Author's response to reviews

Title: Effect of resveratrol on alcohol induced mortality and liver lesions in mice

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Version: 3 Date: 11 October 2006

Author's response to reviews: see over
October 11, 2006

Dear Editor,

In compliance with the suggestions made by the reviewers regarding our manuscript entitled "Effect of resveratrol on alcohol induced mortality and liver lesions in mice" (MS 806903825117846), the following changes have been introduced:

**REVISOR 1**
Revisor – Vladillav Eybl

**Major Compulsory Revisions**

1. Complete literature references with papers published in 2006, results from paragraph "Laboratory findings" would be better to put into the figures or tables.

   *New and more references have been added. Laboratory findings and results are adapted and included in a figure.*

**Minor Essential Revisions**

1. Improve the explanatory note to figures, more details and necessary

   *Explanatory note of the figures is improved.*

**REVISOR 2**
Carani V. V Anuradha

**General**

**Points of concern**

1. Introduction does not focus on the need/importance for the study and is vague.
A new paragraph has been added in the Introduction in order to notice the importance of this study.

2. What is the amount of alcohol and resveratrol consumed by the animals? This should be determined from fluid intake. Alcohol-treated animals drank less water and this could decrease the amount of alcohol & resveratrol consumed as compared to those not given alcohol.

Each mice on the alcohol and alcohol plus resveratrol groups did intake approximately 3 c.c. that is around 30 mgrs of resveratrol and 0.24 grms of alcohol at day. Both alcohol and alcohol plus resveratrol groups decreased there intake along the days but without differences between them. On the Discussion a paragraph dealing with this event has been added.

3. Photographs to show histological changes are needed. Data presented show only mild changes as presented in discussion, which are not consistent with high mortality (survival 20%) at the 7th week.

Two pictures illustrating the procedure are added.

We agree with the reviewer on the fact that we also surprised of the mortality observed on mice with slight hepatic damage. Our expectation was to find a more severe liver damage. This is an important point and that is why it is discussed on the Discussion chapter pointing on how difficult is to promote hepatic damage on mice only with alcohol and comparison is made with other models like Tsukamoto’s (reference 22). That is why we speculate on the existence of a multiple organ failure (MOF) produced by the alcohol and due to many factors including starvation, blood coagulation changes, increased susceptibility to infection, sepsis, etc).

4. The number of animals used for each study (biochemical, histology, mortality) should be specified.

Reviewer is right. In order to confirm pathology or laboratory results, they were repeated on three instances (not two as previously stated. Text has been changed). Animals for pathology studies were 18 per group, same number as for laboratories studies.

5. The appropriate dosage of resveratrol for human consumption as an outcome of the study could be suggested while considering prophylaxis.

As suggested by the reviewer dose has been added on the discussion. This dose should be the equivalent to the one needed in humans to prevent mortality and alcohol-induced damage (last paragraph). Dose was selected according to resveratrol concentration found on red wine (3-12 mgrs/L).

6. What is the reason for the absence of TNF-alfa in plasma in response to ethanol? This should be discussed along with the rise in pro-inflammatory cytokine, IL-1. What are the consequences of a rise in IL-1?

TNF-alfa levels in humans has been related with mortality and the seriousness of the hepatic damage induced by alcohol. That is the reason why we expected to find
elevated levels of TNF-alfa in the alcohol intake group, but expectations failed. More recently other studies on mice have shown that TNF-alfa plasma levels are poor diagnostic markers for the severity of the TNF-alfa dependent liver inflammation. Moreover TNF-alfa plasma levels in response to lipopolysaccharide (LPS) remains elevated a very short period of time. However IL-1 remains elevated for a longer period. This can be due to an augmented clearance of TNF-alfa not involving IL-1, a smaller systemic inflammatory response, to neutralization of TNF-alfa by its type 1 soluble receptor resulting in a marked attenuation of the late phase of the alcohol action.

Both IL-1 and TNF-alfa are elaborated after macrophage activation and produce an endothelial activation process leading to an increase of adhesion molecules expression, secretion of other cytokines, growth factors, echosanoid production, nitric oxide and increased endothelial thrombogenicity. Both cytokines produce fibroblast activation leading to proliferation and augmented extracellular matrix synthesis. IL-1 and TNF-alfa produces stimulation of the acute phase systemic manifestation including somnolence, fever, changes liver protein synthesis, changes in metabolism (caquexia), PMN migration, ACTH liberation and augmented steroid liberation (Robins).

This aspects have been included in the Discussion chapter as well as those produced by IL-1 and supporting references have added.

7. The authors should consider and report data on the mechanisms by which ethanol could reduce survival other than malnutrition. Since some of the benefits of resveratrol suggested in the discussion are a block in nitric oxide production, COX-2 and NF-κB pathway.

In the Discussion other mechanisms why alcohol produces increased mortality not only due to malnutrition have also been included. Alcohol intake in large amounts, like done in this model, has deleterious effects on circulatory system, immune system, neurological system and muscle an others systems and organs (liver, pancreas, blood elements and components, endocrine glands, and so on). All this lead to an increased mortality.

Endothelial nitric oxide synthase (eNOS) and inflammatory mediators, including the widely expressed cyclooxygenase-2 (COX-2), has been implicated in various pathologies such as renal failure, heart failure, stroke or sepsis. Hepatic production of nitric oxide is associated with alcohol-induced liver injury. In alcohol-treated rats hepatic NOS activity was significantly reduced. Cyclooxygenase 2 (COX-2) also have been implicated in tissue injury and fibrogenesis in animal models. Resveratrol increased the stimulation of eNOS expression and activity and it may contribute to reduce mortality in mice. Additionally resveratrol is a potent inhibitor of the cyclooxygenase-2.

This comments have included in the Discussion. Also the effects of IL-1 have been reviewed and appropriate referentes have added.

Thank you for your attention. We remain if any additional modification has to be made according to your criteria.

Yours sincerely,

Luis Bujanda