Alcoholic Hepatitis: A Paradigm shift of Prevention and Treatment Analyzed at Cellular level by Histo/Pathological Micrometric Assessment.

Raja Faisal Zulfiqar[1*], Muhammad Yousuf Khoso[2], Farheen Hameed[3], Adeel Zafar[4], Zen-ul-Abideen[5], Raheela Adil[6].

ABSTRACT:

Objective: To evaluate role of Vitamin C and Naproxen in Alcohol-induced liver toxicity by micrometric technique (hepatocyte count, size and nuclear diameter) in albino rats.

Methodology: This experimental interventional study was conducted at the sheikh Zayed medical college and hospital Rahim yar khan from December 2022 to March 2023. 60 albino rats with equal gender distribution were selected based on the random probability sampling technique and placed in three groups of 20, again with an equal gender distribution (1:1). Group A (positive control) was given purified Ethanol for 10 days., Group B was given Vitamin C and Naproxen Prophylaxis for 7 days and later intoxicated with Ethanol for 10 days and Group C was simultaneously Administered Vitamin C, Naproxen, and Ethanol for 10 days. The hepatocyte count, size and nuclear diameter of all the groups were recorded by micrometric technique using ocular and stage micrometric scales. Data analysis was conducted using SPSS 24.0 and P-Value of ≤0.05 considered Statistically Significant.

Results: Group A animals experienced decreased number of hepatocyte count, increase in size of cell and decrease in nucleus size; Group B showed similar picture as group "A" but less in intensity and Group C showed nearly normal hepatic architecture with hepatocyte count, size and nuclear size close to normal ranges.

Conclusion: The anti-oxidative and anti-inflammatory effects of Vitamin C and Naproxen demonstrated significant hepatoprotective effects on liver histologic architecture, providing safety from alcohol-induced liver injury.

Keywords: Naproxen, Prophylaxis, liver toxicity, alcoholic liver disease, vitamin C.

Introduction:

Alcoholic liver disease (ALD) is the second most common cause of total human death every year. According to published studies, ALD includes alcoholic fatty liver; alcoholic hepatitis, steatohepatitis, liver fibrosis, cirrhosis, and liver cancer.[1] Alcohol intake can lead to changes in gut microbiota composition, even before liver disease development. These alterations worsen with advancing disease and could be complicit in disease progression.[2] The progression of ALD is mainly associated with the amount and duration of alcohol usage; however, it is also influenced by genetic, epigenetic, and environmental factors.[3] Apart from the classic knowledge that ethanol mediates its hepatotoxicity through its metabolism to acetaldehyde, recent research has proved other mechanisms like generation of free radicals, activation of Kupffer cells, and alterations to the human bacterial and fungal microbiome.[4] The molecular mechanisms of alcoholic liver disease, such as the important role of genes, risk factors, pathogenicity, and role of micro RNA, the role of inflammation in the liver, and alcoholic fibrosis in the liver.[5] Abstinence is the most important strategy to prevent disease progression. Corticosteroids improve the one-month survival in patients with severe alcoholic hepatitis, but it was not effective on long-term survival. An N-acetylcysteine treatment combined with corticosteroids may provide a short-term survival benefit than corticosteroids alone. Pentoxifylline is unlikely to affect short-term survival.[6] Alcoholic hepatitis (AH) is manifested as abnormal liver function (scleral yellow staining, etc.), and fewer patients may have thrombocytopenia as the clinical manifestation. Thrombocytopenia is often considered as primary immune thrombocytopenia.[7] Vitamin C is an essential nutrient that serves as antioxidant and plays a major role as co-factor and modulator of various pathways of the immune system.[8] vitamin C and selenium, which have antioxidant, anti-inflammatory, and anti-apoptotic properties.[9] Vitamin C, also known as ascorbate, is a hydrophilic vitamin obtained through diet.[10] Naproxen (2*S*)-2-(6-methoxynaphthalen-2-yl)propanoic acid) is the flagship representative of non-steroidal anti-inflammatory drug (NSAIDs), used in the treatment of arthritic pain and rheumatoid syndromes.[11] As alcoholic liver disease (ALD) is a rapidly rising public health issue with enormous medical, economical and social strain[12] therefore, we conducted a research to find out any possible preventive effect of vitamin C in combination with Naproxen in Alcoholic liver injury.

Methodology:

This experimental interventional study was conducted by the Department of Anatomy, Sheikh Zayed Medical College and Hospital in collaboration with Rahim Yar Khan (RYK) Medical College from December 2022 to March 2023 after ethical approval vide letter no: ERC/SZMCH/SZH/16/2022, dated 18th November, 2022. A sample size of 60 healthy male albino rats, locally sourced and weighing between 180 and 200 grams, was determined using Andrew Fisher's formula, which accounted for a standard deviation of 0.5 and an 80% confidence interval. Female rats and those weighing less than 180 grams or more than 200 grams were excluded from the study. A probability ran-

1. RYK Medical College، Abu Dhabi Road near KFUEIT Rahim Yar Khan, Pakistan.
2. Al Tibri Medical College،Near Al-Ibrahim Eye Hospital, Old Thana Gadap Town Karachi, Pakistan.
3. Shahida Islam Medical and Dental College، Main National Highway 100 M Lodhran, Pakistan.
4. Shahida Islam Medical and Dental College، Main National Highway 100 M Lodhran, Pakistan.
5. CMH Institute of Medical Sciences (CIMS) ، Central Library Road Bahawalpur Cantt, Pakistan.
6. RYK Medical College، Abu Dhabi Road near KFUEIT Rahim Yar Khan, Pakistan.

**=corresponding author:*
Email: rajafaisal76@hotmail.com

dom sampling technique was employed, and the animals were housed in labelled plastic cages, with each cage accommodating five rats. The cages were maintained under a monitored and regulated atmospheric temperature of 30°C, with consistent light-dark cycles of 12 hours each. All animals chosen for observation were provided with a standard rat diet and had access to water ad libitum. Ethanol, 500 mg Naproxen tablets, and 500 mg vitamin C tablets were procured from the hospital's pharmacy. A total of 60 albino rats were then allocated into three groups based on their dosing treatment, with each group consisting of 20 rats. Group A served as the positive control and was administered purified Ethanol at a dosage of 8 ml/kg body weight[13] for a duration of 10 days. Group B received prophylactic treatment with vitamin C and Naproxen Sodium for seven days, at doses of 100 mg/kg[14] and 5 mg/kg[15], respectively, followed by administration of Ethanol for an additional 10 days. Group C was given vitamin C, Naproxen, and purified Ethanol concurrently, at doses of 100 mg/kg, 5 mg/kg, and 8 ml/kg, respectively, over a period of 10 days. The rats were administered vitamin C and Naproxen Sodium between 10 AM and 11 AM after being subjected to overnight fasting, while Ethanol was administered via gastric gavage at one-hour intervals. Upon completion of the dosing regimen, the rats were euthanized under anaesthesia, and a central midline incision was made to access the thoracolumbar organs for the careful extraction of the liver. Histopathological parameters, including hepatocyte count, hepatocyte diameter, and nuclear diameter, were assessed using micrometric techniques.

Data analysis was conducted using SPSS Version 24, with results presented as mean ±SD to evaluate variations among the groups. Statistical analysis was performed using ANOVA with post hoc Tukey's test, as well as student's t-test, with a p-value of ≤0.05 considered statistically significant.

Results:

Mean hepatocyte count per reticule in among rats of A, B and C groups was 7.03, 8.69, 15.08 respectively. Mean hepatocyte count in group A was significantly decreased up to 7.03 cells per reticule, while hepatocyte count in group B & C was 8.69 and 15.08 respectively. Reduced hepatocyte count in group A and B was probably due to inflammation related swelling of hepatocyte which increased the diameter of cell. It's very clear that the highest hepatocyte count was seen in group C. The mean difference of hepatocyte count per reticula among different groups compared statistically and result shown in table 1.

Table No 1: statistical analysis of difference in hepatic cell count per reticule of rats between different groups.

Comparison	Statistical comparison	Mean difference	p-value
A vs B	Positive control vs vitamin C & naproxen (prophylactic)	1.66-	0.000>*
A vs C	Positive control vs vitamin C, naproxen & ethanol (simultaneous)	8.05-	0.000>*
B vs C	vitamin C & naproxen (prophylactic) vs vitamin C & naproxen (simultaneous)	6.39-	0.000>*

P<0.05 considered significant using Tukey's HSD test.
Statistical analysis revealed that all the comparison values among the groups were highly significant and the highest difference of mean (-8.05) was found between the group A vs C.

Mean hepatocyte diameter in group A was greatest (17.08) while it was observed least in group C (14.02). The mean nuclear diameter was highest in group C (7.41) that is almost within normal limits. While group B showed mild changes in both the mean hepatocyte diameter (15.04) and mean nuclear diameter(6.38). Statistical analysis of mean hepatocytes diameter among and nuclear diameter between three graoups is shown in table 2 and 3 respectively.

Table No 2: Statistical analysis of difference in hepatocyte diameter of rats between different groups.

Comparison	Statistical comparison	Difference of mean	p-value
A vs B	Positive control vs vitamin C & naproxen (prophylactic)	2.04	*0.000>
A vs C	Positive control vs vitamin C, naproxen & ethanol (simultaneous)	3.06	*0.000>
B vs C	vitamin C & naproxen (prophylactic) vs vitamin C, naproxen & ethanol (simultaneous)	1.02	*0.000>

Analysis revealed that all the comparison values were highly significant. The biggest difference of mean was observed between group A & C. Group A showed severely disturbed values from normal, while group C showed measurements in normal range. Group B had values out of normal ranges but still better than group A.

Table No 3. Statistical analysis of difference in nuclear diameter of rats between different groups.

Comparison	Statistical comparison	Difference of mean	p-value
A vs B	Positive control vs vitamin C & naproxen (prophylactic)	-0.98	<0.000*
A vs C	Positive control vs vitamin C, naproxen & ethanol (simultaneous)	-2.01	<0.000*
B vs C	vitamin C & naproxen (prophylactic) vs vitamin C , naproxen & ethanol (simultaneous)	-1.02	<0.000*

P<0.05 considered significant using Tukey's HSD test. Statistically all the comparison values were highly significant.
Biggest difference of mean was evident in comparison of group A and C. least difference of mean was observed in comparison of group A and B but still with highly significant P value.

Discussion:

The liver is the center of metabolism of our body. It is important that it is saved and not pushed to the limit with toxins that can deteriorate it, thereby producing disturbance in detoxification, metabolism, and production of compulsory body proteins. Alcohol is known for destructing the liver and producing alcoholic liver disease and fatty liver disease in people with long term alcohol consumption. Treatment is usually not satisfactory, and in most situations, the ultimate probable result is to perform a liver transplant. The drugs used in our study may prove to have a supporting effect on the liver for prevention of insult. The oxidative stress produced by the free radical can start hepatotoxicity; however, the significant antioxidant effect of vitamin C supplies a hepatoprotective effect on the liver, also naproxen is an important medication used to reduce inflammation caused by alcohol. It is also a plus that naproxen has very minor, if any, side effects and is a very safe drug to prescribe. We used both Vitamin C and Naproxen and assessed if they may have any safety action when it came to alcohol-induced liver toxicity. To analyze their positive effect, we studied the histopathologic parameters of rats. In Group A animals, we observed a reduction in the hepatocyte count, a finding very similar to what Tauba et al. 2023 also showed in their study when studying the hepatotoxic effects of Chromium.[16] The cause of the decrease in hepatocyte count in Group A can be associated to the inflammation and destruction due to alcohol consumption. The preventive activity of naproxen and Vitamin C protected any significant decrease in hepatocyte count in Group B. However, analysis of Group C rats; showed that hepatocyte count was almost normal, similar to what Hussain et al. 2020 that went onto report similar findings in predicting direct hepatocyte toxicity.[17] We studied hepatocyte diameter and nuclear diameter as well. Group A showed an increase in hepatocyte diameter which may be consequence of inflammation produced by alcohol, identical finding reported by Hou et al, who studied the hepatotoxicity induced rifampicin via free radicals in 2022.[18] Group B animals showed little increase in hepatocyte diameter. This is probably due to anti-inflammatory effects of naproxen and antioxidative effect of vitamin C. Another study conducted by Hajjar et al, showed that the antioxidant and anti-inflammatory potential having substances such as vitamin C caused significant degree of protection against the toxicity produced by cyclophosphamide an anti-neoplastic drug.[19]

Group C demonstrated the best outcomes, as the liver histologic architecture was almost healthy. Alcohol shrinks the nuclear diameter of hepatocytes and antioxidants can have a very supportive role on the liver, and so can anti-inflammatory role. This is what was evident in our study and supported by a study conducted by Satomoto et al. in which he demonstrated how the repeated dose liver micronucleus can be produced.[20] Overall the best results were observed in Group C as both the agents were infused alongside alcohol in rats; therefore, the group displayed a more beneficial outcome. The best results could be interpreted from Group C rats, as during intoxication with ethanol, there was also the administration of both Vitamin C and naproxen, which helped provide a hepatoprotective effect on the organ. Group B, because of the prophylactic administration, also showed some positive results. However, they were not as better as compared to what was seen in Group C animals.

Limitation and future Perspective: Our investigation focused on a limited number of histopathological parameters; however, a broader range of parameters should be examined to gain a more comprehensive understanding of the role of Vitamin C and Naproxen in maintaining the normal histological structure of the liver. Future research should include biochemical evaluations and additional gross parameters to assess the potential of Vitamin C and Naproxen in mitigating the detrimental effects of alcohol on liver health. The findings indicated that both Groups B and C, which received Vitamin C and Naproxen, exhibited superior preservation of normal histological architecture compared to Group A, thereby highlighting the beneficial effects of these two agents. This may be attributed to the antioxidant and anti-inflammatory properties inherent in Vitamin C and Naproxen.

Conflict of Interest: Authors declare none.

Funding: No funding was received from any source.

References:

1. Kong LZ, Chandimali N, Han YH, Lee DH, Kim JS et al. Pathogenesis, Early Diagnosis, and Therapeutic Management of Alcoholic Liver Disease. Int J Mol Sci. 2019 Jun 2;20(11):2712. doi: 10.3390/ijms20112712. PMID: 31159489; PMCID: PMC6600448. doi:10.3390/ijms20112712
2. Bajaj JS. Alcohol, liver disease and the gut microbiota. Nat Rev Gastroenterol Hepatol. 2019 Apr;16(4):235-246. doi: 10.1038/s41575-018-0099-1. PMID: 30643227. doi:10.1038/s41575-018-0099-1
3. Liu SY, Tsai IT, Hsu YC. Alcohol-Related Liver Disease: Basic Mechanisms and Clinical Perspectives. Int J Mol Sci. 2021 May 13;22(10):5170. doi: doi:10.3390/ijms22105170. PMID: 34068269; PMCID: PMC8153142
4. Kourkoumpetis T, Sood G. Pathogenesis of Alcoholic Liver Disease: An Update. Clin Liver Dis. 2019 Feb;23(1):71-80. doi: 10.1016/j.cld.2018.09.006. PMID: 30454834.
5. Namachivayam A, Valsala Gopalakrishnan A. A review on molecular mechanism of alcoholic liver disease. Life Sci. 2021 Jun 1;274:119328. doi: doi:10.1016/j.lfs.2021.119328 Epub 2021 Mar 10. PMID: 33711388.
6. Song DS. [Medical Treatment of Alcoholic Liver Disease]. Korean J Gastroenterol. 2020 Aug 25;76(2):65-70. Korean. doi:10.4166/kjg.2020.76.2.65. PMID: 32839368.
7. Luo Y, Pei Q, Chen Y, Ye T. Alcoholic liver disease with thrombocytopenia. Asian J Surg. 2022 Dec;45(12):2755-2756. doi: 10.1016/j.asjsur.2022.06.024. Epub 2022 Jun 17. PMID: 35718614.
8. Milani GP, Macchi M, Guz-Mark A. Vitamin C in the Treatment of COVID-19. Nutrients. 2021 Apr 1;13(4):1172. doi: 10.3390/nu13041172. PMID: 33916257; PMCID:PMC8065688
9. Zheng S, Hameed Sultan A, Kurtas PT, Kareem LA, Akbari A. Comparison of the effect of vitamin C and selenium nanoparticles on gentamicin-induced renal impairment in male rats: a biochemical, molecular and histological study. Toxicol Mech Methods. 2023 May;33(4):260-270. doi: 10.1080/15376516.2022.2124136. Epub 2022 Sep 18. PMID: 36093943

10. Collins BJ, Mukherjee MS, Miller MD, Delaney CL. Effect of Dietary or Supplemental Vitamin C Intake on Vitamin C Levels in Patients with and without cardiovascular disease: A Systematic Review. Nutrients. 2021 Jul 8;13(7):2330. doi: doi:10.3390/nu13072330 10.3390/nu13072330. PMID: 34371840; PMCID:PMC8308513
11. Przykaza K, Jurak M, Wiącek AE. Effect of naproxen on the model lipid membrane formed on the water-chitosan subphase. Biochim Biophys Acta Biomembr. 2023 Mar;1865(3):184099. doi:10.1016/j.bbamem.2022.184099. Epub 2022 Dec 6. PMID: 36493856
12. Cao P, Wu Y, Li Y, Xiang L, Cheng B, Hu Y, Jiang X, Wang Z, Wu S, Si L, Yang Q, Xu J, Huang J. The important role of glycerophospholipid metabolism in the protective effects of polyphenol-enriched Tartary buckwheat extract against alcoholic liver disease. Food Funct. 2022 Oct 17;13(20):10415-10425. doi:10.1039/D2FO01518H. PMID: 36149348.
13. Park JH, Lee DH, Park MS, Jung YS, Hong JT. C-C chemokine receptor type 5 deficiency exacerbates alcoholic fatty liver disease through pro-inflammatory cytokines and chemokines-induced hepatic inflammation. J Gastroenterol Hepatol. 2017 Jun;32(6):1258-1264. doi:10.1111/jgh.13657. PMID: 27859576.
14. Levine M, Padayatty SJ, Espey MG. Vitamin C: a concentration-function approach yields pharmacology and therapeutic discoveries. Adv Nutr. 2011 Mar;2(2):78-88. doi:10.3945/an.110.000109. Epub 2011 Mar 10. PMID: 22332036; PMCID: PMC3065766 PMC3065766
15. Azizian H, Mousavi Z, Faraji H, Tajik M, Bagherzadeh K et al. Arylhydrazone derivatives of naproxen as new analgesic and anti-inflammatory agents: Design, synthesis and molecular docking studies. J Mol Graph Model. 2016 Jun;67:127-36. doi: 10.1016/j.jmgm.2016.05.009 Epub 2016 May 24. PMID: 27311100
16. Nauroze T, Ali S, Kanwal L, Ara C, Akbar Mughal T, Andleeb S. Ameliorative effect of *Nigella sativa* conjugated silver nanoparticles against chromium-induced hepatotoxicity and renal toxicity in mice. Saudi J Biol Sci. 2023 Mar;30(3):103571. doi:10.1016/j.sjbs.2023.103571 Epub 2023 Jan 25. PMID: 36844642; PMCID: PMC9944502
17. Hussain F, Basu S, Heng JJH, Loo LH, Zink D. Predicting direct hepatocyte toxicity in humans by combining high-throughput imaging of HepaRG cells and machine learning-based phenotypic profiling. Arch Toxicol. 2020 Aug;94(8):2749-2767. doi:10.1007/s00204-020-02778-3 Epub 2020 Jun 12. PMID: 32533217
18. Hou W, Nsengimana B, Yan C, Nashan B, Han S. Involvement of endoplasmic reticulum stress in rifampicin -induced liver injury. Front Pharmacol. 2022 Oct 20;13:1022809. doi:10.3389/fphar.2022.1022809 . PMID: 36339603; PMCID:PMC9630567
19. Hajjar T, Soleymani F, Vatanchian M. Protective Effect of Vitamin C and Zinc as an Antioxidant Against Chemotherapy-Induced Male Reproductive Toxicity. J Med Life. 2020 Apr-Jun;13(2):138-143. doi: 10.25122/jml-2019-0107. PMID: 32742504; PMCID: PMC7378338.
20. Satomoto K, Suzuki I, Mita K, Wakita A, Yamagata H, Mitsumoto T, Hamada S. The effect of aging on the repeated-dose liver micronucleus assay using diethylnitrosamine. Genes Environ. 2022 Aug 18;44(1):21. doi: 10.1186/s41021-022-00250-5. PMID: 35982479; PMCID: PMC9387043.