RESEARCH ARTICLE

The Protective Potential effect of Metformin during Acetaminophen Hepatotoxicity through Nrf2 Activation

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ABSTRACT

Acetaminophen (N-acetyl-para-aminophenol [APAP]) is the most commonly used medication for the relief of pain and fever around the world. Although APAP is safe and effective at a therapeutic level, acute overdose causes hepatotoxicity and severe liver damage. The hepatotoxicity induced by APAP is a persistent global problem that results in hepatotoxicity cases; acute liver failure and even death worldwide. This toxicity is characterized by extensive oxidant stress which consequently causes hepatocyte cell death. On the other hand, scientific studies have proven that MET shows hepatoprotective effect against hepatotoxicity induced by APAP through numerous mechanisms, e.g., antioxidant activity that alleviate the hepatotoxicity by activating Nrf2 pathway. The activation of Nrf2 pathway is anticipated to protect cells from oxidative stress that forms during hepatotoxicity. The beneficial of using MET to protect against hepatotoxicity after APAP has been performed in an *in-vitro* experiment using Hep2G cell culture. However, up to our knowledge non, an *in-vivo* study (experimental animal) has been used to approve the benefit of MET.

Justification: Nrf2 pathway activation by MET is one of the most important issues that need to be explained and followed up in laboratory animals since it has been previously studied *in-vitro*. Our study focuses on studying Nrf2 pathway *in-vivo*, since the results of *in-vivo* study are more relevant and similar to that of human beings.

Aim of the study: To examine the possible stimulant effect of MET on Nrf2 pathway in experimental animals and its role in protecting the liver from oxidative stress that formed during APAP-induced hepatotoxicity.

Methodology: Twenty-four wistar rats were divided randomly into four groups (six rats/Grp). Grp1; normal saline orally, Grp2; toxic dose of APAP (1000 mg/kg) orally; Grp3; 200 mg/kg of MET ip after 1-hour of APAP (1000 mg/kg) orally, Grp 4; 200 mg/kg MET ip for 24 hours. Then sera from experimental animals were collected for subsequent assessments of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme activities. Liver tissue was harvested to detect the expression of keap1 which is the negative regulator of Nrf2, and HO-1 and GST A1, which are related to Nrf2 pathway, by western blotting technique.

Results: The results were showed a significant elevation in ALT and AST activities in APAP treated group while MET normalized these biomarkers (AST and ALT). Western blotting assay showed that keap1 expression increased in APAP-treated animals while MET showed a significant decrement in keap1 expression. The expression of antioxidant proteins HO-1 and GST A1 are decreased significantly in APAP-treated animals while increased significantly by MET treatment.

Conclusion: The present results demonstrated that MET has a hepatoprotective effect in experimental animals against hepatotoxicity induced by APAP through activating Nrf2 pathway.

Keywords: Metformin, Acetaminophen, Nrf2, Liver Tissues

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INTRODUCTION

Acetaminophen (N-acetyl-para-aminophenol [APAP], paracetamol) is the most commonly used medication for relieving pain and fever around the world.[1] Although APAP is safe and effective at therapeutic doses, but remain one of the most common cause of acute liver injury that may progress to acute liver failure.[2] Liver necrosis is the well-evidenced and frequent adverse effect of APAP; this toxicity could occur in acute APAP overdose at 250 mg/kg, approximately more than 10 gm for a 60 kg adult human.[3] Oxidation (phase I)

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pathway of APAP *via* hepatic cytochrome CYP 450 system (especially CYP2E1, CYP1A2 and 3A4) are responsible for production of a highly reactive toxic metabolite of APAP, N-acetyl-para-benzo-quinone imine (NAPQI). NAPQI is rapidly converted to nontoxic metabolites by glutathione (GSH) conjugation, forming cysteine and mercapturic acid conjugate which are eliminated by renal excretion under normal conditions.[4,5] However, GSH is depleted during attempts to eliminate the toxic metabolite during APAP overdose. As a result, large quantities of NAPQI are accumulated inside the hepatocyte.[6,7] The binding of NAPQI to the mitochondrial electron transport chain (ETC) causes electrons to escape towards oxygen molecules, forming superoxide free radicals ($O2^{-}$). Consequently, other reactive oxygen (ROS) and reactive nitrogen species (RNS) formed, causing mitochondrial DNA damage.[8,9] Mitochondrial oxidant stress was additionally amplified through c-Jun-N- terminal kinase (JNK) activation. Continuous activation of JNK intensifies mitochondrial ROS. Furthermore, activates the opening of the mitochondrial permeability transition pore (MPTP).[10] One of the most harmful consequences of MPTP is swelling of the mitochondrial matrix, which leads to the rupture of the outer mitochondrial membrane and accelerates the release of mitochondrial intermembrane proteins, including endonuclease G and apoptosis-inducing factor. These proteins translocate to the nucleus and causes DNA fragmentation and cell necrosis.[11] During hepatotoxicity of APAP, oxidative stress can stimulate protective signaling pathways such as Nrf2 (nuclear factor erythroid 2-related factor 2) pathway as a compensatory mechanism. Nrf2 is likely stimulated in response to redox status changes of the kelch-like ECH associated protein 1 (Keap1) induced by NAPQI.[12] Nrf2 is a basic leucine zipper transcription factor that positively regulates the expression and activity of cytoprotective genes encoding inducible cytoprotective proteins that allow mammalian cells and organisms to adapt and survive under various conditions of stress.[13-15] On the other hand, scientific studies have proven that metformin (MET) shows a hepatoprotective effect against the hepatotoxicity of APAP through its antioxidant activity. MET was first introduced to clinical trial and established as a safe and effective medication before full mechanistic studies became possible. Generally, MET is the drug of choice for Type 2 diabetes mellitus (T2DM), which is given alone or combined with other medications.[16] Recently, it displays a novel pleiotropic effect which spans from its role in controlling T2DM to numerous regulatory properties, including nephro- and cardio-protection, as well as anti-proliferative, anti-fibrotic, antioxidant, anticancer, anti-inflammatory and anti-aging properties.[17] Regardless of the controversy over the molecular mechanism of MET hepatoprotection against APAP, all previous studies have shown that pre- and post-treatment with MET can effectively mitigate APAP liver injury, as recognized by the significant decrease in markers and histopathological features associated with hepatotoxicity.[18] The antioxidant activity of MET through the activation Nrf2 pathway is one of the most important protective mechanisms against oxidative stress.[19] This Nrf2 activation by MET causes transcriptional activation of several antioxidant genes, including heme-oxygenase-1 (HO-1) and glutathione S-transferase (GST).[12] As a result, this activation can protect cells from oxidative stresses, such as ROS and RNS that formed during hepatotoxicity of APAP as well as increases the anti-stress capacity and stabilize the oxidant level inside the cell to avoid DNA damage and the possibly harmful disruptions caused by oxidative stress.[20]

Animals and Study Design

Healthy 24 male wistar rats of approximately (8–12 weeks old) with weight of (100–180 gm) were used in this research. Animals were group housed under routine conditions of humidity and temperature and divided randomly into four groups (six rats/Grp). Dose levels were carefully chosen depending on published literature and pilot study.

- Control group: rats receiving normal saline orally
- APAP treated group: rats receiving a toxic APAP dose (1000 mg/kg) orally
- APAP + MET treated group: rats receiving a dose of 200 mg/kg MET ip after 1-hour of APAP toxic dose.
- MET treated group: rats receiving a 200 mg/kg dose of MET ip

After 24 hours treatment, blood sample was collected from all study groups by cardiac puncture after anesthesia with chloroform inhalation. Sera were separated by centrifugation at 3000 rpm at 4°C for 15 minutes for subsequent measurement of ALT and AST activities. Liver tissue was harvested, rinsed with cold phosphate buffer saline (0.01 M, pH 7.4) to remove the superficial blood. Then liver tissue samples were stored in a cryovial inside container of liquid nitrogen.

Serum Biomarkers of Liver Function

Serum ALT activity was measured using the (#GWB-AXR344, San Diego, USA) kit while AST activity was identified using the (MAK055, Sigma-aldrich, USA) kit.

Western Blot Analysis

Liver tissue (75 mg) was prepared into homogenate samples and lysed in (0.25 mL) of RIPA lysis buffer with shaking under ice bath followed by centrifugation (12,000 rpm, for 10 minutes). The supernatant was separated to determine the protein concentration by a BCA protein assay kit (Colorimetric Assay Kit, Elabscience, E-BC-K318). Total 30 µg of protein were loaded on 10% SDS-PAGE gels (SDS-PAGE Gel kit, Elabscience, E-AB-R305) and transferred onto polyvinylidene fluoride (PVDF) membranes (Elabscience, E-BC-R266). Membranes are further blocked and incubated overnight with primary antibodies at 4°C using 1:1000 dilutions of Keap1 polyclonal antibody (E-AB-19309), HMOX1 polyclonal antibody (E-AB-18231) with 1:1000 dilution and GST A1 polyclonal antibody (E-AB-40439) with 1:1000 dilution. Then, HRP- conjugated secondary antibodies (E-AB-1003 at 1:5000 dilution) were applied at room temperature for one hour, and signals were detected by enhanced Amersham™ ECL™ substrate (GE Life Science). PVDF membranes were placed inside a developing cassette in a dark room for film-based

development. Super RX X-ray film (Kodak, Rochester, NY, USA) was exposed to the membranes for 10 seconds to 1 minute as required for adequate exposure, followed by development and fixation (Kodak R X-ray film).

Statistical Analysis

All ALT and AST, enzyme activity results, were expressed as the Mean ± SD, while expressed as fold change in western blotting results. Western blot statistical analysis was done by using the imag J program and Graph pad prism 8 software. Statistical significance is specified by (*) whenever the *p-value* is < 0.05 and highly significant (**) whenever $p < 0.01$, while (***) means $p < 0.001$ and (****) means $p < 0.0001$. One way ANOVA test was used to detect the significant difference between study groups

RESULTS

Measurement of Liver Injury Factors

The result of ALT and AST activities in the studied groups were performed as shown in Figures 1, and 2, respectively.

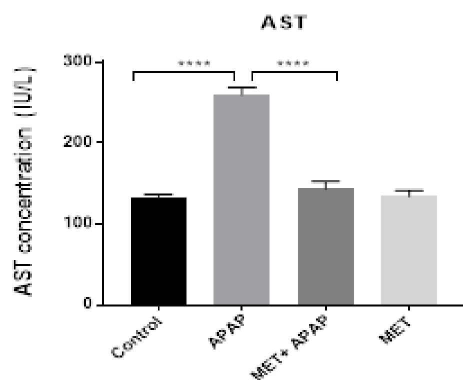

Figure 1: The effect of MET on serum ALT activity in acute APAP-induced hepatotoxicity. Results were expressed as Mean ± SD. Significant differences between groups are expressed as * represents ($p < 0.05$), ** represents ($p < 0.01$), *** represents ($p < 0.001$) and **** represents ($p < 0.0001$).

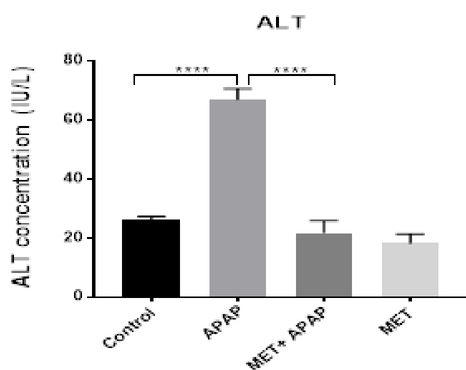

Figure 2: The effect of MET on serum AST activity in acute APAP-induced hepatotoxicity. Results were expressed as Mean ± SD. Significant differences between groups are expressed as * represents ($p < 0.05$), ** represents ($p < 0.01$), *** represents ($p < 0.001$) and **** represents ($p < 0.0001$)

The results showed a significant increase in the serum levels of the two enzyme activities in APAP treated animals compared to control ($p < 0.0001$). MET treated animals showed a significant decrease in AST and ALT activities ($p < 0.0001$) in comparison to APAP treated animals while the levels of two enzymes activities observed to be non-significantly different in both MET+APAP and MET alone treated animals in comparison to that of control animals.

Identification of Hepatic Keap1, HO-1 & GSTA1 Expression by Western Blotting

To investigate the mechanism of MET action as an antioxidant and its role in hepatic protection against toxicity of APAP, protein expression of keap1, HO-1, GST A1 were assessed using the western blot technique.

Keap1 Expression

The western blot results showed a higher density of keap1 in the APAP group compared with control as shown in Figure 3. On the other hand, APAP + MET group showed significantly reduced in the density of keap1 protein band compared to APAP group. By statistical analysis in Figure 4, fold of keap1 expression in APAP group is 3.08 times greater than that of control. The APAP + MET group is 1.5 times greater than that of control and the MET group is about 2.7 times greater than that of control.

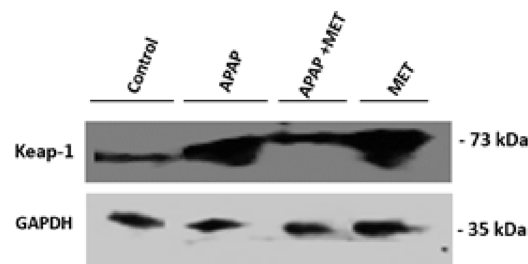

Figure 3: changes in keap1 protein expression in liver homogenate in current study groups. The relative densities of keap1 bands were normalized against GAPDH using Imag J program

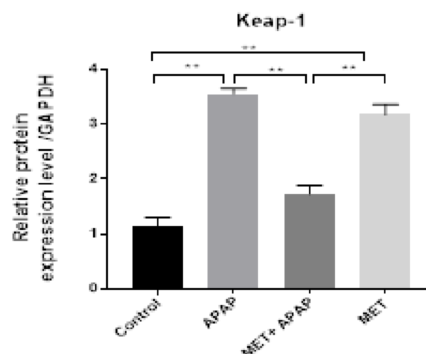

Figure 4: Relative protein expression of keap1 in current study groups**.** Results are expressed as fold changes. Fold changes between groups are considered significant at * represents ($p < 0.05$), ** represents ($p < 0.01$), *** represents ($p < 0.001$) and **** represents ($p < 0.0001$)

Heme-Oxygenase 1 Expression

As shown in Figure 5, the western blot results showed a decrease in the density of HO-1 band in APAP group in comparison with the control while both APAP+MET and MET group showed a significantly increased in the density of HO-1 protein band. By statistical analysis in Figure 6, fold of HO-1expression in APAP group is 0.003 than that of control. For APAP + MET treated group is 0.48 than that of control while in MET group, fold of HO-1 expression is 1.45 times than that of control.

GST A1 Expression

As shown in Figure 7 the western blot results showed a decrease in the density of GSTA1 protein band in APAP group in comparison with control while both APAP+ MET and MET group showed a significantly increased in the density of GST protein band. By statistical analysis in Figure 8, fold of GST A1 expression in APAP group is 0.5 than that of the control. The APAP + MET group is 1.7 times greater than that for control while the MET group is 0.7 times that for the control.

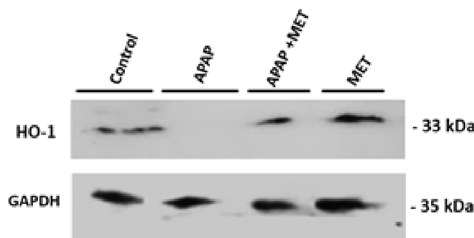

Figure 5: Changes in HO-1 protein expression in liver homogenate in the current study groups. The relative densities of HO-1 bands were normalized against GAPDH using Imag J program

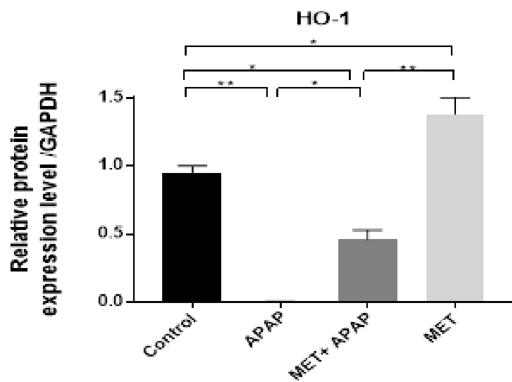

Figure 6: Relative protein expression of HO-1 in current study groups. Results are expressed as fold changes. Fold changes between groups are considered significant at * represents ($p < 0.05$), ** represents ($p < 0.01$), *** represents ($p < 0.001$) and **** represents ($p < 0.0001$)

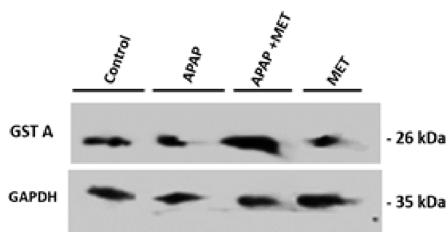

Figure 7: Changes in GSTA1 protein expression in liver homogenate in current study groups. The relative densities of GST A1 bands were normalized against GAPDH using Imag J program

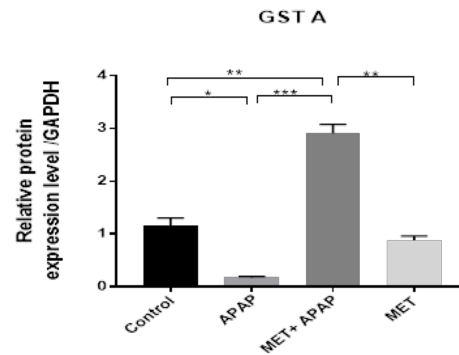

Figure 8: Relative protein expression of GST A1 in current study groups. Results are expressed as fold changes. Fold changes between groups are considered significant at * represents ($p < 0.05$), ** represents ($p < 0.01$), *** represents ($p < 0.001$) and **** represents ($p < 0.0001$)

DISCUSSION

The objective of the present study was to investigate the hypothesis that MET exhibits a protective effect against hepatotoxicity of APAP *via* Nrf2 pathway activation in experimental animals. After APAP overdose, there are several events such as massive hepatic tissue necrosis, inflammation and fatty degeneration, as well as an increased serum level of several biochemical markers, such as ALT/AST activities.[21] Treatment with 200 mg/kg of MET significantly normalized ALT and AST values. Therefore, there is evidence that MET may protect hepatocytes against acute APAP toxicity. Previous studies demonstrated that Nrf2 was negatively regulated by Keap1 protein.[22] Our results showed that Keap1 expression significantly increased by APAP. Previous data demonstrated that APAP administration resulted in a significant increase of keap1 protein expression which is the negative regulator of Nrf2 and thus resulting in decrement of Nrf2 expression and its target downstream antioxidant proteins such as GST and HO^{-1}.[23] The present hypothesis is that MET can positively regulate Nrf2 which directly regulates ARE and increase the antioxidant enzyme expression which reverses hepatotoxicity. In the current study, MET treatment showed a decrement in keap1 expression, this may indicate lower keap1-Nrf2 binding and thus increase the translocation and activation of Nrf2 to compact toxicity of APAP. Our finding in accordance with a previous study revealed that up-regulation of Nrf2 and, consequently, AREs may play a major role in MET hepatoprotection against APAP. Consequently, showed an elevation in the expression of several antioxidant protein.[15]

On the other hand, HO^{-1} is one of the cytoprotective enzymes which its expression is regulated by Nrf2. Studying

the role of HO^{-1} has become increasingly relevant in recent years owing to previous evidence that induction of the HO^{-1} system may prevent the hepatic inflammation associated with ethanol ingestion, besides this enzyme's role in alleviating the oxidative damage in hepatocytes.[24,25] During the toxicity of APAP, we observed that HO^{-1} protein level is declining, due to its consumption in compacting the hepatotoxicity. This finding agrees with a previous study that demonstrated that APAP causes a decrement in Nrf2 related protein including HO^{-1}.[23] However, MET treatment may increase the protein expression of HO^{-1} in hepatocyte. This elevation is owing to possible Nrf2 activation in liver tissue. MET treatment showed a considerable increase in the expression of antioxidant enzymes regulated by Nrf2/AREs, confirming the activation of Nrf2/AREs by MET against repression by APAP.[26] MET alone treated group shows significant elevation in HO^{-1} expression when compared to other study groups because this animal group does not suffer from hepatotoxicity which utilizes the HO^{-1} to compact oxidative stress. Other an essential cytoprotective enzyme in the detoxification of reactive electrophiles and products of lipid peroxidation is GSTA1. GSTA1 serves as a crucial role in both GSH binding reaction and the anti-oxidative defense system.[27] We demonstrated that APAP treated animals showed significant decrease in GST A1 expression. This decrement could be discussed as that in the APAP model, the protein content and expression of GSTA1 significantly decreased in accordance with previous predictions that GSTA1 is affected by APAP-induced hepatic injury.[27,28] The significant decrease of GSTA1 suggests that it may be released as an antioxidant to protect liver tissue against various hepatotoxins. The probable reason is that the injury primarily affected the central lobuli hepatis where GSTA1 is abundant and can be released easily.[29] Therefore, increasing GSTA1 content in liver tissue is an important mechanism leading to the hepatoprotective effect. This can be seen in MET treated group of our study.

CONCLUSION

In conclusion, the hepatoprotective effect of MET was revealed in the APAP-induced toxicity in experimental rats. The study has shown that MET can exert antioxidant activity through Nrf2 pathway activation and limit the incidence of oxidant stress and subsequent hepatocyte necrosis. The research was performed to present a new treatment to attenuate the APAP-induced hepatotoxicity for further interpretation to humans.

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