

DICLOFENAC INHIBITS PROLIFERATION BUT NOT NGF-INDUCED DIFFERENTIATION OF PC12 CELLS

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ABSTRACT

Diclofenac is a non-steroidal anti-inflammatory drug that is prescribed for treatment of rheumatic diseases and as an analgesic. Although the information about these side effects has been widely reported, little is known about the effect of diclofenac on the neural cells. In this study, we investigated the effects of diclofenac on the proliferation and differentiation of PC12 cells. The cell proliferation was evaluated by using XTT assay in the both free-serum neurobasal medium supplemented with B27 supplement and DMEM/F12 medium containing 10% FBS. The nerve growth factor (NGF)-induced differentiation was assessed by measuring the neurite length. The drug toxicity was exhibited at the concentrations more than 310 μ M in the supplemented neurobasal medium. The treatment of cells in the DMEM/F12 medium increased their sensitivity to diclofenac, with 40% and 75% growth inhibition at the 155 and 310 μ M concentrations, respectively. The NGF-induced differentiation was not reduced by toxic and subtoxic concentrations of diclofenac. The results of this study indicated that diclofenac may be able to exhibit its neurotoxic effects through growth inhibition, but not differentiation inhibition. Supplement of B27 has several antioxidant compounds. Therefore, the difference of diclofenac cytotoxic effects in two culture media suggest that drug cytotoxicity may be related to the oxidative stress.

Keywords: Diclofenac, cytotoxicity, differentiation, NGF, PC12.

INTRODUCTION

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) widely used for treatment of rheumatic diseases and as an analgesic. Diclofenac is a lipophilic and weakly acidic compound that features two twisted aromatic rings. A major mechanism of the action of this drug is generally thought to be inhibition of cyclooxygenase (Sallmann 1986; Laneuville 1994; Brogden *et al.*, 1980).

Recent evidence suggests that diclofenac metabolism involves in the production of reactive oxygen species leading to oxidative stress and genomic DNA fragmentation (Hickey *et al.*, 2001; Inoue *et al.*, 2004). In addition, the role of mitochondrial inner membrane permeabilization and activity of caspases in the pathogenesis of diclofenac has been reported (Gomez-lechon *et al.*, 2003a; Gomez-lechon *et al.*, 2003b). Furthermore, it has been reported that the extensive use of diclofenac increases the risk of acute myocardial infarction (Jick *et al.*, 2007) and several cases of severe local reactions associated with intramuscular injection of diclofenac have been reported (Rygnestad *et al.*, 1995; Pillans *et al.*, 1995; Giovannetti *et al.*, 1993). Although the information about these side effects has been widely reported, little is known about the effect of diclofenac on the development of the central nervous system. Therefore it is important to investigate the effects of this drug on the

differentiation of neural cells as a means of predicting the potential risk of toxicity in the central nervous system.

PC12 cell line derived from rat pheochromocytoma, exhibit a variety of neuronal properties resembling those of sympathetic adrenal neurons in response to nerve growth factor (NGF) and is considered to be a useful model for studying neuronal differentiation (Isom *et al.*, 1993). In this study, we investigated the effects of diclofenac on the proliferation and differentiation of PC12 cells.

MATERIALS AND METHODS

Materials

Diclofenac (Sigma-Aldrich Co., USA) dissolved in absolute ethanol. Other materials used in this study are nerve growth factor (NGF), DMEM/F12 (Sigma-Aldrich Co., USA), Neurobasal Medium, B27 supplement, fetal bovine serum (FBS) (Gibco-BRL, UK), XTT cell proliferation kit (Roch Co., Germany), tissue culture microplates and flasks (NUNC Co., Denmark). PC12 cell line was purchased from national cell bank of Iran (NCBI).

Cell culture and preparation of PC12 cells

PC12 cells were maintained in DMEM/F12 without phenolred supplemented with 10% heat – inactivated

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FBS, 100u/ml penicillin and 100µg/ml streptomycin (complete culture medium) in a water-saturated atmosphere of 5% CO₂, 95% air at 37°C.

Drug toxicity assay

The drug toxicity was assessed using the XTT cell proliferation kit to measure the viability of cells (Roehm *et al.*, 1991). Briefly, the cultures in the exponential growth phase were trypsinised, rinsed and diluted in both, serum-free neurobasal medium supplemented with B27 supplement (NBB medium) and complete DMEM/F12 media, to give a total cell count of 10×10^5 cells/ml. One hundred microlitres of cell suspension was added to each well of 96-well plate, and plate was incubated for overnight. Fifty microliters of increasing concentrations of diclofenac was added per well for a total volume/well of 150µl. The plate was then incubated at 37°C in a humidified atmosphere with 5% CO₂ for 72h. After incubation, cell viability was determined by XTT assay according to manufacture's instruction. The plate was read on a microplate reader using a test wavelength of 492nm. The survival rate was calculated using the following formula: test absorbance-background absorbance / control absorbance - background absorbance $\times 100$.

Evaluation of differentiation

PC12 cells in the absence of external NGF stimulation do not exhibit neurites, but they are able to respond to NGF treatment by differentiating into a neuron-like phenotype over the course of several days (Brewer *et al.*, 1993). Briefly, the subconfluence cultures were trypsinised, rinsed and replated in 35mm tissue culture plates (5×10^4 cells/plate) in complete DMEM/F12 medium. After 24 h, the medium replaced with 100ng/ml NGF- supplemented complete DMEM/F12 medium containing 0, 31, 155 and 310 µM diclofenac. On the fourth day following the initial plating, culture medium replaced with complete DMEM/F12 medium containing 100ng/ml NGF. Two days later the plates were incubated and examined for neurite outgrowth. The response to NGF was microscopically examined by a micrometer eyepiece and the longest neurite length of 200 cells in two sister plates was averaged to evaluate the neurite outgrowth for each treatment.

Statistics

The data were expressed as the means \pm S.E.M and analyzed using a one-way analysis of variance (ANOVA). A P-value of <0.05 was considered statistically significant.

RESULTS

Cytotoxicity of diclofenac on PC12 cells

We examined the effects of diclofenac on the proliferation of PC12 cells. As shown in fig.1, the drug decreased the

cell growth by more than 95% in NBB medium at a concentration of 930µM. In this culture medium, the maximal concentration of the drug not causing cytotoxic effect was 310 µM. Surprisingly, the drug decreased the cell growth by 75% ($p<0.001$) and 40% ($p<0.01$) in complete DMEM/F12 medium at the concentrations of 310 µM and 155 µM, respectively. In this condition, the maximal concentration of the drug not causing cytotoxic effect was 31 µM.

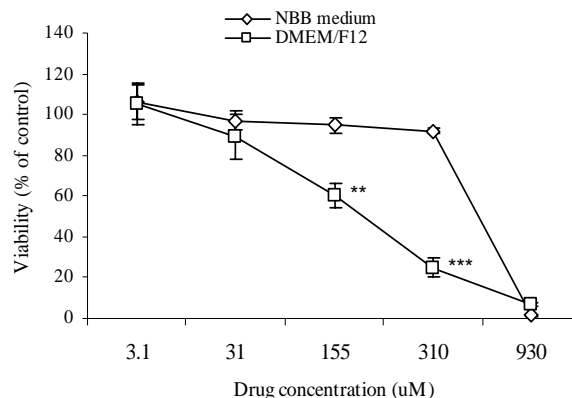


Fig. 1: Effect of diclofenac on proliferation of PC12 cells. PC12 cells were exposed with the different concentrations of diclofenac for three days, then, the viability was assessed by XTT assay. The data were expressed as the means \pm S.E.M. **= $p<0.01$, ***= $p<0.001$; compared with control culture medium.

Effect of diclofenac on cell differentiation

Next, we examined whether treatment with diclofenac during exposure to NGF affected the ability of surviving cells to extend neurites in response to NGF. Under these conditions, treatment with NGF alone induced neurite outgrowth where the average neurite length was 152 ± 52 µm. In contrast, the average neurite length in cells treated with diclofenac (31, 155 and 310 µM) during exposure to NGF was 165 ± 68 µm, 166 ± 49 and 160 ± 69 µm, respectively (fig. 2). A statistically significant difference could not be demonstrated between these results ($p<0.05$).

DISCUSSION

Diclofenac is a widely prescribed non-steroidal anti inflammatory drug. In this study, we investigated the effects of diclofenac on the proliferation and differentiation of PC12 cells. Our results indicated that the drug exhibited high toxic effects in the serum supplemented DMEM/F12 medium in comparison to serum-free neurobasal culture medium supplemented with B27 supplement. The different toxic effects of diclofenac in the two culture media are discussable. Several studies suggest that active metabolites of the drug, oxidative stress and apoptosis are the causes of the drug toxicity (Hickey *et al.*, 2001; Inoue *et al.*, 2004; Gomez-lechon *et al.*, 2003a). B27 supplement contains antioxidant

components including vitamin E, superoxid dismutase, catalase, vitamin E acetate and glutathione (Brewer *et al.*, 1993). Therefore, these antioxidants may be responsible for the decrease of diclofenac toxicity in B27 supplemented neurobasal medium. Our results are in agreement with the previous studies which reported the decrease in diclofenac toxicity in the presence of antioxidant components such as vitamin E and superoxid dismutase (Cantoni *et al.*, 2003; Inoue *et al.*, 2004; Gomez-Lechon *et al.*, 2003a; Gomez-lechon *et al.*, 2003b). Also, our results are consistent with earlier findings which reported the protective effect of the *Rhizophora mangle* against diclofenac-induced gastric ulcer (Berenguer *et al.*, 2006). They indicated that the protective effect of the plant is related with its antioxidant properties which increases the activity of the antioxidant enzymes such as glutathione peroxidase and superoxide dismutase.

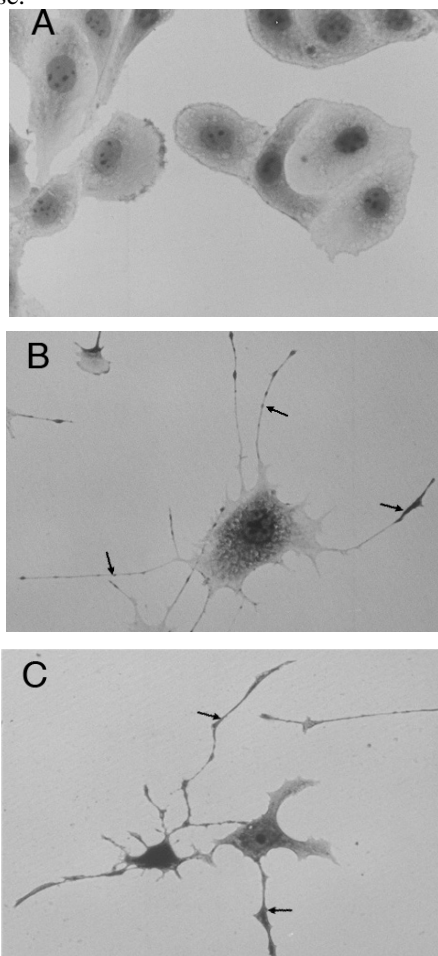


Fig. 2: Effect of diclofenac on NGF-induced differentiation of PC12 cells.

PC12 cells were incubated in DMEM/F12 medium containing NGF in the presence and absence of Diclofenac as described in the materials and methods. The arrows indicate the neurite outgrowth. A) culture medium, B) culture medium containing NGF only, C) culture medium containing NGF plus 310µM diclofenac. Hematoxylin staining, original magnification 200×.

In the present study, diclofenac did not affect NGF-induced differentiation of PC12 cells even in the toxic concentrations. In disagreement with our findings, Kudo *et al.* (2003) showed that diclofenac inhibited the differentiation of neural stem cell into neurons (Kudo *et al.*, 2003). This difference could be due to differences in the cell type and/or culture media. We evaluated the effect of diclofenac on NGF-induced differentiation of PC12 cells in serum supplemented DMEM/F12 medium while Kudo *et al.* (2003) investigated the effect of the drug on the differentiation of neural stem cell in a defined culture medium in the absence of NGF. Our findings indicated that diclofenac may be able to exhibit its neurotoxic effect through growth inhibition, but not differentiation inhibition. Furthermore, the results suggest that the drug cytotoxicity may be related to the oxidative stress.

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