Author’s response to reviews

Title: Xylazine-induced Reduction of Tissue Sensitivity to Insulin Leads to Acute Hyperglycemia in Diabetic and Non-diabetic Monkeys

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Author’s response to reviews: see over
Dear Dr. Rowels,

We are submitting our revised manuscript entitled: Xylazine-induced Reduction of Tissue Sensitivity to Insulin Leads to Acute Hyperglycemia in Diabetic and Non-diabetic Monkeys. Please kindly review it for publication in *BMC Anesthesiology*.

We are grateful to the reviewers for their time and effort to help us to improve our manuscript. The reviewers positively commented on our manuscript which is valuable to the specific research area. We revised it according to the reviewers’ suggestions and comments. With our resubmission the detailed point-by-point response letter is also included. Our revised manuscript has been checked by two native English speakers for potential grammatical errors.

We hope that our manuscript is now suitable for publication in *BMC Anesthesiology*; however, if further changes are in order, please do not hesitate to contact me directly. Thank you in advance for your time and consideration.

Sincerely,

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Reviewer 1

Reviewer's report:
Xylazine is widely used in combination with ketamine for anesthesia of animals during veterinary care and laboratory research procedures. However, xylazine has a poorly understood hyperglycemic effect which contraindicates its use in animal experimental studies of diabetes. The goal of this work is to understand the hyperglycemic action of xylazine. Because xylazine is a drug of abuse, this work also has toxicological significance to man. Therefore, the authors studied the hyperglycemic effect of xylazine in normoglycemic cynomolgus and rhesus monkeys. In addition, they studied a line of cynomolgus monkeys with congenital insulin-dependent diabetes to test the hypothesis that xylazine-induced hyperglycemia results from reduced insulin secretion. Overall, the manuscript is easy to read. Minor corrections are suggested at the end of this review. However, Revisions are needed to enhance the manuscript’s clarity.

Thank the reviewer for his helpful comments. We revised our manuscript according to the reviewer’s suggestions and comments. We also addressed the reviewer’s comments one by one as the following.

Compulsory Revisions
1. Figure 1. Are these all data derived from the diabetic and non-diabetic cynomolgus monkeys whose baseline data are presented in Table 1? Indicate the numbers of monkeys studied to produce the data of Figure 1. The error bars on the data points of B as well as the histogram bars of C and D should be made larger.

Yes, the data in Figure 1 are collected from the animals listed in Table 1. We indicated the source and the numbers of the monkeys in the figure legends of Figure 1 in this revised version. We revised the figure and made the changes as the reviewer suggested. Thanks the reviewer for his helpful suggestion and comment.

2. Figure 2 A. Are these data derived from the same monkeys presented in Table 1 and Figure 1? In lines 5-7 of paragraph 2 on page 9 the authors suggests that hyperglycemia reversed much more slowly for diabetic as opposed to normoglycemic monkeys; this conclusion is repeated in the discussion. However, the data do not support a statistically significant difference in time recovery time. Furthermore, the authors go on to state that “Only one out of 5 diabetic monkeys gradually returned to the pre-xylazine glucose level at 2 hr after xylazine administration.” The authors should discuss why this diabetic monkey recovered whereas the remaining 4 monkeys required yohimbine to recover. Does yohimbine increase the recovery rate from xylazine-induced hyperglycemia for normoglycemic cynomolgus monkeys?

Yes, these data are derived from the same monkeys presented in Table 1 and Figure 1. The data in Fig. 2A clearly show that the blood glucose level in diabetic monkeys was still high at 120 min after xylazine injection whereas the glucose level at that time point was already back to pre-xylazine level in control animals. We added two dash lines in panel A to show the recovery level of blood glucose in both diabetic and normoglycemic groups. The data show that compare with normoglycemic monkeys, xylazine-induce hyperglycemia subsided much slowly in diabetic ones.
Individual variability among the experimental animals might be able to explain that blood glucose in one out of 5 diabetic monkeys returned to its pre-xylazine level. However, the remaining 4 monkeys were administered with yohimbine to recover blood glucose from xylazine hyperglycemia to the pre-xylazine levels. Unfortunately, we did not test whether yohimbine increases the recovering rate from xylazine-induced hyperglycemia for normoglycemic cynomolgus monkeys. However, based on literature and the pharmacological properties of yohimbine and xylazine, it is predictable that yohimbine should increase the recovering rate from xylazine-induced hyperglycemia for normoglycemic cynomolgus monkeys.

3. The data of Figure 2B indicate that xylazine did decrease plasma insulin level for normoglycemic monkeys; this effect occurred at approximately the time when blood glucose concentration increased. These observations contradict the authors' argument that xylazine-induced hyperglycemia does not involve decreased secretion of insulin. Were tests of significance performed for the open circle data points of Figure 2B? The statements noted in paragraph 1 of page 10 need to be clarified. That is, the authors state that insulin levels of normoglycemic monkeys initially decreased after xylazine “…but remained unchanged at all other time points.” Compared to pre-xylazine level, the initial decrease in plasma insulin was not statistically different. The sentence has been reworded (please see the revised version on page xx).

4. On page 9 the authors assess the effects of xylazine on glucoregulatory hormones. The last sentence of paragraph 2 should be more clearly written. The sentence has been reworded.

5. Since the authors had serum samples, it is unclear why they did not measure the concentration of other hormones (e.g., epinephrine, corticosterone, somatostatin, growth hormone) which regulate blood glucose concentration. These measurements should be made to improve understanding of the hyperglycemic action of xylazine. Thanks for the reviewer’s comment. The analyzed samples were measured in a local city hospital lab. The assays for all these hormones listed by the reviewer above are technically challenging and the lab does not have the capability to run those tests. Also, in this study we focused on those hormones which more directly and strongly affect blood glucose and could be measured by the lab.

6. In line 3 from the bottom of page 10 the authors indicate that hyperinsulinemic-euglycemic clamp experiments were made in 8 normoglycemic rhesus monkeys. Representative records for 2 of these monkeys are presented in Figure 3. Because blood glucose concentration was not stable, the authors did not challenge with xylazine until approximately 200 min after beginning the clamp. At this time, xylazine produced a mild increase of blood glucose from 55 to 83 and 64 to 99 mg/dL for these two monkeys; Figure 2 indicates that xylazine increased glucose in normoglycemic cynomolgus monkeys from 58 ± 3 mg/dL to 108 ± 12 mg/dL. Thus, during the clamp experiment xylazine is approximately 45% as effective in increasing plasma glucose. In order to strongly support the authors’ conclusion presented in the last sentence of paragraph 1 on page 11 all of the euglycemic experimental data should be presented and analyzed for
statistical significance of blood glucose changes.

These are two different experiments under different conditions.

1) In the clamp experiment each animal was treated with insulin and glucose for about 2 to 3 hrs to balance body glucose regulation, including hormones, glycogenesis, glycogenolysis and gluconeogenesis.

2) In the clamp experiments each animal constantly received both insulin and glucose infusion before and after xylazine challenge.

3) Rhesus monkeys were used in the clamp experiments which might have some variation between strains.

4) Actually, during the clamp experiment xylazine is approximately 63% as effective in increasing plasma glucose as those animals without clamp \( \frac{((83-55)+(99-64))/2}{(108-58)} = 0.63 \).

The hyperinsulinemic-euglycemic clamp experiments were performed in 8 normoglycemic rhesus monkeys. Two of them received xylazine challenge at approximately 200 min after 40 min stabilization of blood glucose levels in a range from 55 to 75 mg/dL. After xylazine challenge these two animals continuously received glucose infusion at a constant rate to look at the effect of xylazine on blood glucose concentration. Xylazine still increased blood glucose level. The other 6 animals received hyperinsulinemic-euglycemic clamp for 135 ± 11 min \( (M \pm SE, \text{including last 40 min stabilization of blood glucose in a range of 55 to 75 mg/dL}) \) and then giving xylazine challenge. After xylazine challenge these animals continuously received glucose infusion with rate change to maintain blood glucose level in a range of 55 to 75 mg/dL. Xylazine statistically significantly reduced glucose infusion rate, which indicates that xylazine significantly decreased glucose uptake and usage by the body \( (M \text{ rate}) \). As the experiments were conducted under the hyperinsulinemic-euglycemic clamp, xylazine-induced decrease in glucose uptake and usage likely resulted from the reduction of tissue sensitivity to insulin, because the clamp constantly maintained the blood hyper-insulin level.

7. In line 4 of paragraph 2 on page 11 the authors indicate that “…blood glucose in these animals (ie., those studied with euglycemic clamp) reached 40-min stabilization…”. However, in the representative records of Figure 3, blood glucose was apparently judged to be stable only after 200 min and then xylazine was given. Why was there a time difference for glucose stabilization in these 2 sets of experiments?

We reworded the sentence. Actually, it took 135 ± 11 min \( (M \pm SE, \text{including last 40 min stabilization of blood glucose in a range of 55 to 75 mg/dL}) \) to stabilize blood glucose before xylazine challenge in the other 6 rhesus monkeys. The hyperinsulinemic-euglycemic clamp usually takes a 100 to 150 min infusion period with adjustable rates to let the body adjust and balance its blood glucose to a stable period. After the stabilization period (usually 40 min), physiological or drug challenge can be applied. Such a period \( (90 \text{ to } 150 \text{ min}) \) is required for the body to balance its glucose regulation which involves hormones, glycogenesis, glycogenolysis and gluconeogenesis.

8. The hyperinsulinemic-euglycemic clamp was performed on 8 normoglycemic rhesus monkeys which had blood glucose of 86 ± 4.6 mg/dL as compared to 47.9 ± 5.4 mg/dL
for the 5 cynomolgus monkeys of Table 1. Is this difference in blood glucose significant for these two populations of normoglycemic rhesus and cynomolgus monkeys? Yes, the blood glucose levels in the rhesus monkeys were significantly higher than those in the normoglycemic cynomolgus monkeys (6.8 years old), most likely due to the old age (10.7 years old). We added the result in the revised version. We added the information to the revised version (Page 12, the 2nd paragraph).

9. The reduced glucose infusion rate of animals which were first clamped with exogenous insulin to minimize endogenous insulin glucoregulation prior to xylazine administration suggested to the authors that “…xylazine-induced hyperglycemia potentially resulted from a reduction of tissue sensitivity to insulin.” The authors should discuss alternative explanations and means to test the validity of their hypothesis. The sentence has been reworded in the revised version as “These results support our hypothesis that xylazine-induced hyperglycemia most likely resulted from a reduction of tissue sensitivity to insulin, because blood insulin was stably maintained at a high level by constant infusion” (Page 13, the 1st paragraph.

Discretionary Revisions
Page 2 line 4: “… becomes clinically relevant…”
Page 8 line 8 from page bottom: “…insulin AUC was significantly ….”
Page 20: Figure 2 legend line 4 “…. B and C. ?????”.

Thank the reviewer for pointing out the grammar errors. We made the corrections in our revised version.
Reviewer 2

Reviewer's report:
This study tested the impact of the exposure of monkeys to xylazine on blood glucose and its regulatory system. Authors confirmed a transient stimulating effect in this non-human primate as well as in many other species demonstrated before. On the comparative study, especially veterinary clinician and researchers, points of view, the findings may increase our understanding of anesthetics’ side effects and would cause the attention in the field of nutritional endocrinology and metabolism. However, the potential issue about how xylazine increases blood glucose is not resolved and well accounted for. The general (non-metabolic and endocrine) impacts of increased glucose seem limited in clinics and human drug-users though authors claimed some in the discussion.

Thank the reviewer for his helpful comments. We revised our manuscript and also addressed the reviewer’s questions/comments one by one in the letter.

Major revisions
1) The authors concluded that tissue sensitivity to insulin is the most probable cause. But the receptor and signaling caused by this hormone are not investigated at all. This is the core of the mechanistic of this paper and thus needs more investigation.

Our conclusion that xylazine-induced hyperglycemia resulted from reduction of tissue sensitivity to insulin is based on the following results.

- Xylazine administration caused hyperglycemia with no significant change in blood insulin in non-diabetic monkeys.
- Xylazine administration still caused hyperglycemia in insulin-dependent diabetic monkeys who had seriously impaired capability to secret insulin and had very low blood insulin level.
- Xylazine administration still caused hyperglycemia in the animals who were under the hyperinsulinemic-euglycemic clamp. During the clamp the blood insulin level was controlled and maintained constantly at a Hyperinsulinemic level via exogenous insulin infusion. The endogenous insulin secretion was negligible during the clamp.

It is widely recognized and accepted that the glucose clamp is the standard gold method for studying insulin sensitivity or tolerance in diabetic research. In this study we used this method to test tissue sensitivity to insulin after xylazine challenge. The results should be very evidential.

The comment raised by the reviewer “to investigate the receptor and signaling caused by this hormone” is an interesting one and may be worth for another study. However, this question is out of the scope of our current study which focused on the effects of xylazine on blood glucose. In addition, xylazine is a traditional anesthetic being used in animals for several decades. Its pharmacological effects have been well investigated and reported.

We are not sure what “this hormone” means here, insulin or xylazine. If insulin, its receptor and signaling are also out of the scope of our current study and also have been well investigated and published.
2) Xylazine-induced side effects including some mortal ones are well described, but the aims of studying blood glucose and using monkeys as experimental models are not sufficiently explained.

The reasons why we used monkeys in this study are as followings:

a) Non-human primates (NHPs) are more close to humans in physiology and pharmacology than other species;
b) The naturally developed diabetic model in NHPs is one of the most valuable models for diabetic research;
c) The glucose clamp method requires frequently sampling of blood which can be difficult in small animals;
d) Collecting enough blood samples for ivGTT, insulin, glucagon and GLP-1 assays may also be difficult in small animals;
e) The effects of xylazine needs to be looked at in diabetic NHPs, because diabetic NHP models have been used widely in diabetic research and xylazine is often used as an anesthesia purpose;
f) We just had the diabetic model available and the opportunity of using the animals for this study.

3) The comparison of diabetic and non-diabetic animals does not make significant xylazine-dependent difference and thus authors do not present meaningful insight from the data.

It is true that xylazine administration caused hyperglycemia in both diabetic and non-diabetic animals. However, the recovery time of hyperglycemia was longer in diabetic animals. Extra precaution may be needed if xylazine is used as anesthetic in diabetic animals in veterinary clinic. Also, xylazine use in diabetic research may complicate the outcomes and data interpretation because of its hyperglycemic effect. The diabetic animals used in this study were insulin-dependent and endogenous insulin secretion was impaired and very limited. Therefore, the diabetic animals used in the study could help us to understand if xylazine-induced hyperglycemia was related insulin secretion or tolerance.

Minor revisions
1) There are some grammatical errors throughout the text.
2) Some descriptions in Result section should be moved to M and M section or be deleted.
3) Preparation of diabetic monkeys and evaluation of its pathological status are not described.

Thank the reviewer for his/her constructive comments. We carefully checked the grammar of the manuscript and asked others (native English speakers) to read over.

We revised Results section as the reviewer suggested.

We indicated that the diabetes model is naturally spontaneously developed and their pathological conditions we validated are briefly introduced in the revised Methods section.
Associate Editor comments:

General Comments
This manuscript describes a series of experiments using non-human primates of 2 different species. The experiments were conducted to determine the hyperglycemic effects and mechanisms of xylazine. The experiments appear to be well-conceived and carefully conducted. The interpretation of the results is reasoned and explained. The authors could focus more on the reasons why their results may differ from those of previous investigations. The authors admit that they did not measure counter-regulatory hormones and that ketamine could have important interactions with these.

Thank the Associate Editor for his/her time and effort to review our manuscript and for his/her helpful comments. We revised our manuscript and addressed the associate editor’s questions/comments either in the revised manuscript and/or in the itemized letter.

Major Criticisms.
The Reviewer’s major concern is the clinical applicability of this work of this manuscript. The authors make the claim that there has been a recent increase in the human abuse of xylazine that make its study clinically compelling. Most of the descriptions of xylazine use are several years old and the most recent (23) does not provide evidence that xylazine use is increasing in a large area. In addition the toxicology and clinical descriptions of xylazine abuse speak against the significance of its hyperglycemic effects as a major concern. Finally, the results of the study themselves call the significance of xylazine’s effect on glucose in primates into question. While the glycemic response to xylazine in primates appears to be robust and significant it’s pathologic significance in normal primates seems minor and while greater in diabetic monkeys its clinical (human) relevance can again be questioned.

Xylazine is widely used for anesthesia in animal research and veterinary clinic. In some geographic areas xylazine has been used in drug abusers. Xylazine-induced hyperglycemia is one of its side effects, which are thus clinically relevant, at least in some xylazine abusers. It is unclear whether the noticeable open skin ulcers in xylazine abusers result from its hyperglycemia. We have modified our Discussion section in the revised manuscript as the editor commented.

Indeed as the authors point out xylazine is related to other alpha 2 agonists such as clonidine. Human studies with clonidine should be referenced and are of major comparative importance. For example, Lattermann R, Schricker T, Georgieff M, Schreiber M. Low dose clonidine premedication accentuates the hyperglycemic response to surgery. CAN J ANESTH 2001 ,48 (8) pp 755?759.

Thank the editor for making a nice point. We cited the paper and added the comparative importance in the revised Discussion section.

There is no reason given as to why there are 2 types of monkeys used. Can the IV GTT be used to call a NHP insulin dependent are these monkeys really supported by insulin? The authors don’t really describe the selection of diabetic monkeys. it appears the these are monkeys that have been previously characterized and have been selected for this study based on this ? for example they are significantly older than the non-diabetic
monkeys. The authors should better describe either how these monkeys were selected or reference such a description. The explanation of using cynomolgus and rhesus monkeys has been added to the Methods section. We also added how these diabetic monkeys were selected in our revised version.

The Discussion paragraph that describes the nature of xylazine abuse amongst humans seems irrelevant and is a major component of the Discussion section. We have modified that part in Discussion section, but believe that there is potential relevance between its hyperglycemia and skin ulcer xylazine abusers.

Minor Criticisms:
Doses used should be given in the results and not just in the figure legends.
Recently, this veterinary anesthetic compound has gained popularity as a new recreational drug among drug abusers during the past decade [20-24]
Page 8 xylazine has not been studied in NHP then it is redundant to state, especially not in diabetic animals?.
Page 11? minimize endogenous the insulin glucoregulation
Thank the editor very much for his time and effort to help us to improve the manuscript.
We made the corrections by adding or deleting in our revised manuscript as the editor pointed out.

Page 13 authors state that ,? Our results suggest that xylazine-induced hyperglycemia results from the decrease of tissue sensitivity to insulin, which leads to a reduction of glucose uptake and utilization by various tissues.? But they have already stated that this may not be the case in other animals.
That is the main finding of this study evidenced by the xylazine hyperglycemic results from the insulin-impaired diabetic animals and during hyperinsulinemic clamp.

The authors state that , ?Therefore, it is possible that the cause of xylazine-induced hyperglycemia results from stimulation of ?2-adrenoceptors and then modifying other stress hormones, such as ACTH and GH. Although these hormones were not measured in our study, xylazine has been reported to increase the release of ACTH and GH in cattle and dogs.? Understanding the mechanism of insulin resistance would be helpful and one wonders why one of these was not measured. (page 13)
Please see the response to the 1st reviewer, No. 5.