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Time-Dependent Assessment Of Diazepam Overdosing On Serum Levels Of Marker Enzymes Of Toxicity In A Mice Model

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Abstract: Diazepam (Dz) is considered to be very safe initially, but later on it has been revealed that it may cause dependency. Since Dz is a drug of abuse, too, it may also cause intentional dependency. This intentional dependency is more serious due to the undefined nature of Dz consumption (Dose and frequency) and subsequent possible toxicity, which are generally not associated with therapeutic use of Dz. To evaluate the cellular toxic impact of Dz abuse, this study aimed to assess the impact of the number of defined overdoses of Dz on the cellular level of established enzyme markers for toxicity, i.e. TRAP-5b, ALT, AST, LDH and GST in the Swiss albino mice model. This study is unique as it monitors the serum level of these enzymes at various time intervals after discontinuation of Dz overdosing. The results of this study established a link between the number of Dz overdoses and irregularities in the serum levels of enzymes. It has been observed that the appearance of irregularities in the serum level of the chosen enzymes has been delayed after discontinuation of Dz abuse. Additionally, it is also evident that the persistence of this abnormality in the serum level of enzymes may depend on the number of doses of Dz (the higher the number of Dz overdoses, the longer the persistence of abnormalities). These irregularities are directly linked to Dz abuse with cellular toxicity as well as bone health.

Keywords: Diazepam, Drug abuse, Drug toxicity, Drug dependency, Bone health

I. INTRODUCTION

Diazepam (Dz), commonly known as valium, belongs to the class of Benzodiazepines (BZDs). Due to its quick response profile, higher efficacy, low toxicity, and cost-effectiveness, Dz got wider acceptance as a wonder drug against a broad spectrum of neuro-physiological conditions, such as anxiety, epilepsy, and depression (Greenblatt et al., 2020; M. Zhang et al., 2022). Dz has high bioavailability as well as high plasma protein binding capacity (96-99%), and plasma levels peak very quickly after consumption. (Wang et al., 2022). Most of the pharmacological effects of Dz have been attributed to the $GABA_A$ Receptor channel. The binding site of Dz on the $GABA_A$ Receptor is different from the receptor's natural ligand GABA and hence Dz works as a positive allosteric modulator(Costa et al., 1978; Everlien et al., 2022; M. Zhang et al., 2022). CYP450 enzymes of the liver are responsible for the metabolism of Dz. In the liver, Dz converted into temazepam and nor-diazepam. These metabolites of Dz are then converted into oxazepam, which undergoes glucuronidation, leading to the subsequent excretion of Dz (Wang et al., 2020; Zubiaur et al., 2022).

Since high therapeutic as well as low toxicity were associated with Dz, the safety profile of Dz was not much investigated before its release as a psychotropic drug. By the 1990s, it had been established that Dz causes

addiction (Wick, 2013). The addiction caused by Dz was linked with chronic use of low levels of the drug rather than high doses of the drug (Zaami et al., 2022). The addiction caused by Dz is intentional (chronic Dz abuse) as well as unintentional (therapeutic chronic use)(O'Brien, 2005). Due to significant morbidity and adverse effects on society, the WHO has categorised Dz under the list of Schedule IV drugs (Edinoff et al., 2022) . The problem with the intentional abuse of Dz is the uncontrolled dose, and this heavy dose of Dz may cause severe toxicity, which may not be the case in the therapeutic use of Dz. Although the toxicity profile of Dz is acceptable, chronic overdose of Dz may cause some significant physiological alterations. To evaluate the gravity of extent of toxicity associated with Dz overdose, this work evaluated the impact of Dz on serum level of various enzymes such as Tartrate-resistant acid phosphatase type 5b (TRAP 5b), Alanine Transaminase (ALT), Aspartate Aminotransferase (AST), Glutathione S-transferase A1 (GSTA1), and D-Lactate Dehydrogenase (D-LDH) in a mouse model. These enzymes have been linked with drug toxicity.

II. MATERIALS AND METHODS

A. Ethical Clearance

The ethical clearance for this study was taken from the Animal Ethical Committee of the Department of Pharmacy, Guru Ghasidas Vishwavidyalaya, Chhattisgarh, India (Ethical Certificate no. 52/IAEC/Pharmacy/2023)

B. Chemicals and Reagents

All the chemicals and reagents are purchased from reputable manufacturers and supplied by local vendors.

C. Animal and Grouping

25 Healthy adult Swiss albino male mice, weighing 25-30 gm and 05-06 weeks of age, were procured from a reputed animal breeder and supplier. These animals were acclimatised and kept under room temperature, humidity, normal conditions and light-dark cycles. The animals have been provided standard mice food pellets as food with free access to drinking water. These experimental animals were divided into five groups (five animals in each group) as follows a) Placebo; only Saline, b) 02 Doses of Dz, c) 04 Doses of Dz, d) 06 doses of Dz and e) 08 Doses of Dz.

D. Dosing of Animals

The Diazepam tablet was dissolved in 0.9% normal saline and administered at 3 mg/kg body weight (b.w.) through the intraperitoneal route on every alternative day, i.e., after 48 hours, until the respective dose had been achieved in every designated group. The dose chosen for this study was 50% higher than the normal reported dose, i.e. 02mg/kg b.w., to develop an overdosed mouse model.

E. Blood collection and serum separation from mouse

The mice of the designated group were kept in the restrainer with gentle heating of the tail vein as described earlier (Rathkolb et al., 2013). The blood was collected from the tail vein (200 µL) from each group on days 2nd, 7th, 14th, and 21st. The day on which the designated dose was completed was considered day 0. The blood was collected in sterile microcentrifuge tubes and kept at room temperature for 01 hour, and then centrifuged at 1000g for 20 minutes. The supernatant containing serum was collected in separate sterile labelled centrifuge tubes and kept at -20℃ for further use.

F. Assessment of Serum Level of Enzymes

The serum levels of all selected enzymes, TRAP 5b, ALT, AST, Glutathione S-transferase A1 GSTA1, and D-LDH were measured using a Sandwich ELISA method, following the manufacturer's protocol (FineTest Incorporation). The sample dilution was 1/50 for this analysis, and ELISAs were performed in triplicate. The detection limit for 0.313-20mlU/ml, 0.156-10ng/ml,0.156-10ng/ml, 0.625-40mlU/ml and 4.688-300mlU/ml respectively.

G. Statistical Analysis

To analyse the link between Dz dose and serum levels of chosen enzymes, One-way ANOVA was performed using GraphPad Prism (Version 5).

III. RESULTS

A. Assessment of Tartrate Resistance Acid Phosphatase 5 (TRAP5) in response to different doses of Diazepam:

Since Tartrate Resistance Acid Phosphatase 5 (TRAP5) is an essential physiological marker. The statistical analysis reveals that the serum level of TRAP was significantly lower ($p < 0.05$) in group D and group E animals (Fig. 1A-D) compared to the placebo after 2 days of stopping the respective dose. Group B and C animals did not show any changes in the level of serum TRAP5 compared to the placebo, but the level in these groups remained significantly high compared to groups D and E ($p < 0.05$). After 7 days of stopping the drug, the level of serum TRAP5 significantly ($p < 0.05$) decreased in all groups compared to the placebo. The day 14 analysis revealed that the level of serum TRAP5 remains considerably lower in group C, D, and E animals compared to the placebo. Still, the level attains normalcy in group B animals. Day 21 analysis clearly indicates the attainment of normal serum TRAP5 levels in group B and C animals; however, this level remains low in group D and group E animals, even at this point.

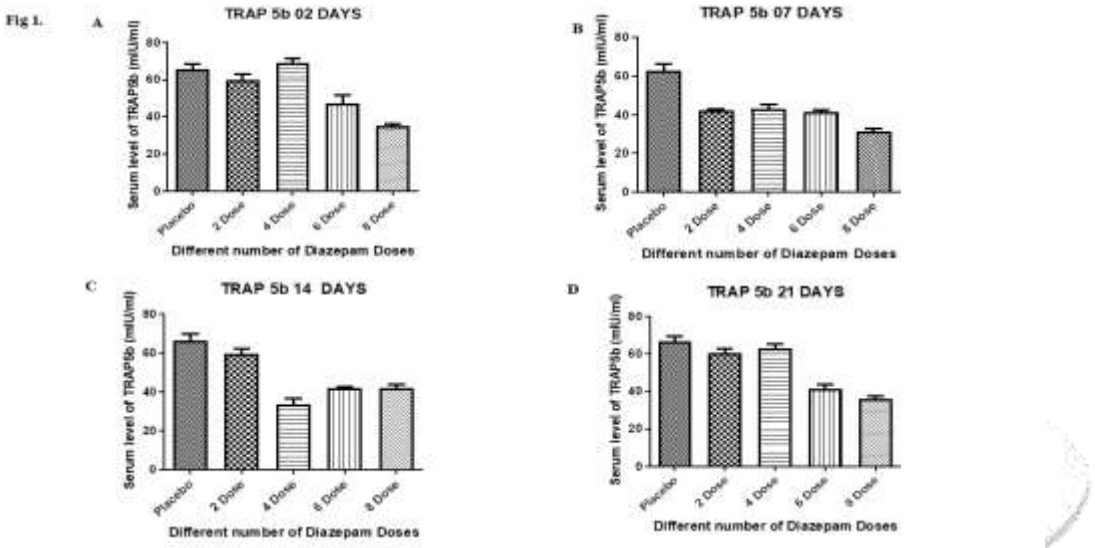

Fig 1. Serum level of Tartrate-resistant acid phosphatase type 5(ACP5) in different experimental group at (A) Day 02, (B) Day 07, (C) Day 14, (D) Day 21 after stopping the respective dose.

B. Evaluation of Serum Alanine Amino Transferase (sALT) levels to link the Diazepam dose and duration with liver toxicity:

One-way ANOVA analysis of the results indicates that no significant changes occurred in the level of sALT in all groups, and trends remain similar at 07 days too. The effect of different doses on the level of sALT was not evident even on day 14. The trends observed on day 14 remain similar on day 21 observation of sALT, which clearly indicates that the DZ consumption has not altered sALT level, irrespective of the number of doses. (Fig 2-A-D).

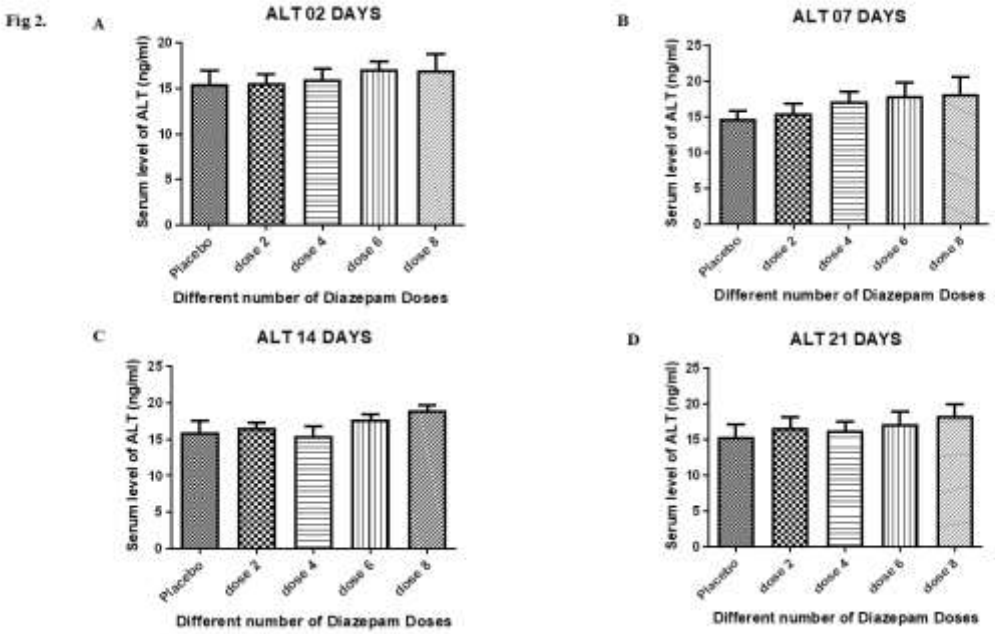

Fig 2. Assessment of liver toxicity; serum level of Alanine Transaminase (ALT) in different experimental group at (A) Day 02, (B) Day 07, (C) Day 14, (D) Day 21 after discontinuation of doses in different mice group.

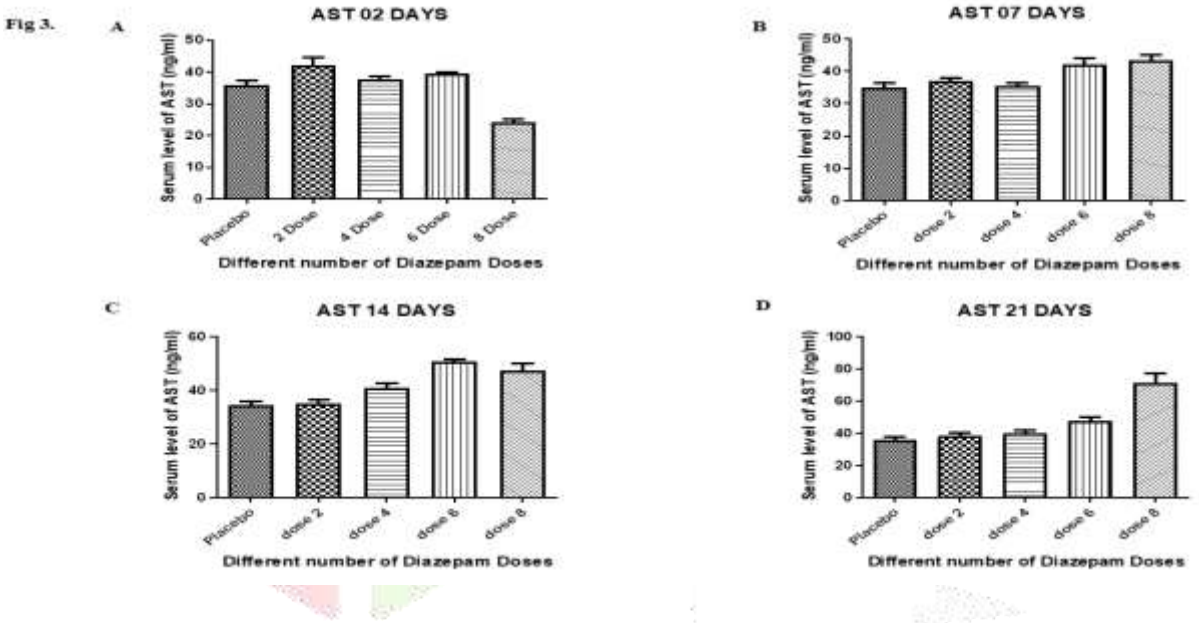

Fig 3. Evaluation of Serum level of Aspartate Aminotransferase (AST) to assess the liver toxicity; S(AST) level was measured through sandwich ELISA in different experimental group at different time interval i.e. (A) Day 02, (B) Day 07, (C) Day 14, (D) Day 21 after cessation the respective dose.

C. Evaluation of Serum Aspartate Amino Transferase (sAST) in different groups of animals:

Interpretation of statistical analysis results indicates that the level of sAST became significantly lower ($p < 0.05$) in Group E animals on day 2 after stopping the respective dose, compared to Group A, i.e., the placebo. This difference is not significant in other groups. On day 07 after stopping the dose, a slightly substantial increase in the level of group D and group E animals was observed in comparison to the placebo, and this trend continues on day 14 too. The evaluation of sAST at day 21 after stopping the dose reveals that group E animals have an elevated level of sAST ($p< 0.05$) in comparison to placebo (Group A), but all other groups did not show any significant difference in the level of sAST at day 21(Fig.3 A-D).

D. Serum D-Lactate Dehydrogenase (sD-LDH) analysis at different time interval in animals having different number of doses of diazepam:

Statistical analysis of ELISA data by one-way ANOVA for sD-LDH reveals that the level of sD-LDH significantly ($p< 0.05$) increased in group B and C animals in comparison to group A but the value was significantly 0.05) in group D and E in comparison to A on day 2 after stopping the dose. There was no significant decrease in ($p< 0.05$*) in group C and a very significant decrease in groups D and E ($p< 0.05$***) on day 14 after stopping the dose. The observation of sD-LDH levels on day 21 showed that the level remains very significantly low ($p< 0.05$***) in group D and E animals, while group C animals attain the level of placebo. (Fig 4. A-D)

E. Evaluation of level of sGST in different animal group in response to different doses of diazepam at different time interval:

The analysis of sGST after 02 days of stopping of the respective doses of Diazepam reveals that there is no significant difference between groups A, B, C and D but very significant difference ($p< 0.05$***) in the level of sGST observed in group E mice in comparison to group A i.e. placebo but remarkably on day 07 after stopping the doses all groups shown very significant decrease ($p< 0.05$***) in the level of sGST in comparison to group A. These trends remain the same for group C and group E, where sGST remain very significantly ($p< 0.05$***) low in comparison to group A animals, but this lowering is less significant in group B (<0.05*) and non-significant in group D in comparison to group A. This difference seems to be clearly linked with the number of doses of Diazepam, as it was evident at 21 days. After stopping the dose of diazepam, the level of sGST was lowered with an increased level of significance with the growing number of doses. (Fig.5 A-D)

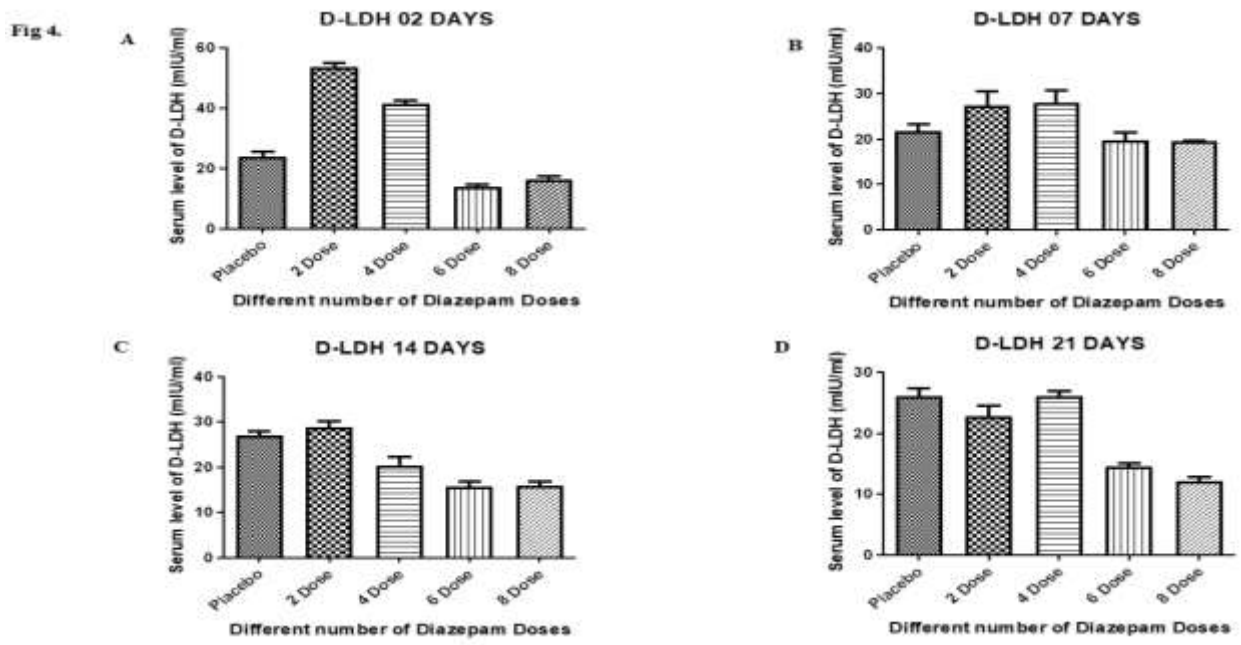

Fig 4. D-Lactate Dehydrogenase (D-LDH) as an indicator of internal tissue injuries; to assess the impact of varied doses of Dz on tissue injuries of D-LDH were performed. The one-way ANOVA results for chosen time interval (A) Day 02, (B) Day 07, (C) Day 14, (D) Day 21 after stopping the respective dose.

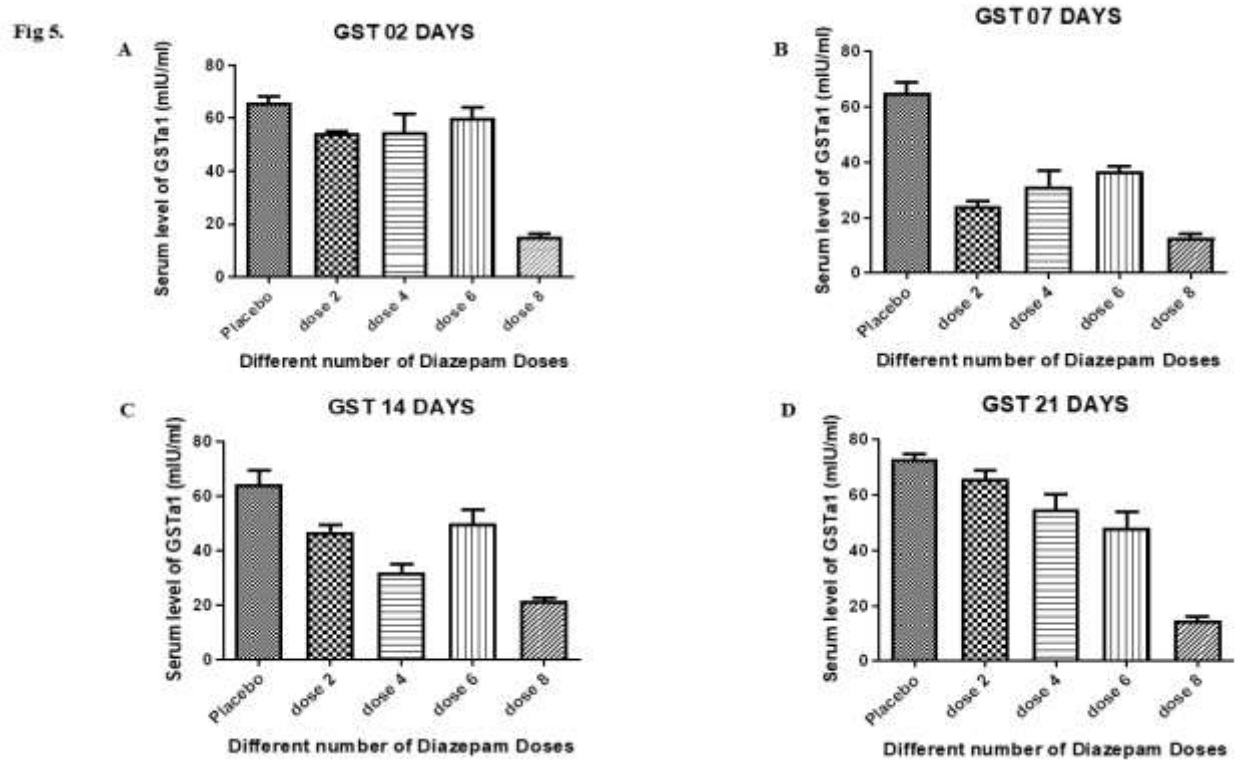

Fig 5. Evaluation of serum level of Glutathione S-transferase A1 (GSTA1); no significant changes were observed in different time interval (A) Day 02, (B) Day 07, (C) Day 14, (D) Day 21 after stopping the varied number of chosen dose i.e. 03mg/kg b.w.

IV. Discussion

Although BZDs is a very effective class of psychoactive drugs, it causes dependency (Intentional or unintentional) (O'Brien, 2005). Generally, abusers use BZDs as a supplement to other, more potent abusive drugs to enhance their potency and duration of influence(Jones et al., 2012). In the current study, the researchers aim to evaluate the levels of five different enzymes in the serum of experimental animals at various time points after the cessation of Dz doses in designated groups. The enzymes chosen in this study are crucial for normal physiological functions and are generally associated with drug toxicity. In this study, the researcher was trying to elucidate the link between the level of these enzymes and the frequency of diazepam overdosing or abuse and the cellular toxicity. Various reports are available which suggest that Dz-induced sleep is different from normal sleep (Kopp et al., 2003; Panagiotou et al., 2025; Pérez Francisco & Vallejo de la Cueva, 2024; Tobler et al., 2001). There is a set pattern associated with natural sleep like slow wave sleep (SWS) followed by rapid eye movement sleep (REM) but Dz inhibits REM and this property of Dz was attributed to being induced by other $GABA_A$ receptors (α_2, α_3 or α_5) instead of usual α_1 $GABA_A$ receptors.(Tobler et al., 2001). It is a well-established fact that normal sleep is crucial for the physiological homeostasis, including the normal functioning of the musculoskeletal system (Okan, 2023). The Tartrate-resistant acid phosphatase (TRAP)-5 is a class of metalloenzymes which mainly perform the catalysis of phosphate ester and anhydrides catalysis in acidic conditions(Janckila & Yam, 2009; Oddie et al., 2000). In mammalian cells, TRAP is used as a marker for osteoclast activities due to its involvement in the reabsorption process by osteoclasts (Lerner, 2000). It has been reported that TRAP isoform 5b is better indicative marker of bone reabsorption (Galliera et al., 2012; Seibel & Meier, 2010; Shidara et al., 2008; Vervloet et al., 2017). The results of this study clearly suggested that the effect of Dz on serum level of TRAP-5b after stopping the dose was not immediate but started to appear after 7 days of and this downregulation in TRAP-5b was significant even after 21 days for a higher number of DZ doses. These observations were in agreement with the previous reports which suggested that chronic use of Dz can affect bone health (Fan et al., 2016; Meier & Kraenzlin, 2011; Van Der Hooft et al., 2008; Verrotti et al., 2010).

Drug abuse has adverse effect on organs functioning specially on liver. The liver is the site for the metabolism of drugs, including abused drugs. As like most of the xenobiotics Dz metabolism occurs in liver through the process of hydroxylation and its glunorid derivatives excreted through urine (Dinis-Oliveira, 2017; Hooper et al., 1992). Although, DZ profile in regards of liver toxicity is considered safer

but chronic overdose or abusive dose of Dz may causes liver injuries. A study in animal model has reported that DZ overdosing had increased the oxidative stress in liver (Abdelmajeed, 2009; Ogueji et al., 2017). To investigate whether the sudden discontinuation of DZ overdosing has any implications on normal liver functioning, the serum levels of ALT and AST were evaluated in this study. ALT and AST are established indicators of hepatotoxicity, and the results of this study were mostly in agreement with previous reports. There is no significant alteration in the serum level of ALT in all groups of animals in comparison to the control, but there are some irregularities in the serum level of AST (At day 2 and at day 21) after discontinuation of the highest number of doses, with opposite directionality in comparison to the control. The one source of these irregularities may be due to the increased bone reabsorption by osteoclasts (Bolam et al., 2012; Y. Zhang et al., 2025), which has been evident through the evaluation of serum TRAP-5b in this study. In continuation of this, the analysis of serum D-LDH was performed to assess the level of total serum D-LDH to analyse the extent of tissue injury caused by the overdosing of Dz. D-LDH is distributed in different types of tissue and is essential for cellular respiration, where LDH catalyses the conversion of pyruvate to lactate (J. Li et al., 2025; X. Li et al., 2022). A clinically high level of LDH in the blood is linked with tissue injury. In this study, the level of serum D-LDH increases in all groups of animals in comparison to the control but these effects were more prominent in animals with a higher number of DZ overdoses and persisted for a longer duration even after discontinuation of doses. GSTs are a group of enzymes that are generally linked with drug metabolism and detoxification(Kumar, 2025; Silva & Carvalho, 2018).Although there is lack of studies which clearly link DZ detoxification with the GST but since, GST have wide substrate specificity, it may be indirectly influence by Dz overdosing. It has been speculated that GST may be indirectly involved in Phase II detoxification of DZ (Alexander & Perry, 1991; Correia, 2018; Crettol et al., 2010). The rationale behind this hypothesis is that DZ has been reported to cause cellular oxidative stress(Abdelmajeed, 2009; Ogueji et al., 2017) and GST is a major component of the defense mechanism against oxidative stress (Sharma et al., 2004). Usually, the level of GST is elevated in response to stress, but the results of this study clearly indicated that DZ overdosing for a longer duration may cause a very significant reduction in serum GST level. These effects persisted even after 21 days of stopping the DZ overdosing. These results are supportive of previous reports, which suggested that chronic use of DZ may impair the protective mechanism against oxidative stress(Kośmider et al., 2023). Since its discovery, Dz has remained one of the most effective psychoactive drugs, which is generally associated with a safer toxicity profile. It has been proven that Dz causes dependency. Since the doses are undetermined in the cases of Dz abuse, the toxicity profile of Dz may be different from therapeutic doses of Dz. Abrupt discontinuation of Dz consumption causes withdrawal syndrome, which may be physical, psychological, as well as physiological (Fluyau et al., 2018; Jones et al., 2012; O'Brien, 2005). The results of this study clearly suggested that long duration of Dz abuse may cause significant physiological alteration, and persistence of this alteration is linked with the number of Dz overdoses. This study focused on physiological alternation after stopping the DZ abuse. It may help design a holistic approach towards the rehabilitation of drug abusers, which not only focuses on physical and psychological withdrawal symptoms but also on physiological parameters. The findings of this study give a new perspective to evaluate the toxicity profile of Dz in case of its abuse.

V. DISCLOSURE STATEMENT: The authors declare no competing Interests.

VI. FUNDING DETAILS: This work was funded by Guru Ghasidas Vishwavidyalaya, Bilaspur (C.G.)

VII. ETHICAL STATEMENT: Ethical clearance was required for this study due to the use of mice. The clearance certification had been obtained from the Animal Ethical Committee of the Department of Pharmacy, Guru Ghasidas Vishwavidyalaya, Chhattisgarh, India (Ethical Certificate no. 52/IAEC/Pharmacy/2023)

VIII. AUTHORS' CONTRIBUTIONS: MY and YP conducted the experiments. AA, MY, and BNU had designed and planned the experiments. AA, MY, and BNU prepared the manuscript. AA BNU, and MY performed the statistical work and analysed the results.

IX. DATA AVAILABILITY STATEMENT: the authors declare that all the data related to this work have been discussed and mentioned appropriately in the manuscript.

X. ACKNOWLEDGEMENTS: The authors acknowledge the institutional and administrative support of Guru Ghasidas Vishwavidyalaya (GGV), Bilaspur, Chhattisgarh, India.

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