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Activity of exoglycosidases in blood, urine, cerebrospinal fluid, and vitreous humor in individuals who died from ethyl alcohol poisoning

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Lysosomal exoglycosidases, namely α-mannosidase (MAN), α-fucosidase (FUC), β-galactosidase (GAL), and β-glucuronidase (GLU), are of crucial importance in breaking down the oligosaccharide chains of multiple glycoconjugates. Those enzymes liberate monosaccharides from non-reducing ends of oligosaccharide chains. In this study, we have aimed to assess the potential utility of MAN, FUC, GAL, and GLU activities as indicators of ethanol abuse in individuals who died from ethanol intoxication, while also investigating the mechanisms underlying their deaths. The study group comprised 22 fatal ethanol-intoxicated individuals, while the control group included 30 deceased individuals whose body fluids showed no traces of alcohol. We measured the activities (pKat/mL) of MAN, FUC, GAL, and GLU in the supernatants of blood, urine, cerebrospinal fluid as well as vitreous humor. The results indicated significantly lower activities of MAN ($p = 0.003$), FUC ($p = 0.008$), GAL ($p = 0.014$), and GLU ($p = 0.004$) in the urine of individuals poisoned by ethanol as compared to the control group. Additionally, there was a significantly lower activity of MAN in the vitreous of those affected by ethyl alcohol poisoning ($p = 0.016$).

Abbreviations

MAN	α-Mannosidase
FUC	α-Fucosidase
GAL	β-Galactosidase
GLU	β-Glucuronidase

At present, consumption of alcoholic drinks gives rise to a notable public health concern associated with increased mortality rates. It is estimated that approximately 4.9% of the global adult population is affected by alcohol abuse. As an illustration, each year, around 88,000 fatalities occur in the United States due to alcohol misuse[1–3]. Additionally, alcohol addiction impacts more than 2% of the world's population, with prevalence rates of 3.4% in the United States and 4% in Europe[2,4]. According to the data provided by the World Health Organisation (WHO), excessive alcohol consumption affects over 7% of the global populace, with prevalence rates surpassing 13% in the Americas and exceeding 16% in Europe. Among adults, binge drinking emerges as a predominant pattern of alcohol consumption, with one in every three alcohol consumers displaying this behavior. However, it is essential

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to acknowledge that alcohol abuse may also lead to fatal consequences[3,5]. Furthermore, in 2015 the estimated prevalence rate of heavy episodic alcohol consumption among the adult population equaled 18.4%. In Europe, the prevalence rate of heavy episodic alcohol use is observed to be the highest worldwide[2].

The consumption of ethanol in high amounts may adversely affect various bodily organs (such as heart, brain, liver, and kidneys)[6–9]. Chronic alcohol use may lead to specific behavioral and psychological changes, causing functional and structural abnormalities in the nervous, circulatory, or immune systems[7,10,11]. Moreover, alcohol may harm the salivary glands, liver, pancreas, and other endocrine organs[8,12,13]. The exact mechanism of ethanol's toxic effects in poisoning remains incompletely understood[14]. There is substantial evidence pointing to kidney damage resulting from ethanol poisoning[15].

The recent data indicate that acute alcohol intoxication causes significant changes in the cerebral cytokine profile, and the stress associated with alcohol use profoundly affects the central biosynthesis of cytokines[16]. Additionally, certain enzymes like α-mannosidase (MAN; EC 3.2.1.24), α-fucosidase (FUC; EC 3.2.1.51), β-galactosidase (GAL; EC 3.2.1.23), and β-glucuronidase (GLU; 3.2.1.31) are considered lysosomal exoglycosidases responsible for breaking down oligosaccharide chains in glycoconjugates (including glycoproteins, glycolipids, and proteoglycans) by releasing monosaccharides from their non-reducing ends[17–20]. However, no investigation has been conducted on the activities of MAN, FUC, GAL, and GLU in biological materials from individuals who died from alcohol intoxication. Thus, the information on the research of MAN, FUC, GAL, and GLU activities in the fluids of deceased individuals due to ethanol intoxication is still lacking.

Materials and methods

The experiment involved two categories of deceased individuals. The study group comprised 22 subjects (2 women and 20 men) who had died as a result of acute fatal alcohol intoxication. The diagnostic criteria for qualifying the deceased for this study as having died of acute alcohol intoxication included the demonstration of the blood ethanol concentration of ≥ 4 g/L as well as the exclusion at autopsy of any other causes of death not related to ethanol intoxication. The control group involved 30 individuals (8 women and 22 men) who had died due to suicides, traffic accidents, or other unfortunate incidents, and no traces of alcohol had been detected in their bodies. The age of the deceased ranged from 15 to 83 years, with the mean age of 49 years. The individuals with kidney, liver, cancer, and rheumatoid arthritis illnesses were excluded from the research. In order to minimize the risk of postmortem interval influencing the activity of the enzymes under consideration, the corpses affected by postmortem processes of a decomposed nature were also excluded from the study. The study protocol was approved by the Local Ethical Committee of the Medical University of Bialystok (No. R-I-002/82/2013). All of the procedures were conducted in adherence to the applicable guidelines and regulations, including the Declaration of Helsinki. The informed consent was secured from the legally authorized representative or guardians of the deceased individual. Samples were taken at the forensic autopsy as soon as possible, approximately 12 h after death. Such a rapid time of sampling allowed us to minimize the risk of the post-mortem interval affecting the activity of the enzymes at issue. Post-mortem samples were collected by means of a syringe from various locations: blood from the femoral vein, urine from the bladder, cerebrospinal fluid from the lateral ventricle, and vitreous from the eyeball. Ethanol concentration in each sample was determined by means of the head-space analysis. After having determined the ethanol concentration, the remaining biological samples were centrifuged to eliminate cells and debris at 3000 g for 20 min at a temperature of 4 °C. Furthermore, the obtained supernatants were aliquoted, frozen, and stored at a temperature of – 80 °C in Eppendorf tubes pending the biological analysis[21].

The activities (pKat/mL) of MAN, FUC, GAL, and GLU in the urine, cerebrospinal fluid as well as vitreous supernatants were assessed by means of the method developed by Marciniak et al.[22]. Following that method, p-nitrophenol (formed from p-nitrophenyl derivatives of several carbohydrates) was assayed colorimetrically. Specific substrates (obtained from Fluka Chemie; Sigma, St. Louis, MO, USA) were utilized for assessing each exoglycosidase: p-nitrophenyl-α-D-mannopyranoside (MAN), p-nitrophenyl-α-fucopyranoside (FUC), p-nitrophenyl-β-D-galactopyranoside (GAL), and p-nitrophenyl-β-D-glucuronide (GLU). The analytical procedure consisted of combining 10 µl of the tested material (vitreous humor / cerebrospinal fluid / urine supernatants) with 40 µl of the appropriate buffer (phosphate-citrate buffer, pH 4.3 for FUC, GAL and MAN, and acetate buffer, pH 4.5 for GLU). The reaction mixtures were incubated at 37 °C for 60 min, being stirred constantly. The enzymatic reaction was stopped by adding 200 µL of 0.2 mol/L borate buffer, pH 9.8. The absorbance of p-nitrophenol (released by the respective exoglycosidases) was assessed at a wavelength of 405 nm using the Elx800 microplate reader (BioTek, Winooski, Vermont, USA)[22].

Since the blood of the deceased subjects bears traces of hemolysis, and the hemoglobin released from the erythrocytes could affect the results of colorimetric determination, in this study, a modification of the method for determining lysosomal exoglycosidases developed by Chojnowska et al. was used for determining the activity (pKat/mL) of MAN, FUC, GAL, and GLU in the hemolyzed blood[23]. The modified method proceeded as follows: 50 µl of serum bearing traces of hemolysis, 200 µl of the appropriate buffer (phosphate-citrate buffer pH 4.3 for FUC, GAL and MAN and acetate buffer pH 4.5 for GLU) and 150 µl of the appropriate substrate for the respective lysosomal exoglycosidase (p-nitrophenyl-α-D-mannopyranoside for MAN, p-nitrophenyl-α-fucopyranoside for FUC, p-nitrophenyl-β-D-galactopyranoside for GAL, and p-nitrophenyl-β-D-glucuronide for GLU) were added to 2 ml tubes. The content of the tubes was mixed, and the tubes were placed at 37 °C for 60 min. After incubation, hemoglobin, and other proteins were precipitated with 5 µl of saturated aqueous trichloroacetic acid (TCA) solution, after which the mixture was centrifuged for 5 min at 14,500 *rpm*. 80 µl of each supernatant was transferred onto a microplate (96 well U Transparent) and then 200 µl of 0.4 mol/L borate buffer at pH 9.8 was added to each well. The amount of released p-nitrophenol was measured at 410 nm twice: first—against the reagent mixture (10 µl of distilled water + 70 µl of the corresponding buffer + 200 µl of 0.2 mol/L

borate buffer, pH 9.8), second—against a blank of hemolyzed serum (10 µL of hemolyzed serum after precipitation of hemoglobin and other proteins with saturated TCA + 70 µL of the appropriate buffer for the respective lysosomal exoglycosidase + 200 µL of 0.2 mol/L borate buffer, pH 9.8)[23]. All the surveys were performed twice for each sample. The means were considered to be the final values. The activities of MAN, FUC, GAL, and GLU underwent the statistical analysis by means of Statistica 12.5 (StatSoft, Cracow, Poland). Normal distribution was checked for the entire dataset that presented as mean ± standard deviation. In order to evaluate the differences between the study and control groups, the U Mann–Whitney test was used. Additionally, the Kruskal–Wallis analysis with post hoc tests was conducted to compare those two groups. The statistical dependence between the two variables was measured by means of the Spearman's rank correlation coefficient. $p < 0.05$ was set as a statistical significance[22–25].

Human experiment statements

The research received acceptance No. R-I-002/82/2013 from the Local Ethical Committee of the Medical University of Bialystok. All procedures were conducted in adherence to applicable guidelines and regulations, including the Declaration of Helsinki. Informed consent was secured from the legally authorized representative or guardians of the deceased individual.

Results

The control group consisted of sober individuals in whom ethanol was not found in their body liquids. The study group comprised deceased individuals in whom very high levels of alcohol were detected in their bodies, as follows: 4–4.5 g/L in blood, 4–6.1 g/L in urine, 4–5.3 g/L in cerebrospinal fluid, and 4–4.9 g/L in vitreous humor. The statistical analysis with the division into genders was not carried out due to a very small number of women.

The control group was compared to the study group in terms of the age of the deceased and the activity of MAN, FUC, GAL, and GLU enzymes in blood, urine, cerebrospinal fluid, and vitreous humor. The analysis did not show any meaningful differences in terms of age ($p = 0.416$). Significantly lower activities of MAN ($p = 0.003$), FUC ($p = 0.008$), GAL ($p = 0.014$), and GLU ($p = 0.004$) were found in the urine of people poisoned with ethanol as compared to the control group. The significantly reduced MAN activity ($p = 0.016$) was also found in the vitreous humor poisoned by ethyl alcohol. In the remaining samples, marked differences were not found (Table 1).

In blood all of the enzymes demonstrated a significantly higher activity as compared to the other samples of both groups under consideration. In the control group in the vitreous humor, higher activities of MAN ($p = 0.005$), GAL ($p < 0.001$), and GLU ($p = 0.001$) were found as compared to urine. In the study group, there were no significant differences between the vitreous humor and urine in all the enzymes tested. Effective differences between the vitreous humor and cerebrospinal fluid were found in terms of the FUC concentration, both in the control and study group ($p = 0.049$ as well as $p = 0.026$, respectively). There was also a substantial difference in the GAL activity between the cerebrospinal fluid and urine ($p < 0.001$) (Fig. 1).

The study has also shown the existence of dependency among the examined enzymes: MAN, FUC, GAL, and GLU within a given sample in the study groups. The highest correlations were found between all the enzymes in the cerebrospinal fluid, similar in the control and study groups (all r coefficients were found to be positive and

Variable	Sample	C group	A group	p
Age		54 (15–83)	46 (26–82)	0.416
MAN [pKat/ml]	Blood	409.62 (54.48–2987.75)	510.01 (103.58–4066.15)	0.535
	Vitreous humor	15.40 (3.40–21.12)	11.35 (3.30–24.01)	**0.016***
	Cerebrospinal fluid	27.72 (5.38–210.11)	17.76 (7.20–109.69)	0.162
	Urine	39.30 (10.16–968.61)	14.46 (2.09–125.58)	**0.003****
FUC [pKat/ml]	Blood	309.06 (35.65–1781.10)	267.20 (21.19–3262.65)	0.331
	Vitreous humor	26.55 (3.67–61.41)	24.65 (5.41–51.08)	0.279
	Cerebrospinal fluid	61.14 (15.20–272.77)	64.15 (11.64–140.01)	0.828
	Urine	47.55 (12.81–1193.35)	16.93 (5.25–467.47)	**0.008****
GAL [pKat/ml]	Blood	358.16 (100.89–3058.20)	357.49 (109.97–4969.25)	0.637
	Vitreous humor	21.61 (9.99–42.58)	17.42 (6.66–31.41)	0.173
	Cerebrospinal fluid	29.14 (2.29–213.29)	31.85 (5.89–111.37)	0.992
	Urine	84.22 (30.33–1774.95)	33.07 (12.98–234.11)	**0.014***
GLU [pKat/ml]	Blood	227.68 (52.46–2492.30)	225.66 (78.69–2388.30)	1.000
	Vitreous humor	23.41 (6.59–53.61)	20.57 (4.54–33.56)	0.151
	Cerebrospinal fluid	24.65 (4.57–183.26)	22.60 (11.77–93.12)	0.598
	Urine	67.26 (16.95–1002.24)	26.50 (7.16–179.52)	**0.004****

Table 1. The activity of exoglycosidases in the blood, vitreous humor, cerebrospinal fluid, and urine of the deceased with no ethanol intoxication (C) and intoxicated with ethanol (A). *A* the deceased intoxicated with ethanol, *C* the deceased with no ethanol intoxication, *FUC* α-fucosidase, *GAL* β-galactosidase, *GLU* β-glucuronidase, *MAN* α-mannosidase. The asterisks above the bolded value mean meaningful differences among A and C groups; statistical significance: *$p < 0.05$, **$p < 0.01$.

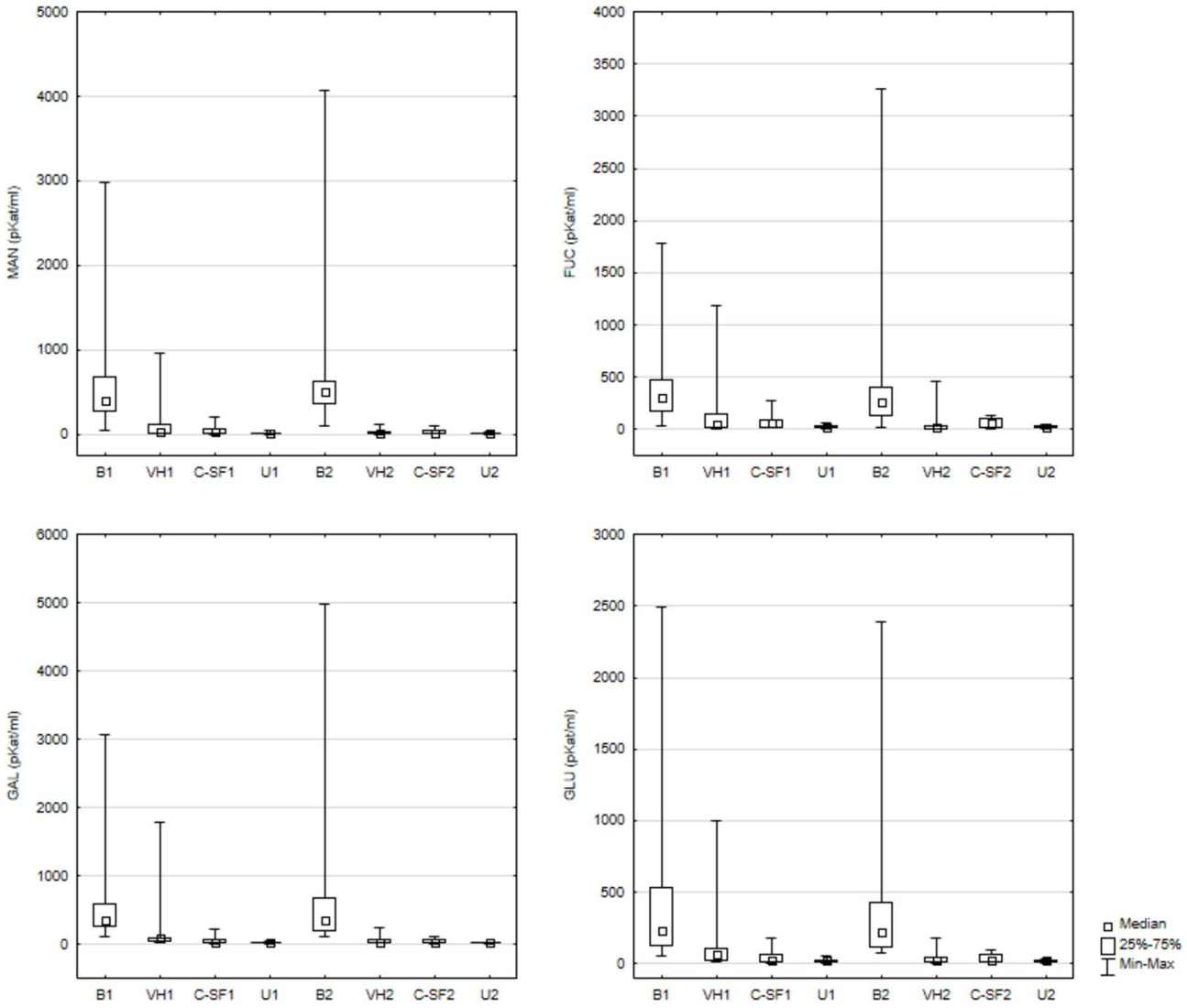

Figure 1. The activity of exoglycosidases in the blood (B), vitreous humor (VH), cerebrospinal fluid (C-SF), and urine (U) of the deceased individuals: without ethanol intoxication (1) and intoxicated with ethanol (2). 1: C: deceased with no ethanol intoxication; 2: deceased intoxicated with ethanol; *B* blood, *CS-F* cerebrospinal fluid, *FUC* α-fucosidase, *GAL* β-galactosidase, *GLU* β-glucuronidase, *MAN* α-mannosidase, *U* urine, *VH* vitreous humor. Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

most of them were contained within the range of 0.8–0.9, $p < 0.001$). Strong correlations were also found between the levels of the tested MAN, FUC, GAL, and GLU enzymes determined in the blood. The strength and direction of the relationship between the levels of those enzymes in the blood and their levels in the other samples under consideration were also evaluated. In the control group, statistically significant positive correlations were found between all the four enzymes (MAN, FUC, GAL, and GLU) in the cerebrospinal fluid and between FUC and GAL in the blood. By contrast, no statistically significant correlations were found between those variables in the study group. Another interesting juxtaposition was found in the level of FUC in the urine and GAL in the blood. In both groups, the correlation coefficients were statistically significant, but in the control group it was positive ($r = 0.491$, $p < 0.05$), while in the study group it was negative ($r = -0.514$, $p < 0.05$). In other findings significant correlation coefficients exceeding 0.5 were observed in one group, while the other group did not show any statistically significant relationship (Table 2). In addition to the correlation between the tested enzymes in each biological fluid, the relationships between the enzymes and the blood alcohol concentration in the study group were also examined. However, no significant correlation was found (Table 3).

Discussion

Alcohol diffusion is commonly acknowledged to follow Fick's law, involving simple diffusion. Trela (1989) proposed a model wherein alcohol diffuses through the following sequence: from blood, through cerebrospinal fluid and vitreous humor, to perilymph, and then in the reverse order during its disappearance. According to Trela's research, instances, where the alcohol level in the blood was lower than in the vitreous humor, indicated the absorption phase[26]. When the concentrations in blood and vitreous humor were similar, it was considered the

Variable	Sample	C group Blood				A group Blood			
		MAN	FUC	GAL	GLU	MAN	FUC	GAL	GLU
MAN	Blood	–	**0.694*****	**0.787*****	**0.766*****	–	**0.441***	**0.426***	**0.609****
	Vitreous humor	0.019	−0.212	0.091	−0.055	**0.457***	−0.135	−0.040	0.173
	Cerebrospinal fluid	0.357	**0.551****	**0.547****	**0.447***	0.219	−0.214	−0.076	0.217
	Urine	**0.439***	0.188	**0.510***	**0.438***	−0.186	**−0.510***	**−0.494***	−0.160
FUC	Blood	**0.694*****	–	**0.774*****	**0.681*****	**0.441***	–	**0.885*****	**0.636****
	Vitreous humor	**0.381***	0.149	0.305	0.213	0.249	−0.260	−0.178	−0.071
	Cerebrospinal fluid	0.218	**0.523****	**0.473***	0.309	0.171	−0.012	0.120	0.321
	Urine	**0.510***	0.241	**0.491***	**0.478***	−0.110	**−0.599****	**−0.514***	−0.249
GAL	Blood	**0.787*****	**0.774*****	–	**0.851*****	**0.426***	**0.885*****	–	**0.675*****
	Vitreous humor	-0.297	0.139	0.071	−0.074	−0.080	**0.574****	**0.556****	0.217
	Cerebrospinal fluid	0.162	**0.468***	**0.427***	0.380	0.057	−0.142	−0.005	0.195
	Urine	0.328	−0.074	0.046	0.215	−0.290	−0.239	−0.260	−0.100
GLU	Blood	**0.766*****	**0.681*****	**0.851*****	–	**0.609****	**0.636****	**0.675*****	–
	Vitreous humor	**0.444***	0.104	0.293	0.205	0.344	−0.380	−0.223	0.205
	Cerebrospinal fluid	0.278	**0.454***	**0.504****	**0.390***	0.294	−0.007	0.152	0.285
	Urine	0.388	0.054	0.409	**0.485***	−0.087	**−0.531***	**−0.512***	−0.187

Table 2. Spearman's rank correlation coefficients among the activity of exoglycosidases in the blood and other body fluids of the deceased who were (A) and were not (C) intoxicated by the ethanol. *A* the deceased with ethanol intoxication, *C* the deceased with no ethanol intoxication, *FUC* α-fucosidase, *GAL* β-galactosidase, *GLU* β-glucuronidase, *MAN* α-mannosidase. The asterisks above the bolded value mean statistically meaningful correlations: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Variable Sample	MAN		FUC		GAL		GLU	
	r	p	r	p	r	p	r	p
Blood	0.160	0.476	0.419	0.052	0.389	0.074	0.175	0.437
Vitreous humor	−0.409	0.059	−0.122	0.587	0.351	0.109	−0.358	0.102
Celebrospinal fluid	−0.212	0.343	−0.068	0.764	0.014	0.952	0.051	0.822
Urine	−0.209	0.362	−0.170	0.462	−0.024	0.917	−0.190	0.408

Table 3. Correlation coefficients among the tested enzymes and the concentration of alcohol in the blood in the study group. *FUC* α-fucosidase, *GAL* β-galactosidase, *GLU* β-glucuronidase, *MAN* α-mannosidase.

concentration equalization phase, while lower blood alcohol concentrations indicated the elimination phase[27]. Remarkably, do some individuals exhibit tolerance to high blood alcohol concentrations. Studies have shown that ethanol concentrations higher than 4 g/L are observed in blood, cerebrospinal fluid, and urine[28–30]. Ethanol is also retained in bladder, leading to its higher urinary level, even for hours after having been sensed in blood[31–33].

Waszkiewicz et al. (2018) investigated whether lysosomal exoglycosidases, previously found elevated in binge-drinking young adults, could serve as indicators of toxic alcohol consumption (by both children as well as adolescents). They discovered lower activities of MAN, FUC, GAL, and GLU in the blood of individuals admitted to hospital due to alcohol intoxication. The study also revealed a reliable diagnostic accuracy for a decreased MAN (admission day); good accuracy for MAN, FUC, GLU (next day), and GAL (admission and the following day); and excellent accuracy for the FUC activity (admission day)[34]. However, the above results differ from those obtained by us. The study groups in the two research projects differ dramatically. In this study, the study group consists of deceased adults who succumbed to acute fatal ethanol poisoning. Therefore, that group of subjects may have been chronically consuming ethanol. Waszkiewicz et al. (2018) studied children and adolescents not addicted to ethanol or cigarettes, admitted to hospital with acute but non-fatal ethanol intoxication. Thus, the differences in the results may have been influenced by the age of the subjects, past alcohol exposure or lack thereof as well as the fact that the biochemistry of fatal intoxication may differ from that of acute, non-fatal intoxication[34].

It is noteworthy that acetaldehyde in urine may be significantly elevated in urine even when ethanol levels are no longer detectable[35]. Moreover, urinary metabolites of ethanol, such as acetaldehyde, may be found in higher levels in urine than in blood[36]. Highly reactive acetaldehyde is capable of forming adducts with amino groups (Schiff bases) and sulfhydryl groups of proteins, interfering with some of glycoconjugate metabolic steps, such as decreasing the activity of glycosyltransferases and/or glycosidases[37]. Therefore, we may find lower enzyme activity in urine stored in the bladder.

In the case of alcohol intoxication, higher levels of serum exoglycosidases are due to increased production and secretion, rather than decreased elimination or leakage from damaged cells[37]. Therefore, the fact they don't

increase in our study seems to be due to the lack of their production and secretion in the liver after death. Alcohol abuse may cause renal dysfunction/damage. We have observed lower urinary activity of exoglycosidases in the alcohol-intoxicated group as compared to the non-intoxicated group, which might be due to the alcohol/metabolite-induced renal dysfunction[38]. However, the lack of other signs/markers of renal dysfunction may be a limitation of our study.

In this study, strong correlations were found amongst MAN, FUC, GAL, and GLU as the tested enzymes in blood. The control group exhibited higher correlations as compared to the study group. Additionally, a positive correlation trend was noted between the alcohol level in the blood and the blood activity of FUC and GAL, too. Another research project revealed not only excellent sensitivity as well as specificity but also high accuracy for the activities of MAN, FUC, GAL, and also GLU in saliva, indicating their potential as chronic alcohol consumption biomarkers[17].

However, ethanol intoxication may impair those protective mechanisms. The absence of correlation between the ethyl alcohol level and the activities of lysosomal exoglycosidases in various biofluids (excluding blood) may be attributed to the significant impact of ethanol on the liver and subsequent release into the blood. Instead, the dysfunction of the brain, vitreous humor, and kidney tissues might be influenced by the metabolites of ethanol.

Conclusions

The damage to bodily organs like the brain, vitreous humor, and kidneys could potentially be attributed to ethanol byproducts. Individuals poisoned with ethanol showed notably reduced levels of MAN, FUC, GAL, and GLU in their urine. Additionally, the MAN activity was significantly lower in the vitreous humor. Nevertheless, more extensive studies are needed to assess the appropriateness of exoglycosidases in elucidating the mechanisms of mortality in the case of acute fatal ethanol intoxication.

Data availability

The data will be made available upon request from the corresponding author (I.P.-S.).

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Competing interests

The authors declare no competing interests.

Additional information

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