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## Review

# Role of cyclin-dependent kinase 5 in neurodegenerative disorders

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**Alzheimer's disease (AD) is a neurodegenerative confusion associated with dementia. AD is indicated by progressive loss of memory. It is having characteristic evidence of  $\beta$ -amyloid extracellularly and neurofibrillary tangle's development intracellularly. Neurons lose the capability of cell division after they attain full development. Cyclin dependent kinase 5 (Cdk5) is a kinase protein which is neuron specific and plays a vital role in the movement of newly developed neurons. When Cdk5 is dysregulated, then several diseases like AD, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) may occur. The Cdk5 phosphorylation takes place as a result of change of N-methyl-D-aspartate (NMDA) receptor activity and expression, neurotransmitter release, degradation of synaptic proteins, or in-gene expression modulation, which leads to the activation of Cdk5. The activated calpain proteins convert p35 activator of Cdk5 into p25, which causes remarkable activation of the Cdk5. This highly activates Cdk5-p25 complex hyperphosphorylates, the Tau protein, which causes the release of microtubules and gathers as cytoplasmic filaments. This leads to tangle formation that leads to neuronal cell death. In AD brain, the Cdk5 is present in a highly activated form. This review article emphasizes the role of cyclin dependent kinase 5 in AD.**

**Key words:** Alzheimer's disease,  $\beta$ -amyloid plaques, CDK5, neurodegeneration, neurofibrillary tangle.

## INTRODUCTION

AD is one of the main causes of dementia. The occurrence of AD increases with age (James et al., 2008; Kang et al., 1987). AD is characterized by the progressive loss of memory related to the decline in language, visuospatial function, estimation and decision. Finally, it leads to major behavioral and functional disability (James et al., 2008; Chung, 2009). AD was originally defined as presenile dementia, but it now appears that the same pathology underlies the dementia irrespective of the age of onset (Rang and Dale, 2007). AD is characterized by a

common functional disorder of the brain of humans (Kang et al., 1987). The dementia is mainly due to AD in 60% and vascular reasons (VaD) in 20% (Beghi et al., 2004). In addition to marked neuronal loss, AD is pathologically characterized by deposition of  $\beta$ -amyloid ( $A\beta$ ) in senile plaques (SPs) extracellularly and development of intracellular NFTs (Garga et al., 2011). The NFTs are primarily composed of hyperphosphorylated tau protein associated with microtubules (Rademakers et al., 2005; Sun et al., 2009; Liu et al., 2006). Normally, the formation of the neurons in the hippocampus of mammals occur throughout life and is vital for the functioning of the brain while in the persons having the disease like AD, PD, and epilepsy, the capability of hippocampus to form neurons is decreased (Albert et al., 2009; Crews et al., 2010). Neurogenesis is the process in which the division of neural stem cells (NSCs) and progenitor cells into daughter cells occur which migrate to other sites and

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**Abbreviations:** AD, Alzheimer's disease; NFTs, neurofibrillary tangles; sAPP, secreted amyloid precursor protein; HD, Huntington's disease; ALS, amyotrophic lateral sclerosis.

gives rise to new neurons (Jessberger et al., 2009). Cdk5 is involved in the movement of newly born neurons of the granule cell layer (GCL). Cdk5 also takes part in exact targeting of dendrites from newly born granule cells (GC) into the molecular layer (ML) of the dentate gyrus (DG). Throughout life the newly born neurons are added to dentate circuitry, with the hippocampal neural progenitor cells NPCs (Albert et al., 2009). Permanent production of new neurons is vital for brain function. The newly formed cells play two distinct roles, firstly the new neurons take part in maintenance of tissue in the olfactory system and secondly they are essential for the development of memory like associative and spatial memory in the hippocampus (Imayoshi et al., 2008). The role of cdk5 in guiding new neurons to their proper place has been discovered, which has increased the understanding of adult neurogenesis, improved understanding of its involvement in cognitive function and brightened the expectations for brain cell therapy (Albert et al., 2009). Also, scarcity in hippocampal neurogenesis has been related with a number of neurological diseases, including AD, epilepsy, major depressions (Albert et al., 2009) and neuropsychiatric illnesses, such as addiction (Hawasli et al., 2009). The abnormal insoluble fibrous proteins are deposited with different proportion inside brain. The localization of fibrous insoluble proteins inside the brain is a characteristic features of several disorders, including AD, PD and Lewy body dementia (LBD) (Mierczak et al., 2011).

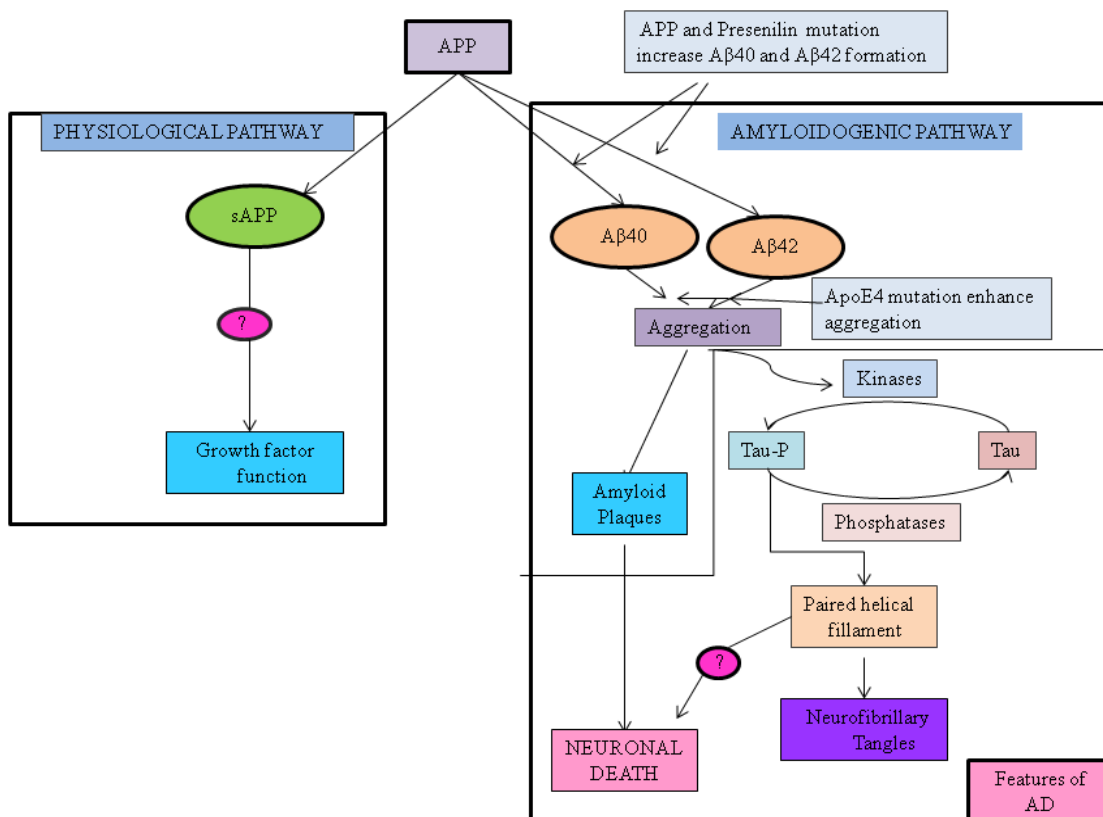
## PATHOGENESIS OF AD

AD is a progressive and slowly occurring disorder which degrades the central nervous system (CNS) characterized by impairment of cognitive function and appearance of neuropathological characters, including amyloid plaques. These amyloid plaques are composed of A $\beta$ , NFT and linked to cholinergic neuronal loss in selective brain parts (Nakdooka et al., 2010). The major component of amyloid plaques is A $\beta$ , which is considered as a key molecule in AD pathogenesis (Uetsuki et al., 1999). Inflammation is the third significant pathological feature in AD, apart from NFTs and amyloid plaques (Muyllaert et al., 2008). The oxidative stress hypothesis of AD pathogenesis is based on A $\beta$  peptide, which initiates oxidative stress in both *in vitro* and *in vivo* studies (Sultana et al., 2009). In a normal physiological pathway, amyloid precursor protein (APP) is converted and secreted as amyloid precursor protein (sAPP) which is responsible for the function of growth factor. However, in amyloidogenic pathway, the mutation in APP and presenilin increases the formation of A $\beta$ 40 and A $\beta$ 42 (Chen et al., 2012). They form aggregates due to mutation in lipid transport protein, that is ApolipoproteinE4 (ApoE4) gene. The production of A $\beta$ 40 usually occurs in small amounts, while A $\beta$ 42 is produced in higher amounts as a result of the genetic

genetic mutations mentioned above (Mann et al., 2011). Both A $\beta$ 40 and A $\beta$ 42 proteins aggregate to form amyloid plaques, but A $\beta$ 42 shows a stronger affinity than A $\beta$ 40 to do so, and appears to be the main cause in amyloid formation (Rang et al., 2007). The A $\beta$ 40 and A $\beta$  42 are formed by proteolytic cleavage of a much larger (770 amino acid) APP (Figure 1). The A $\beta$  accumulation is the cause of neurodegeneration, but whether the damage is done by soluble A $\beta$  monomers or by amyloid plaques remains uncertain. Appearance of Alzheimer mutations in transgenic animal results in development of plaque and neurodegeneration (Rang et al., 2007). The aggregation of A $\beta$ 40 and A $\beta$ 42 also activate the kinase that causes the phosphorylation of Tau protein (Figure 1). Tau, a usual part of neurons, is intracellular microtubules binding protein (Chatterjee et al., 2009). In AD and other tauopathies, phosphorylated tau protein is deposited within the cell as paired helical filaments which have typical microscopic features. After the destruction of cells, these filaments are combined as extracellular neurofibrillary tangles (Crews and Masliah, 2010). It may be possible, but not proven, that the phosphorylation of tau protein is improved by the presence of A $\beta$  plaques. However, it is not sure that hyperphosphorylation and intracellular deposition of tau harm the cells. Although it is known that tau phosphorylation damage fast axonal transport, which depends on microtubules (Rang et al., 2007). Nineteen specific amino acid sequences throughout its 441 amino acids have been recognized in tau, for its phosphorylation, (Augustinack et al., 2001) associated with paired helical filaments. CDK5 has been considered a main tau kinase that takes part in tau pathology (Alvarez et al., 1999), the other most important tau kinases that takes part in tau pathologies are GSK3 $\alpha$ , GSK3 $\beta$  and Casein kinase 1 $\alpha$  (CK1 $\alpha$ ) (Martin et al., 2013).

## NEURONAL CELL CYCLE IN PATHOGENESIS OF AD

There are four main successive phases in a eukaryotic cell cycle: G1 phase (first gap), S phase (DNA synthesis), G2 phase (second gap) and M phase (mitosis) (Figure 2). Change between the various phases and consecutive progression through the mitotic cycle is modulated by a group of protein kinases whose activity is essential to this process. The cyclin-dependent kinase (CDKs) requires the binding of their activating partner cyclins; whose levels of appearance vary throughout the cycle. Two important checkpoints (G1/S and G2/M) direct CDKs activity and manage the order and timing of cell-cycle transitions to ensure that DNA replication and chromosome segregation are finished correctly before allowing additional progress throughout the cycle (Currais et al., 2009). Neurons are born throughout the entire life in limited brain areas of mammals, including humans (Jessberger et al., 2009). After the formation of a neuron, it loses the capability for cell division and differentiation, contributing



**Figure 1.** Processing of APP in Pathogenesis of AD. The main 'physiological' path gives rise to sAPP that exerts a number of trophic functions. Cleavage of APP at different places gives rise to A $\beta$ , the major form typically being A $\beta$ 40, which is faintly amyloidogenic. Mutations in APP or presenilins raise the amount of APP, which is spoiled via the amyloidogenic pathway, and also raise the proportion changed to the much more powerfully amyloidogenic form A $\beta$ 42. A $\beta$  aggregation is occurred by mutations in the apoE4 gene followed by Aggregation of A $\beta$  and forms amyloid plaque, which causes neuronal death. A $\beta$  aggregation activates the kinase which phosphorylates tau to phosphorylated tau, ultimately form neurofibrillary tangles and cause neuronal death. The figure represents the APP' role in AD  
Source: Rang and Dale (1999).

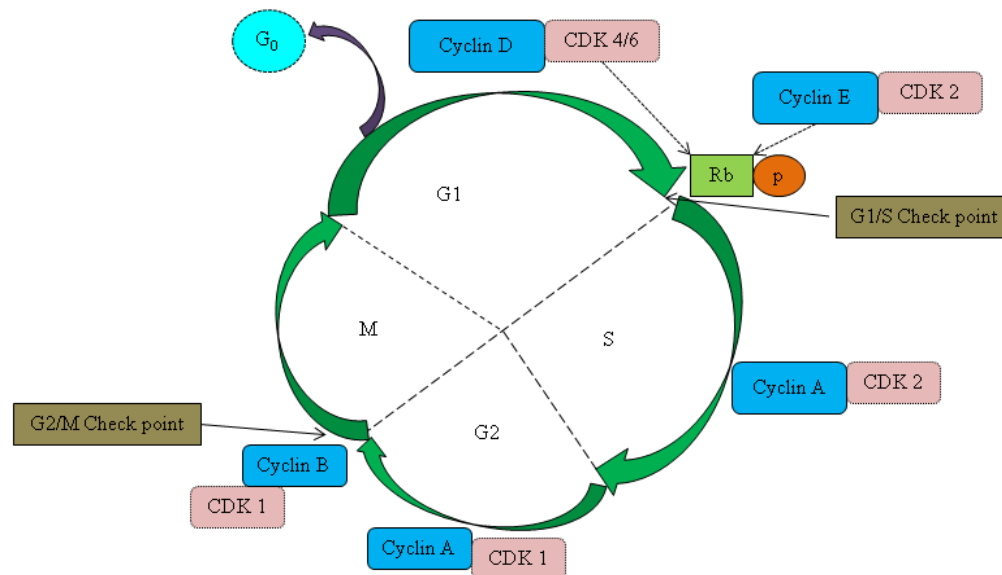
individually to the plasticity of the basic wiring model that defines a neuronal system. The conservation of this pattern is essential for the overall generation and storage of memories, as well as for gaining of other advanced brain skills. Some researcher have reported that neuronal apoptosis is accompanied by the appearance of cell cycle markers. Mainly, cyclins and cyclin-dependent kinases (CDKs) take part in cell cycle machinery (Figure 2). The cell cycle may be up regulated after exposure to severe conditions, like oxidative stress and trophic factor deficiency (Currais et al., 2009; Zhang et al., 2008).

## THE CDK FAMILY

The 9 small serine/threonine kinases take part in the formation of Cdk family. They are numbered based on their discovery, that is from Cdk1 to Cdk9. The biological functions of Cdk are many which ranges from mitosis to the regulation of cellular processes (Cardone et al., 2010).

2010). Cdk are involved in functions like differentiation, senescence and programmed cell death, via modification of gene transcription. In proliferating cells, the tumor production is mainly linked with Cdk dysregulation (Zafonte et al., 2000). The disappearance or inhibition of neuronal precursors takes place with terminal differentiation (Okano et al., 1993). Normally, in order to be activated, Cdk require connecting with regulatory subunits named cyclins. Although specific Cdk are linked to various phases of the cell cycle, sometimes their activities overlap, depending on the association with different cyclins. Cdk action can also be regulated by two other distinct mechanisms. A set of phosphorylation and dephosphorylation actions make ready Cdk for activation by regulatory subunits, as in the case of the Cdk4/cyclin D1 complex, which are activated only after phosphorylation by the Cdk-activating kinase (CAK).

Additionally, a family of Cdk-inhibitory subunits (CKIs) can bind to it and inactivate the Cdk-cyclin complex (Lopes and Agostinho, 2011).



**Figure 2. Schematic representation of the eukaryotic cell cycle** – Cyclin A-CDK2 phosphorylates a variety of substrates throughout S phase, allowing DNA replication. When S phase completed, DNA replication ceases and then cells enter the G2 phase of the cell cycle. Then CDK2 is replaced by CDK1 that linked with cyclin A and control the phosphorylation of G2 and M phases specific proteins together with cyclin B-CDK1, that appears in late G2 phase and triggers the G2/M transition. Cyclin A is ruined and the cycle is reorganized, re-establishing the condition for mitogenic cues to provoke D-type cyclins for the next cell cycle. In M phase, cells physically divide and originate two separate daughter cells  
Source: Currais et al. (2009).

## CDK5 IN CELL CYCLE REGULATION

Initially, it was said that Cdk5 had no role in the cell cycle (Jessberger et al., 2009). In an abnormal position, the expression of Cdk5 does not encourage development of a cell cycle in yeast or in mammalian cells (Jessberger et al., 2009). It can regulate several cell cycle proteins, mostly, phosphorylating retinoblastoma (Rb) protein, which is an important step in cell cycle exit. The absence of Cdk5 activity in dividing cells in the CNS indicates that it does not have a typical role in cell cycle regulation, which is clearly a significant step in embryonic and also in adult neurogenesis (Jessberger et al., 2009). In the cell cycle, initially the phosphorylation of the Rb protein by Cdk4/cyclin D1 and Cdk6/ cyclin D1-3 and further by Cdk2/cyclin E takes place. A complex is formed by Rb, E2F-1, histone deacetylases (HDAC) between other proteins, at the G1/S check point and blocks' protein transcription and in this way arrest- the cell cycle (Vermeulen et al., 2003). Due to phosphorylation, Rb becomes free from this transcription-blocking complex and allows the transcription of S phase-associated proteins. Sometimes strong stimuli, including excitotoxicity, oxidative stress, ischemia or DNA damage forces the mature neurons to leave a steady G0 state and re-enter the cell cycle (Bonda et al., 2011; Kim et al., 2009; Klein et al., 2003). Cell cycle re-entry has been observed in different neurodegenerative conditions like AD, PD and

amyotrophic lateral sclerosis (ALS) or stroke (Raina AK et al., 2004; Currais et al., 2009; Wang et al., 2009). In these neurons, the G1 phase is directly related to the re-expression of a cell cycle Cdk, namely Cdk2, 4 and 6. A very important role is played by Rb protein in the unsuccessful cell cycle re-entry. The phosphorylation/inactivation of Rb causes recycling neurons to rise above the G1/S checkpoint and DNA synthesis will occur. However, these neurons never reach to the M phase and degenerate by apoptosis anywhere between the S and the G2 phases (Lopes and Agostinho, 2011).

## LOSS OF NEURONAL CELL CYCLE CONTROL IN AD

Exposure to stress may cause an unsuccessful cell cycle in neurons. AD brain is characterized by the presence of cyclins, CDKs and additional cell cycle proteins (Currais et al., 2009). It is well known that oxidative stress and free radicals play role in pathogenesis of AD. The state of cell cycle is also controlled by free radicals, free radical generators and antioxidant functions. The accumulation of p35 in AD inhibits the cell cycle at G1 phase of cell cycle, which is secondary to oxidative stress. The mitochondria are powerful sources of free radicals and redox dysfunction. Therefore an increase in the number of mitochondria in the same neurons show cell cycle related abnormalities and undergo successive oxidative

harm and cell death in AD. Thus, when the mitochondrial mass is highest, the cell cycle arrest at a point, poses an elevated, chronic, oxidative damage to the cell (Raina et al., 2004).

## THE NEUROGENESIS PROCESS

In the adult brain, new neurons are frequently being generated in controlled areas of the mammalian brain, from endogenous pools of neural stem cells during life (Lledo et al., 2006). The process of neurogenesis can be divided into three different phases in the dentate gyrus in mammals. First, neural precursor cells that are located at the border between the hilus, and the granule cell layer (GCL) undergo cell division. Second, newborn cells start to migrate into the GCL and extend neuronal processes. Third, the cells add into the GCL and begin to express the neuronal marker neuron specific enolase (Kuhn et al., 1996). The dentate gyrus (DG) is a precise brain region to which newly formed neurons are added during adulthood (McDonald and Wojtowicz, 2005).

## THE CDK5

Cdk5 is a most versatile kinase, and its activity is not stimulated by an associated cyclin but is activated by its specific activators, p35 and p39 (Albert et al., 2009; Valin et al., 2009; Kanungo et al., 2009; Pareek et al., 2010; Changa et al., 2011). Cdk5 is considered as a multi-functional kinase whose activity is limited to the nervous and muscular system (Pareek et al., 2010; Zheng et al., 2010). Cdk5 is a serine/threonine kinase which is governed by proline (Pareek et al., 2007; Hawasli et al., 2009; Takahashi et al., 2010; Hisanaga et al., 2010; Arif et al., 2011). Unlike the other kinases, the cell cycle is not directly controlled with this serine–threonine kinase. Cdk5 can phosphorylate the Rb proteins, which have a major intervenient role in cell cycle development. Like the other members of this group, Cdk5 requires to combine with a regulatory subunit for its activation. Although, Cdk5 does not combine with cyclins (Zhang et al., 2012), it combines with the neuron specific activators p35 and p39 which are structurally similar to cyclins (Dhavan et al., 2002; Crews et al., 2011). Further, Cdk5 does not require any additional phosphorylation for its activation. However, the phosphorylation at tyrosine 15 (Tyr15) by enzyme tyrosine kinases-Src can increase the action of this protein (Cancino et al., 2011). The enzymatic activity of Cdk5 is more important in the CNS, since the appearance of this kinase and its activators is maximum in postmitotic neurons (Lopes and Agostinho, 2011; Kusakawa et al., 2000). Cdk5 activators p35 and p39 are degraded easily. The levels of these proteins are governed by their synthesis and degradation, and the appearance of p35 is exposed to be induced by an

extracellular stimulus. Neurotrophic factors, like nerve growth factor (NGF) also cause an enhancement of p35 appearance. The phosphorylation condition of p35 as well affects the membrane association of the Cdk5/p35 complex. The communication of this complex with the membrane is a possible regulatory method of Cdk5. It has been shown that when the Cdk5/p35 complex binds with membrane; it becomes inactive, while the complex in the free form in the cytoplasm is the active form (Lopes and Agostinho, 2011). Cdk5 appearance was found mainly in the brain, and Cdk5 activity, found only in the nervous tissues (Ohshima et al., 1996). Cdk5-null mutants indicate a more accurate disturbance in the cerebral cortex, cerebellum and hippocampus (Ko et al., 2001).

## ROLE OF CDK5 IN NEUROBIOLOGICAL PROCESS

Normally, the Cdk5 is essential for proper movement of nerve cells, synapse formation, and survival of neuronal cells. However, the severe neurodegenerative disorders like AD, ALS, PD and HD are mainly linked with hyper-activation of Cdk5 (Pareek et al., 2010; Shukla et al., 2011). Cdk5 takes part in different neurobiological processes; such as homeostatic synaptic plasticity, dopamine signaling, neuronal degeneration, and learning and memory. Cdk5 has also been concerned in normal adult neurophysiology, and its inhibition in the hippocampus leads to marked disturbance in associative learning and memory (Albert et al., 2009). Cdk5 plays a significant role in distinct aspects of cortical growth, which includes neuronal migration, neurite development, and axonal path finding. Different from other Cdks which are major regulators of cell-cycle progression, Cdk5 mainly causes phosphorylation of tau proteins in neurons. If the Cdk5 gene is deleted from neurons of the forebrain, by expression of cre-recombinase (Type 1 Topoisomerase), then this approach leads to generation of viable mice with decreased expression of Cdk5 in the forebrain. The expression of cre-recombinase is governed by the supporter of the CaMKII (calcium/calmodulin dependent kinase II) (Takahashi et al., 2010). It can be suggested that when neuronal Cdk5 from the developing forebrain is removed, it may result in the complex neurological loss, growth retardation, and premature mortality in mice (Takahashi et al., 2010). Recently, Cdk5-p35 has been linked with the initiation of disease in nonneuronal lineages for example malignant alteration in cancer; stimulation of inflammatory pain, and other pain mediated disorders. Cdk5–p35 activity has been, reported in human leukemic cell and supposed to play a role in monocytic differentiation (Pareek et al., 2010). The Cdk5–p35 complex is important for activation of T cell and for the initiation of experimental autoimmune encephalomyelitis (EAE) (Pareek et al., 2010). In the absence of Cdk5 mice show perinatal lethality due to having abnormal positioning of neurons in the brain (Hisanaga and

(Hisanaga and Endo, 2010). Cdk5 phosphorylation of tau inhibits its binding to microtubules and promotes microtubule disassembly. The Cdk5 phosphorylates tau at two major sites like Thr231 and Ser262, which are followed by GSK-3 that prevents binding of tau to MT (Sengupta et al., 2006). The surface expression of NMDA receptors (NMDARs) regulated by Cdk5, are the most important group of ionotropic glutamate receptors, which contains various heteromeric complexes like NR1, NR2, and NR3 subunits. These NMDARs are also concerned in development of neurons, learning and memory, synaptic plasticity and addiction in addition to psychiatric and neurodegenerative disorders (Zhang et al., 2008; Li et al., 2001).

## CDK5 IN NEURODEGENERATION

### CDK5 dysregulation

Dysregulation of this Cdk5 is responsible for the neurodegenerative processes of several diseases, like AD, PD, prion-related encephalopathies (PRE), amyotrophic lateral sclerosis (ALS) or acute neuronal injury, which are produced by ischemia or stroke (Figure 3). The activity of Cdk5 increases in various neurodegenerative disorders like AD, PD, PRE, ALS, etc. (Lopes et al., 2009).

### Role of Cdk5 in AD

AD brain is identified by three main markers, that is amyloid plaque deposition, neurofibrillary tangle production and severe selective neuronal loss. Cdk5 act as an attractive candidate for preventing A $\beta$  toxicity, tau pathology and neurodegeneration. In AD affected brains of human, the activity of Cdk5 increases appreciably as compared with same age control brains of human (Tandon et al., 2003). In AD brains, the levels of p25 and activated calpain are increased (Tandon et al., 2003; Muyllaert et al., 2008).

### Cdk5 in A $\beta$ generation

All the mutations which cause AD are situated either in the APP gene or in the genes encoding presenilins 1 (PS1) and 2 (PS2). PS1 and PS2 are the two proteins which are part of the  $\gamma$ -secretase ( $\gamma$ -sec) complex in which one of the secretases is responsible for APP cleavage to A $\beta$ . Further it is also proved that A $\beta$  can activate Cdk5 dysregulation (Lopes and Agostinho, 2011). *In vivo*, study of A $\beta$  established that the production of A $\beta$  involves enhancing intraneuronal calcium levels, that mainly cause calpain activation and enhancement of Cdk5 activity because of the cleavage of p35 to p25 (Dolan and Johnson, 2010).

## Cdk5 in Tau pathology

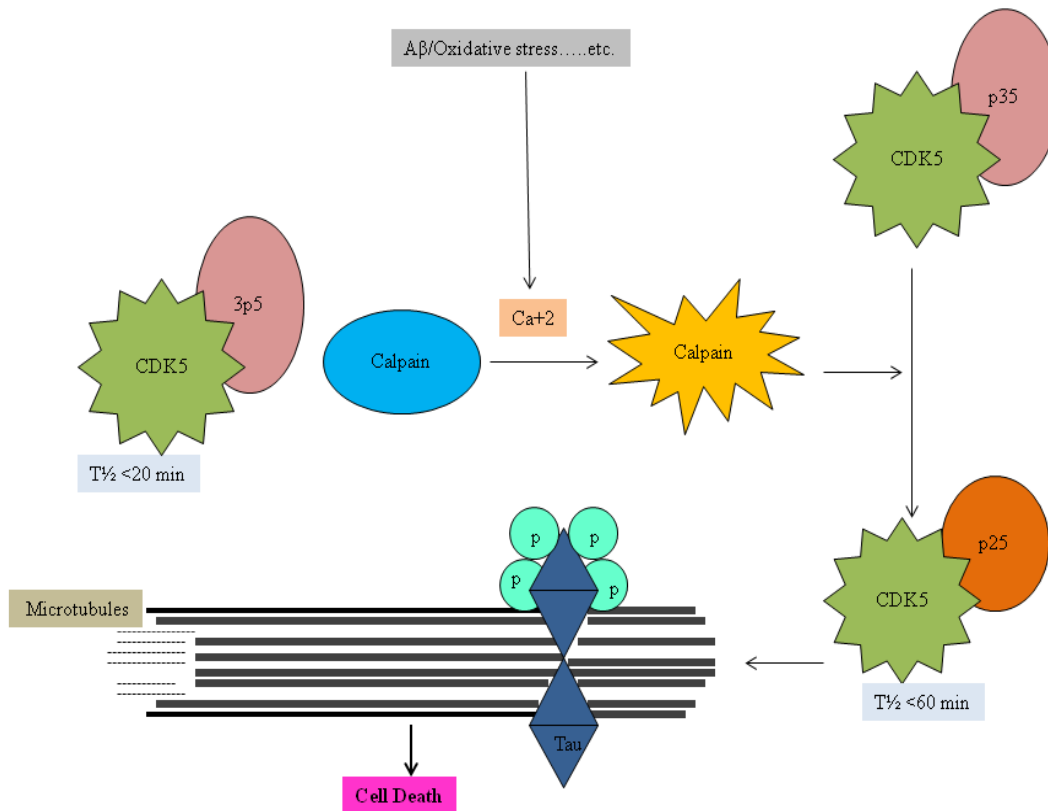
Tau is associated with proteins known as microtubule associated proteins (MAP) so tau is referred as a member of MAP family (Sergeant et al., 2004). The main function of tau proteins are binding and stabilization of the cellular microtubular network (Mandelkow and Mandelkow, 1995). So, tau is necessary to vital processes like axonal transport (Torroja et al., 1999), cytoskeletal organization or mitotic division (Preuss and Mandelkow, 1998). Phosphorylation of tau protein is controlled by numerous kinases, mainly glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), cAMP-dependent protein kinase (PKA), Cdk5 and c-Abelson (c-Abl) kinase or Abl-related gene (Arg) kinase (Martin et al., 2013). GSK-3 $\beta$  is referred as tau kinase I and Cdk5 is referred as tau kinase II (Liu et al., 2006). Tau is a phosphoprotein and the fetal brain tau is more heavily phosphorylated than adult brain tau (Goedert et al., 1993). It is confirmed that any change in hyper phosphorylation of tau is critical to neurofibrillary degeneration (Liu et al., 2006). Tau hyper phosphorylation may be associated with decrease in phosphatase activity while the tau phosphorylation is associated with an increase in protein kinase activity (Buee et al., 2000). GSK-3 $\alpha$ , GSK-3 $\beta$  and Cdk-5 are the members of the MAP kinase family and GSK-3 $\beta$ -mediates the phosphorylation of tau and reduce its affinity for microtubules (Wagner et al., 1996).

### Role of CDK5 in Parkinson disease

PD is the second most common neurodegenerative disease, which is due to loss of dopamine neurons (Cookson, 2009). The dysfunctioning of the dopaminergic and glutamatergic neurotransmitter systems results in Parkinson's disease. In the striatum, CDK5 decreases the postsynaptic release of dopamine. CDK5 inhibitors increase evoked dopamine release. The glutamatergic transmission is also controlled by the presynaptic action of CDK5. In fact, CDK5 inhibition increases the activity and phosphorylation of *N*-methyl-D-aspartate (NMDA) receptors. On other hand, these effects are reduced by dopamine D1 receptor antagonist. Inhibitors of CDK5 enhance dopaminergic transmission at both presynaptic and postsynaptic locations. The ability of CDK5 inhibitors to prevent degeneration of dopaminergic neurons, indicate that the compounds of this class could potentially be used as a new treatment for disorders connected with dopamine deficiency, such as Parkinson's disease (Chergui et al., 2004).

## ROLE OF CDK5 IN AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease, an adult-onset disease. It leads to selective loss



**Figure 3.** Dysregulation of CDK5 through activation of Calpains and hyperphosphorylation of Tau protein. The activator proteins like p35 and p39 are broken in the process of Cdk5 dysregulation by calpains. Calpains are the group of  $\text{Ca}^{2+}$  activated cytosolic proteases. The production of p25 and p29 occurs through the cleavage of p35 and p39 by calpains, respectively. These reduced Cdk5 activators show different features from their original precursors. The half-life of p35 and p39 are significantly shorter, around the 3-fold than p25 and p29 and the binding affinity of these newly formed activators to the kinase is stronger than previous activators. Finally the Cdk5 activity increases as compared to Cdk5/p35 (or p39). The formation of Cdk5/p25 complex are self regulated. After dysregulation, Cdk5 hyperphosphorylates the cytoskeleton protein tau, then this cause the release of tau protein from the microtubules and gathering in the form of cytoplasmic filaments and tangles occurs (Martin et al., 2013; Zhang et al., 2008).

of motor neurons in the spinal cord, brainstem and cerebral cortex, ultimately resulting in paralysis and death over 1 to five year time course. Approximately, 10% of ALS patients are familial cases, 20% of which are caused by missense mutations in the enzyme Cu/Zn superoxide dismutase 1 (SOD1) (Patzke and Tsai, 2002). Nguyen et al. (2001) developed a mechanism to explain the degeneration of motor neurons caused by mutant SOD1. For this purpose SOD1<sup>G37R</sup>, mice were used; they observed mislocalization and hyperactivation of the Ser/Thr kinase cdk5. The increase in the p25/p35 ratio in SOD1<sup>G37R</sup> mice indicates an up regulation of calpain activity, suggestive of elevated  $\text{Ca}^{2+}$  levels in the affected cells. Motor neurons expressing mutant SOD1 are more susceptible to glutamate-mediated cell death than are wild-type neurons (Patzke and Tsai, 2002; Nguyen et al., 2001). The increased Cdk5 activity in SOD1<sup>G37R</sup> mice was associated with hyper phosphorylation of tau and NF proteins, which are Cdk5 cytoskeletal substrates. The

hyperphosphorylation of these proteins has been associated with Alzheimer's disease (Nguyen et al., 2001).

### ACTIVATION OF CDK-5

The activation of Cdk5 occurs with tunable activation threshold-p25 (TAT-p25) which is Temporal Activator of Cdk5 in Primary Neurons. TAT-p25 is formed by fusion of TAT sequence with p25, which cause temporal activation of Cdk5, which is not dependent on other stimuli (Sun et al., 2008).

### CDK5 IN DIFFERENT PATHWAY OF NEURODEGENERATION

The Cdk5 dysregulation depends on the disturbance of



intracellular calcium homeostasis. Normally, disturbance of intracellular calcium homeostasis is caused by an extreme activation of ionotropic glutamate receptors (Lopes and Agostinho, 2011). The glutamate receptor over activation occurs due to various triggering stimuli. Over activation of Cdk5 results in too many phosphorylation of the cytoskeleton protein tau, which correlates with the synaptic loss and production of neurofibrillary tangles in AD, ultimate results are neuronal death (Kerokoski et al., 2002). Cdk5 also phosphorylates  $\alpha$ -synuclein and parkin, two proteins, which take part in the pathogenesis of PD (Avraham et al., 2007; Duka et al., 2006). Cdk5 furthermore, regulates an event that causes synaptic dysfunction via the phosphorylation of Postsynaptic density-95 (PSD-95) which results in the internalization and degradation of NMDA receptors (Roselli et al., 2005). Caspase-3 activation also results in neuronal death (Samuel et al., 2007). The capacity of the cells to bear oxidative stress is also affected by Cdk5 dysregulation, which is confirmed by the inactivation of the peroxidase Prx2, via Cdk5 phosphorylation, in PD and ALS (Shukla et al., 2011, Rashidian et al., 2009). All these events clearly indicate that Cdk5 dysregulation is a major step in the neurodegeneration pathways of various neurological disorders (Shukla et al., 2011).

## RECENT ADVANCES AND FUTURE ASPECTS OF THE CDK5 IN AD

Cdk5 is an important target for CNS disease. It may become possible that by inhibition of Cdk5, the phosphorylation of tau and formation of neurofibrillary tangles is prevented in both AD and tauopathies. There are various potent Cdk5 chemical inhibitors discovered, but they mostly compete with the ATP binding site which may cause lack of specificity in other Cdks and other ATP dependent kinases (Glicksman et al., 2007). It is assumed that specific inhibitors may inhibit the interaction of tau and Cdk5, which binds to a site other than ATP binding site (Glicksman et al., 2007). The calpain also convert the Cdk5-p39 to Cdk5-p25, which is more active than Cdk5-p39 (Zhang et al., 2008). Therefore, it may be possible to treat the AD by inhibition of calpains through calpain inhibitors, which inhibit the change of Cdk5-p39 to Cdk5-p25. Very recently three drugs have been developed by two companies, i.e.; cysteyleucyl-argininal (Trade name: Neurodur) from CepTor Corp., aminocarnityl-glutaryl-leucyl-argininal (Trade name: Myodur) from CepTor Corp., in the USA and BDA- 410 from Mitsubishi-Tokyo Pharmaceuticals in Japan (Rosa et al., 2002).

## DISADVANTAGES OF CDK5 ACTIVITY

It is found that the overactive Cdk5 cause neuronal death under oxidative stress from a variety of sources, such as amyloid- $\beta$ -peptide and the increase of intra-neuronal

calcium level. It is due to p25, which is a more powerful activator of Cdk5 as compared to p35 and causes Cdk5 over activation with neuronal apoptosis (Lin, 2009).

## CONCLUSION

In this review, it is concluded that the Cdk5 plays a vital role in the brain development through its involvement in the processes of neuronal migration. This review not only focused on development of AD, PD, and ALS as a result of Cdk5 dysregulation, but also framed the role of Cdk5 in other neurodegenerative pathologies, such as prion encephalopathies or PD. Overactivation and mislocalization of Cdk5 due to  $Ca^{2+}$  induced calpain activation mediates tau hyperphosphorylation and apoptotic neuronal death. In different neurodegenerative disorders, it may be assumed that Cdk5 can be a superior pharmacological target to prevent these pathologies. The normal activity of Cdk5 is essential to provide neuroprotection. It includes cognition and memory, neuronal survival, neuronal development and migration, etc. For these purposes, two main strategies have been used, first is direct inhibition by using Cdk5 inhibitors to prevent over activation of Cdk5 and second is indirect action by preventing the excessive production of the pathogenesis-associated activator p25 by using the calpain inhibitors.

Therefore, finally we assume that over activation of Cdk5 and calpains enhance neurodegeneration and can become a major cause of memory loss. However, the inhibition of Cdk5 overactivation and calpains through specific inhibitors prevent neurodegeneration and help in memory development.

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Full Length Research Paper

# Pharmacokinetic/Pharmacodynamic (PK/PD) modeling of antipyretic effect of meloxicam: A preferential cyclooxygenase inhibitor in rat

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The main purpose of this study was to predict the efficacy, potency and sensitivity of meloxicam (a preferential cyclooxygenase-2 (COX-2) inhibitor) antipyretic effect by using a simple indirect response model in rat, evaluated by Brewer's yeast induced model. The rats received 1, 3, 7 and 10 mg/kg of meloxicam, after subcutaneous (sc) injection of Brewer's yeast. The plasma concentrations of meloxicam were determined by high performance liquid chromatography-ultraviolet (HPLC-UV) method. Rectal temperature ( $T_a$ ) was measured for the assessment of the pharmacodynamic (PD) of the meloxicam. Before injection of yeast, basal fever mediator's synthesis (prostaglandin  $E_2$ ;  $PGE_2$ ) is maintained by physiological mechanism to regulate body temperature which is described by a constant rate synthesis ( $K_{syn}$ ) and a first order degradation of  $K_{out}$ .  $K_{syn}$  is calculated by the equation,  $K_{syn} = E_0 / K_{out}$ , where  $E_0$  is the baseline body temperature. After injection of yeast, the additional fever mediators' synthesis is regulated by input rate (IR (t)). This process is governed by a first order rate constant ( $K_{IN}$ ), which can be inhibited by meloxicam. The pharmacokinetic (PK) parameters showed dose proportionality, with a  $V_d$  (4124.52, 4236.73, 4657.15, and 5912.1 ml/kg), CL (78.55, 149.25, 1313.57, and 1519.41 ml/h/kg), and  $C_{max}$  (84.72, 258.29, 547.74, and 617.85 ng/ml). Indirect response PD model (inhibitory  $E_{max}$  model), estimated  $K_{IN}$  (1.43, 0.63, 0.51, and 0.42 1/h),  $K_{out}$  (0.005, 0.008, 0.015, and 0.028 1/h), and  $K_{syn}$  (0.29, 0.42, 0.076, and 0.03 h); estimates for  $IC_{50}$  (concentration of meloxicam in plasma eliciting half of maximum inhibition of IR(t) or  $K_{IN}$ ) were 146.19, 379.51, 645.05, and 676.44 ng/ml of 1, 3, 7 and 10 mg/kg dose received by groups, respectively. This model appropriately describes the time course of pharmacological response to meloxicam to various doses, in terms of its mechanism of action and pharmacokinetics.

**Key words:** Brewer's yeast, cyclo-oxygenase-2, fever mediators ( $PGE_2$ ), meloxicam, pharmacokinetic/pharmacodynamic modeling.

## INTRODUCTION

Like other nonsteroidal anti-inflammatory drugs (NSAIDs), meloxicam has been extensively used for the treatment of rheumatoid arthritis (Chen et al., 2008), osteoarthritis (Chen et al., 2008), Alzheimer's disease (Goverdhan et al., 2012) and cancer (Tsubouchi et al., 2000). Meloxicam inhibits preferentially cyclooxygenase 2 (COX-2) isoform

than COX-1 isoform which prevents gastrointestinal bleeding (Hernández-Díaz and García-Rodríguez, 2001; Patringnani et al., 1997). Also, there is a less chance of cardiotoxicity, which may be caused by the selective COX-2 inhibitors (Engelhardt et al., 1995). This preferential inhibition on COX-2 isoform leads to decreased production of prostaglandins which has a crucial role in inflammation, pain, etc. Selection of effective and safe dose for a dosage regimen is very crucial for clinical use. *In vivo* preclinical pharmacokinetic (what drug dose to the body)/pharmacodynamic (what drug dose to the body) (PK/PD) modeling

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is a powerful approach which determines the pharmacodynamic properties of a dosage regimen and explores the safe and effective dose for clinical use. Also, PK/PD modeling can be used to find out sensitive, efficacious and toxic dose. Only limited insights on *in vivo* NSAIDs pharmacokinetics and pharmacodynamics (Toutain et al., 1994, 2001; Lees, 2003) are available, a few preclinical studies have been conducted to model blood or plasma concentration-time profiles. Especially for antipyretic activity, ibuprofen has been modeled in children to biophase concentration (Kelly et al., 1992; Brown et al., 1988) and to plasma concentration (Garg and Jusko, 1994) with indirect response models. To the best of our knowledge, there are only a few reported studies on PK/PD modeling of meloxicam in cat (Giraudel et al., 2005) and pharmacokinetics and pharmacodynamic studies in piglets (Fosse et al., 2008), but till date, there is no modeling studies of meloxicam in rat model.

Injection of various doses of Brewer's yeast into animals induces fever (Kluger, 1991), which is mediated by enhanced formation of cytokines and tumor necrosis factor (TNF- $\alpha$ ). Cytokines increase the synthesis of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in circumventricular organs and organs close to the preoptic hypothalamic area. This PGE<sub>2</sub> via cyclic adenosine monophosphate (AMP) triggers the hypothalamus to increase body temperature (T<sup>a</sup>). NSAIDs (meloxicam) suppress this action by inhibiting the PGE<sub>2</sub> synthesis (Engelhardt et al., 1995; Mariona et al., 2001; Oka et al., 1997).

Therefore, the main objectives of the current study was to assess and develop a suitable preclinical PK/PD model for the antipyretic effect of meloxicam and characterization of the full pharmacological profile to predict suitable dosage regimen for animal and other clinical use. For meloxicam's antipyretic effect, an indirect response PK/PD model was hypothesized and Brewer's yeast was used as a pyretic inducer.

## MATERIALS AND METHODS

### Animals

Male Wistar rats, n = 30, weighing 180 to 270 g were used. Animals were kept under laboratory standard conditions on a 12 h light/dark cycle with light from 8:00 am to 8:00 pm, in a temperature (22°C) controlled room, and were acclimatized for a minimum of 2 days before experiments were performed. They were housed in cages with free access to water. Food was withheld for 12 h before the start of experiments. The experimental protocol of the study was approved by the Institutional Animal Ethical Committee.

### Chemicals

Meloxicam and piroxicam (internal standard) were gifted by Dr. Reddy's laboratory, Hyderabad, A.P. India. Acetonitrile, methanol and acetic acid high performance liquid chromatography (HPLC) grade solvents were purchased from Merk India Ltd. Brewer's yeast was purchased from Sigma Aldrich, India.

## Experimental protocol

### Induction of fever

Animals (n = 30) were randomly divided into five groups. Sterile saline solution (0.5 ml) containing 0.5 mg/kg Brewer's yeast was given subcutaneously (sc) to the groups (I to V) 1 h before the test drug administration (Oforah and Nweke, 2007). Only animals with 0.5 to 1°C and above with increased rectal temperature were used for this study. The yeast solution was prepared at the beginning of the experiment and was injected at 37°C to animals.

### PD data collection

Temperature (T<sup>a</sup>) was monitored in the rectum once every 30 min for 12 h just before and after the injection of the yeast with a rectal thermometer.

### Drug administration

Meloxicam was administered after fever induction as an oral suspension with 0.5% sodium carboxy methyl cellulose at a different doses to the respective groups like 1 mg/kg (group-II), 3 mg/kg (group-III), 7 mg/kg (group-IV) and 10 mg/kg (group-V), whereas group-I (control) receives only 0.5% sodium carboxy methyl cellulose.

### PK data collection

From all groups, blood samples (n = 6) of 100 to 200  $\mu$ l were withdrawn from the retro-orbital at selected time points up to 12 h. Plasma was obtained by centrifugation at 1000 g/20 min, frozen, and kept at -20°C until analysis. The same volume of withdrawn blood was replaced with sterile saline.

### Sample extraction

Meloxicam was extracted from plasma samples by adding 0.5 ml of acetonitrile to 0.5 ml of plasma in 1:1 ratio. This was subjected to vortex mixing at high speed for 1 min, and then centrifuged for 10 min at 9000  $\times$ g. The clear supernatant thus obtained was transferred to clean tube. To 0.5 ml of the supernatant, 0.5 ml of HPLC grade water was added and mixed well. The aliquot was filtered through 0.22  $\mu$ m nylon filter and 10  $\mu$ l of the aliquot was injected into HPLC system for analysis.

### Drug analysis

Measurements of meloxicam in plasma were carried out using HPLC-UV method previously described (Manoj et al., 2007) with some modifications. Briefly, meloxicam and the internal standard (piroxicam) separation were achieved by using the aforementioned method. The mobile phase consisted of a mixture of 65% water:acetic acid (99:1, v/v) and 35% acetonitrile. The flow rate of the mobile phase was adjusted to 0.8 ml/min. Oven temperature was set at 35°C. Meloxicam and piroxicam were detected at 360 nm wavelength (UV-detector). The method was validated prior to the analysis of samples. Stock solution of meloxicam at 1 mg/ml concentration was prepared in acetonitrile:acetic acid (1:1, v/v) and was stored at 4°C. The working standard solutions of meloxicam with internal standard (piroxicam) at 100  $\mu$ g/ml prepared daily were used to spike blank plasma samples of rat. Plasma standards at 1, 0.5, 0.25, 0.1, 0.05, 0.01, 0.005, and 0.001  $\mu$ g/ml for meloxicam

(external standard) were prepared and extracted as described for the experimental samples. Meloxicam was quantified from its respective peak area and the concentrations in plasma samples were determined by means of calibration curves obtained on analysis of blank plasma samples spiked with meloxicam. The retention time for meloxicam and piroxicam were 5.90 and 5.0, respectively at 1 ml/min flow rate of mobile phase. The limits of detection and quantification in plasma for meloxicam were 0.005 and 0.01 µg/ml, respectively. The signal showed linearity over the range of 50 to 1000 ng/ml with  $r^2 = 0.986$ . The intra- and inter day coefficients of variation of the assay (meloxicam standards) were 3.14 and 4.94%, respectively. The respective limits of detection and quantification were determined as 3 and 10 times the signal to noise ratio at the time of elution of the meloxicam. No endogenous interferences were detected in the chromatograms of blank plasma samples of the control group at the retention time of meloxicam.

### Data analysis

#### Pharmacokinetic model

Pharmacokinetic parameters for plasma meloxicam were determined by nonlinear least square regression analysis using Phoenix WinNonlin Professional version 6.2.0 (Pharsight Corporation, Cary, NC, USA). A one-compartmental model is enough to describe the pharmacokinetics of orally administered meloxicam.

#### Pharmacodynamic model

In antipyretic model (Figure 1), after injection with Brewer's yeast, the control group showed a time-varying response. It was modeled using indirect pharmacodynamic response model (Gomathi and Dheeraj, 2012).

$$\frac{dR}{dt} = K_{syn} + IR(t) - K_{out} \times R \quad (1)$$

where  $dR/dt$  is the rate of change of the response over time ( $T^a$ ),  $K_{syn}$  represents the zero-order rate constant for production of the response and  $K_{out}$  is the first-order rate constant for loss of the response,  $IR(t)$  is the input rate function representing the increase in the formation of fever mediators accounting for the temporal increase in response.  $R$  is the measured model response which is assumed to be the result from factors controlling either the input or the dissipation of the measured response.

This model assumes that meloxicam (Drug) exerts action by inhibition of the yeast induced fever mediators and this drug effect (Drug) is included in Equation 1 and the resulted equation as follows (Mariona et al., 2001).

$$\frac{dR}{dt} = K_{syn} + IR(t) \times (1 - DRUG) - K_{out} \times R \quad (2)$$

For this different models were tested for the DRUG: linear model,  $E_{max}$  (maximum effect) model and sigmoidal  $E_{max}$  models.

### Statistical analysis

Results are shown as mean data with their corresponding standard deviations. Comparisons of the observed responses between different groups were made by one way analysis of variance (ANOVA) followed by Tukey's posteriori test. Statistical significance

was set at  $P < 0.05$ .

## RESULTS

### Pharmacokinetics of the observed responses

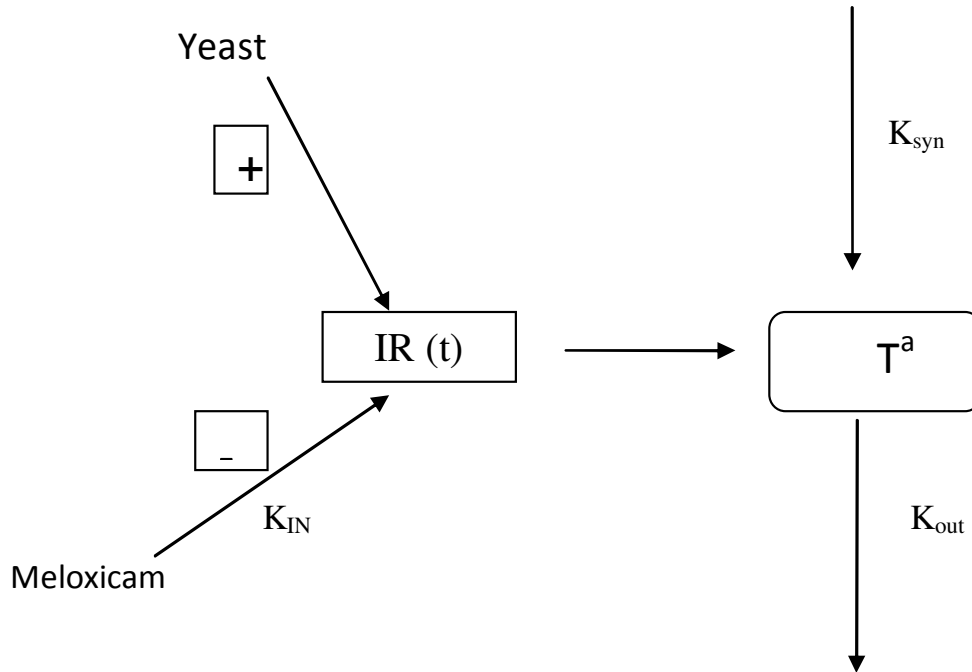
A one-compartment model was used to describe the kinetics of meloxicam in plasma when the drug was given orally. Estimates (from the experiment) of the typical PK parameters and their values of inter-animal variability are listed in Table 1. Mean observed and typical model predicted plasma concentration versus time profiles are as shown in Figure 2. Mean observed peak plasma concentrations of meloxicam was observed after 3 h of the drug administration in all groups with the values  $84.72 \pm 6.75$ ,  $258.29 \pm 23.60$ ,  $547.74 \pm 29.24$  and  $617.85 \pm 55.05$  ng/ml for 1, 3, 7 and 10 mg/kg, respectively.

### Pharmacodynamics of the observed responses

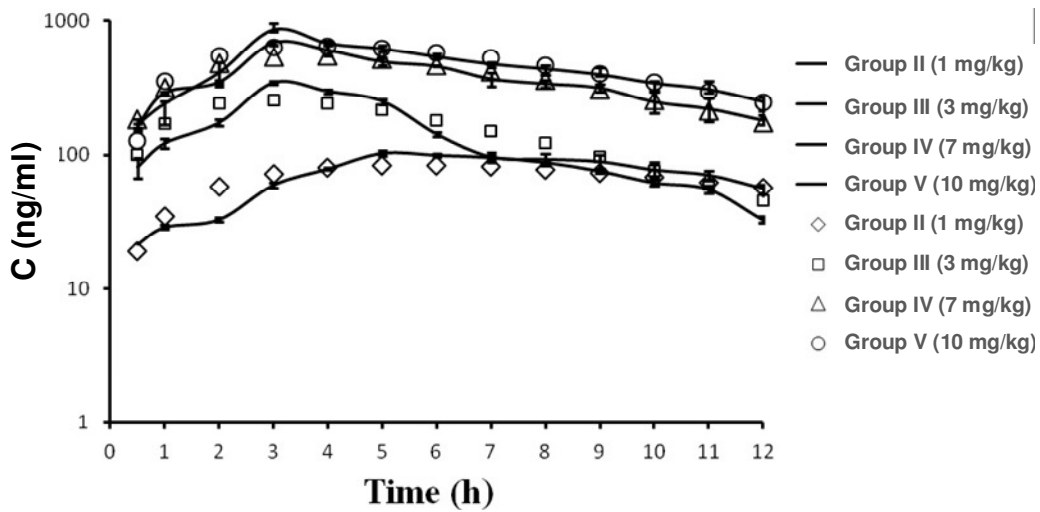
Figure 3 shows the mean observed  $T^a$  versus time profiles for all groups injected with Brewer's yeast. Baseline group showed a constant basal body  $T^a$  over a 12 h period with a mean  $\pm$  standard deviation (SD) value of  $38.96 \pm 0.14^\circ\text{C}$ . Basal  $T^a$  recorded at the time of yeast injection did not differ statistically among groups I to V ( $P > 0.05$ ). In addition, at the times,  $T^a$  were recorded between yeast injection and the start of the drug administration; no statistical differences in  $T^a$  ( $P > 0.05$ ) were found among groups II to V. A mean maximal increase in body  $T^a$  of  $38.55 \pm 0.12$ ,  $38.48 \pm 0.15$ ,  $38.24 \pm 0.05$ , and  $38.11 \pm 0.12^\circ\text{C}$  located at 4, 2, 2, and 2 h after yeast injection was found for group II, group III, group IV and group V, respectively;  $T^a$  then returned gradually to baseline in group V at 12 h after yeast injection, while the remaining groups took their time to get to baseline  $T^a$ . The onset of the antipyretic effects was fast in the four groups. However,  $T^a$  returned to baseline with a 2 to 3 h delay with respect to time to peak plasma concentrations, indicating that the observed effects and plasma drug concentrations could not be related directly.

### Pharmacokinetic/Pharmacodynamic modeling

Figure 3 shows the typical model-predicted time course of  $T^a$  in all groups on the basis of the model described in Figure 2 (top) and by Equations 1 and 2. It can be observed that model predictions for groups II, III, IV, and V are almost super imposable; the fact that plasma drug concentrations for both groups at early times after administration of doses were 9 to 11 times higher than the estimated value of  $IC_{50}$  (model predicted), together with the high inter-individual variability, could explain this issue. This result should be interpreted as an almost instantaneous increase in the synthesis of fever mediators



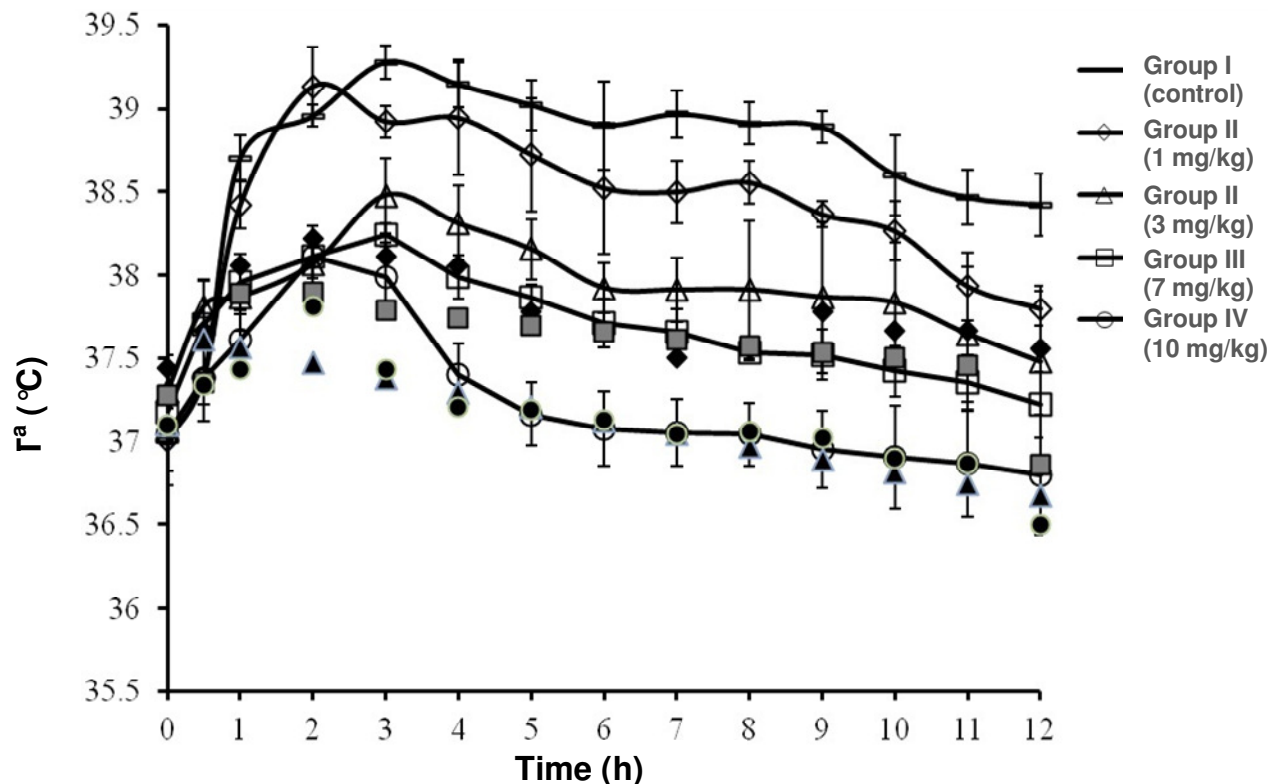
**Figure 1.** Schematic representation of the pharmacodynamic model used to describe the data of antipyretic study. IR(t), input rate function of fever mediators; K<sub>syn</sub> zero order rate constant of formation of basal fever; K<sub>out</sub>, first order rate constant of degradation of fever response.



**Figure 2.** Time course of plasma concentrations of meloxicam in different groups. Symbols represent typical model predictions; line represents mean observed data; and vertical lines represent standard deviation.

after the yeast injection. The effect of meloxicam plasma concentrations on the inhibition of IR (t) was described by an inhibitory E<sub>max</sub> model. Estimates of the parameters of the linear spline and PD parameter with their inter-animal variability are listed in Table 2 with an adequate precision.

During the model building process, E<sub>max</sub> was estimated close to the 1; for that reason, its value was fixed. At times before yeast injection  $dT/dt = 0 = K_{syn} - K_{out} \cdot E_0$ , where E<sub>0</sub> is the basal temperature (T<sup>a</sup>); then  $K_{syn} = K_{out} \cdot E_0$ . The typical value of the K<sub>syn</sub> is computed using the estimates of K<sub>out</sub> and E<sub>0</sub> (Table 2).



**Figure 3.** Time course of rectal temperature  $T^a$  after yeast injection and meloxicam administration; line with data points represents mean observed data; filled symbols represent typical model predictions, and vertical lines represent standard deviation.

## DISCUSSION

### Pharmacokinetics

The estimates obtained for pharmacokinetic parameters are difficult to compare across different study of the compound in the rat model, because of different design and doses. Computed area under the plasma concentrations ( $AUC_{0-\infty}$ ) of the mean plasma concentration of the different doses versus time profile showed linearity when they were plotted  $AUC_{dose}$  against dose administered. These  $AUC_{dose}$  predicted values are 1271.9, 2013.8, 5328.9, and 6607.0 ng/h/ml for 1, 3, 7, and 10 mg/kg doses, respectively showing dose dependent. Obtained results showed that the time ( $T_{max}$ ) to reach peak plasma concentrations ( $C_{max}$ ) is achieved rapidly in all the doses ranging from 3 to 4 h. These results show dose dependent bioavailability where the lowest dose has low bioavailability when compared with higher doses indicating that high AUC values showed longer duration of action.

### Pharmacodynamics

For the anti-pyretic effect evaluation, Brewer's yeast

induced pyresis (sc injection) model has been used. We observed maximum mean increase of body temperature at 39.2°C located at 5 h after yeast injection in the control group. This maximum mean temperature is less in the drug treated groups which is indicative of the effect of treatment on the body temperature. Among all groups, higher dose (10 mg/kg) received group showed less increase in body temperature while other groups showed a slight high in body temperature. At the same time, time taken to reach maximum body temperature varies with the administered dose; this is probably because of the pronounced effect of meloxicam on the synthesis of fever mediators. The percent of reduction in body temperature at the end of the experiment, that is, at 12 h is more in the higher doses when compared with lower doses. This reveals that the effect of meloxicam is in dose dependent manner which is proved in the earlier published study (Engelhardt et al., 1995).

Based on observed response versus time profiles, a suitable model should take into consideration the following factors: (i) there is no circadian variation in  $T_a$  after yeast injection; (ii) the transient increase in  $T^a$  is mainly by an increase in  $PGE_2$ ; (iii) adequate concentration of meloxicam produced inhibition on the increase in  $T^a$ ; and (iv) there is no rebound effects.

In our proposed model, from the following equation,



**Table 1.** Pharmacokinetic parameter estimates of meloxicam given into groups II, III, IV, and V with 1, 3, 7, and 10 mg/kg, respectively.

Group	V (ml/kg)	IAV	CL (mL/h/kg)	IAV	T <sub>max</sub> (h)	IAV	C <sub>max</sub> (ng/ml)	IAV	AUC <sub>0-∞</sub> (h/ng/ml)	IAV
II (1 mg/kg)	4124.52 (0.03)	30.34 (0.43)	78.55 (0.49)	49.57 (0.78)	3.5 (0.24)	15.06 (0.05)	84.72 (0.08)	7.977 (0.08)	1271.96 (0.48)	48.59 (12.71)
III (3 mg/kg)	4236.73 (0.04)	23.43 (0.42)	149.25 (0.15)	14.74 (14.9)	3.86 (0.02)	13.49 (0.03)	258.29 (0.09)	9.14 (2.58)	2013.88 (0.14)	14.72 (20.15)
IV (7 mg/kg)	4657.15 (0.22)	21.88 (0.46)	1313.57 (0.12)	12.07 (13.2)	3.5 (0.11)	11.14 (0.11)	547.74 (0.05)	5.33 (5.58)	5328.98 (0.12)	12.06 (53.39)
V (10 mg/kg)	5912.1 (0.57)	57.06 (0.59)	1519.41 (0.25)	24.81 (15.13)	3.9 (0.2)	20.26 (0.04)	617.85 (0.09)	8.91 (6.18)	6607.05 (0.25)	24.78 (66.08)

Estimates of inter animal variability (IAV) are expressed as coefficients of variation (%). Precision of the estimates is expressed as relative standard error in parentheses. Relative standard error is standard error divided by the parameter estimate. V, volume of distribution; CL, total plasma clearance; T<sub>max</sub>, time taken.

**Table 2.** Pharmacodynamic results obtained from the pharmacokinetic/pharmacodynamic modeling of the antipyretic effect of meloxicam given to different groups of rats; groups II, III, IV and V with 1, 3, 7, and 10 mg/kg respectively.

Group	K <sub>IN</sub> (1/h)	IAV	K <sub>OUT</sub> (1/h)	IAV	K <sub>syn</sub> (h)	IAV	E <sub>0</sub> (°C)	IAV	IC <sub>50</sub> (ng/ml)	IAV
II (1 mg/kg)	1.43 (0.57)	72.51 (0.011)	0.005 (0.57)	72.6 (0.0001)	0.29 (0.70)	0.18 (0.002)	38.38 (0.71)	0.27 (3.81)	146.19 (0.70)	6.48 (15.95)
III (3 mg/kg)	0.63 (0.70)	39.64 (0.010)	0.008 (0.71)	14.84 (0.0004)	0.42 (0.70)	2.13 (0.004)	38.13 (0.71)	0.98 (11.0)	379.51 (0.71)	4.03 (66.57)
IV (7 mg/kg)	0.51 (0.57)	114.99 (0.002)	0.015 (0.58)	114.88 (0.0004)	0.076 (0.58)	0.06 (0.0007)	37.68 (0.58)	0.28 (3.07)	645.05 (0.58)	8.40 (64.97)
V (10 mg/kg)	0.42 (0.71)	34.28 (0.056)	0.028 (0.71)	34.51 (0.0006)	0.03 (0.70)	0.17 (0.003)	37.28 (0.70)	0.25 (4.09)	676.44 (0.71)	11.86 (40.33)

Estimates of inter animal variability (IAV) are expressed as coefficients of variation (%). Precision of the estimates is expressed as relative standard error in parentheses. Relative standard error is standard error divided by the parameter estimate. K<sub>IN</sub>, first order rate constant for release of fever mediators; K<sub>OUT</sub>, first order degradation of fever mediators; K<sub>syn</sub>, duration of fever mediators synthesis; E<sub>0</sub>, baseline T<sub>a</sub>; IC<sub>50</sub>, meloxicam plasma concentration eliciting half of maximum IR(t) inhibition; IAV, inter animal variability.

$dT/dt=0=K_{syn} - K_{out}$ , E<sub>0</sub> predict a time invariant baseline T<sub>a</sub>. The estimate of K<sub>out</sub> (0.028) is very rapid in higher dose (10 mg/kg) than the other doses. Mechanism of action of NSAIDs is to inhibit the synthesis of PGE<sub>2</sub> (Engelhardt et al., 1995). It is estimated by an inhibitory E<sub>max</sub> model and the values of the IC<sub>50</sub> are represented in Table 2.

## Conclusion

Conclusively, the use of PK/PD modeling enables accurate assessment of clinical dose. Anti-pyretic

effect of meloxicam was modeled by a simple indirect response model. Raised body temperature (fever) is mainly because of the production of PGE<sub>2</sub> in the brain and inhibition of fever mediator's synthesis is indicative of the level of antipyretic activity of meloxicam (blood brain crossing nature is very important). In spite of this, all the estimated pharmacodynamic (efficacy, potency and sensitivity) and pharmacokinetic parameters describing meloxicam properties were in a dose dependent manner and they showed significant pharmacodynamic properties when administered at high doses. This comparison in different doses demonstrated the usefulness of preclinical PK/PD

modeling approach for predicting a dosage regimen. It is suggested that PK/PD modeling can provide a more robust rationale for dose selection of COX inhibitors, not only in the target species but also in the humans.

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## ABBREVIATIONS

**Vd**, Volume of distribution; **Cmax**, maximum plasma concentration; **CL**, clearance; **COX-2**, cyclo-oxygenase-2; **HPLC-UV**, high performance liquid chromatography-ultraviolet.

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*Full Length Research Paper*

# Proteomic analysis of the diagnostic biomarker for childhood infectious mononucleosis

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To investigate the different expressions of protein spectra in sera from children with infectious mononucleosis (IM) at acute stage and recovery stage in order to screen out potential protein biomarkers for children IM, the fingerprints of serum protein were obtained from the healthy (controls), acute upper respiratory infection (AURI), acute IM and recovery IM children using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) and gold chip technique. Data were analyzed by Biomarker Wizard 3.1 and diagnostic models were established by Biomarker Patterns System 5.0. Within the mass to charge ( $m/z$ ) ratios, there were six protein peaks (five were down-expressed and one over-expressed) showing significant differences between the acute IM group and the control group ( $P < 0.05$ ). One down-expressed protein peak was found with differential expression levels in the acute and recover IM groups ( $P < 0.05$ ). Two protein peaks were found significant differences between the acute IM and AURI ( $P < 0.05$ ), one was down-expressed and the other was over-expressed. No significant difference in protein expression between the recovery IM and the controls ( $P > 0.05$ ). The established diagnostic model based on significant peak test, the specificity and the sensitivity of IM. All the analytical results suggested that the protein at 6421.5 ( $M/Z$  value) may be the serum biomarker for IM; the protein bank showed that the protein at 6421.5 is a new protein; the diagnostic models based on this peak could accurately distinguish acute IM from normal children, the recovery IM children. SELI-TOF-MS technology is an effective tool to search for disease-related proteins.

**Key words:** Infectious mononucleosis (IM), proteomics, biomarker, artificial neural network.

## INTRODUCTION

Infectious mononucleosis (IM) is a kind of acute infectious disease caused by Epstein-Barr virus (EBV) infection. IM is commonly seen in children and adolescents.

Normally, the course of acute IM lasts 2 to 3 weeks. Because of the recessive latent infectious characteristic of EBV, the symptoms (such as low-grade fever, lymphadenopathy, fatigue and so on) can last several weeks to several months. Even for those abnormalities detected at laboratory in some cases, the recovery is very slow. It is a self-limited disease and has good prognosis except for the cases with complications.

IM can be complicated with upper respiratory tract

obstruction, spontaneous rupture of spleen, acute diffuse type encephalomyelitis, etc. Though, the incidence of these complications is very low, they can lead to fatal consequence (Bahadori et al., 2007; Khoo et al., 2007; Stephenson and Dubois, 2007). It is also found that some IM cases can be complicated with cholecystitis, appendix mass and other system symptoms (Daffinoti et al., 2011; Keramidas et al., 2007; Lagona et al., 2007). Moreover, it has attracted more and more attention that IM can be complicated with hemophagocytic syndrome in recent years. EBV is also an important tumor-related virus. It is closely correlated with lymphadenoma, gastric carcinoma, nasopharyngeal carcinoma, post-graft lymphoproliferative syndrome, etc. Thus, IM poses seriously threat to children's health.

Research has shown that 1% of population suffers from

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EBV-related tumor worldwide (Kimura et al., 2008; Delecluse et al., 2007). The main methods of diagnosis of EBV infections are serological methods that detect certain specific antibodies such as IgG and IgM. But these antibodies cannot express positive results or reach to the detected antibody titer using molecular biological methods such as PCR or *in situ* hybridization. However, due to the lack of specific signs and symptoms, missed diagnosis and misdiagnosis of IM often occur. Therefore, it is urgent to find a specific biomarker as a laboratory parameter for early IM diagnosis, patients' condition monitoring and follow-up.

Serum proteomics is a solution to this problem. Proteomics is a newly-rising discipline in recent year, which studies all the proteins expressed in a particular cell, tissue or organism as well as their activities (Petricoin et al., 2002). Proteins are the end products of gene expression. As the physiological changes of organic tissues will lead to the proteomic changes in blood and different diseases have different serum polypeptide spectra, the study from the perspective of proteomics may help us obtain the biomarker for IM directly (Petricoin and Liotta, 2004; Wulfskuhle et al., 2003).

All diseases will lead to dynamic changes of proteins, and those proteins whose early changes can be affirmed have the potentials to become the clinical early diagnosis indexes of diseases (Garrisi et al., 2008; Mehrotra and Dwijendra, 2011; Somasundaram et al., 2009; Whelan et al., 2008). Thus, the dynamic observation of proteins can screen out the indexes of diseases at their early stage. At present, the main techniques in proteomics include two dimensional gel electrophoresis, mass spectrographic analysis, bioinformatics, SELDI-TOF-MS and so on (Marshall et al., 2003; Merrell et al., 2004; Pandey and Mann, 2000). SELDI-TOF-MS is a newly-emerging proteomic technique in recent years. It can directly be used for the detection of samples without any special treatment such as serum, urine, cerebrospinal fluid, serous cavity effusion, etc, which has accomplished a great leap in the application of spectrography for clinical use (Cadron et al., 2009; Wu et al., 2006). Based on the above, sera from the healthy, AURI (acute upper respiratory infection), acute IM and recovery IM children were detected by using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) in this study.

The pathogenesis of IM was investigated from the perspective of protein expression, and the specific biomarker was screened out for early IM diagnosis, patients' condition monitoring after treatment and follow-up.

## MATERIALS AND METHODS

### Patients, controls and serum samples

All the cases were collected from January 2010 to March 2011. Four groups were divided in this study: the control group (stochastic collected from children received the physical examination test in the Department of Paediatrics and Child Health); AURI (acute upper

respiratory infection), acute IM and recovery IM groups. The latter three groups chose the patients in Department of Paediatrics of Affiliated Hospital of Luzhou Medical College, Sichuan Province, China. The control group: 11 healthy children (5 boys and 6 girls) with the average age of  $3.6 \pm 1.6$  years. The AURI group: 12 children (7 boys and 5 girls) with the average age of  $3.7 \pm 1.5$  years, lymphocytes  $> 50\%$  and atypical lymphocytes within 1 to 9%, the serological test was negative. The acute IM group: 26 children (12 boys and 14 girls) with the average age of  $3.5 \pm 1.4$  years. The classification of the acute IM group was based on the diagnostic criteria for IM provided by the seventh version of Zhu Futang Textbook of Pediatrics (Hu and Jiang, 2005) and children were newly diagnosed with any treatment. The recovery group: 18 children (8 boys and 10 girls) with the average age of  $4.5 \pm 4.7$  years. They were all diagnosed with IM and had received symptomatic treatment with ganciclovir and other drug for 14 days, blood routine results in the recovery group showed that the percentage of atypical lymphocytes was less than 10% and the clinical symptoms were recovered (fever, angina and deradenoncus disappeared). There were no significant differences in age or sex among these four groups.

The diagnostic criteria for IM were based on Zhu Futang Textbook of Pediatrics (the 7th version) (Hu and Jiang, 2005). Clinical symptoms (more than three types are positive at least as following): (1) Fever; (2) Sore throat, (3) Swollen lymph glands, (4) An enlarged liver, (5) An enlarged spleen. Hemogram: the presence of 50% lymphocytes at least or total number lymphocytes greater than  $5.0 \times 10^9/L$  in peripheral blood. At least 10% atypical lymphocytes or total number atypical lymphocytes greater than  $1.0 \times 10^9/L$  in peripheral blood. Epstein-Barr virus antibodies test: the antibody of Epstein-Barr nuclear antigen (EBNA) is negative in acute phase with one type as follows: (1) The antibody of VCA-IgG is positive in initial stage, then turn to negative later; (2) Paired serum VCA-IgG antibody titers  $> 1:4$ ; (3) There is a transient increase of EA antibody; (4) The antibody of VCA-IgG is positive in initial stage, the antibody of EBNA turn to positive latterly.

### Serum collection and processing

3 ml of venous blood was collected and placed at  $4^\circ C$ . 1 h later, the blood sample was centrifuged at 3000 rpm ( $4^\circ C$  for 5 min). Serum was transferred into an Eppendorf tube, centrifuged for 5 min again. 50  $\mu l$  was transferred into a tube and kept at  $-80^\circ C$  for later use. Before use, the serum sample was taken out, thawed out on the ice and then centrifuged at 10000 rpm ( $4^\circ C$  for 2 min). The isolated serum was mixed up with 10  $\mu l$  of Phosphate buffer saline (PBS) buffer according to a certain ratio, and meanwhile, half-saturation sinapinic acid (SPA) in twice volume was added, mixed well and then allow to stand for 5 to 10 min.

### Chip pretreatment and sample detection

Some preliminary experiment had been done to find out a suitable chip which could reveal perfect protein separated result and distinct difference between protein peaks. The result showed that Gold chip is good at differentiating protein peaks with ease to operate and low cost. Gold chips were mounted in the sample injector and the chip numbers were recorded. 50  $\mu l$  of acetone was respectively applied into each sample injection hole in the chip, placed in the chromatography freezer and patted dry 5 min later (600 rpm). 50  $\mu l$  of hydrochloric acid was added, placed in the chromatography freezer and patted dry 5 min later (600 rpm). Then, 50  $\mu l$  of hydrochloric acid/methanol mixture was added, placed in the chromatography freezer and patted dry 5 min later (600 rpm). Lastly, 50  $\mu l$  of methanol liquid was added, placed in the chromatography freezer, patted dry 5 min later (600 rpm), and then dried in the air for later

**Table 1.** The expressions of differently expressed proteins in different groups based on serum protein spectra.

M/Z	Controls $\bar{x} \pm s$	AURI $\bar{x} \pm s$	Acute IM $\bar{x} \pm s$	Recovery $\bar{x} \pm s$
4250.7	7.50±3.35	3.67±2.33	4.06±3.86	3.60±2.22
4985.1	15.97±11.09	5.56±4.40	7.41±6.79	8.29±8.16
6421.5	4.48±1.54	7.31±5.24	14.38±4.04	9.93±5.90
7840.6	7.96±2.15	7.39±3.91	4.82±4.38	5.33±3.07
7926.1	7.18±1.02	3.59±1.76	4.34±1.09	4.08±2.98
8836.3	9.45±4.56	6.18±4.11	4.70±3.94	4.00±2.45

Use (the sample must be injected within 10 min). 2  $\mu$ l of serum solution was added onto the activated Au chip hole. Holes for the controls were preserved.

After drying, 1  $\mu$ l of half-saturation SPA was added into each hole. Samples were detected after drying.

#### Data collection

All-in-one-protein standard molecular chip was used for correction with the mass deviation before testing all samples. The serum proteins adhered to the surface of the chip were detected by PBSII/C (CIPHERGEN Biosystems, Fremont, CA, USA) protein fingerprint spectrometer. The optimized range was set to 2000 to 20000 with the highest detectable molecular weight of 100000, the laser intensity was set to 210 and the detector sensitivity to 9. All-in-one protein standard molecule chips (CIPHERGEN, USA) were used for correction with the mass deviation  $\leq 0.1\%$ . Raw data were automatically collected and stored by ProteinChip Biomarker Software version 3.1 (CIPHERGEN Biosystems, Fremont, CA, USA). All obtained serum protein spectra were pre-processed.

#### The establishment of IM serum fingerprint screening models

The marking protein peak was employed for the construction of the diagnostic models for acute IM, IM staging (acute and recovery) and recovery IM, respectively. During model establishment, samples were divided into the modeling and verification groups by the blind method. The value of the marking protein peak in the modeling groups was input into Biomarker Pattern Software (BPS) for the establishment of diagnostic models, and then the value of the marking protein peak in verification groups was input into their corresponding diagnostic models for verification, respectively.

#### Statistical analysis and differently expressed protein screening

The serum protein fingerprints in four groups were analyzed by Biomarker Wizard 3.1 software. S/N was respectively set to 5 and 2 for filtration.

The threshold of frequency for a significant protein peak was 15%. F test was carried out for comparisons of preliminarily screened protein peaks among different groups, and  $P < 0.05$  was considered statistically significant. Grouping data were further analyzed by SPSS 11.5 software and the M/Z value at the protein peak which was statistically significant and can be taken as the serum biomarker was screened out. Meanwhile, data were provided for BP artificial neural network to establish the neural network diagnostic models.

#### Verification of differently expressed proteins

The M/Z values which showed significant differences were input into Protein Data Bank (PDB), and the corresponding proteins to these values were obtained.

## RESULTS

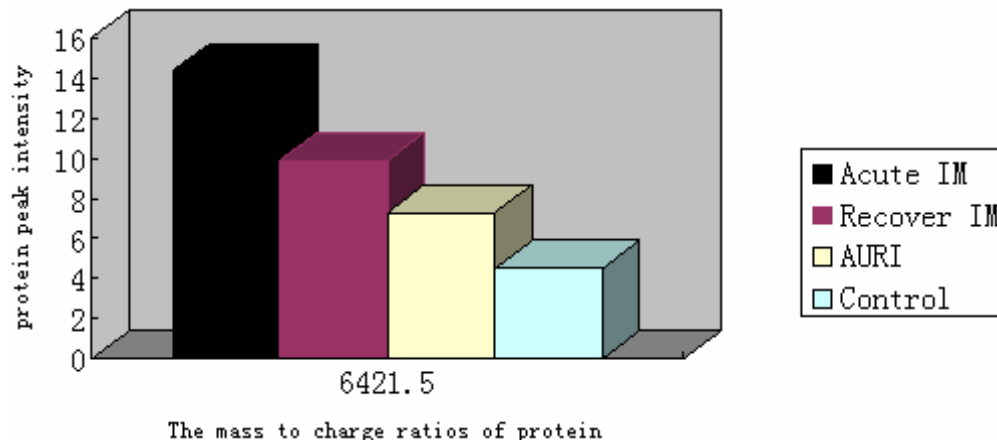
#### Analysis of mass spectrometric detection

The optimized M/Z value range for 67 serum sample detection was 2000 to 20000. The protein spectra of all serum samples were obtained based on the detection by PBS II/C protein fingerprint spectrometer as well as the proteins adhered to the gold chips. These spectra were analyzed. There were six protein peaks showing significant differences between the acute IM group and the control ( $P < 0.05$ ), among which one was over-expressed and five were down-expressed. One protein peak in the acute IM group was significantly higher than that in the recovery group ( $P < 0.05$ ). Five protein peaks in the recovery group showed significant differences compared to the control ( $P < 0.05$ ), among which one was over-expressed and four were down-expressed. There were two protein peaks exhibiting significant differences between the acute IM group and the AURI group ( $P < 0.05$ ), in which one was over-expressed, but no significant difference was found between the recovery group and the AURI group ( $P > 0.05$ ). Meanwhile, results showed that the M/Z value of all over-expressed protein peaks was 6421.5. The protein peak at M/Z value of 6421.5 was low in the control, higher in the AURI group and the highest in the acute IM group.

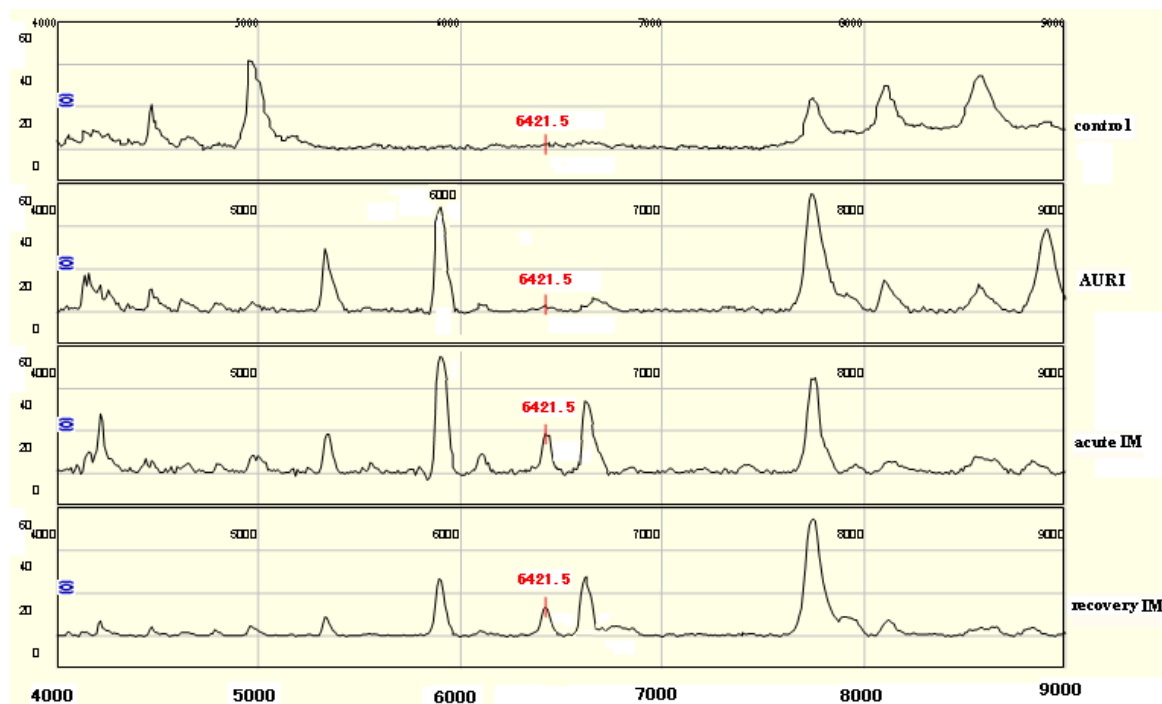
In addition, the protein peak at 6421.5 (M/Z value) in the recovery group was lower than that in the acute IM group but higher than that in the control or AURI group (Table 1, Figures 1 and 2).

#### Protein identification

M/Z values of the protein peaks showing significant differences among different groups were respectively input into PDB, and the corresponded proteins were obtained (Table 2).



**Figure 1.** The protein peak expressions at the M/Z value of 6421.5 in different groups. Black represents the acute IM group; wine represents the recovery IM group; white represents the AURI group; wathet blue represents the control group.



**Figure 2.** The typical expression spectra of the protein peaks at 6421.5 in different groups. From top to bottom, the four spectra represented the control group, the AURI group, the acute IM group and the recovery IM group.

### Establishment of diagnostic models

Analyses by Biomarker Wizard 3.1 software showed that the protein peak at 6421.5 was low in the control, increased in the recovery group and reached the highest in the acute IM group (Figure 1). Pairwise comparisons among these three groups showed that there were significant differences at this value. Thus, the corresponded protein at 6421.5 was screened out as the serum biomarker in this study, based on which diagnostic models were

established. The diagnostic model for acute IM was constructed based on the protein peak at 6421.5. 37 samples were divided into the modeling group ( $n = 19$ ) and the verification group ( $n = 18$ ). The peak values at 6421.5 (M/Z) of 19 modeling samples were input into BPS and the diagnostic model of acute IM was constructed. This model included an input layer, a hidden layer and an output layer. The output value bound at between 0 to 1.1 and 0 were respectively corresponding to the desired output value of the acute IM patients and the

**Table 2.** The corresponding proteins to the M/Z values.

M/Z	Number	MW	PI	Proteins
4250.7	2	4245	8.63	Isoform 2 of CDC42 small effector protein.
		4258	9.51	Neuropeptide NPSF,
4985.1	2	4984	6.92	Gastric inhibitory polypeptide
		4986	9.02	Beta-defensin 107
6421.5				A possible new protein
7840.6	3	7839	4.50	Isoform 2 of Putative uncharacterized protein
		7841	10.69	40S ribosomal protein S28
		7844	4.20	Isoform 3 of Fibronectin type III domain-containing prote
7926.1	3	7921	8.89	Putative uncharacterized protein LOC100133313
		7926	9.84	Isoform 3 of Nuclear protein
		7930	6.00	Isoform 3 of Protein YIPF7
8836.3	5	8834	5.83	Plasminogen-related protein B,
		8835	5.61	SS18-like protein 2.
		8838	8.74	Prostate and testis expressed protein 4
		8839	12.00	Putative uncharacterized protein C1orf191
		8841	9.80	Isoform 2 of p53-regulated apoptosis-inducing protein 1

Note: M/Z, values of mass electron ration (M/Z); Number, the number of protein corresponds to one peak; MW, molecular weight of protein; PI, isoelectric point of each protein.

**Table 3.** Prediction results of 18 blind test samples by BPS.

Group	Cases	Prediction of acute IM children (1)	Prediction of normal children (0)	Accuracy rate (%)
Acute IM group	13	13	0	100
The control group	5	0	5	100

controls (that is, the output value close to 1 judged as patient, the value close to 0 judged as healthy, 0.5 was taken as the criterion).

Our results showed that BPS could discriminate the 19 modeling samples from each other when the input layer was 1, the hidden layer was 2 and the output layer was 1. And after 18, verification samples were tested by the blind method, sensitivity of 100% and specificity of 100% were obtained without any miscarriage of justice (Table 3). The diagnostic model for IM staging was constructed based on the protein peak at 6421.5. 44 samples were divided into the modeling group (n = 22) and the verification group (n = 22). The peak values at 6421.5 (M/Z) of 22 modeling samples were input into BPS and the diagnostic model for IM staging was constructed. This model included an input, a hidden and an output layers. The desired outputs for recovery IM and acute IM were respectively set to 1 and 0, and 0.5 was taken as the criterion. Our results showed that BPS could discriminate

the 22 modeling samples from each other when the input layer was 1, the hidden layer was 2 and the output layer was 1. And after 22, verification samples were tested using the blind method, sensitivity of 88.9% and specificity of 84.6% were obtained with three miscarriages of justice (Table 4).

The diagnostic model for recovery IM was constructed based on the protein peak at 6421.5. 29 samples were divided into the modeling group (n = 15) and the verification group (n = 14). The peak values at 6421.5 (M/Z) of 15 modeling samples were input into BPS and the diagnostic model of recovery IM was constructed. This model included an input, a hidden and an output layers. The desired output for recovery IM children and normal children were set to 1, 0 and 0.5 was taken as the watershed. Our results showed that BPS could discriminate these 15 modeling samples from each other when the input layer was 1, the hidden layer was 2 and the output layer was 1. And after 14, verification samples

**Table 4.** Prediction results of 22 blind test samples by BPS.

Group	Cases	Prediction of recovery IM (1)	Prediction of acute IM (0)	Accuracy rate (%)
Recovery group	9	8	1	88.9
Acute IM group	13	2	11	84.6

**Table 5.** Prediction results of 14 blind test samples by BPS.

Group	Cases	Prediction of recovery IM (1)	Prediction of acute IM (0)	Accuracy rate (%)
Recovery group	9	9	0	100
Control group	5	0	5	100

were tested using the blind method, sensitivity of 100% and specificity of 100% was obtained without any miscarriages of justice (Table 5).

## DISCUSSION

Infectious mononucleosis (IM) represents an uncommon benign self-limiting lymphoproliferative disorder characterized by primary EBV infection of B lymphocytes and massive proliferation of activated cytotoxic T cells (Verbeke et al., 2000). EBV is the principal etiological agent of infectious mononucleosis (IM) and is associated with several human lymphoproliferative malignant diseases such as Hodgkin's lymphoma, nasopharyngeal carcinoma, gastric carcinoma and carcinomas. There are at least 125,000 new cases of IM reported in the United States each year, and 200,000 new cases of EBV-associated malignancies are reported each year worldwide (Cohen et al., 2011). Participants at the February 2011 meeting at the U.S. National Institutes of Health on Epstein-Barr virus (EBV) vaccine research recommend that future clinical trials have two goals: prevention of infectious mononucleosis and EBV-associated cancers, facilitated by identification of disease-predictive surrogate markers (Cohen et al., 2011). The reliable bases for IM diagnosis include epidemiological data, typical clinical manifestations (fever, adenopharyngitis, lymphadenectasis and splenohepatomegalia), atypical lymphocytes in peripheral blood > 10%, positive specific IgM (that is, VCA- IgM), etc (Bell et al., 2006; Cheng et al., 2007; Siennicka and Trzcinska, 2007), in which the increase of atypical lymphocytes and positive specific IgM are the two important bases for IM diagnosis. However, due to the diversity of serological responses to EBV infection, some cases may not display the increase of atypical lymphocytes (< 10%).

Study showed that patients with atypical lymphocytes > 10% accounted for 41.8% in IM patients, and children with atypical lymphocytes > 10% accounted for 21.39% in the first week of IM course and reached 71.94% in the second week (Tsai et al., 2005). Meanwhile, the positive rate of specific VCA-IgM was 25% in children with acute

IM, and some cases might show delay, lasting absence or long-time existence of anti-VCA-IgM (Dohno et al., 2010). Besides the main methods of diagnosis of EBV infection based on detecting certain specific antibodies, recently more attention are drawing to molecular biological methods such as PCR or *in situ* hybridization (Bocian et al., 2011). The review which was analyzed with the data from articles providing diagnosis of IM found that the evaluated diagnostic methods were real-time PCR (RT-PCR), IgM/IgG antibodies [measurement of Epstein-Barr virus viral load (EBV-VL) in peripheral blood, neutrophil/lymphocyte/monocyte counts, C-reactive protein values and monospot test].

RT-PCR and measurement of EBV-VL may provide useful tools for the early diagnosis of infectious mononucleosis in cases with inconclusive serological results (Vouloumanou et al., 2012). Flow cytometric (FC) immunophenotyping is a method of choice in the diagnosis of lymphoproliferative disorders. The lymphocytes showed good expression of HLADR along with partial down regulation of CD5 from FC analysis of a case of acute IM. Serological testing has shown IgM antibodies against EBV-N1 antigen for EBV with significant titer confirming the diagnosis of acute IM due to EBV infection (Tembhare et al., 2010). Children infectious of IM expressed higher level of CCR3 + and lower level of CCR5 + and there was a tendency of Th2 polarization with over production of T helper cell divide imbalance. CCR3 + and CCR5 + may be important targets to judge the degree of seriousness of IM (Qi et al., 2011). Another research showed that IL-18 was markedly elevated during acute EBV infections and EBV-associated diseases, while ferritin concentrations were also elevated during acute EBV infection and correlate with IL-18. Therefore, IL-18 and ferritin may represent infection markers for viral infections such as EBV, similar to CRP for bacterial infections (van de Veerdonk et al., 2012). A study analyzed the genotypes of infectious mononucleosis (IM) and acute lymphocytic leukemia (ALL) in children; children carrying GSTT1 or GSTM1 null genotype have a high risk of suffering from IM or ALL. GSTT1 and GSTM1 might play a potential role in the pathogenesis of both IM and ALL (Li et al., 2012). Epstein-Barr virus (EBV) genotypes can be distinguished



based on gene sequence differences in EBV nuclear antigens 2, 3A, 3B, and 3C, and the BZLF1 promoter zone (Zp).

A novel variant previously identified in Chinese children with infectious mononucleosis, Zp-V1 was also found in 3 of 18 samples of infectious mononucleosis, where it coexisted with the Zp-P prototype. The expression levels of 29 chronic active EBV infection-associated cellular genes were also compared in the three EBV-related disorders, using quantitative real-time reverse transcription polymerase chain reaction analysis. Two upregulated genes, RIPK2 and CDH9 were identified as common specific markers for chronic active EBV infection in both *in vitro* and *in vivo* studies (Imajoh et al., 2012). As there are no specific signs and symptoms for IM diagnosis, it is not feasible to diagnose IM using one reference parameter in laboratory, which obviously increases the difficulty in IM diagnosis. Different diagnosis methods for IM are continuously exploring to find significant serum biomarkers for early IM diagnosis, patients' condition monitoring after treatment and follow-up. A study showed indicated that the rapid and simple IMFA is suitable for point-of-care testing, and it may be used as a first-line assay for the diagnosis of EBV IM, especially in young children (Bravo et al., 2009). In this study, we obtained serum protein mass spectra among the acute IM group, the recovery IM group, the control group and the AURI group using SELDI-TOF-MS.

Our results showed that a total of six significantly different protein peaks were screened out among four groups. There were six protein peaks showing significant differences between the acute IM group and the control group ( $P < 0.05$ ), among which one was over-expressed and other five were down-expressed. There lies the cellular immune dysfunctions in the children with infectious mononucleosis, the protein with high expressive protein peak in the initial stage may be a number of cytokines secreted by cells EBV infected, including EBV early antibody components, while the protein corresponding to low expressive protein peak may be attributed to decreasing secretion of normal cell infected EBV. It is useful for studying the pathogenesis and the early diagnosis of IM by further analysis and identification of those proteins. There was one protein peak showing a significant difference in the acute IM group compared to the recovery IM group and the M/Z value of this peak was 6421.5. Meanwhile, results also showed that the expression of this protein peak was correspondingly reduced with the relief of clinical symptoms after treatment. The study showed patients with IM have secondary humoral immunosuppression, which continued for a long time after the recovery of the disease (Wang et al., 2008). There were still a lot of low expressive protein peaks in the recovery stage that indicated that EBV persistent affected the normal cell secretive function and induced the low level cytokine.

To detect and identify the low proteins will reveal the pathological mechanisms of the recovery stage, monitor

the situation after treatment and provide theoretical basis for the late follow-up and the medication. Robertson et al. (2003) reported that anti-VCA-IgG low-affinity antibodies could be detected within 10 day after the occurrence of clinical symptoms in more than 90% of primary acute EBV infection cases, and the anti-VCA-IgG low-affinity antibodies can still be detected in 50% of cases even 30 days later (Robertson et al., 2003).

Results in our study showed that the protein peak at 6421.5 notably increased in the acute IM group compared to the recovery IM group. After screening, a diagnostic model for acute IM, a model for IM staging (acute and recovery stages) and a model for recovery IM were established, taking the protein peak at 6421.5 as the serum biomarker for IM, the models based on this peak could accurately discriminate the acute IM, the recovery IM and the controls. And research result in protein data suggested that the corresponded protein at 6421.5 is very likely to be a new protein. Thus, further analysis and verification of the expression intensity of proteins at different IM stages as well as in IM-related diseases will be of great significance for the early IM diagnosis, condition monitoring after treatment, follow-up and prognosis.

In summary, we discovery a protein peaks that could discriminate pediatric IM from healthy controls. This panel of markers is likely to be limited to distinguishing pediatric IM from healthy controls. Further studies with additional populations or using pre-diagnostic sera are needed to confirm the importance of these findings as diagnostic markers of pediatric IM.

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*Full Length Research Paper*

# Homocysteine, not high-sensitivity C-reactive protein, associates with the intima-media thickness of carotid artery and arterial stiffness

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The study objective was to assess the relationship between carotid intima-media thickness (IMT) and arterial stiffness in subjects with hypertension, and to investigate the risk factors for increasing carotid IMT and arterial stiffening. 336 eligible residents participated in a community-based survey. Arterial stiffness and carotid intima-media thickness (IMT) were evaluated using carotid arterial stiffness parameter and high-resolution B-mode ultrasonography in 232 subjects with hypertension and 104 control subjects. Serum homocysteine and hs-C reactive protein (CRP) were measured. Arterial stiffness parameter and carotid IMT were significantly higher in the subjects with hypertension than those in the control ( $P < 0.05$ ). There was a significant positive correlation between the carotid IMT and arterial stiffness parameter in both the subjects with hypertension ( $r=0.421$ ,  $P < 0.001$ ) and from the control group ( $r=0.337$ ,  $P < 0.001$ ). Homocysteine and high-sensitivity C-reactive protein (hs-CRP) increased with arterial stiffness and IMT ( $P < 0.05$ ). Homocysteine, but not hs-CRP, was independently related to arterial stiffness parameter ( $P < 0.01$ ) when age, smoking, hypertension, estimated glomerular filtration rate (eGFR) and the roles of high density lipoprotein (HDL)- cholesterol were accounted for ( $R^2=0.330$ ,  $P < 0.001$ ). On the other hand, not only homocysteine but hs-CRP were independently related to IMT ( $P < 0.01$ , respectively) when age, smoking, hypertension and the roles of low density lipoprotein (LDL)-cholesterol were accounted for ( $R^2=0.361$ ,  $P < 0.001$ ). The results indicate that there was association between arterial atherotic (structural) and sclerotic (functional) changes. Homocysteine, but not hs-CRP, associates with the changes of carotid artery IMT and arterial stiffness.

**Key words:** Arterial stiffness, intima-media thickness, homocysteine, hs-CRP.

## INTRODUCTION

Subjects with hypertension and ageing have accelerated atherosclerotic vascular changes (Eren et al., 2004). Arterial wall thickening and stiffening often appear at the same time, which may partly delineate different atherosclerotic processes (Eren et al., 2004; Ikonomidis et al.,

et al., 2008). Atherosclerosis involves a combination of fatty degeneration (atherosis) and of vessel stiffening (sclerosis) of the arterial wall (Sharrett et al., 2006). Therefore, atherosclerosis should be evaluated based on two aspects: Atherosclerosis, which reflects structural changes in the intima and media of vascular walls and provides information about vessel wall anatomy; and sclerosis, which reflects changes in vascular distensibility and relates to vessel function (Jourdan et al., 2005).

In animal studies, a direct relationship has been

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established between regression of atherosclerosis and an increase in arterial distensibility (Farrar et al., 1980). Some clinical trials also demonstrated that carotid arterial stiffness was associated with the morphological changes (Niki et al., 2002; Taniwaki et al., 1999). The association of arterial stiffness and structural changes in the intima and media of vascular walls in different age periods are required to be studied in detail and with priority remains to be elucidated. Furthermore, the study tried to explore the predictors for this process from metabolic factors and chronic subclinical inflammation.

Chronic subclinical inflammation, commonly assessed using high-sensitivity C-reactive protein (hs-CRP), has been associated with obesity and coronary heart disease (Bahrami et al., 2008; Niki et al., 2002; Taniwaki et al., 1999). As we know, inflammation is important in the pathogenesis and progression of endothelial dysfunction and fatty degeneration (atherosclerosis) (Chrysohoou et al., 2009), and hs-CRP has been established as an independent prognostic predictor for IMT (Braunwald, 2008). Controversy surrounds, at present, regarding the association between hs-CRP and arterial stiffness indices (Tomiya et al., 2004). Homocysteine, as a marker for metabolism, has recently been shown to be independent of cardiovascular events (Robinson, 2002). Increased plasma homocysteine levels have been considered as an independent risk factor for cardiovascular disease (Welch and Loscalzo, 1998; Vasan et al., 2003; Vollset et al., 2001). However, the influence of homocysteine in the development of IMT and arterial stiffness has not been clarified at present (de Bree et al., 2006; Anan et al., 2007).

The aims of the present study were to assess the relationship between carotid IMT and arterial stiffness, and to explore a possible approach to determine the association between regression of atherosclerosis and an increase in arterial distensibility by the way of identifying markers for metabolic disorders and chronic subclinical inflammation.

## MATERIALS AND METHODS

This study was conducted in accordance with the declaration of Helsinki and with approval from the Ethics Committee of Chinese PLA General Hospital. Written informed consent was obtained from all participants. Participants with hypertension ( $n=232$ ) were enrolled into the study consecutively. The community has a population of 2334 residents, and 1867 residents participated in the health survey. Of these participants, 452 subjects were defined as hypertension according to the following criteria, and 232 subjects had not taken medicines for hypertension in recent three weeks. Hypertension was defined as a resting systolic blood pressure  $\geq 140$  or a resting diastolic blood pressure  $\geq 90$ , and the systolic pressure and the diastolic pressure were the means of measurements on three occasions. We had excluded subjects with evidence of cardiovascular complications as indicated by medical history or physical examination, with history of diabetes or fasting plasma glucose  $>7.0$  mmol/l, with taking statins and with any condition

preventing echocardiography evaluation. Age and gender-matched normotensive control subjects ( $n = 104$ ) were recruited randomly from the same community during the same period. All participants were grouped by age according to their corresponding arterial stiffness (Niki et al., 2002): the group of  $<45$  years, 45 to 55 years, 56 to 65 years and  $>65$  years.

All the participants completed a self-administered questionnaire about smoking habits and diet. Exposure to smoking was estimated as the product of the number of years of smoking and the number of tobacco products smoked daily at the time of the study. That product was expressed in statistical analysis as cigarette-years. The intakes of folate, vitB6 and vitB12 daily were gotten from three days averaged through diet questionnaire survey and food-B vitamins conversion system. We used a brief food frequency questionnaire (FFQ) designed specifically for estimating intakes of folate, vitamins B(6) and B(12), and covering 21 food items (Yoshino et al., 2010). Blood pressure was determined with a standard mercury sphygmomanometer and cuffs adapted to the arm circumference after the subject had rested for at least 15 min by professional doctors. The systolic blood pressure was taken as the point of appearance of Korotkoff sounds, and the diastolic blood pressure as the point of disappearance of the sounds. Results are reported as averages of three measurements (Table 1).

### Measurement of arterial stiffness parameter

Arterial wall stiffness was expressed by the arterial stiffness parameter  $\beta$ , which was measured through e-tracking technology of ultrasound on the right carotid artery by the machine ( $\alpha$ -10, Aloka, Tokyo, Japan) equipped with a 7.5-MHz linear array probe and calculated according to the formula:  $\ln [(Ps-Pd)]/[(Ds-Dd)/Dd]$ , where Ps and Pd were the mean values of three times of simultaneously measured brachial systolic blood pressure and diastolic blood pressure, respectively. Ps and Pd were measured using an automated sphygmomanometer (705CP, Omron, Dalian, China), and Ds and Dd are the maximal and minimal diameters of the common carotid artery, respectively measured using ultrasonic high-resolution wall tracking. Measurements were taken as a mean of five beats (Figure 4).

### Ultrasonographic measurements of carotid IMT

B-mode imaging of the carotid artery was performed with a high resolution real-time ultrasonograph ( $\alpha$ -10, Aloka, Tokyo, Japan). All the participants were examined in the supine position. The examination included the bilateral common carotid artery and the carotid bulb. These regions were scanned in the longitudinal and the transverse projections. In each longitudinal projection, the IMT was measured at the site with the greatest thickness. IMT was defined as the distance between the places with the strongest ultrasound reflection, from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface. Three still images from the same section of the artery were used for the measurement, and the mean value was calculated.

### Biochemistry parameters measurements

Venipuncture was performed in the morning on patients and healthy control subjects after overnight fasting and maintained at  $4^{\circ}\text{C}$  for  $\leq 2$  h before being centrifuged at 1200 g for 15 min. Serum aliquots were frozen at  $-80^{\circ}\text{C}$  until assays were performed. Concentrations of fasting glucose, total cholesterol, triglyceride, HDL-c and LDL-c were determined using the Roche enzymatic assays (Roche

**Table 1.** Clinical characteristics of hypertensive and control subjects.

Characteristic	Hypertensive subject (n=232)	Control subject (n=104)
Age (years)	56.7±13.7	57.5±14.4
Sex (M/F)	128/94	54/50
Body mass index(kg/m <sup>2</sup> )	24.8±3.3 <sup>#</sup>	22.9±2.8
Cigarette-years	474±411 <sup>#</sup>	248±178
Systolic blood pressure(mmHg)	145±16 <sup>#</sup>	126±15
Diastolic blood pressure(mmHg)	80±8 <sup>#</sup>	73±10
Total cholesterol (mmol/L )	5.23±1.08*	4.86±0.75
HDL-cholesterol (mmol/L)	1.21±0.38 <sup>#</sup>	1.39±0.27
LDL-cholesterol (mmol/L)	3.50±1.11*	3.09±1.05
Triglycerides (mmol/L)	1.48±0.89	1.34±0.78
Fasting plasma glucose (mmol/L)	5.36±0.80*	5.10±0.76
Creatinine(mmol/L)	80.9(66.2-81.0) *	65.7(51.8-72.2)
eGFR(ml/min/1.73 m <sup>2</sup> )	82.8±16.5 <sup>#</sup>	91.2±15.2
Homocysteine ((μmol/L))	17.6(13.8-27.9) <sup>#</sup>	8.22(6.01-12.6)
hs-CRP(mg/dL)	0.24(0.11-0.37) <sup>#</sup>	0.14(0.08-0.25)
Carotid IMT(mm)	0.88±0.38 <sup>#</sup>	0.62±0.16
Arterial stiffness parameter	9.05±2.19 <sup>#</sup>	7.65±1.18
Folate (μg/d)	217.3±97.7 <sup>#</sup>	308±113.2
Vitamin B <sub>6</sub> (mg/d)	0.50±0.14 <sup>#</sup>	0.83±0.26
Vitamin B <sub>12</sub> (μg/d)	0.69±0.18 <sup>#</sup>	1.00±0.22

Exposure to smoking was estimated as the product of the number of years of smoking and the number of tobacco products smoked daily at the time of the study. That product was expressed in statistical analysis as cigarette-years. HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein. \**P*<0.05, #*P*<0.001 vs. control subjects.

Diagnosics GmbH, Mannheim, Germany) on a Roche autoanalyser (Roche Diagnostics, Indianapolis, Indiana). Homocysteine was determined by enzymatic assay (Roche Diagnostics GmbH, Germany). hs-CRP was measured using immunoturbidometry (Siemens Healthcare Diagnostics Inc.) with the help of -Siemens RXL-MAX, (Germany). The inter-assay variations for the biomarkers were as follows: hs-CRP, 4.6% and homocysteine, 4.1%. Concentrations of serum creatinine were measured by enzymatic assay (Roche Diagnostics GmbH) on a Hitachi 7600 autoanalyser (Hitachi, Tokyo, Japan). The plasma creatinine was standardized using a calibration equation: Jaffe's kinetic method Scr (mg/dL)=0.795×[enzymatic method Scr (mg/dL)]+0.29. The estimated glomerular filtration rate (eGFR) was calculated with the Chinese modified Modification of Diet in Renal Disease (C-MDRD) equation:

$$eGFR \text{ (mL/min/1.73 m}^2\text{)} = 175 \times \text{standardized creatinine (mg/dL)}^{-1.234} \times \text{age (year)}^{-0.179} \times 0.79 \text{ (if female).}$$

All testing was performed by well-trained personnel blinded to clinical data in the Department of Biochemistry of Chinese PLA General Hospital.

#### Statistical analysis

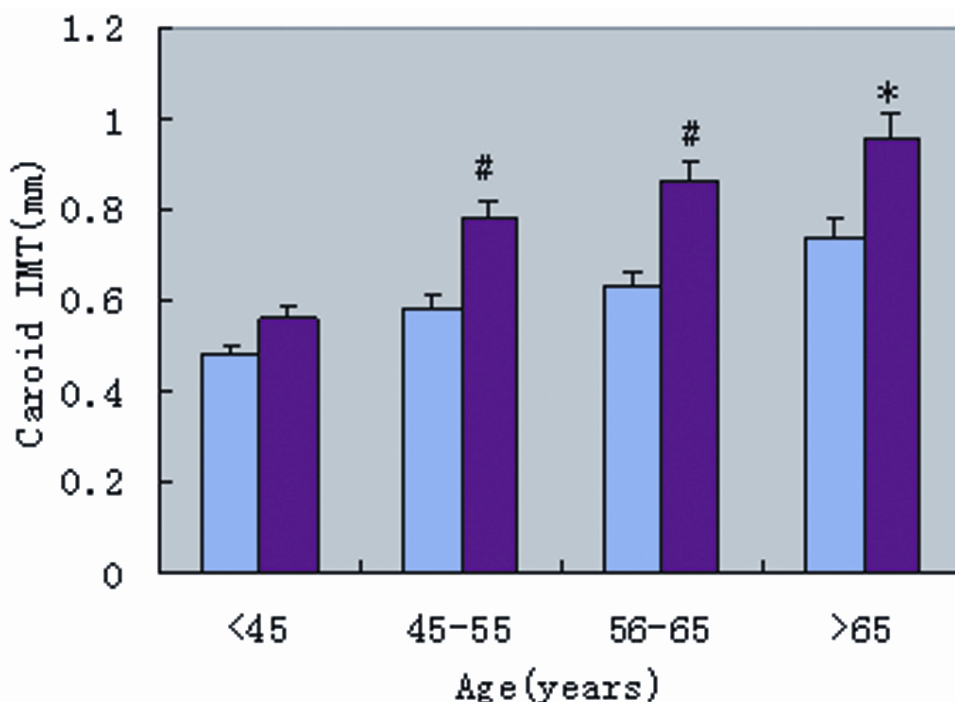
Characteristics are reported as percentages for categorical variables and mean ± standard deviation (SD) or median (with interquartile range) for continuous variables. Student's *t* test or Chi-square test was used to compare differences between groups. The relationship between IMT values and arterial stiffness in the control and hypertensive subjects were examined by linear regression

analysis. The relationship of homocysteine and hs-CRP with IMT values and arterial stiffness were examined by spearman analysis. The relationship of homocysteine with B vitamins and smoking (present as cigarette-years) were also analyzed by spearman analysis. Multiple regression analyses were performed to identify the predictors of homocysteine, carotid IMT and arterial stiffness. All statistical procedures were performed using SPSS statistical package, version 13.0 (SPSS Inc., Chicago, Illinois). Intercorrelated independent variables were removed from the regression model if multicollinearity was detected by a tolerance<0.10 or a variance inflation factor>10. *P*<0.05 was considered significant.

## RESULTS

### Clinical characteristics

The clinical characteristics of subjects are shown in Table 1. The subjects aged from 40 to 72 year. There were no differences in age or the male-female ratio between the control and hypertensive subjects. BMI, cigarette-years, blood pressure, the levels of plasma lipids excepting for HDL-C, blood glucose and renal function were significantly higher in the hypertensive subjects than in the control subjects. All of the participants had not taken B vitamins supplements, containing folate, VitB6 and other B vitamins in the six months. Homocysteine and hs-CRP were significantly higher in the hypertensive subjects than in the control subjects. The level of HDL cholesterol and



**Figure 1.** Bar graphs showing mean values of IMT of carotid artery in control subjects (n =104 blue bar) and patients with hypertension (n = 232 purple bar) by age group. \*P<0.05, #P<0.001 vs. control subjects.

and the intakes of B vitamins were lower in the hypertensive subjects than in the control subjects.

### Carotid IMT in different age durations

The IMT values of the carotid artery in the groups of 45- to 55-year-old ( $0.78 \pm 0.32$  mm, n =88), 56- to 65-year-old ( $0.86 \pm 0.28$  mm, n = 72), and > 65 years ( $0.96 \pm 0.30$  mm, n = 46) were significantly higher than those in the age-matched control subjects ( $0.58 \pm 0.12$  mm, n =34;  $0.63 \pm 0.17$  mm, n = 36;  $0.74 \pm 0.20$  mm, n =18, respectively) with the exception of the group of less than 45 years ( $0.56 \pm 0.14$  mm, n = 26 vs.  $0.48 \pm 0.11$  mm, n=16) (Figure1). The carotid IMT was significantly higher in the hypertensive subjects ( $0.88 \pm 0.38$  mm, n = 232) than in the control subjects ( $0.62 \pm 0.16$  mm, n = 104) ( $P < 0.001$ ). There is no gender difference in the presence of carotid IMT.

### Arterial stiffness in different age durations

The arterial stiffness values of the hypertensive subjects in the group of < 45 years ( $6.94 \pm 0.63$  m/s, n = 26), 45–55 years ( $8.78 \pm 1.33$  m/s, n = 88), 56–65 years ( $9.37 \pm 1.81$  m/s, n = 72), or >65 years ( $10.75 \pm 1.09$  m/s, n =46)

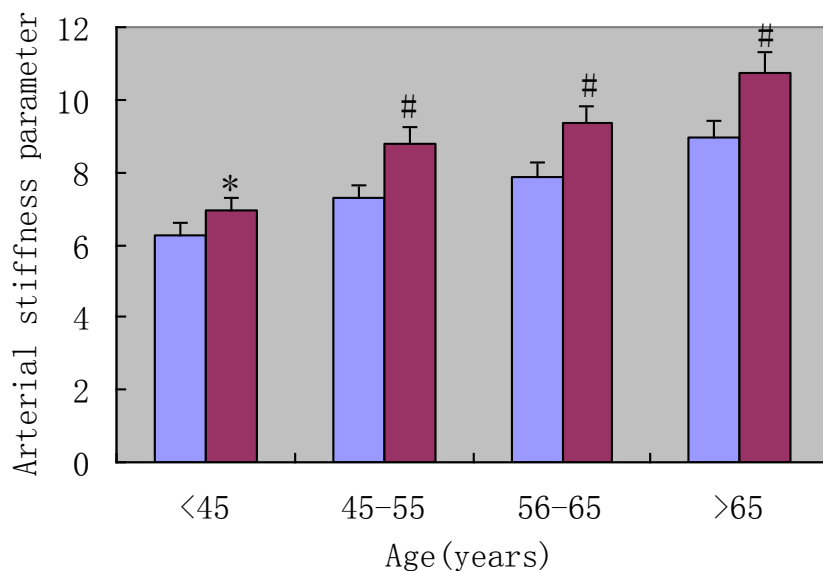
significantly exceeded those in age-matched control subjects ( $6.27 \pm 0.44$  m/s, n=16;  $7.30 \pm 0.54$  mm, n = 34;  $7.88 \pm 0.59$  m/s, n=36;  $8.96 \pm 1.16$  m/s, n=18, respectively) (Figure 2). The arterial stiffness was significantly higher in the hypertensive subjects than in the control subjects ( $P < 0.001$ ). There is no gender difference in the presence of arterial stiffness.

### Relationship between the carotid IMT and arterial stiffness

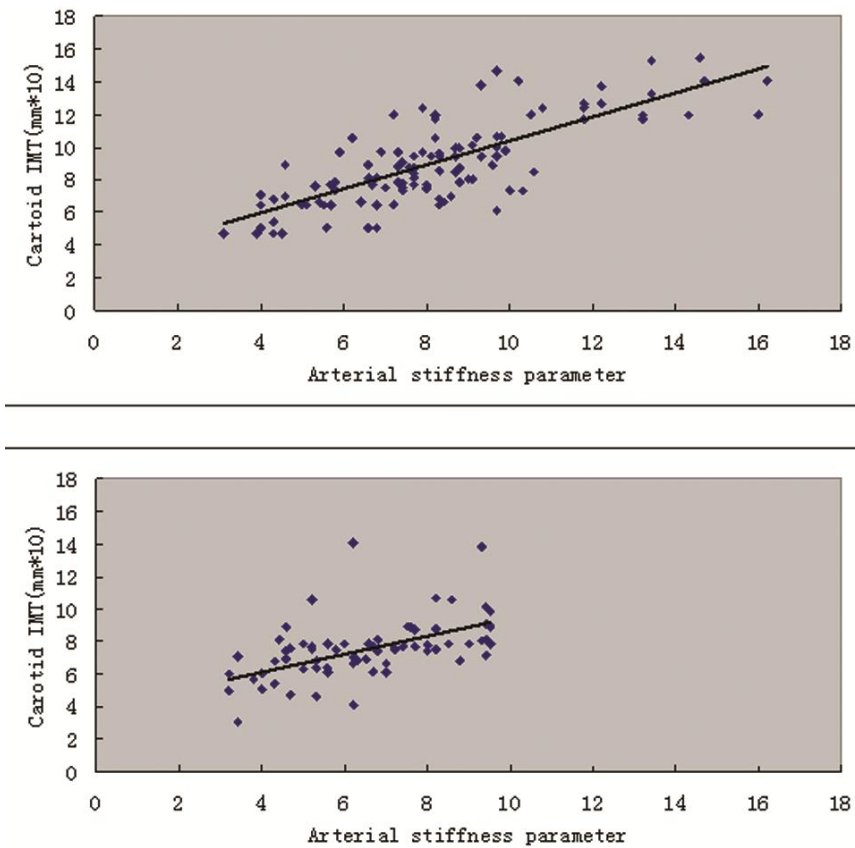
A significant positive correlation between the carotid IMT and arterial stiffness was found in the control subjects ( $r = 0.337$ ,  $P < 0.001$ ). We also observed a significant positive correlation between the carotid IMT and arterial stiffness in the hypertensive subjects ( $r = 0.421$ ,  $P < 0.001$ ) (Figure 3). Moreover, the slope of the regression line between the carotid IMT to the arterial stiffness was significantly steeper in the hypertensive patients than in the control subjects ( $P < 0.05$ ).

### Risk factors for increased arterial stiffness

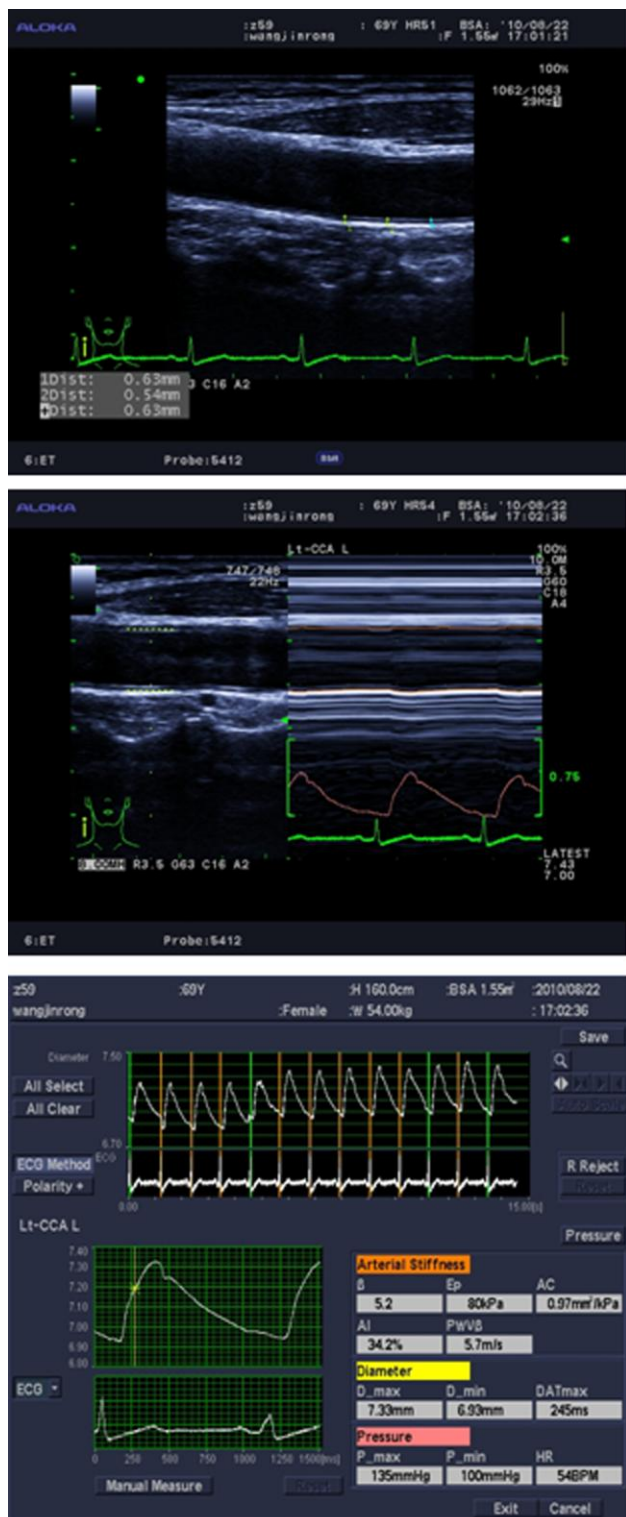
Multiple regression analysis showed that homocysteine was independently related to arterial stiffness parameter



**Figure 2.** Bar graphs showing mean values of the arterial stiffness parameter in control subjects (n = 104 blue bar) and patients with hypertension (n = 232 purple bar) by age group. \*P<0.05, #P<0.001 vs. control subjects



**Figure 3.** The relationship between the IMT of the carotid artery and arterial stiffness in patients with hypertension (n = 232; r = 0.421, P<0.001, see the above figure) and control subjects (n = 104; r = 0.337, P<0.001, see the below figure).



**Figure 4.** The collecting image of arterial stiffness measured by echo-tracking technology on carotid ultrasonography.

(standardized regression parameter 0.155,  $P < 0.01$ )

besides that age, smoking, hypertension, eGFR and the roles of HDL-cholesterol were accounted for (Table 2).

### Risk factors for increased carotid IMT

Multiple regression analysis showed not only homocysteine but hs-CRP were independently related to IMT (standardized regression parameters 0.102,  $P < 0.01$ ; 0.117  $P < 0.01$ ; respectively) besides that age, smoking, hypertension and the roles of LDL-cholesterol were accounted for (Table 3).

### Multivariate predictors of log-transformed homocysteine levels

The associations of these vitamins including folate, Vit. B6 and Vit. B12, intakes with homocysteine concentrations were examined using multiple linear regression analysis after adjusting for age, gender, cigarette-years, body mass index, hypertension, systolic blood pressure, diastolic blood pressure, total cholesterol, triglyceride, HDL cholesterol levels, LDL cholesterol and fasting blood glucose. Homocysteine were independently related with age, gender, smoking, hypertension, intakes of folate and eGFR (Table 4).

### DISCUSSION

We demonstrated that carotid IMT and arterial stiffness were significantly higher in subjects with hypertension than in age-matched control subjects and found significant correlations between structural and functional changes (as indicated by IMT and arterial stiffness parameter, respectively) in hypertensive patients and control subjects in the present study. It is notable that carotid IMT presented no difference between hypertensive and control groups younger than 45 years of age. On the contrary, arterial stiffness parameter showed obvious increase in the hypertensive group younger than 45 years of age. This finding is consisted with the fact that vascular functional (sclerotic) changes occur prior to structural (atherotic) changes (Alan et al., 2003; Davignon and Ganz, 2004; Yambe et al., 2004).

An association between arterial atherotic (structural) and sclerotic (functional) changes is present not only in hypertensive patients but also in senior healthy subjects aged over 45 years. A comprehensive histopathologic study of medium-to-large caliber blood vessels showed vascular fibrosis was a complex process (Selvin et al., 2010). Aging, hypertension, diabetes, poor renal function and dyslipidemia were significantly associated with increased systemic vascular fibrosis and fatty degeneration (atherosis) (Aronson, 2003; Greenwald, 2007). Such



**Table 2.** Risk factors affecting arterial stiffness.

Independent variable	Standard regression coefficient	P-value
Hypertension	0.316	<0.001
Systolic blood pressure	0.221	<0.001
Age	0.169	<0.001
cigarette-years	0.142	0.0076
HDL-cholesterol	-0.113	0.0175
Homocysteine	0.155	0.0035

$R^2=0.330$  ( $P<0.001$ ). Variables assessed in all subjects included age, gender, BMI, cigarette-years, hypertension, blood pressure, fasting plasma glucose, total cholesterol, triglyceride, HDL cholesterol levels, LDL cholesterol, eGFR, homocysteine, hs-CRP, intake of folate, Vitamin B<sub>6</sub> and Vitamin B<sub>12</sub>. eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein.  $R^2$ , multiple coefficient of determination.

**Table 3.** Risk factors affecting carotid IMT.

Independent variable	Standard regression coefficient	P-value
Hypertension	0.320	<0.001
Systolic blood pressure	0.262	<0.001
Age	0.191	<0.001
cigarette-years	0.174	0.0019
LDL-cholesterol	0.227	<0.001
Hs-CRP	0.117	0.0067
Homocysteine	0.102	0.0078

$R^2=0.361$  ( $P<0.001$ ). Variables assessed in all subjects included age, gender, BMI, cigarette-years, hypertension, blood pressure, fasting plasma glucose, total cholesterol, triglyceride, HDL cholesterol levels, LDL cholesterol, eGFR, homocysteine, hs-CRP, intake of folate, Vitamin B<sub>6</sub> and Vitamin B<sub>12</sub>. eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein.  $R^2$ , multiple coefficient of determination.

**Table 4.** Multivariate predictors of log-transformed homocysteine levels.

Independent variable	Standard regression coefficient	P-value
Age	0.218	<0.001
Gender(male=0,femal=1)	-0.147	<0.001
Hypertension	0.175	<0.001
Cigarette-years	0.126	0.0065
Folate ( $\mu\text{g/d}$ )	0.117	0.0142
eGFR( $\text{ml}/\text{mi n}/1.73 \text{ m}^2$ )	-0.112	0.0365

$R^2=0.318$  ( $P<0.001$ ), Homocysteine levels were natural logarithm transformed. Abbreviations are listed in Table 1. Variables assessed in all subjects included age, gender, BMI, cigarette-years, body mass index, hypertension, systolic and diastolic blood pressure, fasting plasma glucose, total cholesterol, triglyceride, HDL cholesterol levels, LDL cholesterol.

atherotic and sclerotic changes may be commonly associated with homocysteine, which should be considered to be a potential target for intervention of arterial stiffening and IMT thickening.

Because high plasma homocysteine was considered as

a biomarker for metabolic disorders, a positive association of homocysteine with arterial stiffness parameter may support the association between metabolic disorder and increased arterial stiffness (Di Minno et al., 2010). Earlier data suggest that homocysteine levels are associated

with aortic stiffness in patients with type II diabetes mellitus (Anan et al., 2007) and arterial hypertension (Vyssoulis et al., 2010). In another study (de Bree et al., 2006), such an association between homocysteine levels and arterial stiffness indices was not indicated, pointing to a clear controversy at present. Our study showed the independent relationship between circulating homocysteine levels and arterial stiffness on carotid artery. The possible mechanisms of the relationship between homocysteine and arterial stiffness are not yet fully well established (Cohen et al., 2001; Spoelstra-De Man et al., 2005). However, it is known that elevated homocysteine levels induce oxidative injury to vascular endothelial cells and impair the production of nitric oxide, a strong relaxing factor by the endothelium (Tawakol et al., 1997; van den Bosch et al., 2003; Anan et al., 2007; Cohen et al., 2001; de Bree et al., 2006; Di Minno et al., 2010; Spoelstra-De Man et al., 2005; Vyssoulis et al., 2010).

A significant positive association between hs-CRP and pulse wave velocity has been shown in healthy Japanese men, but the association may become insignificant after adjusting for conventional cardiovascular risk factor (Tomiyama et al., 2004). In the present study, only homocysteine, but not hs-CRP, was associated with arterial stiffness parameter independently of age, gender, BMI, blood pressure and metabolic components. High arterial stiffness was mainly determined by high homocysteine, and not high hs-CRP.

The complex metabolic factors, especially homocysteine, had an important role in this complex development of IMT and arterial stiffness, but the exact mechanism may be a hopeful illuminating factor for intervention in future. There were many complex influence factors of homocysteine, for example dietary factors (Scuteri et al., 2004). Especially, folate intake, but not other B vitamins, significantly negative correlated with homocysteine level. Folate was the needed substrate of the homocysteine metabolism, while vitB6 and vitB12 were only as coenzymes in the process (Nygard et al., 1995). In China, especially in the north, folate levels in the elderly people were below those in the European and American countries. So, it should be strengthened in the elder of nutrition knowledge to pay attention to adequate B vitamins foods and supplements. There was a correlation between cigarette smoking and increased homocysteine level. Some research showed the increased formation of reactive oxygen species in smokers led to sulfhydryl oxidation and reduction. Additionally, smoking could inhibit the vigor of methionine synthase and decreased homocysteine concentrations. Smoking could also affect the absorption of B vitamins (Kim et al., 2002). Lastly, even if the intake of folate was a predictor for the homocysteine, the intake of folate is not independent for the development of IMT and arterial stiffness, which was not like the role of homocysteine level. The complex influence factors and the potential

mechanism would be explored in the future studies. In conclusion, the study confirmed the association between arterial atherotic (structural) and sclerotic (functional) changes. Homocysteine, but not hs-CRP, associated with the changes of carotid artery IMT and arterial stiffness.

### Clinical implications

The independent association of the level of serum homocysteine with increased arterial stiffness and IMT supports the biologic and mechanistic links between these two entities. Because arterial stiffness may be modifiable, it can be considered a potential target for intervention of IMT. Complex metabolic factors, especially homocysteine, have important roles in this process. However, the deep and exact mechanisms of the roles, including folate, may be focused on in our future investigations.

### Potential limitations

Because of the cross-sectional design, the present study could not explore the causal associations among arterial stiffness, LV diastolic function, hs-CRP and homocysteine. Additional studies with designs to address the relevant questions will be conducted in future.

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### DISCLOSURE OF INTEREST

The authors declare that they have no conflicts of interest concerning this article.

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Full Length Research Paper

# Some pharmacological actions of *Myrica rubra* part 1: Effect on experimentally-induced gastric ulcers, inflammation and haemorrhoids in rats

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Chinese arbutus (*Myrica rubra*) fruits or Yumberry, are stony red fruits with berry-like edible portions growing in China, India, Japan and some other south eastern Asian countries. A 50% juice under the trade name Yumberry is available in some countries. It is rich in polyphenols and proanthocyanidins. This study investigated the effect of 4 weeks administration of the juice as a substitute for the normal rats' drinking water on ethanol- and stress-indomethacin-induced ulcer, carrageenan-induced paw inflammation and croton oil-induced hemorrhoids in male Wistar rats. Consumption of Yumberry drink for 4 weeks almost completely protected the rats against the alcohol-induced gastric ulcers ( $p < 0.05$ ) with no significant effect against the combined cold stress-indomethacin-induced ulcers ( $p < 0.05$ ). The treatment significantly suppressed carrageenan-induced oedema in the rat's paw in a time-dependent manner ( $p < 0.05$  to  $p < 0.001$ ,  $N = 8$ ). The treatment also significantly suppressed experimentally-induced hemorrhoids by  $74 \pm 5.9\%$  ( $p < 0.001$ ,  $N = 8$ ). The different mechanisms of actions of the observed beneficial actions are discussed. The results of this study pointed for the first time the direct protective effects of *M. rubra* beverage on the gastric mucosa and its direct anti-inflammatory effect against skin and rectal inflammations.

**Key words:** Bayberry, gastric ulcers, inflammation, hemorrhoids.

## INTRODUCTION

*Myrica rubra* sieb et zucc. fruits, family Myricaceae, are stony red fruits with berry-like edible portions. The fruit is grown in China, India, Japan and some other south eastern Asian countries such as, Vietnam, Burma and Thailand. It is known by various other names such as Yangmei, Bayberry and Chinese arbutus. It is also known as Wax-myrtle and Yamamoto in Japan.

Phytochemical investigations of the fruit juice revealed the presence of high concentrations of polyphenols and proanthocyanidins (Fang et al., 2006). The latter are con-

densed tannins (polymers) composed of various flavan-3-ol or catechin units. The most available are the reddish procyanidins (Saito et al., 1998). In addition to the fruits, both of the leaves and the bark of the tree constituents were analyzed (Tong et al., 2009; Wang et al., 2010; Tao et al., 2002). The proanthocyanidins were reported to possess various actions that included anti-oxidant (Yokozawa et al., 2012; Dixon et al., 2005), anti-viral (Yokozawa et al., 2012; Cheng et al., 2003), hypolipidemic (Lee et al., 2008), anti-cancer (Kuo et al., 2004), depigmenting (Yamakoshi et al., 2003) and anti-inflammatory actions (Lee et al., 2007; Wang, 2010). The flavonoids and polyphenols were proven to express anti-inflammatory properties by which they inhibit the proliferation and activity of lymphocytes (Sima et al., 2012).

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Peptic ulcer is a chronic disease that impairs the quality of life (Sai et al., 2011). It occurs as a result of the imbalance between the defense and the aggressive factors (Sai et al., 2011). Inflammatory processes play a major role in the etiology of ulcer. *M. rubra* juice is now widely distributed worldwide as a 50% refreshing drink and also as a carbonated beverage under the trade name Yumberry. Thus, the objective of this study was to investigate the influence of the non-carbonated juice drink in experimentally-induced gastric ulcers and inflammation in rats, in order to explore its antiulcer activity and the anti-inflammatory involvement of any antiulcer activity that may arise.

## MATERIALS AND METHODS

### Yumberry juice drink

Yumberry juice bottled drink was purchased from the local market in Riyadh city, Kingdom of Saudi Arabia. The bottled drink is a product of China (Zhejiang Yumberry Juice Co.).

### Chemicals used and their sources

Ethanol, diethyl ether, pyridine and sodium carboxymethylcellulose (BDH, UK), Yumberry drink (Zhejiang Yumberry juice Co., Ltd, China), and Carrageenan (Fluka, Switzerland) were used for this study.

### Animals

In this study, male Wistar rats (body weight  $250 \pm 10$  g) were used. The animals were provided with standard chow diet supplied by Silo and Flour Mills Organization, Feed Mill, Riyadh, Saudi Arabia. All animals were housed at a temperature of  $22 \pm 1^\circ\text{C}$  and relative humidity of  $50 \pm 5\%$ . The light: dark cycle was 12 h each. The animals' treatment was conducted in accordance with the Guide for the Care and Use of Laboratory Animals. The protocol of the current study was approved by the ethics Committee of the College of Pharmacy, King Saud University, Riyadh, Kingdom of Saudi Arabia (KSA).

### Treatment of the animals

Control group was allowed tap water *ad libitum*. All treatment groups ( $N = 8$  animals/group) were administered Yumberry drink (50%) as the sole source of drinking water for 4 weeks.

### Induction of alcohol gastric ulcers

In both Yumberry-treated and the control rats, gastric ulcers were induced by the oral administration of 1 ml 80% ethanol per rat by gavage. One hour after administration of alcohol, all rats were killed by cervical dislocation. The abdomen of each rat was opened, the stomach was removed and opened along the greater curvature and gently rinsed with isotonic saline. The stomach was pinned out flat on a flat surface with the mucosal surface uppermost. The induced gastric ulcers were assessed by calculating an ulcer index using a modification of the scoring system used by Stroff et al. (1993), Gretzer et al. (1998), Lambrecht et al. (1993) and Schmassmann et

al. (1998). The severity factor of the ulcer was defined according to the length of the ulcer (lesion) as follows:

1. Severity factor 1 = no longitudinal ulcers but only hyperemia;
2. Severity factor 2 = lesion < 2 mm in length;
3. Severity factor 3 = lesion 2 to 4 mm in length;
4. Severity factor 4 = lesion 4.1 to 10 mm;
5. Severity factor 5 = lesion 10.1 to 15 mm.

The ulcer index was calculated as the total number of ulcers per stomach multiplied by their respective severity factor.

### Induction of stress-indomethacin ulcers

Stress-indomethacin ulcers were induced in male Wistar rats by a modification of the methods described by Senay and Levine (1967), Levine (1971), Bhargava et al. (1975), Beck et al. (1990) and Schmassmann et al. (1998). In brief, all animals were fasted for 48 h from food only with the respective drinking liquids *ad libitum*. Each animal in the control and the treated group was treated with indomethacin in a dose of 20 mg/kg by gavage. Indomethacin was suspended in a 0.25 % sodium carboxymethylcellulose (w/v). The volume of liquid administered was 4 ml/kg body weight. Then all of the animals were immobilized in restrained cages and placed inside a ventilated refrigerator maintained at a temperature of  $4^\circ\text{C}$ . Three hours later, the rats were killed by cervical dislocation, stomachs removed and the ulcer index was calculated following the scoring system that depends upon the approximate diameter of the induced ulcer. The scoring system was 1, 2, 3, 4, 5 and 6 for ulcers with diameters of 1, 2, 3, 4, 5, and 6 mm. the ulcer index was calculated by multiplying the number of ulcers by their respective scores and summing up.

### Induction of paw inflammation

In both control and treated rats, paw inflammation was induced by the modification of the method described by Winter et al. 1962 and Sima et al. 2012). In brief, initially, each animal was marked around its right ankle in a circular manner using a non-erasable blue ink. The volume of each paw up to the ankle mark was then measured using a plyphesmometer (Ugo, Basil, Switzerland). The displacement fluid used was 0.45% sodium chloride solution. The volume of the displaced paw was then read in the digital display of the instrument. The paw was then injected intraplantarly with 0.2 ml of 2% aqueous Carrageenan solution using 1 ml syringe fitted with a fine hypodermic needle. Thereafter, the paw volume was measured after 1, 2 and 3 h. The net volumes of oedemas formed were then calculated. The percentage decrease in oedemas in the treated animals was calculated relative to the oedemas in the control group.

### Induction of hemorrhoids

Hemorrhoids were induced in both control and treated animals by a modification of the methods described by Nishiki et al. (1988) and Okumura et al. (1995). In brief, male Wistar rats (body weight  $250 \pm 10$  g) were used in this study. To induce hemorrhoids, a mixture of 6% croton oil in diethyl ether, pyridine, diethyl ether and water in a ratio of 10:4:5:1 (v/v) was used. All rats were fasted from food for 3 days but water and Yumberry drink were allowed *ad libitum*.

On the day of experiment, each rat was immobilized in a restrainer cage. The tampon of an ear cotton bud (produced by Septona SA, Greece and purchased from the local market in Riyadh, Kingdom of Saudi Arabia) was dipped in 10 ml of the above inducer contained in a 10 ml vial for 60 s. Then, it was inserted into

the rectum and allowed to contact the mucosa to a depth of 2 cm for 60 s and then removed smoothly. The animals were freed from their restrainer and placed in cages with no food or water for complete 4 h. The volume of the inducer absorbed by each tampon was 333  $\mu$ l. The animals were then sacrificed by an overdose of diethyl ether. The lower abdomen was opened, all of the reproductive organs removed, the rectum bone cut and the lower rectum cleared from any adhering tissue. Then a rectum-anus portion measuring 2 cm was cut from each rat starting from the circular hairline on the anus epithelium using a pair of compass. The cut piece was then opened longitudinally, blotted on a piece of tissue-paper and weighed immediately. The weight of tissue corresponding to 150 g body weight was then calculated in mg. Similarly, the percentage ratio of the whole 2 cm piece relative to the total body weight of the animal was also calculated. The percentage reductions in the weights of the recti of the treated animals in relation to the control weights were then calculated. A third group of animals was administered cotton buds immersed in de-ionized water only as described above.

### Statistics

The values reported in this study were mean  $\pm$  standard error of mean, with  $N$  = number of animals used. Statistical significant differences were calculated using non-paired Student's 't' test. Differences with  $p$  values less than 0.05 were considered significant. Normality test was done using Kolmogorove Smirnov test.

## RESULTS

### Effect of yumberry on gastric ulcers

#### *Effect on alcohol-induced ulcers*

Administration of 80% alcohol by gavage into rats induced excessive hemorrhages and ulcers. The ulcer index was  $18.6 \pm 1.4$  ( $N = 8$ ). Continuous treatment of the rats with Yumberry drink for 4 weeks almost completely prevented the induction of ulcers. The maximum effect observed in the eight rats was only occasional hyperemia. The ulcer index was only  $1.0 \pm 0.5$ . Yumberry drink significantly blunted alcohol-induced ulcers.

#### *Effect on stress-Indomethacin-induced ulcers*

Exposure of rats to the combined hypothermic stress ( $4^{\circ}\text{C}$ ) and oral indomethacin induced excessive number of small ulcers. The ulcer index was  $46.4 \pm 4.1$ . Treatment of rats with Yumberry for 4 weeks continuously resulted in slight protection of the animals against the induced ulcers. The ulcer index was  $41.6 \pm 5.9$  ( $p > 0.05$ ,  $N = 8$ ).

### Effect of yumberry on carrageenan-induced paw oedema

Intraplantar administration of carrageenan into the rats

paw resulted in time-dependent inflammation as reflected by the paw volume (Table 1). Similar intraplantar administration of carrageenan into Yumberry drink-treated rats resulted in highly significant and time-dependent reduction in the paw oedema ( $P < 0.05$  to  $< 0.001$ ), ( $N = 8$ ) (Table 1). The percentage decreases in the induced oedemas were  $31.5 \pm 1.7$ ,  $65.3 \pm 4.9$  and  $78.0 \pm 3.4\%$ , 1, 2, and 3 h, following administration of carrageenan.

### Effect of yumberry on experimentally-induced haemorrhoids

Intra-rectal administration of a mixture of croton oil, pyridine, diethyl ether and water into rats resulted in significant haemorrhage and oedemas that led to significant increases in the recti pieces weights compared with non-treated animals (Table 2). In fact, the haemorrhoids-inducer resulted in more than doubling of the weight. Induction of haemorrhoids in rats that drank Yumberry drink for four weeks revealed the inherent anti-haemorrhoids activity of Yumberry. The treatment suppressed the induced haemorrhoids by  $74.0 \pm 5.9\%$  ( $p < 0.001$ ,  $N = 8$ ) (Table 2).

## DISCUSSION

The results of this study clearly demonstrated that consumption of Yumberry drink protects against alcohol-induced ulcers and suppresses inflammations, whether skin or rectal types (haemorrhoids). Such actions seemed to be due to some Yumberry drink constituents. The juice of *M. rubra*, from which Yumberry drink is made, is known to contain proanthocyanidins such as cyanidin-3-glucoside, flavonoids such as quercetin-3-o-glucoside and various polyphenols such as gallic acid, and various vitamins of the B, C, and E groups (Zhongxiang et al., 2007; Fang et al., 2006).

Alcohol-induced ulcers are known to be due to stimulation of production of oxygen-derived free radicals (Wallace and Whittle, 1985; Oates and Hakkinen, 1988) and a decrease in mucosal level of non-protein sulfhydryl compounds (Szabo et al., 1981). Previous studies using pure proanthocyanidins revealed the ability of these compounds to inhibit production of oxygen free radicals (Dixon et al., 2005; Xie and Dixon, 2005; Yokozawa et al., 2012) and to elevate reduced glutathione level (Yokozawa et al., 2012).

Flavonoids (Kim and Uyamay, 2005) and polyphenols (No et al., 1999) were also reported to be anti-oxidants. Thus, the observed ability of Yumberry drink to protect against alcohol-induced ulcers seemed to be due to the presence of anthocyanidins, flavonoids and polyphenols in the drink. Furthermore, an additional physical mechanism such as binding of the proanthocyanidins to the gastric mucosa proteins cannot be ruled out since procyanidins can bind to bovine serum albumin (Saito et

**Table 1.** Effect of yumberry on carrageenan-induced paw oedema in rats.

Treatment	Net oedema volume (ml) after,		
	1 h	2 h	3 h
Carrageenan	0.57±0.1	1.45±0.13	2.1±0.2
Yumberry + carrageenan	0.39±0.03*	0.50±0.05**	0.46±0.1*

\* $p < 0.05$ ,  $N = 8$ , \*\* $p < 0.001$ ,  $N = 8$ .

**Table 2.** Effect of yumberry drink on experimentally-induced haemorrhoids in rats.

Treatment	Weight of 2 cm rectum per 150 g body weight (mg)	Net haemorrhoids weight	Percentage of the 2 cm rectum to the whole body weight
Normal rectum	178.1±9.7	-	0.071
Haemorrhoids inducer	362.4±13.9	184.3±2.5	0.145
Haemorrhoids inducer + yumberry	226.0±8.4*	47.9±1.3*	0.09

\*Compared with haemorrhoids inducer ( $N = 8$ ) ( $p < 0.001$ ).

al., 1998). On the other hand, indomethacin-induced ulcers seemed to involve inhibition of gastric prostaglandins derived mainly via COX-1, with some involvement of COX-2 (El-Bayer et al., 1985; Beck et al., 1990; Iseki, 1995; Wallace, 1997; Schmassmann et al., 1998).

Stress-induced ulcers are believed to result from an increase in gastric acid secretion (Kitagawa et al., 1979; Marrone, 1984), a decrease in gastric PGE<sub>2</sub> production (Moody, 1976; Arakawa et al., 1981) and disturbed micro-circulation in the gastric mucosa (Murakami et al., 1985). Proanthocyanidins are shown to suppress COX-2 enzyme (Yokozawa et al., 2012). Thus, the success of Yumberry drink to selectively suppress alcohol-induced ulcers but not the indomethacin cold stress-induced ulcers seemed to be due to the difference in the mechanisms of the induced ulcers. Previous reports have shown the ability of some plants containing proanthocyanidins, for example, grape seed extract to suppress gastric ulcers (Saito et al., 1998). Furthermore, Grisham et al. (1986) related the gastric protective effect of proanthocyanidins to their ability to decrease myeloperoxidase activity (Grisham et al., 1986).

The results of this study revealed another inherent property for *M. rubra* drink (Yumberry), an anti-inflammatory and antihemorrhoidal action as shown by the inhibition of carrageenan-induced paw oedema (Winter et al., 1962) and croton oil mixture-induced inflammatory hemorrhoids (Nishiki et al., 1988; Okumura et al., 1995). Carrageenan-induced inflammation is shown to be due to release of various mediators, majorities of which are TNF $\alpha$  (Gong et al., 2009; Bhavin et al., 2010), IL-1 $\beta$  (Chou, 2003; Loram et al., 2007), nitrogen oxide (NO) (Bilici et al., 2002), prostaglandins, kinins, histamine and serotonin (Bhukya et al., 2009). The time-dependent inhibitory effect of Yumberry drink on the

induced inflammation may be due to its constituent proanthocyanidins. Indeed, these are shown to inhibit NO and the PGs via inhibition of their producing enzymes NO synthase and COX-2 (Yokozawa et al., 2012). An additional inhibitory effect on TNF $\alpha$  cannot be ruled out since a proanthocyanidin induced inhibition of their inducer NF $\kappa$ B has been previously reported (Yokozawa et al., 2012). In this connection, it is pertinent to recall that NF $\kappa$ B stimulates the transcription of the genes that encode the enzymes NO synthase and COX-2 (Touyza and Schiffrin, 2004). Furthermore, proanthocyanidins do indeed inhibit TNF $\alpha$  in the rat's paw (Li et al., 2001). Croton oil mixture-induced inflammation and hemorrhoids seemed to involve mainly prostaglandins and IL-6, with no effect on NO or TNF $\alpha$  (Shin et al., 2010). Thus, proanthocyanidins contained in Yumberry drink may also be involved in the observed inhibition of croton oil mixture-induced inflammation (Yokozawa et al., 2012).

On a broad basis, the results of this study pointed for the first time the direct protective effect of *M. rubra* juice beverage on the gastric mucosa and its direct anti-inflammatory effect against skin and rectal inflammations. Thus, *M. rubra* juice drink can be considered as a useful functional food.

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*Full Length Research Paper*

## Pharmacological activities evaluation of some new pyrazolo-pyrimidino-pyridazine derivatives

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**Pyrazolo-pyrimidino-pyridazine derivatives are potent compounds with pharmacological activities like anti-inflammatory, analgesic and anti-microbial. Pharmacological screenings proved that the compounds 1 to 6 had high activities comparable with reference drugs. Anti-inflammatory evaluation of the tested compounds comparable with Indomethacin showed that edema inhibition increased with time. All the tested compounds had no gastric ulcerogenic effect on mice. Also, analgesic activity of the tested compounds comparable with valdecoxib showed that these compounds had activities. Analgesic activities of the tested compounds increased with time. The tested compounds were able to inhibit the growth of the Gram-positive, Gram-negative bacteria and fungi.**

**Key words:** New hetero cyclic compounds, pharmacological screening, anti-inflammatory, analgesic, anti-microbial.

### INTRODUCTION

In previous works we reported that certain substituted pyrazoles, pyridazines and their derivatives had antibacterial, antifungal (Fayed et al., 2009; Nagawade et al., 2009; Bahashwan et al., 2010) and anti-inflammatory (Al-Harbi et al., 2010) activities. Also, pyrazolo, pyrimidino and pyridazino derivatives have been reported to possess a variety of other pharmacological activities such as central nervous system (CNS) depressant (Julino et al., 1998; Abdou et al., 2004), neuroleptic, tuberculostatic (Ali, 2009; Ghorab et al., 2004) and anti-parkinsonism (Bahashwan, 2011). Pyrazolo derivatives were reported as inhibitors of glycogen synthase kinase-

3(GSK-3) (Witherington et al., 2003) and potent antitumor agents (Lin et al., 2007). In view of these observations, we have synthesized some new poly heterocyclic fused ring systems containing pyridazine nuclei, and tested them for analgesic, anti-inflammatory and anti-microbial activities, in comparison to some reference drugs.

### MATERIALS AND METHODS

#### Animals

Female albino Sprague Dawley mice 16 to 18 g were purchased from Theodor Bilharz Research Institute (TBRI), Egypt. Approval of the institutional animal ethical committee for the animals' studies was obtained from the Office of Environmental Health and Radiation Safety, ACUC Protocol # 1096-5. The animals were maintained according to accepted standards of animal care

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(Mahmoud, 1984).

#### Anti-inflammatory activity

Compounds 1 to 6 (Figure 1) (Amer et al., 2011) were dissolved in 0.5% carboxymethyl cellulose (CMC) as a homogeneous solution. One hundred and eight mice were divided into eighteen groups (six animals each). Anti-inflammatory activity of the compounds was studied in mice using carrageenan paw oedema method. A suspension of the tested compound and the reference drug, Indomethacin in aqueous solution, was administered orally at a dose 5 mg/kg. Control animals were treated with 0.5 % CMC only. After 30 min., 0.01ml of freshly prepared 1.0% carrageenan solution (in formol saline) was injected into the sub-plantar region of the right hind paw according to Hernandez-Perez et al. (1995). The right paw volume was measured using a digital plethysmometer (Model 7150) directly before and after 1, 2, 3 h intervals after administration of the tested compounds.

#### Ulcerogenic activity

Seventy-two mice were divided into twelve groups. Ulcerogenic activity was evaluated after oral administration of the tested compounds or indomethacin at doses of 10, 50, and 100 mg/kg. Control mice received 0.5% CMC. Food but not water was removed 24 h before administration of the test compounds. After 6 h, the mice were sacrificed; the stomach was removed and opened along the greater curvature, washed with distilled water and cleaned gently by dipping in saline. The mucosa damage for each stomach was examined using a stereoscopic microscope and compared with the reference drug indomethacin according to reported procedure (Ikuta et al., 1987).

#### Acute toxicity

The median lethal doses (LD<sub>50</sub>) of the most active compounds 5 to 6 were determined in mice (Sztaricskai et al., 1999). Groups of male adult mice, each of six animals, were injected Intraperitoneally (i.p.) with graded doses of each of the test compounds. The percentage of mortality in each group of animals was determined 24 h after injection. Computation of LD<sub>50</sub> was processed by a graphical method.

#### Analgesic activity

Sixty mice of both sexes were divided into 10 groups. One group was kept as control (received formol saline), the second group received vehicle (gum acacia) and the third one received valdecoxib as a reference drug, whereas the other groups received the test compounds subcutaneously (S.C.). Mice were dropped gently in a dry glass beaker of 1 Liter capacity maintained at 55 to 55.5°C. Normal reaction time in seconds for all animals was determined at time intervals of 10, 30, 60 and 120 min. This is the interval extending from the instant the mouse reaches the hot beaker till the animals licks its feet or jumps out of the beaker (dose 5mg/kg) (Tjolsen et al., 1991). The relative potencies to valdecoxib were determined (Table 3).

#### Anti-microbial screening

All the compounds were evaluated *in vitro* for their anti-microbial

activities. The anti-microbial activity was evaluated against three bacterial strains *Staphylococcus aureus*, *S. epidermidis* and *Escherichia coli* and three fungal strains *Aspergillus fumigates*, *A. niger* and *A. alternate*, employing the nutrient agar disc diffusion method (Arthington et al., 2000) at 100 µg/ml concentration. Dimethyl sulphoxide (DMSO) was used as blank and it exhibited no activity against any of the used organisms. The anti-microbial activity was determined by measuring the inhibition zone (Table 4), after 16 to 20 h of incubation at 37°C for bacterial strains and 3 to 4 days at 37°C for fungal strains. Tetracycline and Ketoconazole were used as standard drugs bacterial and fungal strains, respectively at 30 µg/ml concentration.

#### The minimum inhibitory concentration (MIC)

A current definition of MIC is "the lowest concentration which results in maintenance or reduction of inoculums viability". The determination of the MIC involves a semi-quantitative test procedure which gives an approximation to the least concentration of antimicrobial agent needed to prevent microbial growth. The method displays tubes of growth broth containing a test level of pre-servatives, into which inoculums of microbes was added. The end result of the test was the minimum concentration of anti-microbial.

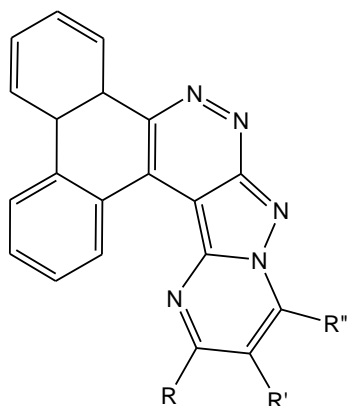
The serial dilution technique (Mostahar et al., 2006) was applied for the determination of MIC of the tested compounds 1 to 6 against two species of bacterial strains (*S. aureus* and *E. coli*) and two species of fungal strains (*A. niger* and *A. alternata*). Dilution series were set up with 6.25, 12.5, 25, 50 and 100 µg/ml of nutrient broth medium to each tube; 100 µl of standardized suspension of the test microbes (10<sup>7</sup> cells/ ml) were added and incubated at 37°C for 24 h (Table 5).

#### Cytotoxicity bioassay

Brine shrimp lethality bioassay (Jaki et al., 1999; Mayer et al., 1982) is a recent development in the assay which indicates cytotoxicity as well as a wide range of pharmacological activities (for example, antimicrobial, anticancer, antiviral, insecticidal, pesticidal, acquired immunodeficiency syndrome (AIDS), etc). In this method, the eggs of the brine shrimp, *Artemia salina* (Leach), were hatched for 48 h to mice shrimp, 38 g of sea salt was weighed, dissolved in one liter of distilled water, filtered off and was kept in a small tank. The eggs were then added to the divided tank. Constant oxygen supply was provided and temperature of 37°C was maintained for 48 h to hatch the shrimp known as nauplii (Larvae). The solutions of compounds 1 to 6 were prepared by dissolving 10 mg of each compound in 2 ml of DMSO. From this stock, a series of solution 5, 10, 20, 40 and 80 µg/ml were transferred to fifteen vials (three for each dilutions were used for each test sample and LC<sub>50</sub> is the mean of three values) and one vial was kept as control having 2 ml of DMSO. Then, about 10 brine shrimp nauplii were applied to each of all experimental vials and control vial. The number of the nauplii that died after 24 h was counted. The resulting data were transformed to the probit analysis for the determination of LC<sub>50</sub> values for the five tested compounds (Table 6).

#### Statistical analysis

Assay results are shown as mean ± standard error (SE). Statistical analyses were carried out with Sigma Plot software (SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) followed by Tukey's posthoc test was used to assess the presence of significant differences. Differences were considered statistically significant at p ≤ 0.05.



**Figure 1.** Structure of the tested compounds 1 to 6.

## RESULTS AND DISCUSSION

Three pharmacological activities namely; anti-inflammatory, analgesic and anti-microbial activities were tested despite their different biological receptors. Six representative compounds 1 to 6 (Figure 1), were studied with respect to their anti-inflammatory, analgesic and anti-microbial activities. The activities of these compounds are different according to the structure and function groups (Tables 1 to 7).

### Anti-inflammatory activity

The results of anti-inflammatory activity after 1 h showing the percent inhibition of edema obtained by the reference drug and tested compounds, respectively is shown in Table 1. Results show that compounds 3 and 5 possess weak anti-inflammatory activity ( $7.3 \pm 1.2$ ,  $14.3 \pm 1.7\%$  of inhibition), respectively in comparison to that of indomethacin ( $44.7 \pm 1.8\%$ ). Compounds 1, 2 and 6 possess intermediate activity ( $34.3 \pm 1.3$ ,  $28.4 \pm 1.3$ ,  $31.3 \pm 1.4\%$  of inhibition). Compound 4 showed activity of  $41.7 \pm 1.6\%$ , nearly equal to that of indomethacin. The results of anti-inflammatory screening after 2 h showed the percent inhibition of edema obtained by the reference drug and tested compounds, respectively. Results show that compounds 3 and 5 possess weak anti-inflammatory activity ( $13.5 \pm 1.1$ ,  $16.7 \pm 1.6\%$  of inhibition), respectively in comparison to that of indomethacin ( $52.4 \pm 1.7\%$ ). Compounds 1, 2 and 6 possess intermediate activity ( $37.0 \pm 1.4$ ,  $24.3 \pm 1.8$ ,  $35.4 \pm 1.7\%$  of inhibition). Compound 4 showed activity of  $45.1 \pm 1.3\%$ , nearly equal to that of indomethacin. The results of anti-inflammatory screening after 3 h showed the percent inhibition of edema obtained by the reference drug and tested compounds, respectively. Results show that compounds 3 and 5 possess weak anti-inflammatory activity ( $14.3 \pm$

$1.8$ ,  $15.3 \pm 1.1\%$  of inhibition), respectively in comparison to that of indomethacin ( $61.2 \pm 1.4\%$ ). Compounds 1, 2 and 6 possess intermediate activity ( $39.5 \pm 1.8$ ,  $29.6 \pm 1.3$ ,  $36.7 \pm 1.7\%$  of inhibition). Compound 4 showed activity of  $49.1 \pm 1.9\%$ , nearly equal to that of indomethacin (Table 1).

### Ulcerogenicity and acute toxicity

Compounds 1 and 4 were screened for their ulcerogenic activity at dose levels of 10, 50 and 100 mg/kg (Table 2). The tested compounds 1 and 4 showed no ulcerogenic activity of 1.4 to 2.1 mm.

### Acute toxicity

LD<sub>50</sub> of compounds 1 and 4 was found to be 145, 155 and 165 mg/kg, respectively, whereas, LD<sub>50</sub> of indomethacin was 50 mg/kg.

### Analgesic activity

All tested compounds exhibited analgesic activity in the hot plate assay. Ten min after administration, compounds 3 and 5 exhibited weak analgesic activities ( $0.31 \pm 0.01$ ,  $0.41 \pm 0.03$ ), respectively in comparison to that of valdecoxib ( $1.00 \pm 0.01$ ). Compounds 1 and 2 were found to possess intermediate activities ( $0.64 \pm 0.01$ ,  $0.53 \pm 0.02$ ), respectively. Compounds 4 and 6 exhibited strong activities ( $0.72 \pm 0.03$ ,  $0.78 \pm 0.02$ ), respectively. Comparison of analgesic potency of test compounds to valdecoxib after 30 min showed that compound 3 to possessed weak analgesic activities ( $0.37 \pm 0.01$ ) in comparison to that of valdecoxib ( $1.00 \pm 0.01$ ). Compounds 2 and 5 possess intermediate activities ( $0.61 \pm 0.04$ ,  $0.47 \pm 0.03$ ), respectively. Compounds 1 and 6 possess strong activities ( $0.77 \pm 0.03$ ,  $0.89 \pm 0.02$ ), respectively. Compound 4 possessed very strong activities ( $0.91 \pm 0.08$ ). Comparison of analgesic potency of test compounds to valdecoxib after 60 min showed that compound 3 to possessed weak analgesic activities ( $0.42 \pm 0.04$ ) in comparison to that of valdecoxib ( $1.00 \pm 0.01$ ). Compounds 2 and 5 possess intermediate activities ( $0.67 \pm 0.01$ ,  $0.53 \pm 0.03$ ), respectively. Compounds 1 and 6 possess strong activities ( $0.83 \pm 0.02$ ,  $0.97 \pm 0.02$ ), respectively. Compound 4 possessed very strong activity which was more than the activity of valdecoxib ( $1.12 \pm 0.01$ ).

Comparison of analgesic potency of test compounds to valdecoxib after 120 min showed compound 3 to possess weak analgesic activities ( $0.49 \pm 0.03$ ) in comparison to that of valdecoxib ( $1.00 \pm 0.01$ ). Compounds 2 and 5 possessed intermediate activities ( $0.69 \pm 0.02$ ,  $0.59 \pm 0.01$ ), respectively. Compound 1 possessed strong

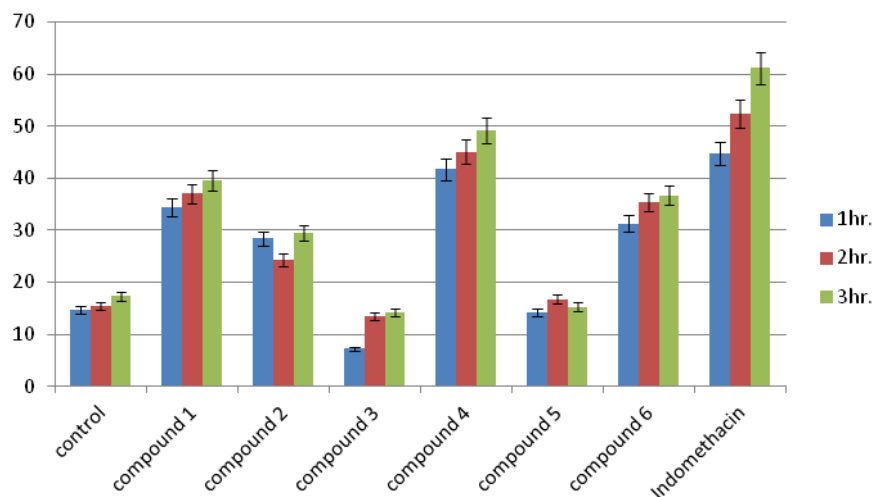


Figure 2. Anti-inflammatory activity of the tested compounds.

activities ( $1.02 \pm 0.03$ ) which was equal to the activity of valdecoxib. Compounds 4 and 6 possessed very strong activity which was more than the activity of valdecoxib ( $1.27 \pm 0.04$ ,  $1.23 \pm 0.04$ ), respectively (Table 3).

### Anti-microbial activity

The organic compounds 1 to 6 were evaluated *in vitro* for their anti-microbial activity. The antimicrobial activities were carried out against three bacterial strains (*S. aureus*, *S. epidermidis* and *E. coli*) and three fungal strains (*A. fumigates*, *A. niger* and *A. alternata*). The preliminary screening results indicated that most of the compounds showed moderate to good antimicrobial activity. From the inhibition zone diameter data analysis, It was found that compound 1 showed in general, moderate inhibition against *S. aureus* and *S. epidermidis*. However, compound 1 showed lower inhibition against *E. coli* and the three species of fungal strains. Compounds 2, 3 and 5 showed in general, good inhibitions against the species of bacterial and fungal strains but compound 2 showed only moderate inhibitions against *A. alternata*, compound 3 exhibited lower inhibitions against *A. niger* and *A. alternata*. Compound 4 showed moderate inhibitions against *S. aureus* and *A. fumigates*, but compound 4 showed lower inhibitions against *S. epidermidis*, *E. coli*, *A. niger* and *A. alternata*. Compound 6 showed moderate inhibitions against *E. coli*. However, compound 6 showed lower inhibitions against other species of bacterial and fungal strains (Table 4).

### The minimum inhibitory concentration (MIC)

The MIC ( $\mu\text{g/ml}$ ) of the most active compounds 2, 3 and 5

against two species of bacteria (*S. epidermidis* and *E. coli*) and also two species of fungi (*A. niger* and *A. alternata*) were determined (Table 5). Compounds 3, 4 and 5 demonstrated good inhibitions against the selected bacterial and fungal strains.

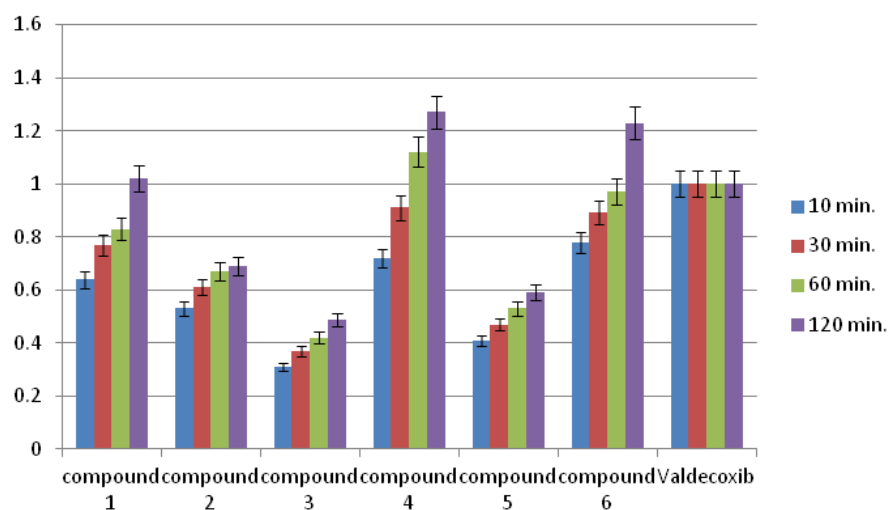
### Cytotoxicity activity

The  $\text{LC}_{50}$  values of tested compounds 2, 3 and 5 were found to be 3.54, 6.49 and 2.31  $\mu\text{g/ml}$ , respectively (Table 6). The standard drug Bleomycin has  $\text{LC}_{50}$  value at 0.41 g/ml. The lowest  $\text{LC}_{50}$  value was found in the case of compound 3, indicating higher cytotoxicity than the other compounds. Compounds 2 and 5 showed potent biocidal activity against brine shrimp

## DISCUSSION

Three pharmacological activities namely; anti-inflammatory, analgesic and antimicrobial activities were tested despite their different biological receptors. Six representative compounds 1 to 6 (Figure 1) were studied for the above purpose. The activities of these compounds are different according to the structure and functional groups (Tables 1 to 6, Figures 2 and 3). Compounds 4 showed activity nearly equal to that of indomethacin. Evaluation results of anti-inflammatory activity of the test compound was comparable with indomethacin over time periods of 1, 2 and 3 h.

The data generated showed that edema inhibition increased with time. Edema inhibition of compounds 4 and 6 by time from  $31.3 \pm 1.4$  to  $49.1 \pm 1.9$  respectively nearly equal the activity of Indomethacin (Table 1) and



**Figure 3.** Analgesic activities of tested compounds 1-6.

**Table 1.** Anti-inflammatory activity of the tested compounds.

Compound number	Edema inhibition (mean $\pm$ SEM) <sup>a,b</sup> (%)		
	1 h	2 h	3 h
Control	14.6 $\pm$ 1.2	15.5 $\pm$ 1.2	17.3 $\pm$ 1.6
1	34.3 $\pm$ 1.3	37.0 $\pm$ 1.4	39.5 $\pm$ 1.8
2	28.4 $\pm$ 1.3	24.3 $\pm$ 1.8	29.6 $\pm$ 1.3
3	7.3 $\pm$ 1.2	13.5 $\pm$ 1.1	14.3 $\pm$ 1.8
4	41.7 $\pm$ 1.6	45.1 $\pm$ 1.3	49.1 $\pm$ 1.9
5	14.3 $\pm$ 1.7	16.7 $\pm$ 1.6	15.3 $\pm$ 1.1
6	31.3 $\pm$ 1.4	35.4 $\pm$ 1.7	36.7 $\pm$ 1.7
Indomethacin	44.7 $\pm$ 1.8	52.4 $\pm$ 1.3	61.2 $\pm$ 1.4

<sup>a</sup>Dose 5 mg/kg b.m (p.o.), <sup>b</sup>n = 6

**Table 2.** Gastric ulceration in mice.

Compound number	Dose(mg/kg)		
	10	50	100
Control	0/6	0/6	0/6
1	0/6	0/6	0/6
4	0/6	0/6	0/6
Indomethacin	3/6 (1.5 $\pm$ 0.2) <sup>b,c</sup>	5/6 (1.9 $\pm$ 0.2) <sup>b,c</sup>	6/6 (2.2 $\pm$ 0.2) <sup>b,c</sup>

<sup>a</sup>number of rats lesions bigger than 0.5 mm in length per total no of rb, <sup>b</sup>mean ulcer lesions  $\pm$  SEM (mm) (n = 6) in parentheses, <sup>c</sup>significant difference at p < 0.05 compared to the control.

the tested compound showed no ulcerogenic activity of 1.4 to 2.1 mm. All tested compounds exhibited analgesic activity in the hot plate assay. Interestingly, compound 4 showed more activity than valdecoxib after 1 h, and compound 6 showed equal activity of valdecoxib after 1

h. Also, compound 1 showed activity equal to valdecoxib after 2 h, but compounds 4 and 6 showed more activity than valdecoxib after 2 h. The analgesic activities of the tested compounds were comparable to valdecoxib over time 10, 30, 60 and 120 min. It can be observed that the

**Table 3.** Analgesic activities of tested compounds 1 to 6.

Compound number	Comparative analgesic potency to valdecoxib after time			
	10 min	30 min	60 min	120 min
1	0.64±0.01	0.77±0.03	0.83±0.02	1.02±0.03
2	0.53±0.02	0.61±0.04	0.67±0.01	0.69±0.02
3	0.31±0.01	0.37±0.01	0.42±0.04	0.49±0.03
4	0.72±0.03	0.91±0.08	1.12±0.01	1.27±0.04
5	0.41±0.01	0.47±0.03	0.53±0.03	0.59±0.01
6	0.78±0.03	0.89±0.02	0.97±0.02	1.23±0.04
Valdecoxib	1.00±0.01	1.00±0.01	1.00±0.01	1.00±0.01

All results were significantly different from the standard and control value at  $p \leq 0.05$ .\*

**Table 4.** The Antimicrobial activity of tested compounds.

Compound number	Diameter of the inhibition zonea (mm)					
	Bacteria			Fungi		
	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>E. coli</i>	<i>A. fumigatus</i>	<i>A. niger</i>	<i>A. althernata</i>
1	16	13	9	9	10	5
2	24	22	20	20	18	11
3	27	27	26	17	12	8
4	14	11	5	17	9	10
5	26	23	20	21	19	19
6	9	11	13	11	7	2
Tetracyclineb	30	25	28	-	-	-
Ketoconazoleb	-	-	-	18	20	21

<sup>a</sup>15 mm or less: resistant or no inhibition, 16 to 20 mm: moderate inhibition, 20 mm or more: maximum inhibition. <sup>b</sup>The concentration of used standard drugs was 30 µg/ml

**Table 5.** The minimum inhibitory concentration (MIC, µg/ml) of tested compounds 2,3 and 5.

Organism	The minimum inhibitory concentration (MIC)			
	2	3	5	Standard <sup>a</sup>
<i>S. aureus</i>	50	50	50	6.25
<i>E. coli</i>	25	12.5	>100	12.5
<i>A. niger</i>	50	2.5	>100	6.25
<i>A. althernata</i>	50	>100	25	6.25

<sup>a</sup>Tetracycline and ketoconazole were used as standard drugs against bacterial and fungal strains, respectively.

**Table 6.** Cytotoxicity activity of tested compounds 2, 3 and 5.

Sample	95% confidence limite ppm			Regression equation	X <sup>2</sup> (df)
	LC <sub>50</sub>	Lower	Upper		
2	3.54	2.08	6.02	Y = 3.98 + 1.85X	3.38 (2)
3	6.49	4.15	10.15	Y = 3.17 + 2.27X	0.35 (2)
5	2.31	1.3	4.1	Y = 4.36 + 1.78X	0.32 (2)
Bleomycin <sup>a</sup>	0.41	0.27	0.62	Y = 3.16 + 1.98X	0.62 (2)
Gallic acid <sup>a</sup>	4.53	3.33	6.15	Y = 3.93 + 1.62X	1.25 (2)

<sup>a</sup>Bleomycin and gallic acid were used as standard drugs in cytotoxicity activity.

**Table 7.** Various functional groups present in compounds 1 to 6.

Compound	R	R'	R''
1	-C <sub>6</sub> H <sub>5</sub>	-H	-NH <sub>2</sub>
2	-C <sub>6</sub> H <sub>5</sub>	-H	-C <sub>6</sub> H <sub>5</sub>
3	-C <sub>6</sub> H <sub>5</sub>	-H	-p(OCH <sub>3</sub> )C <sub>6</sub> H <sub>4</sub>
4	-H	-Coo C <sub>2</sub> H <sub>5</sub>	-NH <sub>2</sub>
5	-C <sub>6</sub> H <sub>5</sub>	-Co C <sub>6</sub> H <sub>5</sub>	-NH <sub>2</sub>
6	-C <sub>6</sub> H <sub>5</sub>	-Coo C <sub>2</sub> H <sub>5</sub>	-NH <sub>2</sub>

## Conclusion

The objective of the present study was to investigate the anti-inflammatory, analgesic and anti-microbial activities of new pyrazolo-pyridazino-pyrimidino derivatives 1 to 6. The newly tested compounds 1 to 6 and the standard drug Indomethacin were found to exhibit essentially equipotent anti-inflammatory activity ( $7.3 \pm 1.2$  to  $49.1 \pm 1.9\%$ ). Also, the newly tested synthesized compounds 1 to 6 and the standard drug valdecoxib were found to exhibit essentially equipotent analgesic activity ( $0.31 \pm 0.01$  to  $1.23 \pm 0.01$ ). Further newly synthesized 1 to 6 were able to inhibit the growth of the Gram-positive, Gram-negative bacteria and fungi. Compound 5 could be identified as most biologically active as anti-inflammatory compound, compound 6 as analgesic and compound 5 as antimicrobial. Activity of compound 5 may be due to the existence of aromatic carbonyl (-COC<sub>6</sub>H<sub>5</sub>) and amino group (NH<sub>2</sub>) and also the activity of compound 6 due to ester group (-COOC<sub>2</sub>H<sub>5</sub>).

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## Full Length Research Paper

# Studies on chronic administration of chloroquine on gastrocnemius muscle and spleen of Swiss albino mice

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**The aim of the present study was to investigate the influence of chloroquine on some vital tissues of mice (gastrocnemius muscle and spleen). Healthy adult male Swiss albino mice weighing between 30 and 40 g were used for the study. Treated group was exposed to 200 mg/kg body weight/day of chloroquine phosphate given orally for 45 days. Control animals were given distilled water for the same period. Microscopic examination of muscle and spleen revealed concomitant changes in histology. Thus, the use of chloroquine for longer periods requires strict monitoring as chronic usage may lead to many detrimental effects in the host.**

**Key words:** Malaria, Chloroquine, toxicity, gastrocnemius muscle, spleen.

## INTRODUCTION

Malaria is a major global health problem, with an estimated 300 to 500 million clinical cases occurring annually and 1.5 to 2.7 million deaths, predominantly in children, mostly among the children of Sub-Saharan Africa (Breman, 2001).

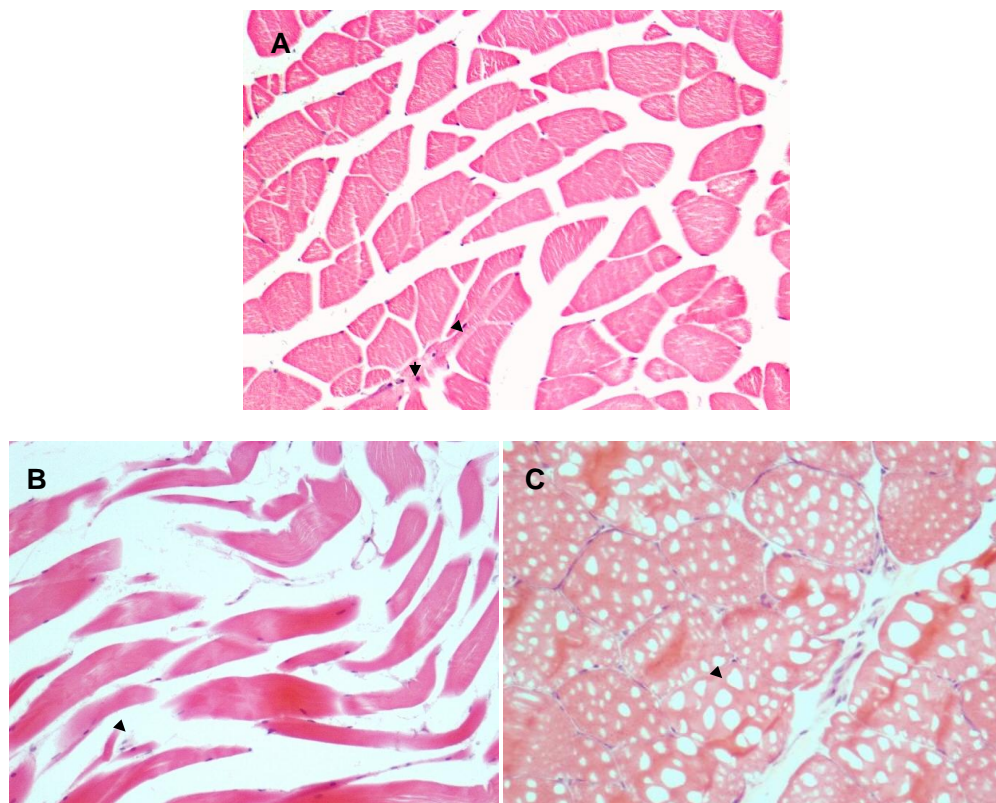
Chloroquine was first synthesized in Germany, but was not recognized as a potent antimalarial drug until the 1940s as part of the US World War II military effort. By 1946, it was found to be far superior to other contemporary synthetic antimalarial drug (Coggeshall and Craige, 1949). It became the corner stone of anti-malarial chemotherapy for the next 40 years. Chloroquine quickly became the main drug of choice globally to treat uncomplicated *Plasmodium falciparum* infections, for instance as part of the Global Malaria Eradication Campaign, launched by the WHO in 1955. It is one of the least expensive antimalarial drugs available and is still in widespread use. Chloroquine can be taken both as prophylactic and as a treatment.

Despite much research during the last 40 years, the exact mechanism by which chloroquine kills the malaria parasite remains controversial (Foley and Tilly, 1997; Foote and Cowman, 1994; Peters, 1998). The drug chloroquine inhibits DNA and RNA biosynthesis and produces rapid degradation of ribosomes and dissimulation

of ribosomal RNA. Inhibition of protein synthesis is also observed, evidently as a secondary effect. Inhibition of DNA replication is proposed as a general mechanism of the antimicrobial action of chloroquine. Chloroquine accumulates in very high concentrations in the parasite food vacuole (Geary et al., 1990). Once in a food vacuole, chloroquine is thought to inhibit the detoxification of heme. Chloroquine then becomes protonated (to CQ<sup>2+</sup>) as the digestive vacuole is known to be acidic (pH 4.7); chloroquine then cannot leave by diffusion. Chloroquine caps hemozoin molecules to prevent further biocrystallization of heme, thus leading to heme buildup. Chloroquine binds to heme (FP) to form what is known as the FP-Chloroquine complex; this complex is highly toxic to the cell and disrupts membrane function. Action of the toxic FP-Chloroquine and FP results in cell lyses and ultimately parasite cell auto digestion. In essence, the parasite cell drowns in its own metabolic products.

Earlier studies show many adverse effects of chloroquine on tissue (Okpako and Aziba, 1989; Warhurst and Robinson, 1996; Ebong et al., 1999). Chloroquine is a potent autophagic drug that may lead to cellular degradation of hepatocytes in the liver with the concurrent production of vacuoles (Abraham et al., 1968). Observed increases in the number of lysosomes suggest





**Figure 1.** L.S. of control muscle and treated muscle under high magnification (40 $\times$ ). (A) Control muscle: showing distinct nuclei on peripheral and parallel fibers; (B) Treated muscle: showing disorganization of muscle fibers with nuclear pycnosis and nuclear proliferation at the periphery; (C) Treated muscle: showing nuclear disorganization with evidence of nuclear pycnosis and vacuolation of muscles fibres.

further cellular degradation. This is accompanied by fusion of lysosomes with autophagic vacuoles resulting in the biogenesis of new lysosomes (Ericsson, 1969). The reported accumulation of chloroquine in lysosomes has an apparent destabilizing effect on lysosomal membranes. Toxic manifestations appear rapidly within 1 to 3 h after ingestion (Jaeger and Flesch, 1994). Thus, information is needed about its effects on organs where the drug accumulates so as to gain insight into the impact of the long term administration of this drug.

## MATERIALS AND METHODS

Twenty four adult male Swiss albino mice weighing between 30 and 40 g were selected for the study. Animals were housed in well ventilated wire meshed cages, exposed to a 12 h light cycle in an air conditioned atmosphere at a temperature of  $26\pm 2^{\circ}\text{C}$  and provided with food and water *ad libitum*. Animals were divided into two groups, Groups I and II; where Group I served as an untreated control and Group II as the chloroquine treated test group.

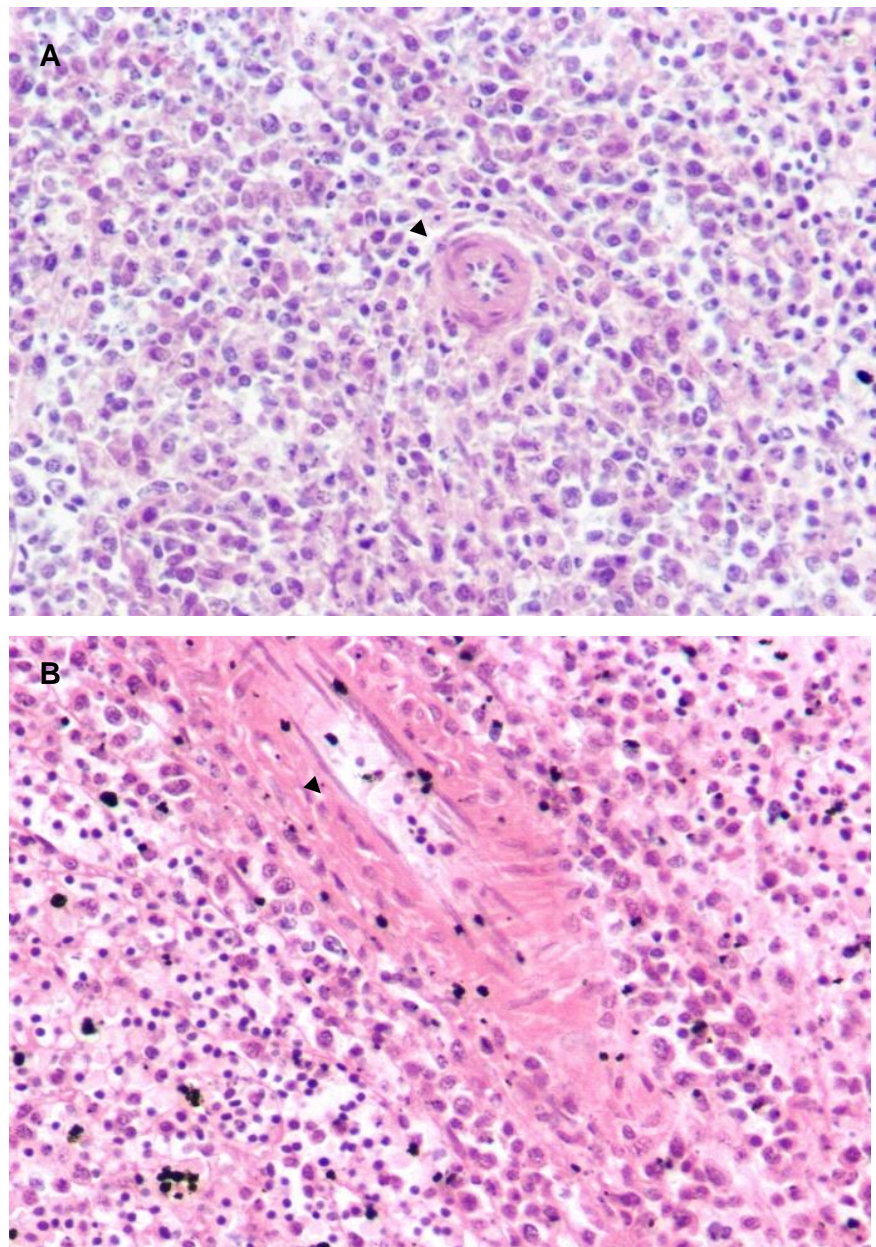
Chloroquine phosphate (99.3% Pure) and other chemicals were obtained from Sigma Aldrich (UK). The prophylactic drug chloroquine phosphate was dissolved in single distilled water. The dose of the drug was selected on the basis of its oral  $\text{LD}_{50}$ , that is, 500 mg/kg body weight for mice (Walum, 1998). The test drug was

administered orally at a dose level of 200 mg/kg body weight for 45 days. A dose less than 200 mg/kg body weight did not produce significant results in other tissues while a higher dose resulted in significant toxicity (Dattani et al., 2009). Hence, to evaluate the impact of an intermediate dose in the present study, the aforementioned dosage was selected.

At the end of treatment on the 46th day, animals were sacrificed. Spleen and gastrocnemius muscle of control and treated animals were dissected out. Histological studies of spleen and muscle were carried out by using the standard technique of haematoxylin and eosin staining.

## RESULTS

Histological examination of the muscle of treated animals under low magnification showed disorganized muscle bands with increased muscle fibre diameter, disrupted muscle fibres in the fasciculae bundle with alteration and broadening of the fibre diameter. Under high magnification, nuclear disorganization and nuclear pycnosis were evident in transverse section (T.S) and alteration of the diameter with a distinct nucleus was exhibited in the longitudinal section (L.S). (Figure 1; A to C). Disorganization and vacuolation of muscle fibres with



**Figure 2.** L.S. of control spleen and treated spleen under high magnification (40 $\times$ ). (A) Control spleen: showing normal organization of red pulp with megakaryocytes; (B) Treated spleen: showing red pulp disorganization and an increased number of megakaryocytes with an increased number of mast cells.

nuclear pycnosis and nuclear proliferation at the periphery was also visible in the L.S. under high magnification. However, no significant changes were observed in the sarcolemma of muscle from treated animals when compared with the control group. The spleen of treated animals showed disorganization of megakaryocytes with cells around the trabaculae, disorganization of red pulp and an elevated number of mast cells under high magnification. Corpuscles were also seen to be spread uniformly (Figure 2; A and B).

## DISCUSSION

Malaria is a disease that was on the verge of eradication once, but has recently returned with greater vigor. This calls for greater preventive and curative treatments and better procedures for disease control. Widespread use of antimalarial drugs further demands a critical evaluation of drug toxicity as well as damage to the tissues. In the present study, administration of chloroquine for 45 days revealed anomalies which could be clearly attributed to

the toxicity of this drug. The histological results confirmed the lysomotropic nature of chloroquine, where disarray of muscle fibre with alteration and broadening of the fibre diameter and nuclear pycnosis were observed, which is attributed to lipidosis. Histochemically, McDonald and Engel (1970) have shown that extensive phospholipid accumulation takes place in muscle fibers. Such an increase was noted earlier in the liver and kidney of miniature pigs (Lullmann et al., 1970; Mastuzawa and Hostelter, 1980). Histologically in spleen, corpuscle degradation around sinusoids, scattering of cells and degradation of red pulp was observed. The increased megakaryocytes, blast cells and mast cells suggest a possible alteration in haemopoiesis (Othman and Arowolo, 1998).

Conclusively, the results of our study suggest that prolonged exposure to the antimalarial drug chloroquine phosphate potentiates adverse effects on vital tissues of the host. Contemplating the risks to humans due to the widespread use of these quinoline derivatives, these findings suggest the necessity of proper instructions and careful monitoring by doctors when prescribing chloroquine for longer duration as it can produce a number of undesirable effects. Furthermore, this work also identifies the need for more of such studies in future which could throw light on other aspects of antimalarial drug toxicity and its therapeutic treatments.

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