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The Role of Two Antidotes in the Prevention of Acetaminophen - induced Toxicity in Male Rabbits

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The research was conducted to investigate some physiological, biochemical and histopathological side effects induced by the administration of an overdose (900mg kg^{-1} b.wt.) of the analgesic and antipyretic drug acetaminophen (APAP) on male albino rabbit. The work was also designed to compare the influence of L-methionine (37.7mg L-methionine kg^{-1} b.wt. repeated every 4 for 12hr. up to a total dose of 142.8mg kg^{-1} b.wt.) and/or phenethyl isothiocyanate (150μmol kg^{-1} b.wt.) on the hazardous side effects of this drug. The results indicated that the administration of an overdose of APAP induced remarkable decreases in the number of red blood cells, haemoglobin contents (Hb), and haematocrit values (Ht). In addition to the elevations in the total bilirubin in the plasma. Also it induced leucopenia, granulocytopenia, and monocytopenia, which may be due to suppression in the organs necessary for their production (bone marrow and lymphatic organs) on the other hand, it induced lymphocytosis. It also caused liver dysfunction, as indicated by the elevation in the activities of alanine and aspartate aminotransferase (AST, ALT) and alkaline phosphatase (ALP) in plasma and the histopathological studies. It resulted in perturbations in the renal functions as indicated by marked rises in the concentrations of plasma creatinine, urea and uric acid. The histopathological examination indicated alterations in the cortex and S_3 segment (pars recta). Overdose of the drug caused hypoglycaemia, increases in the concentration of total lipids and triglycerides and hypocholesterolaemia. The administration of L-methionine following an overdose of APAP succeeded in preventing all the disturbances in the tested hepatic and renal functions. It also increased the plasma and urinary levels of the detoxified metabolites of APAP (APAP-GSH, APAP-Cys, and APAP-merc) and decreased the plasma level of the parent APAP. The administration of PEITC following an overdose of drug succeeded in decreasing the haemato- and hepatotoxicities of APAP but it has serious effects on the renal function. The estimation of the drug and its metabolites indicated increases in the plasma and urinary concentration of APAP-gluc and APAP-sulphate as compared with APAP-treated group, while the concentration of the other studied metabolites decreased. The level of the parent APAP decreased in the plasma and administration of L-methionine together with PEITC succeeded completely in preventing the hepatotoxicity and in modulating the haematotoxicity but not the renal toxicity of APAP. The prominent outcome of this investigation that L-methionine is still superior to either PEITC alone or when administrated in combination with PEITC in modulating most of the toxic side effects of APAP.

Key words: APAP, PEITC, antidotes, L-methionine, hepatotoxicity, histopathological examination

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Introduction

Acetaminophen is one of the safest and most widely used medications for minor illness, pain, and fever (Whitcomb and Block, 1994). Its mechanism of action is mediated by interference with prostaglandin synthesis through inhibition of prostaglandin synthetase in the arachidonic acid cascade in the CNS. APAP has a very little activity as an inhibitor of the peripheral prostaglandin synthetase enzyme, which explains its weak anti-inflammatory action. Its antipyretic action is direct on the hypothalamus. While remarkably safe in ordinary doses, APAP may cause adverse effects when taken in large amounts. Its overdose caused perturbation in the liver enzyme system. When it was injected to mice at a dosage of 5.0m mol kg^{-1} (755mg kg^{-1}), AST and ALT activities were elevated 20-100 fold above control values indicating severe liver injury (Hazelton*et al.*,1986).

A single dose of APAP (500mg kg^{-1} b.wt.) caused a severe hepatocellular injury as indicated by the massive increase of serum ALT activity. The damage started about 3hr. after APAP administration; maximal ALT activities in plasma were reached after 9hr. (Jaeschke, 1990). In mice, Li *et al.* (1997) and Wang *et al.* (1997) stated histological examination of fixed liver sections revealed a significant necrosis after treatment with paracetamol. The histopathological examination of kidney from experimental animals or humans after receiving a high doses of drug revealed that cellular damage in kidney was evident, either moderately or severe, lesions of the kidney were confined to the cortical regions of the proximal tubules and consisted of single tubular cell necrosis (Akça *et al.*, 1999). Gemborys and Mudge (1981) have demonstrated that urinary excretion of APAP and conjugation products accurately reflected the metabolic profile of drug disposition. There is no established form of therapy that has proved effective in preventing organ damage caused by APAP overdose. Several antidotes were previously administrated to reduce the toxic effects of APAP in humans and animals including cobaltous chloride (Tephly and Hibbeln, 1971), activated charcoal (Levy and Houston, 1976), cysteamine (Prescott *et al.*, 1976), syrup of ipecacuanha (Neuvonen *et al.*, 1983), *p*-aminophenol (Newton *et al.*, 1985)butylated hydroxyanisole (Hazelton *et al.*, 1986), piperonyl butoxide (Bartolone *et al.*, 1989), cimetidine (Slattery *et al.*, 1989), N-acetyl cysteine (Harrison *et al*, 1990), allopurinol (Jaeschke, 1990), oltipraz (Davis and Schnell, 1991), pregnenolona 16 αcarbonitrele (Madhu and Klaassen, 1991), peroxisome prolifirators (Manautou *et al.*, 1994), σ-glutamyl transpeptidase inhibitor (Emeigh – Hart *et al.*, 1996) and oleanolic acid (Lin *et al.*, 1996).

L-methionine, is a naturally occurring amino acid, has numerous well documented biological activities that include acting as the principal methyl donor in mammalian tissues and reducing hepatic fat as a so-called "lipotropic" factor (shapiro and schlenk, 1965). Addition of L-methionine to the oral paracetamol dose protect against death and liver injury, and it is suggested that this may be a useful technique for making paracetamol safe against the danger of overdose (McLean and Day, 1974). Another investigation done by Prescott *et al.* (1976) revealed that when L-methionine was given within 10hr. of paracetamol ingestion, it protected against liver damage, renal failure, and death, but when treatment was delayed for 10-24hr., the incidence of these complications was the same as that in the supportive-therapy group (i.e. given APAP only). However, relatively fewer supportive-therapy patients were at high risk.

A paracetamol and L-methionine combination preparation remains available in the United Kingdom: paradote (500mg paracetamol, 100mg L-methionine). The L-methionine in this formulation is the dl racemate, but it is unlikely in that the d-form can be converted into the l-form *in vivo* (Printen *et al.*, 1979).

Unfortunately, L-methionine is metabolized to homocysteine and *vice versa* in the methylation cycle, and raised plasma homocysteine concentrations have been associated with peripheral vascular disease, ischaemic heart disease and stroke (Perry *et al.*, 1995). Cruciferous vegetables such as broccoli, cabbage, cauliflower, turnipes, kale, mustard greens, etc.) are rich in phytonutrients and that have anti-cancer effects (Wang *et al.*, 1997). Among these are phenethyl isothiocyanate (PEITC) (Fig. 1). When PEITC (19-150μmol kg^{-1}) was given to mice intragastrically 1hr before or immediately prior to a toxic dose of APAP and the induced hepatotoxicity was significantly decreased or completely prevented (Wang *et al.*, 1997).

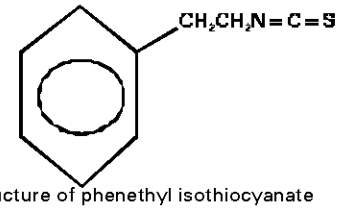

Fig. 1: Structure of phenethyl isothiocyanate

The objective of this investigation was to evaluate the additional information about the deleterious haematological, biochemical and histopathological changes induced by APAP in an animal model (rabbit). It was also designed to compare the effectiveness of L-methionine, which was reported to have a powerful protective effect against toxicity of APAP, and PEITC or both on modulating the side effects induced by APAP.

Materials and Methods

Animal's care: Newzealand white male rabbits (1000 ± 50g) were used throughout the investigation. They were obtained from Abees farm and were acclimated for at least one week prior to experiments. Animals were housed in groups in stainless steel cages at room temperature (22-25 °C) and a photoperiod of 12h d^{-1}. They were permitted for free access to food (Purina chow) and tap water. In order to minimize the possible nutritional effects, both control and experimental animals were fasted 10hr. before sacrifice.

Chemicals:

a Acetaminophen: [N-acetyl-*p*-aminophenol, 4-hydroxyacetanilide, $C_8H_9No_2$].

b L-methionine: [L-2-amino-4-methylthiobutanoic acid, CH_3-S-(CH_2)$_2$-CH(NH_2)COOH].

c Phenethyl isothiocyanate: [C_6H_5-CH_2-CH_2-NCS].

All these chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

In this investigation, an overdose of the antipyretic analgesic drug (APAP) was given postorally. For the prevention of its side effects, L-methionine and / or PEITC were given to the same animals by the same route of administration.

Experimental design: Ninety eight rabbits were divided into seven groups. Table 1 describes the doses given to the animals of these groups. The used dose of APAP was mentioned by Newton *et al.* (1985). The adminstered dose of L-methionine was repeated every 4hr. up to a total dose of 142.8mg kg^{-1} b.wt. (Crome *et al.*, 1976) and the given

Table 1: Doses of APAP, L-methionine and PEITC administrations.

Group Number	Treatment	Dose		
		APAP mg Kg^{-1}	L-methionine mg kg^{-1}	PEITC μmol Kg^{-1}
1	Control	-	-	-
2	APAP	900	-	-
3	L-methionine	-	35.7	-
4	PEITC	-	-	150
5	APAP + L-methionine	900	35.7	-
6	APAP + PEITC	900	-	150
7	APAP + L-methionine + PEITC	900	35.7	150

dose of PEITC was determined according to Li *et al.* (1997).

Preparation of blood and plasma: After 12 or 24hr. of the initial drug administration, 7 rabbits were collected from each group were put under light ether anesthesia, dissected and the blood was collected from the femoral vein into heparinized tubes. The heparinized blood samples were used for haematological determinations. For plasma preparation, the blood was centrifuged at 3000 rpm for 15 min. and the supernatant plasma was collected in capped sterile tubes.

Collection of Urine: For urine collection, the bladder was emptied in the hour before APAP ingestion and Urine was collected over 3gm ascorbic acid for 24hr. (Slattery *et al.*, 1989) after APAP administration. The collection vessels were maintained in refrigerator. Urine was thoroughly mixed at the end of collection and was frozen for analysis.
This investigation comprises:

I) Haematological examinations: Determinations of the erythrocytes count (RBCs), haemoglobin content (Hb), haematocrit value (packed cell volume P.C.V.) and total as well as differential leucocytic count (WBC_s) were formed as described by Ward *et al.* (1982), the international committe for standardization in haematology (1978), Pearson and Guthrie (1982) and Miale (1972), respectively.

II) Biochemical examinations: Estimations of AST and ALT activities in plasma were undertaken according to Wallonofer *et al.* (1974), While the activity of ALP was measured as described by Mathieu (1980). Measurements of creatinine, urea, uric acid, total bilirubin, glucose, total lipid, triglycerides and cholesterol levels in plasma were estimated according to Henry (1974), Young (1990), Caraway (1965), Jendrassik and Grof (1983), Barham and Trinder (1972), Zollner and Kirsch (1962), Wahlefeld (1974) and Roeschlau *et al.*(1974) respectively. Measurement of APAP and its metabolites in plasma and urine were perfromed. A Waters Millennium 2010 HPLC system (Waters Assoc., Milford, MA) was used.

III) Histopathological studies: At the end of experimental period, livers and kidneys were excised from the dissected animals, fixed in Bouin's solution and prepared for histological examination. Sections were stained with Ehrlich's haematoxlin and counter stained with Eosin (Kierman, 1981).

IV) Statistical analysis and test of significant variance: Results were expressed as means of seven experiments ±S.E. The significance of differences were analysed statistically using the student's t-test (Clarke and Cooke, 1983).The test significance of differences were calculated between:
Means of control group and means of either of these treated groups: APAP group (2), L-methionine group (3), PEITC group (4), APAP +L-methionine group (5), APAP + PEITC group (6), or APAP + L-methionine + PEITC group (7) and were referred by letter (a). Means of APAP group and either of these experimental groups: APAP + L-methionine group (5), APAP +PEITC group (6) or APAP + L-methionine + PEITC group (7) and were referred by letter (b). The case of the determination of the parent APAP and its metabolites, the test significance of differences were calculated between means of APAP groups and either of these experimental groups: APAP + L-methionine group (5), APAP +PEITC group (6) or APAP L-methionine + PEITC group (7) and were referred by *. The level of Significance was reported at $P < 0.05$.

Results

The adverse side effects of the postoral administration of a high dose (900mg kg^{-1} b.wt.) of drug APAP were investigated. The efficacy of the co-administration of L-methionine (35.7mg kg^{-1} b.wt.; this dose was repeated every 4 for 12hr up to a total dose of 142.8mg kg^{-1} b.wt.) and/or a single dose of PEITC (150μmol kg^{-1} b.wt.) on modulating the toxic side effects of this drug was also studied.

Effect on haematological parameters: Figs. 2,3 and 4 indicated that the administration of 900mg APAP kg^{-1} b.wt. induced marked depression in the numbers of red blood cells, Hb and ht. values as compared with the control group. Its administration caused also severe reduction in the number of total leucocytes, granulocytes and monocytes. While the number of lymphocytes increased, as compared with the control group (Figs. 5-8).
The data indicated that L-methionine (37.5mg kg^{-1}) for 4 consecutive doses did not provide any protective effect against APAP-induced haematological changes (animals in group 5). While the administration of either PEITC alone (150μmol kg^{-1}) or with L-methionine after APAP administration offered partial protective effects since there were a significant differences between the values of these groups, both the control and APAP-treated groups.

II. Effect on biochemical indices: Estimations of the enzyme activities of AST, ALT and ALP indicated that, the post oral administration of APAP produced significant elevations in the activities of all the tested enzymes during the experimental period in correspondence to control values (Figs. 9, 10 and 11). The administration of L-methionine alone or with PEITC following the post oral administration of the overdose of APAP succeeded completely in returning the activities of the previously mentioned enzymes back to their normal values at the end of the experiment since there were no significant differences between the control (group 1) and groups (5 and 7). On the other hand, the administrated dose of PEITC succeeded only partially in preventing these disturbances. The results depict that APAP administration induced elevations in creatinine, urea, uric acid, total bilirubin, total lipids and triglycerides levels. On the other hand it caused hypocholesterolaemia and hypoglycaemia (Figs. 12-19). It is clear that L-methionine offered a complete protection against the previous toxic effects of APAP at the end of experimental period.

The administration of PEITC did not provide any protective effects against the induced renal toxicity, while it offered partial improvement in the side effects of APAP all over the experimental period towards the other studied parameters (glucose, total lipids, triglycerides and cholesterol), except for total bilirubin. It offered complete protection towards this parameter. Administration of PEITC or PEITC + L-methionine following APAP did not improve the elevations in creatinine, urea and uric acid concentrations, since there still significant differences between values of either group (6) or group (7) and these of group (1). On the other hand it prevented perturbations in the rest of parameters. It is worthy to mention that the administration of L-methionine alone induced no significant effect on all the studied parameters. While the administration of PEITC alone caused a severe toxic effects on kidney (Creatinine, Urea, Uric acid).

Figs. (20- 25) demonstrates the changes in the concentrations of APAP and its metabolites (APAP-gluc, APAP-sulph, APAP-GSH, APAP-cys, and APAP-merc) in the plasma and urine under the influence of APAP + L-methionine, APAP + PEITC and APAP + L-methionine + PEITC. In respect to these Figures, the concentration of APAP-glucuronide was the predominant metabolite in the plasma of APAP-treated animals after 24hr. of administration. APAP concentration was also high. Sulphate, glutathione and cysteine conjugates were relatively low. Mercapturate conjugate was the minor metabolite and in urine, the glucuronic acid conjugate was also, predominate, the concentration of the unchanged APAP was less than any of its metabolites. Simultaneous administration of L-methionine with APAP significantly increased the plasma and urinary levels of APAP-GSH, APAP-cys and APAP-merc concentrations. While the plasma and urinary concentrations of APAP-glucuronide and APAP-sulphate did not showd significant changes as compared to the control. The plasma concentration of the unchanged APAP decreased significantly as compared to that of APAP group, while its concentration in the urine is more or less similar to that of APAP-treated group. Administration of PEITC + APAP increased the plasma and urinary levels of APAP-gluc and APAP-sulph, while concentrations of APAP-GSH, APAP-cys and APAP-merc were decreased as compared with APAP-treated group. The level of the unchanged drug was significantly decreased in the plasma in comparison to APAP-treated group. On the other hand there were no difference in the APAP concentration in the urine of APAP and APAP + PEITC treated groups. Co-administration of L-methionine and PEITC with APAP resulted in significant increases in plasma and urine levels of all APAP-conjugated metabolites as compared to APAP-treated group. In addition, the unchanged drug concentration decreased significantly in the plasma. On the other hand, the urinary concentration of the parent drug did not showed any significant alterations.

III- Histopathological examinations

The liver: Microscopical examinations of liver of APAP-treated animals (Figs. 27-A, B, C and D) revealed the presence of degeneration of hepatocytes (DHC), massive centrilobular hepatocellular necrosis and degeneration (CHND), resulting in clear demarcation of the centrilobular areas from the rest of the liver. In addition, dilatation of blood sinusoids (DBS) which were studded with RBCs, intrahepatic bleeding (IHB), swolling of parenchyma cells (intercellular oedema), vacuolization of some hepatocytes and infiltration of lymphocytes (L). Confluent zones of pyknotic or anuclear cells (ZAC) were also noticed.

The co-administration of L-methionine alone or in addition to PEITC with APAP prevented completely the histopathological drawback changes of liver induced by the drug administration (Figs. 28, 30). On the other hand, simultaneous administration of PEITC with APAP reduced the degenerative changes which occurred in the liver (Fig. 29). These Figures showed less CHND, DBS with decreased number of RBCs inside them, DHC and less IHB. Lymphocyte infiltration, swolling or vacuolization of some hepatocytes are not present. It is worthy to note that the administration of either L-methionine or PEITC alone did not change the histology of the liver and so they were not represented here.

The kidney

a) Cortex: The most prominant findings were degeneration of epithelial cells of many tubules, dilatation of distal convoluted tubules (DCT) which were lined by marked flattened and fragmented epithelial cells, dilatation of proximal convoluted tubules, which were filled with hyaline casts (HC), and shrinkage of glomeruli (SG) (Figs. 33-A, B).

b) Pars recta: The most obvious changes were degeneration of epithelial cells of distal (DED) and proximal tubules (DEP), dilatation of distal convoluted tubules (DDT) which were lined by flattened and fragmented epithelial cells (FEC, FEDT), infiltration of lymphocytes, and formation of hyaline casts filling the lumen of these tubules (HC) (Fig. 34). The administration of PEITC alone resulted in sever histologic alterations in the kidney. Figs. 35 , 36 demonstrated that the kidney of rabbit treated with PEITC (150μmol kg^{-1} b.wt.) showed degeneration of some distal and proximal convoluted tubules and HC (Fig. 35). In addition, degeneration of some distal and proximal tubules were noticed in pars recta (Fig. 36). The administration of L-methionine in combination with APAP offered a good protective action against the histological drawback induced by postoral administration of the latter. It is clear that cortex and pars recta exhibited negligible changes. The number of the injured tubules were negligible as compared with those in rabbits treated with APAP alone (Figs. 37 , 38).

On the other hand, simultaneous administration of PEITC with APAP increased the histopathological drawback changes of the kidney when compared with those induced by APAP alone (Figs. 39 A, B and 40). Infiltration of L and detachments of the basement membranes of tubules were noticed in the cortex in addition to the previously observed histopathological drawback that were caused by APAP administration. In addition, DED and DEP as well as HC were noticed in pars recta. The co-administration of L-methionine + PEITC with APAP offers no protective action against the histological drawback induced by postoral administration of APAP. It is clear that cortex and pars recta still exhibited most of the histopathological changes that were induced by the treatment of APAP alone (Figs. 41, 42). The administration of L-methionine alone did not alter the histology of either the cortex or the pars recta of the kidney and so they were not represented here.

Discussion

Acetaminophen is a well-known effective antipyretic and mild analgesic agent which is widely used in pediatric and adult medicine. While remarkably safe in ordinary doses, it can cause severe side effects when taken in overdoses. Data indicated that the administration of an overdose of APAP to rabbits resulted in dramatic decreases in the number of RBCs, Hb and Ht. In other words, it caused severe anaemia after administration. Similar results were also reported by Henreting *et al.* (1989). The anaemia reported in this work may be ascribed through:

(i) the suppression of normal mechanisms promoting erythropoiesis,
(ii) the shortened red cell life span, and/or
(iii) loss of blood.

Erythropoietin (a glycoprotein with a molecular weight 39.000 which is responsible for normal erythropoiesis) is produced by endothelial cells of the peritubular capillaries in the renal cortex. In the liver, both Küpffer cells (KC) and hepatocytes have been claimed to produce erythropoietin (EPO). When renal mass is reduced by renal disease or nephroctomy, the liver can not compensate and anaemia developed (Ganong, 1993). Thus, the vital function of the kidney is the production of EPO whose deficiency results in renal anaemia. In this work, both the hepatotoxic and the nephrotoxic effects of APAP later harm the EPO-producing cells resulting in a normocytic anaemia. Decreases in the number of the RBCs may also be due to inhibition of the mitotic activity of the bone marrow; i.e. APAP may have a suppressive effect on the uncommitted stem cells. Marrow failure is an important feature of acute leukaemias (Bunch, 1995).

Shortened red-cell life span can be due to abnormalities in the red cell membrane, the cytoplasmic enzyme system, and/or Hb. These defects make the mature erythrocytes more susceptible to damage during their passage through the circulation and thus to the development of a haemolytic anaemia. Increased red-cell destruction releases large quantities of haem into the cells of the reticuloendothelial system which is subsequently converted into bilirubin (Campbell *et al.*, 1984). This explanation is further confirmed in this investigation by the determination of the total bilirubin. Clark *et al.* (1973) noticed severe bleeding from the gastrointestinal tract, requiring transfusion, in 5 patients. At necropsy, it was found to be due to oesophageal and gastric erosions. Thornton and Losowsky (1989) reported a case of a patient who developed oesophageal varices and bled 13 days after paracetamol overdose.

The bleeding was unresponsive to medical management and was proved fatal. There was no evidence that the patient had pre-existing liver disease. At necropsy the liver showed severe acute parenchymal necrosis but chronic lesions were absent. Both the portal and hepatic veins were patent. Bleeding in patients with acute liver failure, after paracetamol administration, may easily be assumed to be caused by the impaired synthesis of liver-derived clotting factors (Thornton and Losowsky, 1990).

The administration of APAP caused acute leucocytopenia, granulocytopenia, monocytopenia, and lymphocytosis. granulocytopenia which was noticed in this work may be due to damage in the bone marrow stem cells as a result of the administration of paracetamol. Many drugs may also causes its by switching off stem cell turnover. Another cause of granulocytopenia is the destruction of cells by an enlarged spleen. The monocytopenia and the lymphocytosis that were noticed in the work were also previously reported by Henreting *et al.* (1989). They are occasionally observed in the blood of a subject, but nothing is known concerning the reasons that might cause them or their consequences. (Wyngaarden and Smith, 1985). The data revealed that APAP induces liver dysfunction which was indicated by increases in the activities of AST, ALT and ALP. The AST and ALT are all intracellular enzymes involved in amino acid or carbohydrate metabolism. These enzymes are present in high concentrations in muscle, liver, and brain. Elevation in the concentrations of these enzymes in the blood indicated necrosis or disease especially of these tissues. They elevated in myocardial infarction, acute infections or toxic hepatitis, cirrhosis of liver i.e. when the liver parenchyma was damaged (Hawcroft, 1987). ALP is a membrane associated enzyme that hydrolyzes phosphoric esters with the liberation of inorganic phosphate at optimum pH between 9 and 10 (Meyer *et al.*, 1992). It may be derived from bone, placenta, kidney, gut or liver.

In this research work postoral administration of paracetamol (900mg kg^{-1} b.wt.) revealed histopathological changes in liver of male albino rabbit. These changes are represented by DHC, massive CHND, resulting in clear demarcation of the centrilobular areas from the rest of the liver. In addition, DBS which were studded with RBCs, IHB, hepatocytes (SHC) (interacellularoedema), vacuolization of some hepatocytes and infiltration of L. Confluent zones of pyknotic or anuclear cells were also noticed. The nature of the vacuoles in the centrilobular hepatocytes of mice is unknown. Dixon *et al.* (1971) showed that similar vacuoles in the rat did not contain fat and they came to a conclusion that they were resulted from hydropic degeneration.

A number of reports indicated that massive overdoses of APAP can result in similar biochemical and histopathological changes in both man and experimental animals (Litovitz *et al.*, 1995; Jones *et al.*, 1997; Li *et al.*, 1997 and Zaher *et al.*, 1998). Results of this investigation indicated that the administration of an overdose of APAP to male rabbits caused marked rises in the concentrations of plasma creatinine, urea, and uric acid. Creatinine is more readily excreted by kidney than urea or uric acid. An increased its concentration is evidence of marked impairment of kidney function and its retention is thus an index of glomerular insufficiency (Murray *et al.*, 1990). Plasma urea level elevated in renal insufficiency, nephritis (tubular necrosis) and urinary tract obstruction. An increase in uric acid concentration in plasma may accompany an increased nucleoprotein catabolism or decreased renal excretion (Marcus and Milton, 1982). The elevations of plasma creatinine, urea, and uric acid concentrations recorded indicate the incidence of severe renal dysfunction. The histopathological studies indicate alterations in the cortex and S_3 segment (pars recta). APAP nephrotoxicity has also been evaluated in several species of laboratory animals and in humans as well (Zaher *et al.*, 1998). It is clear now that the administration of an overdose causes deleterious effects on the haematopoietic system as well as the hepatic and renal organs. It may also cause perturbations in the functions of other tissues or glands. Hypoglycaemia has been noticed in this investigation throughout the experimental period. The decrease in glucose level in plasma in response to APAP administration may be due to:

(i) Decreased glycogenlysis and/or:
(ii) The activities of the enzymes involved in gluconeogenesis e.g. pyruvate carboxylase, phosphoenol pyruvate carboxykinase, fructose-1, 6-diphosphatase and glucose-6-phosphate, may be inhibited under such conditions and/or
(iii) Hypoglycaemia which occurred after the administration of APAP may have been the result of stress-induced hormonal-mediated control mechanism. Two contributing factors are probably responsible, although others may also play some role. Firstly, in the absence of glucocorticoids, the liver responds poorly, or not at all, to normal gluconeogenic and glycogenolytic stimuli such as the increase in the glycogen, insulin ratio or catecholamines. Secondly, lack of glucocorticoid results in decreased rate of amino acid release from the periphery. Thus, the main substrates for gluconeogenesis

are supplied in inadequate amounts. The net result of both effects is decreased hepatic gluconeogenesis and subsequent hypoglycaemia (Campbell *et al.*,1984).

The concentration of plasma total lipids and triglycerides were also increased after the administration of an overdose of APAP, while its administration resulted in hypocholesterolaemia. Hyperlipidaemia is the hallmark of the lipoprotein disorders. Clinical delineation of these disorders is important because of the association of some with premature coronary artery disease and others with recurrent pancreatitis. Also, it has long been recognized as a complication of some forms of parenchymal liver disease. In addition, it is common in the nephrotic syndrome. The primary mechanism appeared to increase the hepatic synthesis and decrease catabolism of lipids. Triglyceride concentration is elevated in cases of liver dysfunction, chronic uremia, dialysis, and nephrotic syndrome. Also diuretic agents may associated with small increases in triglyceride levels (Wyngaarden and Smith, 1985).

Cholesterol concentrations are determined by metabolic functions which are influenced by integrity of vital organs such as liver and kidney (Marcus and Milton, 1982). Hypocholesterolaemia may be secondary to end-stage liver disease, the distal malabsorption syndrome, sever intestinal protein loss, and sideroblastic anaemia (Lewis, 1972). Although many chemically stable foreign compounds are converted in the body to biologically inactive metabolites that are readily excreted in urine, some of these compounds are converted to chemically active metabolites that react with various tissue macromolecules, including nucleic acids and proteins. Many investigators think that this binding of chemically reactive metabolites accounts for the toxic effects of a wide variety of chemically inert substances.

Examination of plasma and urinary levels of APAP and its conjugated metabolites indicated that APAP-glucuronide is the prominent metabolite in the plasma and urine. This conjugate is the major metabolic product after high doses of APAP (Fischer *et al.*, 1981). The unchanged APAP concentration is very high in the plasma. The concentration of the detoxified oxidized forms (APAP-GSH, APAP-Cys, and APAP-merc) in plasma and urine are low as compared with APAP-glucouronide and APAP-sulphate concentrations. When it is ingested in pharmacologic doses, 5-10% is excreted in the urine as a mercapturate. As the ingested dose increases, glucuronide and sulphate conjugation processes become saturated and an increasing proportion is metabolized through the cytochrome P450 system. This leads to increasing amounts of mercapturic acid and cysteine derivatives appearing in urine (Davis *et al.*, 1976 a and b). Eventually, the glutathione content of hepatocytes available for detoxification of the toxic metabolite is exhausted and the hepatocyte becomes vulnerable to the noxious effects of the metabolite, liver-cell necrosis ensure. Gregus *et al.* (1988) mentioned that APAP is actually biotransformed to a toxic electrophile, N-acetyl-*p*-benzoquinoneimine (NAPQI), that produces liver injury unless it is conjugated with endogenous glutathione. In contrast, conjugation of APAP with sulphate and glucuronic acid are biosynthetic reactions rendering APAP inactive and easily excretable. Thus Fig. 43 may be the postulated mechanism for APAP biotransfromation.

Following APAP administration, Tirmenstein and Nelson (1989) mentioned that both the mitochondrial and cytosolic pools of glutathione (GSH) are depleted. Once GSH is depleted, cellular proteins are arylated, presumably, by the reactive metabolite NAPQI. In addition, cytosolic glutathione peroxidase and thiol-transferase activities are impaired in APAP-treated animals. The inhibition of these enzymes as well as the depletion of GSH may make the cell very vulnerable to the deleterious effects of activated oxygen species. Even under normal circumstances, hydrogen peroxide (H_2O_2)and superoxide anions (O^{-2}) are generated in cell from a variety of sources. This background production of the activated oxygen may become significant if the GSH-glutathione peroxidase detoxification pathway is compromised. Under such circumstances, the production of activated oxygen species may lead to the oxidation of sensitive protein thiols. The inhibition of thiol transferase activity may also contribute to the increased levels of oxidized protein thiols in APAP-treated animals by inhibiting the reduction of oxidized protein thiols.

Tirmenstein and Nelson (1989) reported that APAP administration disrupted calcium homeostasis in the liver. APAP-treated mice display decreased the plasma membrane calcium-ATPase activity and impaired mitochondrial calcium sequestration 1hr. after receiving the drug. The influx of extracellular calcium as a result of this plasma membrane calcium-ATPase inhibition in addition to the loss of the ability of mitochondria to sequester calcium may lead to a large-scale calcium cycling by mitochondria. This calcium cycling may have several important consequences for cell function. Thomas and Reed (1988) suggested that calcium cycling may be involved in the production of oxidative stress in isolated hepatocytes by an unknown mechanism. Calcium had also been showed to increase H_2O_2 production by isolated rat heart mitochondria (Cadenas and Boveris, 1980). The uptake of Ca by mitochondria is known to require respiratory energy and to take precedence over ATP formation (Vercesi *et al.*, 1978). This decreased ATP synthesis may lead to the increased breakdown of adenine nucleotides in response to energy requiring cellular metabolism (Fig. 44).

Snawdr *et al.* (1994) suggested that CYP2E1 played a key role in APAP bioactivation and toxicity *in vitro* and *in vivo*. Cytochrome P450 (CYP1A2) which had lower APAP affinity than CYP2E1, also been implicated in APAP toxicity *in vitro*. When CYP2E1 knockout, mice were challenged with the common analgesic APAP, they were found to be considerably less sensitive to its hepatotoxic effects than wild-type animals, indicating that this P450 is the principal enzyme responsible for the metabolic conversion of the drug to its active hepatotoxic metabolite (Lee *et al.*, 1996).

The data of this study clearly showed that APAP has sever renal toxic effects. There are several theoretical explanations for the development of acute renal cellular damage after paracetamol administration. Morais and Wells (1988) mentioned that APAP causes renal necrosis either via the cytochrome P450 pathway in the renal cortex or a prostaglandin synthetase-catalyzed pathway in the renal medulla. The expression of APAP toxicity is dictated by a balance among the pathways of elimination, bioactivation and detoxification. An impairment in a major pathway of elimination, such as glucuronidation may allowed more drug to be bioactivated and hence cause more toxicity. Bartolone *et al.* (1989) mentioned that pretreatment with the mixed-function oxidase (MFO) inhibitor, piperonyl butoxide, significantly alleviated the severity of the APAP-induced necrosis not only in the liver but also in lung and kidney. This suggested that the toxicity in lung and kidney at least in part results from mixed-function oxidase system (MFO)-mediated activation of APAP.

Depletion of glutathione by buthionine sulfoximine, which inhibits the glutathione synthesis, completely protects rats against *p*-aminophenol-induced nephrotoxicity. Also, biliary cannulation partially protects rats from *p*-aminophenol-induced

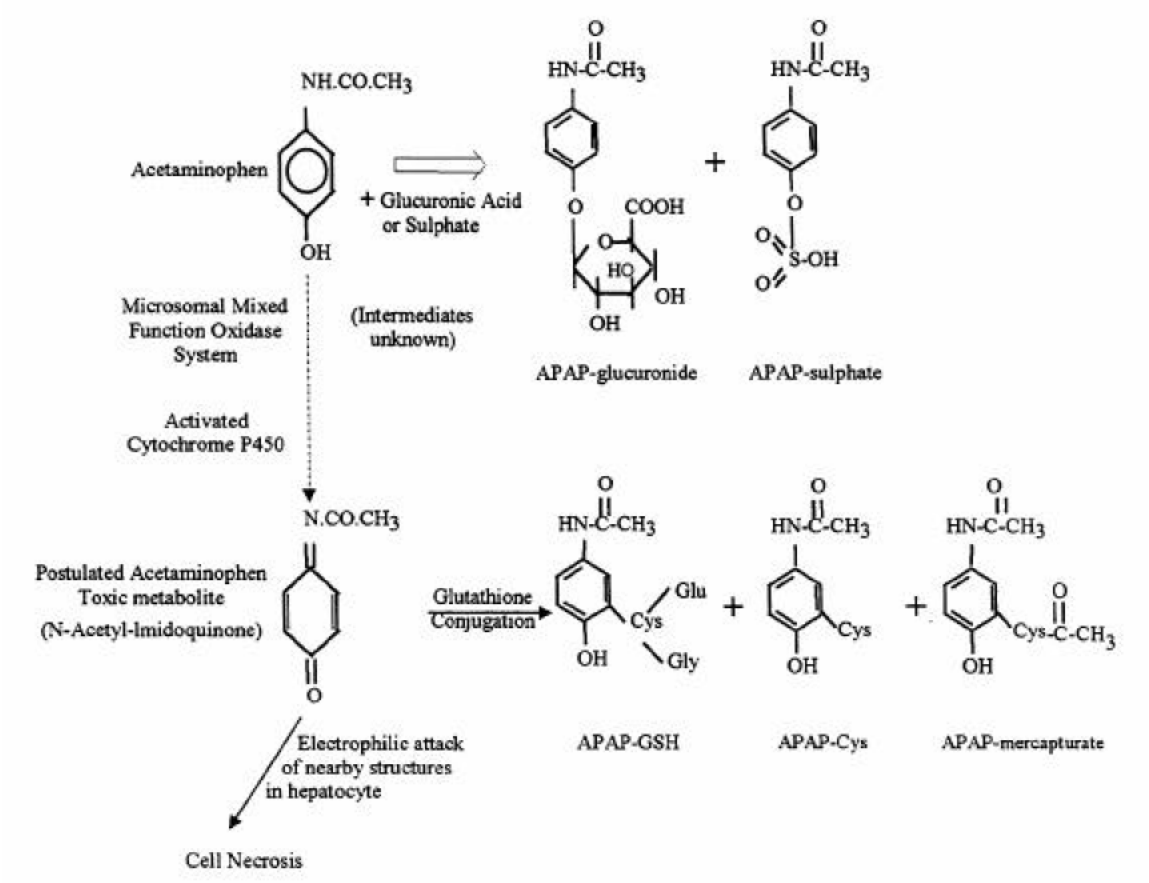

Fig. 43: Main pathways of APAP biotransformation

nephrotoxicity (Gartland *et al.*, 1990). Möller-Hartmann and Siegers (1991) suggested that the APAP-GSH conjugate or other APAP-S conjugates exported from the liver to the kidney might be responsible for the APAP nephrotoxicity. Klos *et al.* (1992) demonstrated that *p*-aminophenol is metabolically transformed to toxic-glutathione S-conjugates. These S-conjugates are excreted from the liver with bile and are, intact and after ð-glutamyltranspeptidase-dependent cleavage, translocated to the kidney. The kidney accumulates *p*-aminophenol-derived S-conjugates in the same way as demonstrated for toxic S-conjugates derived from other xenobiotics. Finally, metabolites formed by oxidation of *p*-aminophenol S-conjugates bind to cellular macromolecules in the kidney to cause toxicity.

Female mice were pretreated with testosterone propionate and then challenged 6 days later with APAP. Western blot analysis of microsomes showed that testosterone increased renal CYP2E1 levels without altering hepatic CYP2E1. Testosterone pretreatment, *in vivo*, also resulted in increased activation of CYP2E1 *in vitro* in kidney microsomes with no effect on the *in vitro* activation of APAP in liver microsomes. These data suggested that APAP-mediated GSH depletion, covalent binding and toxicity in the kidney of testosterone-pretreated females results from increased APAP activation by the testosterone-induced renal CYP2E1. This further suggested that renal, rather than hepatic biotransformation of APAP to a toxic electrophile is central to APAP-induced nephrotoxicity in the mouse (Hoivik *et al.*, 1995).

The results of the present investigation clearly showed that the administration of 142.8mg kg^{-1} b.wt. of L-methionine for 24hr. succeeded in preventing most of the toxic side effects of APAP and in protecting both the liver and kidney from these side effects i.e. it prevents hepatic and renal toxicities. L-methionine is a substrate for the cystathionine pathway in the liver, thus serving as a source for hepatic cysteine and GSH synthesis. GSH presents in high concentrations (~ 5mM) in the liver and plays an important role in many redox and detoxification reactions (Reed and Beatty, 1980).

Data obtained from this work indicated that the administration of L-methionine + APAP increased the plasma and urinary levels of the detoxified metabolites of it (APAP-GSH, APAP-Cys, and APAP-merc) while the plasma and urinary levels of APAP-glucuronide and APAP-sulphate were unchanged. The plasma concentration of the parent APAP decreased significantly as compared to that of the APAP group, while its concentration in the urine was more or less similar to that of the APAP-treated group.

L-methionine acts as the sulphur source both for inorganic sulphate and cysteine production and GSH generation. When administrated shortly after overdose of APAP, L-methionine is thought to act primarily by replenishing mitochondrial and cytosolic glutathione stores that have been

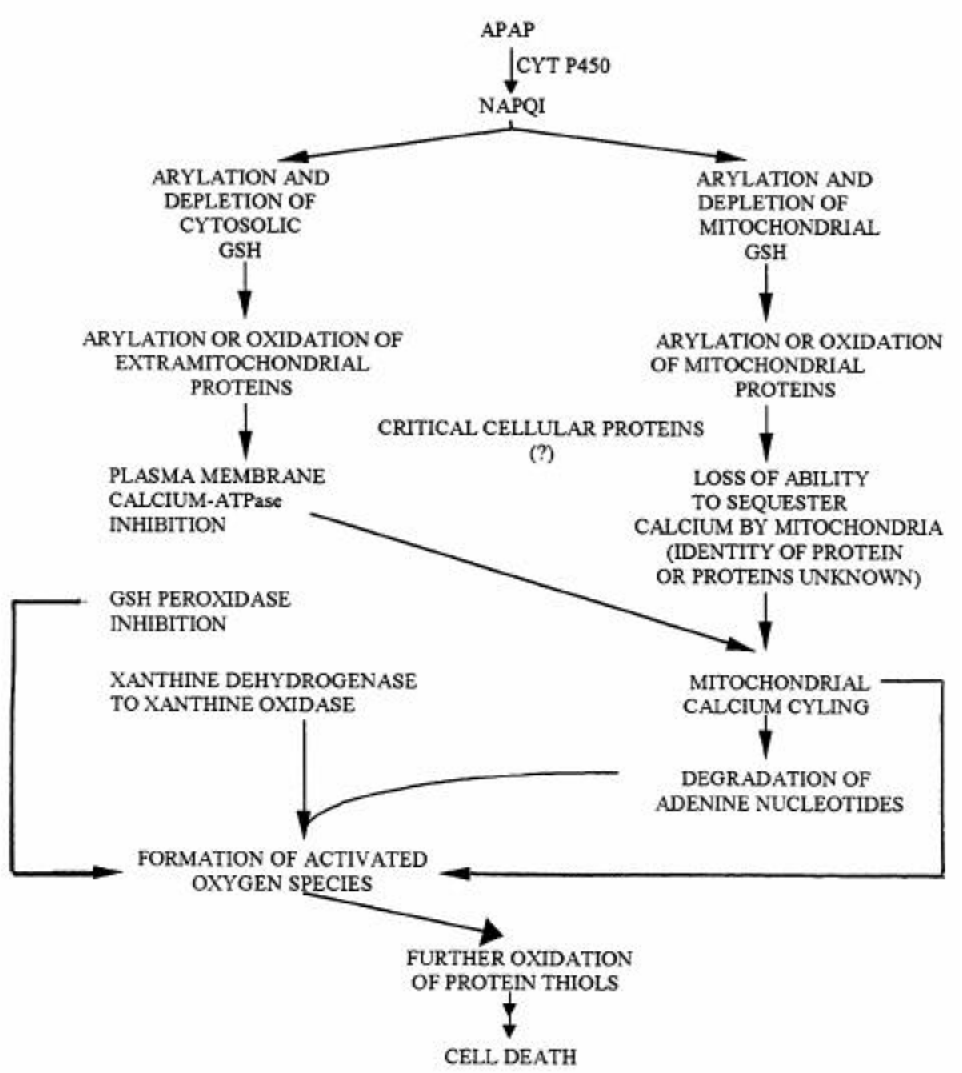

Fig. 44: Proposed mechanism of acetaminophen-induced cell death

depleted by the highly reactive paracetamol metabolite N-acetyl-*p*-benzoquinoneimine (Vale and Proudfoot, 1995). L-methionine may ameliorate APAP toxicity by enhancing liver glu tathione content. Hepatic reduced GSH plays not only an important role in protecting against oxidative injury, but it also serves as scavenger for the generated cellular electrophiles (Manautou *et al*., 1994). Regimens that produce changes in the availability of reduced glutathione may have a significant effect on the capacity of liver to handle reactive intermediates such as NAPQI and conjugate with it forming APAP-GSH, APAP-Cys, and APAP-mercapturate that were increased in plasma and urine in this study.

The postoral administration of an overdose of APAP followed by the postoral dose of L-methionine could not neither modulate nor prevent the haematotoxicity of the former. This may be due to the inability of the postoral administration of L-methionine to protect the oesophagus and gastrointestinal tract from erosions and varices that may cause severe bleeding by APAP (Thornton and Losowsky, 1989). On the other hand, the concentration of total bilirubin did not suffer from any change. i.e. it still at its normal level (group 5). This may be due to the reduction in the parent APAP concentration in the plasma which caused destruction in the RBCs and haemolysis in the APAP-treated group, as the result of this investigation indicated.

The effects of the postoral administration of PEITC on preventing or modulating the haemato-, hepato- and renal toxicities of an overdose of APAP were also investigated. The results indicated that the administration of PEITC following an overdose of APAP succeeded partially in preventing the haemato- and hepatotoxicities of the latter. Cells have at least 200 P450 enzymes to handle the toxic chemicals. Normally, the enzymes convert the toxins into substances that the cell either eliminates or uses for its own needs. But when P450s attack some toxic chemicals, the enzymes produce by-products that have dangerous toxic effects. Several P450

enzymes are known to play an important role in APAP bioactivation to NAPQI, and their importance is dependent on the relative amount of each enzyme and catalytic efficiency. Rabbit and rat P450s 2E1 and 1A1/2 have been suggested to be primary catalysts in APAP bioactivation (Harrison *et al.*, 1988).

PEITC is a highly reactive compound which reacts with amino, histidyl, and cysteinyl groups of proteins to form covalent adducts (Drobrica and Gemeiner, 1976). This covalent binding to P450 can result in a modification of the structure and a loss of activity. Feeding mice on 3μmol of PEITC g^{-1} caused a 25% decrease in the hepatic P450 content. The estimation of the concentrations of APAP and its metabolites in the PEITC-treated group was also studied. The results indicated that increases in the plasma and urine concentrations of the non-toxic metabolites (APAP-gluc and APAP-sulph). While the concentrations of APAP-GSH, APAP-Cys and APAP-merc were decreased as compared with APAP-treated group. The levels of the unchanged drug was significantly decreased in the plasma. On the other hand, there was no difference in the APAP concentration in the urine of PEITC and APAP-treated groups. These results demonstrate that the mechanism of PEITC-induced partial protection from APAP hepatotoxicity is through induction of conjugative pathways in the liver such as glucuronidation or sulphation. Thus the protection resulted from a diminution of net availability of the toxic electrophil NAPQI for reacting with critical cellular constituents.

Stoner *et al.* (1991) reported that there was a second system of enzymes, Phase 2 enzymes, that help to eject toxic chemicals from the cell after coupling them to other molecules that make the toxin easier to eliminate. PEITC increases the activity of these enzymes. Thus, the protective mechanism of PEITC may be through the inhibition of P450 enzymes and/or through stimulation of the Phase 2 enzymes. The incomplete i.e. partial protection induced by PEITC may be dose-dependent. The postoral administration of PEITC provided partial protection in the studied haematological parameters that were induced by the postoral administration of a toxic dose of APAP. It may be due to a somewhat coating protection against APAP-induced oesophageal and gastrointestinal erosions and varices.

Although the co-administration of PEITC to APAP-treated animals was useful in modulating the haemato- and hepatotoxicities of APAP, it has a serious effects on the renal functions. This may be due to the fact that PEITC has a renal-toxic effect as indicated in the present results in animals of group 4. The administration of L-methionine together with PEITC succeeded completely in preventing the hepatotoxicity and in modulating the haematotoxicity but not the renal toxicity of APAP. Their administration resulted in increases in the plasma and urine concentrations of the non-toxic as well as the oxidized pathways. The plasma level of the parent APAP was low, while its urinary concentration was still nearly at the same levels as those of APAP-treated animal.

It is worthy to note here that the histopathological results are confirming both the physiological and biochemical studies. This finding indicated that L-methionine co-administration along with APAP is more effective and advantageous in protecting against hepatic and renal toxicities of APAP, than when it was given with PEITC , and also L-methionine is more potent than PEITC in preventing the APAP-induced hepatic and renal toxic effects. This suggest that L-methionine is still superior to either PEITC alone or when it is administrated in combination with PEITC on modulating most of the toxic side effects of APAP.

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