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RESEARCH ARTICLE

Hepatoprotective and anti-inflammatory effects of entecavir or probiotics on Oxaliplatin-Induced Liver Injury in the rats.

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ABSTRACT

Oxaliplatin (OXA), a current cancer chemotherapeutic, has low efficacy and is linked to serious adverse effects, including liver damage. We anticipated that probiotics and entecavir would help reduce OXA-induced liver damage because the pathophysiology of drug-induced liver damage is thought to be related to the disordered gut microbiota. Twenty-four rats were used in this study and divided into 4 groups: control group (n=6), OXA group (n=6), entecavir (ENT) group (n=6), and probiotics (PRO) group (n=6). After 3 weeks, all rats were sacrificed, and blood samples were analyzed for alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), interleukin 6 (IL-6), and IL-1. Serum measurement of biochemical parameters showed a significant increase in ALP in the OXA group compared to the control group ($p<0.0001$). The treatment with ENT or PRO along with OXA alleviated these changes. Significant elevation of serum ALT ($p=0.041$) and non-significant ($p=0.210$) increase of AST was observed in OXA-treated rats as compared with control rats. The administration of ENT or PRO along OXA restored these changes, but they did not reach the levels of control rats. Significant elevations of serum IL-1 ($p=0.024$) and a non-significant ($p=0.114$) increase of IL-6 were observed in OXA-treated rats compared to control rats. The administration of ENT or PRO along OXA reduced inflammatory cytokines' levels but they did not return to the baseline. The treatment with ENT or PRO was beneficial in reducing the severity of OXA-induced liver injury, likely by reducing inflammatory responses and liver function tests.

Keywords: oxaliplatin, entecavir, probiotic, liver function tests, and inflammatory cytokines.

1.0 INTRODUCTION

Chemotherapy is one of the treatments that can fight cancer cells; chemotherapy can not discriminate between malignant cells and non-malignant rapidly dividing cells (Bai *et al.*, 2016). For this reason, chemotherapy has many severe side effects, including renal, gastrointestinal, renal, and hepatic problems (Florea and Büsselberg, 2011). OXA is platinum-based therapy having many disadvantages, including vomiting, anorexia, and severe diarrhea (Zhang *et al.*, 2010). Many researchers aim to apply other protective drugs and adjuvant therapies along with OXA to reduce the drawbacks of chemotherapy.

The mechanism of killing the malignant cell by OXA is due to their metabolite oxalates and dichloro platinum.

Gastrointestinal problem is the main side effect of OXA. Hepatic damage is the main complication of OXA, and the disturbance of the liver function tests (LFTs) is another adverse reaction of OXA (Waseem *et al.*, 2015). The hepatotoxicity of OXA may be due to increased free radicals by OXA, culminating in the damage of mitochondria of hepatocytes (Zicca *et al.*, 2002, Correia *et al.*, 2010). For this reason, the application of drugs with OXA to reduce hepatic damage is mandatory. Other downsides of OXA include the disruption of natural flora and microflora dysbiogensis. The drug's disruption of the gut-liver axis may be the cause of the OXA-induced inflammation. OXA reduces the prevotella, a healthy member of the typical gut flora, and raises the opportunistic micro-organisms, which causes

inflammation to rise (Stojanovska *et al.*, 2018).

Entecavir is one of the antiviral drugs that fight the hepatitis B virus (HBV). It is a guanosine nucleotide analog that decreases the polymerase activity of the virus leading to clearance of the virus, reversing the damage of hepatocytes, and restoring the LFTs, including ALP, ALT, and AST (NCBI, 2018). Probiotics are living beneficial microorganisms that can be given to patients who suffer from imbalances of normal flora imbalances, which are the main side effects of chemotherapy (Azad *et al.*, 2018). To the best of our knowledge, few papers are present for applying entecavir and probiotics along with OXA chemotherapy to decrease the side effects of chemotherapy, such as live injury and inflammation (Yoo *et al.*, 2016, Feng *et al.*, 2022); therefore, the hepatoprotective and anti-inflammatory effects of entecavir and probiotics are our aims in this study.

2.0 Materials and methods

2.1. Setting of experiments

The study was carried out at Hawler Medical University in the experimental animal house of the College of Pharmacy in the pharmacology and toxicology department from (5^{th} January 2022) to (1^{st} March 2022)

2.2. Study Population

2.2.1 *Animal*

The study was performed on 24 female albino rats weighting (220±20g), each purchased from an animal house in the college of pharmacy, Hawler Medical University. The animals were housed in plastic cages and randomly divided into four groups of 6 rats per cage for at least 2 weeks before the start of the experiment and were kept in a room with a 12-hr light/12-hr dark cycle and under conditions of the controlled ambient temperature of 26±2 C°. All animal procedures were approved by the Animal ethical committee college of pharmacy/Hawler Medical University (Number 214-HMU-PH-EC on 22-8-2021).

2.3. Dose selection

This study used the following doses: OXA 100mg/20ml, ENT 0.5mg, and advanced PRO 1250mg. The dose that should be given to the rats was: OXA 10mg/kg (Hubert et al., 2015). ENT 0.09mg/kg (Fei-Yan et al., 2013) and PRO $2.5 * 10^9$ colony form unit (CFU) (Skrypnik et al., 2019).

2.4. Experimental design

The rats were allocated into four groups as follows:

Group 1: (Negative control group)

The group included 6 rats and served as a negative control group; they received 1ml distilled water orally and 0.5ml of normal saline intraperitoneal for 3 weeks.

Group 2: (Positive control OXA group)

The group included 6 rats and served as model-induced hepatotoxicity. The animals were injected with 10mg/kg of OXA dissolved in 0.5ml 0.9% NaCl IP once weekly for 3 weeks. (Hubert et al., 2015).

Group 3 (OXA- ENT group)

The group included 6 rats and served as the treatment group; they were given OXA 10mg/kg dissolved in 0.5ml 0.9% sodium chloride (NaCl) IP once weekly for 3 weeks along with ENT 0.09mg/ kg orally gavage daily for 3 weeks (Li *et al.*, 2013, Hubert *et al.*, 2015).

Group 4. (OXA + PRO group)

The group included 6 rats and served as the treatment group; the animals were injected with 10mg/kg of OXA dissolved in 0.5ml 0.9% NaCl IP once weekly for 3 weeks, along with $2.5 * 10^9$ CFU of PRO daily for 3 weeks by oral gavage (Hubert *et al.*, 2015, Skrypnik *et al.*, 2019).

2.5. Method

Firstly, all rats were weighed before being given the drugs then the control group was given 1ml distilled water by oral gavage and 0.5 ml NaCl IP. The Treatment Groups Immediately after the injection of drugs (OXA), the rats received the corresponding treatment drugs (PRO or ENT) according to the started dose for each group (except for the control group and OXA groups), and the treatment drugs dissolved in distilled water immediately before administration by oral gavage.

The OXA is given intraperitoneal to (OXA, OXA+PRO, and OXA+ENT) groups by intraperitoneal. After 3 weeks, 24 hrs. of the last dose, the rats from (OXA, OXA+PRO, and OXA+ENT) groups and the control group were anesthetized by injecting ketamine 50mg/kg and xylazine 5mg/kg IP (Konecny, 2021), to render them unconscious which required approximately 3-5 min. Then, using a 5ml syringe, blood was withdrawn by direct cardiac puncture.

The 3ml of drawn blood was placed in ordinary (gel and clot activators) tubes and allowed to settle for 30-45 min, then centrifuged at 3000 RPM for 10 min. The obtained serum was placed in Eppendorf tubes and stored at (– 20C°) for further analysis.

2.6. Measurement of LFTs

The LFTs compromising ALP, AST and ALT were measured by Cobas c311 according to the manufacturer's instructions (Roche, Germany).

2.7 Determination of inflammatory cytokines (IL-1 and IL-

6)

IL-1 and IL-6 were measured using relevant commercial kits recommended by the manufacturer (KPG, USA).

2.8 Statistical analysis

Graph Pad prism 9 was used for the analysis of data. The data performed the normality tests, so they are parametric data. It was expressed as mean+ standard error of the mean (SEM). One-way ANOVA analyzed the data, and Tukey was used as a post hoc test for multiple comparisons. Probability (P)-value less than 0.05 is considered statistically significant.

3.0 RESULTS

3.1 Immunomodulatory effects of entecavir and probiotic on hepatic damage induced by OXA

As shown in (Table 1, Figure 1a), OXA administration caused a significant increase in IL-1 levels (18.42 ± 1.661) when compared with the control group (10.31 ± 0.5869) (P= 0.024). A non-significant alteration was observed in IL-1 levels after ENT treatment (14.99 ± 2.127) and PRO treatment (13.88 ± 1.956) when compared with the OXA group (10.31 ± 0.5869).

In addition, IL-6 level increased non-significantly after OXA exposure (38.12 ± 6.154) when compared with the control group (20.45 ± 3.241) (P=0.114) (Figure 1b). Statistical analysis revealed that ENT and PRO treatment did not significantly restore IL-6 after OXA exposure.

Table 1. Liver function tests and inflammatory markers between rat groups

One-way ANOVA compared parameters between groups and Tukey was used as post-hoc test for multiple comparisons. Probability (P)-value less than 0.05 is considered statistically significant. ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; IL-1: interleukin-1; IL-6: interleukin-6; M: mean; SEM: standard error of the mean.

Parameters	*Control (n=6) (M ± SEM)*	*OXA (n=6) (M ± SEM)*	*OXA+ENT (n=6) (M ± SEM)*	*OXA+PRO (n=6) (M ± SEM)*	*P-value*
IL-1 (pg/ml)	10.31 ± 0.5869	18.42 ± 1.661	14.99 ± 2.127	13.88 ± 1.956	0.024
IL-6 (pg/ml)	20.45 ± 3.241	38.12 ± 6.154	31.62 ± 5.129	27.85 ± 4.724	0.114
AST (U/L)	64.77 ± 9.134	103.3 ± 16.63	83.30 ± 4.971	81.50 ± 14.78	0.210
ALT (U/L)	27.92 ± 1.976	47.70 ± 8.022	29.87 ± 4.245	31.67 ± 3.664	0.041
ALP (U/L)	75.78 ± 17.79	206.0 ± 12.69	100.5 ± 11.60	79.07 ± 13.94	<0.0001

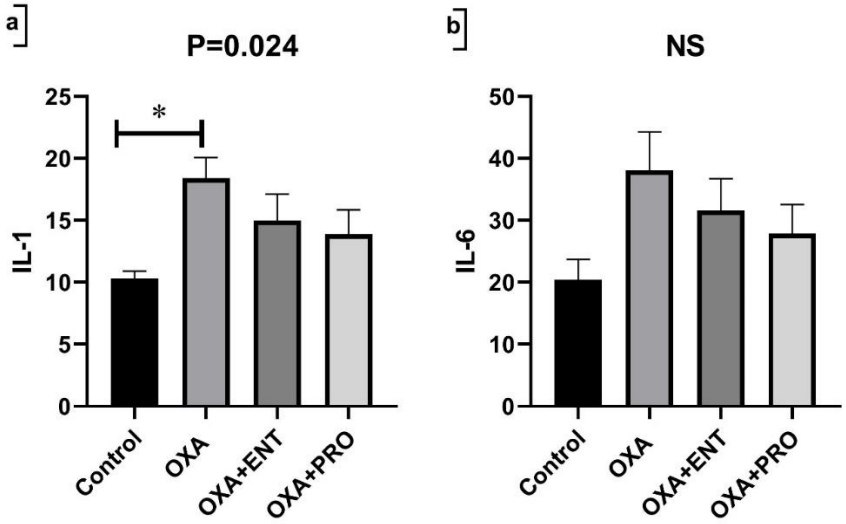

Figure 1: Comparison of inflammatory markers between different rat groups. a) IL-1, b) IL-6. One-way ANOVA compared parameters between groups and Tukey was used as a post hoc test for multiple comparisons. Probability (P)-value less than 0.05 is considered statistically significant. ENT: entecavir; IL-1: interleukin-1; IL-6: interleukin-6; OXA: oxaliplatin; PRO: probiotic.

3.2 Hepatoprotective effects of entecavir and probiotic on hepatic damage after exposure to OXA

The protective effect of entecavir against acute liver injury induced by OXA was confirmed by analysis of serum aminotransferase activity. The results in (Table 1, and Figure 2a) show that OXA administration increased plasma AST activity non-significantly (103.3 ± 16.63) when compared with the control group (64.77 ± 9.134) (P=0.210). However, ENT and PRO administration could decrease AST but they didn't reach a significant level (P=0.210). Statistical analysis in (Table 1, Figure 2b) revealed that OXA raised ALT significantly (P=0.041) if compared to the control group while treatment with ENT or PRO reduced the activity of enzymes ALT (ALT of OXA+ENT: 29.87 ± 4.245; ALT of OXA+PRO: 31.67 ± 3.664), when compared to ALT of OXA induced liver damage group (47.70 ± 8.022) but they also did not return it to the normal level. OXA administration could significantly increase ALP in the rat (206.0 ± 12.69) compared to ALP in the control group (75.78 ± 17.79). On the other hand, the analysis of the results revealed that along with OXA therapy, the treatment with ENT or PRO significantly (P<0.0001) reversed the hepatic ALP levels increased by OXA (Table 1, Figure 2c).

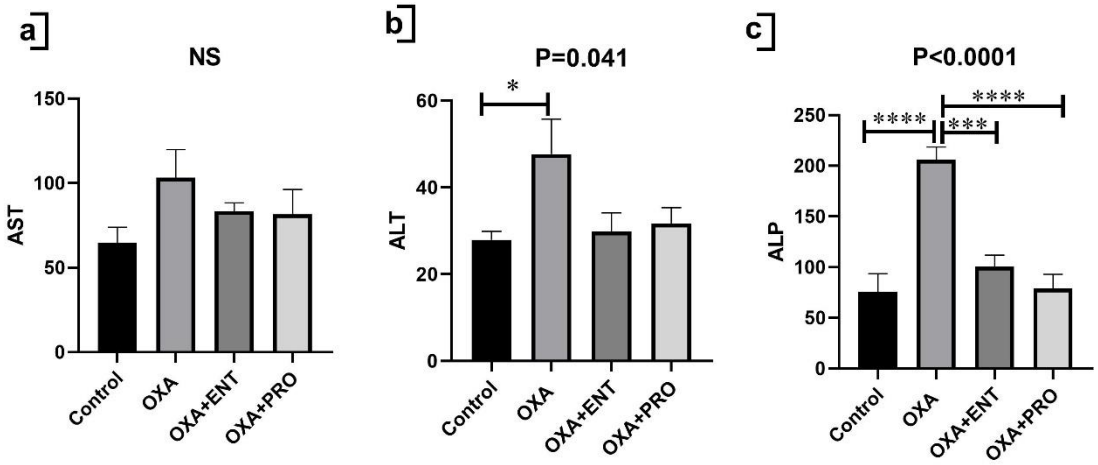

Figure 2: Comparison of liver function tests between different rat groups. a) AST, b) ALT, and c) ALP. One-way ANOVA compared parameters between groups and Tukey was used as a post-doc test for multiple comparisons. Probability (P)-value less than 0.05 is considered statistically significant. ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

4.0 DISCUSSION

OXA is a platinum-based therapy used frequently in a combination regime for treating cancer, especially gastrointestinal cancer. Liver damage, vomiting, anorexia, and diarrhea are the most common side effects of OXA (Badary *et al.*, 2005).

After giving the rat OXA, the inflammatory cytokines (IL-1 and IL-6) and LFTs were increased in this study, this result was not in line with Luo *et al.* (2020) who showed that OXA could decrease inflammatory cytokines. The inflammatory and liver effects of OXA are not fully understood. There are many theories put out, including the possibility that OXA interferes with the intestinal barrier and disturbs normal flora. When the germs enter the bloodstream, they interact with the macrophage's toll-like receptor and cause it to release the pro-inflammatory cytokines IL-1 and IL-6. These inflammatory cytokines cause inflammation and liver damage by causing the release of acute phase protein (CRP) in the liver.(Inokuchi *et al.*, 2011, Hong *et al.*, 2015). The other theory for OXA-induced inflammation and hepatic damage is that the OXA may change the nature of normal flora, leading to the dominance of Bacteroides over prevotella, the opportunistic microbes cause damage to the intestinal vill and inflammation (Forsgård *et al.*, 2017). The changing normal flora also changes the "gut-liver axis. The term "gut-liver axis" was first used by Marshall in 1998, explaining that changing the bulk of flora in the gut causes inflammation in the liver inducing hepatic damage (Wang *et al.*, 2021). The last theory for

increasing inflammation and hepatic damage is that OXA may increase oxidative stress in the liver, resulting in liver damage and inflammation. The evidence for increased oxidative stress is that SOD3, HO1, and Mt1 were upregulated after OXA administration, as confirmed in the study published by Robinson *et al*, 2013 (Robinson *et al.*, 2013). Lin et al, 2018 (18) also reported oxidative stress-induced liver damage. The increased free radicals by OXA culminated in the damage of mitochondria of hepatocytes (Zicca *et al.*, 2002, Chun *et al.*, 2009, Correia *et al.*, 2010).

The LFTs and inflammatory cytokines were restored to nearly normal after administration of entecavir, one of the antiviral medications used to treat HBV. Entecavir is a guanosine nucleotide analog that reverses hepatocyte damage, inhibits the virus's polymerase activity, and increases the virus's clearance. This result parallels the finding, which documented that entecavir could restore LFTs compromising ALP, ALT, and AST (NCBI, 2018). Entecavir can also decrease inflammation in the liver by increasing the clearance of HBsAg and HBeAg and preventing the progression of hepatitis to liver cirrhosis and hepatocellular carcinoma (Woodhouse *et al.*, 2018, Wang *et al.*, 2020). Lu *et al.* (2021) unveiled that the inflammatory cytokines (IL-6 and TNF-α) and ALT were decreased after entecavir treatment. Entecavir can also decrease liver inflammation by restoring normal flora, as confirmed by Lu *et al.* (2021).

After the adjuvant drug was administered probiotic, the LFTs and inflammatory cytokines returned to almost normal levels. The mechanism of probiotics' hepatoprotective and anti-inflammatory effects may be due to rebalancing normal flora, which was dysregulated after OXA administration. Restoring normal flora leads to an improved gut-liver axis and the integrity of the gut barrier. Probiotics have many functions, such as anti-inflammatory, anti-cancer, and antioxidant effects (Azad *et al.*, 2018). Probiotics may alleviate liver damage by decreasing free radicals. Probiotics can restore normal flora by decreasing the number of harmful flora (Bacteroides), and decreasing inflammation and oxidative stress (Feng *et al.*, 2020).

This study has potential limitations. Firstly, the sample size is small, which may affect the increasing SEM of the study. Secondly, The duration of study and drug administration is short, which is different compared to the chemotherapy administration in humans. Lastly, this study did not measure oxidative stress markers such as MDA, SOD, NO, and GSH. Their measurement in another study is our recommendation.

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5.0 CONCLUSION

The rats in the ENT and PRO groups had much lower levels of ALT, ALP, AST, IL-1, and IL-6 compared to the rats with OXA therapy alone, which revealed that the therapy with ENT or PRO has a significant influence on alleviating liver injury and inflammation. The current study also showed that ENT or PRO therapy enhanced rats' immunity. The ENT or PRO therapy contributed to the relief of inflammatory responses in rats. The administration of entecavir and probiotics should add to a combination anti-cancer regime that contains OXA.

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Conflict of interests

The authors declare that there is no conflict of interest.

Author contributions

AAA and ANA contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

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