UNIVERSITI PUTRA MALAYSIA

# INVOLVEMENT OF MITOCHONDRIA IN DICLOFENAC – AND IBUPROFEN- INDUCED HEPATOTOXICITY

MOHANAMBAL MOORTHY

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# INVOLVEMENT OF MITOCHONDRIA IN DICLOFENAC - AND IBUPROFEN- INDUCED HEPATOTOXICITY

By

MOHANAMBAL MOORTHY

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfilment of the Requirement for the Degree of Masters Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Masters Science

# INVOLVEMENT OF MITOCHONDRIA IN DICLOFENAC - AND IBUPROFEN- INDUCED HEPATOTOXICITY

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**June 2008**

**Chair : Sharida Fakurazi, PhD**

**Faculty : Medicine and Health Sciences**

Diclofenac and ibuprofen are commonly used non-steroidal anti-inflammatory drugs (NSAIDs) in the treatment of rheumatic diseases. However, these drugs are known to cause hepatotoxicity in patients. Recent *in vitro* studies indicated that the hepatotoxic effects of these NSAIDs are related to their ability to induce apoptosis by targeting the mitochondria. This study was carried out to investigate and to compare possible liver perturbation following diclofenac and ibuprofen administration to rats. Male Sprague-Dawley rats (n=144) were treated with 3mg/kg, 5mg/kg and 10mg/kg diclofenac and ibuprofen in normal saline, intraperitoneally at 500µl/rat/day for 15 days. The control group was administered with saline in a similar manner. Four rats from each group were euthanised every 3 consecutive days. While 200mg/kg diclofenac and ibuprofen-treated rats (n=4) were euthanised following a single dose 10 hours post-treatment. Upon euthanisation, the livers were removed and cleaned with normal saline. A section across the right lobe was taken and fixed in 10% (v/v %) formal saline and 4% (v/v) glutaraldehyde for light (H&E

staining and TUNEL assay) and transmission electron microscopy, respectively. The remaining samples were kept under -80°C for Western blotting analysis. The three mg/kg diclofenac administered group at day 15 showed significant presence of microvesicles and lymphocytic infiltration. The five mg/kg diclofenac-treated rats revealed significant presence of microvesicles, lymphocytic and neutrophilic infiltrations at day 15. Liver sections obtained from rats administered with 10 mg/kg diclofenac showed significant presence of microvesicles, mild lymphocytic and neutrophilic infiltration and inflammation. The five mg/kg and 10mg/kg ibuprofen-injected rats showed significant presence of microvesicles and mild focal lymphocytic and neutrophilic infiltrations. These observations were mainly seen around central veins (CVs). In TUNEL assay, 5mg/kg and 10mg/kg diclofenac and 10mg/kg ibuprofen administered rats, showed apoptotic cells around the CVs at day 15. Ultrastructural study revealed swollen and ruptured mitochondrial membranes in rats treated with 5mg/kg diclofenac, 10mg/kg diclofenac and 10mg/kg ibuprofen on day 15. Western blotting analysis showed constant expression of cytochrome c in liver homogenate and mitochondrial fraction on day 3, 6, 9, 12 and 15. However no cytochrome c expression was detected in the cytosolic fraction. In 200 mg/kg diclofenac and ibuprofen-treated rats, cytochrome c was detected in all 3 fractions; homogenate, mitochondrial and cytosol. The expression of cytochrome c is higher density in the cytosol from rats administered with diclofenac when compared to the expression in cytosol from rats treated with ibuprofen. It can be concluded that diclofenac is probably more potent in inducing changes in mitochondrial membrane leading to apoptosis. However, at therapeutic dosage both drugs did not induce prominent alteration in the mitochondria and the hepatocytes in general.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Masters sains

# KAJIAN MENGENAI KESAN TOKSIK TERHADAP SEL-SEL HEPAR SELEPAS PENGAMBILAN DICLOFENAC DAN IBUPROFEN SERTA KAITANNYA DENGAN MITOKONDRIA

Oleh

**MOHANAMBAL MOORTHY**

**Jun 2008**

**Pengerusi : Sharida Fakurazi, PhD**

**Fakulti : Perubatan dan Sains Kesihatan**

Diclofenac dan ibuprofen merupakan antara ubat non-steroidal anti-inflammatroy (NSAIDs) yang biasa digunakan untuk rawatan penyakit tulang dan sendi. Namun demikian, ubat-ubatan ini boleh menyebabkan kesan toksik pada hati di kalangan pesakit yang mengambilnya. Kajian *in vitro* yang terbaru mengaitkan kesan toksik tersebut dengan kebolehan ubat-ubatan ini untuk menyebabkan apoptosis terhadap sel-sel hepar dengan memberi kesan ke atas mitokondria. Kajian ini dijalankan dengan tujuan untuk menganalisa dan membandingkan kesan diclofenac and ibuprofen ke atas hati tikus. Tikus 'Sprague Dawley' (n=148) telah diberi 3mg/kg, 5mg/kg dan 10mg/kg diclofenac dan ibuprofen dalam normal saline melalui intraperitonium pada 500µl setiap hari sehingga hari ke 15. Kumpulan kawalan telah disuntik dengan normal saline sama seperti kumpulan yang diuji. Manakala, 200mg/kg diclofenac dan ibuprofen telah diberi secara intraperitonium pada tikus

(n=4) dan diautopsi selepas 10 jam. Empat tikus dari setiap kumpulan (kumpulan kawalan dan kumpulan ujian) telah diautopsi setiap 3 hari sehingga hari ke 15. Kemudian, organ hati dikeluarkan dan dibersihkan. Bahagian lobus kanan hati telah diletak dalam 10% (v/v) formalin dan 4% (v/v) glutaraldehyde untuk analisa di bawah mikroskop cahaya (celupan H&E dan esei TUNEL) dan mikroskop elektron. Sampel selebihnya telah disimpan pada suhu -80°C untuk ujikaji 'Western blotting'. Kajian menerusi pewarnaan 'H&E' pada 3mg/kg diclofenac menunjukkan kehadiran mikrovesikel dan serangan limfosit yang signifikan pada hari yang ke-15. Kumpulan yang diberi 5mg/kg diclofenac pula menunjukkan mikrovesikel, serangan limfosit and neutrofil yang signifikan pada hari yang ke-15 juga. Kumpulan yang disuntik 10 mg/kg diclofenac juga menunjukkan kehadiran mikrovesikel, serangan limfosit, neutrofil dan inflamasi yang signifikan pada hari yang ke-15 berbanding dengan normal saline. Kumpulan tikus yang diberi 5mg/kg dan 10mg/kg ibuprofen pula menunjukkan kehadiran mikrovesikel, limfosit dan neutrofil yang tertumpu selepas hari ke-15. Pemerhatian ini telah di buat terutamanya di sekitar kawasan PVs. Dalam esei TUNEL, 5mg/kg diclofenac, 10mg/kg diclofenac dan 10mg/kg ibuprofen telah menunjukkan kehadiran sel apoptosis di sekitar PV sahaja. Kajian ultrastruktur ke atas mitokondria menunjukkan kehadiran mitokondria yang membesar dan mitokondria dengan membran yang pecah pada 5mg/kg diclofenac, 10mg/kg diclofenac dan 10mg/kg ibuprofen pada hari ke 15. Analisa 'Western blot' menunjukkan kehadiran sitokrom c dalam homogenat hati dan fraksi mitochondria pada hari ke-3, 6, 9, 12 dan 15. Tetapi, tiada sitokrom c di kesan dalam fraksi sitosol pada semua masa. Kumpulan yang diberi 200mg/kg diclofenac dan ibuprofen, menunjukkan kehadiran sitokrom c dalam homogenat, fraksi mitokondria dan fraksi sitosol. Ekspresi sitokrom c dalam fraksi sitosol adalah lebih ketara dalam kumpulan

yang diberi 200mg/kg diclofenac berbanding ibuprofen. Kesimpulannya, diclofenac mungkin menyebabkan perubahan yang lebih ketara terhadap mitokondria berbanding ibuprofen dan seterusnya membawa kepada apoptosis sel-sel hepar. Namun demikian, diclofenac dan ibuprofen tidak menyebabkan perubahan yang mendadak terhadap mitokondria dan sel-sel hepar apabila diuji di bawah dos terapeutik.

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I certify that an Examination Committee has met on 6 June 2008 to conduct the final examination of Mohanambal a/p Moorthy on her Master of Science thesis entitled "Involvement of Mitochondria in Diclofenac- and Ibuprofen-Induced Hepatotoxicity" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the student be awarded the Master of Science.

Members of the Examination Committee were as follows:

**Arifah Abdul Kadir, PhD**
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

**Roslida Abd. Hamid, PhD**
Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

**Noordin Mohamed Mustapha, PhD**
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

**Md Anuar Osman, PhD**
Associate Professor
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
(External Examiner)

**HASANAH MOHD. GHAZALI, PhD**
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

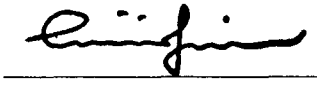
Date: 26 August 2008

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

**Sharida Fakurazi, PhD**
Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

**Fauziah Othman, Ph.D**
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

**Hairuszah Ithnin, MD, MPath, AM**
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

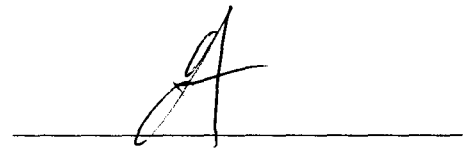


**AINI IDERIS, PhD**
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11th September 2008

## DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



**MOHANAMBAL MOORTHY**

Date: 6/10/2008

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| | |
|---|---|
| acyl-CoA | Acyl Coenzyme A |
| ANT | Adenine Nucleotide Transporter |
| ADP | Adenosine Diphosphate |
| ATP | Adenosine Tri-Phosphate |
| ADR | Adverse Drug Reaction |
| AIF | Apoptosis Inducing Factor |
| Apaf-1 | Apoptosis Protease Activating Factor-1 |
| (CARD) | Caspase Recruitment Domain |
| CV | Central Vein |
| COX-1 | Cyclooxygenase-1 |
| COX-2 | Cyclooxygenase-2 |
| CsA | Cyclosporine A |
| CYP | Cytochrome |
| DD | Death Domain |
| DED | Death Effector Domain |
| DISC | Death-Inducing Signaling Complex |
| DNA | Deoxyribonucleic Acid |
| DPPIV | Dipeptidyl Peptidase IV |
| fasL | Fas Ligand |
| FADD | Fas-Associated Death Domain |
| GI | Gastrointestinal |
| FDA | Food and Drug Administration |
| GSH | Glutathione |
| H&E staining | Haemotoxylin and Eosin |



| | |
|---|---|
| HO-1 | Hemeoxygenase-1 |
| LDH | Lactate Dehydrogenase |
| MPT | Mitochondrial Permeability Transition |
| MPTP | Mitochondrial Permeability Transition Pore |
| DMTU | *N,N*-Dimethylthiurea |
| NADH | Nicotinamide Adenine Dinucleotide |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |
| NSAIDs | Non-Steroidal Anti-Inflammatory Drugs |
| OTC | Over The Counter |
| PV | Perivenular Region |
| PT | Portal Triad/tract |
| ROS | Reactive Oxygen Species |
| rER | Rough Endoplasmic Reticulum |
| sER | Smooth Endoplasmic Reticulum |
| $O^{2-}$ | Superoxide Anions |
| SOD | Superoxide Dismutase |
| TNF | Tumor Necrosis Factor |
| UDPGT | UDP-Glucuronosyltransferase |
| VDAC | Voltage- Dependent Anion Channel |

# CHAPTER 1

# INTRODUCTION

## 1.1 GENERAL INTRODUCTION

Drug-induced liver injury is conceived as a major health problem affecting patients and therefore a major concern to health care professionals and pharmaceutical industry (Holt and Ju, 2006) and it is the most common cause for withdrawal of drugs from the market (Brind, 2006). The pivotal role of liver in drug metabolism often predisposes the liver to injury due to accumulation of drugs or formation of toxic metabolites. The most common cause of hepatotoxicity in United States has been attributed to drug-induced liver injury (Lee, 2003) of which non-steroidal anti-inflammatory drugs (NSAIDs) are the major class (Talley *et al*, 1995; Laine, 2001; Galati *et al*, 2002).

NSAIDs are a group of widely used drugs for the treatment of rheumatoid diseases and relief pain and inflammation (Galati *et al*, 2002). Occurrence of NSAIDs-induced hepatotoxicity is identified to result in 2.2 hospitalisation per 100 000 population per year (Fry and Seeff, 1995). Hepatic injury due to NSAIDs became a central focus following introduction of benoxaprofen in 1982, which killed almost seventy patients worldwide (Jurima-Romet *et al*, 1994). This causes withdrawal of the drug from the market within few months of its introduction (Lewis, 1984). Besides benoxaprofen, NSAIDs such as pirprofen, sudoxicam and bromfenac were also withdrawn from the market due to unacceptable level of hepatic injury (Tolman, 1998). Following review by the United States Food and Drug Administration (FDA)