Histopathological and histomorphometric analysis of the small intestine in wistar rats subjected to daily alcohol intake

Análise histopatológica e histomorfométrica do intestino delgado de ratos wistar submetidos à ingestão diária de álcool

Análisis histopatológico e histomorfométrico del intestino delgado en ratas wistar sometidas a la ingesta diaria de alcohol

Thaís Santa Cruz Moraes
ORCID: https://orcid.org/0009-0004-8975-0912
Universidade do Vale do Sapucaí, Brazil
E-mail: thais.scmoraes@gmail.com
Sara Semaan Silveira
ORCID: https://orcid.org/0009-0005-2672-9558
Faculdade Ciências Médicas de Minas Gerais, Brazil
E-mail: sarassemaan@hotmail.com
Thiago Jardim de Oliveira
ORCID: https://orcid.org/0009-0002-1151-8510
Universidade do Vale do Sapucaí, Brazil
E-mail: gardenambiental@yahoo.com.br
Adriana Rodrigues dos Anjos Mendonça
ORCID: https://orcid.org/0000-0003-0526-6636
Universidade do Vale do Sapucaí, Brazil
E-mail: adrianjos@univas.edu.br
Paulo Roberto Maia
ORCID: https://orcid.org/0000-0003-0044-195X
Universidade do Vale do Sapucaí, Brazil
E-mail: PauloMaia@univas.edu.br
Fiorita Gonzales Lopes Mundim
ORCID: https://orcid.org/0000-0002-7375-4108
Universidade do Vale do Sapucaí, Brazil
E-mail: hjmundim@univas.edu.br
Rodrigo Machado Pereira
ORCID: https://orcid.org/0000-0001-9525-043X
Universidade do Vale do Sapucaí, Brazil
E-mail: rodrigom@univas.edu.br

Abstract
The excessive consumption of alcohol poses a significant public health concern due to its detrimental effects on individual well-being, particularly on the gastrointestinal (GI) tract. Chronic ethanol ingestion leads to morphological and functional alterations in the GI tract, affecting epithelial cells and resident microbial flora, thus impairing intestinal absorption. This study aimed to assess the impact of chronic ethanol consumption on small intestinal histological morphology. Animals were divided into control (water), 5% ethanol, and 20% ethanol groups, with 10 animals in each. Over 80 days, ethanol consumption was monitored ad libitum. Histopathological and histomorphometric analyses of the small intestine were conducted. Results revealed similar daily liquid consumption among the groups. However, alcoholic groups exhibited increased mononuclear cells, eosinophils, and villus degeneration compared to the control group. Ethanol-treated animals also displayed reduced villi extension, indicating structural damage to the intestine. Chronic ethanol consumption induced a chronic inflammatory pattern and deterioration of intestinal structures. In conclusion, regardless of alcohol content, chronic ethanol consumption adversely affects the gastrointestinal tract. This study underscores the importance of understanding the pathological effects of alcohol on the GI system and emphasizes the need for interventions to mitigate its impact on public health.
Keywords: Ethanol; Alcoholism; Small intestine; Absorption; Nutrition.

Resumo
O consumo excessivo de álcool representa uma preocupação significativa para a saúde pública devido aos seus efeitos prejudiciais no bem-estar individual, especialmente no trato gastrointestinal (GI). A ingestão crônica de etanol resulta em alterações morfológicas e funcionais no trato GI, afetando células epiteliais e a flora microbiana residente,

prejudicando assim a absorção intestinal. Este estudo teve como objetivo avaliar o impacto do consumo crônico de etanol na morfologia histológica do intestino delgado. Os animais foram divididos em grupos de controle (água), 5% de etanol e 20% de etanol, com 10 animais em cada grupo. Ao longo de 80 dias, o consumo de etanol foi monitorado ad libitum. Análises histopatológicas e histomorfométricas do intestino delgado foram realizadas. Os resultados revelaram consumo diário de líquidos semelhante entre os grupos. No entanto, os grupos alcoólicos apresentaram aumento de células mononucleares, eosinófilos e degeneração vilosa em comparação com o grupo de controle. Os animais tratados com etanol também exibiram redução na extensão das vilosidades, indicando danos estruturais ao intestino. O consumo crônico de etanol induziu um padrão inflamatório crônico e deterioração das estruturas intestinais. Em conclusão, independentemente do teor alcoólico, o consumo crônico de etanol afeta negativamente o trato gastrointestinal. Este estudo destaca a importância de compreender os efeitos patológicos do álcool no sistema GI e enfatiza a necessidade de intervenções para mitigar seu impacto na saúde pública.
Palavras-chave: Etanol; Alcoolismo; Intestino delgado; Absorção; Nutrição.

Resumen
El consumo excesivo de alcohol representa una preocupación significativa para la salud pública debido a sus efectos perjudiciales en el bienestar individual, especialmente en el tracto gastrointestinal (GI). La ingesta crónica de etanol resulta en alteraciones morfológicas y funcionales en el tracto GI, afectando las células epiteliales y la flora microbiana residente, lo que dificulta la absorción intestinal. Este estudio tuvo como objetivo evaluar el impacto del consumo crónico de etanol en la morfología histológica del intestino delgado. Los animales se dividieron en grupos de control (agua), 5% de etanol y 20% de etanol, con 10 animales en cada grupo. A lo largo de 80 días, se monitorizó la ingesta de etanol ad libitum. Se realizaron análisis histopatológicos e histomorfométricos del intestino delgado. Los resultados revelaron un consumo diario de líquidos similar entre los grupos. Sin embargo, los grupos alcohólicos mostraron un aumento de células mononucleares, eosinófilos y degeneración vilosa en comparación con el grupo de control. Los animales tratados con etanol también mostraron una reducción en la extensión de las vellosidades, lo que indica daños estructurales en el intestino. El consumo crónico de etanol indujo un patrón inflamatorio crónico y deterioro de las estructuras intestinales. En conclusión, independientemente del contenido alcohólico, el consumo crónico de etanol afecta negativamente al tracto gastrointestinal. Este estudio destaca la importancia de comprender los efectos patológicos del alcohol en el sistema GI y enfatiza la necesidad de intervenciones para mitigar su impacto en la salud pública.
Palabras clave: Etanol; Alcoholismo; Intestino delgado; Absorción; Nutrición.

1. Introduction

Alcoholism is defined by the World Health Organization as the dependence on alcohol and/or problems related to the consumption of alcoholic beverages. It is considered a chronic disease, associated with innumerous factors that contribute to its development, such as the individual's health conditions, genetic factors, metabolic disturbances, psychosocial factors and the environment. Excessive alcohol consumption is directly related to deleterious effects on the individual's health and well-being, which classifies it as one of the major public health problems today (Franklin et al., 2021; WHO, 2018).

Ethanol consumption exerts a very toxic effects on all tissues of the body and affects a large part of the vital functions, because it is a small and soluble molecule, both in aqueous and lipidic ambience. After the ingestion of this drug, about 20% of it is absorbed in the stomach and the rest in the early portions of the small intestine, where absorption is extremely rapid, complete, and independent of ethanol concentration or the presence of food. Only about 2 to 10% of ethanol is eliminated via the lungs and kidneys, while the rest is oxidized in the liver, since it cannot be stored (Amadieu et al., 2022). Morphological and functional alterations of the gastrointestinal tract after the ingestion of ethanol have been described in humans and experimental animals, characterized mainly by interference in the epithelial cells by increasing the permeability between these cells and allowing the passage of toxic substances, which increase the risk of developing inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, besides diarrhoea, malabsorption and nutritional deficiencies, in addition to affecting the maintenance of resident microbial flora, impairing intestinal absorption (Haber & Kortt, 2021).

In this context, the negative gastrointestinal consequences of continuous alcohol ingestion are varied. Most recent scientific studies focus on demonstrating the adverse effects that repeated exposures to ethanol can cause to the morpho-physiology of the small intestinal mucosa and establish relation with the already known acute toxicity. In addition, alcohol can

cause various forms of malnutrition: inappropriate consumption can cause primary malnutrition, as it can displace nutrients from the diet, as well as secondary malnutrition, as it is responsible for malabsorption and aggression to intestinal cells due to its cytotoxicity. This damage to intestine health affects numerous aspects of life quality, including physical, psychological, family, and social conditions (Heilbron et al., 2020). The objective of this study was to evaluate the effects of chronic ethanol ingestion on small intestinal cell morphology through histopathological and histomorphometric analyses, taking into consideration the different concentrations of alcohol, 5% and 20%, and the amount of alcohol ingested.

2. Methodology

2.1 Animals and experiment

The study is of analytical, individual, interventional, longitudinal, prospective, clinical trial format. It was conducted in accordance with the Ethical Principles for Animal Experimentation, adopted by the National Council for the Control of Animal Experimentation in Brazil. The project was concomitantly submitted to and approved by the Ethics Committee on Animal Use of Sapucai Valley University (protocol 280/12).

Twenty-seven male rats (Rattus norvegicus) of the Wistar strain, sixty days years old and with body weight between 150 and 250 grams at the beginning of the experiment, were used, distributed in the following groups: EtOH 0% (control, n=9), EtOH 5% (n=9) and EtOH 20% (n=9) (Macieira et al., 1997). Absolute ethyl alcohol (ISOFAR, RJ, Brazil) was diluted in water (v/v) at concentrations of 5% and 20%, which are close to those usually found in beer and fortified wine, respectively. The alcoholic solutions were offered through spontaneous and intermittent ingestion as part of the liquid diet. The animals received the solutions at the end of the day, and it was available during the night for a period of 12-14h. Only at weekends the alcoholic solution was available day and night. The volume of liquid intake was measured in the morning.

2.2 Sedation and euthanasia

For removal of the small intestine, the animals were anesthetized with an intramuscular injection of a standardized solution, dosed by weight, of Xylazine (1.1mg/kg), a sedative, analgesic and myorelaxant (Konig do Brasil Ltda. - Santana de Paranaíba, SP, Brazil); and Ketamine (80mg/kg.), a dissociative agent (Konig do Brasil Ltda. - Santana de Paranaíba, SP, Brazil). This anesthetic technique has a neuroleptic characteristic with latency of 5 minutes and effectiveness in the period of 50-80 minutes, allowing bloodless manipulations.

The animals were sacrificed after all the procedure described in this project, still under general anesthesia. Euthanasia occurred with an intracardiac injection of potassium chloride into the myocardium (Conselho Federal de Medicina Veterinária, 2012).

2.3 Collection and fixation of the material

The small intestine was collected along with the other constituent organs of the monobloc, which were immediately placed in Carson's Formalin in Millonig's Buffer for fixation. For this purpose, a plastic containers were used sealed and properly identified. They were kept in this solution for a minimum of 24 hours.

2.4 Histological processing

After the fixation, the organs were transversely cleaved about 3 mm, packed in high density polymer cassettes and soaked in 10% formalin solution. The samples were submitted to increasing solutions of ethanol 70%, 95% and 100% (three times) for dehydration, followed by diaphanization in three xylene baths for one hour each. Paraffin impregnation was executed in an oven at 65°C, for one and two hours respectively, and embedded.

Histological sections were obtained on a rotating paraffin microtome at 3 micrometers thickness. They were distended in water at 50ºC in a water bath. They were also collected on clean and identified glass slides for staining procedures. The slides were stained using four techniques: Hematoxylin and Eosin, Masson's Trichrome and Periodic Acid-Schiff (PAS).

2.5 Histopathological analysis

The analysis of variance was performed based on a semi-quantitative examination, in which values from 0 to 3 points were assigned regarding the presence and intensity of the parameters: hemorrhage, cellular desquamation, denudation, villous degeneration, ulcer, epithelial necrosis, mucosal edema, fibrosis, mononuclear cells, neutrophils and eosinophils, correlating with the amount of alcohol ingested. The scale was determined as 0 being the absence of the factor, 1 as weak, 2 as moderate and 3 as severe (Krawisz et al., 1984; Lambert et al., 2004; Söderholm et al., 2002). For each pathological pattern analyzed, it was used the staining slide that most favored the investigation of each factor.

2.6 Histomorphometric analysis

For morphometry, images were collected with a digital camera (Eurekam, 5.0MP, BEL Engineering) attached to the optical microscope. The analysis of the cut was made through the projection of the slides with the cuts stained with Hematoxylin and Eosin. The luminosity, the aperture of the diaphragm, and the use of filters were standardized as needed.

From the images, pixel lengths of the intestinal villi were measured using Bel Capture software (BEL Engineering), which were later converted to micrometers. This method was adapted from Seyyedin & Nazem (2017).

2.7 Statistical analysis

Quantitative variables are classified using measures of central tendency and for categorical variables, absolute and relative frequency. Data analysis was performed using the Statistical Package for the Social Sciences, Inc. (SPSS) (Chicago, USA, version 26.0). The significance level used as acceptance or rejection criteria in the statistical tests is 5% ($p < 0.05$). Spearman's ordinal correlation test is used to assess correlations between histopathological variables in alcohol consumption and staining. The values obtained in histomorphometric analysis and comparison of liquid consumption were individually examined by one way analysis of variance (ANOVA) and the morphometry of villus length by the Kruskal-Wallis test and then subjected to Tukey's and Bonferroni's post-tests, honestly significant difference test.

3. Results

A comparative study was made between the daily averages of the amount of liquids ingested by each group of rats. As for the average daily fluid intake, the daily water intake for the control group was 28.21 ml/day, 26.913 ml/day of 5% ethanol and 26.74 ml/day of 20% ethanol. There was no difference between the average daily liquid consumption for the 3 groups (p=0.504) (Figure 1).

Figure 1 - Comparison between liquid consumption for the three groups.
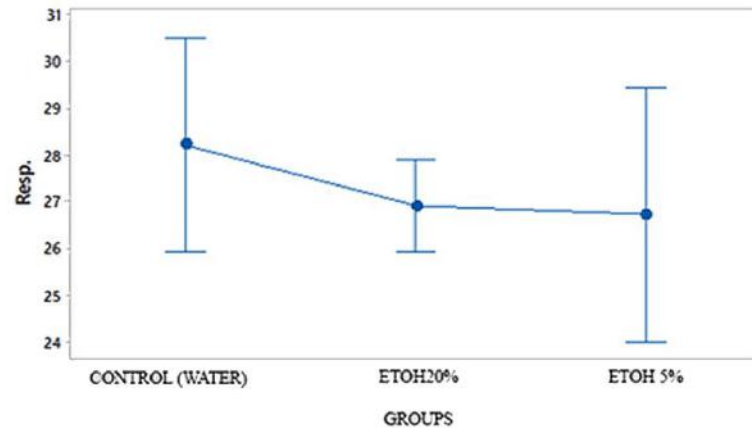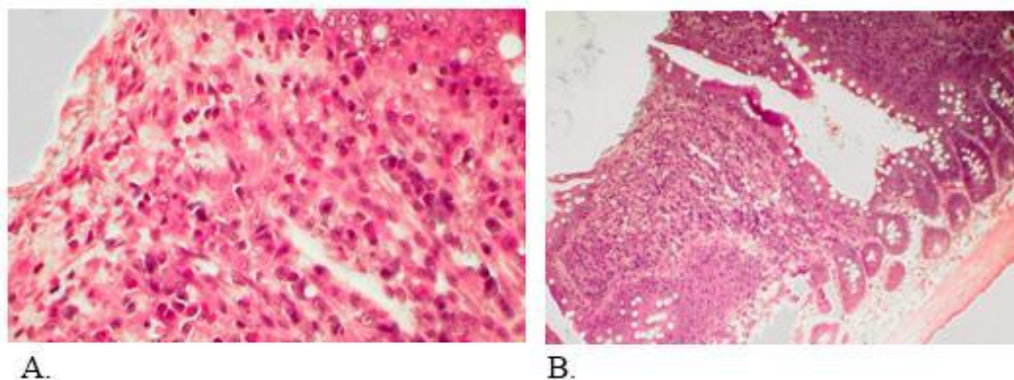

Source: Authors (2024).

There is a correlation between chronic alcohol intake and histopathological variables. Animals from ethanolic groups demonstrated intestine villus degeneration and inflamation (Figures 2 and 3).

Figure 2 – Intestine villi in hematoxylin and eosin staining at 10x magnification. (A) control group (no alcohol), (B) ETOH 5% and (C) ETOH 20%.
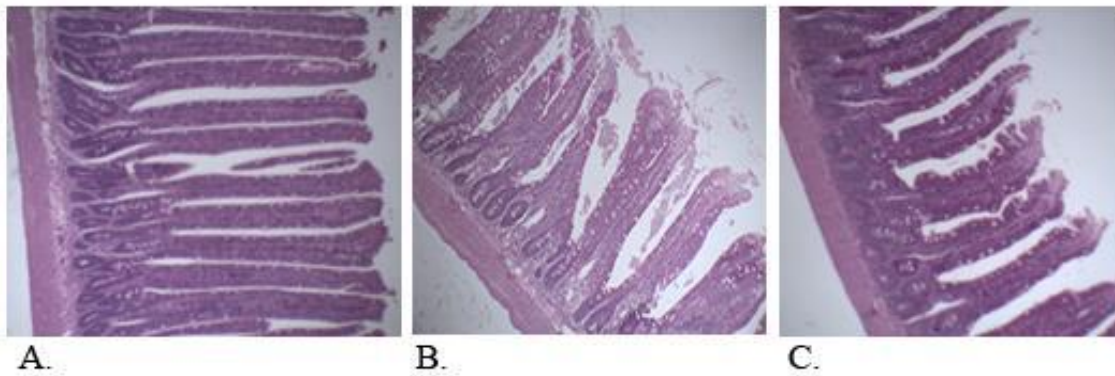
Source: Authors (2024).

Figure 3 - Mononuclear cell infiltrate and eosinophils in the intestinal mucosa of the rats in the 20% ETOH group at (A) 100x and (B) 40x microscopic magnification.

Source: Authors (2024).

Villi degeneration was proportionally reported to the ethanol ingestion. This alteration was found in 5 rats of control group, 8 animals that ingested 5% EtOH, and 9 of the 20% EtOH group. In these correlations the p value are significant. As for the other parameters there was no relevant difference (Table 1).

Mononuclear cells (MNC) and eosinophils were found in different amount between animals, excepting 2 rats of control. In this group, MNC and eosinophils were absent to moderate. Ethanolic treated groups (EtOH 5% and EtOH 20%) animals had moderate to severe amount of these cells. Although, massive infiltration of inflammatory cells was not found.

Table 1 - Histopathological variables in alcohol consumption according to each staining.

Histopathological variables	Masson's trichrome		PAS		Hematoxylin-eosin	
	Correlation	p value	Correlation	p value	Correlation	p value
Bleeding	-0.109	0.581	-0.103	0.611	-0.109	0.581
Peeling cells	-0.272	0.161	0.168	0.403	-0.272	0.161
Desnudation	0.197	0.314	0.198	0.322	0.088	0.655
Villous degeneration	**0.559**	**0.002**	**0.491**	**0.009**	**0.487**	**0.009**
Ulceration	0	0	0	0	0	0
Necrosis	-0.230	0.239	-0.231	0.247	-0.230	0.239
Mononuclear cells	**0.285**	**0.141**	**0.420**	**0.029**	**0.552**	**0.002**
Neutrophils	0	0	0	0	-0.230	0.239
Eosinophils	**0.656**	**0.000**	**0.504**	**0.007**	**0.490**	**0.008**
Mucosa edema	0	0	0	0	-0.230	0.239
Erosion	0	0	0	0	0	0
Fibrosis	0.029	0.882	0.025	0.902	0.029	0.882

Source: Authors (2024).

In the histomorphometric analysis, the average villus length in micrometers (µm) was, for the group without alcohol 855,3 µm; for the ETOH 5% group 660,0 µm; and for the ETOH 20% group 666,0 µm. There is no difference in villus lengths between the 5% and 20% alcohol groups, p value=0.437. The difference occurs in villus lengths between the non-alcohol and the 5% alcohol groups, and between the non-alcohol and the 20% alcohol groups, both p-value=0.000 (Figure 4).

Figure 4 - Villus length average between the three groups of rats (µm).
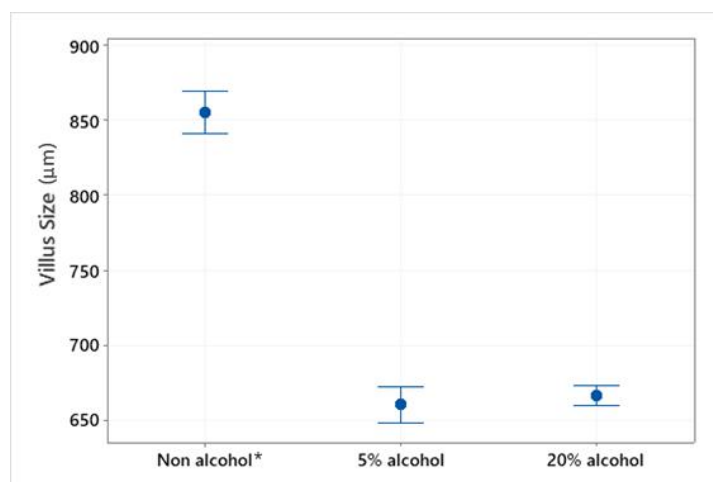
Source: Authors (2024).

Analyzing the food consumption of the three groups, the average feed intake of the control group was 17,279g per day, while in the ETOH 5% group it was 14,237g per day and in the ETOH 20% group it was 10,884g per day. There was a significant difference between the control and alcohol groups (p=0.000) (Figure 5).

Figure 5 –Difference between feed consumption among the three groups of rats.
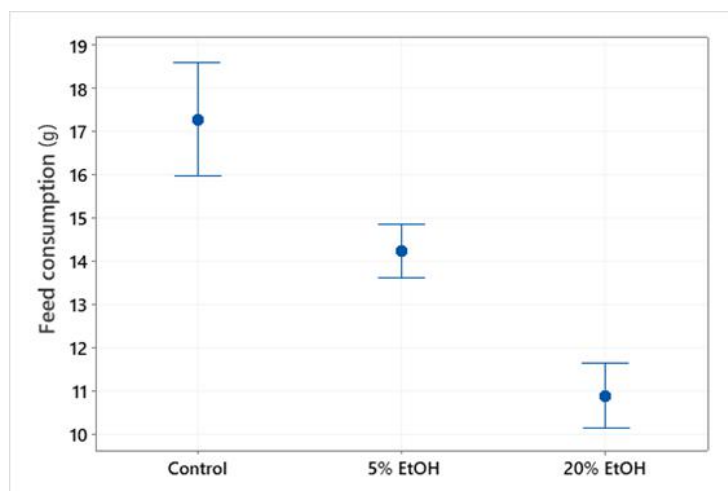
Source: Authors (2024).

Therefore, a variation was also observed in the average weight of the three groups during the experiment period. In the control group there was a significant increase of 12.444 grams, and 10.1 grams increase in the 5% ETOH group, with no statistically significant difference between them. In the 20% ETOH group there was a notable decrease of 15.6 grams, which, when compared to the other groups, showed a statistical difference (p = 0.019) compared to the control group and the 5% ETOH group. When subtracting the mean weight of the 20% ETOH group and the control group, this difference was negative, showing a statistically significant loss of body mass in the 20% ETOH group. When comparing the 5% ETOH group with the 20% ETOH group, the difference between the means was positive, also showing an increase in body mass in the 5% ETOH group and a decrease in the 20% ETOH group. When comparing the 5% ETOH group with the control group, although the control group's mass gain was slightly greater, there was no statistical difference in body weights between these two groups. When the three groups were compared about their weight, the variance was significant (p = 0.024).

4. Discussion

The presence of mononuclear cells and eosinophils in the intestinal mucosa of ethyl alcohol rats indicate a chronic inflammatory pattern of these cells (Krawisz et al., 1984). Furthermore, degeneration of these villi was evidenced, suggesting a deterioration in the function of these structures, conferring alcohol a toxic character to the gastrointestinal system.

Another studies have proven the consequences of prolonged alcohol intake on the small intestine. Diarrhea is among the most common effects, present both in acute and chronic use of the beverage. As for the microbiota, the damage reduces in quantity and quality, leading to inflammation and hyperpermeability of the intestinal epithelium, as well as compromised innate and adaptive immune responses. In addition, it inhibits the active transport of sugar, dipeptides, and amino acids, which causes absorptive defects of water, carbohydrates, lipids, vitamins such as thiamine and folate, and minerals such as calcium, zinc, and selenium (Haber & Kortt, 2021). Furthermore, the dysbiosis alcohol induced makes it easy the colonization and proliferation of opportunities pathogenic bacteria, like *Clostridioides difficile* (Ramos & Kane, 2021).

The eosinophils are an important part of the innate immune system of the gastrointestinal tract, and eosinophilic

infiltration of the intestine mucosa is common in many disorders, like parasitic infections, inflammatory intestine disorder, hypereosinophilic syndrome and drug hypersensitivity (Vuyyuru et al., 2022). The relation between chronic alcohol consumption and impaired absorption occurs because when there is villous degeneration, there is increased permeability of the epithelium, which causes activation of the mediated immune response, leading to inflammation (Attauabi et al., 2022; White et al., 2022). This activates proinflammatory mediators as tumor necrosis factor–α, interleukin (IL)-1 and IL-6. This mechanism is the same involved at Inflamatory Bowel disease's pathogenesis (Neri et al., 2021; Ramos & Kane, 2021).

Another finding of this study was about mononucler cells, that is, macrophagues, dentritic cells and lymphocytes, that are part of the innate immunity. The hyperinflamatory response is related to the onset of inflammatory bowel diseases, in particular macrophages, that causes an exaggerated inflammatory immune reaction. (Viola & Boeckxstaens, 2020). The T-cell disorder is related to uncontrolled inflammation that is presented as Inflammatory Bowel Diseases group, even though the changes about these cells are not clear. The natural killer T cells from the alcoholic group rat show a less mature phenotype and lower activation by antigens than the control group in an infection. In humans, natural killer cells are the most abundant T-cell subtype in the intestine, lung, and peripheral blood. As alcohol consumption induces intestine dysbiosis and the natural killer cell development depends on the gut flora, we hypothesized that alcohol-mediated dysbiosis reduces the prevalence and dysregulates the function of this cell in the intestinal tract and in distal organs. Natural killer cells counts were significantly reduced in the intestine and were hyperactivated by alcohol. This effect is related by the reduction of riboflavina (vitamin B) produced on gut microbiota in alcohol consumption, that activeates these cells, and with the hyperactivation of natural killer T and consequently apoptosis of them in an infection (Gu et al., 2021; Vuyyuru et al., 2022).

Besides altering the immune response of the gastrointestinal tract, the dysbiosis caused by chronic alcohol consumption is also related to alterations in the metabolic response, especially when related to nutrient-poor dietary habits. Similar to the present study, other experimental models in animals exposed to alcohol ingestion and diets rich in fat and carbohydrates showed cellular alteration of the intestinal epithelium, promoting change in the local microbiota, increased permeability of these cells and translocation of metabolites, as already mentioned, favoring fat deposition and steatotic disease, whether alcoholic or non-alcoholic, being the most effective therapeutic measure known today the abstention of alcohol consumption (Thoen, 2022).

The absorption of alcohol from the gastrointestinal tract depends on a number of conditions, such as its concentration, previous ingestion of ethanol, concomitant consumption with food or drugs, gender, age, or the body mass of the individual. Since ethanol is a water-soluble substance and cannot be stored in the body, it is rapidly metabolized, changing metabolic pathways, such as lipid oxidation, increasing the body's fat stock, with preferential abdominal deposition (Lucey et al., 1999; Stevens et al., 2022).

Furthermore, excessive alcohol consumption is associated with poor diet. Energy intake from increased chronic alcohol consumption can impair adequate macronutrient intake, reducing healthy carbohydrate, especially dietary fiber, which is crucial for optimal digestive health, and protein intake and leading to reduced weight and body fat and malnutrition. Reduced body fat has been associated with loss of interest in food and decreased nutrient absorption and metabolism (Joseph et al., 2022).

On the one hand, alcohol consumption has been shown to promote fat retention by reducing lipid oxidation, favoring lipogenesis, inhibiting glycolysis, and stimulating neurochemical and peripheral systems, which may lead to overheating, which alters the body's mitochondria-dependent enzymatic metabolism process. On the other hand, alcohol consumption can hinder caloric absorption and increase energy expenditure when consumed concomitantly with meals, leading to weight loss. This latter effect can be partially attributed to alcohol's high thermogenic effect, especially with long-term consumption (Larsen et al., 2022; Souza-Silva et al., 2022).

There are studies elucidating a direct association of visceral fat mass and chronic beer consumption, which may be attributed to the energy composition of beer in conjunction with differences in consumption patterns by alcohol classes.

Although, beer contains the lowest alcohol value by volume, it contributes to predominantly carbohydrate consumption, which contributes to an increase in visceral fat mass. For chronic wine drinkers, adiposity can be correlated with the type of wine consumed. Specifically, higher red wine consumption showed inverse relationships with visceral adipose mass and subcutaneous adipose mass, due to the presence of anti-inflammatory substances that may decrease human adipocyte fat. In contrast, white wine consumption showed no association with adiposity (Larsen et al., 2022).

In this study, the inflammatory pattern and degeneration was observed in both ethyl groups, with no significant difference between them. Therefore, cellular injury is related to chronic alcohol intake, not to its content. In addition, there was weight reduction in the group that drank alcohol at 20%, due to an appetite suppression and the fact that drinks with higher alcohol content decrease the absorption of macronutrients. Since the amount of alcohol ingested by the three groups was the same, the relationship between the findings is direct.

5. Final Considerations

This study demonstrates, according to the histological pattern observed, that habitual alcohol intake has a deleterious effect on the gastrointestinal tract, by the presence of mucosa chronic inflammation and the increase of eosinophils, mononuclear cells activation and decreased villus length, in other words, it alters the intestine microbiota composition, structure and function which predispose to inflammatory bowel diseases. In addition, it shows that there was weight reduction in the higher alcohol group and appetite suppression; as to a reduction in the absorption of macronutrients, further work is needed to prove this relationship. This result demonstrates the toxicity of the daily consumption of ethanol, regardless its alcohol content.

Future studies must be considered to investigate changes in molecular level of intestinal epithelial cells through this intermittent alcohol consumption model. Likewise, it is suggested to evaluate changes in the immune system using this experimental treatment regimen.

Acknowledgments

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