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Review article

HISTOPATHOLOGICAL TOXICITY OF DIAZEPAM: AN IN-DEPTH REVIEW

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ABSTRACT

Background -Diazepam is a primary drug for treating neurological diseases, muscle spasms, and alcohol withdrawal symptoms. People usually evaluate and use the sedative effect of the drug on their own without prescription or supervision. Surprisingly, the effect of diazepam on tissue organs is relatively scarce compared to its well-documented pharmacological efficacy. It's on this background that the present study was carried out. This review was conducted following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for Scoping Review (PRISMA-ScR). Most studies on benzodiazepine-induced toxicity were based on case reports, case series, or retrospective analyses, which often lack the rigor of controlled clinical trials. However, Diazepam of 5 mg / kg body weight was characterized as a high dose in experimental animals. The liver often cannot metabolize the entire amount of the applied diazepam and thus accumulates it on prolong use. Histopathological analysis of the liver, kidney, spleen, and thymus treated with diazepam showed markedly thickened capsules and disorganization of cells. There were increased interstitial gaps in the lungs and kidney parenchyma, and abnormal intercalated discs in the cardiac muscles. Intra-sinusoidal macrophages and many cells with karyolytic nuclei were also seen in the kidney. In addition to the increase in the number of hepatocytes and increase in their mitotic index, the number in apoptosis in treated animals organs is also increased. As with any medication, individual patient factors, such as preexisting renal impairment or concurrent use of other drugs, may increase the risk of adverse effects.

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Introduction

Diazepam, first marketed as Valium, is a medicine of the benzodiazepine family that acts as an anxiolytic. It is commonly used to treat a range of conditions, including anxiety, seizures, alcohol withdrawal syndrome, muscle spasms, insomnia, and restless legs syndrome [1]. Its pharmacological actions are mediated through the potentiation of gamma-aminobutyric acid type A (GABA), which is the primary inhibitory neurotransmitter in the central nervous system (CNS) [2]. Diazepam acts by modifying the GABA receptor complex [3], an ionotropic receptor responsible for chloride ion influx upon activation commonly used for several neurological disorders like seizures, anxiety, benzodiazepine withdrawal syndrome, alcohol withdrawal syndrome, sleeping disorder, restless legs syndrome, and spasm of muscle [1,4].

It is also used for purposeful loss of memory during various medical surgeries [5]. In the management of anxiety disorders, the drug offers rapid relief of symptoms and is often used for short-term treatment, particularly during acute exacerbations [6].

Diazepam's muscle relaxant properties make it valuable in alleviating skeletal muscle spasms caused by conditions such as musculoskeletal injuries and spastic disorders. Moreover, it is an effective anticonvulsant agent, both in the treatment of acute seizures and as adjunctive therapy for chronic epilepsy, owing to its ability to enhance GABAergic inhibition [7].

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However, limited information is available on diazepam toxicity. The effect of diazepam on tissue organs is relatively scarce compared to its well-documented pharmacological effects. Most studies on benzodiazepine-induced toxicity are based on case reports, case series, or retrospective analyses, which often lack the rigor of controlled clinical trials.

In addition, many reports involve patients who have taken multiple medications concurrently, making it challenging to attribute organ damage specifically to diazepam. Given the lack of robust evidence linking diazepam to liver or renal toxicity, it is reasonable to conclude that significant adverse effects on organs like liver or kidney function directly caused by diazepam are rare [8]. As with any medication, individual patient factors, such as preexisting renal impairment or concurrent use of other nephrotoxic drugs, may increase the risk of adverse effects. Hence, the present study is needed to elucidate the histopathological changes and assess potential implications for long-term diazepam use.

METHOD

This review was conducted following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for Scoping Review (PRISMA-ScR). We retrieved and examined scientific papers published in electronic databases such as PubMed, Scopus, and Google Scholar. Documents that were published in these databases but could not be downloaded were excluded from the review.

Diazepam Mechanism of Action

The principal way in which the benzodiazepine drug diazepam affects the central nervous system is by altering the gamma-aminobutyric acid (GABA) neurotransmitter system [9]. GABA, which is the primary inhibitory neurotransmitter in the brain, is essential for controlling the

excitability of neurons. By binding to particular locations on the GABA-A receptor, diazepam amplifies the inhibitory effects of GABA, potentiating the GABAergic neurotransmitter and producing a range of therapeutic effects [10]. The ligand-gated chloride ion channel known as the GABA-A receptor has distinct allosteric sites bound by diazepam [9]. Diazepam causes an influx of chloride ions into the neuron by activating the GABA-A receptor, which causes the cell membrane to become hyperpolarized [10].

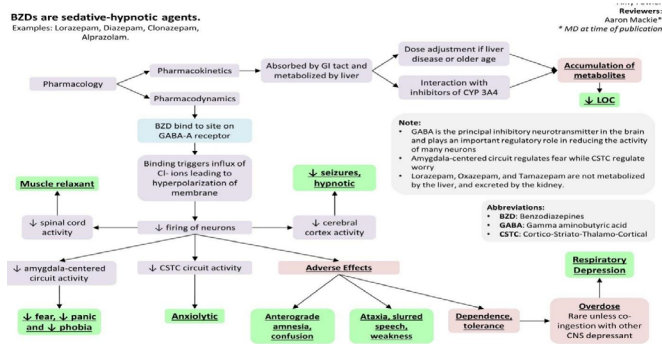


Fig. 1. Mechanism of Benzodiazepines

Diazepam may also modulate the release of other neurotransmitters, including dopamine, serotonin, and norepinephrine, although the precise mechanisms underlying these effects are not fully understood [11]. These neurotransmitter systems are involved in various central nervous system functions, and diazepam modulation of their activity may contribute to its therapeutic and side effects. Multiple sub-units constitute the GABA-A receptor, and different sub-types have different functional and geographical distributions in the brain [12]. The selective binding affinity that Diazepam demonstrates for particular sub-types of the GABA-A receptor is one of the factors that contribute to its range of clinical effects. For instance, diazepam preferentially binds to GABA-A receptors with $\alpha 1$ subunits, widely distributed in brain areas related to anxiolysis and sedation [13]. Diazepam works by allosterically modulating the GABA-A receptor, which increases the GABAergic neurotransmitter, suppresses the central nervous system, and has several other therapeutic benefits [10].

TOXICOLOGICAL EFFECT OF DIAZEPAM

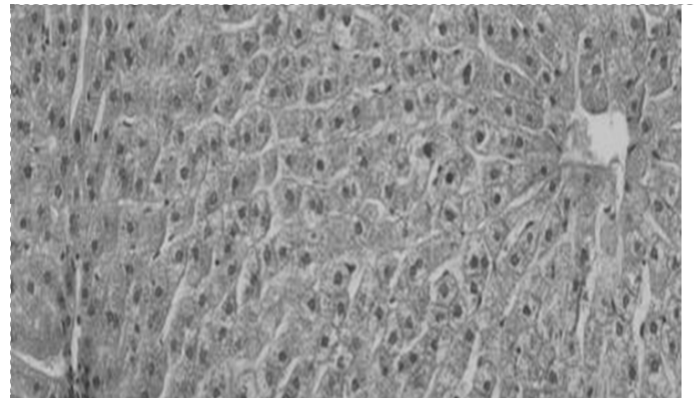
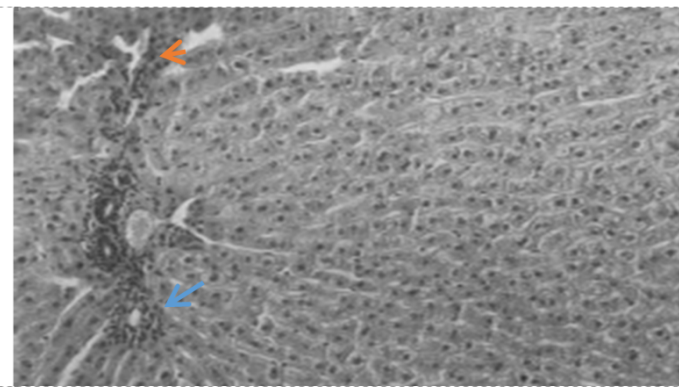
Despite its efficacy, diazepam is not devoid of adverse effects, particularly concerning its potential for organ toxicity. Organ toxicity associated with diazepam primarily revolves around the liver and kidneys, the key organs responsible for its metabolism and excretion. The oral LD50 (lethal dose in 50% of the population) of diazepam is 720 mg/kg in mice and 1240 mg/kg in rats [14]. D. J. Greenblatt and colleagues reported in 1978 that two patients who had taken 500 mg and 2000 mg of diazepam, respectively, went into moderately deep comas and were discharged within 48 hours without having experienced any important complications, despite having high concentrations of diazepam and its metabolites, desmethyldiazepam, oxazepam, and temazepam, according to samples taken in the hospital and as follow-up [15].

The Liver

There is limited evidence to suggest that diazepam, a benzodiazepine medication primarily used for treating anxiety, seizures, and muscle spasms, directly causes hepatotoxicity (liver damage) in humans. Diazepam is metabolised in the liver by the cytochrome P450 enzyme system, primarily via the CYP3A4 pathway, into active metabolites, including desmethyldiazepam and oxazepam. While diazepam and its metabolites undergo hepatic metabolism, they are generally considered to have a low potential for hepatotoxicity compared to other medications like acetaminophen or certain antibiotics [16]. However, it is essential to note that adverse effects on the liver, including hepatotoxicity, have been reported with the use of various benzodiazepines, although they are rare. These effects are typically idiosyncratic reactions, rather than dose-dependent toxicity. Idiosyncratic drug reactions are unpredictable adverse reactions to medications that are not related to the drug's pharmacological action and occur in only a small subset of patients [17]. While the mechanism of benzodiazepine-induced hepatotoxicity is not fully understood, it is believed to involve immune-mediated responses or

metabolic idiosyncrasy rather than direct toxicity.

Fig. 2. Histopathological sections of the normal liver control (a) and liver treated with diazepam (b). Macrophages were detected (red arrow). Close to the central vein, individual mastocytes were seen (blue arrow) [18].



Furthermore, a small number of cases of hepatic injury have been described in patients on oral diazepam, but the clinical pattern has varied. The onset of injury has ranged from 1 to 6 months, and the pattern of serum enzyme elevations has typically been cholestatic or mixed. Fever and rash are uncommon, as is autoantibody formation.

In large surveys and case series of clinically apparent drug-induced liver injury, diazepam and other benzodiazepines are usually not listed. There have been no case reports of hepatotoxicity from diazepam since the 1980s and before the general availability of tests for acute hepatitis A and C and advanced methods of hepatic and biliary imaging [19].

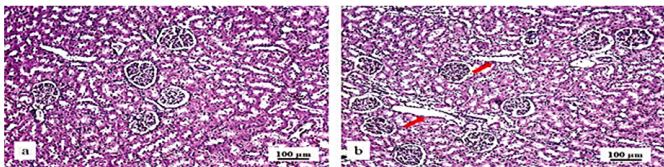
Another study published in the journal "Gut and Liver" in 2011 examined drug-induced liver injury (DILI) cases reported to a Korean national registry. Among the implicated drugs, benzodiazepines were responsible for only a small percentage of DILI cases, with diazepam specifically being infrequently reported [20]. In an experimental study, in liver tissue (Fig. 2) around intralobular vessels, individual macrophages were detected. Moreover, around the central vein, individual mastocytes were observed. We concluded that this was an effect of the mobilisation of specific cells of the immune system of the liver, the so-called stellate cell. The mastocytes present in the perivascular zone of the liver might be used to measure the activation of cells.

The Kidney

While renal excretion of diazepam and its metabolites occurs, significant adverse effects on kidney function related to diazepam use are rare. Study on the renal toxicity of diazepam is limited, and there is a paucity of studies directly addressing this issue. Most information on diazepam potential effects on renal function comes from studies investigating the overall safety profile of benzodiazepines or case reports of adverse drug reactions [21]. Given the lack of robust evidence linking diazepam to

renal toxicity, it is reasonable to conclude that significant adverse effects on kidney function directly caused by diazepam are rare. However, as with any medication, individual patient factors, such as preexisting renal impairment or concurrent use of other nephrotoxic medications, may increase the risk of adverse renal effects. A light microscopic examination of the adrenal cortex of adult male albino rats treated with diazepam showed a markedly thickened capsule with congested blood vessels and disorganization of cells in the zona glomerulosa. Some cells with flat nuclei were noticed in-between the capsule and zona glomerulosa.

Figure 3. Histological cross-section of the rats' kidney, Dilatation of collecting duct (red arrows); control (a) and diazepam-treated rats' tissue. (H&E. X400) [22].

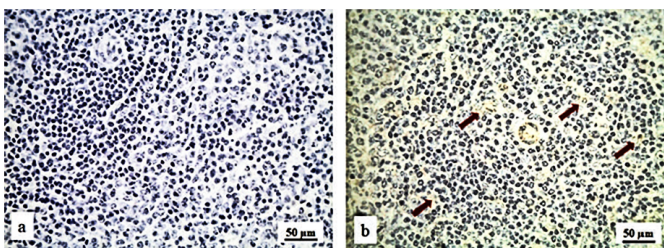


Most of the zona glomerulosa cells exhibited swollen, vacuolated cytoplasm and multiple deeply stained nuclei. Some zona fasciculata cells were observed with dark pyknotic nuclei and cytoplasmic vacuolation. Others showed deeply eosinophilic cytoplasm and deeply stained nuclei. Intra-sinusoidal macrophages and many cells with karyolytic nuclei were also seen. (Figure 2a). Zona reticularis showed congested blood sinusoids. Some cells had dark-stained nuclei (Figure 2b) [23].

The spleen

In our study (Fig. 4) diazepam administration increased inflammation and lymphopoiesis in the rat spleen tissue. documented results in which histopathological analysis of rats spleen, treated for eight weeks with 0.1 mg/kg diazepam, confirm excessive infiltration of the lymphocytes in the red pulp and depletion of lymph tissue of white spleen pulp.. Reason for this increase is increased vascularization and increased number of erythrocytes in the liver tissue of treated rats [22].

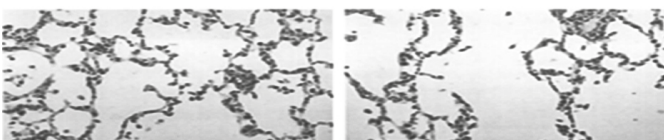
Fig. 4: Histological section of the spleen showing lymphocytes, epithelial cell in apoptosis (red arrows); control (a) and diazepam-treated rats' tissue [22].



The lung

In the lungs (Fig. 5), the cells of macrophages in the parafollicular septum and in close vicinity of the respiratory epithelium, both individual and, in some cases, clustered in groups, were encountered. In the connective endothelial tissue of precapillary blood vessels and in the adventitia of capillaries, individual mastocytes were observed.

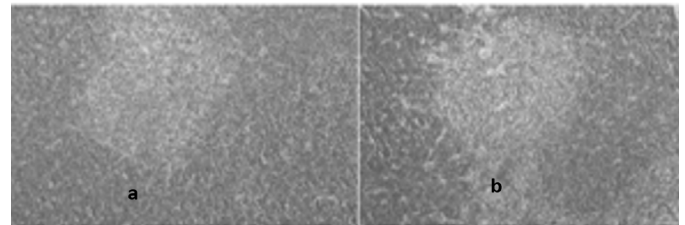
Fig. 5. Histopathological sections of the lungs treated with diazepam (b) and control (a). Cells of macrophages in the parafollicular septum and in close vicinity of the respiratory epithelium [18].



The thymus

In the thymus (Fig. 6), the process of degradation of cells in the stroma of the thymus was observed due to the presence of thymus cells (Hassall).

Fig. 6. Histopathological sections of the thymus treated with diazepam (b) and control (a). Degradation of the stroma cells was observed [18].



The Brain

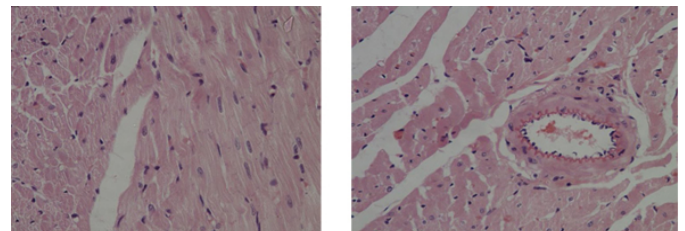
Brain diazepam, a benzodiazepine medication, exerts its primary effects on the central nervous system (CNS) by enhancing the activity of gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain [9]. Through its interaction with the GABA-A receptor, diazepam potentiates the inhibitory effects of GABA, leading to CNS depression, muscle relaxation, anxiolysis, anticonvulsant activity, and sedation [10]. Despite its therapeutic benefits, diazepam also carries the risk of CNS-related adverse effects and toxicity, particularly when used inappropriately or at high doses. Central nervous system depression is a common side effect of diazepam, manifested by sedation, drowsiness, and impaired cognitive and psychomotor function [9]. Diazepam could also cause respiratory depression, particularly when administered intravenously or in high doses [24]. Furthermore, paradoxical reactions, including agitation, aggression, dis-inhibition, and hallucinations, may occur with diazepam use, particularly in susceptible individuals such as children, older adults, or those with psychiatric disorders [25]. Moreover, prolonged use of diazepam could lead to the development of tolerance, requiring higher doses to achieve the desired therapeutic effect [26].

The Heart

While diazepam can cause side effects such as hypotension and bradycardia, severe cardiovascular toxicity is rare and is typically associated with an overdose or concurrent use of other medications affecting cardiovascular function.

Fig 6. Illustrates normal cardiocytes (A) with normal tissue orientation and reduced fat deposits with no extra intercellular space. Diazepam-control rats exhibited more interstitial gaps and abnormal intercalated discs (Fig. 1C) compared to normal control [27].

One of the potential cardiovascular side effects of diazepam is



A. Normal control

c. Diazepam Control

hypotension, or low blood pressure [9]. This effect is primarily due to the medication's CNS depressant properties, leading to vasodilation and decreased systemic vascular resistance. Hypotension may result in dizziness, lightheaded, and syncope, particularly in susceptible individuals. Diazepam may also cause bradycardia, or a slow heart rate, in some patients [28]. This effect is thought to result from the medication's influence on the autonomic nervous system, leading to decreased sympathetic tone and a reduced heart rate. While mild bradycardia is relatively common and usually asymptomatic, severe bradycardia requiring intervention is rare. While these cardiovascular effects are possible with diazepam use,

severe cardiovascular toxicity requiring medical intervention is rare and typically occurs in the context of overdose or patients with preexisting cardiovascular conditions [28]. However, it is essential to note that most evidence regarding Diazepam's cardiovascular effects comes from case reports, case series, or retrospective analyses, which may lack the rigor of controlled clinical trials. In addition, many of these reports involve patients who have taken multiple medications concurrently, making it challenging to attribute cardiovascular effects specifically to diazepam. Despite extensive research, there were few relevant references specifically discussing the cardiovascular toxicity of diazepam. The information provided is based on established pharmacological principles and clinical experience with benzodiazepines as a class of medications used in the management of CNS disorders. However, experimental study has shown that diazepam-control rats (Fig. 6) exhibited more interstitial gaps and abnormal intercalated discs (compared to normal control [27].

HISTOLOGICAL MARKERS FOR DIAZEPAM TOXICOLOGICAL EFFECT

Histological markers are cellular or tissue features commonly used in histopathology to assess tissue damage, changes, or specific cellular characteristics. These markers are visualised through histological techniques such as staining, immunohistochemistry, and electron microscopy. They provide valuable information about the morphology, structure, and function of tissues under investigation [29,30]. Some common histological markers used to assess tissue damage or changes include: Hematoxylin and eosin (H&E) staining. It's a widely used histological technique that stains cell nuclei blue with hematoxylin, cytoplasm, and extracellular matrix pink with eosin. This staining method allows for visualising cellular morphology, tissue architecture, and abnormalities such as inflammation, necrosis, or fibrosis [29].

Proliferating cell nuclear antigen (PCNA): PCNA is a nuclear protein involved in DNA replication and repair. Increased expression of PCNA indicates active cell proliferation, which may indicate tissue regeneration or neoplastic growth [30].

Cleaved caspase-3: Cleaved caspase-3 is an apoptosis-related protein that plays a central role in the execution phase of apoptosis. The detection of cleaved caspase-3 by immunohistochemistry can indicate ongoing apoptotic cell death within tissues [30].

Ki-67 antigen: Ki-67 is a cellular marker of proliferation expressed during all active cell cycle phases but is absent in resting cells (G0 phase). Ki-67 immunostaining is commonly used to assess the proliferative activity of cells within tissues [30].

Collagen deposition: Increased collagen deposition, visualised by techniques such as Masson's trichrome or Sirius red staining, can indicate tissue fibrosis or scarring in response to injury or chronic inflammation [29].

CONCLUSION

The review study provides a comprehensive overview of the adverse effects associated with diazepam, ranging from immediate concerns such as drowsiness and impaired cognitive function to longer-term issues. Diazepam of 5 mg / kg body weight was characterized as a high dose in experimental animals. The liver often cannot metabolize the entire amount and thus accumulates on prolonged use. Histopathological analysis of the liver, kidney, spleen, and thymus treated with diazepam showed disorganization of cells. It underscores the importance of weighing the benefits against the risks when prescribing diazepam, especially for prolonged periods or in vulnerable populations.

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Authors' Contribution: All authors were equally involved in data collection, analysis, manuscript preparation, revision and finalization

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