11.05.2016
The Editor
Behavioral Neurology, Hindawi Publishing Corporation
Subject: Submission of research manuscript

Dear Sir,

Here you find the revised manuscript entitled **"The effect of chronic Alprazolam intake on memory, attention and psychomotor performance in male healthy human male volunteers of Bangladesh"** in your reputed journal. This research manuscript has never been published previously in whole or part. This article has not been submitted in any other place for publication. Intitutional ethical permission was taken and the study followed the International Conference of Harmonization (ICH) for Good Clinical Practice (GCP) and it was conducted in compliance with the Declaration of Helsinki and its further amendments. We declare that we have no competing interests.

We have addressed the points raised by the honorable reviewers as mentioned in subsequent portion.

Therefore, I hope that you would be kind enough to accept our appeal to the points raised by honorable reviwers and take necessary action to publish our study in your journal.

Regards

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Manuscript without reference: 4692 words
Manuscript with Reference: 6475words
Figure: Figure 1-2
Table: Table 0
Pages: 21 (with references and Figures)
Supplementary file: 1

Author's Response to Reviewer comment

REVIWER#1 (Initial recommendation: reject)

In their paper entitled 'The effect of chronic alprazolam intake on memory, attention and psychomotor performance in healthy human volunteers of Bangladesh' the authors investigate the effects of a dose of 0.5 gram Alprazolam, administered daily over a 2 week period. Effects on cognitive performance were assessed by several measures from the CANTAB battery.
They observe some impairing effects on measures for attention and memory, and they observe reversal of effects on attention and psychomotor performance, that they attribute to the development of tolerance.
The topic is important to study as many people take benzodiazepines on a daily basis.
However, I find the paper not clearly written and should be much improved before it can be published.

Author's Response: >>A1. We have addressed all the points raised by the honorable reviewers and our responses are mentioned as follows.

ABSTRACT

Q1. Authors claim the no study has been conducted investigating the effects of long term clinical dose intake of Alprazolam. However, a quick search resulted in at least the following publication:Kiliç C, Curran HV, Noshirvani H, Marks IM, Başoğlu M (January 1999). "Long-term effects of alprazolam on memory: a 3.5 year follow-up of agoraphobia/panic patients". Psychol Med 29 (1): 225–31. doi:10.1017/S003329179800734X. PMID 10077311.
Besides they mention 4 studies in the discussion (20,21,22,26) that they claim have administered Alprazolam chronically.

Author's Response: >>A1. We have re-written the section

INTRODUCTION

Q2. Mention how short the half-life of Alprazolam is.

Author's Response: >>A2. Modified and highlighted. See page 3 line 21.

Q3. Please rephrase sentence 1 of the 2nd paragraph

Author's Response: >>A3. Modified and highlighted.

Q4. It's not clear what the purpose of the second half (from 'Alprazolam has been….') of the second paragraph is meant to convey.

Author's Response: >>A4. Primarily to show the varieties of side effects due to alprazolam intake. However, the section is modified and highlighted. See page 4 line 10-12.

Q5. In the former to last paragraph of the introduction the authors claim that other studies failed to mimic the clinical situation of patients. I believe this is phrased incorrectly as some of these studies did not aim to mimic the clinical situation.

Author's Response: >>A5. Modified and highlighted. See page 4 line 32-33.

Q6. Many grammatically incorrect phrases are in the last paragraph, please revise.

Author's Response: >>A6. Modified and highlighted. See page 5 line 15-17.

Q7. Please refer to a publication showing CANTAB validity.

Author's Response: >>A7. Modified and highlighted. See page 5. Line 21-27.

Q8. Explain why 0.5 mg of Alprazolam was chosen as this seems to be the lower end of the dose range.

Author's Response: >>A8. A low dose was selected to avoid subsequent dose tapering after completion of the study. This section is modified and highlighted. See page 6 line 25-26.

METHODS AND ASSESSMENT

Q9. Please explain why only males were included.

Author's Response: >>A9. Institutional review board approved the study only for males. However, we have made changes in the title of the paper.

Q10. Were the screened for usage of any drugs and how was this ensured?

Author's Response: >>A10. Modified and highlighted. See page 6 line 10-13.

Q11. Please rephrase the second sentence of the methods and assessment

Author's Response: >>A11. Modified and highlighted. See page 6 line 23-24.

TREATMENT AND DESIGN

Q12. Please elaborate of the dose choice

Author's Response: >>A12. Modified and highlighted. See page 6 line 25-26.

Q13. Please elaborate on why participants were instructed to take the drugs at night.

Author's Response: >>A13. Modified and highlighted. See page 6 line 29-31.

Q14. How was it ensured that the participants did not know which treatments they were taking?

Author's Response: >>A14. Modified and highlighted. See page 6 line 29-31 and page 7 line 3-4.

ASSESSMENT

Q15. Please add a reference to a study showing CANTAB subtest validity.

Author's Response: >>A15. Modified and highlighted. See page 7 line 25-26.

Q16. All task descriptions lack the required details of the stimulus presentation (ITI, number of trials, etc.). This should most certainly be more clear.

Author's Response: >>A16. Modified and re-written.

Q17. There is no clear reasoning on why some of the measures are chosen.

Author's Response: >>A17.The primary goal was to measure memory, attention and psychomotor function. There were several tests in CANTAB software for these testing. We selected the battery that could be finished within 40 minute. From our previous experience we found that testing more than 50 minutes give stress to subjects and many abandon the test. However, the manuscript is modified and highlighted. See page 7 line 31-33 and page 8 line 16-18, and page 9 line 9.

Q18. Some sentences seem to convey a message, but this is not clear. For example, 'This test is sensitive to dysfunction in the parietal and frontal lobe areas of the brain' Why is this mentioned? I believe this does not apply to the currently studied population.

Author's Response: >>A18. Deleted

Q19. Explain how RVP A and RVP B were calculated.

Author's Response: >>A19. The RVP test outcome was predetermined on the CANTAB software which for rapid visual processing measures the outcome in RVPA', RVPB' and RVP total hits. RVP section is modified and highlighted in the manuscript.

Q20. The authors claim that the CRT is a measure for alertness and motor speed. While these faculties should operate well in order to perform well, good performance is not restricted the good functioning of these faculties. There are many processes that cause bad performance on CRT tasks, e.g. visual function (may be applicable to benzodiazepine's effects), response choice processes.

Author's Response: >>A20. We hypothesized that the design and operation mode of CRT is simple enough to measure impairment in alertness and motor speed response. Every test module is preceded by practice sessions to familiarize the subjects with test environment and to limit the possibility of other functions such as visual to impact the outcome.

STATISTICAL ANALYSIS

Q21. I don't understand the need for a regression analysis, when they can do a multivariate ANOVA for repeated measures ('Time of assessment') and a between subjects factor called 'Group'. There is no mentioning of the significance level for the regression and ANOVA. There is no mentioning of a correction for multiple comparisons.

Author's Response: >>A21. The section is re-written. We consulted statistician and applied statistics that is in compliance with our previously peer reviewed papers. (PMID: 26351508, PMID: 24412554). We had age and IQ as the variables other the psychological variable. Regardless of age and IQ; similar results were obtained and therefore was not mentioned.

RESULTS

Q22. How was IQ estimated?
Author's Response: >>A22. National Adult Reading Scale is used with slight modification as mentioned in the previously published paper (Bin Sayeed et al., 2013. PMID: 23707331)

Q23. For every section of the results: I believe the crucial effect is an interaction between Time and Group. Where after the effects should be tested per level of a factor. The way it is written now, it's not clear what all the effects refer to. Are these differences between Group A and Group B at time point 2, or differences between time point 1 and time point 2 in group B.
Author's Response: >>A23. In this investigations, Repeated measure ANOVA was used and the result mentioned in the result section is the over all F test value as well as Univariate tests results as obtained from the SPSS section. In the discussion section, further clarification is provided.

DISCUSSION

Q24. I really don't understand what is conveyed in the second part of the first paragraph (from: 'It has been proposed….').

Author's Response: >>A24. Modified and highlighted. See page 12 line 8-10.

Q25. The second paragraph contain new findings that should be either in the results section as a priori analyses, or discussed in the discussion with a clear reason why further analyses were performed and provide an explanation of the current results.

Author's Response: >>A25. Modified and highlighted. See page 12. Line 2-3.

Q26. I miss a discussion of the reasonable possibility that the dose of 0.5 mg failed to induce effects on some measures, as it is a dose that is low.

Author's Response: >>A26. Modified and highlighted. See Page 12 line 27-28, page 14 line 21-23 and 31-33.

REVIWER#2 (Consider after major changes)

There was some strength to this paper. Alprazolam was administered for 14 days, which would appear to be the longest period of time that it has been administered and then tested for psychomotor and cognitive effects in the peer-reviewed literature. The CANTAB is well recognized as a battery of tests that accurately assesses different parameters of psychomotor and cognitive performance. Safety measures were employed (constant communication with subjects, ability of subjects to contact study center in case of emergency).
There are several concerns this reviewer has that need to be addressed before s/je would be favorably disposed to recommending acceptance of this paper. At the very least the concerns raised should be placed into a limitations paragraph or paragraphs in the Discussion section of the paper.

Author's Response: >> We have followed the suggestion of the honorable reviewer. We have mentioned the limitation of the study at the end of discussion and We also proposed future study.

Q1.One significant concern is the external validity of the study. The authors themselves say that alprazolam is used for treatment of GAD and panic disorder. According to my reading of the literature the drug is dosed initially at 0.25 – 0.5 mg three times daily. In this study the drug was given only once. And it was given at a time of day in which, if one wanted to make statements about effects of the drug being used in patients being treated for GAD or panic disorder, does not make sense.

Author's Response: >>A1. First, the dose of the drug was minimized to confirm that whether alprazolam can induce memory, attention and psychomotor performance impairment at 0.5 mg daily for 14 days given that CANTAB tests are sensitive enough. We agree that the dose does not fully comply with regular treatment. However, multiple dosing of alprazolam may result in dose missing by participants and higher doses might cause drowsiness in one test group, which may reveal that they are taking an active drug, which may affect the outcome of the study.

Q2. It was being given as a hypnotic (sleep inducing) agent (i.e., 9-10 pm). That raises a second issue: when were the CANTAB tests administered? Thirty to 60 minutes after alprazolam administration? This reviewer has doubts about that, but will await the authors to state when testing was done relative to drug administration. If the testing was not done until the next morning then they were testing the residual effects of alprazolam in which, given its short half-life, one would not expect to see much impairment. The manuscript will have to be rewritten in which it is clear that acute effects of a drug were not being measured but residual effects were. But the authors need to make it clear how this paper makes a significant contribution to the extant literature on alprazolam effects when it was given just once daily and given at night.

Author's Response: >>A2. Modified Page 6 line 29-34.

Q3. A third concern is experimental design. If one wants to say anything about tolerance development to a drug (or a drug over time actually resulting in improved performance) the effects of the drug has to be tested after initial administration and

after chronic administration. This was not done in this study. Drug effects were only tested one time, and so any mention of tolerance has to be used cautiously in this paper. For example, in the last sentence of the abstract the word "tolerance" is mentioned. The authors are assuming that memory was impaired after the first dose but they do not know that. The investigators also missed the opportunity to actually detect tolerance that may have occurred. All the tests in which there were no significant differences between the groups after 14 days tells us that no impairment was evident from alprazolam. But it may have been the case that those tests on the CANTAB might have been adversely affected by alprazolam after the first couple of days of alprazolam administration but by the 14th day tolerance developed to many of the cognitive/psychomotor parameters that the CANTAB measured. Given the experimental design of this study one will not know if this was the case or not. This is a shortcoming of the paper.

Author's Response: >>A3. The deleterious effect of acute alprazolam treatment has already been reported earlier (Curran et al., 1994 PMID: 7892364). Repetition could have been done but due to difficulty in getting volunteers to sit for the test for 3 times, we chose to study the chronic effect of two weeks alprazolam intake. However, we modified and highlighted manuscript and mentioned the limitation at the end of discussion section. page 13. Line 11-14.

Reviewer's other comments section:

Q4. When discussing abuse liability studies please do a PubMed search using 'Griffiths RR' and 'alprazolam' as search terms. Zacny et al. (2012) studied the drug in subjects without a history of sedative abuse. Dr. Griffiths as well as others have studied abuse liability in a group more likely to be sensitive to abuse liability effects of this class of drugs, i.e., sedative abusers. It is not appropriate to compare ref. 8 with ref. 9 without mentioning the differences in the nature of the sample's drug use/abuse history.

Author's Response: >>A4. We highly appreciate the kind suggestions by the honorable reviewer. Based on the suggestions, the manuscript is modified and highlighted. Page 4 line 9-12.

Q5. In the Introduction please give a range of number of days other studies have used examining subchronic effects of alprazolam. One of the significant aspects of this study was supposed to be the length of time alprazolam was administered but no references are made to length of time in which subjects were administered the drug in other studies.

Author's Response: >>A5. Manuscript is modified and highlighted. See page 4 line 25-30 and page 6 line 6-11.

Q6. This study was done only in males. Different results might have occurred in females. The word "males" should be used in the title of the paper. It is unclear why "Bangladesh" is in the title of this paper.

Author's Response: >>A6. Title is Modified

Q7. On the RVP were the numbers ‘2’ ‘4’ and ‘6’ shown on the screen simultaneously or were they shown sequentially (one at a time)?

Author’s Response: >>A7. Manuscript is modified and highlighted. See page 9 line 11-15.

Q8. In second paragraph of Discussion the authors state that analysis of DMS test showed that the probability of making an error either following error, or following correct response, did not change significantly ($p>0.05$) over two weeks for alprazolam ingestion in test group. How can this be said if testing was not done after the first dose of alprazolam? Similarly on the next page when talking about attention in RVP, how can the authors state that “it was evident that upon long-term ingestion, tolerance developed against poor attention”? Tolerance implies impaired attention after the first dose of alprazolam and then either attenuated or complete lack of impairment over time but testing was not done after initial dosing.

Author’s Response: >>A8. This section is modified and highlighted. See page 13 line 11-14.

Q9. In the last paragraph of the Discussion the first sentence may have to be revised depending on when testing occurred after the 14th day of alprazolam administration. If tested 30-60 minutes afterwards this reviewer would agree with the statement as written. If tested 8 hours or so afterwards the authors have to talk about residual effects or lack of residual effects of alprazolam depending on what aspect of psychomotor or cognitive performance was being assessed.

Author’s Response: >>A9. This section is modified and highlighted.

REVIEWER#3 (Publish unaltered)

An interesting and timely study
Author's Response: >> We thank the honorable reviewer for the appreciation.

REVIEWER#4 (Consider after minor changes)

This is a worthwhile study that uses generally sensitive measures of memory, attention and motor performance and tackles an important question. The authors make an interesting contribution from a part of the world most researchers rarely consider as a site of good research. The long term effects of chronic benzodiazepines (e.g. alprazolam) have been inadequately studied (e.g., poor design, insensitive measures). This study is really only a start - future studies in patients; with higher doses; longer treatment will be worthwhile. The finding of lack of tolerance on memory effect could be clinically important.

Q1. The limitations of the study need to be elaborated a bit more (low dose; once a day; healthy Ss; relatively short-term; small sample size)

Author's Response: >>A1. Modified. See page 14, line 31-34 and page 15, line 1-4.

Q2. The 2 choice CRT is not very sensitive - future should go up as high as 5 or 6 choice. A delayed memory task could be added

Author's Response: >>A2. It could be but we wanted to limit the duration of study no more than 50 minutes. From our experience in several other experiments, we found that test battery over 50 min put stress to the subjects and many of them abandon the test. Therefore, we made the battery that could be finished within about 40 minutes.

Q3. The introduction needs some critical revisions e.g., alprazolam half-life is not as short as some benzos; ?? is powerful anxiolytic correct??; statement of faster onset is pretty questionable (diazpam absorbed faster); alprazolam clearly has abuse potential - scheduled drug by WHO and CSA in USA and clearly produces physical dependence; all benzos are a risk factor to falls in elderly; is true that few studies have looked at chronic dosing - might suggest a few reasons not done??

Author's Response: >>A3. Modified and highlighted. See page 3 line 18-20

Q4. Have the authors considered that taking the drug at bedtime might be the reason tolerance did not develop?? The phenomenon of learned tolerance i.e., when a task has to be done while drug is on board might show that tolerance does develop (an example of where this can occur is with alcohol). I have no idea what would happen with alprazolam

Author's Response: >>A4. Learned tolerance can only be measured if the same tests have been conducted multiple times to observe the effects, which in turn may alter outcome by inducing learning effect itself given that the dose of alprazolam is not so high. The nature of tasks in CANTAB battery in different time is helpful to minimize such incidents.

Q5. Table #1 could be just put in text

Author's Response: >>A5. The information is put in the man manuscript main body and Table 1 is deleted

Q6. The Tables 2,3 and 4 should be supplemented by a Figure showing key findings - will be easier to digest.

Author's Response: >>A6. Tables 2 to 4 is put in the supplementary files.

REVIEWER#5 (Consider after major changes)

This paper describes an experimental study in healthy volunteers whereby they were randomly assigned to receive either 0.5 mg alprazolam or placebo to take once daily for two weeks. Various tasks measuring cognitive performance were performed at baseline and again at the end of the 2-week medication exposure. The authors state there was a significant decrease in cognitive performance in the alprazolam group compared to the placebo group on some measures.

Q1. While this appears to be an adequately designed and well-run study, I question the statistical methods used to analyze the data. The design is a simple 2x2 with drug condition as the between-subjects factor and time (baseline and post-treatment) as the within-subjects factor. The data should have been analyzed using a repeated measure ANOVA. It is not at all clear to this reviewer why a Poisson regression was used sometimes and an ANOVA other times. This needs to be more clearly justifies or the Poisson regression needs to be deleted. Also, there does not appear to be any correction for multiple comparisons. For example, for the DMS only 3 out 9 'tests' were significant but only barely and would not survive correction.

Author's Response: >>A1. We highly appreciate honorable reviewer for this and we modified and highlighted the section accordingly.

Q2. I have a hard time believing that the RVP total hits is statistically significant between groups – the data are virtually identical.

Author's Response: >>A2. There is significant difference in either group over two weeks of treatments.

Q3. Table 1. Were age and IQ the only demographics collected? What about Gender, weight, psychiatric history, alcohol and other drug use, tobacco dependence?

Author's Response: >>A3. Modified. See page 6 line 14-16.

Q4. Throughout the manuscript please change Group A and Group B to Alprazolam and Placebo.

Author's Response: >>A4. The manuscript is re-written.

Q5. The Table titles need more description.

Author's Response: >>A5. Modified and placed either in the main document or in the supplementary file as suggested by another honorable reviewer.

DISCUSSION

Q6. I assume that the hypothesis was that those given alprazolam would show a decrease in cognitive performance from baseline compared to those given placebo. Eyeballing the data presented, this does not appear to be the case. Both groups

improved their performance on the task. Therefore the clinical significance of any marginally significant between group differences needs to be discussed.

Author's Response: >>A6. Discussion part is re-written considering the comment of the honorable reviewer.

REVIEWER#6 (Consider after major changes)

Comment: I commend the authors for choosing this topic as benzodiazepines are often misused and abused by patients with mood disorders and remitted substance users. They also impair both cognitive abilities and global functioning and their use should therefore be better monitored or minimized. I therefore think that this paper deserves to be published but a stronger rationale and additional background information need to be provided. Further, the conclusions need to be restructured to include a more critical explanation of the findings by referring to chemical/physiological changes induced by benzodiazepine use, what future research needs to achieve in this field, clinical relevance of the findings and identification of limitations and weaknesses of the study.

ABSTRACT

Q1. I would highly recommend that the authors mention means/SDs for the age of the participants for each group. p-values are usually not mentioned in the abstract.

Author's Response: >>A1. Modified according to honorable reviewer's suggestions.

Q2. I would provide additional background information on the effects of Alprazolam (or benzodiazepine in general) on the brain in terms of cognitive function and mood

Author's Response: >>A2. Modified and highlighted. See page 4 line 1-6.

Q3. Since benzodiazepines are often administrated in case of panic disorder, generalized anxiety disorder (GAD) or social anxiety disorder (SAD) and alongside mood-stabilizing drugs in MDD/BDD I would briefly discuss this too.

Author's Response: >>A3. Modified and highlighted. See page 3. Line 11-20.

Q4. I would also recommend that the authors provide additional information on the pharmacokinetic and pharmacodynamics profile of alprazolam.

Author's Response: >>A4. Modified and highlighted. See page 3 line 21-34 and page 4 line 1-8.

Q5. I would provide 1-2 references at the end of their introduction when they mention CANTAB, selection of the study period and drug dosage. Overall the authors should provide a more thorough explanation of what previous studies did in terms of duration and dosage. 1 sentence at the end of the introduction is not sufficient.

Author's Response: >>A5. Modified and highlighted. Page 5. Line 6-14 and 21-26.

Q6. In the methods section I would highly recommend to mention Table 1 and provide means and SDs for age in each group. Why only men? Inclusion/exclusion criteria should also be mentioned, specifically in terms of medical health status, lifetime of psychiatric disorders, family history of psychiatric disorders, medication etc.

Author's Response: >>A6. Institutional ethical committee approved the protocol only for male. The manuscript is modified and highlighted. Page 6. Line 1-21.

Q7. Cognitive assessment: could the authors mention IQ values, education level or estimated intellectual abilities (e.g. based on the local WRAT questionnaire etc.), and employment status

Author's Response: >>A7. Modified and highlighted. Page 6. Line 1-21.

Q8. Statistical analyses: could the authors please mention how they corrected for multiple comparisons. (per test and/or experiment-wise approach?). Also did the authors assess the severity of potential mood symptoms related to depression/hypomania (e.g. MADRS, HAMD, YMRS) in these participants?

Author's Response: >>A8. We modified the statistical analysis section. We assumed that alprazolam intake may cause change in the mood. So we ran another test from CANTAB named ERT, emotion recognition test to check that whether volunteers kept on alprazolam can identify the correct emotion shown on the computer display. The accuracy rate was decreased after treatment although not significantly (data not shown).

Q9. Given their findings did the authors think about calculating an RT to accuracy ratio to see if individuals on alprazolam compensated for reduced alertness by taking additional time to complete the task? Have other studies in individuals using opiates, alcohol etc. shown similar findings? addressing these points will make your conclusions more effective and compelling.

Author's Response: >>A9. Can be done. Psychomotor performance was not affected by alprazolam intake. Given that the CRT is a simply designed experiment the accuracy ratio did not change after alprazolam intake (data not shown). But the mean latency to make a choice actually decreased (although not significantly) after alprazolam treatment. So, we believe after presenting the stimulus the subjects did not take additional time. Rather they responded earlier than control subjects. Similarly in our DMS test subjects have to match abstract images after presenting the stimulus. There we found that memory score was affected in all delay situations significantly but latency time was not, although it reduced after alprazolam intake. In chronic alcohol intake subjects, it was reported that cognitive function is not associated with cognitive decline. However, there is no study reporting cognitive defects with alcohol intake with only 14 days.

Q10. Their findings make me think of what is usually observed in individuals with past history of substance use. This may not be surprising since both substances act on GABA receptors but it is not that clear. Although abstinence is associated with the recovery of cognitive functions, authors have observed that even after a few years individuals still show problems with non-verbal abstract reasoning, mental flexibility and visual-spatial abilities. Given that all these functions are related to attention I wonder if the authors could comment further in terms of brain regions that may be more susceptible to the potential up regulation of NMDA receptors leading to excessive glutamate activity and excitotoxicity (due to benzodiazepine) which may result in neurodegeneration. Have there been studies showing how quickly such

changes may occur after starting patients with benzodiazepines? Please comment on this in your discussion.

Author's Response: >>A10. Modified and highlighted. See page 13 line 28-34 and page 14 line 1-14.

Q11. One of the primary problems with benzodiazepines is tolerance and dependence. Could the authors comment on potential long-terms effects of benzodiazepine use on cognition? Also since mood state is closely linked to cognitive abilities how would the authors expect to see in populations with anxiety or mood disorders? This should also be mentioned in the discussion.

Author's Response: >>A11. Modified and highlighted. See page 14, Line 24-30.