Alterations in Biochemical Markers of Liver and Kidney Function and Oxidative Stress Indices on Sub-acute Exposure of Meloxicam in Albino Rats

Snehal Wasnik¹, Vidhi Gautam¹, Prateek Mishra¹, Rajesh Sharma¹, Sachin Jain¹, Vijay K. Gond¹

ABSTRACT

Background: The study was performed to study the alterations in biochemical markers of liver and kidney function and oxidative stress indices on sub-acute exposure of meloxicam in albino rats.

Methods: The study was performed in three groups of rats, consisting six rats in each group. The rats of group I were served as control. However, rats of group II, III were treated with meloxicam @ 0.2 mg/kg b.wt. meloxicam @ 0.6 mg/kg b.wt. respectively.

Result: Meloxicam administration induced oxidative stress in rats as indicated by significant decrease in concentration of catalase, superoxide dismutase and reduced glutathione in blood as compared to control. However, lipid peroxidation was significantly enhanced as indicated by increased level of MDA in blood as compared to control. Meloxicam altered the functions of liver and kidney as indicated by the alteration in the biochemical markers of liver and kidney function. Meloxicam significantly enhanced the concentration of biochemical markers of liver function viz. ALT, AST, GGT, ALP, albumin and bilirubin as compared to control. The concentration of biochemical markers of kidney function viz. BUN and creatinine was significantly enhanced after meloxicam administration as compared to control.

Key words: Albino rats, Maloxicam, Oxidative stress.

INTRODUCTION

Non steroidal anti-inflammatory drugs (NSAID) are widely used drugs in both veterinary and human medicine for various inflammatory conditions. NSAIDs are one of the best therapeutic choices to prevent and treat postoperative pain (Pizzino et al., 2017). Meloxicam, a non-steroidal anti-inflammatory drug is very popular in both veterinary and human medicine as anti-inflammatory, analgesic and antipyretic drug (Burukoglu et al., 2014). Meloxicam, either alone or with antimicrobial drugs, is indicated for the treatment of pneumonia, pleuritis, laminitis, myositis, sprain, mastitis, prolapse of uterus, premature labour etc. in animals (Jadhav et al., 2014). It produces its effect by inhibiting cylooxygenase (COX) enzyme which is found in two isoforms i.e COX-1 and COX-2. Meloxicam is selective for COX-2 than COX-1 in lower doses, but in higher doses it can inhibit COX-1 enzyme also and produces serious adverse effects (Mahaprabhu et al., 2011).

Meloxicam administration leads to renal vasoconstriction and decreased renal perfusion, which is responsible for acute renal abnormalities. It also induced liver toxicity, as liver is the major target for drug metabolism and hepatic biotransformation reaction (Amin et al., 2017). Meloxicam have been reported to induce oxidative stress to various organisms (oxidative stress occurs as a result of imbalance between preoxidants and anti-oxidants which may lead to excessive production of reactive oxygen species (ROS). The ROS produced may interact with biomolecules and may result in an increase in the LPO, protein denaturation and alterations in the activities of anti-oxidant enzymes such as CAT, SOD and other non-enzymatic biomarkers such as GSH (Villegas et al., 2001).

MATERIALS AND METHODS

Location and place of work

The proposed work was conducted in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University (NDVSU), Jabalpur, Madhya Pradesh, India.
Experimental animals
The study was conducted on healthy albino rats of 6-7 weeks age, weighing around 150-200 gm. The experiment was approved by the Institutional Animal Ethical Committee (IAEC) of College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University (NDVSU), Jabalpur. Before the start of the experiment the rats were kept in laboratory condition for a period of 7 days for acclimatization. The rats were maintained with good hygienic conditions and kept in colony cages under standard managemental conditions and provided with standard feed and water ad libitum.

Drugs
Meloxicam was administered orally for subacute exposure.

Experimental design
Eighteen rats were randomly divided into seven groups with six rats in each group to the oxidative stress and organ damage induced by sub-acute exposure of meloxicam the experiment was conducted for 28 days (Table 1).

Collection of blood sample
Blood was collected on day 0 and day 28 from the retro-orbital plexus with the help of capillary tube as described by Archer and Riley (1981). Blood was collected in heparinised vials and used for study of biochemical and oxidative stress parameters.

Biochemical studies
Plasma was separated from heparinised blood samples and refrigerated at 4°C for biochemical studies. The following biochemical markers of liver and kidney function were estimated by using Semi-auto analyzer with respective commercially available kits of ERBA, manufactured by Transasia Bio-Medicals Ltd., Daman.

1. Aspartate aminotransferase (AST) (IU/L). 
2. Alanine transaminase (ALT) (IU/L). 
3. Alkaline phosphatase (ALP) (IU/L). 
4. Bilirubin (mg/dl).
5. GGT - Gamma glutamyl transpeptidase (U/L).
6. Albumin (g/dl).
7. Creatinine (mg/dl).
8. Blood urea nitrogen (BUN) (mg/dl).

Assessment of oxidative stress indices
After blood collection the samples were centrifuged at 2000 rpm for 15 min to separate plasma. The layer of white blood cells above the packed erythrocytes was discarded. Erythrocyte pellet was washed three times with 0.15 M NaCl, diluted (33 per cent) in phosphate buffer saline (mM: NaCl, 136.9, KCl, 2.68; KH₂PO₄, 1.47; and Na₂HPO₄, 6.62; pH 7.4) and kept at 4°C until further analysis. The 33 per cent packed erythrocytes were used for the estimation of LPO, GSH, Glutathione reductase, Catalase and Superoxide dismutase activity by using Helios double beam spectrophotometer. LPO and GSH were measured on the day of blood collection (Das and Vasudevan, 2005).

Lipid peroxidation
Membrane peroxidative damage in erythrocytes was determined in terms of malondialdehyde (MDA) production as method suggested by Rehman (1984).

Reagents
1) Trichloroacetic acid (TCA) (10 per cent solution): 10 gm of TCA was dissolved in distilled water and the volume was made up to 100 ml with distilled water.
2) Thiobarbituric acid (TBA) (0.67 percent solution): 0.67 gm of TBA in 100 ml of distilled water and warm up for dissolving TBA.

Procedure
One ml of 33 per cent of erythrocyte was incubated at 37°C for 2 hrs. To each sample, 1ml of 10 per cent w/v TCA was added. After thorough mixing, the reaction mixture was centrifuged at 2000 rpm for 10 minutes. Then 1 ml of 0.67 per cent w/v of TBA was added in 1 ml of supernatant and kept in boiling water bath for 10 minutes. Reaction was cooled and diluted with 1ml of distilled water. Blank was made by adding all the reagents except the packed erythrocyte. The absorbance of these samples was read at wavelength 535 nm.

Calculation
Calculation was done by using molar extinction coefficient (EC) of MDA-TBA complex at 535 nm, i.e., 1.56 × 10⁸M/cm.

LPO level (nM MDA/g blood sample) = 
\[ \frac{OD \times \text{Total volume of reaction mixture} \times 10^4 \times \text{DF} \times \text{Time of incubation}}{\text{OD of sample taken}} \]

OD = Optical density.
DF = Dilution factor.

Reduced glutathione (GSH)
GSH was estimated by the 5, 5′ dithiobis (2-nitrobenzoic acid) (DTNB) method suggested by Prins and Loos (1969).

Reagents
1) H₂SO₄ 0.08 N in distilled water.
2) Tungstate solution, containing 0.3 M Na₃WO₄ and 0.1 M ethylene diamine tetra acetic acid.
3) TRIS buffer, pH 8.0, a solution containing 1M tris-hydroxymethyl amino methane and adjusted with HCl to pH 8.0.
4) DTNB reagent, a solution containing 0.14 M NaCl, 0.009M Na₂HPO₄, 0.000013 M, NaH₂PO₄ and 40 mg/100 ml DTNB (prepared fresh).

Procedure
RBCs pack (0.2 ml) (33 per cent dilution in PBS) was added to 4 ml of 0.08 N H₂SO₄ and mixed carefully. After 10 minutes of standing at room temperature, 0.5 ml of Tungstate solution was added to clear haemolysate. The tube was
stopped and mixture was shaken vigorously for 5 minutes. The stopper was removed and suspension was allowed to stand for 5 minutes in order to avoid bubble formation on top of the supernatant. The suspension was then centrifuged for 15 minutes at 2000 rpm at room temperature. After centrifugation, 2 ml of supernatant was added to 2.5 ml of tris-buffer, 0.2 ml of DNTB reagent was added and mixed well. Within a minute, absorbance was measured at 412 nm against blank by using Spectrophotometer in which 2 ml of distilled water was substituted for supernatant.

Calculation
Calculation was done by using the following formula and results were expressed as milimole (mM) of GSH per ml of packed RBCs.

\[
\text{GSH level (mM/g of blood sample) = } \frac{\text{OD of test}}{\text{EC}} \times \frac{\text{Total volume of reaction mixture}}{\text{Volume of sample taken}} \times \frac{1}{\text{mg of Haemoglobin}}
\]

EC - Extinction coefficient i.e., 13100/M/cm.
OD - Optical density.

Superoxide dismutase
Superoxide dismutase (SOD) activity was determined in the blood supernatant according to the method of Madesh and Balasubramanian (1998). A calorimetric assay involving generation of superoxide by pyrogallol auto-oxidation and the inhibition of superoxide dependent reduction of tetrazolium dye (MTT) \([3-(4-5\text{ dimethyl thiazol-2-4}) 2, 5\text{-diphenyl tetrazolium bromide}\]. One unit of SOD was that which caused a 50 per cent inhibition of reduction rate of MTT.

Reagent
1) Sample: 1:20 haemolysate.
2) Phosphate buffer saline.
3) MTT (1.25 mM) fresh 12.61 mg/10ml.
4) Pyrogallol(100 mM) fresh 12.61 mg/100 ml distilled water.
5) DMSO (Dimethyl sulfoxide).

Procedure
In a set of tubes 0.65 ml PBS (pH 7.4) was taken. To this 30 μl (MTT) was added. Then, 75 μl of pyrogallol was added to both the tubes. This mixture was incubated for 5 min at room temperature and the reaction was stopped by adding 0.75 ml of dimethyl sulfoxide and finally 10 μl of sample was added to second tube to find out the percentage of reduction of MTT formation. The absorbance was read at 570 nm and the activity has been expressed as Unit. One unit of SOD was defined as the micrograms of hemoglobin causing 50 per cent inhibition in the MTT reduction.

Catalase
Catalase activity in erythrocytes was assayed by the spectrophotometric method of Brannan et al. (1981).

Results and Discussion
Biochemical studies
In the present research work, biochemical study was carried out to study the alterations in biochemical markers of liver and kidney function on sub-acute exposure of meloxicam in albino rats.

Biochemical markers of liver function
The mean values of biochemical markers of liver function viz. Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Gamma-glutamyl transferase (GGT), Alkaline phosphatase (ALP), albumin and bilirubin in rats have been presented in (Table 2).

| Reagents | 1) Phosphate buffer saline. 2) Haemolysate (10 per cent). 3) Hydrogen peroxide (10 mM). |
| Procedure | Two ml of phosphate buffer (50 mM, pH 7.0) and 10 μl hemolysate (10 per cent) were taken in a cuvette. Reaction was started by adding 1 ml H₂O₂ (10 mM) and the absorbance was recorded at every 10 sec for 1 min at 240 nm against water blank. The activity of catalase has been expressed as mmol H₂O₂ utilized/min/mg hemoglobin. |
| Statistical analysis | Means and standard error were obtained as per standard procedure. Parameters were analyzed by using the method of complete randomized design with seven treatments allotted to groups of six animals each. The difference between treatments was tested statistically for their significance (Snedecor and Cochran, 1994). |

Table 1: Design of experiment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>I</td>
<td>Control</td>
</tr>
<tr>
<td>II</td>
<td>Meloxicam @ 0.2 mg/kg b. wt. once daily, orally for 28 days.</td>
</tr>
<tr>
<td>III</td>
<td>Meloxicam @ 0.6 mg/kg b. wt. once daily, orally for 28 days.</td>
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</table>
In the present investigation, elevated levels of serum ALT and AST after sub-acute exposure of meloxicam are in agreement with the result of Gretzer et al. (2001) and Wallace et al. (2000). ALT and AST are liver enzyme markers used as an indicator of meloxicam induced hepatic changes in albino rats (Burukoglu et al., 2014). Amin et al. (2017) suggested that meloxicam administration in rats significantly increased levels of liver enzymes AST and ALT after sub-acute treatment.

**GGT (Gamma glutamyl transferase)**

The concentration of GGT (Gamma glutamyl transferase) in control was 7.18±0.07 IU/L. Meloxicam, significantly increased the concentration of GGT and this increase was 7.43±0.75 IU/L after the sub-acute exposure of meloxicam at the dose rate of 0.2 mg/kg b.wt., orally for 28 days. In higher doses i.e. 0.6 mg/kg b.wt., orally for 28 days, meloxicam further increased the concentration of GGT to 7.87±0.52 IU/L.

Serum GGT is sensitive, highly specific and indicative enzyme for liver function. Fayed et al. (1998) suggested that meloxicam after the sub-acute administration increased the level of enzyme GGT in serum. Karatopuk and Gökçimen (2010) found the elevation in serum GGT level after the administration of meloxicam in rats by the production of oxidative stress.

**ALP (Alkaline phosphatase)**

The concentration of alkaline phosphatase in control was 454.30±2.81. Meloxicam, significantly increased the concentration of ALP and this increase was 526.30±1.71IU/L after the sub-acute exposure of meloxicam at the dose rate of 0.2 mg/kg b.wt., orally for 28 days. In higher doses i.e. 0.6 mg/kg b.wt., orally for 28 days, meloxicam further increased the concentration of ALP to 543.70±3.54 IU/L.

ALP is an enzyme that helps in protein breakdown and plays important role in liver function. Luna et al. (2007) showed that administration of meloxicam @ 2 mg/kg body weight daily for 15 days caused elevation of serum ALP when given orally. Ahmad et al. (2015) showed that oral administration of meloxicam @ 0.4 mg for 28 consecutive days caused elevation in serum ALP level in mice by the production of oxidative stress.

**Albumin and bilirubin**

The concentration of albumin in control was 4.08±0.09 g/dl. Meloxicam, significantly increased the concentration of albumin and this increase was 4.38±0.10 g/dl after the administration of meloxicam at the dose rate of 0.2 mg/kg b.wt. orally for 28 days. In higher doses i.e. 0.6 mg/kg b.wt., orally for 28 days, meloxicam further increased the concentration of albumin to 4.93±0.07 g/dl.

The concentration of Bilirubin in control was 0.115±0.025 mg/dl. Meloxicam, significantly increased the concentration of bilirubin and this increase was 0.125±0.025 mg/dl after the administration of meloxicam at the dose rate of 0.2 mg/kg b.wt., orally for 28 days. In higher doses i.e. 0.6 mg/kg b.wt. orally for 28 days, meloxicam further increased the concentration of bilirubin to 0.135±0.025 mg/dl.

The present study has shown that, meloxicam administration resulted in significant increase in albumin and bilirubin. Alencar et al. (2003) also suggested that meloxicam, after the sub-acute administration increased the level of albumin and bilirubin in dogs. Jadav et al. (2014) and Abatan et al. (2006) also reported the elevation in serum albumin and bilirubin level after the administration of meloxicam. Meloxicam induced oxidative stress contributes to liver injury and elevates concentration of albumin and bilirubin.

**Biochemical markers of kidney function**

The mean values of biochemical markers of kidney function viz. Blood urea nitrogen and creatinine in rats treated with meloxicam have been presented in (Table 3).

**Creatinine and blood urea nitrogen**

The concentration of creatinine in control was 0.654±0.05 mg/dl. Meloxicam, at the dose rate of 0.2 mg/kg b.wt., orally for 28 days did not affect the creatinine level in serum significantly and the mean value was 0.852±0.03. However,
meloxicam in higher doses i.e. 0.6 mg/kg b.wt., orally for 28 days, significantly increased the concentration of creatinine to 1.057±0.06 mg/dl.

The concentration of blood urea nitrogen in control was 14.58±0.35 mg/dl. Meloxicam, at the dose rate of 0.2 mg/kg b.wt., orally for 28 days did not affect the BUN level in serum significantly and the mean value was 14.75±0.51. However, meloxicam in higher doses i.e. 0.6 mg/kg b.wt., orally for 28 days, significantly increased the concentration of BUN to 21.73±0.52 mg/dl. In group IV, where rats were treated with meloxicam at the dose rate of 0.2 mg/kg b.wt.

Creatinine and blood urea nitrogen are potent indicators of renal impairment. In this study meloxicam administration significantly increased the concentration of creatinine and BUN in serum as compared to control. El-Syaed et al. (2017), mentioned meloxicam induced changes in biomarkers of kidney function and found increased concentration of creatinine and blood urea nitrogen.

**Oxidative stress indices**

The mean values of oxidative stress indices Viz. Lipid peroxidation (MDA), Superoxide dismutase (SOD), reduced glutathione (GSH) and catalase in rats treated with meloxicam alone have been presented in (Table 4).

**Lipid peroxidation (MDA)**

Lipid peroxidation was calculated in terms of nM MDA/gm of blood on day 28 of experiment in albino rats. The concentration of LPO in control was 4.50±0.02 nM MDA/gm of blood. However, meloxicam at dose of 0.2 mg/kg b.wt and 0.6 mg/kg b.wt. significantly increased the concentration of LPO and the mean values were 5.44±0.09 and 5.71±0.11 nM MDA/gm of blood, respectively.

MDA is the end product of lipid peroxidation, due to oxidation of polyunsaturated fatty acids by free radicals. The increase in its concentrations in the kidney tissue revealed an enhanced lipid peroxidation leading to tissue damage as well as the failure of the antioxidants to prevent excessive production of free radicals (Karadeniz et al., 2008). In the present study, the concentration of LPO was increased significantly in meloxicam treated group as compared to control group. This indicates that meloxicam induced oxidative stress since MDA, which is the last product in lipid peroxidation process, is considered as oxidative stress marker Ayala et al. (2014). However, similar elevation in MDA level in the plasma associated with meloxicam treatment was also recorded by previous studies Villegas et al. (2001).

**SOD (Superoxide dismutase)**

Superoxide dismutase was calculated in terms of U/g of Hb on day 28 of experiment in albino rats. The concentration of SOD in control was 1.34±0.09 U/g of Hb, however meloxicam at dose of 0.2 mg/kg b.wt and 0.6 mg/kg b.wt. significantly decreased the concentration of SOD and the mean values were 0.85±0.06 and 0.79±0.04 U/g of Hb respectively.

Ergul et al. (2010) indicated that meloxicam administration reduced superoxide dismutase (SOD). Amin et al. (2017) indicated that meloxicam administration caused formation of reactive oxygen species and reduces the level of superoxide dismutase. The findings of present study are in agreement with the findings of Ergul et al. (2010) and Amin et al. (2017).

**GSH (Reduced glutathione)**

Reduced glutathione was calculated in terms of µmol/ml of blood on day 28 of experiment in albino rats. The concentration of GSH in control was 356.78±0.67 µmol/ml of blood. However, meloxicam at dose of 0.2 mg/kg b.wt and 0.6 mg/kg b.wt. significantly decreased the concentration of GSH and the mean values were 331.03±1.49 and 323.53±1.49 µmol/ml of blood respectively.

Mahaprabhlu et al. (2011) suggested that metabolism of meloxicam residues, generate free radicals and contribute to an increase in oxidative stress, promoting inhibition of cellular enzymes by the reduction in glutathione peroxidase (GSHPx). In agreement with that GSH concentration was significantly lower in meloxicam treatment compared to control group in present study. Beutler et al. (1963) and Villegas et al. (2001) indicated that administration of meloxicam in rats produced reactive oxygen species and increased oxidative stress.

**Catalase**

Catalase was calculated in terms of µmol of H$_2$O$_2$ decomposed/min/gm Hb on day 28 of experiment in albino rats. The concentration of CAT in control was 243.04±0.29 µmol of H$_2$O$_2$ decomposed. However, meloxicam at dose of 0.2 mg/kg b.wt and 0.6 mg/kg b.wt., significantly decreased the concentration of CAT and the mean values were 173.30±1.96 and 141.31±2.90 µmol H$_2$O$_2$ decomposed, respectively. Decrease in catalase activity would increase

### Table 4: Effect of *Phyllanthus niruri* on oxidative stress indices in meloxicam induced oxidative stress in albino rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Oxidative stress indices (Mean±SE)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>MDA (nM MDA/gm)</td>
</tr>
<tr>
<td>G1</td>
<td>Control</td>
<td>4.50±0.02</td>
</tr>
<tr>
<td>G2</td>
<td>Meloxicam @ 0.2 mg/kg b. wt. once daily, orally for 28 days.</td>
<td>5.44±0.09</td>
</tr>
<tr>
<td>G3</td>
<td>Meloxicam @ 0.6 mg/kg b. wt. once daily, orally for 28 days.</td>
<td>5.71±0.11</td>
</tr>
</tbody>
</table>
H₂O₂ concentration in the cell, leading to increased oxidative stress according to Mahaprabhu et al. (2011).

Nazifi et al. (2019) suggested that metabolism of meloxicam residues, generate free radicals and contribute to an increase in oxidative stress, indicated by the reduction in catalase (CAT). The findings of present study are in agreement with Nazifi et al. (2019), as in the present research, CAT concentration was significantly lower in meloxicam treated group as compared to control group.

**CONCLUSION**

Meloxicam at the dose of 0.2 mg/kg body wt. and 0.6 mg/kg body wt., orally, daily for 28 days in albino rats induced the liver impairment as indicated by increased concentration of biochemical markers of liver function viz. ALT, AST, GGT, ALP, albumin and bilirubin. Meloxicam at the dose of 0.2 mg/kg body wt. and 0.6 mg/kg body wt., orally, daily for 28 days in albino rats induced the renal impairment as indicated by increased concentration of biochemical markers of kidney function viz. BUN and creatinine. Meloxicam at the dose of 0.2 mg/kg body wt. and 0.6 mg/kg body wt., orally, daily for 28 days in albino rats induced oxidative stress as indicated by altered oxidative stress indices viz. catalase, superoxide dismutase, reduced glutathione and lipid peroxidation in blood.

**Conflict of interest:** None.

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