Co-abuse of alprazolam augments the hepato-renal toxic effects of methylphenidate

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Abstract:
OBJECTIVE: Methylphenidate (MPH) is a first-line treatment option for attention-deficit hyperactive disorder and narcolepsy. MPH is one of the most abused psychostimulants by the adults and young population to stay awake, perform better, or improve concentration. The scanty reports say that the medical users or abusers mostly consider the administration of benzodiazepines to overcome the adverse effects, i.e., mood- and anxiety-related problems associated with MPH chronic abuse. This work aims to study the effect of alprazolam (ALZ) on MPH-associated adverse effects on liver and kidney.

MATERIALS AND METHODS: Female Wistar rats (n = 58) were administered with MPH (10, 20, and 40 mg/kg) and ALZ (5, 10, and 20 mg/kg) alone and in combination for 28 days. Bodyweight, feed intake, and water intake were monitored weekly. Parameters related to liver and renal function, oxidative stress, and histopathology were performed to evaluate the toxic impacts on the liver and kidneys.

RESULTS: ALZ, along with MPH, increased the serum alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, creatinine, and urea levels. The co-abuse also led to elevated oxidative stress and structural abnormalities in the liver and kidney tissues.

CONCLUSION: The co-abuse of ALZ has amplified the hepato-renal toxic effects of MPH. Therefore, it is a significant concern for public safety, and their co-abuse must be restricted and discouraged.

Keywords: Adverse effects, benzodiazepine, oxidative stress, polydrug abuse, psychostimulant

Introduction

Methylphenidate (MPH) is a psychostimulant widely prescribed as the first-line drug option for the treatment of attention-deficit/hyperactivity disorder. Besides its medical applications, MPH is widely known for its abuse potential. The prevalence of stimulant misuse and abuse has expanded globally, mainly due to the reward effects such as increased self-confidence, wakefulness, euphoria, increased energy, and reduced fatigue.¹ The findings from a national survey on substance abuse and mental health services administration reported 7.6% of prescription stimulant misuse in people aged 18–25 years. In contrast, benzodiazepines misuse/death due to overdose increased by 8.5% from 2006 to 2018.² Chronic use/abuse of MPH exerts considerable adverse effects such as anxiety, aggressive behavior, insomnia, depression, increased suicidality, and dependency. On the other hand, alprazolam (ALZ) is a benzodiazepine, often prescribed for the treatment of anxiety, insomnia, and panic attacks.³ Similar to MPH, ALZ also has a high potential for abuse/misuse. There are cases in which the abusers consume stimulants and depressants together, assuming that they can subsidize each

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other’s side effects\textsuperscript{[4,5]} and co-administration of ALZ and MPH is one such instance. Based on the co-abuse reports of MPH and ALZ, this study was designed to investigate the effects of both drugs, alone and in combination on liver and kidney, which are primarily involved in drug metabolism and elimination, respectively.

**Materials and Methods**

**Animals**
Six to eight-week-old female Wistar rats (200 ± 20 g) were acquired from the Central Animal House Facility of Panjab University and were housed in the standard laboratory animal housing environment (temperature: 25°C ± 2°C; relative humidity: 45%–55%) with 12:12h light: dark cycle and ad libitum access to food (Ashirwad Industries, Chandigarh, India) and water. The use of animals approved by the Institutional Animal Ethics Committee (PU/45/99/CPCSEA/IAEC/2018/126) of the Panjab University, and all the experiments were carried out in compliance with the guidelines laid by the Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

**Drugs and kits**
ALZ and MPH tablet formulations were procured from Elder Pharmaceuticals Ltd. (Mumbai, India) and IPCA Pharmaceuticals (Mumbai, India), respectively. Biochemical kits to estimate aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, and serum urea were purchased from Reckon Diagnostic Pvt. Ltd. (Vadodara, India).

**Experimental design**
A total of 58 female Wistar rats were randomly divided into ten different groups, namely: normal control (NC; \( n = 4 \) group was given water and food, three groups of Alprazolam (ALZ; 5, 10, and 20 mg/kg p.o.; \( n = 6/\) group) and three groups of Methylphenidate (MPH; 10, 20, and 40 mg/kg p.o.; \( n = 6/\) group), rest three groups were administered with the combination of ALZ and MPH (A + M; 5 + 10 mg/kg 10 + 20 mg/kg, and 20 + 40 mg/kg p.o.; \( n = 6/\) group), A 20 µL of tween-20 was added to the powdered tablets and then suspended in distilled water. To simulate the drug overdose/abuse, the doses of MPH (10, 20, and 40 mg/kg) and ALZ (5, 10, and 20 mg/kg) were fixed by increasing the human equivalent animal dose in an arithmetic manner. Furthermore, the selected doses of ALZ and MPH were also observed to simulate the similar pharmacokinetic profile in rats as in clinical conditions.\textsuperscript{[6,7]}

**Physiological parameters**
Body weights, food intake, and water intake of animals were measured weekly.

**Blood collection, dissection, and tissue homogenization**
After an overnight (2200–0900 h) fasting, on day 29, the animals were anesthetized using sodium thiopental (45 mg/kg; i.p.) for blood collection followed by transcardial perfusion using phosphate-buffered saline (pH 7.4). The liver and kidneys were isolated, and 20% tissue homogenate was prepared in phosphate buffer (0.5 M, pH 7.2). Homogenate was centrifuged at 10,500 \( \times g/20 \) min/4°C, and the supernatant was further used for biochemical estimations.

**Serum biochemistry**
Blood was collected from retro-orbital plexus and was subjected to centrifugation at 800 \( \times g/15 \) min/4°C for serum extraction. Serum levels of AST, ALT, ALP, creatinine, and serum urea were estimated using the kit manufacturer.

**Oxidative stress parameters**
Oxidative stress parameters including the levels of malondialdehyde,\textsuperscript{[8]} reduced glutathione (GSH),\textsuperscript{[9]} and superoxide dismutase (SOD)\textsuperscript{[10]} in the liver and kidney homogenates were estimated.

**Protein estimation**
Lowry’s method was used for determining the protein content in the samples.\textsuperscript{[11]}

**Histopathology**
At necropsy, both the kidneys and liver were removed and fixed in 10% phosphate-buffered formaldehyde. Subsequently, the tissue sections were embedded in the paraffin wax. Sections of 5-µm thickness were cut and stained using hematoxylin and eosin.\textsuperscript{[12]} Photomicrographs were captured using a light microscope attached with a digital camera (Nikon-TS100F Charge Coupled Device, Tokyo, Japan).

**Statistical analysis**
The GraphPad Prism 6.01 software (GraphPad Software, San Diego, California, USA) was used to analyze the data. The data were analyzed using one-way ANOVA, followed by Tukey’s multiple comparisons test. A \( P < 0.05 \) was considered statistically significant. The results expressed as the mean ± standard deviation.

**Results**

**Effect of alprazolam and methylphenidate on physiological parameters**
Both the drugs, when administered alone (ALZ and MPH) and together (A + M), have shown a dose-dependent and significant reduction in
bodyweights as compared to the NC [Table 1]. However, no significant change in food intake and water intake was observed. Besides, no mortality was seen in any of the treated groups.

**Effect of alprazolam and methylphenidate on liver function tests**
Apart from the low doses (LDs) of ALZ and MPH individual treatments, all other doses of ALZ and MPH, when administered alone and in the combination, showed a pronounced increase in AST and ALP levels. Furthermore, all the doses, except the LD of ALZ, have significantly increased the serum ALT levels in comparison to the NC group [Table 1].

**Effect of alprazolam and methylphenidate on renal function and tissue damage**
Only high doses (HDs) of MPH, as well as the mid-dose (MD) and HDs of A + M co-administration, significantly increased the serum levels of creatinine and urea, whereas an only HD of ALZ when given alone could elevate the serum creatinine but not the urea levels as compared to the NC. Interestingly, only an HD of ALZ along with the MD and HDs of A + M showed an increase in the serum LDH levels. However, MPH administration did not produce any significant effect on the serum LDH levels at any given dose [Table 1].

**Effect of alprazolam and methylphenidate on Oxidative stress**
All three doses of A + M co-abuse regimens significantly increased the lipid peroxidation and decreased the SOD as well as GSH levels in both kidney and liver tissues [Table 1]. However, individual treatments of ALZ and MPH could not cause any significant lipid peroxidation, but the HDs have moderately reduced the levels of endogenous antioxidant enzyme levels in the liver as well as kidney tissue.

**Histopathology**
Only HDs resulted in noteworthy structural alterations in liver [Figure 1a-c], and in kidney [Figure 1e-g] as compared to the normal control [Figure 1d and h]. To add further, the ALZ + MPH has led to increased necrosis and vacuole formation and changes in a morphological arrangement of hepatocytes in the liver tissue [Figure 1c]. In the renal corpuscle, large urinary spaces were seen due to shrinkage of glomeruli, and a highly dense nuclei population is observed in glomeruli, which signifies the infiltration of inflammatory mediators [Figure 1g].

**Discussion**
Polydrug abuse has become a prominent health challenge in modern society. A very few preclinical and clinical studies on MPH and ALZ have reported their hepatotoxic and nephrotoxic potential. The rationale behind the selection of female rats was that females are more susceptible and severely addicted to psychostimulants as compared to males. Moreover, female rats show rapid and stable dose–response behavior.

Administration of MPH and ALZ alone and in combination for 4 weeks significantly reduced the body weights, which might be due to loss of appetite and increased locomotor activity associated with MPH administration. On the other hand, the fall in bodyweight of ALZ and A + M treated rats might be as a result of increased energy expenditure due to hyperlocomotion and excessive loss of water through increased frequency of defecation and urination. However, there were no significant differences in the food and water intake levels.
### Table 1: Effects of alprazolam, methylphenidate, and A+M on various physiological, serum biochemical, tissue oxidative stress parameters

| Parameter                        | ALZ (LD) | ALZ (MD) | ALZ (HD) | MPH (LD) | MPH (MD) | MPH (HD) | A+M (LD) | A+M (MD) | A+M (HD) | NC (LD) | NC (MD) | NC (HD) |
|----------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|---------|---------|---------|
| Percentage change in body weight| −4.6±0.4 | −9±0.7   | −12±0.5  | 7.5±0.4  | 3.5±0.6  | −4.7±0.6 | 3.5±0.5  | −3.7±0.5 | −8.6±0.9 | 7±0.1  | 6±0.1  | 12±1.2  |
| LDH (IU/l)                       | 56.8±33.8(NS) | 78.7±6.8 (NS) | 130.5±18.5 (NS) | 45.5±6.0 (NS) | 77.1±11.2 (NS) | 97.2±25.3 (NS) | 62.4±10.5 | 122.0±13.6 | 154.1±2.5 | 43.3±11.4 |
| AST (IU/l)                       | 16.0±2.95 (NS) | 33.7±5.3   | 68.6±13.7  | 45.5±6.0 (NS) | 77.1±11.2 (NS) | 97.2±25.3 (NS) | 62.4±10.5 | 122.0±13.6 | 154.1±2.5 | 43.3±11.4 |
| ALT (IU/l)                       | 6.7±0.8 (NS) | 37.7±7.8  | 66.6±6.8  | 11.9±3.1 (NS) | 51.9±10.0 (NS) | 88.6±11.65 (NS) | 25.5±1.8  | 72.7±12.9 (NS) | 101.5±13.8 | 12.5±3.1 |
| Liver MDA (nmol/mg protein)     | 0.7±0.2 (NS) | 0.2±0.04 (NS) | 0.8±0.2 (NS) | 2±0.9 (NS) | 1.4±0.5 (NS) | 3.1±0.1 (NS) | 5.2±2.6 (NS) | 3.2±2.0 (NS) | 7.0±1.5 (NS) | 0.8±0.2 |
| Liver SOD (units/mg protein)    | 194.9±61.8 (NS) | 152.2±13.6 (NS) | 111.7±88.8 (NS) | 269.9±85.3 (NS) | 145.6±74.1 (NS) | 114.5±40.9 (NS) | 225.2±73.6 (NS) | 172.1±122.7 (NS) | 88.1±29.8 (NS) | 317.3±12.4 |
| Liver GSH (µmoles/mg protein)   | 0.02±0.004 (NS) | 0.01±0.007 (NS) | 0.009±0.003 (NS) | 0.02±0.005 (NS) | 0.02±0.01 (NS) | 0.01±0.002 (NS) | 0.013±0.003 (NS) | 0.009±0.002 (NS) | 0.006±0.003 (NS) | 0.03±0.003 |
| Creatinine (mg/dl)              | 0.6±0.2 (NS) | 1.0±0.2   | 1.8±0.5   | 0.7±0.2 (NS) | 1.1±0.2 (NS) | 2.9±0.5 (NS) | 1.2±0.41 (NS) | 1.9±0.3 (NS) | 4.2±0.3 (NS) | 0.7±0.18 |
| Urea (mg/dl)                    | 128.7±8.8 (NS) | 123.7±6.7 (NS) | 138±9.9 (NS) | 134±20.7 (NS) | 161.5±15.9 (NS) | 183±32.1 (NS) | 158.5±7.1 (NS) | 214.0±28.1 (NS) | 245.3±30.4 (NS) | 153.0±7.08 |
| Kidney MDA (nmol/mg protein)    | 0.4±0.4 (NS) | 0.4±0.06 (NS) | 0.5±0.2 (NS) | 1.06±0.4 (NS) | 2.33±1.8 (NS) | 4.7±0.9 (NS) | 3.4±0.23 (NS) | 5.4±0.6 (NS) | 7.6±1.5 (NS) | 0.7±0.2 |
| Kidney SOD (units/mg protein)   | 141.3±77.5 (NS) | 101.2±42.5 (NS) | 67.1±13.5 (NS) | 221.8±5.8 (NS) | 111.9±93.01 (NS) | 95.4±9.8 (NS) | 150.5±92.4 (NS) | 85.5±16.1 (NS) | 74.2±10.6 (NS) | 326.3±13.2 |
| Kidney GSH (µmol/mg protein)    | 0.02±0.02 (NS) | 0.01±0.002 (NS) | 0.01±0.004 (NS) | 0.03±0.007 (NS) | 0.02±0.005 (NS) | 0.01±0.001 (NS) | 0.025±0.002 (NS) | 0.014±0.007 (NS) | 0.008±0.001 (NS) | 0.029±0.003 |

- The significance versus NC.
- The significance MD versus LD of same treatment (ALZ or MPH or A + M).
- The significance HD versus LD of same treatment (ALZ or MPH or A + M).
- The significance between the LD of A + M versus ALZ.
- The significance between the MD of A + M versus MPH.
- The significance between the MD of A + M versus MPH, ALZ.
- The significance between the HD of A + M versus ALZ.
- The significance between the HD of A + M versus MPH.
- ALZ: Alprazolam, MPH: Methylphenidate, LDH: Lactate dehydrogenase, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione, ALP: Alkaline phosphatase, NS: Not significant, LD: Low dose, MD: Mid dose, HD: High dose.
The hepato-renal toxicities of MPH and ALZ are rare at therapeutic doses. However, ALZ-induced reduction in the endogenous GSH and SOD levels, in addition to the MPH’s ability to promote the ROS generation, might have exaggerated the toxic effects of MPH on the liver and kidney. Furthermore, the intercalation of ALZ with genomic and mitochondrial DNA could have led to the activation of the cascades involved in cell death. We also noted a prominent elevation in the serum LDH levels (which is a biomarker of cellular damage) of ALZ and MPH treated (alone and combination) rats.

The histopathological observations from our study substantiated the structural damage associated with ALZ and MPH administration. The increase in liver and kidney serum enzyme markers can be directly correlated to the leakage of the enzymes from cytosol into the bloodstream, followed by cellular damage. Hence, the results suggest that the ALZ-mediated reduction in the antioxidant enzymes and MPH-induced oxidative stress levels are major driving forces of the augmented hepato-renal toxicity [Figure 2]. Further studies of the drug self-administration model are required to mimic the drug abuse cases and to elucidate the underlying mechanisms involved in the enhanced toxicity with co-administration of ALZ and MPH.

Conclusions

The co-abuse of psychostimulants and benzodiazepines may or may not result in any beneficial effects; however, the findings of our study suggest that the administration of prescription or non-prescription ALZ and MPH for a longer duration may result into hepato-renal damage. Therefore, it is concluded that the co-abuse of these drugs is a significant public safety concern, and the co-treatment must be restricted to lower doses, and co-abuse should be discouraged.

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Conflicts of interest

There are no conflicts of interest.

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