Author's response to reviews

Title: Training exercise at the aerobic/anaerobic metabolic transition prevents glucose intolerance in neonatal alloxan treated rats

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Author's response to reviews: see over
Dear reviewer Cheng Juei-Tang,

Title: Exercise training in the aerobic/anaerobic metabolic transition prevents glucose intolerance in alloxan-treated rats,


The authors are very grateful for the comments and suggestions. We tried to briefly make the corrections in a satisfactory way in order to improve the quality of the manuscript. We have applied the modifications to the text whenever possible following the referee's commentaries. The corrections were made as follows:

**Question:** The method using alloxan to induce type-2 diabetes shall be added the reference(s). If authors were the first one to develop this method, please explain the dose of alloxan, the date for injection and the female rats used for injection.

**Answer:** We modified the text in pages 3 and 6 following the suggestions. The experimental model employed in this study was originally proposed by Kodama et al (Diabetes Res Clin Pract, 20:183-189, 1993), but with male rats instead of females and the days of drug administration were set to their 2nd, 4th and 6th days of life (at 200mg/kg of body weight). We have previously found with this dose resulted in the rats presenting spontaneous recovery from the glucose intolerance past 90 days of life of the drug administration (Oliveira et al, Exp. Physiol, 90: 79-86, 2004). Based on this finding, we decided to modify the procedures from the proposed by Kodama et al (1993) to the alloxan dose of 250 mg/Kg of body weight and the administration day to the 6th.

**Question:** The presence of insulin resistance in this animal model shall be characterized. It is hard to find the difference in HOMA values between alloxan-treated group and vehicle-treated control. How do you know this model is reliable?

**Answer:** We chose HOMA index to reflect insulin resistance because of the great number of experimental models in the literature about many diseases in which HOMA index proved efficient to indicate insulin resistance (Sánchez et al.,
Endocrinology. First published ahead of print November 8, as doi:10.1210/en.2007-0630, 2007; Komaki et al., Endocrine Journal 52 (3), 345-351, 2005; Ping et al., J Zhejiang Univ Science B 7(8):627-633, 2006). As this is a test based on mathematical calculations, it turns efficient when applied to experimental models presenting both elevated plasmatic fasting glucose and/or insulin concentrations. In the present study, in spite of the glucose intolerance, the animals did not experience hyperinsulinemia, which could have affected the use of HOMA index as an indicator of insulin resistance.

Regarding the model validation, we are working on finding alternative ways other than HOMA indexes to assess insulin resistance, like the insulin tolerance test.

**Question:** Exercise training employed the different way, but not the running as regular. Please add the reason(s).

**Answer:** We chose to employ training by swimming instead of by running because otherwise many rats would be necessary, considering that not all animals can run in a treadmill. This way, we would have to pick up those able to run out of a lot. In addition, some previous studies of ours and others have showed that training by swimming induces just adequate results to what regards the effects on the aerobic conditions of eutrophic animals (Gobatto et al, Comparative biochemistry and physiology Part A 130, 21-27, 2001) and rat models of metabolic disorders (Hart et al., J Appl Physiol 91:1663-1668, 2001). The use of treadmills is a routine in our lab (Manchado et al., Journal of Exercise Physiologyonline, 8 (4), 29:35, 2005), thus future studies also evaluating its comparative effects are warranted.

**Question:** The blood sugar level in this model shall be indicated. Then, changes of blood sugar need to show in detail.

**Answer:** In order to depict alterations on the serum glucose levels we have added to the results section the corresponding areas under the glucose curves during GTT and the values of HOMA indexes in each day of experiment (28th, 60th, 90th and 120th days of life). See pages 8 and 9.
**Question:** Effort test needs more detailed statements and reference(s). Also, the relationship is reliable in human subjects. But is it also related in animal? Please add more data.

**Answer:** In Figure 2 we presented the results for most animals of each group as to illustrate what in fact took place during the test, because it was impossible plotting a single curve with the mean blood lactate levels and overloads of all animals since they used different loads to reach the metabolic transition. We also added to results section the remaining data of workloads and lactate concentrations during MLSS testing.

Regarding the reliability of the correlation between workload/blood lactate levels of the animals, it was demonstrated in a previous study of our group (Gobatto et al, Comparative biochemistry and physiology Part A 130, 21-27, 2001).

**Question:** The references show too much of human data. But the related and recent references for animals were not indicated. It shall be improved in detail.

**Answer:** We added references to studies using animals. However, it was not always possible since there is a lack of data linking experimental models of type 2 diabetes with swimming sessions.

**Question:** Expression of data in figures need to improve. It is not easy to follow.

**Answer:** To make figures 2 and 3 more intelligible, we have included the obtained figures for each group to the results section. We also modified figure 2 which now shows the MLSS results most of the animals of each group instead of one animal of each group. The HOMA and GTT analyzes carried out at 28th day of life were separated from the remaining results relative to 60th, 90th and 120th days of life, being now depicted as figures 1A and 1B.

Sincerely

Clécia Soares de Alencar Mota
Dear reviewer Helena Barros,

Title: Exercise training in the aerobic/anaerobic metabolic transition prevents glucose intolerance in alloxan-treated rats.

Version: 1 Date: 20 November 2007.

The authors are very grateful for the comments and suggestions. We tried to briefly make the corrections in a satisfactory way in order to improve the quality of the manuscript. We have applied the modifications to the text whenever possible following the referee's commentaries. The corrections were applied as follows:

Question: Background- What is the relevance of the following sentences found in page 3 -4 for the study? These explanations might be important to be summarized and fit into the methods section.

Answer: The mentioned paragraph of the Background section on pages 3 and 4 was included in this section to justify the increased administered drug dose (250mg/Kg) and chosen administration day (6th day of age) for this study. Moreover, Kodama et al (Diabetes Res Clin Pract, 20:183-189, 1993) verified that the 6th day of age would be the best to administrate the drug because of the developmental stage of most of the rats’ organs, increasing the drug’s efficiency. This information in the revised form was moved to page 5.

Question: The maternal deprivation required for 6 days old pups to be in a 16 hs fasting influences the animals behaviors and results in a deregulation of the HPA axis at multiple levels when adults. How did the authors control for that?

Answer: Regarding the forced separation of the pups from their mothers, we have tried to avoid too sudden alterations in the conditions to both pups and mothers. We always kept the baby rats in the same cage in which they previously were with the mothers because of the smell as to attenuate the absence of the mother. The same was made with the animals injected with citrate buffer (controls) so that all were
submitted to the same stressful situations, equalizing the experimental conditions. We used fasted animals since there is information in the literature that shows a decrease in the drug efficiency if animals are fed (Katsumata et al, Horm Metab Res 24: 508-510, 1992; Szkudelski et al, Physiol Res 47: 343-346, 1998).

Question: Substitute ..... followed by Newman-Keuls test or Friedman test, where appropriated.... for followed by Newman-Keuls test or Friedman test, where appropriate.

Answer: The text of page 8 was modified as indicated.

Question: Results- it is important to include the calculated F values for each factor (alloxan AND exercise) and for interaction in the 2 W-ANOVAS.

Answer: As suggested by you the values were added in the results description.

Question: It seems that for water intake and food intake the animals submitted to T consume more than animals not trained irrespective to the diabetes condition.

Answer: Trained animals tended to eat and drink more, with or without being injected with alloxan. This might occur as to renew their energy after the physical activities (5 days, 1 hour/day). In other studies carried out in our lab (Oliveira et al, Exp Physiol, 90: 79-86, 2004) we also observed an increase in water intake and weight reduction in animals that were trained.

Question: Explain the lack of face validity of the model, because the A animals show decreased weight, no change in water intake, no change in insulin resistance (during most of the time).

Answer: As can be checked in the discussion section, it is possible that the weight loss observed in alloxan-injected animals was due to the drug effects in reducing insulin levels on the rats’ first days of life, what might have compromised their body growth and development. There are studies in the literature that show that early in life insulin restrictions retard body weight gain, maturation and growth (Adams, Exercise and Sport Sciences Reviews, v.26, p.31-60, 1998; Luciano et al. Medicine and Science in Sports and Exercise, v.30, p.S24, 1998.1998).
We chose HOMA index to reflect insulin resistance because of the great number of experimental models in the literature about many diseases in which HOMA index proved efficient to indicate insulin resistance (Sánchez et al., Endocrinology. First published ahead of print November 8, as doi:10.1210/en.2007-0630, 2007; Komaki et al., Endocrine Journal 52 (3), 345-351,2005; Ping et al., J Zhejiang Univ Science B 7(8):627-633, 2006 ). As this is a test based on mathematical calculations, it turns efficient when applied to experimental models presenting both elevated plasmatic fasting glucose and/or insulin concentrations. In the present study, in spite of the glucose intolerance, the animals did not experience hyperinsulinemia, which could have affected the use of HOMA index as an indicator of insulin resistance.

Regarding the model validation, we are working on finding alternative ways other than HOMA indexes to assess insulin resistance, like the insulin tolerance test. It seems that the β pancreatic cell mass is compromised in the alloxan treated rats, as was observed with newly born rats treated with streptozotocin, which reduces the production/release of insulin. When low concentrations of insulin were used to calculate HOMA indexes, no significant increases in the indexes occurred, thus not enabling us to state that the animals were insulin resistant.

**Question:** Figure 1- should present group data instead of one animal example for each, control and alloxan groups. Figure 2 legend and others- correct “TC= Treinad control” Fig 3- Not all legends “C=Control; TC= Treinad control; A= Alloxan e TA= Treinad alloxan. “ b = C vs A; c = C vs TA; d = A vs TA; e = TC vs TA.

**Answer:** The figure legends were modified as indicated.

**Question:** make sense-please review if a three way ANOVA is not the best analysis to be applied.

**Answer:** A specialist in statistical analyzes helped us and considered that, based on our dataset, it would be better to keep ANOVA 2-WAY analyzes instead of ANOVA 3-WAY. We should consider three factors to use ANOVA 3-WAY: administrated drug, exercise training and time. As we carried out many experiments,
the evaluations are not necessarily repeated measures of the same animals in away that each experiment set could be analyzed individually (28th, 60th, 90th and 120th days of life). Thus, ANOVA 2-WAY becomes more adequate to the present study.

**Question:** The sentence “Our results show that the sedentary control group (A) showed higher body weight than the other groups while the trained alloxan group (AT) presented the highest food intake without transforming this energy in body weight gain” in discussion is not supported by the results presented and is incorrect when one looks at results in table 1 and the sentence “Group C presented higher area under the curve of body weight than the other groups (CT, A, AT). AT group presented higher food intake when compared with the sedentary groups (C and A).”

**Answer:** On checking the mentioned text, we identified a typographical error in the results section that has been checked on page 9 of the revised ms.

**Question:** Other sentences in discussion are also without the support of evidence. Therefore, the results of the study regarding the effects of exercise on HOMA and glucose tolerance do not support the conclusions of the authors.

**Answer:** We included further references and modified some parts of the Discussion section in order to make it clearer. Moreover, we included a statement about the effectiveness of HOMA indexes in identifying insulin resistance in the model used to the conclusion section. As previously mentioned, in forthcoming studies using this experimental model, we will try to identify insulin resistance by other means.

Sincerely,

Clécia Soares de Alencar Mota