Aromatase Gene Expression in N-Ethyl-Nitrosamine-Induced Hepatocellular Carcinoma: Hepatoprotective Effects of Grape Seed Extract and Letrozole

Sadia Kazi[1], Sadia Tabassum[2], Bushra Sajid[3], Kishwar Lakhair[4], Shahnaz Bano[5], Mozna Talpur[6]

Abstract

Objective: To determine the hepatoprotective properties of Letrozole alone and in combination with grape seed extract on hepatocellular carcinoma induced by N-Ethylnitrosamine in male albino Wistar rats.

Methods: A quasi-experimental study was conducted at Sindh Agriculture University and Diagnostic Laboratory of Isra Hospital. Fifty albino Wistar rats, 200-250 g in weight, were allocated into five equal groups. Group A control group, Group B were induced with N- Ethylnitrosamine of 30mg/kg for 11 weeks, Group C was given N- Ethylnitrosamine of 30mg/kg and Grape Seed Extract of 100mg/kg for 11 weeks, Group D was given N- Ethylnitrosamine of 30mg/kg and Letrozole of 2mg/kg for 11 weeks and E were given N- Ethylnitrosamine of 30mg/kg, Grape Seed Extract 100mg/kg and Letrozole of 2mg/kg for 11 weeks. Blood samples for liver enzymes, albumin, and hepatic tissue samples for histopathology were collected at the end of the study. Data regarding liver enzymes and aromatase gene expression was analyzed using SPSS (version 22, pd"0.05, 95% confidence).

Results: There was a statistically significant ($p < 0.05$) difference in the mean levels of albumin and liver enzymes i.e. AST, ALT, and GGT across experimental groups. A relative aromatase gene expression of 21.13 ± 3.59, 36.22 ± 3.15, 28.61 ± 2.71, 46.66 ± 2.61, and 35.24 ± 3.48 was observed in Groups A, B, C, D, and E, respectively. The difference was statistically significant ($p<0.05$). There was a marked deterioration of the hepatic architecture in Group B. Similar changes with varying degrees were observed in experimental groups and group C showed the best results.

Conclusion: Grape Seed Extract and Letrozole significantly affected liver enzyme levels, hepatic architecture, and relative gene expression of the aromatase gene. Additionally, individual therapy with grape seed extract exerts better effects against hepatocellular carcinoma induced by N-Ethylnitrosamine.

Keywords: Aromatase, Hepatocellular carcinoma, Grape seed extract.

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Introduction

In the world, cancer is the second greatest cause of death, accounting for close to 10 million deaths and more than 18 million cases[1]. Globally, hepatocellular carcinoma (HCC) is among the top five most prevalent primary liver cancers. The incidence rates of HCC in Pakistan are 2.8 per 100,000 women and 7.6 per 100,000 men. In contrast to other Asian nations where infection of chronic hepatitis B virus (HBV) is more common, 60–70% of HCC cases in Pakistan are associated with chronic hepatitis C virus (HCV) infection[2]. This different prevalence trend emphasizes the critical significance of chronic HCV infection in generating HCC occurrences in Pakistan. This surge in HCC incidence underlines the critical need for focused measures to reduce the disease burden and to determine effective intervention strategies. The high

[1,5,6]Department of Pharmacology, Isra University
[2]Department of Biochemistry, SRMC, Tando Adam
[3]Department of Histopathology, Isra University
[4]Department of Physiology, Bilawal Medical College, Jamshoro

Correspondence: Dr. Sadia Kazi
Department of Pharmacology, Isra University
Email: sadia_kazi@hotmail.com
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death rate linked to hepatocellular carcinoma (HCC) is mainly caused by a dismal prognosis and insufficient availability of effective treatment options. Treatment options for HCC that are often used include radiation therapy, chemotherapy, and surgical excision[3].

Chemotherapy appears to be a very promising treatment option for patients with HCC, either as a stand-alone intervention or in combination with other therapeutic modalities, even though the efficacy of the currently available choices is still relatively restricted[4]. Furthermore, radiation therapy is also very important since it tries to reduce the size of the tumor before surgical resection or eliminate any cancer cells that remain after surgery, which improves the efficacy of treatment plans as a whole. Conventional cancer treatments are widely used around the world and have a substantial positive impact on many patients' survival rates. However, some people have partial remission in addition to a range of negative symptoms. In addition, many treatment plans come with hefty costs, which makes them unaffordable for some people, especially those in underdeveloped countries. Furthermore, as several studies have shown, the treatment effectiveness of commonly used medications for hepatocellular carcinoma (HCC) falls short of the intended results. For the majority of HCC patients, the reported 5-year overall survival rate falls between 33% and 44%[5].

The deleterious effects of various nitrosamines on animals and humans are firmly established. Diethylnitrosamine (DEN) or dimethylnitrosamine (DMN) administered orally or parenterally leads to severe liver damage[7]. Both male and female reproductive and non-reproductive roles are modulated by estrogen[6].

Additionally, in the liver estrogen receptors' decreased expression and function are linked to liver dysfunction and obesity. The CYP19A1 gene encodes aromatase, an enzyme that promotes the conversion of androgens into estrogens. A number of biological functions, such as metabolism, hormone signaling, and proliferation, are impacted by aromatase. Furthermore, it induces hormone-dependent tumor expansion and is overexpressed in a variety of malignancies[7]. Letrozole (Femara), an aromatase inhibitor licensed by the Food and Drug Administration (FDA), has been used as a first-line medication to treat malignancies that express aromatase. Aromatase inhibitors block cytochrome P450, hence exhibiting anti-estrogenic action[12]. This process is achieved through immuno- histochemical techniques and reverse transcription polymerase chain reaction (RT-PCR)[8]. Grape Seed Proanthocyanidins (GSPs) have been shown to have significant anticancer effects in HCC. However, the exact mechanisms underlying GSPs' inhibitory actions in HCC are yet unclear[9]. Additionally, research has demonstrated that Grape Seed Proanthocyanidins (GSPs) block tumor angiogenesis, thereby effectively suppressing the growth of HCC[10]. Furthermore, Grape Seed Proanthocyan- idins have hepatoprotective demonstrated by inhibiting effects on phosphatase for liver regeneration. Previous research has demonstrated a strong association between the antiproliferative properties of GSPs on HepG2 cells and mechanisms like autophagy[11]. Likewise, it has been noted that there is an upregulation of the protein's expression that is linked to the pathway mitogen-activated protein kinase (MAPK)[12]. Hence based on previous evidence the present study is aimed to examine the effects of grape seed extract alone and in combination along with Letrozole on hepatocellular carcinoma-induced albino Wistar rats.

Methodology

A non-randomized quasi-experimental study was carried out at the Animal Husbandry and Veterinary Sciences Department of Sindh Agriculture University and Diagnostic Laboratory. The study utilized non-probability purposive sampling to select a sample of 50 mature male albino Wistar rats. The inclusion criteria specified rats weighing between 200-250 grams and in good health. Rats that did not meet the weight criteria, female rats, those not eating properly, or those that were ill or moribund were excluded. Male Albino Wistar rats (N=50) were obtained from the animal house of the Faculty of

Animal Husbandry and Veterinary Sciences, Sindh Agriculture University. The animals were housed and handled according to the provided guidelines by NIH for the Care and Usages of Laboratory Animals. Male Albino Wistar rats (N=50) were accommodated in labeled stainless steel cages into five equal groups each group containing 10 animals. The environment for the adult albino rats was hygienic and well-ventilated. Rats were provided food (lab chow) in feed bottles and tap water in plastic drinkers *ad libitum*. Prior to the experimental protocol the male rats were acclimatized for 1 week by maintaining a light/dark cycle at the 12 hr interval. Group A, served as a control group and received distilled water ad libitum. Group B, was the experimental control group and was injected with N-Ethylnitrosamine diluted in 0.9% normal saline intraperitoneally at the dosage of 30mg/kg body weight two times a week for the duration of 11 weeks[13]. Group C was the experimental group injected with N-Ethylnitrosamine diluted in 0.9% normal saline intraperitoneally at the dosage of 30mg/kg body weight two times a week and grape seed extract orally at the dosage of 100mg/kg every day for the duration of 11 weeks[14]. Group D was also an experimental group injected with N-Ethylnitrosamine diluted in 0.9% normal saline intraperitoneally at the dosage of 30mg/kg body weight two times a week and Letrozole orally at the dosage of 2mg/kg body weight every day for the duration of 11 weeks[15]. Group E was an experimental group injected with N-ethyl nitrosamine diluted in 0.9% normal saline intraperitoneally at the dosage of 30mg/kg body weight two times a week, oral grape seed extract at the dose of 100mg/kg daily and oral Letrozole at the dose of 2mg/kg body weight every day for the duration of 11 weeks.

At week 12, one week after the experiment, all the rats were sedated after one more week of observation. Rats were anesthetized subcutaneously with Xylazine (2 mg/kg) and Ketamine (20 mg/kg) to reduce discomfort and any side effects. After that, blood samples were drawn by heart puncture using plain tubes and EDTA to analyze the results of the liver function tests such as albumin, ALT, AST, and GGT. Transfer the stored samples of liver function test parameters i-e AST, ALT, GT, Albumin into the sampling plate of an automatic analyzer Hitachi Rosche, cobas 311. Start the automatic biochemical analyzer, then confirm the calibration of results. After the measurement, the results were printed on paper. The animals were then all put to sleep via cervical dislocation and laparotomy was executed. Liver tissue fixing was done in formalin. Later, dehydration, clearing, and embedding into the paraffin wax, thin slices were cut and stained with hematoxylin and eosin stains. Microscopic examination of these slides was done for a detailed assessment of liver morphology, cellular changes, inflammation, and pathology. Findings are then documented in a report, providing crucial insights into the liver's health and any observed abnormalities. SPSS version 22 was used for the analysis of data and the level of significance was measured at a p-value of ≤ 0.05

Results

The findings indicated significant differences ($p < 0.001$) in mean levels of albumin and liver enzymes, including serum gamma-glutamyl transferase (GT), serum aspartate aminotransferase (AST), and serum alanine aminotransferase (ALT), among the five experimental groups. The subsequent ANOVA results, as shown in Table 1, confirmed these significant differences. Detailed examinations revealed notable variations in levels of albumin and liver enzyme concentrations among the experimental groups. Specifically, at week 11, albumin levels in Group B were significantly lower at 1.58 ± 0.28g/dl when compared with the control group, which had levels of 3.78 ± 0.28 g/dl. Similar patterns were observed in experimental Groups C, D, and E, which demonstrated that the treatments with Letrozole and grape seed extract alone and in combinations highly influenced the levels of albumin. Furthermore, variations were also observed in the levels of liver enzymes such as ALT, AST, and GT, as detailed in Table 1

Table 1. Differences in liver function tests between experimental groups

Variable	Groups	Mean±SD	Level of Significance
Albumin g/dl	Group A	3.78 ± 0.28	
	Group B	1.58 ± 0.28	
	Group C	2.08 ± 0.48	<0.001
	Group D	1.78 ± 0.29	
	Group E	1.92 ± 0.30	
Serum Aspartate Aminotransferase (AST) IU/L	Group A	88.9 ± 10.54	
	Group B	231.5 ± 14.32	
	Group C	158.9 ± 5.02	<0.001
	Group D	170.4 ± 7.45	
	Group E	162.4 ± 14.19	
Alanine Aminotransferase (ALT) IU/L	Group A	27.8 ± 10.22	
	Group B	75.79 ± 15.69	
	Group C	51.24 ± 5.46	<0.001
	Group D	58.5 ± 3.68	
	Group E	54.3 ± 7.49	
Gamma Glutamyl Transferase (GT) IU/L	Group A	5.16 ± 1.56	
	Group B	71.4 ± 5.73	
	Group C	52.1 ± 8.06	<0.001
	Group D	59.45 ± 5.57	
	Group E	54.8 ± 4.02	

The groups had a significant difference ($p < 0.05$) in the mean gene expression. In particular, Group A had relative aromatase gene expression from liver tissue of 21.13 ± 3.59, which rose to 36.22 ± 3.15 in Group B, 28.61 ± 2.71 in Group C, 46.66 ± 2.61 in Group D, and 35.24 ± 3.48 in Group E. (Table 2)

Table 2. Relative aromatase gene expression between experimental groups

Groups	Relative gene expression	Level of Significance
Group A	21.13 ± 3.59	<0.001
Group B	36.22 ± 3.15	
Group C	28.61 ± 2.71	
Group D	43.66 ± 2.61	
Group E	35.24 ± 3.48	

On histological examination, there was marked deterioration of the hepatic architecture in Group B. This included changes such as necrosis and permeation of immune cells. Groups D and E also showed similar changes but not as severe as observed in group B. However, Group C displayed hepatic cytoarchitecture which resembled the control group the most, as shown in Figure 1.

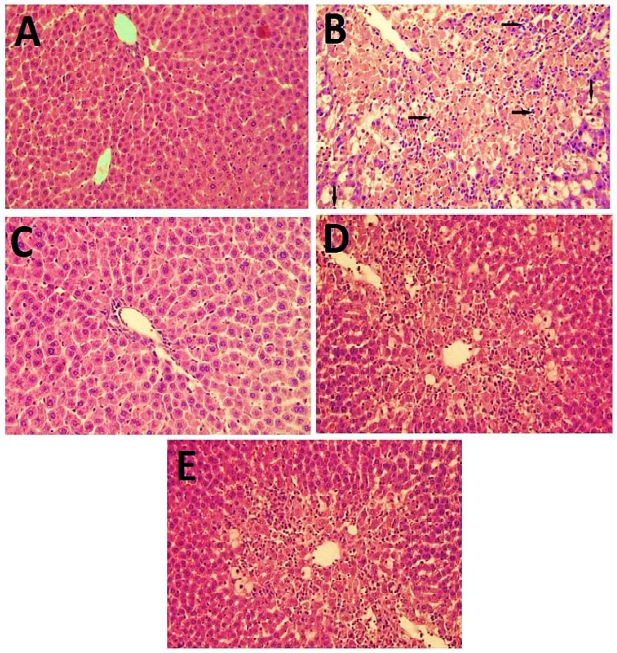

Fig 1. Histological examination of hepatic architecture in experimental groups.

Discussion

The study included five groups of male albino Wistar rats that were administered to various treatments such as control, N-Ethylnitrosamine injection, grape seed extract, Letrozole, and a combination of Letrozole and grape seed extract. The major findings demonstrated substantial differences between groups in blood albumin levels, liver enzyme activity (AST, ALT, GT), histopathology, and aromatase gene expression. Notably, the experimental groups, particularly those given grape seed extract and Letrozole, demonstrated unique patterns in these parameters, indicating possible effects on liver function and gene expression in the setting of N-Ethylnitrosamine exposure. Furthermore, as revealed by ANOVA findings, the baseline weights among the groups were homogeneous, providing a meaningful comparison of treatment effects. Ghufran H, et al., 2021 also explored time-dependent regulatory changes during disease advancement and HCC development by employing a rat model that was injected with N-Ethylnitrosamine at a dose of 30 mg/kg twice a week for 10 weeks, then once a week from the 12th to the 16th week. Their findings corresponded with the current study[13]

The co- or post-treatment of EST-bearing mice with GSE reduced the activities of ALT, AST, and ALP; the level of AFP in serum; and the expressions of PCNA and P53 in the liver, in accordance with the Abd Eldaim MA study. In addition, it lowered the pathogenic modifications and hepatic DNA damage brought on by EST while raising serum albumin and total protein levels. The results of the comparative analysis with our results lined up with the conclusions[16]. According to Yousef MI, et al. (2019), grape seed proanthocyanidin extract has strong antioxidant properties that aid in minimizing liver and heart damage caused by thalidomide and carboplatin. These characteristics include decreased levels of liver enzymes, tumor necrosis factor-á, interleukin-6, tumor suppressor gene P53, and histological alterations of the liver and heart. The results reported are consistent with the results from the current study[17]. In line with Aldubayan MA et al.'s 2019 outcomes, hyperthyroid mice had significant spikes in serum albumin, liver catalase, GSH, SOD, and Bcl2, but significant reductions in serum T3, T4, ALT, AST, ALP, liver MDA, and P53 levels. Using GSPE to treat hyperthyroid animals has the benefit of lessening the adverse outcomes of hyperthyroidism, including its biochemical, histological, and P53 expression, and declared results were in promising to results of the our study[18]. In a similar vein, additional investigations examined the way GSE affected the oxidative stress and growth restriction caused by diquat induction in chicks and found that it improved liver health and antioxidant capacity[19]. Atasever A. et al, 2019 performed a study to determine the hepatoprotective effects of Grape seed oil against liver grazes affected by carbon tetrachloride in rat models. The researchers measured blood levels of alanine aminotransferase (ALT), triglycerides, protein, cholesterol, and malondialdehyde in the liver to see how GSO affected hepatic damage. Caspase-3, caspase-8, and caspase-9 activities in cellular apoptosis were also examined. Later, Histopathological examination revealed that the CCl4 group had significant necrosis, lymphocyte-rich mononuclear cell infiltration in the portal region, mildly portal fibrosis in the parenchyma, and macro- and microvesicular steatosis in hepatocytes. However, grape seed oil treatment largely normalized the abnormal histological alterations as well as the activities of caspase-3, caspase-8, and caspase-9. The effects of Letrozole, a recognized aromatase inhibitor, on breast cancer cells and cisplatin resistance were investigated in relation to the genetic expression of the aromatase gene. Letrozole's success in treating hormone receptor-positive breast cancer is attributed to its capacity to downregulate the expression of the aromatase gene[20]. In 2003, Chen J. et al. researched non-small cell lung cancer (NSCLC) and discovered stearoyl-CoA desaturase 1 (SCD1), a vital enzyme involved in lipid metabolism. Elevation of SCD1 has been proven to affect cell invasion and migration by targeting the CYP19A1 enzyme, which is responsible for producing estrogen. Lower concentrations of CYP19A1, catenin protein, and estrogen have been observed in SCD1 knockdown cells. The results of this work are consistent with Chen J's observations of the impacts of SCD1 inhibitors and grape seed extract on NSCLC cells, which limit cell migration and invasion thereby preventing tumor formation and metastasis[21]. Letrozole is an aromatase inhibitor, suggesting it lowers the synthesis of estrogen. It is used to treat adjuvant, neoadjuvant, and metastatic breast cancer as well as to encourage ovulation in infertile patients, according to a review of research conducted by Mukherjee AG, et al. in 2022. Moreover, it induces fibrosis, necrosis, and apoptosis, all of which lead to the death of cancer cells. Mukherjee AG goes into great detail about the pharmacokinetics, pharmacodynamics, and side effects of Letrozole on several organs such as the heart, kidney, liver, embryo, bone, and ovary. Some anticipated or unforeseen negative effects resulting from using Letrozole over or longer than the suggested therapeutic dose range[22].

The study shows significant advantages in using Wistar rats as a standard model, offering a reliable framework for thorough physiological evaluations. The study is carefully monitoring the effects of Letrozole and grape seed extract on vital markers such as blood albumin, liver enzyme levels, and histopathology. Furthermore, the incorporation of a

variety of criteria, such as blood albumin, liver enzyme levels, and histopathology, permits a thorough assessment of the therapies' outcomes. However, because it largely focuses on short-term repercussions without investigating the durability of observed modifications, the study has drawbacks, including a lack of long-term assessment. The study's failure to explore the underlying molecular mechanisms or signaling pathways connected to the effects further hampered a more complex interpretation of the findings.

Conclusion

In conclusion, Letrozole and grape seed extract have a significant effect on liver enzyme levels, hepatic architecture, and relative gene expression of the aromatase gene. Additionally, individual therapy with grape seed extract exerts hepatoprotective effects against hepatocellular carcinoma induced by N-ethylnitrosamine.

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