In vitro cytotoxic and genotoxic evaluation to ascertain toxicological potential of ketoprofen

Dawood Ahmad Hamdani¹*, Aqeel Javeed¹, Muhammad Ashraf¹, Jawad Nazir², Aamir Ghafoor³, Imran Altaf⁴ and Muhammad Shahbaz yousaf⁵

¹Department of Pharmacology and Toxicology, University of Veterinary and Animal Sciences, Lahore-Pakistan.
²Department of Microbiology, University of Veterinary and Animal Science, Lahore- Pakistan.
³University Diagnostic Lab, University of Veterinary and Animal Sciences, Lahore-Pakistan.
⁴Microbiology section, Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan.
⁵Department of Physiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.

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Analgesic and anti-inflammatory properties of ketoprofen are well documented but little is known about its cytotoxic activity and the potential to damage the DNA. The present study was designed to evaluate the cytotoxic and genotoxic potential of ketoprofen. MTT dye (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was used to assess cytotoxicity in which confluent monolayer of vero cells were incubated in the presence of increasing concentrations of ketoprofen. Genotoxicity was evaluated by single cell gel electrophoresis (SCGE) assay or comet assay. Lymphocytes were separated from the mice blood and treated with different concentrations of ketoprofen. Lymphocytes were incorporated in agarose gel on cavity slides and visualized for strand break to asses DNA damage. Ketoprofen concentrations 8, 6, 4.5, 3.3, 2.5, 1.8, 1.4, 1 and 0.5 mM were used for both cytotoxic and comet assay. The results of cytotoxic assay showed significant (p < 0.001) cytotoxicity at 8 and 6 mM concentrations. The cytotoxic concentration for 50% of cells (CC₅₀) value was calculated at 5.2 mM concentration. In case of the comet assay, ketoprofen presented DNA damaging potency, creating significant (p < 0.001) DNA damage at 8 mM concentration, a moderate damage at 6 mM concentration and a mild damage at 4.5 mM concentration which was evident from the comet tail lengths and changes in head diameter. DNA damage index was calculated for each concentration of ketoprofen and compared with the control. The data advocates that ketoprofen possesses cytotoxic and genotoxic potential at higher concentrations and its dosage should be carefully monitored to avoid its toxicity.

Key words: Ketoprofen, cytotoxic, genotoxic, MTT assay (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), comet assay, DNA damage.

INTRODUCTION

Ketoprofen possess well documented anti-inflammatory, analgesic (Akural et al., 2009), antipyretic and antirheumatic properties (Celebi et al., 2009; Liu et al., 2007; Shinkai et al., 2008). Most of the effects of are ketoprofen due to the

*Corresponding author.  E-mail: davidbhai@hotmail.com. Tel: +92 42 99213697, +92 3214400446. Fax: +92 4299211461. Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License.
inhibition of prostaglandins which are produced by various cell types and regulate various body activities (Hata and Breyer, 2004). Ketoprofen can be given in the form of injectables, taken orally or applied topically to the skin. Photosensitization and contact eczemas are the side effects reported with the use of ketoprofen gel as cutaneous application (Baudot et al., 1998). There are no side effects with the solution containing ketoprofen used as intraoral solution (Liccardi et al., 2003). Ketoprofen developed in the form for nano-emulsion for topical application on the skin is considered very effective (Sakeena et al., 2010).

The cytotoxic assay using MTT dye is considered to be the most sensitive method to assess the nonsteroidal anti-inflammatory drugs (NSAIDs) induced toxicity. MTT assay is mostly used in lab to determine cytotoxicity of various drugs at different concentrations. MTT assay has a number of advantages than clonogenic assay as large number of assays can be performed in one batch (Plumb, 1999). MTT assay is extensively used to study cell viability and cytotoxic effects of the test substance on cell lines under *in vitro* conditions (van Meerloo et al., 2011). Toxicity studies are mostly performed on cell culture models which are an effective ways to evaluate test compounds (Hong et al., 2007). The phosphoramidate derivatives of NSAIDs (fenoprofen, ketoprofen, ibuprofen, indomethacin, diclofenac) were evaluated for their cytostatic and antiviral activity by using malignant tumor cell lines. It was found that phosphoramidate derivatives possessed viral inhibitory activities (Wittine et al., 2009).

Genotoxicity describes that cells integrity is affected due to damaging action on a cell’s genetic material which disrupt cell survival. The comet assay is a micro-electrophoretic technique for the direct visualization of damage to DNA of individual cells (Ostling and Johanson, 1984). Comet assay is unique method for assessing the DNA damage caused by certain drugs, environmental factors and other substances (Blaisak et al., 1999). In comet assay the comets are formed and their tail lengths are measured. These tail lengths give an estimation of extent of DNA damage (Fairbairn et al., 1995). Previously, different NSAIDs were tested for their genotoxicity and comet assay was performed for estimation of DNA damage. Cell of HEC/UGT cell lines were treated with different NSAIDs and the cytotoxicity and genotoxicity caused by these NSAIDs was assessed. In these studies, no cytotoxicity and genotoxicity were observed at ketoprofen (1 mM concentration). It was suggested that ketoprofen at 1 mM concentration is safe for use (Koga et al., 2011).

The objective of present study was to determine the cytotoxic and genotoxic activity of ketoprofen at its various concentrations by adopting various *in vitro* bioassays such as MTT assay and comet assay. Ketoprofen different concentrations were tested both for its cytotoxic and genotoxic potential. The CC<sub>50</sub> value and DNA damage index was also calculated.

**MATERIALS AND METHODS**

**Preparation of drug concentrations**

Ketoprofen was obtained from Sigma Aldrich. Stock solution of drug was prepared by dissolving the drug in sterile PBS. Different concentrations 8, 6, 4.5, 3.3, 2.5, 1.8, 1.40, 1 and 0.5 mM were used for both MTT and comet assay. For MTT assay vero cell lines were used and in comet assay lymphocytes from the mice were exposed to different concentrations of ketoprofen.

**Cell lines**

Vero cell lines were obtained from quality operation laboratory (QOL), University of Veterinary and Animal Sciences, Lahore in freeze dried form and were revived for propagation of cells using Dulbecco’s modified eagle’s medium (DMEM) (Sigma Aldrich, Germany) media and trypsin solution. Cells were counted for their viability by hemocytometer method using the modified neubur chamber. Cell viability was calculated by following formula.

\[
\% \text{ viable cells} = \frac{\text{Number of viable cells} / \text{ml}}{\text{Total number of cells} / \text{ml}} \times 100
\]

**Cytotoxicity assay**

We used MTT dye to assess cytotoxicity of ketoprofen. In the assay, vero cells along with cell culture media were kept as negative control, however vero cells, dimethyl sulphoxide (DMSO) (10%) and cell culture media were taken as positive control, respectively. After development of confluent monolayer in 96 well plate, media in the cells was regularly changed and afterward 100 ul of ketoprofen different concentrations were added in triplicate to the 96 well plates and incubated for 48 h at 37°C. After incubation media was removed and after washing, new media was added to the wells and then 100 ul of MTT solution (98% catalog#194592, MP Biomedical USA) was added to each well and plates were then incubated for 4 h. The MTT solution was afterwards removed and DMSO was added to each well and incubated for 2 h at 37°C. Finally the enzyme linked immunosorbent assay (ELISA) reader (Type 355, Model 2005-05, Thermo, China) was used to measure the optical density at 570 nm (Raheel et al., 2013). Cell survival percentages were calculated.

**Comet assay**

**Preparation of reagents**

Lysing solution, electrophoresis buffer and neutralizing solutions were prepared for comet assay. Staining of slides was carried out by Ethidium bromide solution (Singh et al., 1988).

**Lymphocytes separation**

Lymphocytes for comet assay were separated on the principle of density gradient using Histopaque 1077 (Sigma-Aldrich, USA) (Yildiz et al., 2008).
Preparation of slides

For preparation of slides, low melting point agarose and normal melting agarose were prepared. Slides were dipped in methanol and burn over a blue flame to remove the machine oil and dust. Cavity slides were used for comet assay. Normal melting agarose was added to the cavity of the slide. For quick drying, slides were air dried. After preparing the slides, ketoprofen exposed lymphocytes were placed on them and electrophoresis of micro gel slides and evaluation of DNA damage was carried out (Blasiak et al., 1999). The slides were then stained with 80 μl of ethidium bromide (2 μg/ml) for 10 min and examined at 40× magnification of fluorescent microscope (Nikon, Japan) equipped with excitation filter of 515 to 560 nm and barrier filter of 590 nm. The damage to DNA was analyzed by quantifying the tail length, head diameter changes and was expressed as damage index. To measure the tail length we used the image J software. The images of 100 randomly chosen nuclei (50 cells from each of two replicate slides) were analyzed visually. Depending on the tail length, four damaging categories were established. Those nuclei which did not presented any DNA damage or tail length were considered no damaged nuclei and were labeled class 0. For damage level arbitrary categories were considered as class 1, presenting smaller tail length when tail length are shorter or equal to head diameter, class 2 when tails lengths are greater or equal to head diameter, class 3 when tail lengths are double of head diameter. Using this protocol, the comet assay was used to test which concentration of ketoprofen showed genotoxicity (Parolini et al., 2009).

Damage index

The damage index was calculated for each concentration of ketoprofen and it was compared with the control. The damage index was calculated by the following formula (Sallustio et al., 2006).

\[
\text{Damage index} = \frac{\text{No. of cells in Class.1} + (2 \times \text{No. of cells in Class.2}) + (3 \times \text{No. of cells in Class.3})}{\text{Total number of nuclei}}
\]

Statistical analysis

Data collected was analyzed using the statistical package for social sciences (SPSS) for Windows version 13. One-way analysis of variance (ANOVA) and post hoc tests were applied to see statistical differences between groups. Differences were considered significant at P < 0.05.

RESULTS

Cytotoxicity testing

Cell survival percentages were calculated for determination of cytotoxic activity of different concentrations of ketoprofen. Cell viability % of vero cells was calculated using Trypan blue dye exclusion technique. Viability of vero cells was 92.45% which was suitable for cytotoxicity testing of ketoprofen. Nine different concentrations in the range of 0.5 to 8 mM were used for ketoprofen cytotoxicity analysis. For ketoprofen, with concentrations 8, 6, 4.5, 3.3, 2.5, 1.8, 1.4, 1 and 0.5 mM, the cell survival percentages are shown in Figure 1 which present significant reduction (p < 0.001) in cell survival percentage; however concentration of 1.4 mM with cell survival percentage of 92.75% resulted in significant reduction (p < 0.05) in cytotoxic effect. Ketoprofen concentrations in the range of 0.5 to 4.5 M were non cytotoxic for vero cell line. Ketoprofen showed cytotoxicity at the concentration of 6 and 8 mM because cell survival percentage at these concentrations was less than 50% that is, 30.76 and 14.3%, respectively. Concentrations of 1 and 0.5 mM showed no significant reduction with cell survival (%) of 96.11 and 99.62. The cytotoxic concentration CC50 for 50% of cells was seen at 5.2 mM concentration. The complete data of cell survival percentage of ketoprofen different concentrations is shown in Figure 1.

Comet assay

Genotoxicity of ketoprofen of nine different concentrations ranging from 0.5 to 8 mM was evaluated using the comet assay. Lymphocytes from mice were used for in vitro evaluation of genotoxicity of ketoprofen. Lymphocytes were characterized and their viability was assessed. Ketoprofen showed gentoxicity at 4.5, 6 and 8 mM concentrations. Image J software was calibrated by stage micrometer and was used to evaluate the comet tail lengths and changes in head diameter. The comet tail length and head diameter at different concentrations of ketoprofen are shown in Table 1. Ketoprofen showed concentration dependent DNA damage. No DNA damage was observed at 3.3, 2.5, 1.8, 1.4, 1 and 0.5 mM concentrations of Ketoprofen. There was significant difference (p < 0.001) in DNA tail length between 4.5, 6 and 8 mM concentration of ketoprofen. DNA tail length was 6.25 μm at 4.5 mM, 11.05 μm at 6 mM and 18.06 μm at 8 mM concentration. DNA damage index increased with increase in ketoprofen concentrations, with highest values of damaging index at 8 mM concentration (Table 2). There was no significant difference in damage index with ketoprofen concentration from 0.5 to 3.3 mM when compared with control. The damage observed with 4.5, 6 and 8 mM showed marked difference with control (p < 0.01).

DISCUSSION

Identification of toxicity scale and contamination of certain chemicals which require more investigation are mostly carried out using in vitro testing approach which is a more easy and reliable method for testing the chemicals (Raheel et al., 2013). We also used in vitro methods for evaluating the cytotoxicity and genotoxicity potential of ketoprofen. We used MTT assay for evaluating cytotoxicity...
Figure 1. Cytotoxicity of ketoprofen at different concentrations. Graphical representation of cytotoxic potential of various concentrations of ketoprofen. Cell survival percentage of Vero Cell line after 48hr exposure to different concentrations of ketoprofen with \( CC_{50} \) was observed at 5.2mM concentration. ANOVA, LSD Post hoc test; * \(< 0.05, *** < 0.001 \).

Table 1. Results of genotoxic activity of ketoprofen.

<table>
<thead>
<tr>
<th>Concentrations (mM)</th>
<th>Tail Length (µm)</th>
<th>Head diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>6.25±1.498</td>
<td>16.87±2.574</td>
</tr>
<tr>
<td>6</td>
<td>11.05±2.438</td>
<td>13.89±1.894</td>
</tr>
<tr>
<td>8</td>
<td>18.06±2.572</td>
<td>12.72±2.078</td>
</tr>
</tbody>
</table>

Table 2. DNA damage induced by different concentrations of ketoprofen evidenced from comet assay.

<table>
<thead>
<tr>
<th>Conc. (mM)</th>
<th>Class 0</th>
<th>Class 1</th>
<th>Class 2</th>
<th>Class 3</th>
<th>Damage index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>98</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>0.5</td>
<td>97</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>97</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>1.4</td>
<td>94</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>1.8</td>
<td>91</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>2.5</td>
<td>87</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>3.3</td>
<td>85</td>
<td>11</td>
<td>2</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>4.5</td>
<td>21</td>
<td>73</td>
<td>3</td>
<td>3</td>
<td>88*</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>30</td>
<td>44</td>
<td>5</td>
<td>133*</td>
</tr>
<tr>
<td>8</td>
<td>27</td>
<td>27</td>
<td>24</td>
<td>22</td>
<td>141*</td>
</tr>
</tbody>
</table>

\( n = 100 \) nuclei in two experiments. Nuclei with damage DNA were registered from 0 (undamaged nuclei) to 3 (damaged nuclei). * = significant difference (p<0.01) as compared to control analyzed by SPSS Windows Version 13 Tukey’s test.

of ketoprofen and comet assay for evaluating genotoxicity of ketoprofen. The result showed cytotoxicity and genotoxicity in concentration dependent manner at higher concentrations. Different attempts were previously made using various methods for testing the cytotoxicity and genotoxicity of ketoprofen. Photogenotoxicity potential of ketoprofen was assessed using the alkaline comet assay (Parolini et al., 2009). Comet assay and MTT assay were previously used for evaluating the cytotoxicity and genotoxicity of ketoprofen (Rafael et al., 2012). Sallustio
et al. (2006) used murine hepatocytes for evaluating cytotoxicity and genotoxicity of ketoprofen along with other NSAIDs. The results showed no cytotoxicity and genotoxicity at 0.5 mM concentrations of ketoprofen. They declared that 0.5 mM concentration of ketoprofen is safe and no cytotoxicity and DNA damage was observed at this concentration. Our results suggested that 0.5 mM showed no cytotoxicity when vero cell lines were used. Lymphocyte DNA was also not affected by this concentration.

Allen et al. (1991) used 1 mM concentration of ketoprofen using MTT assay for assessing the ketoprofen cytotoxicity. The results of their study suggested ketoprofen has partial toxicity. Their results did not claim that cytotoxicity is observed at 1 mM concentration, only partial toxicity was observed. Our study suggested that no cytotoxicity or genotoxicity is observable at 1 mM concentration which is in contrast to the result of Allen et al. (1991).

Previously, very few concentrations of ketoprofen were tested for the study of cytotoxic and genotoxic effects of the drug. In our study, we evaluated the cytotoxic potential of ketoprofen and according to our results ketoprofen shows cytotoxic potential at a dose of 5.2 mM against the vero cell lines. The C50 is 5.2 mM. CC50 value of ketoprofen was not previously reported. Our results suggest that ketoprofen cause cytotoxicity at higher concentrations. Various concentrations tested for genotoxicity showed that ketoprofen causes genotoxicity at higher concentration and at 8 mM concentration considerable comet tails can be seen. To the best of our knowledge no previous studies have shown genotoxic concentrations of ketoprofen. Our results suggested that ketoprofen at higher concentrations causes genotoxicity which is evident from comet tails formation and changes in the head diameter of murine lymphocyte DNA.

Conclusion

Ketoprofen, a non-steroidal anti-inflammatory drug, causes cytotoxicity and genotoxicity at higher concentrations. Its dose should be carefully monitored in order to avoid its accumulation in the body which may cause cytotoxicity and genotoxicity.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES


