PHARMACOLOGICAL AND TOXICOLOGICAL EVALUATION OF LOPERAMIDE & NIACIN COMBINATION EITHER CANCER PROMOTER OR INHIBITOR IN DENA INDUCED LIVER CANCER USING WISTAR RATS

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ABSTRACT
Combination effect of Loperamide & Niacin in chemically induced hepatocarcinogenesis was assessed in Wistar rats. Intraperitoneal administration of chemical carcinogen diethyl nitrosamine (DENA) (200 mg/kg) in single dose elevated the levels of Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), Serum Bilirubin (BIL), Total cholesterol (TC), Triglycerides (TG), Total leucocytes count (TLC), Alfa feto protein (AFP) and reduced Haemoglobin (Hb) and High density lipoproteins (HDL) level in tested animals. Histopathological examinations of the liver tissue showed marked carcinogenicity of the chemical carcinogen. Food and water intake, animal weights were also assessed. The animals exposed to DENA showed a significant decrease in the body weights and, there were no significant alterations found in the total leucocytes count (TLC). The elevated levels of serum SGOT, SGPT, BIL, TC, TG and AFP significantly decreased and increased HDL and Haemoglobin levels as compared to disease control group by administration of Loperamide (5 mg/kg) and Niacin ad libitum combination daily for 12 weeks P.O. Physiological and biochemical analysis showed the beneficial and synergic effects of Loperamide and Niacin combination in the animals exposed to DENA.

KEYWORDS: Pharmacological evaluation, toxicological evaluation, cancer promoter, cancer inhibitor, wistar rats, DENA- N-diethyl nitrosamine, Loperamide, Niacin, SGOT- Serum glutamate oxaloacetate transaminase, SGPT - Serum glutamate pyruvate transaminase.
1. INTRODUCTION
Cancer is one among the most serious health problems worldwide, affecting individuals of all sexes, ages, and races. Cancer is the groups of diseases, characterized by uncontrolled cellular proliferation, and invasion from one part to another part that process referred to as Metastasis. The uncontrolled growth and spreading of cancer cells make them dangerous.\(^1\) Liver cancer is among the most lethal cancers (five-year survival rates under 11%), which makes it the third most frequent cause of cancer related deaths in men and the sixth in women.\(^2\) Liver cancer consists of several different primary hepatic malignancies, such as Angiosarcoma, Cholangiocarcinoma, hepatoblastoma and haemangiosarcoma, but hepatocellular carcinoma (HCC) is by far the most common type, accounting for 70\%-85\% of cases.\(^3\)

Hepatocellular carcinoma (HCC) represents a major health problem worldwide\(^4\) and is the fifth most common cause of cancer and the third leading cause of cancer-related deaths worldwide.\(^5\)

Major risk factors for HCC include chronic infection with human hepatitis B virus (HBV) and hepatitis C virus (HCV) and other conditions associated with chronic inflammatory liver disease and cirrhosis, such as alcohol consumption or hepatic metabolic disorders. In some geographic areas, aflatoxin B1 (AFB1) and microcysti are also significant etiologic factors.\(^6\) In almost all populations, males have a higher HCC rate than females.\(^7\) HCC develops as a result of slow and progressive genetic alteration and dedifferentiation of liver cells.\(^8\) The only curative treatment is surgical resection or liver transplantation, but only 10–20\% of patients are eligible for these procedures. The majority of hepatocellular carcinomas that present at an advanced stage are cancer not be cured. Systemic chemotherapy for advanced hepatocellular carcinomas either as single-agent therapy or in combination, radiofrequency ablation or recently introduced tyrosine kinase inhibitors, e.g. sorafenib.\(^9\)

The toxic side effects often limit dose escalation of anticancer drugs, leading to incomplete tumor response, early disease relapse, and ultimately, the development of drug resistance. Several approaches were developed to improve the selective toxicity of anticancer drugs such as encapsulating anticancer drugs in delivery systems and targeting anticancer drugs via monoclonal antibodies\(^10\) or peptide ligands\(^11\), that bind to antigens or receptors that are over expressed or uniquely expressed on the cancer cells.
Given the time course of the disease and the burden of treatment, there is an increase in concerns about the liver diseases and HCC. To consider all above about hepatic cancer therapy and many more literature here the present study is attempt to explore and establish “Loperamide in combination with niacin for the treatment of liver cancer”.

Loperamide is an opioid agent widely used in the clinic to control diarrhea induced by digestive disorders, chemotherapy, and radiotherapy.\(^{[12]}\) It works by inhibiting gut motility and slow down all body secretory chemicals.\(^{[13]}\) Niacin is a hydrophilic vitamin used in combination with loperamide for giving synergistic effects and reduced side effects of one drugs by the other one. Loperamide and niacin both drugs had been prove by numerous researcher as an anti cancer agent.\(^{[14, 15, 16]}\) Several potential mechanisms have been suggested and established for the ability of loperamide and niacin to suppress cancer growth in vitro and in vivo: (1) Induction of cell cycle arrest and/or apoptosis, (2) inhibit angiogenesis process (3) free radicals scavenger activity (4) Anti-inflammation activity (4) regulate lipoprotein level (5) anti-diabetic activity. Evaluation of Loperamide with niacin for the treatment of hepatocarcinogenesis, is a novel approach in the field of chemotherapy.

2. MATERIALS AND METHODS

2.1. Drugs and chemicals

Loperamide and Niacin was provided as a gift sample from Dr. Firoz Anwer Principal & Dean Siddhartha Institute of Pharmacy; DENA was procured from Sigma–Aldrich Chemicals Co., St. Louis, USA and Chloroform and Diethyl ether from S.D. Fine Chem. Ltd., Mumbai. All the chemicals were of analytical grade.

2.2. Animals

Adult, healthy, male Wistar rats weighing 100–125 g were procured from the animal house facility of Siddhartha Institute of Pharmacy for the present protocol. The rats were housed in groups in polypropylene cages under controlled conditions of temperature (22+ 3°C) and light (14:10 h light and dark cycle) and provided with balanced pallet diet and water ad libitum. The protocol was approved by the Institutional Animal Ethics Committee (IAEC) as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA); Ministry of Social Justice and Empowerment, Government of India.

2.3. Induction of hepatocarcinoma:

Liver cancer was induced by a Sub-carcinogenic dose of 200 mg/kg body weight, I.P. DENA when associated with fasting/refeeding.\(^{[17, 18, 19]}\)
2.4. Experimental design
The rats were acclimatized and randomly divided into three groups each having 6 rats for a 12 week study.
Group-I rats served as vehicle control and were treated with saline orally.
Group-II rats were administered a single dose of DENA (200 mg/kg).
Group-III rats were treated with DENA (200 mg/kg) and after 7th day therapeutic treatment (Loperamide (5 mg/kg) and Niacin (ad libitum). The dose of Loperamide and Niacin was selected by performing an effective dose fixation study.

2.5. Estimation of biochemical parameters:
Blood samples were collected on the termination day of the experiment from the retro-orbital plexus under light ether anesthesia without any anticoagulant and were allowed to stand for 30 min at room temperature, centrifuged at 2500 rpm for 10 min to separate the serum. The serum obtained was kept at 2–4 °C for further use. Estimation of serum SGOT, SGPT, BIL, TC, TG, HDL, Hb, TLC, and alpha feto-Protein was performed using standard kits (Nicholas India Pvt. Ltd.) with semi-auto analyzer (photometer 5010, Nicholas India Pvt. Ltd.). Serum a-feto protein (AFP) was estimated by the method described by Premalatha and Sachdanandam (1999).[20]

2.6. Histopathological examination
The liver samples were preserved in phosphate-buffered 10% formalin, embedded in paraffin and used for histopathological examination. Five-lm-thick sections were cut, deparaffinized, hydrated and stained with hematoxylin and eosin. The sections were examined blindly for tubular cell swelling, interstitial edema, tubular dilatation, and moderate to severe necrosis in all treatments.

2.7. Statistical analysis
Statistical analysis was carried out using Graph pad prism 5.0 (Graph pad software, San Diego, CA, USA). The results were expressed as Mean ± S.E.M. Statistical significance between more than two groups was tested using one-way ANOVA followed by Tukey’s multiple comparison tests. Values of p < 0.05 were regarded as significant.
3. RESULTS

3.1. Animal weight
There were no significant alterations observed in the daily food and water intake in experimental animals. DENA control group showed significant reduction in body weight as compared to a normal control group (Table 3).

3.2 Mortality
There was no mortality reported during the study.

3.3. Liver profile study

3.3.1. Serum glutamate pyruvate transaminase (SGPT/ALT)
In DENA control group the SGPT level were significantly increased (P<0.001) as compared to Normal control group, while in therapeutic group (Loperamide + niacin) SGPT level were significantly decreased (P<0.001) as compared to DENA controls (table 1).

3.3.2. Serum glutamate oxaloacetate transaminase (SGOT/AST)
DENA control group the SGOT level were significantly increased (P<0.001) as compared to Normal control group, the elevated SGOT level were significantly decreased (P<0.001) as compared to DENA control group (table 1).

3.3.3. Bilirubin
In DENA control group the Serum Bilirubin level was significantly increased (P<0.001) as compared to Normal control group, while in treated group the serum bilirubin level were slightly decreased (P<0.01) as compared to DENA control group (table 1).

3.4. LIPID PROFILE

3.4.1. Triglycerides (TGs)
Animal exposed to DENA showed an elevation in the levels of TGs Administration of (Loperamide+Niacin) significantly lowered (P<0.001) the elevated levels of serum TGs in all the groups under study (table 2).

3.4.2. Total Cholesterol (TC)
Total cholesterol level was significantly increased (P<0.001) in DENA treated rats when compared to Normal group while in therapeutic group showed significantly decreased (P<0.001) TC level when compared to DC group (table 2).
3.4.3. High Density Lipoproteins (HDLs)
DENA administration decrease the level of HDLs in disease control group while therapeutic group increases the HDLs level significantly (P<0.001) as compared to Disease control group (table 2).

3.5. BLOOD PROFILE
3.5.1. Haemoglobin (Hb)
Administration of DENA in disease control rats decline the level of Hb significantly (P<0.001), whereas therapeutic group significantly increases (P<0.01) the level of Hb as compared to DENA control group (Table 3).

3.5.2. T-Lymphocytes (TLC)
A significant increase (p<0.001) in the TLC level observed in Disease control groups. Animals treated with Loperamide and Niacin combination showed no such alteration was reported in the level of TLC as compared to DENA control (Table 3).

3.6. Alfa feto protein (AFP)
Disease control group exhibited a significant elevation (P<0.001) in serum AFP level as compared to Normal control. Treatment with Loperamide with Niacin decreased the elevated level of AFP significantly (P<0.001) in therapeutic control group as compared to DENA control (table 3).

3.7. Histopathological study
Liver sections of the normal control group showed normal liver histology with unremarkable central veins, no evidence of hepatocyte injury or fibrosis or dysplasia or malignancy noticed. Disease control animals showed central veins surrounded by extensive necrosis and inflammatory infiltrate, clusters of hepatocyte necrosis and the portal tract with bile duct proliferation and marked atypia. The tumor cells resembling hepatocytes show pleomorphism and were seen 2-8 cell, wide trabeculae which are separated by endothelium lined sinusoidal spaces. The prophylactic group showed periportal inflammation with conspicuously dilated blood vessels and ballooning degeneration mononuclear infiltrates associated with regenerative cellular changes of the adjacent hepatocytes, mild bile duct proliferation and intra-acinar inflammatory cell infiltrates was observed. Liver section from loperamide with niacin control group shows the normal architecture of the liver, no necrosis was observed (Figure. 1).
Table 1: Data showing the levels of Serum SGPT, SGOT and Serum Bilirubin in Normal control, DENA control, therapeutic group of animals.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Groups</th>
<th>SGPT (mg/dl)</th>
<th>SGOT (mg/dl)</th>
<th>BIL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal control</td>
<td>46.33±1.838</td>
<td>50±2.221</td>
<td>0.3833±0.0362</td>
</tr>
<tr>
<td>2.</td>
<td>NA control</td>
<td>85.5±2.997###</td>
<td>87.83±3.081###</td>
<td>0.78±0.0568###</td>
</tr>
<tr>
<td>3.</td>
<td>Therapeutic group</td>
<td>65.17±1.973***</td>
<td>62.83±2.197***</td>
<td>0.5667±0.0348**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (N=6). Where NS - not significant, ### P<0.001 Normal Vs Disease control group, * P<0.05 Disease Vs Treated group, ** P<0.01 Disease Vs Treated group, *** P<0.001 Disease Vs Treated group

Table 2: Data showing the levels of Serum lipid profile in Normal control, DENA control and therapeutic group of animals.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Groups</th>
<th>TG (mg/dl)</th>
<th>TC (mg/dl)</th>
<th>HDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal control</td>
<td>55.33±3.28</td>
<td>96.17±1.905</td>
<td>45.67±2.011</td>
</tr>
<tr>
<td>2.</td>
<td>DENA control</td>
<td>141.2±2.71###</td>
<td>141.3±3.062###</td>
<td>18.00±1.065###</td>
</tr>
<tr>
<td>3.</td>
<td>Therapeutic group</td>
<td>107.3±3.293***</td>
<td>115.3±2.616***</td>
<td>33.33±1.706***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (N=6). Where NS - not significant, ### P<0.001 Normal Vs Disease control group, * P<0.05 Disease Vs Treated group, ** P<0.01 Disease Vs Treated group, *** P<0.001 Disease Vs Treated group
Table 3: Data showing the Body weight, Blood level of Haemoglobin (Hb), Total Leucocyte count (TLC) and alpha-feto protein (AFP) in Normal control, DENA control, Therapeutic group of animals.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Hb (g/dl)</th>
<th>TLC (/cumm)</th>
<th>AFP (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal control</td>
<td>103.44±5.562</td>
<td>14.18±0.416</td>
<td>11458±447.4</td>
<td>0.1005±0.0254</td>
</tr>
<tr>
<td>2.</td>
<td>DENA control</td>
<td>75.01±1.55###</td>
<td>10.25±0.425###</td>
<td>16343±599.1###</td>
<td>0.5198±0.0525###</td>
</tr>
<tr>
<td>3.</td>
<td>Therapeutic group</td>
<td>108.29±1.874***</td>
<td>12.17±0.295**</td>
<td>5010±434.9ns</td>
<td>0.2835±0.02264***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (N=6). Where NS - not significant,

### P<0.001 Normal Vs Disease control group,

* P<0.05 Disease Vs Treated group

** P<0.01 Disease Vs Treated group

*** P<0.001 Disease Vs Treated group

HISTOPATHOLOGY OF LIVER

Figure (1a), Normal Control

Figure (1b) DENA control

Figure 1(c) DENA+Loperamide+Niacin

Figure 1: Photomicrographs (original magnification 45x) of histological studies of liver of various groups.
Normal architecture of rat liver with unremarkable central vein in normal control group, while in disease control group hepatocytes necrosis and inflammatory infiltrate, and hepatocellular degeneration of DENA. Liver from rat treated with (DENA+Loperamide+Niacin) had shown lesser damage of hepatocytes and low index of necrosis in therapeutic group.

4. DISCUSSION

Hepatocellular carcinoma (HCC) accounting for 70%-85% of cases of liver cancer\textsuperscript{[21]}, and is the fifth most common cause of cancer and the third leading cause of cancer-related deaths worldwide\textsuperscript{[22]}, Although there are many strategies for the treatment of liver cancer, chemoprevention seems to be the best strategy for lowering the incidence of this disease. Therefore, this study has been initiated to investigate loperamide and Niacin in combination play an important role as a potent anti-cancer activity in hepatocellular carcinomas induced by diethylnitrosamine (DENA), a potent initiator and hepatocarcinogen, in rats.

In the present study, DENA induced Hepatocellular damage clearly demonstrated that DENA significantly ($p<0.001$) elevated the levels of liver enzymes i.e. SGPT, SGOT, and bilirubin and caused severe histopathological lesions in liver tissues. It has also been observed and established by the researchers that SGPT, SGOT, serum bilirubin level elevates significantly after DENA exposure in the experimental animals.\textsuperscript{[23]} In present study serum SGPT and SGOT levels elevated significantly ($p<0.001$) in all groups exposed to DENA as compared to the NC group. While the Therapeutic group loperamide with niacin SGOT SGPT and Serum bilirubin levels brought towards the normal levels. These results firmly established the role of loperamide with niacin as a chemopreventive agent in DENA induced Hepatocellular carcinoma.

The total cholesterol and triglyceride levels elevated significantly ($p<0.001$) in both groups (II and III) exposed to DENA as compared to the NC group. Interestingly, treatment with Loperamide (5mg/kg) and Niacin (ad libitum) combination significantly reduced ($p<0.001$), ($p<.001$) respectively lipid profile. These results are firm indications that Loperamide and niacin combination maintains the lipid profile in DENA induced liver cancer and inhibit the cell proliferation.

Previously the researchers has established that Plasma lipid metabolism are associated with hepatocellular carcinomas\textsuperscript{[24]} alterations in lipid metabolism, affects cellular function and
growth, further development of hepatocyte nodules in rat liver has been found with changes in lipid parameters and oxidative status. Alteration in plasma lipid profile in malignant tissue are of important due to the effect on membrane integrity, fluidity and regulation of cellular process related to growth and cell survival.

The present research concluded that therapeutic group (Loperamide+Niacin) maintained the lipid profile, hence it can be suggested that they may play the role in inhibition of carcinoma progression.

Further to establish the above claim Haematological studies of animals exposed to DENA done. Scientist has stated previously that Haematological changes are associated with Hepatic carcinoma. Present experimental observations concluded, Hb (Haemoglobin) level was decreased in DENA control group; while level of Hb markedly improved by combination therapy. Disease control animals, showed significant increase (P<0.001) in the TLC level when compared with Normal controls. Treatment with loperamide 5 mg/kg + niacin significant decreased (P<0.05) the TLC level as compared to Disease control.

Moreover, AFP is a serum protein, shows higher specificity for HCC. AFP has to be considered as ‘the gold standard’ for HCC serum markers. In present experimental protocol it has been observed that serum AFP level were increased in the disease control group as compared to normal controls, treatment with loperamide 5mg/kg + niacin significantly reduced (p<0.001) the serum AFP levels as compared to disease controls. Moreover Loperamide works as a anti-cancer agents due to their anti-angiogenic and apoptotic properties. And niacin work as anti-cancer agents due to their anti-angiogenic, anti-oxidant, anti-inflammatory, anti diabetic and calcium releasing properties.

From the outcomes of the present research done on the experimental animals it is concluded that the combination may be proved a boon for the treatment of hepatocellular carcinoma. But further exploration of the combination needs to be done i.e the clinical studies.

CONCLUSION
Data from the study suggests that Loperamide and niacin together can posses synergestic chemopreventive action. Combination of Loperamide (5 mg/kg) and niacin suppress the tumour lesions and decrease the biochemical markers which were elevated in HCCs. The clinical application of loperamide and niacin combination would benefit the cancer patients.
due to decrease their therapeutic cost significantly. In conclusion loperamide and niacin combination were found to be a potential anti-tumor agents with apoptosis inducing activity, anti-angiogenesis, anti-proliferation activity and free radicals scavenger. This finding provides new insight into the existing drugs and may help to facilitate the development of anti-tumor agents.

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