<0.001$- compared with Group-III

[d] $P<0.001$- compared with Group-IV

[e] $P<0.01$- compared with Group-IV

Fig. 4.2.5: Levels of sodium and potassium in the liver of normal and experimental groups of rats.



Values are expressed as the mean ± SD for six animals.

[a] $P<0.001$- compared with Group-I, [b] $P<0.001$- compared with Group-II, [c] $P<0.001$- compared with Group-III, [d] $P<0.001$- compared with Group-IV, [e] $P<0.01$- compared with Group-IV

leading to the disturbance in equilibrium between intracellular and extra cellular calcium concentrations. The level of Mg^{2+} was also found to be raised significantly in the hepatotoxic group. The intracellular concentration of calcium regulates the activity of the Mg^{2+}. Bironaite and Ollinger (1997) reported that peroxidation can influence the alteration of Mg^{2+}.

Co-administration of chitosan and glucosamine significantly ($P<0.001$) prevented the isoniazid and rifampicin induced alterations in the levels of these minerals to the near normal level. It may be done by protecting the plasma membrane bound ATPases from the free radical attack by their free radical scavenging capacity. An earlier report (Wada *et al.*, 1997) supports this finding, that chitosan can reduce the whole-body retention of Calcium in rats.

In conclusion, the results of the present study indicate that the co-treatment with chitosan and glucosamine prevent antitubercular drugs-induced hepatotoxicity in rats. The overall hepatoprotective effect of chitosan and glucosamine is probably due to it membrane stabilizing action, or to a counteraction of free radicals by its antioxidant nature, or to its ability to maintain near to the normal status the activities of the free radical scavenging enzymes and the level of reduced glutathione, which protects cell membrane against peroxidative damage by decreasing lipid peroxidation and strengthening the liver cell membrane.

5. SUMMARY AND CONCLUSION

The protective effect of chitosan and glucosamine against ibuprofen-induced gastric ulcer was studied. Administration of ibuprofen had brought about the ulcerated mucosal lesions in the experimental rats. It is evident by the increased acidity, number of lesions and decreased peptic activity. The gross pathological and histopathological observations also confirmed the mucosal tissue damage.

Pre-oral treatment with chitosan and glucosamine significantly decreased the ibuprofen induced gastric lesions and acidity and brought back the peptic activity to the normal level. This shows the cytoprotective effect of these compounds. Chitosan and glucosamine might be neutralized the increased acid secreted by the stomach due to ibuprofen irritant.

The levels of antioxidant enzymes, antiperoxidative enzymes, reduced glutathione, lipid peroxidation and membrane bound ATPases were also altered by the ibuprofen intake. Chitosan and glucosamine pre-treatment maintained these levels to near normalcy. The results of the present study indicate that the pre-oral administration of chitosan and glucosamine maintain near to the normal status the activities of the mucosal antioxidant enzymes and the level of GSH, which protect mucosa against oxidative damage by decreasing the lipid peroxidation and strengthening the mucosal barrier, and which are the first line of defense against exogenous and endogenous ulcerogenic agents. Pre-treatment with chitosan and glucosamine significantly maintained the activities of membrane bound ATPases by the counteraction of ibuprofen-induced free radicals by the free radical scavenging property of chitosan and glucosamine.

Administration of ibuprofen deteriorated protein and glycoprotein levels due to the corrosion of gastric mucosa, resulting in the disruption and disintegration of gastric mucosal cells. This was reflected in the electrophoretic pattern also. Oral pre-treatment with chitosan and glucosamine maintained these levels. This may be because; the incorporation of glucosamine units into mucosal glycoprotein might have resulted in strengthening of the mucosal barrier.

Histopathological study showed that ibuprofen induction was characterized by severe cell necrosis, which is normalized by the administration of chitosan and glucosamine showing their cytoprotectivity. The level of sodium was decreased and potassium, calcium, magnesium and zinc were increased due to ibuprofen administration. The accumulation of intracellular calcium levels also play an important role in the DNA fragmentation and ulcer induced by ibuprofen. Increased peroxidation may be the cause of increment in the magnesium level. Chitosan and glucosamine pre-treatment maintained the levels of these minerals to the normal level. Changes in the amino acid levels bought about by the ibuprofen induced cell damage were also restored to normal level by the pre-oral aministration of chitosan and glucosamine.

Observations in this study indicate that the oral pre-treatment of chitosan and glucosamine can prevent ibuprofen-induced peptic ulcer in rats. The overall antiulcerogenic activity of chitosan and glucosamine is probably related to the ability to neutralize the hydrochloric acid secreted into the stomach, and to the maintenance of GSH level by decreasing lipid peroxidation and to the capability to strengthen the mucosal barrier by increasing mucosal glycoprotein synthesis and the free radical scavenging property. On comparison, glucosamine was found to have better antiulcerogenic property than chitosan.

The protective effect of chitosan and glucosamine against antitubercular drugs-induced hepatotoxicity was also studied. Administration of isoniazid and rifampicin, most widely used antitubercular drugs, caused severe toxicity to liver. It increased the levels of diagnostic marker enzymes in the serum. Enhanced susceptibility of hepatocytes cell membrane to the isoniazid and rifampicin-induced peroxidative damage might have resulted in increased release of these diagnostic marker enzymes into the systemic circulation. In cases of liver damage with hepatocellular lesions and parenchymal cell necrosis, these marker enzymes are released from the damaged tissues into the blood stream. As a result the levels of liver ALT and AST were decreased in the antitubercular drugs-induced rats. Co-administration of chitosan and glucosamine maintained the levels of diagnostic marker enzymes in the serum and liver to near normalcy.

They probably did so by preventing the antitubercular drugs induced necrotic damage by the membrane stabilizing action normalizing the protein metabolism.

Histopathological investigations showed severe vacuolation and degeneration of hepatocytes in the three zones of lobules. Membrane disintegration with intense vacuolation accompanied with cytoplasmic rarefication resulting in loss of polyhedral structure in the liver of rat treated with antitubercular drugs. Treatment with chitosan and glucosamine caused reversal of such pathology with no evidence of necrotic areas.

The oral administration of antitubercular drugs led to periportal infiltration of inflammatory cells, disruption of lobular architecture, and liver cell necrosis and causes severe disturbance of RNA and protein metabolism. Accumulation of metabolites of antitubercular drugs may also contribute to the disturbance in protein metabolism. The eletrophoretic pattern of hepatotoxic rats was also found disturbed especially in albumin fraction. Drug induced hepatotoxicity is associated with failure to maintain serum albumin level whereas serum globulins, particularly γ-globulin, which is formed in the reticuloendothelial system are increased. In hepatotoxic condition, due to the failure of the liver to convert amino acids and ammonia to urea, a significant decrease in urea was observed. The inhibition of protein synthesis in isoniazid and rifampicin-administered rats disturbed the glycoprotein synthesis. Thus, the glycoprotein conjugates (hexose, hexosamine and sialic acid) were found decreased in hepatoxic animals. Alterations in all these factors were maintained to near normal level by chitosan and glucosamine co-administration. Since, chitosan and glucosamine were N-acetyl sugars they induced glycocylation, which helped glycoprotein synthesis. These muco-compounds can decrease the accumulation of reactive metabolites of isoniazid and rifampicin and balance protein metabolism.

Administration of isoniazid and rifampicin was characterized by increased lipid peroxidation and decreased levels of glutathione-dependent antioxidant enzymes, reduced glutathione and antiperoxidative enzymes. Co-administration of chitosan and glucosamine along with antitubercular drugs brought back the

levels of these enzymes, lipid peroxidation and reduced glutathione to normal status. The antioxidant property of chitosan and glucosamine prevented the formation of free radicals and they inhibited some of the deleterious actions of reactive oxygen species that damage lipids, DNA and proteins

The antitubercular drugs-induced hepatitis is characterized by the fatty accumulation in the liver, which develops either due to excessive supply of lipids to the liver or interference with lipid deposition. The levels of total cholesterol and LDL-cholesterol were increased whereas HDL-cholesterol was decreased in antitubercular drugs induced animals. Increased cholesterol levels in the liver might due to increased uptake of LDL from the blood by the tissues. The abnormal cholesterol deposition is favored by the dangerous tendency of cholesterol to passive exchange between the plasma lipoproteins and the cell membranes. Animals co-administerd with chitosan and glucosamine maintained the levels of total, HDL and LDL cholesterol levels to the near normal level. The alterations in triglycerides, free fatty acids and phospholipids in the hepatotoxic rats were also normalized by chitosan and glucosamine co-administration. This may be due to their ability to inhibit the lipid accumulation in the liver tissue by the antilipidemic property and membrane stabilizing effect.

Chitosan and glucosamine co-administration rectified the deviations, caused by the anititubercular drugs administration,in the levels of membrane bound ATPases, hepatic thiols, minerals and fatty acid components.

From all the results obtained in the present study, it can be concluded that co-administration of chitosan and glucosamine can effectively prevent the isoniazid and rifampicin induced hepatotoxicity in rats. Comparatively, chitosan was found to have better results than glucosamine in alleviating the hepatic disorders.

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