ISSN: 1220-3173; E-ISSN: 2457-7618

BEHAVIOURAL ANALYSIS OF WISTAR LABORATORY RATS UNDER THE INFLUENCE OF WINE AND DIAZEPAM

ANALIZA COMPORTAMENTALĂ A ȘOBOLANILOR DE LABORATOR WISTAR SUB INFLUENȚA VINULUI ȘI A DIAZEPAMULUI

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ABSTRACT | REZUMAT

While there are several studies describing the effects of alcohol and benzodiazepines on the physiological and psychological parameters of laboratory rats, few studies describe their overall effects on their social behaviour. Understanding these factors can serve as solid evidence for further research in humans' social behaviour and contribute to future investigations in animal behaviour and welfare. The aim of our study was to determine the effects of alcohol and a type of benzodiazepine on the behaviour of rats over a period of 24 hours, separately. A sample of 21 Wistar laboratory rats kept in a confined environment was used to determine our findings. Obtained data were analysed and compared statistically with a control group described in a previous study. Inferential statistics indicate that by conventional criteria the difference is considered not to be statistically significant (the two-tailed P value equals 0.9509) between the two analysed studies evaluating various behavioural patterns, according to the T-test. The analysis, therefore, revealed that both substances could interfere with the normal social behaviour of laboratory rats, potentially leading to either positive or negative effects on their behaviour; however, these results are not necessarily notable.

Keywords: laboratory rats, wine, diazepam, welfare

Deși există mai multe studii care descriu efectele alcoolului și ale benzodiazepinelor asupra parametrilor fiziologici și psihologici ai șobolanilor de laborator, puține studii descriu efectele globale ale acestora asupra comportamentului lor social. Înțelegerea acestor factori poate servi drept dovezi solide pentru cercetările ulterioare privind comportamentul social al oamenilor și poate contribui la investigațiile viitoare privind comportamentul și bunăstarea animalelor. Scopul studiului nostru a fost de a determina efectele alcoolului și ale unui tip de benzodiazepine asupra comportamentului șobolanilor pe o perioadă de 24 de ore, separat. Un eșantion de 21 de șobolani de laborator Wistar ținuți într-un mediu închis au fost utilizați pentru a determina rezultatele noastre. Datele obținute au fost analizate și comparate statistic cu un grup de control descris într-un studiu anterior. Statisticile inferențiale indică faptul că, în conformitate cu criteriile convenționale, se consideră că diferența nu este semnificativă din punct de vedere statistic (valoarea P este egală cu 0,9509) între cele două studii analizate care evaluează diverse modele comportamentale, conform testului T. Prin urmare, analiza a arătat că ambele substanțe ar putea interfera cu comportamentul social normal al șobolanilor de laborator, putând duce la efecte pozitive sau negative asupra comportamentului acestora, însă aceste rezultate nu sunt neapărat notabile.

Cuvinte cheie: șobolani de laborator, vin, diazepam, bunăstare

Alcohol experiments on rats were first investigated by Richter and Campbell in the 1940s to model human alcohol abuse (24). More than eighty years have passed since then, and according to Hickman et al. (2017), the laboratory rats are still among the most used animals for testing (9). Use of animal subjects such as the rat to model behavioural patterns specific to humans has been justified from the economical point of view and from the fact that they are easily accessible and available to obtain (24). Easy control over factors like housing conditions, nutrition and previous drug exposure played a pivotal role in the selection of this species for experimental purposes (24). Several treatments were developed for disorders affecting humans' health, e.g., by Griffin (2002) (2). According to the same authors: *'an animal model allows for the examination of neurobehavioral, neurochemical and neurophysiological correlates with the behavioural, physiological or neurological state that is modelled'*. Combining these elements led to the development of new pharmacological and behavioural approaches (2), whilst certain procedures (e.g., physiological manipulation) could not be conducted on human sub-

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jects (24) due to ethical reasons.

Rats bred in laboratory facilities, over time were subject to different housing conditions influencing their behaviour; despite that, several countries designed targeted guidelines and implemented legislative measures on minimal housing conditions under which laboratory animals (namely rats) are kept and rules regarding their care (1).

The European Union safeguards the health and welfare of animals used for testing under the European Directive (2010/63/EU) for the Protection of Animals used for Experimental and Scientific Purposes. In addition, Article 13 of the Treaty of the Functioning of the E.U. considers animals as sentient beings, mentioning that *'full regard should be paid to animals' welfare requirements'* (31), whilst respecting each country's traditions, heritage and practices. Apart from that, the principle of 3Rs is fully regarded and respected in the E.U. (through Regulation 2019/1010), adding an extra layer of protection of animals' rights while accelerating the process of replacing animal experimentation with novel techniques (34). Such an ambitious initiative was started by the Dutch government, which proposes to entirely phase out animal experimentation in the country by 2025 (33). Instead of the 'traditional' concept of 3Rs, scientists included an additional R (responsibility), which stands for the ethical treatment of animals considered sentient beings, afterwards assuring that their welfare is not compromised throughout the research journey (32).

The effects of alcohol on the behaviour of observed laboratory rats

Experimental studies on animals suggest that alcohol exposure, apart from the cognitive deficits associated with foetal alcohol syndrome (FAS), can have major implications for their social behaviour (16). FAS is often described in women consuming large quantities of alcohol during pregnancy, leading to potential dysfunctions of the central nervous system (11). People with FAS were considered to be manifesting changes in the social behaviour (15); however, it is still uncertain whether abnormal behaviours, such as committing a crime against another person (25), are characteristic signs of this syndrome. Humans experience the effects of alcohol in adolescence as a means to facilitate social behaviour (29). During this life cycle, social life is centred on peer relationships, so the social context plays a pivotal role in the responsiveness to alcohol consumption (29). These relationships were investigated in adolescent rats as models to humans (29). Results of the investigation revealed that the effects of ethanol consumption were largely dose-dependent and biphasic (29). While low doses seemed to improve the social activity of individuals, lower doses led to reduced social activity and avoidance of a peer. In a non-social context, however, ethanol suppressed their activity at higher doses, whilst lower doses did not activate the activity to an inanimate object (29).

In a very interesting research paper, authors investigated the effects of alcohol on the behaviour of rat pairs. According to the study, the level of social activity is dose dependent (14). Therefore, at a dose of 1.2 g/kg, all social behaviours showed a decreased level with some small exceptions (e.g., crouches) (14). The proportion of approaches, follows, leaves, mounts, full aggressive postures and social sniffing were among the impacted behavioural patterns (14). With regards to individual actions, similar low results were obtained in the frequency of rears and digging, whilst opposite results were recorded in the number of immobile rests (14). By increasing the concentration to 3.0 g/kg, the intensity of all encountered behaviours increased. Individual walking and self-grooming did not show any change, whilst the level of rearing and digging decreased significantly (14). However, there was an increase in the frequency of immobile rests (14).

In a scientific study by Bell et al. (2006), P rats were used to study the effects of ethanol consumption (2). As described by the authors, these rats were less sensitive to the sedative and aversive effects of ethanol, whilst more prone to its stimulatory effects, being 'alcohol-preferring' subjects developed through selective breeding, which has been used as a model for alcoholism and beyond (2). The high level of tolerance to the behavioural and motor changes induced by ethanol consumption could therefore lead to increased quantities of overall alcohol consumption (14).

The effects of benzodiazepines on the behaviour of observed laboratory rats

Nowadays, benzodiazepines are widely used by medical professionals for different scopes (e.g., to induce muscle relaxation in patients). Despite their known side effects, they are also utilised to enhance anaesthesia protocols, manage epileptic seizures, treat anxiety (15) and several other psychological and neurological disorders, depending on their characteristics and severity. According to Weber (1985), intake of benzodiazepines has been found to negatively impact the neural, physiological and behavioural function in clinical and experimental trials (30).

Although the effects of alcohol on the behaviour of humans are well described thanks to many investigations performed on laboratory rats, a scientific study indicated that administration of a 5 mg/kg dose of midazolam in these animals increased the overall consumption of ethanol within two hours after its administration (23). By increasing the dose to 10 mg/kg, similar results were obtained (23). Regardless of dosage, a taste reactivity test indicated that midazolam increased the chance of a hedonic orofacial response whilst suppressing the frequency of passive drippings (23). Similar results were observed during the administration of other benzodiazepines such as diazepam (5 mg/kg) or chlordiazepoxide (10 mg/kg) in alcohol-naïve rats (23). According to Mikulecká et al. (2014), benzodiazepines' (BZDs) effects are particularly mediated by their interaction with the BZD receptor binding site, which alters the effectiveness of the g-aminobutyric acid, the primary inhibitory neurotrans-

mitter (19). Effects of benzodiazepines were blocked by pre-administration of flumazenil (selective $GABA_A$ receptor antagonist), suggesting that agonists may increase the consumption of ethanol by increasing its flavoured hedonic characteristics (23). Mikulecká et al. (2014), suggested in their findings that postnatal exposure to clonazepam (CZP) reduced play behaviour independently from factors such as age or the experimental environment but had no effect on the social investigatory response itself (19). The same authors also described that intense wrestling and inhibition of the genital investigation were recorded in situations when rats were confronted with an intruder in their known environment (represented by the cage) (19). On the other hand, when neonates were exposed to lorazepam, the length and frequencies of submissions to the intruder increased (8). Therefore, short-term post-natal exposure to CZP leads to inhibitory effects on the social play behaviour while altering other patterns depending on the age and environment (19). Another study investigating the persistent behavioural changes in neonatal and adult rats after administration of benzodiazepines indicated that early therapy had little effect on the acquisition or retention of passive avoidance (5). Other effects on the behaviour after diazepam (10 mg/kg, alone or combined with 15 or 30 mg/kg of caffeine) administration in neonate and adolescent rats were time dependent and described as: increased paddling and forward walking (on days 5-7), myoclonic jerks (on day 7) and spontaneous loss of the righting reflex (6). Despite that, only significant intake of benzodiazepines led to disturbed physical development in rats (27).

MATERIALS AND METHODS

This study was performed between the 10th and 11th of February 2024. as a prospective experimental, randomised study based on the Wistar albino rat drawing model. The research was conducted at the vivarium of the University of Sarajevo - Veterinary Faculty, in compliance with the local legal requirements for welfare and protection of animals during trials. In addition, the experiment was subjected to Bioethical Committee approval (registration number 07–03-161–2/23), and all animal welfare standards were in line with the provisions stipulated in Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. For the assessment, 21 Wistar laboratory rats of mixed genders were allocated. All rats weighed within the range of 250 g and 300 g, while their age varied between 16 and 20 weeks each. Rats under the influence of alcohol were marked with picric acid on the head, while those under the effects of diazepam were marked at the base of their tail with the same substance. Based on their body weight, they were given alcohol orally through a tube every day in the morning (red wine-14.0 % alc.). The dose used per rat was 10 ml/kg of body weight. The same procedure was used for the administration of diazepam; however, its dose was standardised according to the pharmacological equations to 5 mg/kg. Rats were housed in a spacious cage located in the vivarium room, which accommodated other cages housing laboratory rats of different age and gender. Access to the room was possible through a wooden door equipped with a metal mosquito net designed to stop potential intruders or escapees. The group of rats was housed under reserved lighting with alternating periods of twelve hours of daylight and darkness. Mean room temperature was 23°±3°C, and recorded humidity was 50%±10% during data collection. Mechanical ventilation was provided by a built-in ventilator, and non-mechanical ventilation was assured through a window equipped with a metal net. The standard laboratory cage was made of horizontal galvanised wire bars to enable animals to stretch and climb, which measured 101 x 46 x 34 cm (L x W x H). The inferior part of the cage was manufactured of non-transparent solid plastic material, which was filled with a layer of at least 5 cm height sawdust. Hygienic conditions were assured by a technician regularly sanitising the cages and providing new bedding to the animals. Rats had ad libitum access to food (a complete feed mixture) and bottled water. Their diet was further supplemented by various cereals, vegetables, fruits, bread, nuts and seeds.

Data collection

Laboratory rats were recorded 24/7 with a Xiaomi Smart Camera C300 360°, resolution 2304 × 1296 2K, 3 megapixels, with a recording angle of 180° shooting. They were monitored in real-time through the Mi Home application (Wi-Fi - Wi-Fi IEEE 802.11b/g/n 2.4GHz) for the entire assessment, then recordings were stored on a Transcend SD card with a storage capacity of 64 GB. Recordings were analysed in the VLC media player program. Once analysed, data were introduced in a Microsoft Excel sheet to prepare the ethogram, with the most prevalent behavioural patterns occurring in rats under the influence of wine and diazepam. Raw data (recordings) were analysed by J.A. and validated by A.A. to determine the inter-observer reliability. Behavioural patterns were analysed every 15 minutes (see Results chapter) at a population level. Despite that, some particularities were observed in the two groups of rats housed together. Further on, the scientific literature was consulted to validate the findings of our study.

Statistical analysis

For the purpose of statistical analysis, the unpaired T-test was used to determine the statistical significance of our sample compared to the results of a previous study. Collected data was analysed quantitatively by using one type of graphical representation: scatter with a smooth line and markers.

In order to determine the inter-observer reliability, we used the same technique (unpaired t-test) and list of behavioural patterns described by Ališah et al. (2024) with some small adaptation (e.g., scratching was classified as grooming in the current study).

Therefore, after each 15 minutes of video analysis, a specific pattern was allocated, and in the end the sum of each of them was divided by 24 (hours). The final results represented the time distribution of each behavioural pattern that occurred during an entire day and their overall proportion. Data was compared to findings of Ališah et al. (2024) to determine the effects of wine and diazepam on laboratory rats' social behaviour. We must mention that since there was no significant statistical difference between the assessments of Ališah et al. (2024) (behaviours recorded in September 2023 compared to December 2023), we calculated their average and compared those results with the findings of this study.

RESULTS AND DISCUSSIONS

Although forced administration of alcohol and benzodiazepines could trigger several animal welfare issues (e.g., handling-related stress, fear, and anxiety) in laboratory rats, the closest and most reliable model would be the voluntary intake of such substances. However, this technique is highly questionable due to rats' different physical, physiological and emotional needs (e.g., some may drink more than the others, while the volume cannot be adjusted) but could represent a great model for mirroring humans' behaviour. According to the results, more than three-quarters (82.30%) of the laboratory rats under the influence of wine and diazepam showed excessive signs of grooming compared to more than half (59.00%) during their normal behaviour (Fig. 1).

Smolinsky et al. (2009) described grooming as an intricate and ethologically rich behaviour in rodents whose activity and patterns can be affected by stress, genetic and pharmacological procedures (22). The same authors also suggest that an effective way to gauge signs of stress and anxiety in wild and experimental animals is by observing grooming behavioural patterns and their underlying microstructure (22). According to Bolles (1960), a proportion of 25%-40% of laboratory rats awake time is spent on grooming, its occurrence being the highest before and after periods of reduced activity (3). These findings of the normal behaviour in rats came to support our findings that wine and diazepam administration led to a significant increase in the frequency of grooming in both groups of laboratory rats, compared to the normal ones (see Ališah et al., 2024). Chasing (5.21% vs. 17.00%) and playing (7.29% vs. 20.00%) seemed to be less frequently encountered behavioural patterns compared to the results of our previous study. Although play has intrigued scientists over the decades, its recognition is easy; however, it is not as easy to define it (28). The reason behind it maybe can be attributed to the multifunctional role of it in various animal species (28). Hole (2010) analysed the frequency of play bouts at different ages, suggesting that play was most frequently encountered at 31-50 days of age. Overall, the frequency of play bouts ranged between 14.9% (at 51-60 days of age) and 38.6% (at 41-50 days of age) (10). In terms of chasing, Ališah et al. (2024) described it as a 'typical behaviour of a rat following one or more conspecifics in order to gather information, steal food and engage in play or fight' (1). Play behaviour is usually associated with so-called 'pouncing' and chasing the other individual/s. A decrease in the number of play and chasing behaviours only comes to support our findings that the use of wine and diazepam in laboratory rats may negatively influence the occurrence of such behavioural patterns. Feeding and drinking behaviour were more accentuated (58.34% vs. 51.00%), while exploring the surroundings and jumping and/or climbing showed a decrease (43.92% vs. 46.00% and 17.92 % vs. 24.00%, respectively), the latter aspects being attributed to the sedative effect of diazepam and alcohol's depressant role in the central nervous system. According to a study by Manzo et al. (2014), studying the oral consumption of chlordiazepoxide (CDP) and ethanol after reward devaluation, the authors suggested that during pre-shift sessions 1-10 there was a considerably higher preference for CDP and ethanol

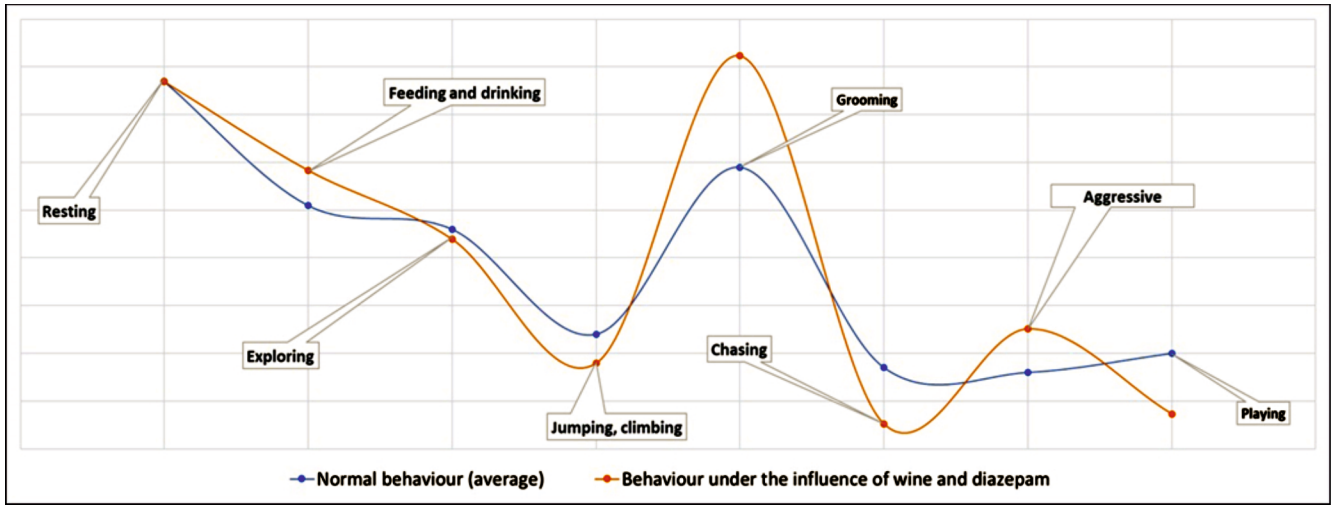

Fig. 1. Comparative behavioural analysis of laboratory rats

compared to water, while during post-shift sessions 11-13 there was a significant increase in the consumption of CDP and ethanol; however, it was not the same case during water consumption (17). In addition, Timberlake et al. (2010) highlighted that forcing laboratory rats to drink alcohol quickly increased their preference for its ingestion in the three high-drinking lines studied, comparable to animals originating from the same line but subject only to the choice condition (26). Regarding food intake, Richardson et al. (1990) demonstrated that when laboratory rats were administered alcohol (or 10% ethanol solution) instead of water, they were capable of maintaining their daily energy intake by reducing the amount of consumed solid food in proportion to the calories attributed to ethanol consumption, suggesting that rats may possess a specific mechanism capable of recognising the caloric properties of ethanol (20). In terms of diazepam's influence on the feeding behaviour, Cooper et al. (1977) indicated that a low dose (2.5 mg/kg) increased the period spent on eating familiar food, whilst higher doses (5 mg/kg) elevated the time expenditure for eating a novel type of food (4 as cited in 7). These results support our theory that alcohol and certain types of benzodiazepines may increase the food intake in laboratory rats; however, this aspect is not applied to water intake, in this situation suggesting exactly the opposite. On the other hand, alcoholised and drugged rats tended to be much more aggressive compared to the results of our previous research (25.13% vs. 16.00 %). According to Miczek et al. (2003), stimulation of GABA receptors suppressed aggression; however, some studies have demonstrated that positive allosteric modulators of $GABA_A$ receptors can elevate the chance of aggressive behaviour (18). Roizen (1994) and various other authors described alcohol as one of the agents that is associated with aggressive and violent behaviour (21). In the meantime, low doses of certain benzodiazepines (e.g., diazepam) were suggested to increase aggressive behaviour in mice or rats, whilst elevated doses decreased it (18). These findings came to support our results. In addition to the analysed list of behaviours, some particularities were observed in each of the two assessed groups (Table 1).

The unpaired T-test revealed a statistically not significant difference (the two-tailed P value equals 0.8948) between the two assessors of behavioural patterns in this study. When results of the two studies were compared (Ališah et al. vs. current study), according to the same test, the difference was not statistically significant (the two-tailed P value equals 0.9509). Although the difference was not statistically significant, which can be indirectly influenced by various reasons, such as, e.g., small sample size, we must not neglect the fact that changes in the normal social behaviour of Wistar laboratory rats can certainly occur if they're under the influence of benzodiazepines or alcohol.

CONCLUSIONS

Laboratory rats are very social animals, like humans, and their behavioural repertoire can be influ-

Table 1

Complementary findings in laboratory rats

Behavioural patterns	
Alcohol related (red wine 14.0% alc.)	Drug related (diazepam)
✓ Yawning	✓ Trampling over other rats
✓ Aggressive licking (cleaning)	✓ Excessive grooming of the genital area
✓ Offensive	✓ Unstable net climbing
✓ Unstable net climbing	✓ Relying on others for grooming
✓ Stumbling across the net	✓ Frozen posture while on the net
✓ Reclining during grooming	✓ Staggering
✓ Digging the substrate	✓ Reduced food intake
✓ Moving (or stealing) food	✓ Defensive
✓ Trampling other rats	✓ Submissive
✓ Jumping out of place	✓ Falling on their backs and laying on the substrate when attacked
✓ Frequent eating and drinking	✓ Climbing on the net over other rats (using them as a support or ladder)
✓ Dominant behaviour	✓ Excessive sleeping
✓ Biting other rats	✓ Resting their heads on the edges of the cage
	✓ Unsteady rear legs
	✓ Sliding the front legs on the edges of the cage

enced by several factors throughout their lifespan. This study highlighted that intake of alcohol or small doses of diazepam could influence laboratory rats' social activity, potentially but not necessarily leading to undesirable behaviours. These situations can be further accentuated by other factors such as housing conditions, stock density or already formed social hierarchy within a specific group of individuals. Therefore, addressing all these aspects in a reliable time manner and with regard to rats physiological, psychological and emotional needs could improve the animal welfare standards. Finally, we must always consider the ethical implications of the use of alcohol and diazepam in animal treatments or experiments, whose benefits should always surpass the disadvantages.

Financial Support and Sponsorship

This research was funded by the Federal Ministry of Education and Science of Bosnia and Herzegovina for the period 2024-2025.

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