Niger. J. Physiol. Sci. 33(June 2018) 001-008
www.njps.com.ng

Niger. J. Physiol. Sci.

Effect of Caffeine on Serum Tumour Necrosis Factor Alpha and Lactate Dehydrogenase in Wistar Rats Exposed to Cerebral Ischaemia-reperfusion Injury

Mubarak Muhammad*[1], Abbas Bubakar El-ta'alu[1], Mohamed Mabrouk A[1], Isyaku Umar Yarube[1], Jibril Muhammad Nuhu[2], Ibrahim Yusuf[3], Usman Aliyu Daneji[4]

Departments of [1]Human Physiology, [2]Physiotherapy, [3]Pathology and [4]Biochemistry, College of Health Science, Bayero University Kano, Nigeria.

Summary: Caffeine is known to confer neuro-protection via A_1 and A_{2A} adenosine receptor antagonism in which adenosine neuro-modulates excitotoxic release of glutamate. Currently, it is unclear whether caffeine modulates inflammation in ischaemic stroke model. The present study examined effects of caffeine following ischaemia-reperfusion injury on neuro-inflammatory tumour necrosis alpha (TNF-α), lactate dehydrogenase (LDH), as well as effect of caffeine against brain ischaemic damage on histology. Thirty three adult male Wistar rats (180-300 g) were used in this study. They were randomly divided into four groups (n=5 each): Group I (Control) that received neither the operation nor any treatment; Group II (Sham/Water) received a *pseudo*-ischaemic-reperfusion and 1ml water for injection; Group III (BCCO/Water) that received complete bilateral common carotid occlusion (BCCO) and 1ml water for injection; Group IV (BCCO/Caffeine) that received complete BCCO and caffeine solution intraperitoneally at a dose of 50% LD_{50} value (144mg/kg); and thirteen rats were used for LD_{50} assessment. Sensory and motor functions significantly ($p<0.05$) decreased in the rat following ischaemia-reperfusion injury when compared to pre-injury state on Garcia neurological score. Caffeine reduced brain ischaemic injury and significantly reduced ($p<0.05$) TNF-α activity. While no significant effects ($p>0.05$) of caffeine was observed on LDH activity. This study has shown neuro-protective roles of caffeine against ischaemia-reperfusion damage to brain tissue, inflammatory TNF-α activity, but not on LDH activity.

Keywords*:* Caffeine, ischaemia-reperfusion injury, cerebral ischaemia, neuro-inflammation, stroke.

**Address for correspondence*: mubarakmahmad@yahoo.com

Manuscript Accepted: May, 2018

INTRODUCTION

Stroke is the world's second-leading cause of death and a major factor in causing disabilities in humans (Kim *et al*., 2015). Stroke incidence as at 2010 worldwide was around 17 million persons per year, with a total of 33 million people being still alive after stroke, with about 70 % of all stroke patients staying with residual symptoms (Douven *et al*., 2016). Brain stroke results from either vessel occlusion (ischemic stroke) or cerebral blood-related neurotoxicity (haemorrhagic stroke). Thus, stroke is classified into ischaemic and haemorrhagic types. While the former type accounts for 85% of all strokes, the latter accounts for 15% of all incidence of strokes (Wang *et al*., 2015). Ischaemia-reperfusion injury is the exacerbation of tissue damage when blood supply is re-established in a previously ischaemic organ (Kalogeris *et al*., 2014). Neuro-inflammation that is mediated by microglia and astrocytes plays a critical role in neuro-degeneration (Melani *et al*., 2009) and is a major factor known to contribute to the expansion of ischaemic lesions following ischaemic stroke (Simats *et al*., 2015). Neuro-inflammation during cerebral ischaemia is associated with a series of cascading cellular events including enhancement of prior glutamate excitotoxicity and production of a plethora of inflammatory mediators that trigger apoptosis and exaggerate neuronal damage (Brothers *et al*., 2010). Biomarkers of neuro-inflammation offer an objective and quantitative data on neurochemical mechanisms of brain inflammation, and a number of potentially neuro-protective strategies are based on mediating inflammatory mediators (Piskunov, 2010). Tumour necrosis factor alpha (TNF-α) in the serum is a sensitive marker of inflammation and tissue injury whose concentration increases rapidly in brain tissue

following hypoxia-ischaemia (Graham *et al*., 2016). Lactate dehydrogenase (LDH) is a cytoplasmic cellular enzyme present in essentially all major organ systems, and in the extracellular space, although of no further metabolic function in this space, are still of benefit because they serve as indicators suggestive of disturbances of the cellular integrity induced by pathological conditions (Kato *et al*., 2006).

Adenosine is an endogenous neuro-modulator, which acts on at least four distinct receptor subtypes: A_1, A_{2A}, A_{2B}, and A_3, all of which are expressed in the brain (Fredholm *et al*., 2001), with A_1 and A_{2A} receptors of critical importance in ischaemic damage after stroke (Chen *et al*., 1999; Yang *et al*., 2013). Adenosine via A_1 and A_{2A} receptors modulates presynaptic release of excitotoxic glutamate. There is concomitant elevation of both adenosine and glutamate in response to cerebral ischaemia (Dai *et al*., 2010) and adenosine neuro-modulation via A_{2A} receptor stimulates glutamate outflow from neurons and glial cells, thereby contributing to glutamate excitotoxicity (Wardas, 2002; Marchi *et al*., 2002; and Pedata, 2008). Caffeine (1, 3, 7-trimethylxanthine) exerts its physiological effects in the central nervous system (CNS) through antagonism of A_1 and A_{2A} adenosine receptors (Nehlig, 2002), and increase in sympathetic nervous system (SNS) activity by stimulating α and β-adrenoreceptors (Belza *et al*., 2009; Joy *et al*., 2016). Caffeine has been well established to offer neuro-protection via A_1 and A_{2A} adenosine receptors antagonism to intervene glutamate excitotoxicity in ischaemic stroke model (Rudolphi et al., 1989; Bona *et al*., 1995; Alexander *et al*., 2013). There is also evidence that adenosine A_{2A} receptors are also involved in regulating factors responsible for the detrimental inflammatory effects in brain ischemia (Melani *et al*., 2009). This evidence seemingly suggested that, post-ischaemic A_{2A} receptor blockade using caffeine might also offer protection against neuro-inflammation, which currently is yet to be investigated. Hence this study examined effects of caffeine following experimentally induced ischaemia-reperfusion injury on neuro-inflammatory tumour necrosis alpha (TNF-α), lactate dehydrogenase (LDH), as well as the effects of caffeine on brain ischaemic damage on histology.

MATERIALS AND METHODS

Chemicals and Equipment: Analytical grade caffeine powder, ketamine hydrochloride and water for injection were sourced commercially from ScP chemical limited. Elisa kit for TNF-α (Catalogue number E-EL-R0019) was sourced from Elabscience Company, China, while Elisa kit for LDH was sourced from Agappe Diagnostics, Switzerland.

Experimental Animals: Thirty three adult male Wistar rats weighing 180-300 g were used for the study. They were housed in plastic cages at room temperature (32^0C), monitored under normal photoperiod and fed with standard animal feed and water *ad libitum*. Animal care and handling were conducted in accordance with ethical provisions of Bayero University Kano.

Acute Toxicity (LD_{50}) Study: An initial acute toxicity study was conducted by the method of Lorke (1983) using 13 adult male Wistar rats to determine the dose range of caffeine to be used for the study. In the first phase, nine rats were divided into three groups of three rats each; and treated with caffeine solution at doses 10, 100, and 1000 mg/kg intraperitoneally, after which they were observed frequently for 24 hours for signs of toxicity. All the animals in the group given 1000 mg/kg developed convulsions and died three hours after caffeine administration. The result obtained from the first phase determined the dose given in the second phase which was 140 mg/kg, 225 mg/kg, 370 mg/kg and 600 mg/kg selected from Lorke's standard table. One animal per group was used in the second phase, and the animal given 370 mg/kg and 600 mg/kg were dead within 24 hours in the second phase. The LD_{50} value was finally calculated to be 228 mg/kg accordingly (Akhila *et al*., 2007).

Experimental Design: Twenty rats were randomly divided into four groups of five rats each (n=5) and treated as follows:

Group I (Control): received neither the operation nor any treatment;
Group II (Sham/Water): received sham operation (i.e. bilateral carotid arteries were exposed but without occluding them) and 1 ml water for injection intraperitoneally (*i.p*);
Group III (BCCO/Water): received complete bilateral common carotid occlusion (BCCO) for 5 minutes and 1 ml water for injection *i.p*;
Group IV (BCCO/Caffeine): received complete BCCO for 5 minutes and caffeine solution intraperitoneally at a dose of 144 mg/kg.

Bilateral common carotid artery occlusion: BCCO was carried out to induce cerebral hypo-perfusion. Rats were food-deprived overnight before surgery in order to stabilize plasma glucose, and surgical procedure was performed between 9.00 am and 12 noon in order to minimize diurnal variations (Aytac *et al*., 2006). Rats were anaesthetised with 50 mg/kg of ketamine hydrochloride *i.p*, and the level of anaesthesia was assessed by the absence of withdrawal reflex, additional anesthesia was administered if necessary. A midline skin incision in the neck was performed and the common carotid arteries were identified lateral to trachea and behind sternomastoid

muscle and carefully isolated from the accompanying nerve. Bilateral carotid arteries were then completely occluded for 5 minutes followed by reperfusion. The wound was then closed with silk sutures and the animals were monitored until consciousness was regained.

All treatments were administered at 1, 3, and 6 hours after rats fully recovered from anaesthesia. After 24-hour following the surgery, rats were sacrificed under anaesthesia and blood was collected by cervical decapitation into plain bottles. The samples were allowed to clot and then centrifuged at 15,000 rpm for 20 minutes and the sera were acquired and used for determination TNF-α and LDH contents.

Neurological Assessment Using Garcia Score

Garcia *et al*., (1995) neurological evaluation of sensory and motor function was carried out in the experimental group that received BCCO prior to and after the BCCO procedure to assess the effect of ischaemia-reperfusion injury. The test consist of six components; spontaneous activity, symmetry in the movement of four limbs, symmetry of fore limb, climbing, body proprioception, and response to vibrissae. Test evaluations were carried out by an observer who was blinded to the experimental procedure and the score assigned to each rat at completion of the evaluation equals to the sum of all six test scores. The highest score possible of Garcia score is 18, while the least is 3.

Laboratory Procedures

Serum level of TNF-α and LDH were analysed using their respective ELISA kits following the manufacturer's manual. The serum level of TNF-α was analysed by method of Sandwich-ELISA which is based on the principle that, optical density (OD) of the standard preparation measured using sphectrophotometer at a wavelength of 450 nm is proportional to the concentration of TNF-α. Briefly, 100 micro litres (µL) of the serum samples were added to micro ELISA plate wells, followed by successive addition of biotinylated detection antibody, avidin-horseradish peroxidase (HRP) and stop solution (sulphuric acid) to each micro ELISA plate well, and OD was then immediately measured at 450 nm wavelength. The LDH kit principle was based on the reversible reaction catalyzed by lactate dehydrogenase enzyme to convert pyruvate to lactate. Accordingly, 24 ml of reagent R1 which composed of Tris buffer (pH 7.4), pyruvate, and sodium chloride, was mixed with 6 ml of reagent R2 which composed of NADH to obtain a working reagent, which was added to each 10 µL of sample and the change in absorbance per minute (ΔOD/min) was measured after 1, 2, and 3 minutes.

Histological Evaluation of the Brain Cortex

One rat brain from each group was randomly selected and used for histological analysis. Immediately after harvesting, the brain was fixed in 10% formalin after which it was allowed to dehydrate in ascending grade of alcohol. Brain tissue was then cleared from alcohol for 2 hours using xylene after which it was embedded in molten wax and allowed to solidify for 2 hrs. Section was cut using microtone at 5 microun after which it was subsequently stained (Chatterjee, 2014).

For staining, each section was de-waxed in xylene for 1 min before dehydrating in descending grades of alcohol starting with absolute alcohol for 30 secs. Slides were then inserted in Harris hematoxylene solution for 10-15 mins after which it was rinsed in distilled water, and then washed in Scotts tap water for 3 min. Slides were then differentiated in acid alcohol and washed in distilled water. They were then counter stained with Eosin, washed with distilled water and then dehydrated in ascending grades of alcohol. Slides were finally cleared in xylene and then mounted using mounting medium DPX (Ravikumar *et al*., 2014).

Statistical Analyses: Obtained data were processed using Statistical Package for Social Science (SPSS), version 20 and expressed as Mean ± Standard Error of Mean (SEM). Mean values of TNF-α and LDH from control and experimental groups were compared for differences using one-way analysis of variance (ANOVA) with Schaffe post hoc test, while mean values of Garcia scores were compared using paired t test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Neurological Evaluation

There was significant neurological deficit following ischaemia-reperfusion injury for the animals in both groups III and IV as indicated by decreased Garcia scores (P = 0.001) (Figure 1).

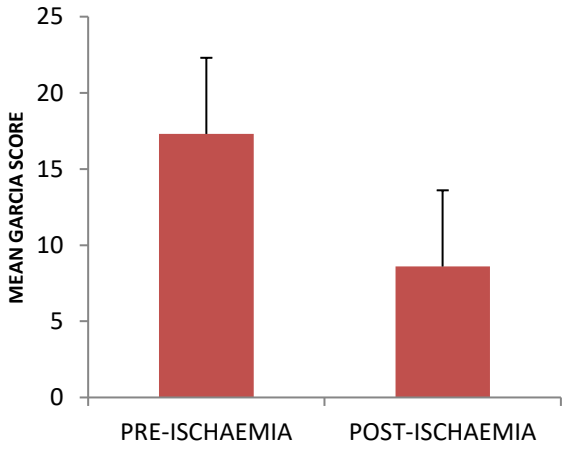

Figure 1: Neurological evaluation on Garcia score following ischaemia-reperfusion injury in rats.Values on each bar represent the means of both group III and IV at pre and post ischaemic reperfusion stage. Mean difference are statistically significant (P = 0.001).

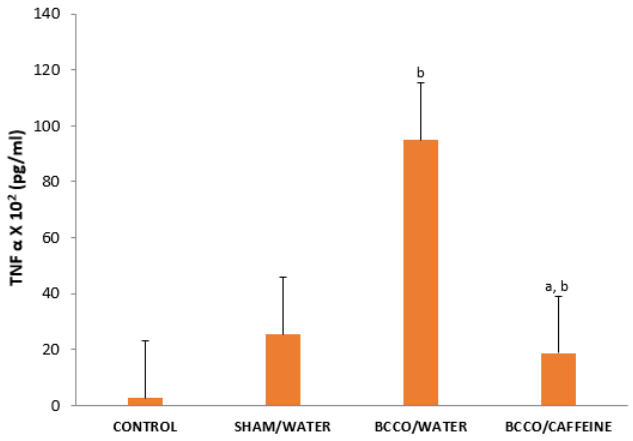

Figure 2: Effect of caffeine (144 mg/kg, i.p) on serum level of TNF-α (pg/ml) following ischaemia-reperfusion injury in rats. [a]Mean differences is statistically significant compared to control (P = 0.001). [b]Mean differences is statistically significant compared to BCCO/WATER (P = 0.001).

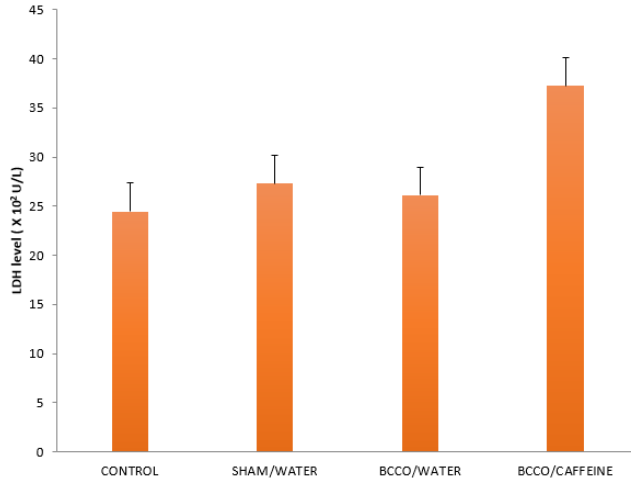

Figure 3: Effect of caffeine (144 mg/kg, i.p) on serum level of LDH following (U/L) ischaemia-reperfusion injury in rats. Mean difference are not statistically significant (P = 0.409).

Tumour Necrosis Factor Alpha Level

TNF-α level was significantly higher in the BCCO/WATER group compared to the control (P = 0.002), indicating that BCCO induced increase in TNF-α. The TNF-α level of BCCO/CAFFEINE group is significantly lower than that of the BCCO/WATER group (P = 0.009). This shows that caffeine treatment has reduced the raised TNF-α value seen in the BOOC/WATER group back to normal (Figure 2).

Lactate Dehydrogenase (LDH) Level

There is no major difference in LDH level of BCCO/WATER group compared to the control (P=0.997). The LDH level of BCCO/CAFFEINE group is non-significantly higher than that of the BCCO/WATER group (P=0.593) (Figure 3).

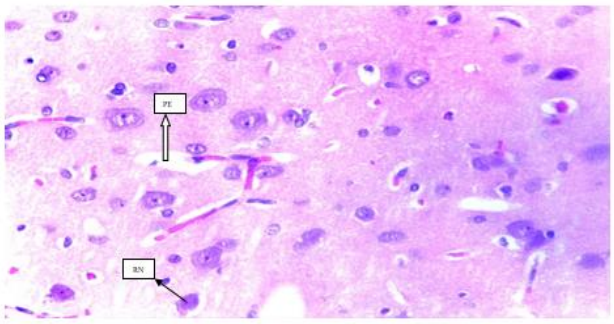

Plate IA: Photomicrograph of cerebral cortex of Control group rat. Note the perivascular edema (PE), red neuron (RN). H & E stain × 400 magnification.

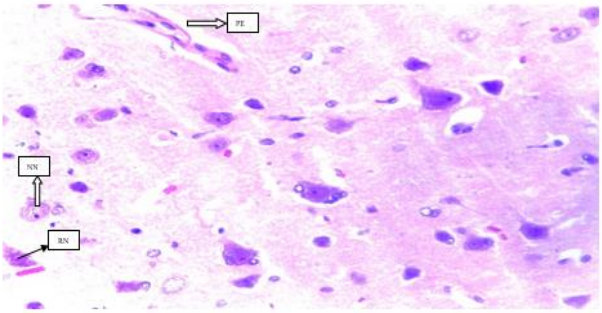

Plate IB: Photomicrograph of cerebral cortex of Sham/Water group rat. Note the perivascular edema (PE), red neuron (RN), normal neuron (NN). H & E stain × 400 magnification.

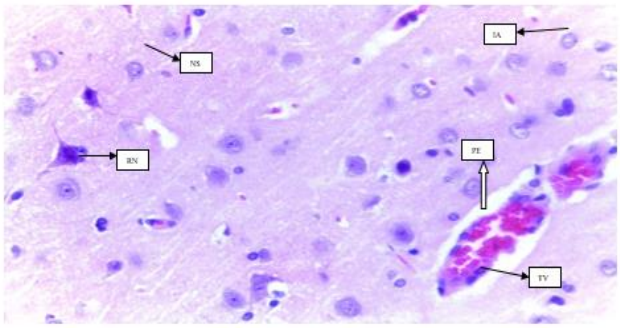

Plate IC: Photomicrograph of cerebral cortex of BCCO/Water group rat. Note the infarct area (IA), perivascular edema (PE), red neuron (RN), thrombosed vessels (TV), neutrophil spongiosis (NS). H & E stain × 400 magnification.

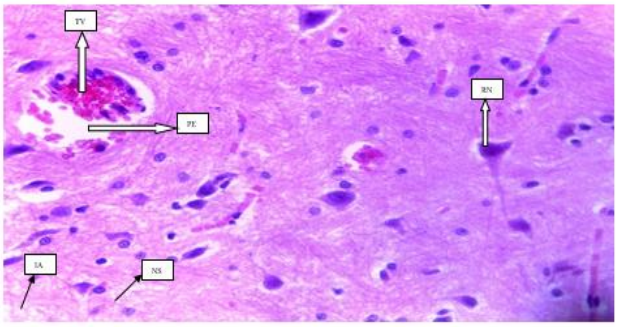

Plate ID: Photomicrograph of cerebral cortex of BCCO/Caffeine group rat. Note the infarct area (IA), perivascular edema (PE), red neuron (RN), thrombosed vessels (TV), neutrophil spongiosis (NS). H & E stain × 400 magnification.

Histological Evaluation of the Brain Cortex:
Plate I shows result of brain cortex histology of rat in control group which received neither the operation nor caffeine (Plate 1A); sham/water group that received sham operation and water (Plate 1B); BCCO/water group that received bilateral common carotid occlusion and water (Plate 1C) and BCCO/Caffeine that received bilateral common carotid occlusion and 144 mg/kg of caffeine (Plate 1E).

DISCUSSION

Pathophysiologic pathways leading to neuronal death during cerebral ischaemia consists of immediate (within minutes) *peri*-infarct depolarization and excitotoxicity. Hours later, inflammation and oxidative stress occurs, while days later, it is followed by apoptosis (Woodruff *et al*., 2011; Muhammad *et al*., 2016). Cerebral ischaemia-reperfusion injury leads to the appearance of different degrees of sensory motor and other impairments, making assessment of neurological symptom in the final outcome of experimental cerebral ischaemic models critical (Zarruk *et al*., 2011; Desland *et al*., 2014). Garcia exam is scored on a scale from 3 to 18 between pre- and post-ischaemic cerebral damage. BCCO ischaemic stroke model has been shown to significantly reduce functional vascular area in the anterior part of the cortex (Gong *et al*., 2013). It has also been reported to moderately decrease cerebral blood flow (CBF) in the forebrain of adult rats into oligaemic range (Schmidt-Kastner *et al*., 2005) and, thus induce sensorimotor deficit. This is in agreement with present finding as Garcia score in post-ischaemic rats was significantly ($p<0.05$) lower in comparison to pre-ischaemic rats scores.

There was significant decrease ($p<0.05$) in serum level of TNF-α at caffeine acute dose of 144 mg/kg administered intraperitoneally, in experimental group compared to control group as revealed by One-way ANOVA (Figure 2). Post-hoc Scheffe test revealed that the significance lies between BCCO/CAFFEINE and BCCO/WATER groups. TNF-α released by microglia is one of pro-inflammatory cytokine that becomes locally expressed in response to acute cerebral ischaemia (Denes *et al*., 2010; Kalogeris *et al*., 2014). TNF-α interacts with two receptors, TNFR1 and TNFR2, to induce extrinsic apoptotic cellular death pathway via Fas associated death domain (FADD) and inflammation via nuclear factor kappa-light- chain enhancer of activated B cells (NFκB), respectively (Sedger and McDermottc, 2014). Numerous neuro-protective agents have shown their neuro-protective functions by attenuating the expression TNF-α in ischaemic stroke models (Mohamed *et al*., 2012; Ewen *et al*., 2013; Yuan *et al*., 2014; Guan *et al*., 2015; Han *et al*., 2016). Caffeine has been reported to provide potentials in modulation of neuro-inflammation by suppressing the generation of pro-inflammatory mediators such as NO, PGE_2 and TNF-α (Kanga *et al*., 2012), and attenuate the number of activated microglia within the hippocampus (Brothers *et al*., 2010) in lipopolysaccharide-induced neuro-inflammation. Hence the finding herein is in conformity with the aforementioned previous findings. The proposed mechanism through which caffeine confers anti-inflammatory effect is through attenuation of glutamate-induced microglia activation (Brothers *et al*., 2010; Kanga *et al*., 2012).

There was no significant difference ($p>0.05$) in serum level of LDH at caffeine acute dose of 144 mg/kg administered intraperitoneally, in the experimental group compared to control group as revealed by One-way ANOVA (Figure 3). Lactate dehydrogenase (LDH) is a ubiquitous cytoplasmic enzyme that catalyses the reversible conversion of pyruvate to lactate within cells during anaerobic glycolysis. It is known to be retained by viable cells with intact plasma membranes and released from cells with damaged membranes (Seetapun *et al*., 2013), and as such elevation of LDH during cerebral ischaemia is primarily due to glutamate induced excitotoxicity (Tikka *et al*., 2001) or secondary to cytokine mediated neuronal damage (Erez *et al*., 2014). Obtained results demonstrated the highest increase in mean value of LDH in experimental rats given the BCCO/CAFFEINE treatment compared to all other groups (Figure 3). This is in sharp contrast to previous findings of numerous neuro-protective agents that were found to reduce LDH release in ischaemic stroke model (Tikka *et al*., 2001; Kagiyama *et al*., 2004; Hurtado *et al*., 2005; Cimarosti *et al*., 2005; Seetapun *et al*., 2013; Cai *et al*., 2016; Ram *et al*., 2016). The reason behind caffeine effect in increasing LDH might be due to its well established physiological effect of phosphodiestrase inhibition (Astrup *et al*., 1990; Acheson *et al*., 2004; Belza *et al*., 2009). It is known that many intracellular signal pathways depend upon production of cyclic adenosine monophosphate (cAMP), and increased cAMP activity is short-lived because it is terminated by an enzyme known as phosphodiesterase which catalyses the conversion cyclic AMP back to AMP. Therefore, inhibition of phosphodiesterase enzyme results in high tissue concentrations of cyclic AMP (cAMP) which has many physiological and pathophysiological implications (Yu *et al*., 2009). One of such implications is sustained activation of protein kinase that results in cAMP-mediated up-regulation/stabilisation of LDH activity. The mechanism is that cAMP activates protein kinases; both protein kinases A (PKA) and C (PKC), which leads to LDH transcriptional gene regulation through stabilisation of LDH-A mRNA and subsequent increase of intracellular LDH-A mRNA levels (Hong *et al*., 2004; Richard *et al*., 2005; Huang *et al*., 2009).

On histological analysis, cerebral ischaemia-reperfusion damage was assessed based on the degree of infarct area (IA), presence of red neurons (RN), perivascular edema (PE), neurophil spongiosis (NS), and thrombosed blood vessels (TV). These were the key histologic features reported in the phase of acute neuronal injury after cerebral infarct (Mena *et al*., 2004). All rats of both experimental and control groups showed the presence of red (eosinophilic) neurons. Red neurons being a manifestation at the cellular level are earliest histological feature in neuronal injury that consisted of increasing cytoplasm eosinophilia, with the nucleus appearing shrunken and darkly basophilic or developing clumped chromatin condensation (Margaritescu *et al*., 2009). The presence of perivascular edema, neutrophil spongiosis and thrombosed vessels indicated vascular changes in acute ischemic stroke. Perivascular edema is secondary to plasma components extravasation in the area of the cerebral infarct, and closely related is neutrophil spongiosis linked to plasma leakage into the adjacent brain. The presence of thrombosed vessels indicates vascular endothelial damage of small arteries adjacent to necrotic zones (Margaritescu *et al*., 2009). The infarct area is due to the necrosis in the hypoperfused cortical regions. Based on revelation of histological finding, the extent of ischaemia-repefusion injury in BCCO/CAFFEINE group rat is reduced in comparison with BCCO/WATER group rat, while the degree of injury in SHAM/WATER group rat is about the same with that of CONTROL group rat.

In conclusion, caffeine significantly reduced serum levels of inflammatory TNF-α as well as manifested noticeable neuro-protective roles against ischaemia-reperfusion damage to the brain tissue, but conversely did not produce significant effect on serum LDH levels.

REFERENCES

Acheson, K.J., Gremaud, G., Meirim, I., Montigon, F., Krebs, Y., Fay, L.B., Gay, L.G., Schneiter, P., Schindler, C. and Tappy, L. (2004). Metabolic effects of caffeine in humans: lipid oxidation or futile cycling? *American Journal of Clinical Nutrition*, 79: 40-46.

Akhila, S.J., Deepa, S.S. and Alwan, V. (2007). Acute toxicity and determination of median lethal dose. *Current Science* 93(7): 917-920.

Alexander, M., Smith, A.L., Rosenkrantz, T.S. and Fitch, R.H. (2013). Therapeutic effect of caffeine treatment immediately following neonatal hypoxic-ischemic injury on spatial memory in male rats. *Brain Science* 3: 177-190.

Astrup, A., Toubro, S., Cannon, S., Hem, P., Breum, L. and Madsen, J. (1990). Caffeine: a double-blind, placebo-controlled study of its thermogenic, metabolic, and cardiovascular effects in healthy volunteers. *American Journal of Clinical Nutrition*, 51: 759-767.

Aytac, E., Seymen, H.O., Uzun, H., Dikmen, G. Altug, T. (2006). Effects of iloprost on visual evoked potentials and brain tissue oxidative stress after bilateral common carotid artery occlusion. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 74; 373-378.

Belza, A., Toubro, S. and Astrup, A. (2009). The effect of caffeine, green tea and tyrosine on thermogenesis and energy intake. *European Journal of Clinical Nutrition* , 63: 57-64.

Bona, E., Aden, U., Fredholm, B.B. and Hagberg, H. (1995). The effect of long term caffeine treatment on hypoxic-ischemic brain damage in the neonate. *Pediatric research* 38(3): 312-318.

Brothers, H.M., Marchalant, Y. Wenk, G.L. (2010). Caffeine attenuates lipopolysaccharide-induced neuroinflammation. *Neuroscience Letters*, 480: 97–100.

Cai, M., Ma, Y., Zhang, W., Wang, S., Wang, Y., Tian, L., Peng, Z., Wang, H. and Qingrong, T. (2016). Apigenin-7-O-β-D-(-6"-p-coumaroyl)-Glucopyranoside treatment elicits neuroprotective effect against experimental ischemic stroke. *International Journal of Biological Sciences*, 12(1): 42-52.

Chatterjee, S. (2014). Artefacts of Histopathology. *J Oral Maxillofac Pathol* 18(1): 111-116.

Chen, J.F. and Pedata, F. (2008). Modulation of ischemic brain injury and neuroinflammation by adenosine A_{2A} receptors. *Curr Pham Des* 14(15): 1490-1499.

Chen,J., Huang, Z., Ma, J., Zhu, J., Moratalla, R., Standaert, D., Moskowitz, M.A., Fink, J.S. and Schwarzschild, M.A. (1999). A_{2A} adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *Journal of Neuroscience* 19(21): 9192-9200.

Cimarosti, H., Siqueira, I.R., Zamin, L.L., Nassif, M., Balk, R., Frozza, R., Dalmaz, C., Netto, C.A. and Salbego, C. (2005). Neuroprotection and protein damage prevention by estradiol replacement in rat hippocampal slices exposed to oxygen-glucose deprivation. *Neurochemical Research*, 30(4): 583–589.

Dai, S., Zhou, Y., An, J., Li, P., Yang, N., Chen, X., Xiong, R., Liu, P., Zhao, Y., Shen, H., Zhu, P. and Chen, J. (2010). Local glutamate level dictates adenosine A2A receptor regulation of neuroinflammation and traumatic brain injury. *Journal of Neuroscience* 30(16):5802-5610.

Denes, A., Thornton, P., Rothwell, N.J. and Allan, S.M. (2010). Inflammation and brain injury: Acute cerebral ischaemia, peripheral and central inflammation. *Brain, Behavior, and Immunity*, 24: 708-723.

Desland, F.A., Afzal, A., Warraich, Z. and Mocco, J. (2014). Manual versus automated rodent behavioral assessment: comparing efficacy and ease of Baderson and Garcia neurological deficit scores to an open field video tracking system. *J Cent Nerv Syst*, 6: 7-14.

Douven, E., Schievink, S., Verhey, F., Oostenbrugge, R., Aalten, P., Staals, J. and Kohle, S. (2016). The cognition and affect after stroke – a prospective evaluation of risks (CASPER) study: rationale and design. *BMC Neurology*, 16(65): 1-11.

Erez, A., Shental, O., Tchebiner, J.Z., Laufer-Perl, M., Wasserman, A., Sella, T. and Guzner-Gur, H. (2014). Diagnostic and prognostic value of very high serum lactate dehydrogenase in admitted medical patients. *IMAJ*, 16: 439-443.

Ewen, T., Qiuting, L., Chaogang, T., Tao, T., Jun, W., Liming, T. and Guanghong, X. (2013). Neuro-protective effect of atorvastatin involves suppression of TNF-α and upregulation of IL-10 in a rat model of intra-cerebral haemorrhage. *Cell Biochemistry and Biophysics*, 66(2):337-346.

Fredholm, B.B., Ijzerman, A.I., Jacobson, K.A., Klotz, K. Linden, J. (2001). International union of pharmacology (XXV) nomenclature and classification of adenosine receptors. *Pharmacological Review* 53: 527-552.

Garcia, J.H., Wagner, S., Liu, K. and Hu, X. (1995). Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. *Stroke*, 26: 627-635.

Gong, H., Shu, L., Xu, H., Chen, B., Mao, R., Zhang, F. and Wang, Y. (2013). Bilateral internal carotid arteries ligation temporary impairs brain vasculaturev in young rats. *Autonomic Neuroscience: Basic and Clinical*, 173: 39-44.

Graham, E.M., Burd, I., Everett, A.D. and Northington, F.J. (2016). Blood biomarkers for evaluation of perinatal encephalopathy. *Frontiersin Pharmacology*, 7(196): 1-12.

Guan, Y., Jin, X., Guan, L., Yan, H., Wang, P., Gong, Z., Li, S., Cao, X. and Xing, Y. (2015). Nicotine inhibits microglial proliferation and is neuro-protective in global ischemia rats. *Molecular Neurobiology*, 51(3): 1480-1488.

Han, J.U., Lee, E., Moon, E., Ryu, J.H., Choi, J.W. and Kim, H. (2016). Matrix metalloproteinase-8 is a novel pathogenetic factor in focal cerebral ischemia. *Molecular Neurology*, 53(1): 231-239.

Hong, S., Gibney, G.T., Esquilin, M., Yu, J. and Xia, Y. (2004). Effect of protein kinases on lactate dehydrogenase activity in cortical neurons during hypoxia. *Brain Research*, 1009(1): 195-202.

Huang, D., Hubbard, C.J. and Jungmann, R.A. (2009). Lactate dehydrogenase A subunit messenger RNA stability is synergistically regulated via the protein kinase A and C signal transduction pathways. *Molecular Endocrinology*, 9(8): 888-889.

Hurtado, O., Moro, M.A., Cardenas, A., Sanchez, V., Fernandez-Tome, P., Leza, J.C., Lorenzo, P., Secades, J.J., Lozano, R., Davalos, A., Castillo, J. and Lizasoaina, I. (2005). Neuro-protection afforded by prior citicoline administration in experimental brain ischemia: effects on glutamate transport. *Neurobiology of Disease*, 18: 336-345.

Joy, J.M., Vogel, R.M., Moon, J.R., Falcone, P.H., Mosman, M.M. and Kim, M.P. (2016). Twelve weeks supplementation with an extended-release caffeine and ATP enhancing supplement may improve body composition without affecting hematology in resistance-trained men. *Journal of the International Society of Sports Nutrition*, 13(25): 1-11.

Kagiyama, T., Glushakov, A.V., Sumners, C., Roose, B., Dennis, D.M., Phillips, M.I., Ozcan, M.S., Seubert, C.N. and Martynyuk, A.E. (2004). Neuroprotective action of halogenated derivatives of L-phenylalanine. *Stroke*, 35: 1192-1196.

Kalogeris, T., Bao, Y. and Korthuis, R.J. (2014). Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biol*, 2: 702–714.

Kanga, C., Jayasooriyaa, R.G., Dilsharaa, M.G., Choib, Y.H., Jeongc, Y., Kimd, N.D. and Kima, G. (2012). Caffeine suppresses lipopolysaccharide-stimulated BV2 microglial cells by suppressing Akt-mediated NF-kB activation and ERK phosphorylation. *Food and Chemical Toxicology*, 50: 4270-4276.

Kato, G.J., McGowan, V., Machado, R.F., Little, J.A., Taylor, J., Morris, C.R., Nichols, J.S., Wang, X., Poljakovic, M., Sidney, M., Morris, J. and Gladwin, M.T. (2006). Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. *Blood*, 107(6): 2279-2285.

Kim, K., Heo, M., CHun, Jun, H., Lee, J., Jegal, H and Yang Y. (2015). The relationship between stroke and quality of life in Korean adults: based on the 2010 Korean community health survey. *J. Phys. Ther. Sci*. 27: 309-312.

Lorke, D. (1983). A new approach to practical acute toxicity testing. *Arch Toxicol*, 54:275-287.

Marchi, M., Raiteri, L., Risso,F., Vallarino,A., Bonfanti,A., Monopoli,A., Ongini,E. and Raiteri, M. (2002). Effects of adenosine A_1 and A_{2A} receptor activation on the evoked release of glutamate from rat cerebrocortical synaptosomes. *British Journal of Pharmacology*, 136(3): 434-440.

Margaritescu, O., Mogoanta, L., Pirici, I., Pirici, D., Cernea, D and Margaritescu, C.L. (2009), Histopathological changes in acute ischemic stroke.

Romanian Journal of Morphology and Embryology, 50(3): 327-339.

Melani, A., Cipriani, S., Giuliana M.V., Daniele Nosi, Donati, C., Bruni, P., Grazia, M.G. and Pedata, F. (2009). Selective adenosine A_{2A} receptor antagonism reduces JNK activation in oligodendrocytes after cerebral ischaemia. *Brain*, 132: 1480-1495.

Mena, H., Cadavid, D., and Rushing, E.J. (2004). Human cerebral infarct: a proposed histopathologic classification based on 137 cases. *Acta Neuropathologica*, 108(6): 524-530.

Mohamed, R.A., Agha, A.M. Nassar, N.N. (2012). SCH58261 the selective adenosine A_{2A} receptor blocker modulates ischemia reperfusion injury following bilateral carotid occlusion: role of inflammatory mediators. *Neurochem Res*, 37: 538-547.

Muhammad, M., El-ta'alu, A.B. and Mabrouk, M.I. (2016).: Pathogenesis and neuro-protective agents of stroke. *Int J Pharm Sci Res*, 7(10): 3907-16

Nehlig, A. (2002). Pharmacological properties and neurophysiological effects of caffeine. *Pharmacopsychoecologia*, 15: 35-70.

Piskunov, A.K. (2010). Neuroinflammation Biomarkers. *Neurochemical Journal*, 4(1): 55-63.

Ram, M., Begum, N., Pathakala, N. and Bakshi, V. (2016). Neuroprotective effect of Nevirapine on cerebral ischaemic stroke by middle cerebral artery occlusion in wistar rats. *International Journal of Applied Pharmaceutical Sciences and Research*, 1(1): 16-24.

Ravikumar, S., Surekha, R., and Thavarajah, R. (2014). Mounting media: An overvieaw. *Journal of NTR University of Health Sciences*, 3(1): 1-8.

Richard, A., Jungmann, and Kiryukhina, O. (2005). Cyclic AMP and AKAP-mediated targeting of protein kinase A regulates lactate dehydrogenase subunit A mRNA stability. *The Journal of Biological Chemistry*, 280(26): 25170-25177.

Rudolphi, K.A., Keil, M.J. Fredholm, B.B. (1989). Ischaemic damage in gerbil hippocampus is reduced following upregulation of adenosine (A_1) receptors by caffeine treatment. *Neuroscience Letters* 103(3): 275-280.

Schmidt-Kastnera, R., Aguirre-Chena, C., Saula, I., Yicka, L., Hamasakib, D., Bustoa, R. and Ginsberga, M.D. (2005). Astrocytes react to oligemia in the forebrain induced by chronic bilateral common carotid artery occlusion in rats. *Brain Research*, 1052: 28-39.

Sedger, L.M. McDermottc, M.F. (2014). TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants – past, present and future. *Cytokine & Growth Factor Reviews*, 25: 453–472.

Seetapun, S., Yaoling, J., Wang, Y. and Zhu, Z. (2013). Neuroprotective effect of Danshensu derivatives as anti-ischaemia agents on SH-SY5Y cells and rat brain. *Biosci Rep*, 33: 677-688.

Simats, A., García-Berrocoso, T. and Montaner, J. (2015). Neuroinflammatory biomarkers: From stroke diagnosis and prognosis to therapy. *Biochimica et Biophysica Acta*, 4: 1-14.

Tikka, T., Fiebich, B.L.,Goldsteins, G., Keinanen, R. and Koistinaho, J. (2001). Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. *The Journal of Neuroscience*, 21(8): 2580-2588.

Wang, Y., Reis, C., Applegate, R., Stier, G., Martin, R. and Zhang, J. (2015). Ischemic conditioning-induced endogenous brain protection: Applications pre-, per- or post-stroke. *Experimental Neurology*, 1-15.

Wardas, J. (2002). Neuroprotective role of adenosine in the CNS. *Polish Journal of Pharmacology*, 54: 313–326.

Woodruff, T.M., Thundyil, J., Tang,S., Sobey, C.G., Taylor, S.M. and Arumugam, T.V. (2011). Pathophysiology, treatment, and animal and cellular models of human ischemic stroke. *Molecular Neurodegeneration*, 6(11): 1-19.

Yang, Z., Wang, B., Kwansa, H., Heitmiller, K.D., Hong, G., Carter, E.L., Jamrogowicz, J.L., Larson, A.C., Lee J Martin, L.J. and Koehler, R.C. (2013). Adenosine A_{2A} receptor contributes to ischemic brain damage in newborn piglet. *Journal of Cerebral Blood Flow & Metabolism*, 33: 1612-1620.

Yu, H., Hsieh, P., Chang, Y., Chung, P., Kuo, L. and Hwang, T. (2009). DSM-RX78, a new phosphodiesterase inhibitor, suppresses superoxide anion production in activated human neutrophils and attenuates hemorrhagic shock-induced lung injury in rats. *Biochemical Pharmacology*, 78: 983–992.

Yuan, Y., Zha, H., Ramgarajan, P., Ling, E. and Wu, C. (2014). Anti inflammatory effects of Edaravone and Scutellarin in activated microglia in experimentally induced ischemia injury in rats and in BV-2 microglia. *BMC Neuroscience*, 15: 125-133.

Zarruk, J.G., García-Yebenes, I., Romera, V.G., Ballesteros, I., Moraga, A., Cuartero, M.I., Hurtado, O., Sobrado, M., Pradillo, J.M., Fernandez-Lopez, D., Serena, J., Castillo-Melendez, M., Moro, M.A. and Lizasoain, I. (2011). Neurological tests for functional outcome assessment in rodent models of ischaemic stroke. *Rev Neurol*, 53(10): 607-618.