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Prophylactic effect of edible bird's nest on acetaminophen-induced liver injury response in mice model

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Abstract: Edible bird's nest (EBN) is one of the natural products believed to pose health-enhancing properties. To provide a better insight into the protective role of EBN from a toxicological perspective, acetaminophen (APAP) as a common hepatotoxicant is chosen. This study focuses on the regenerative response of prophylaxis EBN extract in APAP-induced liver injury (AILI) of mice model. Eighty (80) ICR mice were assigned to groups of control, APAP (500 mg/kg), silymarin (200 mg/kg), and prophylactic EBN (60, 120 and 250mg/kg). The EBN and silymarin were orally administered daily for 7 days followed by an APAP intraperitoneal induction. Animals were sacrificed at 5, 10 and 24 hours post-APAP dosing (hpd). Liver samples were processed for hematoxylin and eosin (H&E) staining and proliferating cell nuclear antigen (PCNA) immunostaining. Significant differences in histological changes between APAP and prophylactic EBN groups were observed at 10 hpd with complete liver recovery for all groups at 24 hpd except for EBN 250 that continuously showed injuries. Hepatocyte proliferation was initiated at 5 hpd in EBN 60 and 120, while at 24 hpd, EBN 120 and 250 expressed higher PCNA-stained hepatocytes. The hepatoprotective role of EBN was shown earlier in EBN 60 and 120, while cellular proliferation delay in EBN 250. In conclusion, EBN has the potential as a prophylactic liver supplement to accelerate hepatic regeneration in the AILI model.

Keywords: Acetaminophen, edible bird's nest, histology, liver injury, liver proliferation.

INTRODUCTION

Edible bird's nest (EBN) has been used as the traditional supplementary food among the Chinese dynasty for over hundreds of years due to its promising health benefits (Ibrahim *et al.*, 2009). In modern sciences, it has been known profoundly to act as an anti-oxidant agent (Yew *et al.*, 2014, Yida *et al.*, 2015), increase proliferation of cells (Abd Rashed and Wan Nazaimoon, 2010, Roh *et al.*, 2012), inhibit influenza infection (Akmal *et al.*, 2017), and increase cognitive function (Zhiping *et al.*, 2015, Careena *et al.*, 2018). Ingestion of APAP at the sub-lethal dose of 500 mg/kg induced liver injury would exhibit centrilobular necrosis (Shahid and Subhan, 2014, Caparrotta et al., 2018) with subsequent liver regeneration at a later time point (Williams *et al.*, 2011, Muhammad-Azam *et al.*, 2019). The usage of N-acetyl-cysteine (NAC) is the approved drug clinically for APAP-induced liver injury (AILI) by reducing oxidative stress and increasing the GSH level (Du *et al.*, 2014). Hence, it is crucial to identify the other alternatives that possess hepatoprotective properties with minimal side effects (Jadeja *et al.*, 2016). The previous studies have mentioned the usage of silymarin as a liver tonic to encounter the toxicity effect of hepatotoxicants by preventing GSH depletion and reducing the oxidative stress level (Uchida *et al.*, 2017, Papackova *et al.*, 2018).

The EBN has been studied widely as natural medicine due to its rising market demand towards health-promoting benefits such as anti-oxidative agents and enhancing regeneration. Currently, some studies reported that EBN helps to reduce oxidative stress by acting as an antioxidant to scavenge free radicals (Yida *et al.*, 2015). Cell proliferation is also associated with EBN consumption on colonic adenocarcinoma cells (Abd Rashed and Wan Nazaimoon, 2010) and adipose-derived stem cells of human cells (Roh *et al.*, 2012). However, the hepatoprotective potential of EBN is poorly understood as the EBN functional effects on the liver are yet to be explored. To discover the benefits of EBN in enhancing cellular proliferation, this study assesses the protective effects of EBN in the drug-induced liver injury model of APAP that is known as an ideal hepatotoxicant. We hypothesised that EBN ameliorates AILI by attenuating the oxidative stress level and minimising the hepatocyte damage and subsequently enhancing cellular regeneration. Therefore, this study aims to assess the prophylactic effect of EBN on the APAP-induced liver injury in mice model towards the histomorphological changes of liver and their regenerative capabilities.

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MATERIALS AND METHODS

List of abbreviations

AILI: acetaminophen-induced liver injury, APAP: acetaminophen, EBN: edible bird's nest, GSH: glutathione, H&E: hematoxylin and eosin, hpd: Hours post-dosing, PCNA: proliferating cell nuclear antigen. Grouping: (i) APAP (500 mg/kg APAP), (ii) Silymarin (200 mg/kg silymarin plus APAP), (iii) EBN 60 (60 mg/kg EBN plus APAP); (iv) EBN 120 (120mg/kg EBN plus APAP); (v) EBN250 (250mg/kg EBN plus APAP).

Ethical approval

All protocols mentioned were undertaken following criteria approved by Universiti Putra Malaysia (UPM), Institutional Animal Care and Use Committee (IACUC) - UPM/IACUC/AUP-R078/2017.

Experimental animals

The animals were purchased from the Animal Resource Unit (ARU), Faculty of Veterinary Medicine, UPM. The procedures were done at the Animal Research Facility, Faculty of Veterinary Medicine, UPM. They were acclimatised for 7 days before the study. The mice were maintained in a 12 hours light-dark cycle at 7am and 7pm with ranges temperature and humidity of 21 to 23°C and 40 to 60%, respectively, with free access to food and drink throughout the experimentation.

Experimental, treatment and sampling protocols

A total of eighty (80) ICR male mice (5-6 weeks old, 20-25 g) was used for this study. Five (5) mice served as controls received normal saline (0.9% NaCl). The remaining animals were allocated randomly into five (5) other groups; (i) APAP (500mg/kg APAP); (ii) silymarin (200 mg/kg silymarin plus APAP); (iii) EBN 60 (60 mg/kg EBN plus APAP); (iv) EBN 120 (120mg/kg EBN plus APAP); and (v) EBN 250 (250mg/kg EBN plus APAP). Meanwhile, silymarin as a conventional liver tonic was assigned as a positive control as reported by Papackova *et al.*, 2018. The APAP group served as the AILI model while the EBN groups as prophylactics. Raw EBN obtained from Nest Excel Resources Sdn. Bhd. was cleaned and dried at room temperature before grounding into powder for the EBN extraction process. The EBN extract solution was prepared based on a previous study (Albishtue *et al.*, 2018). For silymarin and EBN groups, these compounds dissolved in normal saline were orally administered daily for 7 days and followed by APAP induction. The APAP solution was freshly prepared and given intraperitoneally in a single dose based on body weight as published (Muhammad-Azam *et al.*, 2019). In 5 mice per group, they were euthanised humanely using cervical dislocation technique at respective time points; 5, 10, and 24 hours post-APAP dosing (hpd) to harvest the liver samples. Then, the samples were fixed in 10% buffered formalin and left overnight until further histological processing.

Histopathological examination by hematoxylin and eosin (H&E) staining

The fixed liver samples were processed at Histopathology Laboratory, Faculty of Veterinary Medicine, UPM. Briefly, the tissues were embedded into the paraffin wax followed by the dehydration process with a series of increasing concentrations of ethanol. Then, the embedded tissues were sliced using a microtome at 3 to 5 µm in size to be mounted onto the glass slides. For histological assessment, liver slides were stained with Harris' Hematoxylin and Eosin (H&E) staining method for observation under a light microscope. The hepatocellular changes were scored in a range of 0 (no histopathological changes) to 5 (severe damage) based on a grading system described previously by Antoine *et al.* (2009). Degree of hepatocellular changes with histological description were done independently in a blinded fashion by a pathologist.

Proliferating nuclear cell antigen (PCNA) immunostaining

The tissue sections were processed for PCNA immunostaining following a protocol by Foley *et al.,* 2013. The tissues were mounted on charged slides (silanised) and allowed to dry overnight. The deparaffinisation process was done by heating and immersing process into xylene (organic cleaning agent) before rehydrating with a series of increasing ethanol concentrations. Then, the slides were immersed into a 10x Target Retrieval Solution (1 TRS: 9 ddH$_2$O) and heated for 10 minutes at 15 p.s.i (121°C) using a microwave. The staining procedure was done by incubating the slides with biotinylated primary antibody (200µL), peroxidase blocking, streptavidin-peroxidase reagent and DAB + subsrate-chromogen, respectively. Routine hematoxylin counterstain was done by applying the slides into Harris' Hematoxylin staining method. Finally, the slides were mounted and viewed under a light microscope. Positive PCNA hepatocytes were measured based on the method previously described by Eldridge *et al.,* 1993. PCNA-stained cells were quantified in ten (10) random areas for each liver section with a total of 1000 cells per area viewed. Each group was expressed by the mean percentage (%) of PCNA positive cells of liver samples.

STATISTICAL ANALYSIS

All data were expressed as mean ± standard deviation (SD) and analysed for non-normality data using Shapiro-Wilk's test. For normally distributed data, they were analysed using unpaired t-test while non-normally distributed data were analysed using the Mann-Whitney U test. All calculations were performed using Statistical Package for Social Science (SPSS) version 19.0 software where results were considered as significant when p-value<0.05 (*P<0.05, **P<0.01 and ***P<0.005).

RESULTS

Histopathological examination of the liver

Histological findings of liver changes in ICR mice were assessed in all groups. Liver samples were prepared for haematoxylin and eosin (H&E) staining and their histomorphological features were assessed based on a grading score system as previously described (Antoine *et al.*, 2009). An average grading scores of AILI is summarised in fig. 1. It demonstrated the control group had a grading score of 0 as expected. At 5 hpd, EBN 250 showed the highest scoring with statistically significant compared to the rest of the groups (P<0.005). In contrast, the APAP group at 10hpd showed a significant peak of AILI score than the others (P<0.005), before all scores dropped at 24 hpd. Microscopic changes of liver injury after APAP intoxication are shown in figs. 2, 3, and 4, respectively.

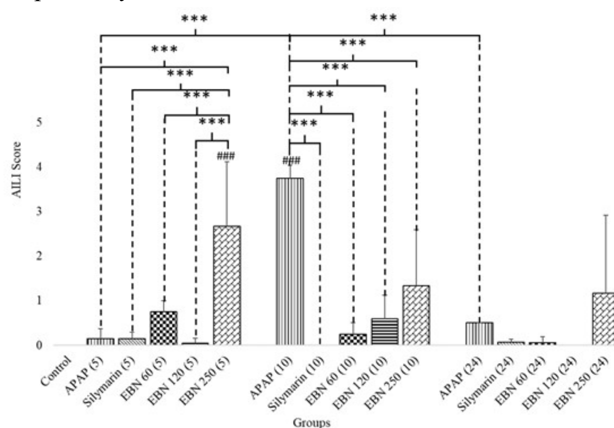

Fig. 1: Histopathological grading score of the liver for all groups at all time points. No liver injury in controls while APAP-treated group had a significant increase in AILI score at 10 hpd (3.75±0.29) followed by hepatocytes recovery at 24 hpd (0.50±0.00). Silymarin-dosed mice revealed minimal hepatic damage throughout experimentation. By 24 hpd, EBN 60 (0.06±0.13) and 120 (0.00±0.00) showed substantially reduced of hepatocytes damage. Almost no hepatocytes improvement was observed in EBN 250 for all-time points. AILI groups: *P<0.05, **P<0.01 and ***P<0.005; Control: ###P<0.005.

Histopathological changes at 5 hpd

Histomorphological features at the early time point were compared between the groups are demonstrated in fig. 2. At 5 hpd, none to minimal hepatocellular changes in APAP-dosed mice (fig. 2a). Fig. 2b revealed early hydropic degeneration was observed that evident by variably sized cytoplasmic vacuoles in silymarin, as similar to the EBN 60 group with scattered degenerated hepatocytes seen at centrilobular areas with the presence of numerous mitotic figs as early as 5 hpd (fig. 2c) than the APAP group which was only observed at 24 hpd. A similar feature was also examined in EBN 120 although no mitosis was observed histologically (fig. 2d). However, severe centrilobular necrosis along with marked haemorrhage was seen in EBN 250 at 5 hpd (fig. 2e).

Histopathological changes at 10 and 24 hpd

Histopathological changes of liver sections at 10 and 24 hpd were illustrated in figs. 3 and 4, respectively. Severe hepatic injury primarily centrilobular haemorrhage observed at 10 hpd as seen in fig. 3a. On the other hand, at the same time point, normal hepatocytes architecture was observed completely in the silymarin group as indicative of complete regeneration of hepatocytes following injury (fig. 3b). EBN 60 and EBN 120 groups showed mild centrilobular necrosis mostly at the central vein with obvious mitotic figs seen in EBN 60 (fig. 3c) but no mitosis observed in EBN 120 (fig. 3d). In fig. 3e, continuous liver abnormalities were seen at 10 hpd exhibited by severe haemorrhage and centrilobular necrosis in EBN 250 group. Although the histological features of the APAP group at 10 hpd revealed highly significant damage than those at 5 hpd, silymarin and EBN 60 groups underwent liver recovery at this same time point. As shown in fig. 4a, mild liver changes persist apparently by swollen hepatocytes in APAP mice at 24 hpd. However, all prophylactic groups of silymarin, EBN 60 and 120 showed significantly lower AILI scores than the APAP group, and their hepatic injuries were relieved by 24 hpd with most areas were covered by regenerated hepatocytes as seen in fig. 4b, 4c and 4d, respectively. In contrast, intense damage was continuously seen in EBN 250 with evidence of haemorrhage and necrosis (fig. 4e).

Quantification of liver proliferation by proliferating cell nuclear antigen (PCNA) immunostaining

A degree of regenerating hepatocytes is determined by PCNA immunohistochemistry to assess the prophylactic effect of EBN in APAP-induced liver injury of mice model. The amounts of PCNA-expressing cells of all groups at all time points are shown in fig. 5. It demonstrated normal liver cells also had a minimal degree of proliferation in the control group although no hepatic injury was seen. EBN 60 revealed the highest expression of PCNA positive cells with highly significant than EBN 60 (P=0.43) at 5 hpd. While hepatocytes proliferation at 10 hpd were similar among groups, at 24 hpd, APAP, EBN 120 and EBN 250 groups showed an increased amount of PCNA staining. Statistically, EBN 120 expressed PCNA cells significantly than controls, EBN 60 and silymarin (P<0.005) (fig. 5). Significant changes of PCNA-stained hepatocytes could be observed in EBN 120 with expression increased at 24 hpd compared to 5 hpd (P = 0.015) and 10 hpd (P=0.000).

Assesments of PCNA-stained liver sections

Photomicrographs of PCNA-stained cells are illustrated in figs. 6 and 7, respectively. In fig. 6, proliferating cells observed in controls (fig. 6a) had the same amount to APAP (fig. 6b) and silymarin (fig. 6c) groups had the

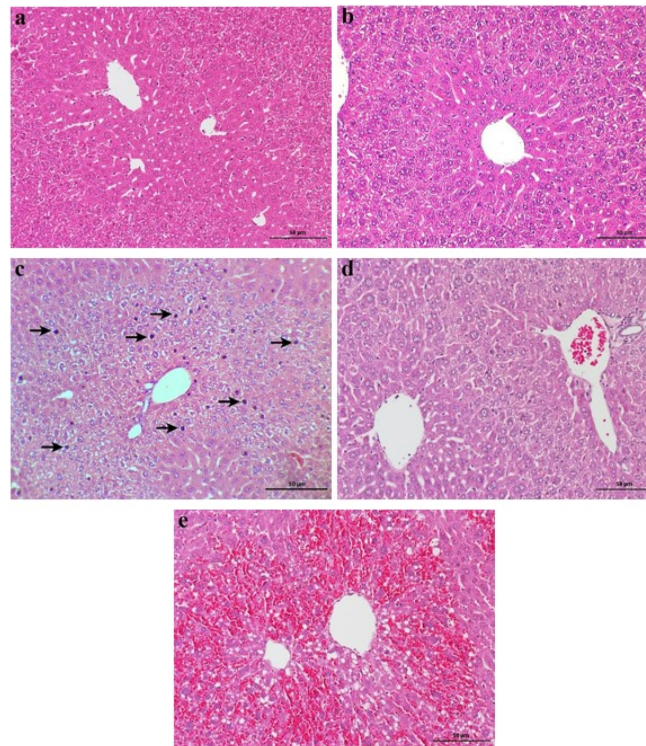

Fig. 2: Photomicrograph of the liver of mice at 5 hpd (H&E). A) APAP-dosed mice showed minimal degree of centrilobular changes (0.15±0.22). B) Variable cytoplasmic vacuoles of early hydropic degeneration observed in silymarin group (0.15±0.14). C) Mild centrilobular hepatocytes degeneration in the EBN 60 group (0.75±0.25) with numerous mitotic figures (arrow). D) EBN 120 group had slight hepatocytes loss (0.05±0.11). E) Intense hepatocytes damage with presence of both centrilobular necrosis and haemorrhage in EBN 250 (2.67±1.44). Bar 100µm.

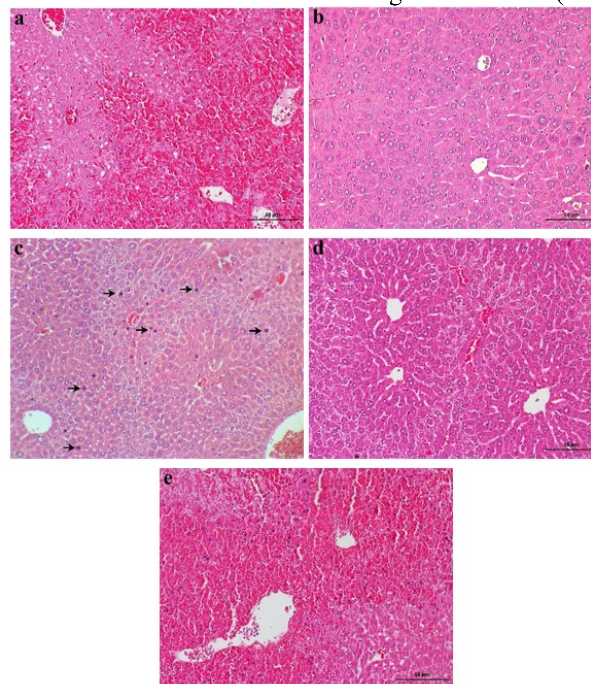

Fig. 3: Photomicrograph of the liver of mice at 10 hpd (H&E). A) Severe liver injury reflected by haemorrhage at centrilobular area (3.75±0.29). B) Silymarin group showed complete hepatocytes regeneration with absence of hepatic injury (0.00±0.00). C) EBN 60 mice exhibited scattered individual injury (0.25±0.25), with evidence of mitotic figures (arrow). D) Liver regeneration with intact hepatocytes resumed in EBN 120 (0.60±0.52). E) EBN 250 showed ongoing liver injury with continuous severe centrilobular necrosis and haemorrhage at 10 hpd (1.33±1.26). Bar 100 µm.

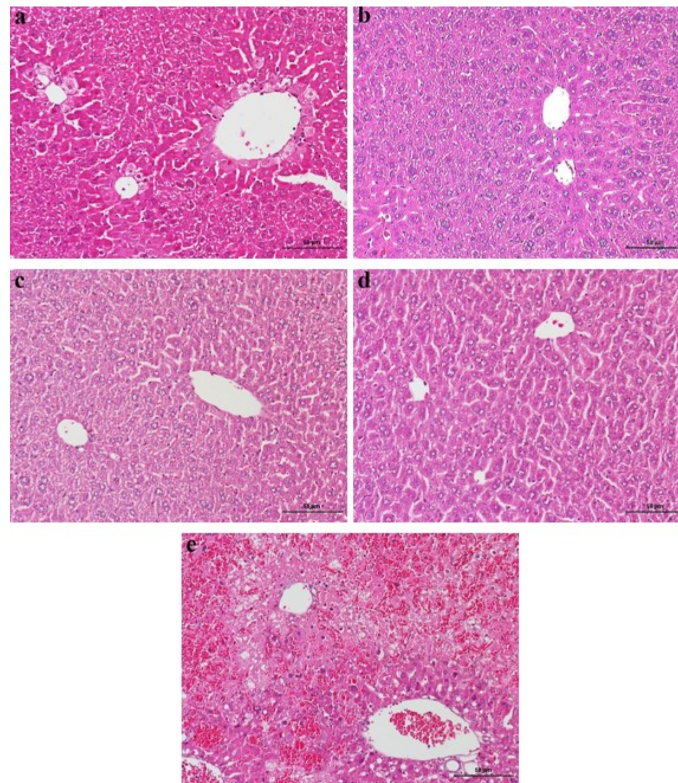

Fig. 4: Photomicrograph of the liver of mice at 24 hpd (H&E). A) APAP group exhibited mild centrilobular necrosis with apparent of ballooning degeneration (arrow) (0.50±0.00). B) Minimal to no liver cell damage in silymarin mice (0.07 ± 0.06). C) Complete regeneration of liver cells with minimal to no damaged cells in EBN 60 (0.06±0.13). D) EBN 120 group showed normal hepatocytes architectures (0.00±0.00). E) Persistent hepatocellular damage reflected by intense necrotic cells and haemorrhage at later time point in the EBN 250 group (1.17±1.75). Bar 100 µm.

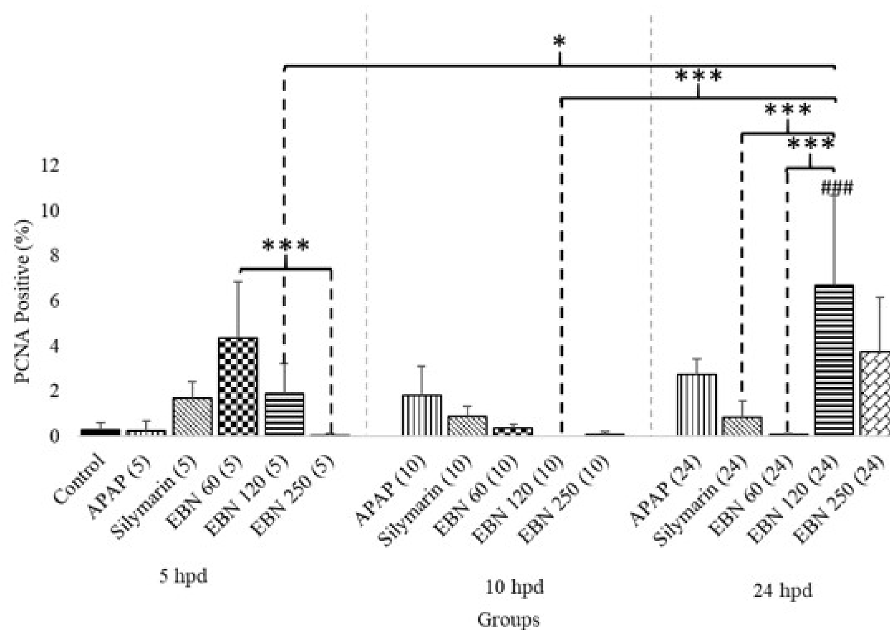

Fig. 5: Quantification of PCNA positive cells for all groups at all time points. Proliferating cells increased proportionally over time in the APAP groups. In contrast, silymarin (1.63±0.72) and EBN 60 (4.37±2.48) groups had the highest proliferative hepatocytes at 5 hpd before both reduced proportionally at the latter. At 24 hpd, both groups dropped significantly than EBN 120, while EBN 120 (6.68±4.00) revealed the highest PCNA expression together with EBN 250 (3.77±2.40) than the other time-points. AILI groups: *P<0.05, **P<0.01, ***P<0.005; Control: ###P<0.005.

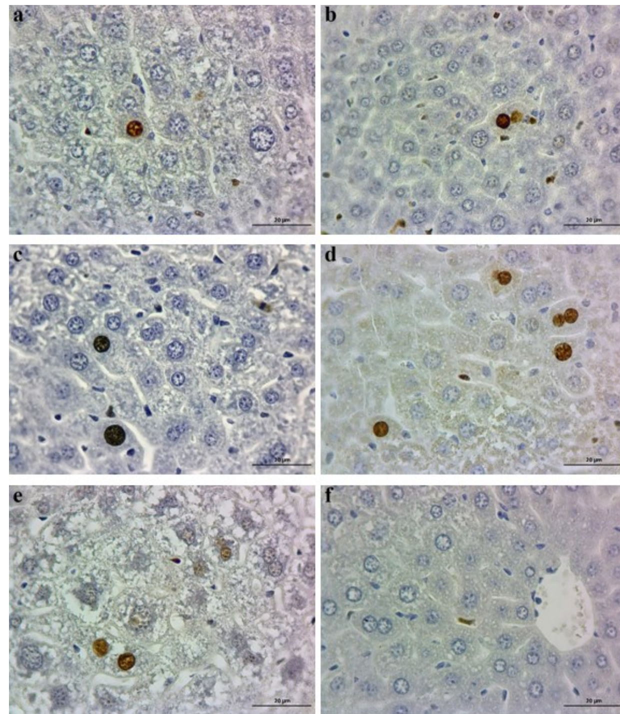

Fig. 6: Photomicrographs of PCNA-stained liver sections of mice at 5 hpd. A) Control group showed low proliferative cell (0.30±0.30). B) Low PCNA expression (0.25±0.44) in APAP-treated mice. C) Silymarin exhibited higher number of PCNA positive cell (1.63±0.72). D) EBN 60 group had the highest number of regenerated cell (4.37±2.48). E) Low PCNA amount in EBN 120 (1.90±1.32). (f) Almost none PCNA cells were detected in EBN 250 (0.05 ± 0.06). Magnification at 400x, bar 50µm.

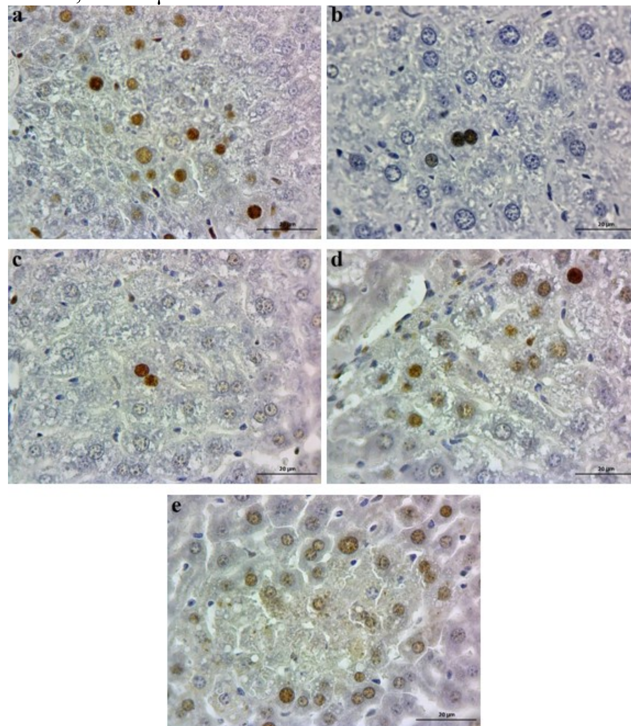

Fig. 7: Photomicrographs of PCNA expression in liver sections during 24 hpd. A) APAP group had variable density of PCNA expressing cells (2.73±2.02). B) Silymarin mice showed consecutive PCNA positive hepatocytes (0.87±0.70). C) EBN 60 group continuously revealed low amount of PCNA (0.07±0.06). In contrast, both EBN 120 (D: 6.68±4.00) and EBN 250 (E: 3.77±2.40) groups increased the amounts of proliferating cells at the end of study. Magnification at 400x, bar 50µm.

same amount. However, PCNA-stained cells were significantly seen in groups of EBN 60 (fig. 6d) and EBN 250 (fig. 6e). Meanwhile, none to the low number of PCNA hepatocytes observed in EBN 250 mice (fig. 6f) as similar to controls. Although the PCNA cells were similar in all groups at 10 hpd, the exponential increment of PCNA positive hepatocytes was observed at 24 hpd in APAP mice as compared to the earlier time points (fig. 7a). Hence, this result showed that PCNA proliferating cells were increased proportionally to the time of toxic induction for the APAP group only while EBN 60 showed the PCNA positive cells were inversely proportional to the time of APAP toxicity. Silymarin mice exhibited continuous proliferating cells (fig. 7b) while EBN 60 group had the lowest proliferation at 24 hpd (fig. 7c). In contrast to EBN 60, EBN 120 (fig. 7d) possesses a markedly high number of PCNA expression at 24 hpd than control, silymarin, and EBN 60 groups but slightly lesser in EBN 250 (fig. 7e). Therefore, these groups with high numbers of regenerated expression indicated the greater occurrence of hepatocytes proliferation at later time points at the doses of EBN 60 and 120.

DISCUSSION

Although EBN is considered a current natural supplement that has been consumed for years, the evidence towards liver health has not been proven scientifically. This study provides insight on EBN's capability to aid in liver regeneration after induction with hepatotoxicant (ie. APAP) in an animal model. The low degree of liver injury of EBN 60 and 120 at early time points are possibly due to the anti-oxidative properties of EBN and its bioactive component that react to NAPQI to prevent its protein macromolecules from binding. However, the actual mechanism of EBN has yet to be understood clearly. Previous studies have suggested that sialic acid in EBN helps in reducing oxidative stress and cytotoxicity of hydrogen peroxide (H_2O_2) by using murine lymphoma cells (Iijima *et al.*, 2004; Vimala *et al.*, 2012). In a study by Papackova *et al.*, 2018, pre-treatment of silymarin alleviates GSH level and reduces oxidative stress in APAP-induced BALB/c mice. In another study, silymarin can reduce hepatotoxicity indicated by decreasing infiltration of neutrophil and serum transaminase level restoration (Freitag *et al.*, 2015). Besides, inflammatory cells recruited to the injury site would remove the necrotic cell debris to allow hepatocytes to regenerate as reported by Woolbright and Jaeschke, 2017. At a later time, liver injuries were settled down and most of the groups showed almost zero hepatocytes damage and normal architecture of liver cells was resumed.

In normal circumstances, cells also undergo proliferation without liver injury as exhibited by the controls due to the absence of regenerative signaling to trigger cellular regeneration. The regeneration process in EBN 60 and 120 mg/kg took place as early as 5 hours after APAP-induced liver injury that was supported by the presence of a high number of PCNA positive cells. The proliferating cells spiked at 5 hpd may initiate the hepatocytes regeneration at an earlier period. This finding suggested that these prophylactic groups of EBN 60 and EBN 120 potentially enhance hepatocytes proliferation after APAP intoxication to resume complete hepatic architectures and restore their cellular functions. Apart from being an anti-oxidative agent, EBN also may initiate the other components to accelerate the recovery of hepatocytes such as epidermal growth factor (EGF)-like component in swiftlets nest that was discovered by Kong *et al.*, 1987. This could be assumed that the EGF-like component aids significantly in accelerating the hepatocytes regeneration at an early point. Similar to EBN, silymarin is believed to assist in cellular proliferation, however, the assumption is still unjustified (Vargas-Mendoza *et al.*, 2014). Hence, it is believed that silymarin does not aid in cell proliferation as the exhibition of PCNA cells in the silymarin group remained low throughout the study. However, the insignificant difference results possibly due to the unknown optimum dosage and time of reaction of EBN that can significantly enhance liver proliferation in comparison to the other groups. APAP-induced mice showed recovery of hepatocytes after 24 hpd with evidence of numerous proliferating cells during this time point (Muhammad-Azam *et al.*, 2019). Individual cells will regularly undergo the mitosis process which remains in the G_0 phase of the cell cycle unless trigger by cellular injury (Apte, 2015). In a study by Bhushan and Apte, 2019, severe APAP-induced liver injury inhibited the occurrence of liver regeneration until the clearance of toxic insults.

Unexpectedly, the degree of liver injury in a group of EBN 250 mg/kg tends to be worsening at every time point after APAP induction than the other EBN groups. According to a previous study (Abd Rashed and Wan Nazaimoon, 2010), the EBN toxic effect is due to the excessive amount of N-acetylgalactosamine (galNAC) and N-acetylglucosamine (glcNAC), leading to inhibition of cell proliferation in colorectal adenocarcinoma (Caco-2) cell. The mechanism of regenerative signaling mediators like growth factors and cytokines aid in the liver regeneration process possibly delay. EBN also contains an EGF-like structure that aids in promoting cell regeneration and improving cell recovery at low concentrations (Zainal Abidin *et al.*, 2011). This statement is similar to our finding whereby the highest EBN dosage (250mg/kg) exhibited intense hepatocellular damage than the lower doses at 60 and 120mg/kg.

CONCLUSION

This study discovered that EBN does help in enhancing cell proliferation in the liver as early as 5 hpd at 60 and

120 mg/kg doses. It is also proven that a higher dosage of EBN at 250 mg/kg inhibits hepatocellular proliferation leading to a delayed hepatic regeneration process. To ascertain the health benefit of EBN, the right doses need to be consumed. Therefore, EBN consumption has the capability to intensify liver regeneration and help in subsiding acute hepatoxicity induced by APAP. The potential of cellular proliferation by EBN extract can be an additional value of EBN as a health supplement that can benefit the swiftlets industry. In conclusion, this study highlights the hepatoprotective role of EBN as prophylaxis in APAP-induced liver injury in mice, and similar potential responses are to be further studied and translated in humans.

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