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Flavocoxid, dual inhibitor of cyclooxygenase-2 and 5-lipoxygenase, exhibits neuroprotection in rat model of ischaemic stroke

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ABSTRACT

The efficacy of flavocoxid, a prescription medical food used in osteoarthritis in the USA, containing natural flavonoids, baicalin and catechin in experimentally induced cerebral ischaemia in rats was evaluated. Rationale behind the study was that the transient acute ischaemic attack triggers neuroinflammatory cascade. Global cerebral ischaemia was induced transiently by occluding both common carotid arteries for 15 min followed by restoration of perfusion. Flavocoxid (50, 100, 200 mg/kg; p.o.) pre-treatment was instituted 6 days prior to surgery and fluoxetine (10 mg/kg, p.o.) and rivastigmine (2 mg/kg, p.o.) as a standard treatment for depression and cognition impairment was implied from day 1 after the surgery. Different behavioural, biochemical, neurochemical tests, molecular markers of inflammation e.g. tumour necrosis factor-α, interleukin-1 beta, and nuclear factor-kappa B levels and infarct volume were determined. Flavocoxid's strong antioxidant properties figured out from the decreased level of lipid peroxidation and protection of endogenous antioxidants like reduced glutathione and superoxide dismutase. It also reduced TNF-α, IL-1β, and NF-κB levels, and infarct volume as well as protected the loss of biogenic amines in brain tissue of ischaemic rats. This dual inhibitor of cyclooxygenase-1 and 2 with additional 5-lipoxygenase inhibition activity might be useful as a potential neuroprotectant medical food in ischaemic stroke prone patient population.

1. Introduction

Stroke is a devastating illness second only to cardiac ischaemia as a cause of death worldwide and is a leading cause of long term disability worldwide (Iadecola and Anrather, 2011). Stroke causes a wider range of physical and cognitive disabilities than any other chronic condition (Taylor et al., 2011). The protection of neurons of the penumbra area is a prime goal of the neuroprotection approach in the management of ischaemic attack. This can be achieved by institution of substances which could block one or more pathways of the generation of neurotoxic products. One such approach is to inhibit the inflammatory pathway of neurodegeneration.

The role of dual inhibitor of cyclooxygenase (COX) and lipoxygenase (LOX) is being evaluated continuously in various pathological conditions e.g. for the management of neoplasia (Barry et al., 2009; Shi et al., 2011), neuro-inflammation (Klegeris and McGeer, 2002), and acute pancreatitis (Polito et al., 2010). COX-1 and COX-2 are two isoenzymes: COX-1 is constitutively expressed in virtually all cell types (Phillis et al., 2006) but COX-2 is generally induced upon activation of inflammatory mediators (Minghetti, 2007). COX-2 induction after ischaemia has been observed in neurons, vascular cells and microglia (Nogawa et al., 1997). Active COX-2 induces production of superoxide anions and prostanoids; theoretically, these are the major contributors of neuronal damage. It has been reported that the inhibition of COX alone shunts the arachidonic acid (AA) metabolism towards the leukotriene pathway which produces potent inflammatory leukotrienes such as leukotriene (LT) B_4, LTC_4, LTD4 (Martel-Pelletier et al., 2003). We searched for the molecules which can act on both pathways of AA metabolism. Our search narrowed down to flavocoxid, a dual COX-2 and 5-LOX inhibitor. Rationale behind the selection of this formulation is that it is of botanical origin, having comparable anti-inflammatory property to standard anti-inflammatory drugs (Levy et al., 2009; Messina et al., 2009) and also having additional antioxidant properties by virtue of the presence of flavonoid constituents. The composition of flavocoxid (Limbrel®) is >90% purified mixture of baicalin and catechin at a ratio of approximately 4.5:1 with the remainder being excipient (5–6%) and water ~3%. The key components i.e. baicalin and catechin come under GRAS category. For an ingredient to be recognised as GRAS (US-FDA GRAS notification, accessed on 27/01/2014), it requires technical demonstration of non-toxicity and safety, general recognition of safety through widespread usage, and agreement of the safety by the experts in the field [Volume 21 CFR Section 172.345 (f)]. The formulation is available in the US market since 2004 and after its release an open label post marketing surveillance study was conducted in 2010 (Pillai et al., 2010). There are some reports suggesting that flavocoxid treatment causes liver injury but the biggest limitation of this case report is

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that the frequency and mechanism of this effect was not clear (Chalasani et al., 2012). The report suggests that only in 4 female patients out of 877, the causality assessment suggested that in 3 patients, it is highly likely and in one it is only a possible cause. The product information sheet of Limbrel® also suggested that in that case this medical food should always be administered with the supervision of a physician. In light of these kind of studies the reply of R. M. Levy from Primus Pharmaceutical clarifies the issue that the occurrence of hypersensitivity pneumonitis comes under rare events (as in mid-2010, around 270,000 prescriptions were given and the incident of this event was estimated to be 0.0023%). The package insert has been revised timely that clearly mentions these rare events, continuous physician watch is suggested for the monitoring of any untoward event.

The conventional Indian and American diet usually lacks the presence of specially formulated flavonoid content of Limbrel®. The quantity of daily flavonoid generally would need to be significantly higher for patients with hypochlorhydria or low intrinsic factor, both of which occur most often in the elderly population. So the use of flavocoxid in stroke prone population (elderly population) as a prescribed medical food can provide a better neuro-protective approach for them. Recent clinical efficacy trial of flavocoxid itself demonstrate a renewed interest in the use of natural molecule for the management of osteoarthritis (Levy et al., 2009), and this can be translated to some other diseases which have inflammation as their major mode of pathogenesis e.g. neuro-inflammatory disease (ischaemic stroke, Parkinson's disease, Alzheimer's disease, muscular necrosis, etc.).

2. Materials and methods

2.1. Animals

Male Wistar rats (180–230 g), bred in Central Animal House facility of Panjab University, Chandigarh, India were used. The rats had free access to rodent feed and water and were exposed to natural dark/light cycle. All behavioural experiments were carried out between 0900 and 1600 h. The experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC/282/30/8/2012), Panjab University, Chandigarh, India, and conducted according to the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines on the use and care of experimental animals.

2.2. Drugs

Flavocoxid (Limbrel®) was a kind gift from Primus Pharmaceuticals, Inc. (Scottsdale, AZ, USA). Rivastigmine tartarate was procured from Sun Pharmaceutical Industries Ltd. Silvassa, India. Fluoxetine hydrochloride was procured from Cadila Pharmaceuticals Ltd., Jammu and Kashmir, India.

2.3. Experimental protocol

The animals were randomly divided into eight groups with 8 animals in each group. Group I animals were sham operated; Group II comprised of rats subjected to transient global cerebral ischaemia (TGCI); Groups III, IV and V comprised of TGCI rats receiving flavocoxid in doses of 50, 100, and 200 mg/kg, p.o. respectively; Group VI animals received fluoxetine 10 mg/kg, p.o. as a standard antidepressant drug; similarly Group VII animals received rivastigmine 2 mg/kg, p.o. as a standard nootropic. Group VIII served as per se group for the sham operated group and received flavocoxid 200 mg/kg p.o.

2.3.1. Treatment schedule and experimental procedure

Flavocoxid was administered orally in a constant volume of 0.5 mL/100 g body weight of rat. Sham operated group and TGCI induction group received only 0.9% saline solution. Flavocoxid pre-treatment was given for 6 days before induction of TGCI and continued till the day before the sacrifice of the animals. However in fluoxetine and rivastigmine groups, the treatment was started just after the induction of TGCI (Fig. 1).

2.3.2. Induction of transient global cerebral ischaemia (TGCI) in rats

TGCI was induced in rats by bilateral common carotid arteries occlusion (BCCAO). The animals were anaesthetised with thiopentone sodium (40 mg/kg, i.p.) and occlusion was done for 15 min using clamps followed by continuous reperfusion as per the modified method of Jingtao et al. (1999). Sham operated animals were subjected to surgery but without BCCAO.

2.4. Behavioural assessment

All the animals were subjected to the following behavioural test batteries and nonparametric tests were observed by a test blind observer.

2.4.1. Evaluation of motor, sensory-motor functioning and activity

Brain stem reflex and motor functioning were evaluated by a battery of six tests performed sequentially on each rat for neurological scoring after TGCI (Bederson et al., 1986; Shapira et al., 2002). In the hanging wire test, the cut-off time was kept at 90 s from a wire placed 60 cm above a foam pillowed surface and the fall off time (seconds) from the wire was recorded (Hunter et al., 2000).

The sensory-motor function was evaluated by modified inclined beam walking test, forelimb placing test and corner test. The inclined beam test consists of movement of rat on an inclined beam (160 cm in length, 60° in inclination) and the time to cross the beam was determined and scored accordingly (Jin et al., 2010). In the corner test, turns out of ten trials for each rat were determined; the turns which are not part of rearing movement were not scored. The percentage of the number of turns to total trials was calculated. Gross vestibular motor function after TGCI was evaluated by using beam balance test (Jin et al., 2010). Vibrissae evoked forelimb placing test (Zvejniece et al., 2012) was modified slightly using stimulation of the rat's vibrissae to trigger a placing response.

Motor co-ordination was determined by using a rotarod apparatus (INCO, Ambala, India). Animals were pre-acclimatised to a rotating rod (25 rpm) on the very 1st day when the protocol started. The recording was done on 6th (pre-TGCI) and 11th (post-TGCI) day. 90 s was set as the cut-off time (Dhir et al., 2008). Locomotor activity was recorded by a photoactometer (IMCORP, Ambala, India). Animals were pre-acclimatised for 3 min on day 1 and recording was done on the 6th and 11th day for 5 min with a 3 minute habituation period (Gaur et al., 2009).

2.4.2. Evaluation of depressive status

The hyper-emotionality test includes four behavioural responses viz. attack, startle, struggle and flight. The presence or absence of vocalisation was added to each response. The method and scoring pattern for the test were adopted from Takahashi et al. (2011). The forced swim test (FST) was used to measure the immobility time in a water filled tank providing inescapable condition and was according to the specification provided by the original method of Porsolt et al. (1977).

2.4.3. Evaluation of post-stroke cognition status

For short term memory evaluation after TGCI, a modified passive avoidance response (PAR) test was employed (Zvejniece et al., 2012). The long term memory was evaluated using Morris' water maze (MWM) task (Morris et al., 1982; Tuzcu and Baydas, 2006). All the groups received this water maze training from day 1 to day 4 and the retrieval of escape latencies (in seconds) was determined on day 5 (pre-TGCI). After the TGCI, the retention of memory by discovering escape platform by the rats was determined on the last day of the protocol (19th day). In post-TGCI retrieval, the time spent on target quadrant (TSTQ) (Tiwari and Chopra, 2013; Tuzcu and Baydas, 2006) was also determined to evaluate the consolidation of memory by removing the