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Full Length Research Paper

Histopathological changes and metabolic gene polymorphism in experimental rats exposed to aramite

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A total of 40 experimental rats (*Rattus norvegicus*) were divided into three groups: 10 rats for G1 (normal rats), 15 rats for G2 [rats that were exposed to aramite with daily intake of food for 90 days (5 g aramite/1 kg of pellet diet)] and 15 rats for G3 [rats that were exposed to aramite with daily intake of water for 90 days (5 g aramite dissolved in 1 L tap water)]. The result showed that the rats treated with aramite had significant increase in the activity and level of glutamate pyruvate transaminase (GPT), glutamate oxaloaceate transaminase (GOT), acid phosphatase activity (ACP) and alkaline phosphatase activity (ALP) (P≤0.05) when compared with the control group. The results indicated histopathological changes in the liver and intestine with clear mutation in *GSTM1* gene in rats that were exposed to aramite with diet and water when compared with the normal control rats.

Key words: Aramite, metabolic gene polymorphism, histopathological, GSTM1.

INTRODUCTION

Medical surveillance is indicated whenever exposure to a carcinogen has cytogenetic and/or other/ tests that might become useful or mandatory. Chemical carcinogens aramite is used as acaricide, such as in the control of mites in citrus fruits. During the application of aramite as an acaricide, occupational exposure is possible through dermal contact and inhalation of aerosols and dusts. Aramite can be released directly into the environment through its use as acaricide (miticide); however, this use has apparently been discontinued.

Popper et al. (1960) described the incidence of hyperplastic liver nodules: 2/193, 2/93, 3/100 and 20/90 in rats (male and female data combined) in the control, low, mid and high-dose groups, respectively. The hyperplastic nodules would now be classified as neoplastic liver nodules (Baggs, 1990; Chiu and Singh, 1990). Also, in the high-dose group, two liver carcinomas and five bile duct adenomas were found. These tumor types were not observed in any other groups, in that rats with carcinomas also had neoplastic nodules, but it is unclear whether the rats with the bile duct adenomas also had neoplastic liver nodules. Tumor incidence data were not reported by sex. CFN rats showed a dose-related increased incidence of neoplastic nodules: 5/180, 3/93, 10/90 and 22/96 in the 0, 100, 200 or 400 ppm groups, respectively. No liver carcinomas were observed in CFN rats, but the incidence of bile duct adenomas was 0/180, 2/93, 1/200 and 2/96 in the control, 100, 200 and 400 ppm groups, respectively. It is unclear whether these rats also had neoplastic liver nodules. Only Sprague-Dawley rats that died after 1 year were examined. High mortality (about 60%) (unrelated to treatment) in the ninth and tenth months due to respiratory infections in the Sprague-Dawley rats precluded evaluation for late developing tumors. Respiratory infections also caused many deaths in CFN rats (about 20%) (Popper et al., 1960).

Radomski et al. (1965) and Deichmann et al. (1967) reported that tumors were detected by gross examination at autopsy; however, all tumors and several body organs, including the liver, were examined histologically. Sternberg et al. (1960) identified tumors in the extrahepatic biliary tract of dogs exposed to aramite in the diet. A total of 40 mongrel dogs (17 male and 23 female) were fed diets containing aramite at 0, 500 or 828 to 1429 ppm for 811 to 1220 days (low dose group) and 462 to 1206 days (high dose group). Seven of the 12 low dose dogs and 12/16 high dose dogs appeared moribund or died during treatment and were necropsied

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and examined for tumors. The control dogs and the remaining low dose dogs appeared healthy and were not autopsied. Extra hepatic biliary system adenocarcinomas were found in 7/7 low dose dogs and in 7/12 high dose dogs subjected to necropsy (extrahepatic biliary system adenocarcinomas were observed in all the 7 high dose dogs that survived for more than 715 days). Neoplastic nodules in the liver parenchyma (3/7 and 3/12 in the low and high dose dogs, respectively), and hyperplasia and adenocarcinomas of liver bile ducts (6/7 and 7/12 in the low and high dose dogs, respectively) were also observed in both treated groups. Five of the 12 high dose dogs died early in the experiment (before 715 days) with no signs of cancer. Oser and Oser (1960) showed that histopathologic examinations of the major organs and tissues of rats that died or were sacrificed at the termination showed diverse pathologic changes in both test and control groups, which except for those in the liver, were characteristic of aged rats and not considered to be dose related. Thus, at 1580 and 5000 ppm, the degrees of hepatic injury were graded to dosage, varying from focal hyperplasia and inflammatory reactions to malignancy, except for one rat which showed a hepatic hyperplastic nodule of questionable significance. The histopathologic changes observed at the 500-ppm level were interpreted as not causally related to the test material, blood and urinary findings that were normal throughout, except for a trend toward anemia at the higher dose level during the terminal period of the study. Microscopically, liver changes in the 500 ppm dogs were degenerative in nature and comparable in degree to those seen in the control dogs. However, in the livers of dogs fed 1580 ppm aramite, similar but more marked changes were observed.

MATERIALS AND METHODS

A total of 40 male rats (*Rattus norvegicus*) weighing 200 to 250 g were used in this study. They were reared in the animal house of the science college. The animals were fed *ad libitum* with pellet diet and water.

All chemicals and reagent kits that were used in this experiment were purchased from the traditional agriculture agency and health offices.

Experimental procedure

A total of 40 rats were used in exterminate. The rats were divided into three groups: 10 rats for G1 (normal rats), 15 rats for G2 [that were exposed to aramite with food daily for 90 days (5 g aramite/1 kg of pellet diet)] and 15 rats for G3 [that were exposed to aramite with water daily for 90 days (5 g aramite dissolved in 1 L tap water)]. For every 30 days from the experiment period, blood samples were collected from the heart of each rat at 1 ml volume for estimation of glutamate oxaloacate transaminase, glutamate pyruvate transaminase (Reitman and Frankel, 1957), acid phosphatase activity and alkaline phosphatase activity (Georgatsos, 1989). After 90 days, the animals were killed by decapitation, and blood samples were collected for the purpose of had neoplastic liver nodules. Tumor incidence data were genetics study. So, the liver, small and large intestine were dissected out, washed with cold saline, and prepared for the purpose of histological study (Luna, 1968).

Genetic study

Isolation of genomic DNA was done according to the procedure of Sambrook et al. (1989), while multiplex PCR for *GSTM1* and *GSTT1* genotyping were analyzed by multiplex PCR according to the protocol of Arand et al. (1996). Genomic DNA was amplified by using six sets of primers: *GSTM1* Forward5'-GAA CTC CCT GAA AAG CTA AAG C–3' and Reverse 5'– GTT GGG CTC AAA TAT ACG GTC G– 3', *GSTT1* Forward 5'- TTC CTT ACT GGT CCT CAC ATCT C-3' and Reverse 5'– TCA CCG GAT CAT GGC CAG CA–3' and Albumin Forward 5'– GCC CTC TGC TAA CAA GTC CTA C –3' and Reverse 5'-GCCCTA AAA AGA AAA TCG CCA ATC–3'. Albumin was used as the control. The reaction mix included: green master mix (12 µl), forward primer and reverse primer (each 1.5 µl), DNA (5 µl), D.W. (5 µl) and mineral oil (25 µl). The PCR conditions were: (1) denaturation at 95°C for 5 min and 1 cycle, (2) denaturation at 94°C for 1 min, (3) annealing at 58°C for 1 min, (4) extension at 72°C for 1 min and 30 cycles, and (5) extension at 72°C for 5 min and 1 cycle, after which the PCR product was then subjected to electrophoresis on a 2% agarose gel.

The presence of bands of 480 and 215 bps was indicative of the GSTT1 and *GSTM1* genotypes, whereas the absence indicated the null genotypes for that gene. Albumin, indicated by a 350 bp product, was used as an internal control.

Statistical analysis

The data for various biochemical parameters were analyzed using Fisher exact and T-test (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Glutamate pyruvate transaminase (GPT) and glutamate oxaloaceate transaminase (GOT)

Tables 1 and 2 show the activity of GPT and GOT in the blood of the normal and experimental animals. There was a significant elevation in the activity of GPT and GOT enzymes during the exposure to aramite in diet and water compared with the control group. Serum GPT and GOT are enzymes which reversibly exchange amino and keto groups on alpha carbon positions of serum organic acids. These enzymes are prevalent in the heart, liver, muscle and kidney tissue, and their elevation in serum can be used for differential diagnosis involving these organs and in indicating the integral stress (Jau-Shin, 1996).

Acid phosphatase activity (ACP) and alkaline phosphatase activity (ALP)

Tables 3 and 4 show the activity of (ACP) and (ALP) in the blood of the normal and experimental animals. There was a significant elevation in the activity of (ACP) enzyme during the exposure to aramite in the diet and water when compared with the control group. Acid

Table 1. The level (mean ± standard error) of blood glutamic pyruvic transaminase (GPT) in the experiment period/IU.

Group	1st day before treatment with aramite	1st month post treatment with aramite	2nd month post treatment with aramite	3rd month post treatment with aramite
G.1	29.1±3.2[a]	26.7±2.4[a]	28.6±2.6[a]	25.3±2.2[a]
G.2	28.6±2.7[a]	33.1±3.7[b]	33.3±3.3[b]	33.1±3.1[b]
G.3	29.3±3.4[a]	39.4±4.1[c]	41.1±4.6[c]	38.7±3.8[c]

*Vertical variable small letter refers to significant differences between groups at 5%. **LSD value = 5.084

Table 2. The level (mean ±standard error) of blood glutamate oxaloaceate transaminase (GOT) (GOT) in the experiment period/IU.

Group	1st day before treatment with aramite	1st month post treatment with aramite	2nd month post treatment with aramite	3rd month post treatment with aramite
G.1	76.3±8.9[a]	67.4± 6.7[a]	61.2± 5.9[a]	69.7±7.1[a]
G.2	83.6±10.1[b]	75.1± 8.2[b]	75.7± 8.3[b]	71.0± 8.3[a]
G.3	86.1± 11.3[b]	85.7± 11.8[c]	86.7± 9.6[c]	87.2± 8.9[b]

*Vertical variable small letter refers to significant differences between groups at 5%. **LSD value = 6.294.

Table 3. Demonstration of the level (mean±standard error) of blood acid phosphatase activity (ACP) in the experiment period KAU/ dl.

Group	1 day before treatment with aramite	1st month post treatment with aramite	2nd month post treatment with aramite	3rd month post treatment with aramite
G.1	10.5± 1.3[a]	11.3± 1.7[a]	11.4± 1.6[a]	10.3± 1.4[a]
G.2	9.9 ± 0.9[a]	16.1± 2.7[b]	16.6± 2.7[b]	15.3± 2.2[b]
G.3	10.7± 1.6[a]	22.3± 3.3[c]	24.7± 2.3[c]	21.9± 3.2[c]

*Vertical variable small letter refers to significant differences between groups at 5%. **LSD value = 4.113.

Table 4. The level (mean ±standard error) of blood alkaline phosphatase activity (ALP) in the experiment period KAU/ dl.

Group	1st day before treatment with aramite	1st month post treatment with aramite	2nd month post treatment with aramite	3rd month post treatment with aramite
G.1	32.3± 2.8[a]	30.8± 2.6[a]	27.9± 2.2[a]	25.8± 2.1[a]
G.2	32.3± 3.1[a]	36.7± 3.4[b]	34.6± 3.2[b]	33.2± 3.1[b]
G.3	30.9± 2.6[a]	43.5± 4.5[c]	46.1± 4.8[c]	42.2± 4.3[c]

*Vertical variable small letter refers to significant differences between groups at 5%. **LSD value = 5.102.

phosphatase (ACP), a type of enzyme, is a phosphatase used to free attached phosphate groups from other molecules during digestion. It is stored in lysosomes and functions when these fuse with endosomes, which are acidified while they function; therefore, it has an acid pH optimum (en.wikipedia.org/wiki/Acid_phosphatase). Alkaline phosphatase (ALP) is a hydrolase enzyme responsible for removing phosphate groups in the 5- and 3- positions from many types of molecules, including nucleotides, proteins and alkaloids. The process of removing the phosphate group is called dephosphorylation (en.Wikipedia .org/wiki/Alkaline_ phosphatase). Different forms of acid phosphatase and alkaline phosphatase are found in different organs, and their serum levels are used as a diagnostic for disease in the corresponding organs. Acid-/alkaline balance is a dualistic model representing the two opposite abnormalities of pH control; in that the failure to maintain normal pH may be associated with one or more of seven causative factors, such as water/electrolyte imbalance. In fact, excess alkalinity is just as harmful as excess acidity.

The results of this study showed an increase in the levels of GPT, GOT, ALP and ACP in rats that were exposed to aramite which indicated that the liver was affected by the harmful and toxic substances that were produced as free radicals from metabolite of aramite, such as an increase in hydroxyl radical production. Free radicals may also be formed via the auto-oxidation of

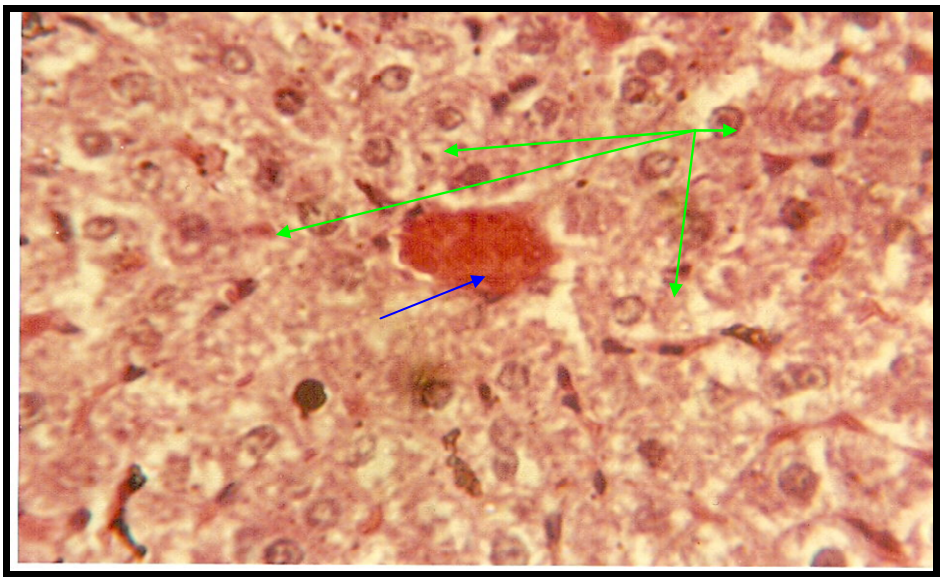

Figure 1. Normal microscopic appearance of liver tissue (G1) showing central vein (→) and normal arrangement of hepatocytes→ (H&E 285X).

unsaturated lipids in plasma and membrane lipids (Integrated Risk Information System, 1991). The free radicals produced may react with polyunsaturated fatty acids in cell membranes leading to lipid peroxidation, while the level of lipid peroxidation in cells is controlled by various cellular defense mechanisms consisting of enzymatic and non enzymatic scavenger systems. The levels of these defense mechanisms are altered and increased when exposed to toxic substances. Therefore, the ineffective scavenging of the free radicals plays a crucial role in determining tissue injury (Oser and Oser, 1960).

Histological changes

Liver

The results observed in this study indicated cardinal histological changes in the liver of rats that were exposed to aramite as compared with the normal control group (G1) (Figure 1). The severity of these changes that occurred in the G3 group (exposed to aramite with water) was slightly more than that which occurred in the G2 group (exposed to aramite with diet). The changes revealed glycogen degeneration, fatty necrosis, mitotic figures in hepatocytes, proliferation of bile ducts and dilation of liver sinusoids (Figures 2, 3, 4 and 5). Liver is the major organ that is affected with various diseases especially in the presence of toxic substances or its metabolites in the absorbent material. Degeneration occurs as a result of retrogressive changes in cells and tissues characterized by abnormal structural changes and decreased functions, and nonspecific responses of cells and tissues following a variety of injuries. Some of these processes may be reversible if the injury is mild. If the injury is severe and persistent, it may progress to the point where the involved cells necroses and dies (Muris, 1991). Glycogen is normally present in the cytoplasm of the cells (particularly in liver cells). Glycogen degeneration involves the presence of an abnormally large amount of glycogen in the cytoplasm of the cells. However, excessive accumulation occurs in some disease processes characterized by prolonged hyperglycemia or the presence of strong free radicals. Further, reduction in food intake will result into a loss of glycogen from hepatocytes and a reduction in RER. The release of intracellular content after cellular membrane damage is the cause of the inflammation in necrosis (Muris, 1991). Severe damage to one essential system in the cell leads to secondary damage of other systems, called "cascade of effects". Necrosis is caused by special enzymes that are released by lysosomes which are capable of digesting the cell components or the entire cell itself. The injuries received by the cell may compromise the lysosome membrane, or may set off an unorganized chain reaction which causes the release in enzymes. Unlike apoptosis, the cells that die by necrosis may release harmful chemicals that damage other cells. The other most common condition seen in the liver is the proliferation of bile ducts and dilation of the liver sinusoids. The severity of the condition varies and may consist of only a few foci of proliferating ducts to large numbers of foci, which may become confluent and infiltration of the stroma by plasma cells and lymphocytes (Oser and Oser, 1960)

The results of this investigation are in agreement with many *in vitro* and *in vivo* metabolism studies in several

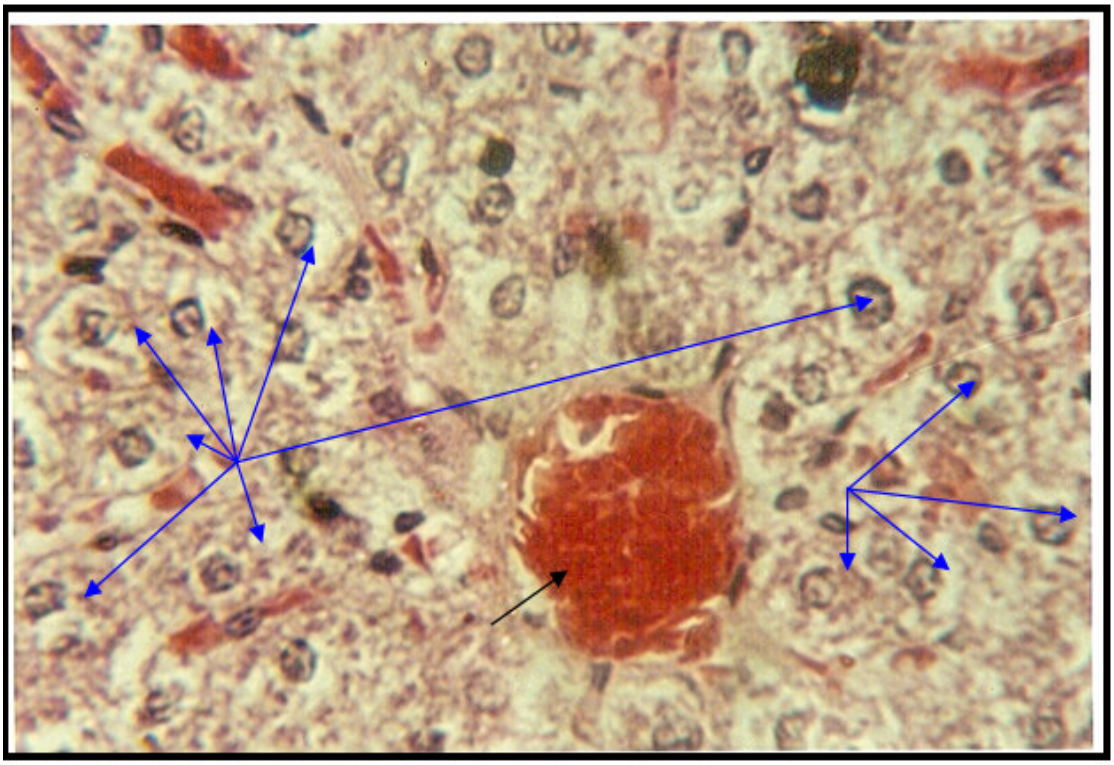

Figure 2. Microscopic appearance of liver tissue (G2) showing dilation in central vein (→) with changes in hepatocytes arrangement and shape that appears as mitotic (figures (→) H&E 285X).

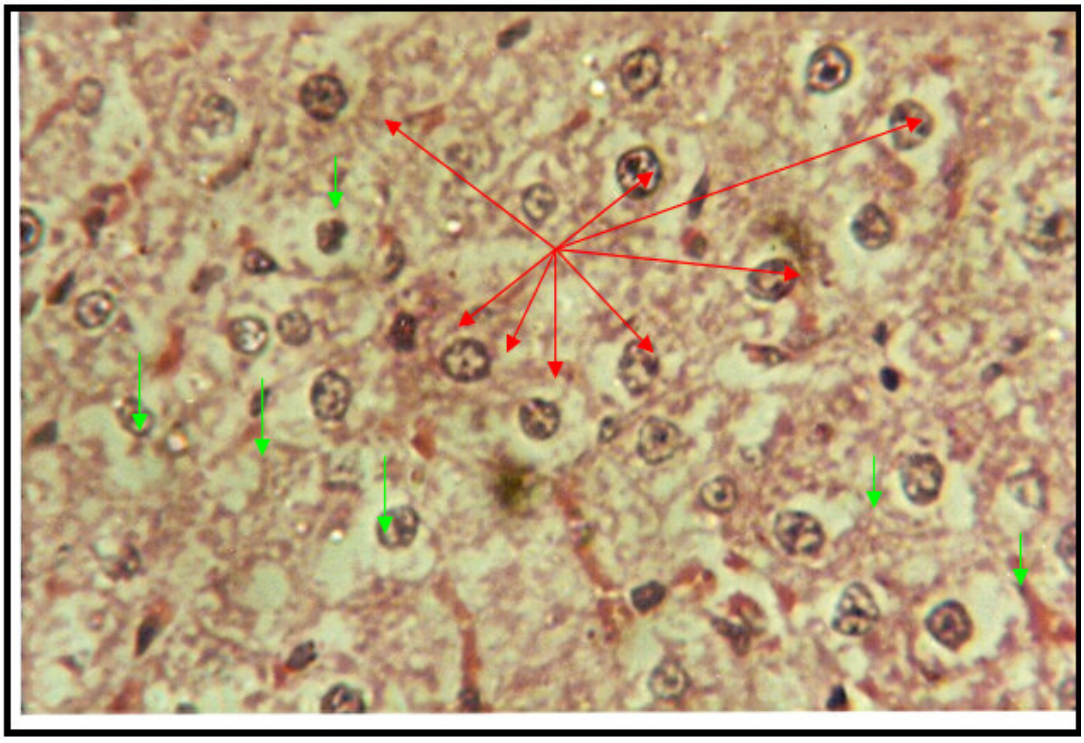

Figure 3. Microscopic appearance of liver tissue (G3) showing changes in hepatocytes arrangement and shape that appears as mitotic figures (→) and clear cell change demonstrated by spaces in the cell cytoplasm where glycogen has been removed during histological processing (→) (H&E 285X).

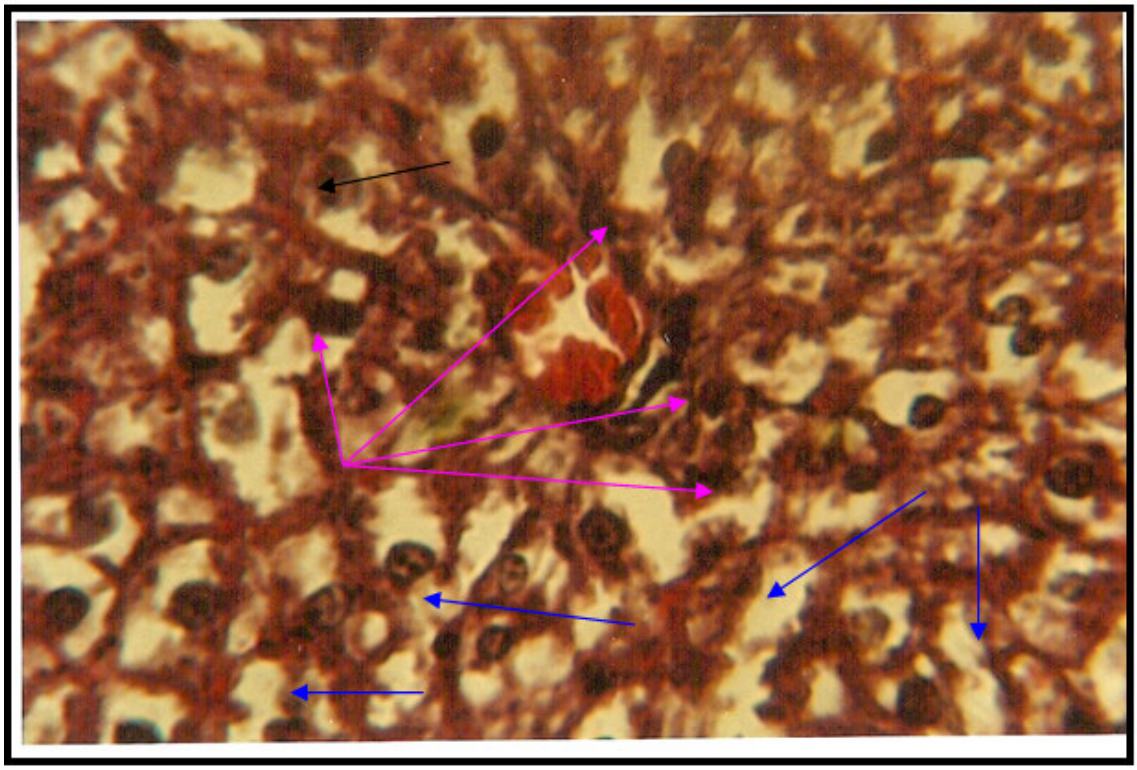

Figure 4. Microscopic appearance of liver tissue (G2) showing large areas of necrosis as karyorrhexis and a few remaining viable cells around blood vessels (→) with proliferation of bile ducts and dilation of liver sinusoids (→) (H&E 285X).

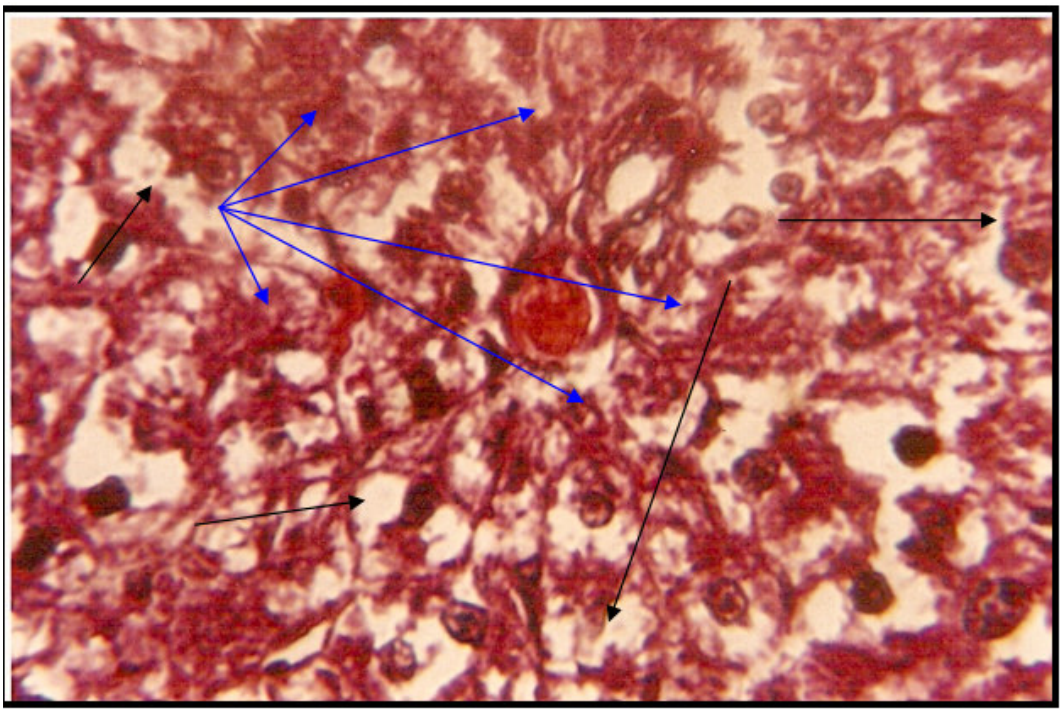

Figure 5. Microscopic appearance of liver tissue (G3) showing large areas of necrosis as karyorrhexis and karyolysis with a few remaining viable cells around blood vessels (→) with proliferation of bile ducts and dilation of liver sinusoids (→) (H&E 285X).

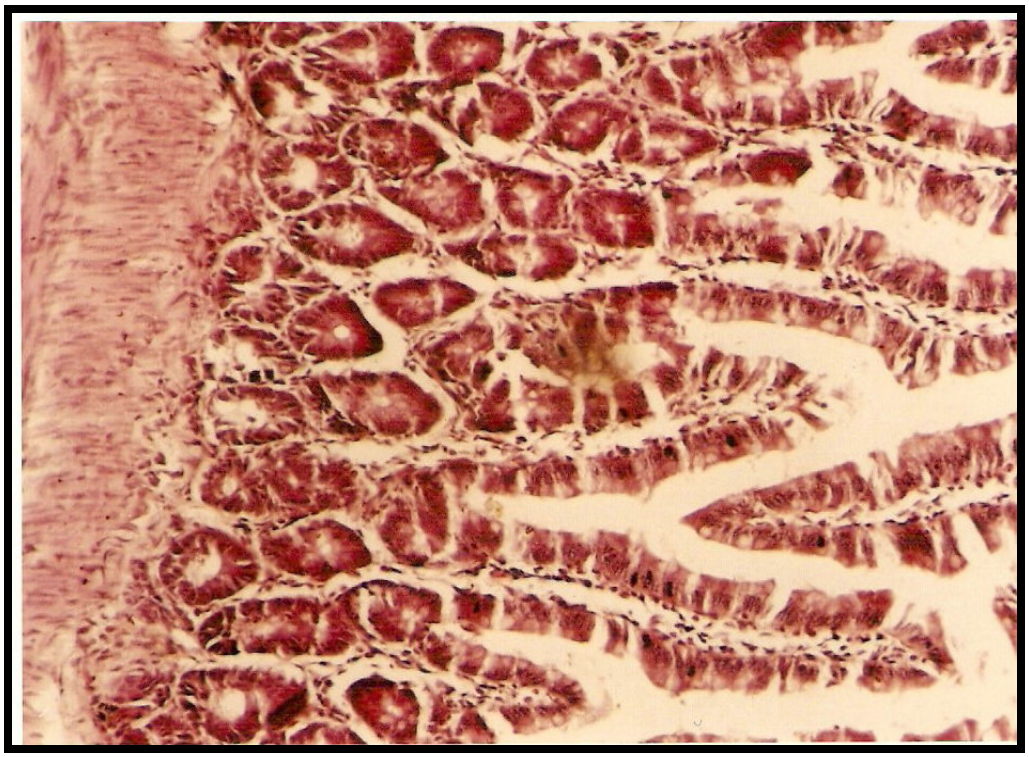

Figure 6. Microscopic appearance of ileum (G1) showing the normal arrangement of layers and villi (H&E 250X).

animal species. These confirmed the rapid degradation and hydrolysis of aramite to form free radical and the parent ester, followed by other cleavage that formed the products which are alcoholic and phenolic compounds that can be rapidly excreted or further metabolized and incorporated into harmful chemical in natural tissue constituents by biochemical reactions (International Agency for Research on Cancer (IARC) Summaries and Evaluations, 1975; Integrated Risk Information System, 1991).

Small and large intestine

Normally, the small and large intestine tissue consist of four clear layers which are mucosa, submucosa, muscularis externa and fibrosa (Figures 6 and 11).

The histopathological examination of intestines showed irregular arrangement of layers with tubular adenocarcinoma of ileum (fibrosarcoma), accumulation of lymphocytes and neutrophils forming a granuloma within the submucosa that may further extend into the muscularis externa, increased of the thickness of the intestenal layer with the hyperplasia of tubular glands and the presence of the fibrohistiocytic sarcoma and lymphoma (Figures 7, 8, 9, 10, 12, 13, 14 and 15). All the parts of the intestines are hypersensitive to certain external chemicals (especially denaturant protein). On ingestion of these chemicals, a large number of lymphocytes, plasma cells, macrophages and eosinophils accumulate within the lamina propria of the intestinal mucosa and result in the loss of normal arrangement of the layer and its consistency. These factors may contribute to the immunologic damage of the intestines (Muris, 1991).

Molecular genetic study

Polymorphism in *GSTM1* and *GSTT1* genes

The detailed analysis of polymorphisms in *GSTM1* and *GSTT1* genes is given in Tables 5 and 6, and Figures 16 and 17. Among the G1, G2 and G3 groups, the frequency of *GSTM1* was 9, 3 and 6 in G1, G2 and G3, respectively. In G1, one case of *GSTM1* null genotype was seen, while in G2, there were 12 cases lacking association with exposure to aramite that was added in the diet with/or 36.95% CI=(3.92-405.915). In G3, *GSTM1* null genotype had 13.5 fold increased risk when exposed to aramite with water. In contrast, the frequency of *GSTT1* was 9, 14 and 14 in G1, G2 and G3, respectively. There was one case of *GSTT1* null genotype in G1, G2 and G3, which appeared as none significant changes when exposed to aramite with diet or water. The glutathione-S-transferases (GSTs) are a

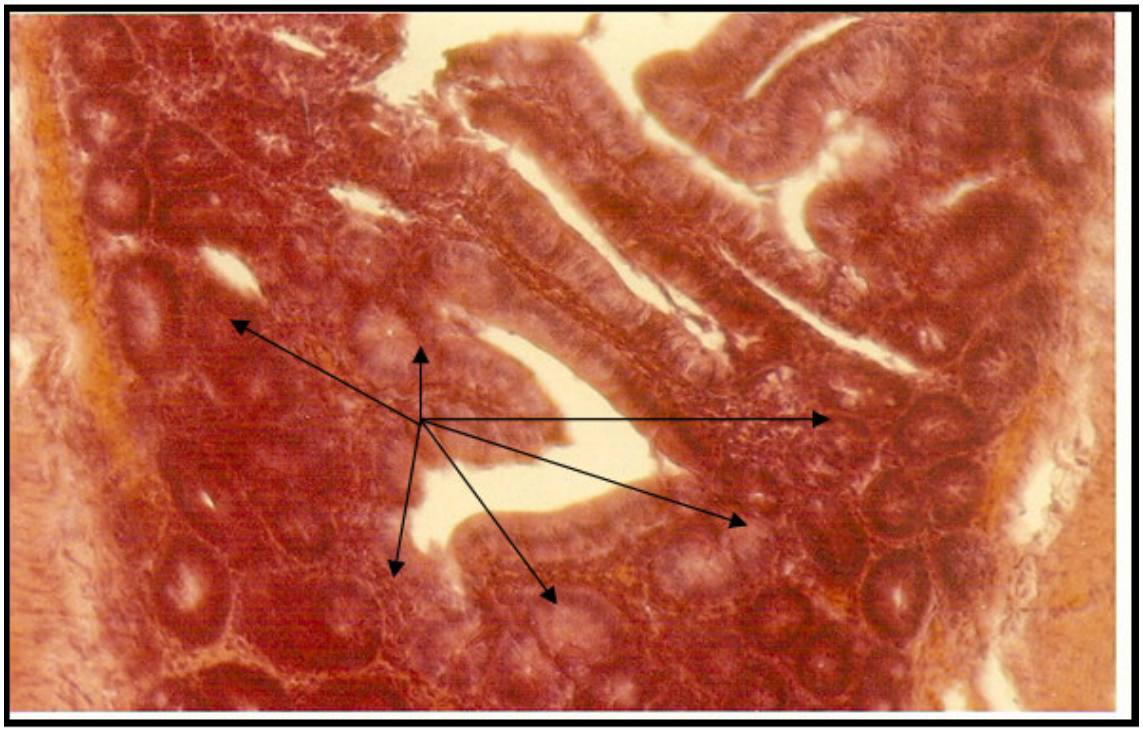

Figure 7. Microscopic appearance of ileum (G2) showing irregular arrangement of layer with tubular adenocarcinoma (→) (H&E 285X).

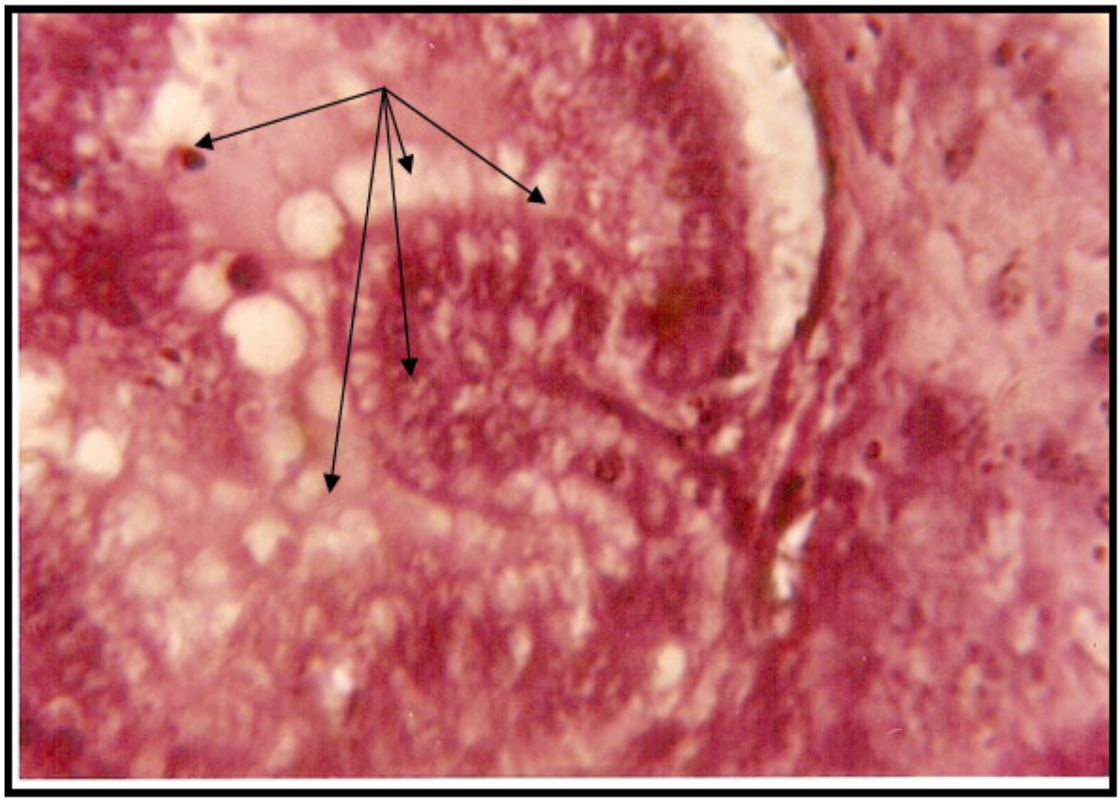

Figure 8. High power microscopic appearance of tubular adenocarcinoma of ileum (G2) showing cells with intracellular accumulations of mucus giving the characteristic of 'signet ring' appearance (→) (H&E 400X).

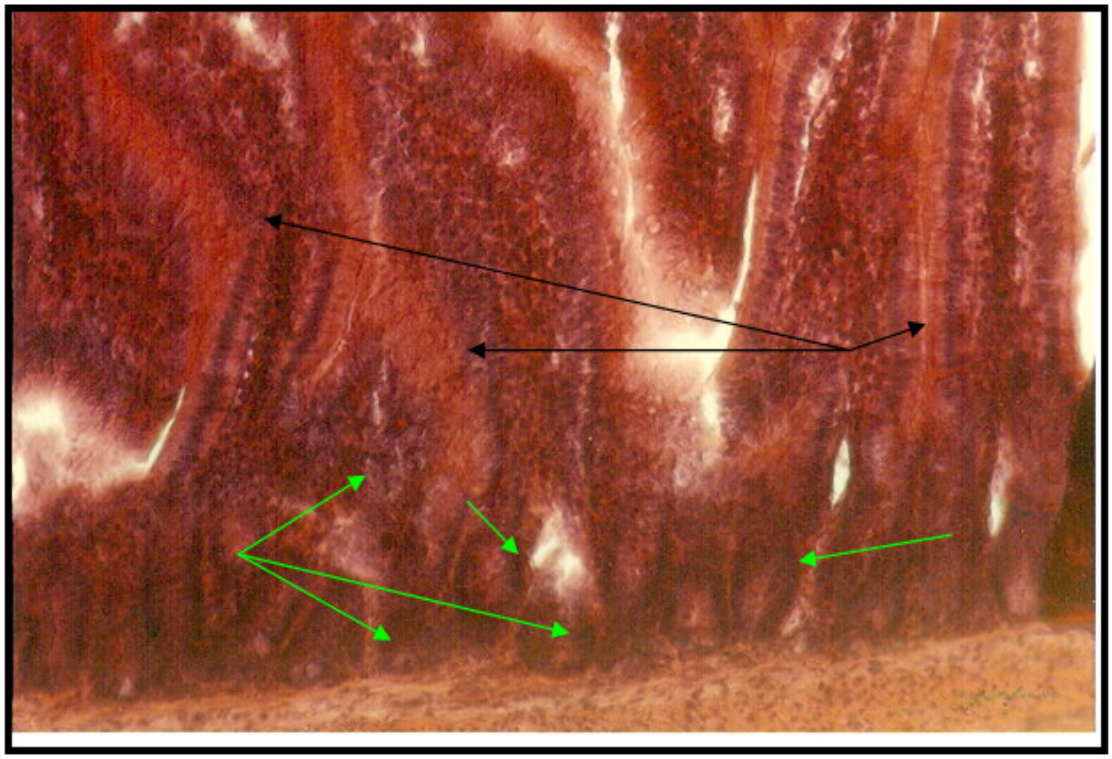

Figure 9. Microscopic appearance of ileum (G3) showing tubular adenocarcinoma (→) with Fibrosarcoma (→) (H&E 285X).

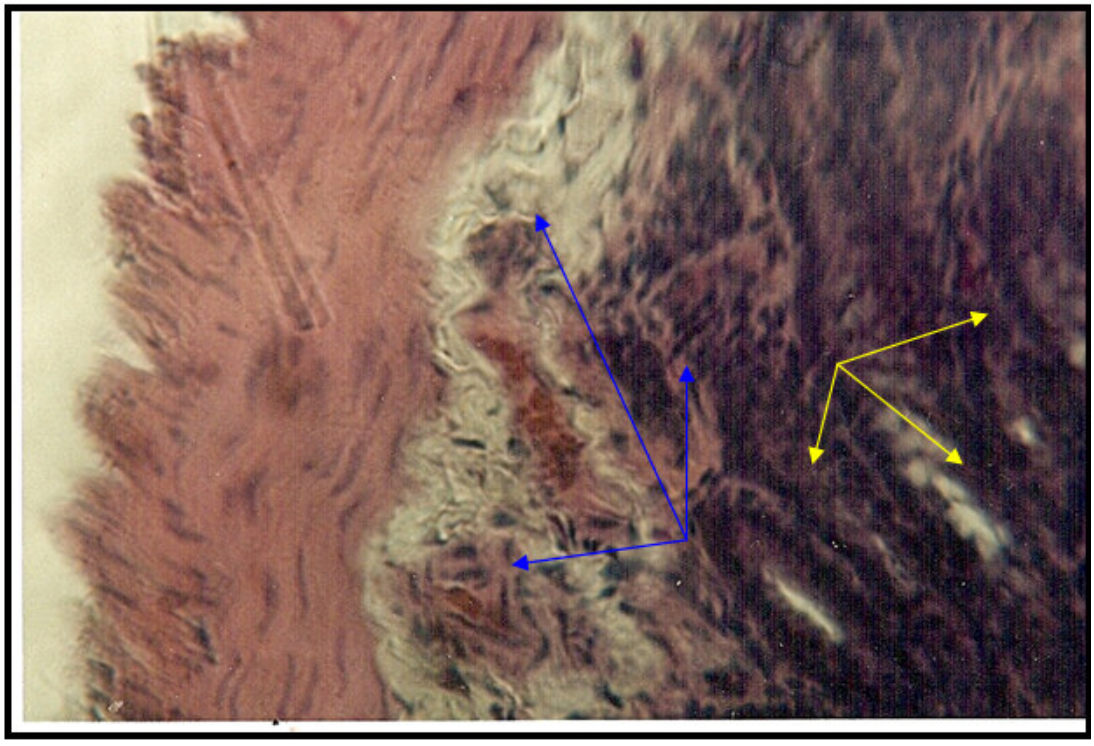

Figure 10. High power microscopic appearance of ileum (G3) showing tubular adenocarcinoma and Fibrosarcoma (→) with accumulation of lymphocytes and neutrophils forming a granuloma within the sub mucosa extending into the muscularis externa (H&E 400X).

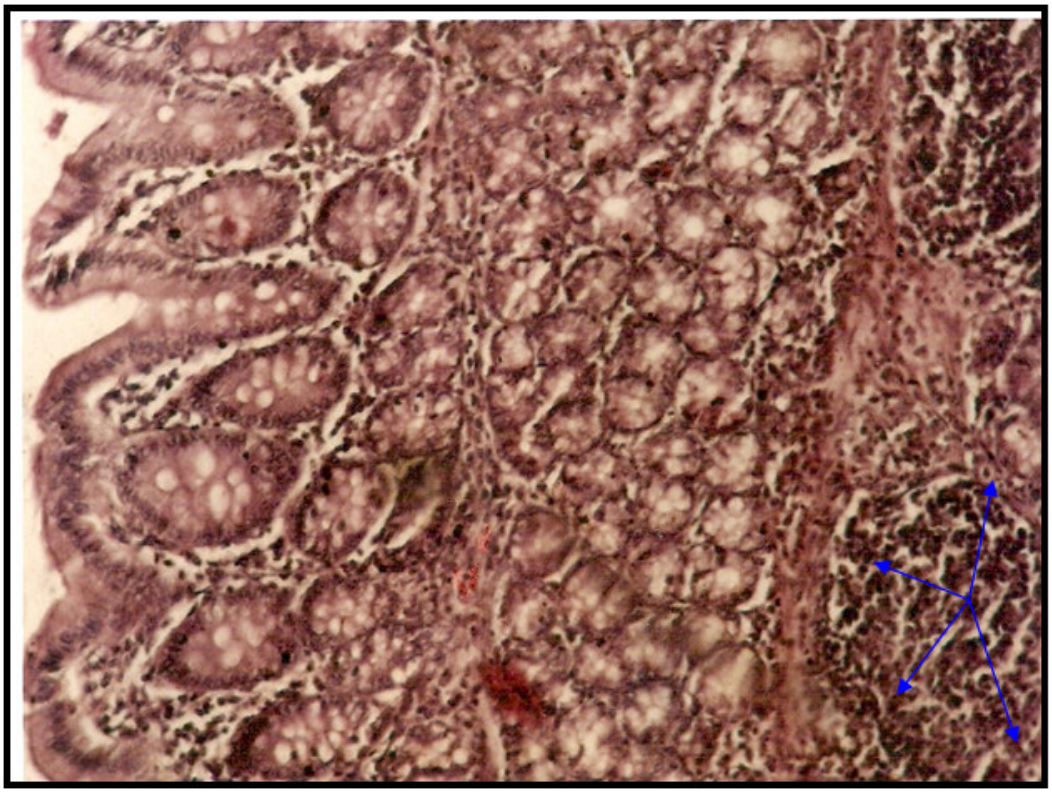

Figure 11. Microscopic appearance of normal colon (G1) showing the normal arrangement of layer and normal size of lymph node (→) (H&E 280X).

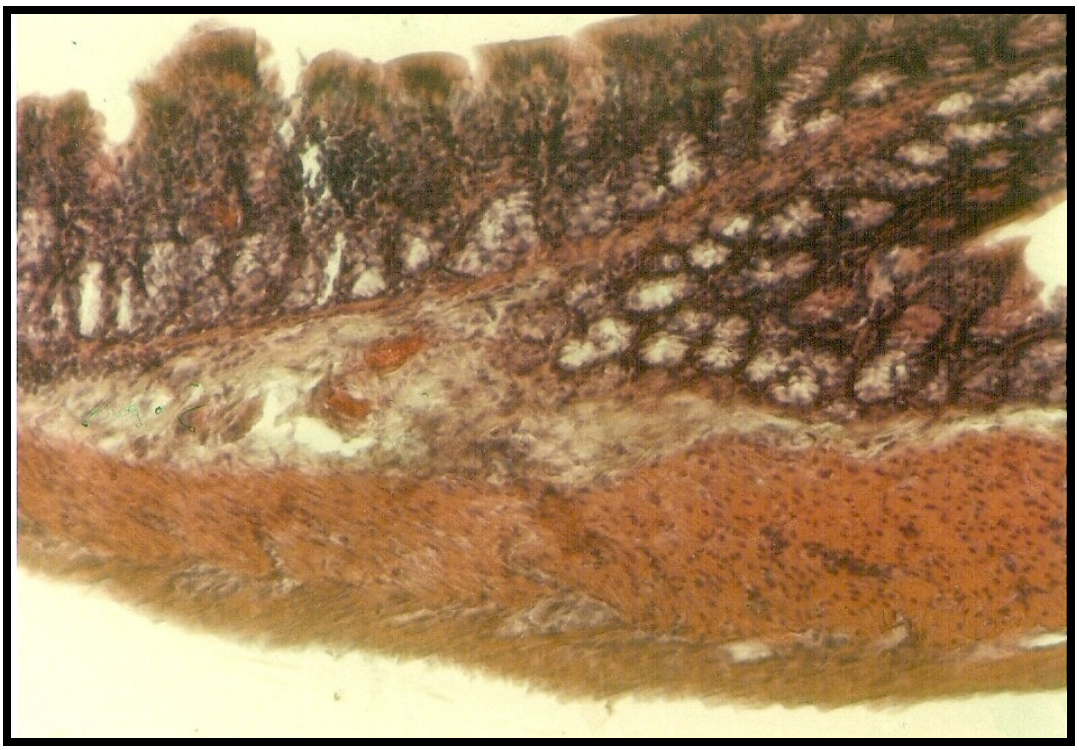

Figure 12. Microscopic appearance of colon (G2) showing increased thickness and irregular arrangement of layer with hyperplasia of tubular glands (→) (H&E 285X).

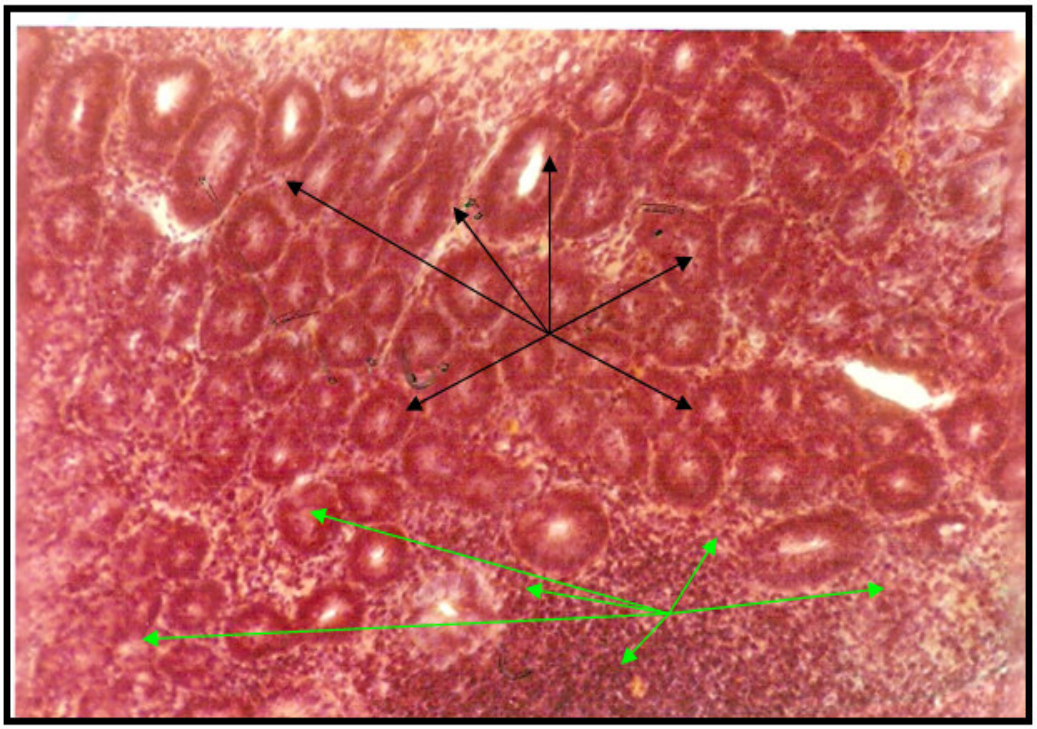

Figure 13. Microscopic appearance of colon (G2) showing increased and irregular arrangement of layer with hyperplasia of tubular glands (→) demarcation between layers and presence of the fibrohistiocytic sarcoma and lymphoma (→) (H&E 285X).

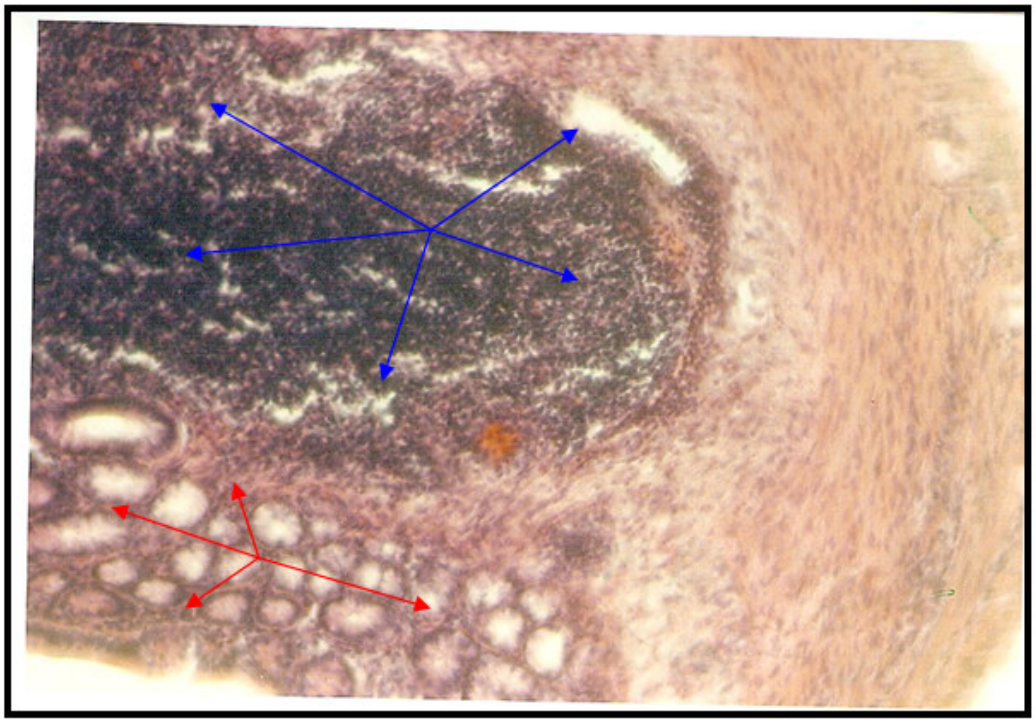

Figure 14. Microscopic appearance of colon (G3) showing increased irregular arrangement of layer hyperplasia of tubular glands (→) and demarcation between layers with the presence of the fibrohistiocytic sarcoma and lymphoma → (H&E 285X).

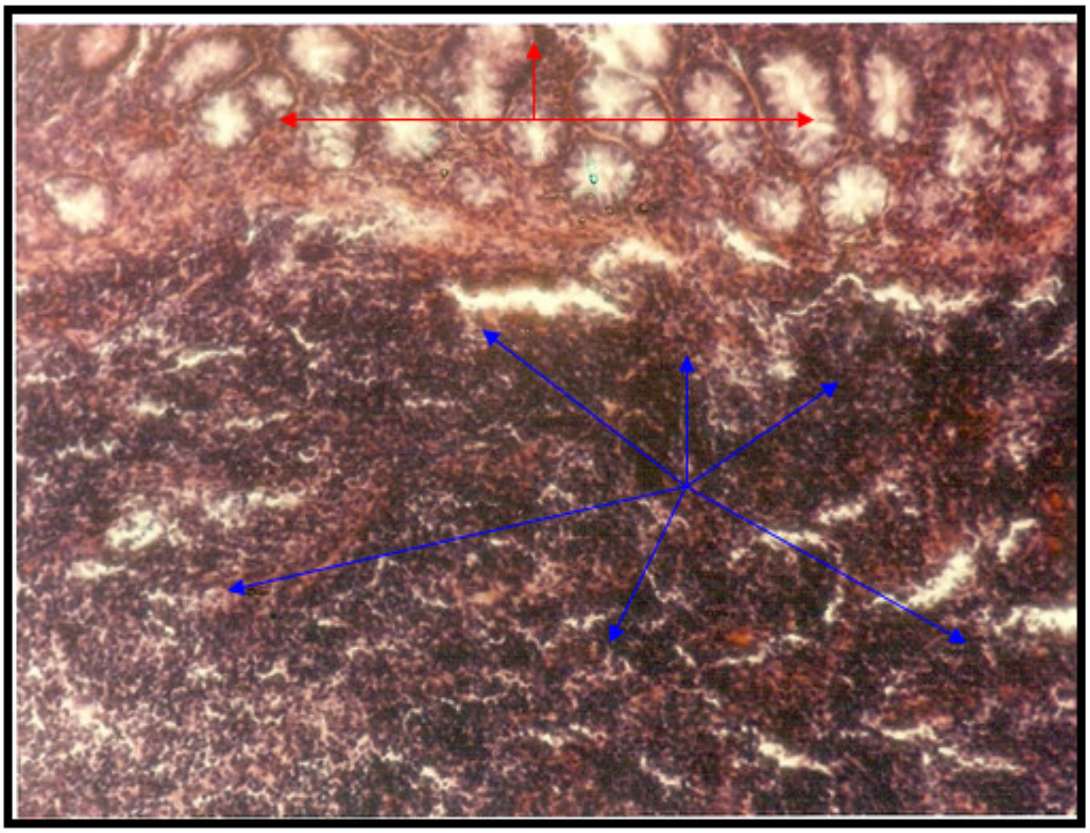

Figure 15. Microscopic appearance of colon (G3) showing increased irregular arrangement of layer hyperplasia of tubular glands (→) and demarcation between layers with the presence of the fibrohistiocytic sarcoma and lymphoma (→) (H&E 285X).

Table 5. Distribution of polymorphism of *GSTM1* gene among control and treated groups.

Genotype	*GSTM1*(+)	*GSTM1*(-)	OR (95%CI)	P value
G1	9	1	1	-
G2	3	12	36(3.92-405.915)*	0.317
G3	6	9	13.5(1.34-135.988)*	0.01

*Significant differences.

Table 6. Distribution of polymorphism of *GSTT1* gene among control and treated groups.

Genotype	*GSTT1*(+)	*GSTT1*(-)	OR (95%CI)	P value
G1	9	1	Null	-
G2	14	1	Null	-
G3	14	1	Null	-

family of enzymes that are important in the metabolism of a wide variety of xenobiotics, including environmental carcinogens, reactive oxygen species and chemotherapeutic agents (Hayes and Pulford, 1995; Raunio et al., 1995). They act as phase II metabolising enzymes, catalysing reactions between glutathione and various electrophilic compounds (Engle et al., 2002). Five classes of GST enzyme, that is, α, γ, θ, μ and π have been identified in humans. Due to their detoxification role, these enzymes and the genes encoding them may play

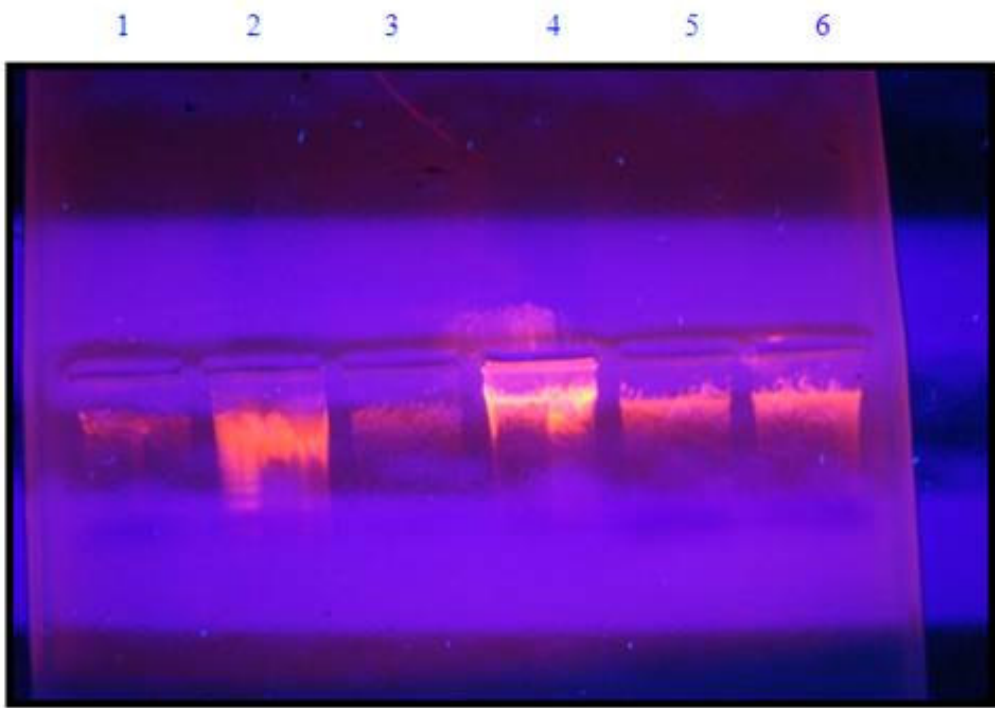

Figure 16. Photograph of agarose gel showing the high molecular weight of DNA. Lanes 1 and 2 , control; Lanes 3 and 4, G2 group; Lanes 5 and 6, G3 group.

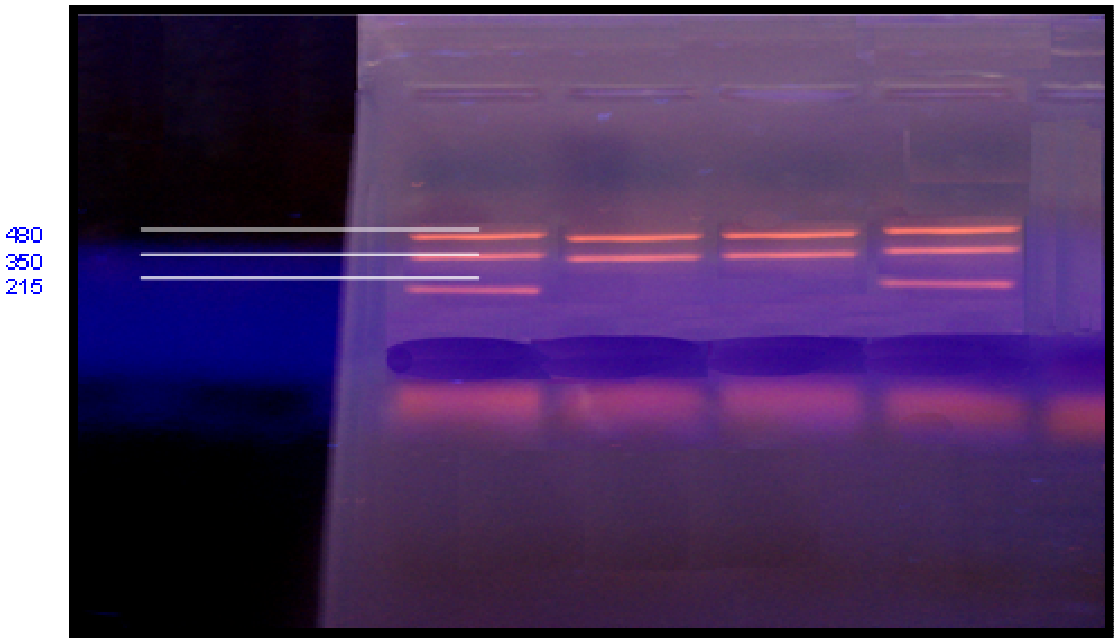

Figure 17. PCR amplification of *GSTM1*, albumin and *GSTT1* genes (PCR product: 215, 350 and 480 bp). Lanes 1 and 4, normal; Lanes 2 and 3, null *GSTM1*.

an important role in cancer susceptibility. Although the vast majority of reactions catalyzed by the GSTs result in detoxification products, there are a few cases in which the reaction is reversible or in which the product or a metabolite of the product is more reactive than the parent compound (Hayes and Pulford, 1995). GSTM1 and GSTP1 can detoxify carcinogenic polycyclic aromatic hydrocarbons, while GSTT1 can detoxify smaller reactive

hydro-carbons, such as ethylene oxide and diepoxybutane (Pemble et al., 1994). In addition, glutathione transferases may have a role in the metabolism of lipid and DNA products of oxidative stress (Strange et al., 1991; Wiencke et al., 1995) and also in the resistance to cancer chemotherapeutic agents (Russo et al., 1994).

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