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The forensic significance of core temperature in identifying primary and secondary hypothermia as a cause of death: A pilot study on Wistar rats

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ABSTRACT

Introduction: Hypothermia is defined as a body core temperature below 35 °C and can be caused by internal or external stress. Primary hypothermia is caused by excessive exposure to low environmental temperature without any medical conditions prior to that. Secondary hypothermia is caused by alteration in thermoregulation by disease, trauma, surgery, drugs, or infections. The aim of the research is to investigate core temperature values in rats subjected to specific water temperatures at five different time points. It focuses on distinguishing between primary and secondary hypothermia in these rats.

Methods: The total 21 Wistar rats were divided into three experimental groups as: Control group rats exposed only to hypothermic condition (n = 7); Alcohol + hypothermia (n = 7); and Benzodiazepines + hypothermia (n = 7). The temperature spots analyzed in the study were: normal core temperature, core temperature during injection of 0,3 ketamine, temperature of immersion and the temperature at the onset of hypothermia and temperature at the time of death.

Results: In our study the comparative analysis of body temperatures at various time points following submersion in water revealed significant differences among the study groups treated with either alcohol or benzodiazepines and the control group. Notable differences were observed in baseline temperature, post-anesthesia induction temperature, and immediate post-submersion temperature. Specifically, significant differences were discovered among the alcohol and benzodiazepine groups ($p < 0.001$) and ranging from the alcohol and control groups ($p < 0.001$). The analysis of survival times following induced hypothermia revealed a statistically significant difference among the three experimental groups ($p = 0.04$), though subsequent post-hoc comparisons did not demonstrate significant differences in mean survival times.

Conclusion: There is a difference in survival time between primary and secondary hypothermia groups, depending on consumption and intoxication with alcohol or benzodiazepines. The analysis of survival times following induced hypothermia showed a statistically significant difference among the groups.

1. Introduction

Hypothermia is a non-specific condition in the forensic field of research. It is defined as a condition that occurs when core internal temperature is approximately 2 °C below physiological body temperature in range 37.5–37.7 °C [1]. Depending on the values of the core temperature, mild (35–32 °C), moderate (32–28 °C) and severe (<28 °C) hypothermia are distinguished. The forensic significance is distinguished by the difference between primary and secondary hypothermia [1]. Primary hypothermia can be the cause of death if the person was directly exposed to low temperature until the failure of the body's thermoregulatory and metabolic residues. In contrast, secondary hypothermia occurs if, along with exposure to low temperature, we find the action of another etiological factor, such as alcohol, drugs in the sense of overdose or acute intoxication, which affect the body's capability to keep its constant inner temperature [2]. Exposure to low

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temperatures can result in localized hypothermia, systemic hypothermic injuries even death [3]. The key factor is the length of exposure of the organism to low temperatures, but also the compensatory ability of the organism in the thermoregulatory struggle for life. Vulnerable groups particularly sensitive to prolonged exposure to cold environments are the geriatric population and adolescents, while infants and children have a biochemical mechanism different from adults that aids in prolonged thermoregulation. During the inspection, different environmental locations can be detected as dry or wet hypothermia [4].

Depending on the length of exposure to cold and based upon pathophysiological variations, hypothermia is classified as acute, subacute, and chronic [5]. Acute hypothermia most commonly happens after being immersed in freezing water. In circumstances where the body is submerged in freezing water, acute hypothermia takes over, intense oxidative stress affects the thermoregulation process, body overcomes heat production cools down in the shortest time possible. In such conditions of extreme oxidative stress, the mechanism explains that the thermal conductivity of water is 20 times higher than the thermal conductivity of air [6]. When the environmental exposure factor is water, it is important during the autopsy to thoroughly assess the mechanism of death and the cause of death, whether it is hypothermia, drowning, or the vagal reflex that can lead to cardiac arrest. In subacute hypothermia, prolonged hypothermia does not exist, and cooling occurs only under stress. In chronic hypothermia, victims are exposed to moderate cold for longer periods of time, which most often occurs in winter months. Since hypothermia sometimes does not provide macroscopically visible evidence forensically, it has become one of the most puzzling questions in the field of forensic pathology for decades. Books and scientific papers of forensic sciences, report signs on autopsy such as red liver, purple spots on the extremities, red blood, Wischnewski erosions in the stomach, pancreas bleeding, reduction of lipids and sometimes vacuolation of liver cells and other similar minor symptoms of hypoxia [7].

In studies of cold exposure and hypothermia, identifying specific physiological markers is crucial for accurate diagnosis. The postmortem diagnosis of hypothermia remains problematic even in the era of molecular and digital diagnostic advances. Gross hemorrhages in iliopsoas muscles have been regarded as a helpful diagnostic sign in hypothermia fatalities; nevertheless, they have received marginal attention since their original description.

During autopsies, psoas muscle bleeding and a positive Puparev sign can serve as significant indicators of severe hypothermia. Psoas muscle bleeding, observed as hemorrhagic areas within the muscle tissue, often results from prolonged exposure to cold, leading to vasoconstriction and compromised blood flow. The Puparev sign, characterized by subcutaneous hemorrhages in response to cold, further corroborates the presence of hypothermia. These findings are instrumental in confirming cold exposure as a contributing factor to death, particularly when coupled with other clinical and environmental evidence. [8].

Consequently, the diagnosis of hypothermic death is determined by a combination of observations, input of exposure history, nonspecific pathologic findings when other lethal factors are present or absent. There is no evidence about core temperature intervals in postmortem analyses. Furthermore, morphological changes are general non-specific and do not allow unequivocal diagnoses of death from the hypothermia [9–13].

The aim of the research is to determine the core temperature values of rats exposed to a target water temperature range of 6–8 °C at five time points: Baseline temperature (°C), Temperature after anesthesia induction (°C), Temperature at 0 min after submersion (°C), Temperature at the onset of hypothermia (°C), Temperature at 10 min after submersion (°C), and Temperature at the time of death (°C) in rats with primary and secondary hypothermia.

2. Methods

The study was conducted as a prospective experimental, randomized study using the albino Wistar rat model of hypothermia. The research took place at the Veterinary Faculty, University of Sarajevo, following the Principles for the Care of Laboratory Animals [14]. After obtaining approval from the Ethics Committee of the Veterinary Faculty of the University of Sarajevo (registration number 07–03-161–2/23), Bosnia and Herzegovina, 21 Wistar rats weighing between 200 to 250 g were divided into three experimental groups:

1. Control group exposed only to hypothermic action (n = 7) – primary hypothermia
2. Alcohol group + hypothermia (n = 7) – secondary hypothermia
3. Benzodiazepines group + hypothermia (n = 7) – secondary hypothermia

2.1. Anesthesia

The rats were anesthetized with ketamine administered intramuscularly into the m. quadriceps. The dosage was numbered using the formula: 1.2 ml/1 kg of body weight, with a variation of +/- 10 %. Ketaminol 10, 100 mg/ml, produced by MSD Animal Health, was utilized for anesthesia [14].

2.2. Probe placement

Before being put in the water, an esophageal probe designed for calculating the inner temperature was inserted into the esophagus (5 cm) of an anesthetized rat [14,15]. The temperature was continuously being monitored using a thermometer (Physitemp Thermalert Model TH-8) and recorded at several points: before being put in the water (Baseline temperature (°C)), after anesthesia induction (Temperature after anesthesia induction (°C)), immediately after immersion (Temperature, 0 min after submersion (°C)), at the onset of hypothermia (Temperature at the start of hypothermia (°C)), 10 min after submersion (Temperature, 10 min after submersion (°C)), and at the time of death (Temperature at time of death (°C)). The thermometer and temperature probe are manufactured by Physitemp Instruments, Clifton, USA.

2.3. Experimental introduction of hypothermia

The water temperature has been continuously monitored using a probe submerged in the water and readings from a thermometer. An anesthetized rat, with its head positioned above the water level and secured on a wooden board, was then immersed in pre-cooled ice water set to the target temperature of 7 °C. The survival time was recorded, starting from the moment the rat was immersed in the 7 °C water until the time of death. Hypothermia was defined as a decrease of 2 °C in the internal temperature, as per previous definitions [2,3,16].

2.4. Statistical analysis

Statistical analysis was conducted in R 4.4.0 (R Foundation for Statistical Computing, Vienna, Austria). Descriptive summaries are reported as mean (SD), median (Q1 – Q3), and range (Min-Max) for numerical variables, as applicable. Significance threshold was set at $\alpha = 0.05$, with lower p-values deemed as statistically significant at this level.

Quantitative variables were assessed for normality via Shapiro-Wilk's test and evaluated visually with QQ plots and histograms, while homogeneity of variances was evaluated via Levene's test prior to use of bivariate tests for differences in means between groups. Differences in means were tested using Welch's one-way ANOVA test for variables with unequal variances.

3. Results

3.1. Core temperature

The comparative analysis of body temperatures at various time points following submersion in water revealed significant differences among the study groups treated with either alcohol or benzodiazepines and the control group (Table 1). Post-hoc analyses showed no difference in temperatures in the range from the benzodiazepine and control groups at any timepoint. No significant differences were seen among the groups at the onset of hypothermia, 10 min post-submersion, and at the time of death regarding body temperature.

Notable differences were observed at baseline temperature (Fig. 1), post-anesthesia induction temperature (Fig. 2), and immediate post-submersion temperature (Fig. 3). Specifically, significant differences were found between the alcohol and benzodiazepine groups ($p < 0.001$) and between the alcohol and control groups ($p < 0.001$).

3.2. Survival time

The analysis of survival times following induced hypothermia revealed a statistically significant difference among the three experimental groups ($p = 0.04$), though subsequent post-hoc comparisons did not demonstrate important differences in mean survival times (Table 2).

Specifically, the alcohol-treated group exhibited the shortest mean survival time of 9.14 min, while the control group demonstrated the longest mean survival time of 11.29 min. The minimum observed survival time was 5 min, recorded in a benzodiazepine-treated subject, and the maximum was 13 min, recorded in a control subject (Fig. 4).

In the evaluation of the time from submersion to the onset of hypothermia (Fig. 5), the benzodiazepine group had the lowest mean time of 2.43 min, whereas the control group had the highest mean time of 4.29 min. This variation was not statistically important. Notably, an outlier in the control group resisted the onset of hypothermia for 16 min. The median times to achieve hypothermia were 2 min for the control group, and 3 min for both the alcohol and benzodiazepine groups.

Regarding overall survival time from submersion to death, the mean times for the alcohol and benzodiazepine groups were both 12.14 min, compared to 15.57 min for the control group (Fig. 6). Median survival times were 12 min for the alcohol group, 13 min for the benzodiazepine group, and 14 min for the control group. These differences were not statistically significant. One control subject exhibited an exceptionally prolonged survival time of 29 min from submersion to death, whereas the shortest survival time observed was just 6 min, recorded in a benzodiazepine-treated subject.

4. Discussion

Given that we are in a decade of the most extreme climate changes, many global burden studies have provided arguments of the correlation between surrounding temperature and death rate [16,17]. The forensic significance of climate change directly linked to sudden and unexpected deaths has increased, along with interest in this subject following weather extremes and in response to reports on climate change [18]. When it comes to climate change, it is important to emphasize that both supremely high and low temperatures result in fatal outcomes, and they are subject to increasingly frequent forensic investigations. The detailed pathophysiological mechanisms connecting exposure to suboptimal temperatures as well as mortality risk have not been explored to the fullest [17].

Study results indicate that environmental temperature is responsible for an increase in the number of deaths worldwide, accounting for 7.71 % of global mortality in the last decade. The majority of this mortality burden is caused by days cooler than the optimal temperature (7.29 %), compared to days warmer than the optimal temperature (0.42 %). In Bosnia and Herzegovina, there have been more cases on autopsy where the cause of death was hypothermia than heatstroke. The report obtained in the literature is that most deaths are caused by long-term exposure to moderately hot and freezing temperatures, with acute exposure to extreme peaks being less common. The largest study to date, published in The Lancet, was based on the largest dataset ever collected to assess the association between temperature, health, and mortality, including over 74 million deaths from 13 countries [17].

The biological processes underlying cold-related mortality mostly involve respiratory and circulatory effects, affecting vasoconstriction, blood viscosity, and inflammatory responses [18–21]. The established physiological body temperature of rats is 37.5–37.7 °C, while hypothermia is defined by a 2-degree temperature decrease [1]. The forensic significance of hypothermia is multifaceted as individual temperatures are vulnerable, and autopsy findings are often nonspecific. Identifying hypothermia as a cause of death was always challenging in forensic pathology due to the lack of specific, consistent, or even negative macro/microscopic findings. Even though the presence of certain indicators like frost erythema, Wischnewski spots, bloody discoloration of synovial fluid in the knee, bleeding into the synovial membrane strongly suggest fatal hypothermia, their absence does not rule it out. Postmortem biochemical research is valuable for detecting adaptation responses to cold stress and metabolic changes following cold exposure. However, ethanol or benzodiazepine intoxication can hinder the occurrence of adapting reactions to cold, making the diagnosis less evident. Despite numerous trials in the field of hypothermia with immunohistochemistry, biochemical analyzes and molecular pathology, there are still no

Table 1
Comparison of temperatures at different timepoints between groups.

	Group				Pairwise comparisons (p-value)		
Variable	**Alcohol (N=7)**[1]	**Benzodiazepines (N=7)**[1]	**Control (N=7)**[1]	**p-value**[2]	**Alcohol vs. Benzodiazepines**[3]	**Alcohol vs. Control**[3]	**Benzodiazepines vs. Control**[3]
Baseline temperature (°C)	38.8 (±0.24)	37.9 (±0.29)	37.6 (±0.39)	**<0.001**	**<0.001**	**<0.001**	0.2
Temperature, after anesthesia induction (°C)	38.6 (±0.30)	37.8 (±0.25)	37.4 (±0.34)	**<0.001**	**0.001**	**<0.001**	0.093
Temperature, 0 min after submersion (°C)	37.8 (±0.46)	37.2 (±0.37)	36.9 (±0.52)	**0.015**	0.073	**0.040**	0.5
Temperature, at the start of hypothermia (°C)	36.3 (±0.38)	35.2 (±0.40)	35.6 (±0.44)	0.074	0.7	0.2	0.3
Temperature, 10 min after submersion (°C)	32.6 (±1.88)	31.6 (±1.86)	31.9 (±1.96)	0.7	>0.9	>0.9	>0.9
Temperature, at time of death (°C)	29.0 (±0.67)	28.4 (±1.84)	28.7 (±0.88)	0.7	>0.9	>0.9	>0.9

[1] Mean (±SD).
[2] One-way analysis of means (not assuming equal variances).
[3] Games-Howell post-hoc test, corrected for FDR by Benjamini-Hochberg.

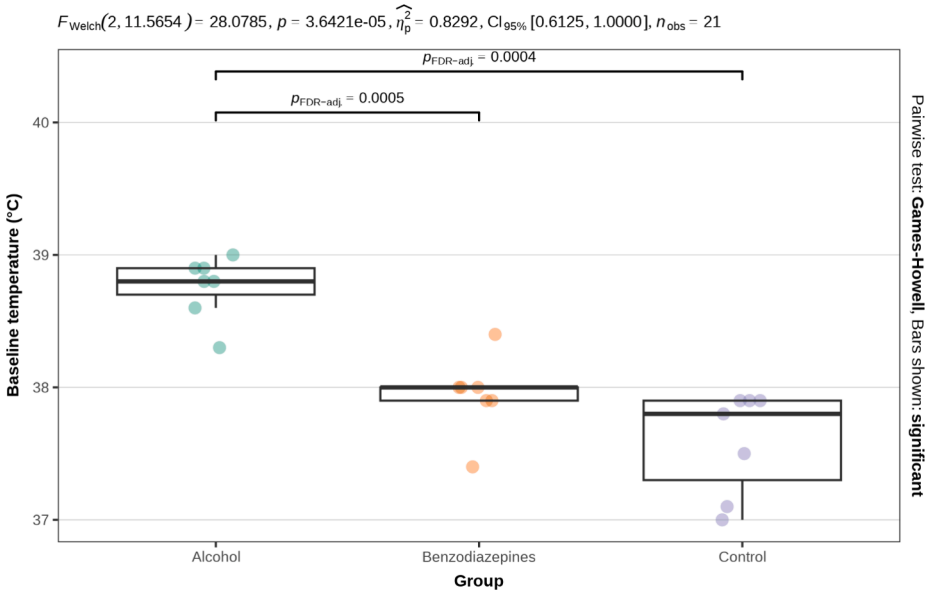

Fig. 1. Baseline temperature between groups.

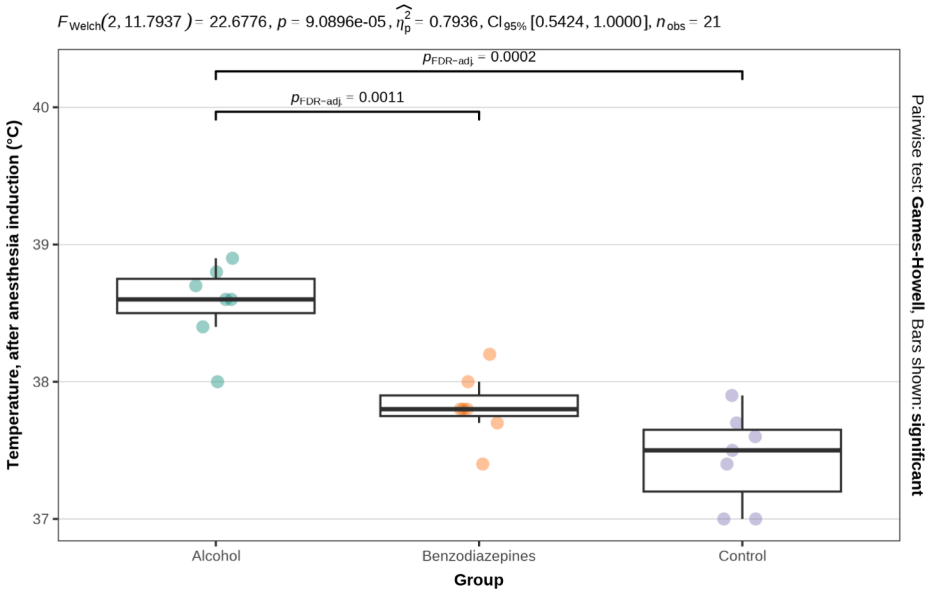

Fig. 2. Temperature, after anesthesia induction between groups.

pathognomonic signs of fatal hypothermia. Our study encompassed three groups: a control group, a group administered benzodiazepines, and a group of alcohol consumers. Before administering substances, the rats were weighed, and based on body weight, alcohol was orally administered daily in the morning via a probe (Red wine, Vranac-14.0 % alcohol). The dose used per rat was 10 ml/kg body weight, calculated using the proportion formula [19]. Additionally, the dose of benzodiazepine (diazepam) was calculated in the same way, proportionally, and administered daily in the morning. The dose of benzodiazepine (diazepam) was standardized according to pharmacological equations. In the literature, reports related to core temperature, changes due to organism exposure to cold, and divided survival time were not found [20–22].

In our study, the comparative analysis of body temperatures at various time points following submersion in water revealed important differences among the study groups treated with either alcohol or benzodiazepines and the control group. Notable differences were observed in baseline temperature, post-anesthesia induction temperature, and immediate post-submersion temperature. Specifically, significant differences were found between the alcohol and benzodiazepine groups ($p < 0.001$) and between the alcohol and control groups ($p < 0.001$). Post-hoc analyses indicated no significant differences in temperatures between the benzodiazepine and control groups at any timepoint. The difference in core temperature among the groups indicated that those who consumed alcohol had higher temperatures compared to the other two groups. There was also a difference in temperature between the benzodiazepine group and the control. Comparable results were obtained by Kukoyi et al. [23], Soares Filho PR et al. [24], and Lazzarini R et al. [25], where there were differences in temperatures among the groups.

It is necessary to help in determining the cause of death considering temperature differences and effects of possible substances consumption when lifeless bodies are found at low temperatures in comparison to lifeless bodies found at low temperatures previously intoxicated with alcohol or benzodiazepines.

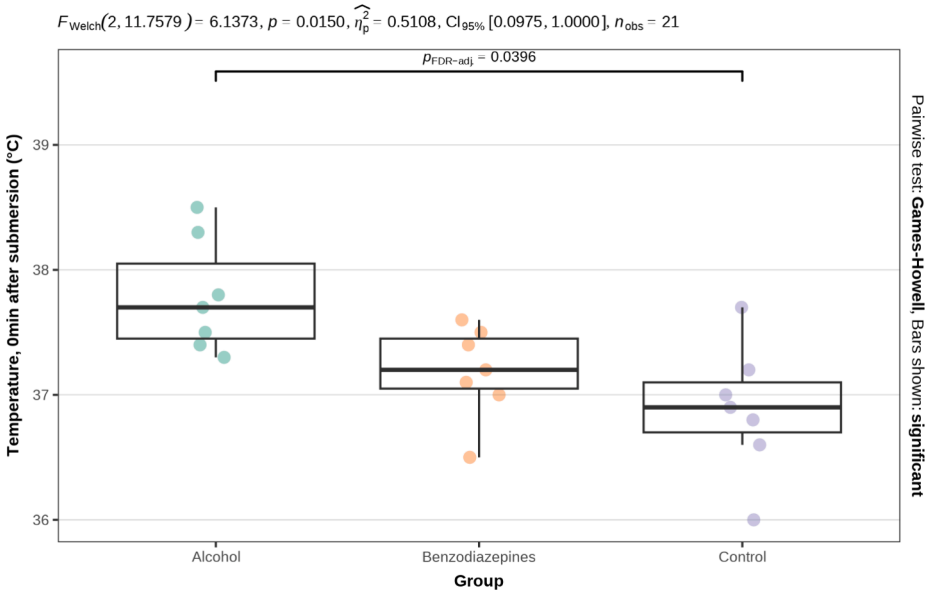

Fig. 3. Temperature, 0 min after submersion between groups.

Table 2
Comparison of time to event between groups.

Variable	Group				Pairwise comparisons (p-value)		
	Alcohol(N=7)	Benzodiazepines (N=7)	Control(N=7)	p-value[1]	Alcohol vs. Benzodiazepines[2]	Alcohol vs. Control[2]	Benzodiazepines vs. Control[2]
Time from hypothermia to death (minutes)				**0.040**	0.9	0.091	0.6
Mean (±SD)	9.14 (±0.90)	9.71 (±2.69)	11.29 (±1.60)				
Median, (IQR)	9.00, (8.50—10.00)	10.00, (8.50—11.50)	12.00, (10.00—12.50)				
Minimum-Maximum	8.00–10.00	5.00–13.00	9.00–13.00				
Time from submersion to hypothermia (minutes)				0.6	0.8	0.8	0.8
Mean (±SD)	3.00 (±1.41)	2.43 (±1.40)	4.29 (±5.35)				
Median, (IQR)	3.00, (2.00—4.00)	3.00, (1.00—3.50)	2.00, (1.50—4.00)				
Minimum-Maximum	1.00–5.00	1.00–4.00	1.00–16.00				
Time from submersion to death (minutes)				0.4	>0.9	0.6	0.6
Mean (±SD)	12.14 (±1.57)	12.14 (±3.02)	15.57 (±6.24)				
Median, (IQR)	12.00, (11.00—13.50)	13.00, (12.00—13.00)	14.00, (12.00—15.50)				
Minimum-Maximum	10.00–14.00	6.00–16.00	11.00–29.00				

[1] One-way analysis of means (not assuming equal variances).
[2] Games-Howell post-hoc test, corrected for FDR by Benjamini-Hochberg.

In older adults, the risk of hypothermia can be increased due to increased heat loss, lowered heat production, or disrupted thermoregulation. This may result from physiological reduction in subcutaneous fat, malnutrition, stress induced illnesses, disorders of the central nervous system, and age-related inactivity. An older person suffering from existing, weakening conditions may experience hypothermia at temperatures of 22 to 24 °C. Other particularly susceptible cases to develop fatal hypothermia include houseless individuals, chronic patients, and individuals suffering from psychiatric illnesses as well as alcohol or drug poisoning. Ethanol is commonly found in the blood of individuals who have died from hypothermia because ethanol accelerates the loss of body heat by causing continuous peripheral vasodilation. Additionally, its intoxicating effects impair decision-making abilities in cold environments and disrupt the body's ability to regulate temperature by affecting the hypothalamus. Additionally, ethanol-induced alterations in the redox potential of hepatocytes impact biochemical responses to cold, such as ketogenesis and gluconeogenesis. Chronic alcoholics, who are often malnourished, are thus more susceptible to hypothermia. Certain pharmacological agents can also influence central thermoregulation, including paracetamol, barbiturates, opioids, tricyclic antidepressants, and benzodiazepines. Diazepam, for instance, can disrupt central thermoregulation and hinder peripheral vasoconstriction in response to cold due to its α-blocking activity. It has been noted that other α-blockers, such as prazosin, cause hypothermia, with older individuals being particularly sensitive to this outcome. Valproic acid has been observed to induce hypothermia in some cases, likely due to a mechanism associated with its agonistic effect on γ-aminobutyric acid [25,26].

The analysis of survival times following induced hypothermia disclosed a statistically significant difference among the three experimental groups ($p = 0.04$), though subsequent post-hoc comparisons did not demonstrate significant differences in mean survival times. Specifically, the alcohol-treated group exhibited the shortest mean survival time of 9.14 min, while the control group demonstrated the longest mean survival time of 11.29 min. The minimum observed survival time was 5

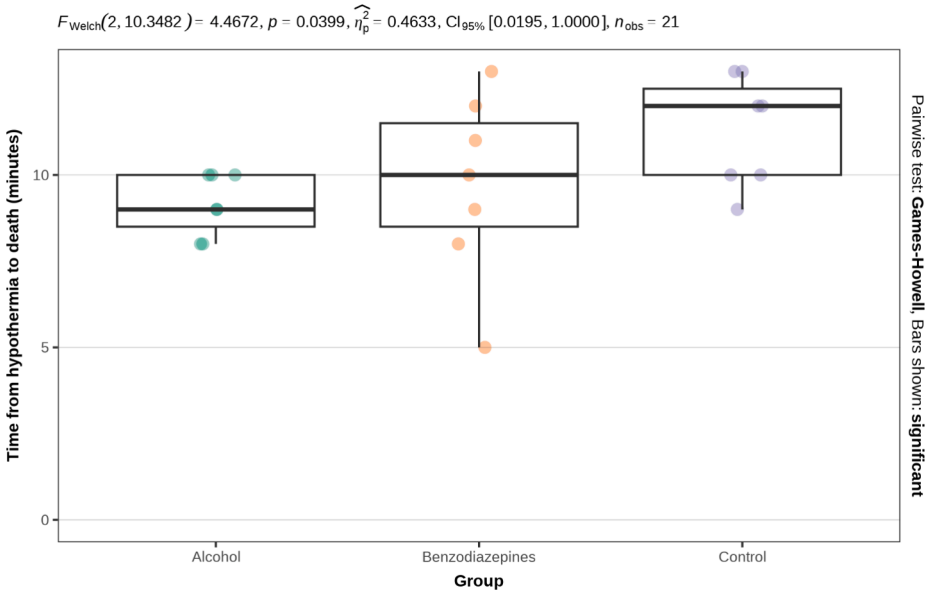

Fig. 4. Time from hypothermia to death between groups.

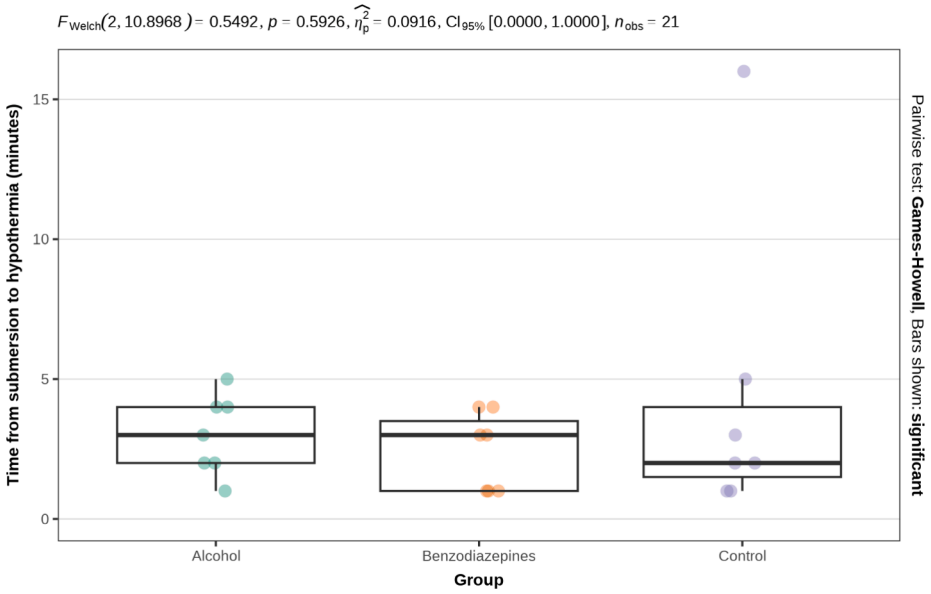

Fig. 5. Time from submersion to hypothermia between groups.

min, recorded in a benzodiazepine-treated subject, and the maximum was 13 min, recorded in a control subject.

In the evaluation of the time from submersion to the onset of hypothermia, the benzodiazepine group had the lowest mean time of 2.43 min, whereas the control group had the highest mean time of 4.29 min. This variation was not statistically significant. Notably, an outlier in the control group resisted the onset of hypothermia for 16 min. The median times to achieve hypothermia were 2 min for the control group, and 3 min for both the alcohol and benzodiazepine groups.

Regarding overall survival time from submersion to death, the mean times for the alcohol and benzodiazepine groups were both 12.14 min, compared to 15.57 min for the control group. Median survival times were 12 min for the alcohol group, 13 min for the benzodiazepine group, and 14 min for the control group. It was concluded that alcohol acutely inhibits all functions of thermoregulation control. Therefore, physiological mechanisms for dissipation of body heat, as well as those for heat production, are disabled by either alcohol or benzodiazepines. Alcohol is a poikilothermic agent; therefore, the observed drop in body temperature in animals exposed to low environmental temperatures is a result of the cold and not a consequence of the assumed "hypothermic" effect of alcohol [27–29]. One control subject exhibited an exceptionally prolonged survival time of 29 min from submersion to death, whereas the shortest survival time observed was just 6 min, recorded in a benzodiazepine-treated subject. Here, it is important to mention the interindividual responses that occur as a true organism's ability to respond differently.

In conclusion, hypothermia can be cause of sudden death of healthy individuals in freezing cold temperatures during winter in cases when they can't get necessary help.

This situation suggests that hypothermia is the only and exclusive cause of death, representing just one aspect of the cases encountered in routine forensic medicine. This is one of the most difficult challenges for forensic pathology since the results so far suggest that we must continue to search for postmortem intervals, survival time, and postmortem

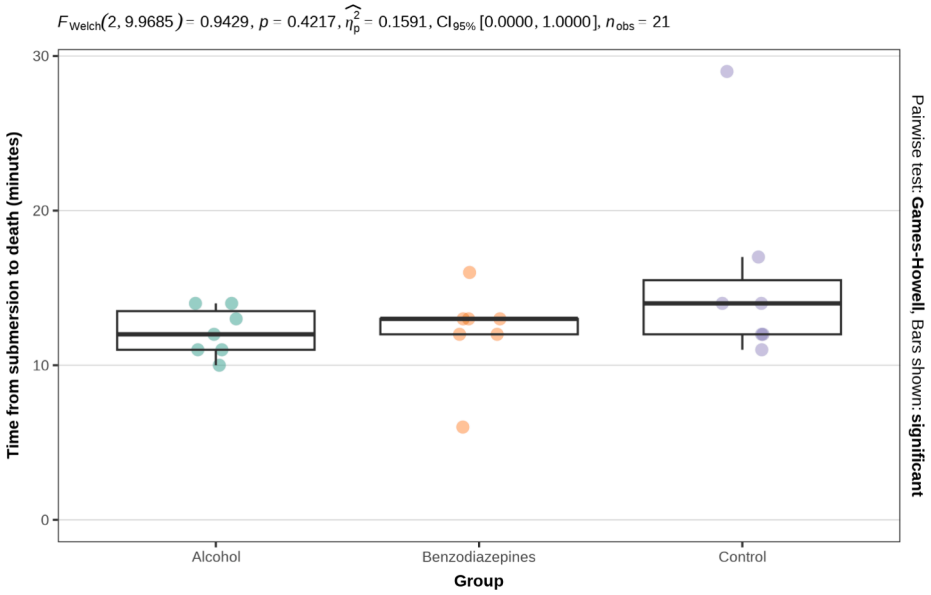

Fig. 6. Time from submersion to death between groups.

biochemical markers to make the diagnosis of hypothermia more specific.

The observation that differences between groups are already present at baseline temperature suggests that inherent physiological or genetic differences might exist prior to the experimental intervention. This could mean that some individuals or groups have pre-existing vulnerabilities or adaptations that affect how they respond to cold exposure.

The lack of significant difference in the time from hypothermia to death between groups, despite baseline temperature differences, indicates that the absolute temperature might not be the sole factor influencing survival. Instead, the rate at which temperature drops or the relative temperature change (temperature difference) could be more critical in triggering hypothermic pathways and affecting survival. This suggests that the relative change in temperature and how quickly the body's thermoregulatory mechanisms are overwhelmed might be more important than just the starting temperature itself in determining outcomes in hypothermia.

Considering previous observations, it is very important to find the most appropriate forensic medical approach to differentiate the ultimate cause of death from low-temperature hypothermia from other secondary causes when exposed to low temperatures. Guidelines should include forensic examination, macroscopic and microscopic analyses, immunohistochemical analyses, biochemistry, and toxicology. A detailed investigation at the scene of death in addition to evidence of prolonged exposure of the body to cold environmental conditions are of paramount importance of ensuring accuracy assessment of all deaths related to hypothermia. Finally, within the forensic pathology community, advocating for a multidomain approach that emphasizes a comprehensive panel of postmortem investigations is essential. This multidisciplinary approach would facilitate a more precise diagnosis of hypothermia in future.

Ethics approval

Ethics Committee of the Veterinary Faculty of the University of Sarajevo (registration number 07-03-161-2/23), Bosnia and Herzegovina.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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