POINT-BY-POINT ANSWERS TO REVIEWERS AND EDITORS:

Manuscript NO.: 76316, Basic Study, entitled :
Hyperglycemia and reduced adiposity of streptozotocin-induced diabetic mice are not alleviated by oral benzylamine supplementation
by: Christian Carpéné, Kristiyan Stiliyanov Atanasov, Francisco Les and Josep Mercader Barcelo

REVIEWER #1:

General comment) The manuscript entitled "Hyperglycemia and reduced adiposity of streptozotocin-induced diabetic mice are not alleviated by oral benzylamine supplementation" describes the effect of oral benzylamine on type 1 diabetes. However，it is difficult with the results presented to conclude that oral benzylamine is invalid in type 1 diabetes. I have some concerns and issues for the authors to address that may enhance the clarity of the manuscript and strengthen the data.

Thank you for your careful perusal. The authors initially thought that benzylamine (Bza) was valid for treatment of type 1 diabetes since it was an hypothesis raised by the corresponding author more than a decade ago and we are glad that reviewer #1 shares the same view. Unfortunately, the current data scientifically collected in the chosen mouse model of drastic insulin-deficient diabetes did not support this hypothesis. As we have already demonstrated that the amine helps in limiting the diabetes in obese and diabetic insulin-resistant db/db mice (Iffiú-Soltész et al. Oral supplementation with benzylamine delays the onset of diabetes in obese and diabetic db-/- mice. Nutrients, 2021, 13, 8, 2622. DOI: 10.3390/nu13082622), we planned that Bza can be also helpful in type 1 diabetes. As it will be argued below and in the revised manuscript, it is not the insulin-deficient state *'per se'* of the streptozotocin-treated mice (STZ-mice) that prevented the efficiency of benzylamine consumption, but rather the extreme atrophy of adipose tissue induced by STZ challenge. Thus the authors have emitted doubt about the efficiency of Bza treatment only in the case of 'skinny' type1 diabetic states. It appears that Bza exerts some insulin-like action on adipose cells only. Perhaps initially bad formulated, we hope this message is now correctly presented in the revised version.

1. the study should experiment with different dose of benzylamine.

The reviewer is right in absolute, and supposes that higher dose could have been efficient. However, the current study was performed as a comparative approach, having as reference the somewhat successful effect of Bza in insulin-resistant, diabetic and obese db/db mice (Iffiú-Soltész et al., 2021, DOI: 10.3390/nu13082622), in which a dose closely similar to that administered here was able to lower the extreme hyperglycaemia. In fact, Bza was given at 0.5% (w/v) in the drinking water of the obese and diabetic treated mice, and the Bza intake was determined to vary between 9300 and 10,100 µmol/kg bw/day in this recent study (see ref above). Here, Bza was also given at 0.5% in the drinking water and the calculated consumption of STZ-mice was 10,850 ± 598 µmol/kg bw/day, as reported in the original version of the Ms. Therefore the similarity between these doses entirely justifies our choice: in the same order of magnitude, Bza was active in one model and inefficient in another, at least regarding glucose homeostasis. However, in regard with its effect on water intake, the same dose of Bza induced almost the same reduction in the two models. In these conditions, what is the value to test any other (higher ?) dose in the model that was non responsive for antihyperglycemic effect? Irrespective of its results, a complementary study with elevated dose will not fit with the scope of Nutrients but rather with that of a Pharmacological and Toxicological Journal. Lastly, other dietary consumptions of Bza have been performed with a similar dose: e.g. 0.44% solution to mice under obesogenic diets (Iffiú-Soltész et al., 2010,

DOI: 10.1016/j.phrs.2009.12.014). We therefore consider that no other dose study is necessary for the revised version to support the focused message we want to disseminate.

2. author wrote in the paper that an insufficient amount of adipocytes isolated from the atrophied WAT of STZ mice hampered the verification of glucose transport responsiveness to insulin and benzylamine in the type 1 diabetic state. However, evaluate the effect of benzylamine in the adipocytes are necessary, lack of this result cannt support the auther's conclusion.

Again thank to the reviewer for such clever remark. The only evaluation that could be performed on the atrophied fat stores of the STZ mice during revision was about Bza oxidation by homogenates of frozen samples of white adipose tissue (WAT), and this has been included since it indicates that Bza is poorly oxidized in this tissue. The conclusion of this additional exploration is in line with our expectations: there was less metabolism of this amine in poorly developed fat depots. Therefore, due to the clear-cut smaller quantity of WAT, as well as to the tendency for a lower metabolism of Bza, its resulting activation of glucose transport was surely quantitatively lower in the STZ mice than in control. Let us remind that BZA stimulatory action on hexose uptake also disappears in mice invalidated for AOC3 gene (encoding for the amine oxidase predominantly involved in Bza oxidation), and after pretreatment with the amine oxidase inhibitor semicarbazide (ref #11). Thus, the authors do not think that the requested unrealisable experiments are mandatory in this situation. A more constructive criticism would have consisted in explaining to authors how to explore adipocyte responsiveness when there is not sufficient available biological material (by miniaturizing the techniques...). It is specified in the Ms that, even after pooling WAT from different anatomical locations, we did not succeed in obtaining sufficient functional adipocytes after tissue digestion. Nevertheless, we reported in the normoglycemic animals the evidence of the direct insulin-like action of Bza on glucose transport. As far as we know, we did not claim that Bza was effective in vivo on the STZ-induced hyperglycemia, on the contrary, we wanted to honestly publish somewhat 'negative' results. It is therefore rather fake to ascertain that our results do not support our conclusions, due to a few missing in vitro explorations.

3. author need more works to prove this opinion. Study is not deep enough, molecular mechanism of benzylamine in STZ diabetic mice is need explore.

We respectfully disagree with reviewer's proposal, especially whether the comment is linked with the molecular mechanisms involved in the insulin-like effect of Bza in hexose uptake activation in adipocytes. Let say that we have already published about the Bza-dependent pathway leading to glucose transporter translocation to the cell surface, after inhibition of tyrosine phosphatase activity, and increased tyrosine phosphorylation of key proteins involved in insulin-signalling. This was examined in rat fat cells (Marti et al. Combined treatment with benzylamine and low doses of vanadate enhances glucose tolerance and reduces hyperglycemia in streptozotocin-induced diabetic rats. Diabetes, 2001, 50, 2061-2068, already quoted as ref #10) and in murine 3T3 adipocytes (Enrique-Tarancon et al. Substrates of semicarbazide-sensitive amine oxidase co-operate with vanadate to stimulate tyrosine phosphorylation of insulin-receptor-substrate proteins, phosphoinositide 3-kinase activity and glucose transporter translocation in adipose cells. Biochem J 2000, 350, 171-180) and is not of utmost interest to be re-explored and re-published in a mouse model that is less responsive than others, with regard to glucose handling.

As noted by another reviewer, there is already another work that let suppose that Bza could be active in type 1 diabetes, but only when this amine is associated with vanadium and when the insulin-deficient animal model is not totally lipoatrophic such as the rats with bw >200 g

treated with STZ at 45-70 mg/kg bw (ref#10). At this stage, it is important to note that we have recently observed that other amines does not exhibit any potentiation with vanadium when looking at their effect on the activation of glucose consumption by in human adipocytes (Carpéné et al. High doses of catecholamines activate glucose transport in human adipocytes independently from adrenoceptor stimulation or vanadium addition. World J. Diabetes, 2022, 13, 37-53. DOI: 10.4239/wjd.v0.i0.0000, quoted as ref #27). Consequently, our interest is now focused on the effect of Bza alone, without combination with vanadium, because this combination appears poorly relevant for humans in spite of the impressive synergism observed in rodents.

REVIEWER #2:

Specific Comments: The authors obtained favorable results for the effect of benzylamine.

Thank you. Indeed, as confirmed by another reviewer, the same dose of Bza chosen for treating STZ-induced diabetic mice and genetically obese/diabetic db/db mice (ref #1) resulted in a clear limitation of their polydipsic behaviour.

The authors must confirm that their diabetic model is type 1 by measuring blood insulin levels in order to improve the manuscript. In addition, the authors must confirm the effect of benzylamine in their animals by measuring the activity of monoamine oxidase.

As recommended by the reviewer, the blood insulin levels are now reported in the revised manuscript. Thank you for noting this omission. Since as it was specified as soon as the Methods that only STZ-treated mice with blood glucose levels > 300 mg/100 mL were included in the control and Bza-treated groups of diabetic animals, we though that we had clarified enough the type 1 diabetes of these mice. As expected a clear-cut difference between control and animals challenged with streptozotocin was evidenced, as it was the case in previous of our works using the same pharmacological induction of diabetes (e.g. Visentin et al. Eur. J. Pharmacol. 2005, 522, 139-146). Moreover, the widely used models of type2 diabetes are not obtained by pharmacologic treatment but rather by nutritional challenge (Diet Induced Obesity, High Sucrose Feeding, etc).

As requested, we have measured the capacity of adipose tissue to oxidize Bza, owing to a radiochemical methods that works on stored frozen samples since most of Bza is oxidized in WAT by an amine oxidase, which is not the monoamine oxidase as supposed by the reviewer, but by a copper-containing enzyme called semicarbazide-sensitive amine oxidase (SSAO also known as AOC3 or VAP-1), which is resistant to cold and functions in thawed samples. A paragraph in Methods, a Figure 5, and sentences in Results and Discussion have been added in the revised version. However due to the scarcity of biological material, we could not perform a complete pharmacological analysis of the amine oxidase involved in this oxidation. But the authors remind that they have already demonstrated that: 1) semicarbazide prevents the antihyperglycemic effect of Bza in nondiabetic rats by inhibiting >90% of SSAO activity (ref #10), 2) SSAO activity is totally abolished and no detectable benzylamine oxidation is found in tissues, including WAT, from two distinct lineages of mice genetically invalidated for AOC3 gene (Jargaud et al. J Physiol Biochem; 2021, 77, 141-154). We therefore suppose that the lowered capacity to oxidize Bza reflects limited SSAO activity, the richness of which was decreased in WAT of STZ mice irrespective of the supply of one of its substrates by the Bza-drinking treatment. Together with the dramatic reduction of WAT mass in STZ mice, the data added during revision further support our interpretations of a poor involvement of WAT in the biotransformation of ingested Bza in animals with lipoatrophy.

Please revise the language throughout the manuscript. Cuproenzyme is one of the strange words in the manuscript. Cu proenzyme should be used.

We totally disagree with the example used by the referee. It is not constructive at all. Indeed, copper is a critical functional component of several essential enzymes known as 'cuproenzymes' since decades. One of the earlier reference mentioned in the PubMed database is from 1976. The review of O'Dell entitled "Biochemistry of copper" published in Med Clin North Am. 1976;60(4):687-703 overtly use the term cuproenzyme. This recognized name is widely used by enzymologists, while your proposed "cu proenzyme" has no known meaning, at least to our knowledge. However, since World Journal of Diabetes has a large audience not limited to enzymologists, we have replaced this term by the less specialized term 'copper-containing enzyme'. Whatsoever, we have tried to improve English style during revision

REVIEWER #3:

Specific Comments: This submission investigated the effects of oral benzylamine on lipoatrophic changes in type 1 diabetic mice.

Thank you for careful perusal and perfect understanding of our goal.

1. A supplementation with 0.5% benzylamine (5 g/L) in the drinking water has been found to prevent the onset of diabetic complications in genetic mice by authors. Is it same for the normal mice?

As specified in an answer to another referee, as well as in the discussion of the original version of the Ms, we quoted a previous of our reports showing that sustained consumption of Bza at 0.44% in the drinking water during 17 weeks reduces body weight gain and non-fasting glycemia in mice fed an obesogenic and diabetogenic diet (ref#22). If by using 'normal mice', the reviewer confers to the normoglycemic mice included in this study: no influence was reported on any of the parameters measured, except a tendency to limit water consumption. Notably, the 'normal' insulin responsiveness was not altered by Bza-drinking in adipocytes. The beneficial effect of Bza, if any, is supposed to be antihyperglycemic, and could not be evidenced in normoglycemic mice since we described that it is devoid of hypoglycemic effect. This issue was shortly mentioned in Discussion for keeping it concise.

2. The in vitro insulin-like actions of benzylamine have been mentioned in previous reports shown in introduction. Thus, authors speculated that benzylamine shall be effective in type-1 diabetes. However, results in current study were not the same. Why?

Thanks for these comments. First, let us tell that if a given researcher is sure to find what was expected in all his/her planned experiments, then this researcher is doing -or excellent research - or more merely only tutorial practical works at the University! More seriously, it is true that the current work fails in demonstrating directly the masterpiece proposed to be lacking in the lipoatrophic and diabetic STZ-mice studied: their low amount of WAT is not sufficient to oxidize ingested Bza and to generate in the close vicinity of adipocytes a threshold of hydrogen peroxide capable of exerting insulin-mimicking effects on glucose disposal. Due to the paucity of fat cells in the STZ mice of the study, we could not directly demonstrate that Bza was no more activating glucose entry in fat cells. Nevertheless we have evidenced clearly: 1) the reduced fat stores, 2) the lower SSAO activity in WAT of STZ mice, 3) the direct insulin-like action of Bza in adipocytes of non-diabetic mice, 4) its lack of change after sustained Bza consumption, 5) the inhibition of such Bza-induced glucose utilization by SSAO pharmacological blockade. All these converging observations led us to propose that a minimal amount of WAT is required to orientate the metabolism of ingested dietary amine into a somewhat insulin-like action consisting in facilitating the glucose storage under the form of lipids. When this pathway is sufficiently developed, then the help in glucose disposal is made at the expense of a noxious hypertrophy of WAT and the onset of obesity-related complications. Curiously, what we remain unable to explain is why

the sustained consumption of the SSAO substrate Bza did not improve the expression of its metabolizing enzyme, SSAO in the atrophied WAT of STZ mice.
Indeed, we were aware that not all the ingested Bza is metabolized by oxidation in WAT, and we have discussed that the dietary amine act on other targets and exhibit other effects, such as the limitation of water intake, found to be equivalent in the extremely lean STZ mice used here and in the obese/diabetic db/db mice (ref #1), which are probably independent from SSAO activity, as documented by the central effects of this amine described by the group of Raimondi et al. (refs # 32,35,36).

3. Success of the diabetic model was not identified in a good way.

We respectfully disagree with this negative remark, which is not constructive. It was clearly specified in Methods that mice were selected after STZ challenge on the basis of their elevated blood glucose (> 300 mg/100mL). In the revised version, it is even clearer for the reader that the STZ-challenged mice were hyperglycaemic (see Methods and Figures 2A &B), insulin-deficient (see Results), and exhibited the characteristic hyperphagic and polydipsic features (see Figure 1). What is required to further define a widely accepted model of type1 diabetes? It was already specified in the Results of the original version that we were prone to examine other surrogate markers of the diabetic STZ mice (e.g. fructosamine, NEFAs, glycation markers, pancreatic insulin stores, etc) but this was rendered useless since Bza treatment did not lower the dramatically elevated blood glucose levels (> 4g/L). We do not think this decision was in the wrong way.

4. Insulin sensitivity was ignored in current study.

What is meant? The insulin sensitivity of the adipocytes regarding hexose uptake activation was not modified by Bza-drinking in normoglyemic mice as shown in Figure 4. EC_{50} values for insulin activation of hexose transport, reported in the original version, remain in the text of Results after revision. Whether the referee is asking for an in vivo test of insulin sensitivity, we unfortunately did not perform such invasive test requiring serial blood sampling since our working hypothesis was to test Bza in the absence of insulin, and not to examine whether Bza could act as an insulin sensitizer. This is an exciting issue, but which probably requires other approaches than those planned in the current study.

5. Bias of benzylamine in the drinking water was not conducted in detail.

Thank you for being well informed about putative changes in the water consumption between control and treated groups. The authors presume that these comments were raised by the details given in Results about Figure 1B. We paid a special attention to measure water intake: while only a tendency to limit water intake was observed in normoglycemic mice, an almost complete correction of the polydipsia was seen in STZ mice. We did not miss this change, and discussed overtly about the real vs planned intake of Bza. Do the reviewer means that an adjustment was necessary? In this case, what arguments are constructively proposed? To correct the supply of control arm by reducing Bza dose to reach the daily consumption of STZ mice, or the reverse? Does the reviewer realize how difficult and dubious these adjustments are, as they are always biased by decay between the prior determination of the driving arm of the mouse groups and its application to the other arm. To this reason, Pair-feeding or pair-drinking is mandatory in treatments that are highly efficient. Not in the precise current case, since treated STZ mice consumed the same amount of Bza than treated db/db obese mice (see ref#1). I the referee wants to refute our conclusions, better than proposing to repeat expensively the same study with pair-drinking, an alternative could have been to propose the test of Bza at different doses in another type 1 diabetic model with larger adiposity, but this is another study /meassage/article.

6. Oral benzylamine may improve glucose handling in high-fat diet fed mice and it directed authors focusing the lipoatrophy of STZ diabetic mice. However, linkage of it with the recovery of hyperphagic and polydipsic behavior in diabetic mice was not discussed.

Exact. We did not use any argument to link lipoatrophy and hyperphagic/polydipsic behaviour in STZ mice. However, in the special case of Bza-drinking, the reduction in water intake was expected to traduce a reduced water and glucose leak in urines, but it was not the case. This was discussed in the light of the findings of the group of Raimondi, who showed that central Bza effects on appetite regulation were independent from its oxidation by SSAO (refs #32, 35, 36). Since urine emission could not be determined, we did not add other succinct comments to avoid further speculations.

7. The dietary amine was effective to reduce hyperglycemia in alloxan-injected rat as shown in Reference 41. Why? Additionally, adipose atrophy induced by alloxan or not that shall be discussed in detail.

We agree with the referee's remark. Initially used to rule out a putative rescue of insulin secretion by ingested tyramine, the work of Lino et al (ref #41) has also been introduced to suggest that other amine oxidase substrates might influence glucose disposal in insulin-deficient states that are not accompanied with a dramatic lipoatrophy as in the case of the STZ mice of our study. Perhaps a preventive effect can be expected -and obtained in the case of alloxan-challenged rats- as long as sufficient fat stores contribute to the dietary amine biotransformation and its improvement of glucose disposal in peripheral tissues (mainly WAT). Moreover it is specified in the Ms that: 1) the reduction of hyperglycemia by tyramine in this quoted work was only about 35-43%, 2) tyramine is the substrate of both MAO and SSAO. In line with the observations of Lino et al., we also observed that tyramine modestly corrects the glucose intolerance of STZ rats, which are less lipoatrophic than the mice of this study (Visentin et al. Effect of prolonged treatment with tyramine on glucose tolerance in streptozotocin-induced diabetic rats. J. Physiol. Biochem, 2003, 59, 225-232). In spite of this partial efficiency, the dietary tyramine cannot be considered on its own as a strong antidiabetic agent.

8. *Another evidence that methylamine inhibited the insulin degradation by adipocytes is a good target for current report.*

We thank the reviewer for understanding our proposal in discussion. However, there is a so defective insulin production in the STZ mice used in this study that one can expect that its low circulating levels are more the consequence of pancreatic failure than increased turn-over. Moreover, considering the low amount of WAT of this model we did not plan to repeat in 'normal' and 'STZ' adipocytes the pioneering investigations performed by Kahn & Baird, in which the methylamine effect likely accounts for the internalization of insulin and its receptors in adipocytes.

9. *WAT was not enough in STZ-Mice that needs evidence while it has been used in the conclusion.*

The figure 3 brings evidence of the WAT atrophy of STZ mice. The additional Figure 5 shows lower Bza oxidation in WAT of STZ mice. Together, these data support our conclusion about a limited biotransformation of Bza in lipoatrophic diabetic mice and . Curiously, they are mirroring the opposite situation seen in obese and type2 diabetic db/db mice, showing high tissue-bound SSAO activity, WAT hypertrophy (refs #1 and 32) , and improved glucose disposal when subjected to Bza consumption (ref#1).

SCIENCE EDITOR:

The manuscript investigated the effects of oral benzylamine supplementation on glucose handling and adiposity in insulin-deficient STZ mice. And they found benzylamine supplementation could not normalize the altered glucose handling of STZ diabetic mice with WAT atrophy. The study is of some interest, but the manuscript still needs substantial revision according to the issues raised by the reviewers to further support the author's conclusion. Especially, insulin secretory function and insulin sensitivity should be evaluated as they both are important indicators of glucose metabolism.

Thanks for this overview, the insulin deficiency induced by the streptozotocin challenge has been documented during revision, while the insulin sensitivity was already demonstrated to be similar in the adipocytes of control and Bza-drinking normoglycemic mice (Figure 4). On frozen WAT samples, we could complete the analysis of Bza oxidation and add it to the revision (Figure 5). Unfortunately no remaining biological material was suitable to further explore the insulin sensitivity of the STZ-treated mice, the altered glucose handling of which was not alleviated by Bza-drinking. During our additions/deletions made in red font, we tried to make efficient language polishing.

COMPANY EDITOR-IN-CHIEF:

I have reviewed the Peer-Review Report, the full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements of the World Journal of Diabetes, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the Criteria for Manuscript Revision by Authors. Before final acceptance, uniform presentation should be used for figures showing the same or similar contents; for example, "Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...". Please provide decomposable Figures (in which all components are movable and editable), organize them into a single PowerPoint file. Please check and confirm whether the figures are original (i.e. generated de novo by the author(s) for this paper).

The figures are original, never published elsewhere and organized as requested. We have included in Results messages for their optimal inclusion in the text. Please not that these messages @@note to production: Fig. 1 inserted about here@@ have to be deleted during the production process.