ACUTE ORAL TOXICITY STUDY OF ITU AND VTU IN SWISS ALBINO MICE

Acute oral toxicity study was performed according to the OECD test guidelines 423- Acute toxic class method. Young healthy adult Swiss albino mice weighing between 18-22 g body weight, were divided into eight groups of 3 animals/group; 4 groups/test drug. Animals were housed in groups (3 animals /cage) in a well ventilated polypropylene cage. A 12-h light/12-h dark artificial photo period was maintained. A room temperature of 22°C (± 3°) and relative humidity of 50–80 % were maintained in the room. Animals had free access to pelleted feed (M/s. Provimi Animal Nutrition Pvt Ltd, India) and Reverse osmosis (Rios, USA) purified water ad libitum. Test drug was administered once orally via gastric intubation to 3 h fasted female mice. Group I and II received a single dose of ITU and VTU, respectively at a dose level of 5 mg/kg b.wt. Since no mortality and toxic clinical signs were observed for all these animals in next 48 h, another six females (3/group) were dosed at 50 mg/kg b.wt. The procedure was repeated for 300 and 2000 mg/kg b.wt.Lethality and abnormal clinical signs were observed on the day of dosing and thereafter for 13 days. Body weight was recorded before dosing and thereafter once in a week till the completion of the experiment. Gross pathological changes were also observed at the end of experiment.
RESULT

- There were no treatment related deaths, abnormal clinical signs or remarkable body weight changes observed in all the experimental animals.

- No gross pathological observation was recorded in all the experimental animals.

- From the above results, LD$_{50}$ of the test drugs “ITU and VTU” was found to be greater than 2000 mg/kg b.wt. Hence, the test drugs fall in the “category-5 or unclassified” in accordance to the globally harmonised system of classification of chemicals.

Table 20. Body weight of the experimental animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>I</td>
<td>ITU (5 mg/kg b.wt.)</td>
<td>20.67±0.33</td>
</tr>
<tr>
<td>II</td>
<td>ITU (50 mg/kg b.wt.)</td>
<td>20.67±0.33</td>
</tr>
<tr>
<td>III</td>
<td>ITU (300 mg/kg b.wt.)</td>
<td>20.67±0.33</td>
</tr>
<tr>
<td>IV</td>
<td>ITU (2000 mg/kg b.wt.)</td>
<td>20.67±0.88</td>
</tr>
<tr>
<td>V</td>
<td>VTU (5 mg/kg b.wt.)</td>
<td>19.67±0.33</td>
</tr>
<tr>
<td>VI</td>
<td>VTU (50 mg/kg b.wt.)</td>
<td>20.33±0.88</td>
</tr>
<tr>
<td>VII</td>
<td>VTU (300 mg/kg b.wt.)</td>
<td>20.00±1.00</td>
</tr>
<tr>
<td>VIII</td>
<td>VTU (2000 mg/kg b.wt.)</td>
<td>19.67±1.20</td>
</tr>
</tbody>
</table>

Values expressed in mean±SEM; n=3
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Animal ID</th>
<th>Organs</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>ITU - (5 mg/kg b.wt.)</td>
<td>H</td>
<td>Skin, eyes, brain, heart, liver, kidney, adrenals, spleen, sex glands and others</td>
<td>No abnormality detected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>ITU - (50 mg/kg b.wt.)</td>
<td>H</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>ITU - (300 mg/kg b.wt.)</td>
<td>H</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>ITU - (2000 mg/kg b.wt.)</td>
<td>H</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>VTU - (5 mg/kg b.wt.)</td>
<td>H</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>No abnormality detected</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>CL</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>VTU - (50 mg/kg b.wt.)</td>
<td>H</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>VTU - (300 mg/kg b.wt.)</td>
<td>H</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>No abnormality detected</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>CL</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>VTU - (2000 mg/kg b.wt.)</td>
<td>H</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>No abnormality detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL</td>
<td>No abnormality detected</td>
<td></td>
</tr>
</tbody>
</table>
CONCLUSION

- From the above tested condition, the test drugs “ITU and VTU” were found to be safe at 2000 mg/kg b.wt. when administered once orally to fasted female Swiss albino mice.

- Hence, the test drugs fall under “Category 5 or unclassified” based on globally harmonised system of classification of chemicals.

REFERENCE

INTRODUCTION

Cancer is a degenerative disease. Accumulation of toxins through carcinogenic food like fast food, colas, habits like smoking, drinking, paan chewing, stressful lifestyle, toxic medicines and environmental pollution lowers immunity and leads to the life threatening disease cancer. There are 10.9 million new cases, 6.7 million deaths, and 24.6 million person alive with cancer. The most commonly diagnosed cancers are lung (1.35 million), breast (1.15 million) and colorectal (1 million); the most common causes of cancer death are lung cancer (1.18 million deaths), stomach cancer (700,000 deaths) and liver cancer (5, 98,000 deaths). Internationally the cancer burden doubled between 1975 and 2000 and is set to double again by 2020 and nearly triple by 2030.

There were around 12 million new cancer cases and 7 million cancer deaths worldwide in 2008, with 20-26 million new cases and 13-17 million deaths projected for 2030. In India every year about 8, 50,000 new cancer cases are being diagnosed, resulting about 5, 80,000 cancers related death every year. The control of cancer, one of the leading causes of death worldwide, may benefit from the potential that resides in alternative therapies. Conventional therapies cause serious side effects and, at best, merely extend the patient's lifespan by a few years. Better cancer treatments with milder side effects are desperately needed. There is thus the need to utilize alternative concepts or approaches to the prevention of cancer.

Prevention of disease is an old and important concept. It has been realized that the focus and emphasis should be shifted to the control of carcinogenesis, rather
than attempting to cure end-stage disease. Chemoprevention, which is a pharmacological approach to intervention in order to arrest or reverse the process of carcinogenesis, attempts to address this issue. Recently, considerable attention has been focused on identifying naturally occurring chemo preventive substances capable of inhibiting, retarding or reversing the pre neo plastic lesion of carcinogenesis. Chemopreventive substances are identified on the basis of their antioxidant, anti mutagenic and anti-inflammatory activities capable of arresting proliferation and enhancing apoptosis and these are the major criteria for their anti carcinogenic activity.

Cancer is a hyper proliferative disorder that involves transformation, deregulation of apoptosis, proliferation, invasion, angiogenesis and metastasis. Colon cancer is one of the leading causes of cancer death in western countries. Globally, colorectal cancer is the third commonest cancer in men since 1975. The present study focuses on the preventive strategies aimed at reducing the incidences and mortality of bowel cancer. Chemoprevention of colon cancer appears to be a very realistic possibility because various intermediate stages have been identified preceding the development of malignant colonic tumours. It has already been proved from various experiments that chemo preventive agents by virtue of their anti-oxidant, anti-inflammatory, anti-proliferative, apoptosis-inducing activity, act at various levels including molecular, cellular, tissue and organ levels to interfere with carcinogens.
Genetics, experimental and epidemiological studies suggest that colorectal cancer results from complex interactions between inherited susceptibility and environmental factors including lifestyle factors such as diet and nutrition.

Aberrant crypt foci (ACF), constellation of morphologically abnormal crypts have been recognized to precede the development of colonic adenoma. Pretlow have shown that these lesions are present in the colonic mucosa of patients with colon cancer and have suggested that aberrant crypts are putative precursor lesions from which adenomas and carcinomas develop in the colon.

**COLON CANCER**

Colon cancer is treated by surgery, radiation therapy, chemotherapy and by other methods. Early detection can be done by the following methods.

**Faecal occult blood testing**

It is tested by colorimetric assay catalysed by pseudoperoxidase present in blood. The method has low specificity. Ascorbic acid must be avoided for several days before the test because ascorbic acid inhibits the guaiac reaction (11).

**Barium enema**

Patients are exposed to radiations during barium examination. Detection of polyp at barium enema necessitates colonoscopy as a second examination for biopsy or polypectomy.
Flexible sigmoidoscopy

It is relatively insensitive at colon cancer or colon polyp detection because the proximal half of the colon is not endoscopically visualised.

Colonoscopy

Colon cancers are rarely missed at colonoscopy because they tend to be larger than adenomatous polyps.

MRI

It is superior to CT in detecting focal liver metastases from colon cancer.

Stool genetic markers, virtual colonoscopy, video capsule endoscopy, molecular screening and genetic testing. If colon cancer is detected at early stage then it is mostly curable.

Colon is the last part of large intestine consisting of cecum, ascending, transverse, descending, and sigmoid colon and the rectum. Absorption of fluid and solutes occur in right colon. Movement and storage of faecal material occur in the left colon. Normal colonic epithelium is converted to an adenomatous polyp and an invasive cancer.

Colon cancer is described by the following terms

Polyp - It is a clinical description of any circumscribed mass of cells that project above the surfaces of surrounding normal mucosa.
Aberrant crypts ACF- It is a cluster of abnormal colonic crypts which can be identified on the mucosal surface after methylene blue staining and observation under low magnification. The pre neoplastic nature of ACF is supported by the finding of APC, K-ras gene mutations and microsatellite instability in minute dysplastic foci of aberrant crypts.

Adenomas are well demarcated, circumscribed lumps of epithelial dysplasia, and are classified into three major histological types: tubular, villous and tubulovillous. Most adenomas remain benign and asymptomatic lesions that can be discovered during lower endoscopy; however, a small fraction of these lesions may evolve into malignancy.

Malignant adenoma: Neoplastic cells pass through the muscularis mucosae and infiltrate the submucosa.

Colon carcinogenesis begins with a generalized disorder of cell replication, differentiation and apoptosis that antedates and then accompanies the development of morphologic lesions (aberrant crypt foci, small adenomas, large adenomas and cancer). Cell replication and differentiation in colon epithelial cells is controlled by two pathways, Wnt signalling pathway and Hedgehog signalling pathway.

Prevention of cancer includes primary and secondary. The primary prevention strategies mainly include identification of the risk factors, eradication of risk factors, lifestyle and diet modification and surgical resection. In primary preventive measures such as dietary modification and chemoprevention are currently
being employed and are undergoing extensive research. Secondary prevention approaches are taken to prevent malignancy in a population.

**Symptoms**

- Partial obstruction produces constipation, nausea, abdominal distention and abdominal pain.
- Distal cancer sometimes causes gross rectal bleeding.
- Bleeding from proximal cancer tends to be occult.
- Advanced cancer, particularly with metastasis, can cause cancer cachexia, feeling of poor health.
- Blood in the stool is the most common sign.
- Hepatomegaly and Jaundice in advanced disease.
- Leg edema as a consequence of lymph node involvement, thrombophlebitis, fistula formation, weight loss and pain in the lower back or radiating down the legs are indicative of widespread disease.

**Laboratory tests**

- Positive guaiac stool test and anaemia from blood loss.
- Elevated carcino embryonic antigen.
- Elevated liver enzymes may be present with metastatic disease.
Fluorouracil is widely used for treatment of colon cancer, both as a single agent and in combination with other drugs. Several studies demonstrated that adjuvant fluorouracil is equivalent to surgery in improving the survival. Clinical studies generally favour continuous infusion of fluorouracil as compared to bolus, due to its short plasma half life and S-phase specificity for optimal Thymidylate synthetase inhibition. Dihydro pyrimidine dehydrogenase is the main enzyme responsible for the catabolism of fluorouracil to inactive metabolites. A rare pharmaco-genetic disorder characterised by complete or near - complete deficiency of this enzyme has been identified in cancer patients. Patients with this enzyme deficiency develop severe toxicity, including death, after fluorouracil administration (1).

**IN VIVO MODELS**

1. **Chemically Induced Models**

   **Advantages**

   Reflection of human pathology, high number of tumors and early end points are available.

   **Disadvantages**

   Physiologically high amounts of carcinogen are required.

2. **Aromatic amines** e.g DMAB by s.c injection for 20 weeks.

   **Advantages**

   Induce adenomas
Disadvantages

Require multiple injection and less potent

3. **Alkyl Nitrosoureido compounds**: By intrarectal injection for 20 weeks.

Advantages

Do not require metabolic activation.

Disadvantages

Intra rectal injection is required from highly skilled technicians.

4. **DMH 1, 2 dimethyl hydrazine** 20 mg/kg once weekly for 20 weeks

Advantages

Cancer produced is very close to human colon cancer.

Disadvantages

Requires multiple injections

5. **AOM Azoxymethane**

Advantages

AOM does not require metabolic activation. It is a metabolite of DMH.

Single injection is required
Disadvantages

High dose causes random DNA and biochemical disturbances in addition to genetic changes.

Azoxy methane is a powerful chemical that induce cancer in rodents.

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Figure 65. Metabolism of azoxy methane
Azoxymethane is N-hydroxylated to methyl azoxy methanol which can be excreted into the bile and transported to the colon or enter directly into the epithelial cells of the colon from the blood stream. Methyl azoxy methanol is chemically unstable at body temperature and decomposes spontaneously \textit{in vitro} to formaldehyde, water and the alkylating agent methyldiazonium which forms a reactive carbonium ion which is capable of methylating DNA, RNA or protein. The carcinogenic action involves methylation of colonic epithelial cell DNA mainly at N\textsubscript{1} and O\textsubscript{6} position of guanine. AOM, a chemical and metabolic derivative of 1, 2-dimethylhydrazine is like the powerful and specific colon carcinogen in rodents. MAM is an unstable compound with a half life of about 12 h under physiological condition. MAM is oxidised to methyl azoxy formaldehyde, specially in a reaction catalysed by alcohol dehydrogenase, a cytosolic enzyme.

The first lesion in the multistep development of colon cancer cannot be seen. They can be identified in histological sections of colon mucosa after the careful histological examination as hyperplastic or dysplastic epithelial lesions.

The development of tumors is relatively of lengthy process, taking around 6-8 months to develop in the DMH/AOM rat model so preneoplastic lesions can be used as biomarkers for assessing the developing colon cancers in short term studies

**MATERIALS AND METHODS**

**Experimental Design**

The experiment was designed to test the chemopreventive efficacy ITU and VTU respectively. It was divided into seven groups negative control, positive control, standard, test compounds low dose and high dose.
Each group in a set consisted of 10 mice. All the groups except the normal were initiated with intra peritoneal injections (5 mg / kg body weight) of AOM (Sigma Chemicals Co. MO, USA) once a week for two weeks. The normal group received no treatment. The carcinogen control group (CC) was given AOM alone; whereas the test compounds treated groups received 200 mg/kg and 100 mg/kg body weight, continuously starting from 1st day of AOM injection. The treatment was continued for 8 weeks from the 1st day of AOM injection and the parameters were studied thereafter (12).

I. Protocol for the Biochemical Assays

Estimation of SGPT in plasma

PRINCIPLE OF THE METHOD

Alanine aminotransferase (ALT or GPT) catalyzes the transfer of the amino group from alanine to 2-oxoglutarate, forming pyruvate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm; by means of the lactate dehydrogenase (LDH) coupled reaction (2).

\[
\text{Alanine + 2 – Oxoglutarate} \rightarrow \text{Pyruvate + Glutamate}
\]

\[
\text{Pyruvate + NADH +H+} \rightarrow \text{Lactate + NAD+}
\]

Reagents used

A. Reagent: Tris 150 mmol/L, L-alanine 750 mmol/L, lactate dehydrogenase > 1350 U/L, pH 7.3.
B. Reagent: NADH 1.9 mmol/L, 2-oxoglutarate 75 mmol/L, Sodium hydroxide 148 mmol/L, sodium azide 9.5 g/L.

C. Reagent (cod 11666): Pyridoxal phosphate 10 mmol/L. 5 mL.

Equipments used

− Analyzer, spectrophotometer or photometer with cell holder thermostatable at 30 ºC or 37ºC which can read at 340 nm. [The path length of cuvettes should be 1 cm light path].

PROCEDURE

1. The working reagent and the instrument was brought to reaction temperature.

2. Pipette into a cuvette: (Note 2)

   Reaction temperature 37ºC, 30ºC

   Working Reagent 1.0 ml, 1.0 ml

   Sample 50 AL, 100 AL

3. The cuvette was mixed and inserted into the photometer. The stopwatch was started.

4. After 1 minute (Note 1), initial absorbance was recorded and at 1 minute interval thereafter for 3 minutes.

5. The difference between consecutive absorbances was calculated and the average absorbance difference per minute (DA/min) was noted
CALCULATIONS

The ALT/GPT concentration in the sample was calculated using the following general formula:

\[ \frac{\Delta A}{\text{min}} \times \frac{V_t \times 10^6}{e \times I \times V_s} = \frac{U}{L} \]

The molar absorbance (e) of NADH at 340 nm was 6300, the lightpath (l) was 1 cm, the total reaction volume (Vt) was 1.05 at 37°C and 1.1 at 30°C, the sample volume (Vs) was 0.05 at 37°C and 0.1 at 30°C, and 1 U/L were 0.0166 μkat/L.

Estimation of SGOT in plasma

Biochemical assays such as SGOT and SGPT were performed at the end of the terminal necropsy by using fully automated biochemical analyzer, Biosystem A15, Spain with Biosystems kit as per the kit instructions (5).

PRINCIPLE OF THE METHOD

Aspartate aminotransferase (AST or GOT) catalyzes the transfer of the amino group from aspartate to 2-oxoglutarate, forming oxalo acetate and glutamate. The catalytic concentration was determined from the rate of decrease of NADH, measured at 340 nm; by means of the malate dehydrogenase (MDH) coupled reaction.
Aspartate + 2 – Oxoglutarate \[\rightarrow\] Oxalacetate + Glutamate

Oxalacetate + NADH + H$^+$ \[\rightarrow\] Malate + NAD$^+$

**COMPOSITION**

A. Reagent: Tris 121 mmol/L, L-aspartate 362 mmol/L, malate dehydrogenase > 460 U/L, lactate dehydrogenase > 660 U/L, Sodium hydroxide 255 mmol/L, pH 7.8. Irritant (Xi): R36/38: Irritating to eyes and skin. S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. S37/39: Wear suitable gloves and eye/face protection.

B. Reagent: NADH 1.9 mmol/L, 2-oxoglutarate 75 mmol/L, Sodium hydroxide 148 mmol/L, sodium azide 9.5 g/L

C. Reagent (cod 11666): Pyridoxal phosphate 10 mmol/L. 5 mL.

**PROCEDURE**

1. The working reagent and the instrument was brought to reaction temperature.

2. Reagents were pipetted in to the cuvette.

   Reaction temperature 37$^\circ$ C, 30$^\circ$ C

   Working Reagent 1.0 mL, 1.0 mL

   Sample 50 AL, 100 AL

3. The cuvette was mixed and inserted into the photometer. The stopwatch was started.
4. After 1 minute (Note 1), initial absorbance was recorded and at 1 minute interval thereafter for every 3 minutes.

5. The difference between consecutive absorbances and the average absorbance difference per minute (DA/min) was calculated.

CALCULATIONS

The AST/GOT concentration in the sample was calculated using the following general formula:

\[ \Delta A/\text{min} \times \frac{V_t 	imes 106}{e 	imes l 	imes V_s} = U/L \]

The molar absorbance (e) of NADH at 340 nm was 6300, the lightpath (l) was 1 cm, the total reaction volume (Vt) was 1.05 at 37° C and 1.1 at 30° C, the sample volume (Vs) is 0.05 at 37° C and 0.1 at 30° C, and 1 U/L are 0.0166 μkat/L.

ROS and Non enzymatic antioxidant estimation

a. Lipid peroxidation (TBARS)

Reagents

1) Thiobarbituric acid (TBA) (0.8 %): 0.8 g in 0.5 N HCl

2) Butylated hydroxyl toluene (0.05 %): 0.05 g in methanol.

3) Saline (0.9 %): 0.9 g in 100 ml distilled water.

Procedure

The method involved heating of homogenized liver and colon sample with 0.8 ml saline, 0.5 ml of BHT and 3.5 ml TBA reagent for 1 and 1/2 hrs in a boiling
water bath. After cooling, the solution was transferred to vial and centrifuged at 3,500 rpm for 10 min and the precipitate obtained was removed. The absorbance of the supernatant was determined at 532 nm using spectrophotometer against a blank that contained all the reagents minus the biological sample. The values were expressed in mg/g tissue (10).

b. **Assay of reduced glutathione**

GSH was determined by the method of Moren et al., 1979.

**Reagents:**

1. 5 % TCA: 5 g in 100 ml of distilled water.
2. Phosphate buffer (pH: 8) 0.2 M
3. DTNB (0.6 mM): 12 mg in 50 ml PO₄ buffer.

**Procedure**

Estimation is based on the reaction of GSH with DTNB to give a compound that absorbs at 412 nm. Liver and colon homogenate was precipitated with 5 % TCA. To an aliquot of the supernatant, 2 ml of DTNB (0.6 mM in 0.2 M sodium phosphate buffer, pH 8.0) and 0.8 ml of sodium phosphate buffer (0.2 M, pH 8.0) were added to make a final volume of 3 ml. The absorbance was read at 412 nm against a reagent blank. A series of standards treated in a similar manner were also run. The amount of GSH was expressed as mg/g tissue.
Estimation of Glutathione-S-transferase (GST) Activity

Spectrophotometric method was adopted to evaluate the activity of GST in rat liver and colon tissues. After 8 weeks of 1st AOM injection, the livers and colons were excised immediately after sacrifice. GST activities were measured in tissue cytosol by determining the increase in absorbance at 340 nm with 1-chloro-2, 4-dinitrobenzene (CDNB) as the substrate and the specific activity of the enzyme was expressed as formation of CDNB-GSH conjugate per minute per milligram of protein.

Estimation of the Level of Lipid Peroxidation

Spectrophotometric method was also applied to estimate the level of lipid peroxidation by measuring the formation of lipid peroxides using thiobarbituric acid. After 8 weeks of 1st AOM injection, the livers and colons were excised immediately after sacrifice. The level of lipid peroxidation was determined by measuring the formation of lipid peroxides using thiobarbituric acid, [TBA (Sigma)] and expressed as Thiobarbituric Acid Reactive Substances (TBARS) formed per milligram of protein using an absorbance of 532 nm.

Histopathological slides Preparation:

At the end of the experimental period all the mice were euthanized using overdose of ether. The livers and colons were collected from mice of all groups and fixed in 10 % neutral buffered formalin solution. Representative tissues of liver and colon were dehydrated in series of graded alcohol and embedded in paraffin wax. Paraffin sections of 3-4 micron thickness were obtained, mounted on glass slides and stained with Hematoxylin and Eosin (H&E) for light microscopic analyses.
## RESULTS

Table 22. In vivo result

<table>
<thead>
<tr>
<th>S. No</th>
<th>Group</th>
<th>Colon LPO Mean ± S D</th>
<th>Liver LPO Mean ± S D</th>
<th>Colon GSH Mean ± S D</th>
<th>Liver GSH Mean ± S D</th>
<th>SGPT plasma Mean ± S D</th>
<th>SGOT plasma Mean ± S D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative control</td>
<td>117.95 ± 15.33</td>
<td>145.90 ±23.44</td>
<td>110.64±92.40</td>
<td>122.38±54.69</td>
<td>44.00±10.81</td>
<td>97.33±13.31</td>
</tr>
<tr>
<td>2</td>
<td>Positive control</td>
<td>147.40 ±48.54</td>
<td>158.99±35.74</td>
<td>144.14±44.73</td>
<td>145.28±66.05</td>
<td>57.33±16.80</td>
<td>112.67±26.10</td>
</tr>
<tr>
<td>3</td>
<td>Standard 5-flourouracil 20mg/kg</td>
<td>140.45 ±28.75</td>
<td>129.71±32.36</td>
<td>133.95±6.70</td>
<td>120.52±36.08</td>
<td>51.67±8.32</td>
<td>95.33±15.27</td>
</tr>
<tr>
<td>4</td>
<td>ITU 200mg/ml</td>
<td>140.20 ±32.00</td>
<td>144.39±5.44</td>
<td>165.66±15.20</td>
<td>179.41±94.35</td>
<td>42.00±4.00</td>
<td>80.33±15.94</td>
</tr>
<tr>
<td>5</td>
<td>ITU 100 mg/ml</td>
<td>147.36±45.14</td>
<td>157.95±15.69</td>
<td>78.31±41.14</td>
<td>201.53±33.22</td>
<td>50.08±11.35</td>
<td>70.55±17.69</td>
</tr>
<tr>
<td>6</td>
<td>VTU 200mg/ml</td>
<td>249.89 ±76.77</td>
<td>244.20±57.60</td>
<td>68.77±9.14</td>
<td>201.53±33.22</td>
<td>57.67±12.66</td>
<td>90.00±11.53</td>
</tr>
<tr>
<td>7</td>
<td>VTU 100mg/ml</td>
<td>232.31 ±8.65</td>
<td>338 ±21.6</td>
<td>71.17±5.88</td>
<td>196.91 ±32.24</td>
<td>40.77±4.50</td>
<td>100.50±13.50</td>
</tr>
</tbody>
</table>
Figure 66. Level of LPO in colon
Groups

a, b significantly different from normal group (p=0.05)
c significantly different from carcinogen control group (p=0.05)

d significantly different from standard group (p=0.05)

Figure 67. Level of LPO in liver
Figure 6.8. Level of GSH in colon
Figure 69. Level of GSH in liver
Figure 70. Level of SGOT and SGPT in plasma
HISTOPATHOLOGICAL STUDIES OF COLON IN NORMAL & TREATED MICE

Figure 71. Normal colon

Reference control standard, 5-Flourouracil 20mg/kg

Figure 72. Colon treated with 5-Flourouracil
Figure 73. Positive control

Figure 74. Colon treated with ITU high dose 200mg/kg
Figure 75. Colon treated with ITU low dose 100mg/kg

Figure 76. Colon treated with VTU low dose 100mg/kg
HISTOPATHOLOGICAL STUDIES OF LIVER IN NORMAL & TREATED MICE

Figure 77. Colon treated with VTU high dose 200mg/kg

Figure 78. Normal liver
Figure 79. Treated liver

Table 23. Body weight of animal in initial & final days of treatment
<table>
<thead>
<tr>
<th>Groups</th>
<th>Name of the group</th>
<th>Body weight in grams [ Initial ]</th>
<th>Body weight in grams [ Final ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>28.3 ±5</td>
<td>31.3 ±0.6</td>
</tr>
<tr>
<td>II</td>
<td>Control</td>
<td>32 ±0.6</td>
<td>29.4±1.2</td>
</tr>
<tr>
<td>III</td>
<td>Standard [ fluorouracil] 20 mg / kg</td>
<td>30.5 ±0.7</td>
<td>33.2±2.4</td>
</tr>
<tr>
<td>IV</td>
<td>ITU high dose 200mg/kg</td>
<td>31.7±4.5</td>
<td>33.1±1.6</td>
</tr>
<tr>
<td>V</td>
<td>ITU low dose 100 mg/ Kg</td>
<td>29.1±1.6</td>
<td>27.4±1.7</td>
</tr>
<tr>
<td>VI</td>
<td>VTU high dose 200mg/kg</td>
<td>31.4±2.3</td>
<td>33.1±2.7</td>
</tr>
<tr>
<td>VII</td>
<td>VTU low dose 100 mg/ Kg</td>
<td>31.5±1.2</td>
<td>31.2±1.9</td>
</tr>
</tbody>
</table>

It is quite evident from the results that treatment with compounds ITU and VTU has lead to an elevation in the level of GST in both liver and colon. After 8 weeks of carcinogen exposure the mean value of GST activity was found to increase in the colon of the carcinogen control group when compared to that of the normal mice. The activity of this phase II xenobiotic metabolizing enzyme was found to increase further in the compounds -treated group. As a result of treatment with ITU 200 mg/kg the mean level of GST was increased. In liver an elevation of GST activity was noted in the carcinogen control group when compared to the corresponding value of the normal group. Treatment with ITU 200 mg/kg caused the mean GST activity to increase. Similarly treatment with VTU 200 mg/kg resulted in significant increase in the GST activity in comparison with the carcinogen control groups.

Results suggest that in both the experimental sets, levels of hepatic and colonic lipid peroxidation had increased quite significantly in the carcinogen control groups with respect to that of the normal mice. In other words, exposure to azoxymethane has lead to a significant
increase in the free radical mediated damage to membrane lipids when compared to that of the normal mice. However, after 8 weeks of treatment, the test compounds could counteract this oxidative damage to cellular membranes quite effectively as is evidenced by comparing the levels of Thio Barbituric Acid Reacting Substances (TBARS) in the treated groups and the carcinogen control group. Assay of lipid peroxidation in the liver reflected a similar observation as in the colon of both the experimental sets.

**STATISTICAL ANALYSIS**

The collected data were analysed with SPSS 16.0 version. To describe about the data descriptive statistics the mean and S.D were used. For the multivariate analysis the Kruskal Walli's test was used. To find the significant difference between the bivariate samples in Independent groups Mann-Whitney U test was used. In both the above statistical tools the probability value 0.05 is considered as the significant level.

**DISCUSSION**

Prevention of cancer remains a primary need and new chemopreventive agents must be developed for this purpose. Towards this goal a chemopreventive study was conducted to evaluate the activity of the test compound. It was assessed using inflammatory cells. Certain carcinogens are believed to be mediated by free radicals mechanism. Hydrazines and its derivatives, DMH and isoniazid which can produce oxygen species have shown to induce DNA damage process and carcinogeneis which can be abolished by free radical scavengers, also provide indirect evidence of the involvement of radicals in carcinogenesis. Human colon carcinogenesis is usually associated with a combined action of various kinds of xenobiotics and autobiotic carcinogenic factors. The use of antioxidants as inhibitors of tumour has received much attention. The precise mechanism of action of these compound is presently
unknown although proposals include, scavenging of ultimate carcinogenic metabolites and alteration of enzyme systems responsible for the activation of procarcinogens.

Liver antioxidant enzymes were also assessed to determine whether impaired activity of the antioxidants might have contributed to oxidative stress. Liver GSH levels were significantly lower in AOM-treated mice as compared with the other treated group and thus might have contributed to the increased liver lipid peroxidation. This antioxidant property of compounds may be responsible for protecting the cells against the oxidative stress, possibly by increasing the endogenous defensive capacity of the liver to combat oxidative stress induced by AOM.

Unlike liver lipid peroxidation, colonic TBARS were not increased on treatment with AOM. Previous studies proved that mice administered with AOM for a long period had decreased colonic lipid peroxidation when compared with the normal mice tissue. Our data also demonstrate reduced lipid peroxidation in AOM treated mice. It is generally believed that there is an inverse relationship between the concentration of lipid peroxides and the rate of cell proliferation and differentiation.

In the present study, test compounds supplementation to AOM-treated mice had higher levels of TBARS. This finding suggests test compounds can protect cells from loss of their oxidative capacity due to the administration of the procarcinogen AOM. The enzyme GSH play a key role in the cellular defense against free radical damage. GSH replenishment on test compound treatment could be important in inhibiting carcinogenesis induced by AOM.

The body weight gain was significantly improved as compared with mice treated with azoxymethane this may be due to the reduced carcinogenicity and consequent tumor burden.
It is colon specific carcinogen which is metabolically activated in the liver and then delivered to the colon via the blood stream or via bile as glucuronide conjugates. After further activation, it methylates DNA mainly at the N\textsuperscript{1} and O\textsuperscript{6} position of guanine. DNA adduct formation is considered to be the initiating step in the formation of tumorigenesis. It is believed that there is an inverse relationship between the concentration of lipid peroxides and the rate of cell proliferation.

The aminotransferases catalyze the formation of glutamic acid from 2-oxoglutarate by transfer of amino groups. AST is found in highest concentration in the liver and heart muscle but it is also abundant in skeletal muscle, kidney and pancreas. The serum concentration of AST is elevated in hepatitis and other forms of hepatic disease associated with necrosis: infectious mononucleosis, cholestasis, cirrhosis, metastatic carcinoma of the liver, delirium tremens.

The aminotransferases catalyze the formation of glutamic acid from 2-oxoglutarate by transfer of amino groups. ALT is normally present in various tissues but its higher concentrations are found in liver and kidney. The serum concentration of ALT is elevated in hepatitis and other forms of hepatic disease associated with necrosis: infectious mononucleosis, cholestasis, cirrhosis, metastatic carcinoma of the liver, delirium tremens.

From a practical point of view, those inhibitors, which act during promotion and progression stages, are more likely to be useful for the prevention of human cancer. Since initiation is rapid, especially in the colon, the duration of treatment with inhibitors is crucial. Promotion, on the other hand, is considered as reversible and generally a slow process influencing the proliferation of initiated cells. Therefore, the possibility of blocking this process is much greater. Thus, agents that inhibit the promotion stage of carcinogenesis, or
both initiation and promotion events could offer one of the most effective methods of cancer prevention.

CONCLUSION

Cumulatively, our results suggest that test compounds can protect colonic tissues against AOM-induced colon carcinogenesis in mice. This protective effect of test compounds against mice colon tumour was more striking when test compounds were supplemented in entire period of the study. This is evident by the reduced inflammatory cells, dilatation of glands and degeneration of mucosa. The chemopreventive effect was more significant when test compounds were administered during the promotion and progression stages of carcinogenesis. Thus test compounds might have practical applications as a chemopreventive agent, providing a scientific basis against human colon carcinogenesis.

Histopathology result of anticancer activity in Swiss albino mice

- Normal Control: The histological profile of the colon revealed normal structure of tunica layers.

- Positive control: The colon revealed moderate degree of dilation of glands with moderate degree of inflammatory cells of mononuclear cells.

- Reference control: The colon revealed moderate degree of inflammatory cells infiltration and degeneration of mucosa and absence of dilation of glands.

- Test drug (ITU – low dose): The mucosa layers remained apparent normal with moderate degree of inflammatory cells infiltration of mononuclear cells.
• Test Drug (ITU-high dose): The mucosa layer remained apparently healthy and mild degree of inflammatory cells infiltration in the lamina propria and muscularis was seen.

• Test drug (VTU-low dose): Erosions and denudation of the mucosa were noticed with moderate degree of infiltration of mononuclear cells.

• Test drug (VTU – high dose): Mild degree of mucosal degeneration with moderate degree of inflammatory mononuclear cells infiltration in the lamina propria and around the tubular glands.

• The liver excised from the control group samples revealed normal histology of central vein, portal triads and hepatocytes.

**SUMMARY**

• The test drug ITU at the high dose level has reduced the inflammatory cells infiltration and the dilatation of the glands in colon tissues when compared to the positive control.

• Livers tissues from positive control, reference control and test drugs (VTU and ITU) at low dose and high dose groups revealed no abnormalities and histological profile remained similar to the control group.
REFERENCES


