Author's response to reviews

Title: Anti-inflammatory and antioxidant effects of Tualang honey in alkali injury on the eyes of rabbits: Experimental animal study

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Dear Editor,

Anti-inflammatory and antioxidant effects of Tualang honey in alkali injury on the eyes of rabbits: Experimental animal study

Response to Reviewer 1

1. Comment
Authors studied the antiinflammatory and antioxidants effects of Tualang honey by a clinical and histopathological examination and evaluating the antioxidant status and the amount of lipoperoxidation products following established methods. However, in order to complete and improve the results it would be recommendable that the authors include some data from animals just with alkali injury (positive control). It is important to know the grading of the features of alkali chemical injury in cornea induced by themselves, with their experimental conditions, not according with previous reports. Otherwise, author should say way they did not include.

Response
We understand the reviewer concern. Initially we have 3 arms of the rabbit to conduct this study (control group with no treatment, conventional treated group and honey treated group) together with preliminary study (no treatment) to establish the alkali chemical injury on rabbit’s cornea however our Animal Ethics Committee only approved 2 arms of the rabbit (conventional treated group and honey treated group) and also the preliminary study. So we used the preliminary study as a control group.

We have added the preliminary study in the method of the manuscript.

Abstract

Methods: A preliminary study was carried out prior to the actual study to establish the alkali chemical injury on rabbit’s cornea and we found that alkali chemical injury with 2 N NaOH showed severe clinical inflammatory features.

Methods

1. Preliminary Study
A preliminary study was carried out prior to the actual study to establish the alkali chemical injury on rabbit’s cornea. Three rabbits were used for the preliminary study.

1.1 Experimental animals
Three New Zealand white adult rabbits (aged 8 - 10 months) weighing between 2.0 and 2.5 kg, with clear cornea were used in this preliminary study. The rabbits were maintained and handled according to the recommendations of the animal ethics guidelines. The animals were housed individually in stainless steel cages under controlled
temperature, humidity and 12 hour light: dark cycle. Food (pellet) and water were provided *ad libitum*.

### 1.2 Induction of alkali injury on cornea

The first rabbit was anaesthetized with an intramuscular dose of Ketamine hydrochloride (Troy Laboratories, New South Wales, Australia) (35 mg/kg of body weight) and Xylazine hydrochloride (Troy Laboratories, New South Wales, Australia) (2.5 mg/kg) approximately 45 minutes prior to induction of alkali injury. The experiment was carried out only on the right eye of each rabbit. A drop of Propacaine hydrochloride 0.5% (Alcaine®, Alcon) was instilled to the right eye followed by insertion of barraquer wire speculum. Surplus moisture was removed with cotton tipped applicator.

Filter paper disc (Whatman 3 filter paper) with a 7.5 mm diameter was produced and immersed in 1 N NaOH (Sodium hydroxide) for 30 seconds \[4,17\]. The alkali soaked filter paper disc was then placed on the central axis of the right cornea, gently held with a forcep for 30 seconds. The cornea then was rinsed with 15 ml of balanced salt solution for 2 minutes. The induction of the alkali chemical injury was done by Investigator A.

The same procedure was repeated for the second rabbit. The size of the filter paper used was 7.5mm and immersed in 2 N NaOH instead of 1 N NaOH. The duration of filter paper contact on the central axis of the cornea remained at 30 seconds. In the third rabbit, the same concentration of NaOH (2 N NaOH) was used but the duration of filter paper contact on the cornea was increased to 60 seconds.

### 1.7 Results of the preliminary study

The result of the preliminary study for clinical inflammatory and histopathological inflammatory features of alkali injury on rabbit’s cornea is shown in Table 2. Rabbit-3 shows severe clinical inflammatory features in terms of conjunctival hyperemia (Figure 1), corneal edema (Figure 1) and corneal epithelial defect (Figure 2). Light-microscopic examination of the cornea found that the number of PMN leucocytes was below 50 (mild grade) for rabbit-2 and rabbit-3 and absent in rabbit-1 (Table 2). Figure 3 shows the histopathological inflammatory features of the rabbit-3 in three adjacent areas.

### 2. Comment

Authors indicates that the histopathological examination of the cornea showed the number of polymorphonuclear leucocytes was below 50 for both groups (mild grade). However, in order to complement the results would be important to include photographs of histopathological analysis showing the presence or absence of inflammatory cells.

Response

We agree with the comment. We have added the table and figures in the manuscript.

Table 2: Results of the preliminary study in alkali chemical injury on rabbit’s cornea
Figure 1: Conjunctival hyperemia and corneal oedema post induction of alkali injury on rabbit’s cornea

Figure 2: Corneal epithelial defect post induction of alkali injury on rabbit’s cornea

Figure 3: Histopathological inflammatory features on day 7 post induction of alkali injury on rabbit’s cornea

3. Comment
Although the authors indicate that there was also no significant difference in the level of total antioxidant status as well as lipid peroxidation. However, again they need to include in Table 3 the values of the oxidative status and the lipid peroxidation observed from animals with alkali injury in order to be more clear and know the changes produced by each treatment.

Response
We understand the reviewer concern. The aim of our preliminary study is to establish the alkali chemical injury on rabbit’s cornea and only to optimize the absorbance value of total antioxidant status and lipid peroxidation products within the range of the standards.

We have added this limitation in the manuscript.

1.7 Results of the preliminary study
(2nd paragraph)

In the preliminary study, the samples from aqueous humour, vitreous humour and serum was used to optimize the absorbance value within the range of the standards for the estimation of total antioxidant status and lipid peroxidation products. We observed that 10 times dilution of the samples (aqueous humour and vitreous humour) gave the optimum results in which the absorbance value of the samples showed absorbance within the range of the standards. The serum however was diluted 20 times as per mentioned in the catalogue and showed absorbance within the range of the standards.

For the estimation of lipid peroxidation products, the samples without dilution gave the optimum results and showed absorbance within the range of the standards.

3. Comment
Authors indicate in Results section that they used a Mann-Whitney for the statistical analysis. It would be convenient that they include in Material and Methods the statistical method and the significance level (p =, < ?).
Response
We agree with the comment. We have added the statistical analysis in the Methods of the manuscript.

4. Statistical Analysis
The data collected were analyzed using Statistical Package for Social Science (SPSS) software version 12.1. Fisher Exact test and Chi square test were used to analyze the results where appropriate. For the data that was not normally distributed, Mann-Whitney test was used for the statistical analysis. Thus the values are expressed as median (interquartile range). The p value of < 0.05 is considered as statistically significant.

Response to Reviewer 2

Comment
The data provided by the investigators are very interesting. I would like the authors to add a comment in their manuscript that replies to the following question: An alkali-injured cornea is very prone to becoming secondarily infected by microorganisms. Honey may facilitate the growth of such microorganisms. Hence, is there any potential danger of secondary microbial infection when using honey for treatment of alkali-injured corneas? Did the authors experience such a complication in the course of their study?

Response
We understand the reviewer concern. In our study, we found that there was no sign of microbial infection (eye discharge) in all of the rabbits (in both preliminary and actual study). We have added the finding of eye discharge in the manuscript and mentioned the possible mechanisms that prevent microbial growth in the discussion.

2. Preliminary Study
1.3 Eye examination for clinical inflammatory features
The right eyes of all the rabbits were examined to evaluate the clinical inflammatory features with a binocular loupe at 12, 24 and 72 hours and on the 5th and 7th days post induction of alkali burn by the Investigator A [4,18]. The rabbits were restrained in a wooden box prior to the examination. The eyes were examined for conjunctival hyperemia and corneal edema. Fluorescein strip 1% was used to evaluate the corneal epithelial defect. The grading of the clinical inflammatory features of the cornea was done as per in Table 1. There was no eye discharge noted in all of the three rabbits.

3. Actual Study
2.4 Eye examination for clinical inflammatory features
The right eyes of all the rabbits were examined to evaluate the clinical inflammatory features with a binocular loupe at 12, 24 and 72 hours and on the 5th and 7th days post
induction of alkali burn as per mentioned in 1.3 by the Investigator A. There was no eye discharge noted in all of the rabbits in the honey treated group as well as in the conventional treated group.

Discussion

(last paragraph)

In this study, we found that there was no clinical sign of infection with the absence of eye discharge in all of the eyes with alkali chemical injury in honey treated group as well as in conventional treated group. Although honey has a high content of sugar but it has a low content of water, together with acidity property [29]. These characteristics will prevent microbial growth. Honey also will generate hydrogen peroxide when diluted [30, 31] and this hydrogen peroxide is the major contributor to the antimicrobial activity [30, 32].