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Research Article

Molecular and Histological Study of Low Molecular Weight Heparin's Effect Against Cerebral Ischemic Injury

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Abstract

Background and Objective: Ischemic injury is one of the major causes of long-term disability and death worldwide. In this context, several studies have been carried out to provide new targets capable of reducing clinical outcomes of ischemia/reperfusion. The work of the present study aims at prospecting and highlighting the other specific properties of enoxaparin. These new antioxidants and anti-inflammatory properties of LMWH certainly constitute a new strategy to overcome the structural alterations of ischemic stroke. **Materials and Methods:** Rats were subjected to the occlusion of the common carotid arteries for 30 min followed by 1 hr of reperfusion to induce ischemia. Low molecular weight heparin injection was performed 30 min before the occlusion. **Results:** Heparin treatment prevented morphological changes in brain tissue induced by ischemic injury. The effect of enoxaparin in altering oxidative stress was assessed based on the maintenance of lactate dehydrogenase level, calcium homeostasis, antioxidant status (Superoxide Dismutase (SOD), catalase and peroxidases (POD) activity) and inhibition of cerebral lipid peroxidation and Reactive Oxygen Species (ROS) and Nitric Oxide (NO) generation. **Conclusion:** Low molecular weight heparin efficiently protected brain cells from ischemia/reperfusion-induced oxidative damage and inflammation.

Key words: Antioxidant activity, ischemia/reperfusion, low molecular weight heparin, oxidative stress, glycosaminoglycans, metastasis, angiogenesis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Heparin is an acidic polyanionic polysaccharide with a linear chain, which belongs to the family of glycosaminoglycans (GAGs) and is mostly known for its anticoagulant activity, but since its discovery, many reports have demonstrated the anti-inflammatory potential of this molecule[1-3]. The endogenous form of heparin proteoglycan is uniquely expressed in mast cells and more precisely in the lumen of secretory granules, attached to the core protein of serglycin[4-6]. Heparin is secreted in association with histamine on the degranulation of mast cells in an allergic response[1]. The chain of this biopolymer is composed of repeating disaccharide sequences, consisting of uronic acid and D-glucosamine connected through glycosidic linkages[2]. Approximately, 80% of uronic acid residues are L-iduronic acid while only 20% contains D-glucuronic acid[5].

Heparin is extracted from animal tissues, almost exclusively from porcine intestinal mucosa[3] or bovine lung[7]. There are three types of commercially prepared heparin based on the molecular weight; unfractionated heparin, LMWHs and ULMWH. Unlike unfractionated heparin which uses is associated with an elevated bleeding rate, LMWH reduces the risk of haemorrhage during the process of treating ischemic stroke. The anticoagulant activity of heparin is independent of the anti-inflammatory effect. There is much theoretical and experimental evidence that stands for the capacity of LMWH to protect the brain from ischemic insult.

One of the reasons behind the multiple functions of heparin is the structural diversity and microheterogeneity due to the variable sulfation patterns exhibited by these molecules[3]. In addition, heparin can bind different type of molecules like cytokines, growth and angiogenic factors, adhesion molecules[8]. These interactions lead to the regulation of many physiological processes like development, cell proliferation, inflammatory response, bacterial and viral infectivity, metastasis and angiogenesis[3,9].

Mary *et al.*[10] have used *in vivo* stroke models of Middle Cerebral Artery Occlusion (MCAO) and demonstrated that the administration of enoxaparin in standard non-haemorrhagic doses decreases lesion size and ameliorates neurological score. Oxygen homeostasis maintains is crucial to ensure the optimal function of the organism. Ischemic stroke is a complicated pathological process induced by a disruption of blood flow and lack of oxygen and glucose delivery to the nervous system[11]. The BBB disruption makes it more permeable, which increases vascular inflammatory cells recruitment and toxic proteins penetration[12]. Neurons become unable to maintain the ionic gradients necessary for cellular function and homeostasis[13]. Ischemia and reperfusion injuries involve excitotoxicity and overactivation of glutamate receptors and can lead to neuronal cell death including apoptosis necrosis, necroptosis and autophagy[10,14].

Excitotoxicity is associated with excessive neuronal depolarization, redox balance disruption and release of excitatory neurotransmitters inducing an increase in intracellular Ca^{2} [10,13]. Depolarized cells in the ischemic core release a huge amount of excitatory amino acid, in particular glutamate, that provokes anoxic depolarization resulting in enhanced neurotransmitter release and reuptake inhibition[14-16]. Thus, glutamate receptors (NMDA, Kainate, AMPA and metabotropic receptors) are excessively activated which triggers calcium release from overstimulated neurons into their cytosol[11,14]. This increase of intracellular calcium levels activates cyclooxygenases (COX-2), calpain protease activity, calcineurin, phospholipases and Nitric Oxide Synthase activity (NOS), which induces free arachidonic acid accumulation and leads as a consequence to the depletion of calcium from intracellular stores[16,17]. Calcium cytotoxicity leads to mitochondrial impairment, which amplifies energy failure and results in the depolarization and swelling of mitochondria[17].

Most of the tissue damage occurs following blood flow reestablishment by reperfusion and thus initiates a cascade of reactions similar to inflammatory response including complement activation, free oxygen radicals release, overexpression of chemokines and cytokines, neutrophils activation and recruitment and endothelial dysfunction[2,18]. Oxidant-antioxidant balance disruption contributes to oxidative damage undergone by lipid, protein and DNA. Accumulation of Reactive Oxygen Species (ROS) alters cellular membranes, thus contributing to the peroxidation of lipids and generating cytotoxic products[19]. In this context, maintain ROS homeostasis and the development of antioxidant-based strategies would be important for cell survival.

To simulate the ischemic cerebral lesion, we used *in vivo* the bilateral occlusion model of the common carotid artery (BCCAO), which is a model validated by several works and which makes it possible to reproduce the same alterations as those of ischemic lesions. Although the effectiveness of heparin as a drug with anti-inflammatory properties has been investigated previously in brain injury models, the exact mechanism underlying its effect still unexplored. To understand the mechanism of action of the neuroprotective effects of heparin, we will attempt to determine the mode of action of heparin, is it a mechanism of scavenging free radicals of oxygen, or it is indeed a possible inhibitory effect, which will eventually block the entry of calcium into the nerve cells and

thus prevent ischemic attacks. In the end, the study aims to elucidate the different pathways involved in the processes of the neuroprotective and/or glioprotective effects of enoxaparin during an ischemic cerebral situation.

MATERIALS AND METHODS

Study area: The study was carried out in the Department of Biology, Neurophysiology, Cellular Physiopathology and Biomolecules Valorisation Laboratory, the unit of Transmission Electron Microscopy of the Department of Anatomic Pathology, Faculty of Medicine, Salah Azaiez Institute, Tunisia from September, 2019-December, 2020.

Chemicals: Heparin (enoxaparin) was purchased from Medis. Formaldehyde, Fluorescein Diacetate-Acetoxymethyl ester (FDA), bovine liver catalase, DL-epinephrine, trichloroacetic acid (TCA) and guaiacol were obtained from Sigma Aldrich (St. Louis, MO, USA). Calcium, total protein and lactate dehydrogenase (LDH; EC 1.1.1.27) assay kits were from Bio-Maghreb (Tunisia). Butylated Hydroxy Toluene (BHT) and 2-Thio-barbituric acid (TBA) were obtained from Loba Chemie PVT.LTD. 2 ,7-dichlorodihydrofluorescein diacetate (H_2 DCFDA) was purchased from Invitrogen and DAF-FM diacetate (4-amino-5-methylamino-2′,7′-Difluorofluorescein diacetate) was from Molecular Probes (Eugene, OR, USA).

Experimental animals and drug administration: Totally 56 adult male rats, weighing between 250-300 g, were selected and housed 2 per cage with free access to food and water and under controlled laboratory conditions. They were maintained at a rearing temperature of 22±2°C and under a controlled light-dark cycle. They were purchased from the Pasteur Institute of Tunis. All experiments carried out on rats were approved by the ethic committee of Tunis University for the care and use of animals in conformity with NIH guidelines.

After a one-week acclimatization period, rats were randomly assigned to four groups:

- Control group (Sham)
- ischemia/Reperfusion group (Isch)
- Heparin pre-treatment group (Hep)
- Group receiving a pre-treatment with heparin and exposed to bilateral common carotid artery occlusion (Isch+Hep)

Drugs were administrated by intravenous injection: sham-operated animals received either saline or heparin (enoxaparin). For the two first groups, the sodium chloride solution (0.9%) was administrated as a vehicle, 30 min before the tests. The two remaining groups (c and d) received heparin injection (200 IU kg^{-1}), 30 min before the occlusion.

Procedure of Bilateral Common Carotid Artery Occlusion (BCCAO): Cerebral ischemia/reperfusion insult was induced using a model of Bilateral Common Carotid Artery Occlusion as previously reported[20] with some changes. Ketamine (100 mg kg^{-1}) was used to anaesthetize rats and both common carotid arteries were exposed through a midline incision. Carefully, we separated each carotid from its adventitial sheath and vagus nerve[21]. Arteries occlusion was performed by placing clamps for 30 min, which were subsequently removed to allow reperfusion for 1 hr.

The animals were euthanized, decapitated and each cortex was removed from the skull immediately for the following tests. An ultra turrax T25 homogenizer (Janke and Kankel, IKA WERK, D.6072 Dreieich-Germany) was used to homogenize the other brain tissues in a solution of tris buffered saline pH 7.4 (Microprocessor pH meter, Hanna Instruments, France). Finally, the centrifugation of the homogenates was performed at 4°C and 10000 rpm for 15 min. Then supernatants were collected for subsequent assays of reactive oxygen species, nitric oxide, lipoperoxidation activity and antioxidant markers.

Tissue's collection for histology: The brain was removed and submerged in 10% saline formaldehyde. Each brain was immersed in paraffin, following that sagittal sections were cut at 5 µm thickness and stained with hematoxylin/eosin (H and E).

LDH assay: LDH activity assay was performed according to Bergmeyer, 1975[22] and following manufacturer's instructions. The reaction catalysed by LDH is the conversion of pyruvate and NADH/H to lactate and NAD. This enzymatic activity was determined based on NADH disappearance at 340 nm within 3 min and calculated using the Eq.[23]:

$$\Delta DO/min*8095$$

Brain lipoperoxidation: Malondialdehyde (MDA) levels were measured to evaluate lipid peroxidation. This test is based on the thiobarbituric acid reactive substance (TBARS) method through the reaction of TBA with MDA, which is one of the end products of peroxidation[21,24]. Tissue homogenates (80 µL) were mixed with 20 µL TBS and a solution of BHT-TCA (62.5 µL) composed of 1% BHT (m/v) added to 20% TCA (m/v).

The centrifugation was performed at 4°C for 10 min at 1000 g. A solution of 0.6 M HCl (16 µL) was added to the supernatant (80 µL) then the reaction initiation occurred following the addition of 120 mmol L^{-1} TBA in 26 mmol Tris (64 µL). After heating the samples for 10 min at 80°C, the reaction was stopped by cooling the samples with tap water. This step is followed by the appearance of pink colour which absorbance was measured at 530 nm. MDA levels were calculated based on the extinction coefficient of the MDA-TBA complex which equals 1.56 10 mol L^{-1} cm^{-1}.

Antioxidant enzyme activities

Superoxide enzyme activity: Superoxide dismutase enzyme activity was evaluated through the autoxidation of epinephrine by superoxide anion using a spectrophotometric assay[25]. Epinephrine is oxidized over time of incubation to adrenochrome in the presence of bovine catalase. Brain homogenates were incubated in a solution of sodium carbonate/sodium bicarbonate ($Na_2CO_3/NaHCO_3$) buffer (pH 10.2), DL-epinephrine (5 mg mL^{-1}) and bovine catalase (0.4 U μL^{-1}). Epinephrine oxidation is inhibited by the superoxide dismutase enzyme, which traps the flow of the superoxide anion. SOD activity was assessed by following the kinetics of inhibition of adrenochrome formation for 5 min and was measured at $\lambda = 480$ nm[21].

Catalase activity: Catalase activity was evaluated through Aebi method[26] based on the decrease of H_2O_2 level at 240 nm. The kinetics of H_2O_2 disappearance was measured every 30 sec for 3 min. Brain homogenates were mixed with 50 mM phosphate buffer (KH_2PO_4/Na_2HPO_4 $12H_2O$) containing 30 mM H_2O_2. Catalase activity was determined using the extinction coefficient of 40 mM cm^{-1} for H_2O_2[23].

Peroxidases activity: Peroxidases activity was assessed following the oxidation of guaiacol at 25°C. The reaction mixture contained 10 µL of brain homogenate, 40 mM guaiacol in 50 mM phosphate buffer pH 7 and finally 50 mM H_2O_2. H_2O_2 addition initiated the reaction and the increase in absorbance was followed at 470 nm using guaiacol as a hydrogen donor. Peroxidases activity was calculated using a molecular extinction factor of 26.6 mM cm^{-1} and expressed as nmol guaiacol oxidized per minute[23].

Reactive oxygen species measurement: Reactive Oxygen Species (ROS) generation was monitored by quantifying the fluorescence of 2',7'-dichlorofluorescein (DCF)[27]. Cortex homogenates were incubated with 5 µM cell-permeant DCFH-DA_2 for 30 min at 37°C and ROS were detected through the deacetylation and oxidation of DCFH-DA to fluorescent DCF.

The conversion of DCFDA into DCF was determined using an FL800TBI fluorescence microplate reader (Bio-Tek Instruments, Winooski, VT, USA) with excitation and emission wavelengths at 485 and 538 nm, respectively.

Intracellular mediator

Ionizable calcium measurement: Ionizable Ca^2 level was measured using a commercially available kit from Biomaghreb, based on a protocol by Stern and Lewis[28]. Calcium forms with cresolphthalein, at an alkaline medium, a purple component which intensity is proportional to calcium concentration and is detected at 570 nm. Total 20 µL of tissue homogenate was added to 1 mL of reaction mixture composed of 2-Amino-2-methyl 1-propanol buffer (500 mmol L^{-1}), hydroxyl-8 quinoline (69 mmol) and cresolphthalein (0-62 mmol L^{-1}). Absorbance was measured after incubating the mixture for five min at room temperature.

Nitric oxide (NO) levels: Changes in NO content were evaluated spectrofluorimetrically using the NO-sensitive fluorescent dye 4-Amino-5-Methylamino-2′,7′-Difluorofluorescein Diacetate (DAF-FM Diacetate). When this probe enters the cells, it is deacetylated by intracellular esterase, becoming DAF-FM. This later becomes fluorescent subsequently to its interaction with NO to form a fluorescent benzotriazole. Cortex homogenates were incubated with 3 µM cell-permeant DAF-FM DA at 37°C for 30 min. Fluorescence was measured using an FL800TBI fluorescence microplate reader (Bio-Tek Instruments) with excitation at 485 nm and emission at 538 nm.

Statistical analysis: All data found in this study were analysed with Graphpad Prism 7 software using the One Way ANOVA test for multiple comparisons. Results were expressed by Mean±SEM.

RESULTS

Histopathological evaluation: Histopathological changes in brain tissue induced by ischemic insult were evaluated by hematoxylin and eosin staining (H and E). Following 30 min of global ischemia, there is the death of some of the CA1 pyramidal neurons in the hippocampus. In Fig. 1a, the microscopical structure presents different areas of the hippocampal section especially the region CA3 and CA4. In

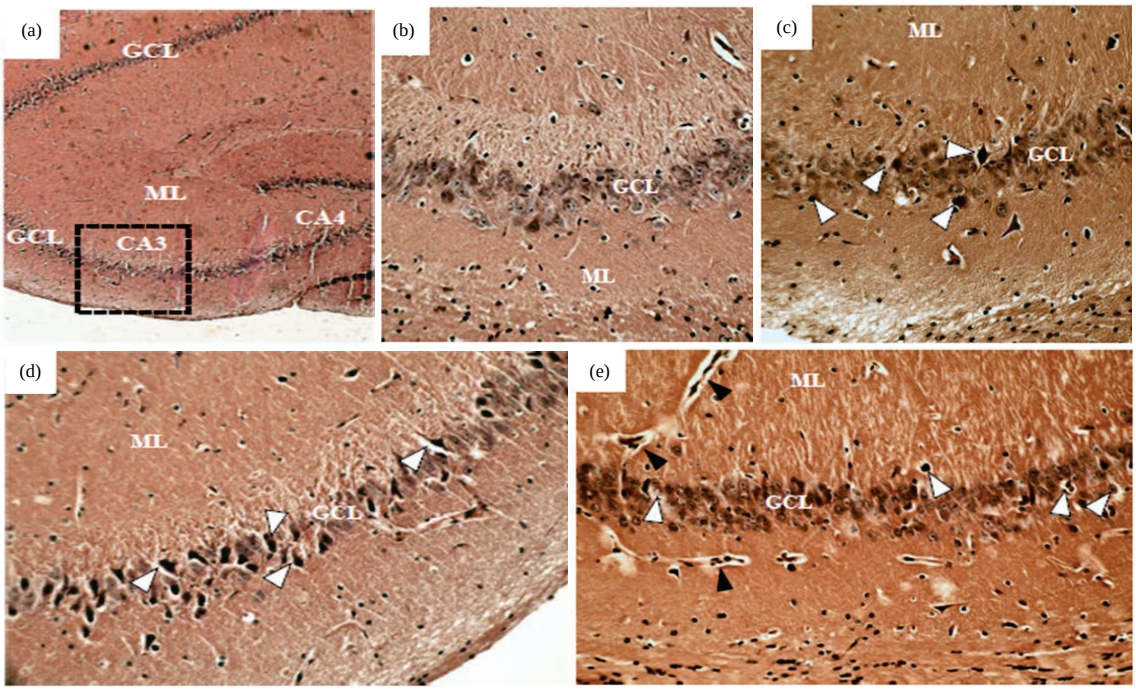

Fig. 1(a-e): Histologic changes following ischemia/reperfusion and heparin treatment

(a) Location at the hippocampal section of the observed region (black square) (X200), (b-c) Examination of the pyramidal-like cells of CA3 field of the hippocampus showed respectively normal appearance of most cells in normal rats (B) and a few cells which have darkly stained nuclei (white arrows) in rats treated with Heparin (C) (X400), (d) Inspection of hippocampus cells of CA3 field, in Ischemic rats, showed neuronal death with areas awarding dead neurons (white arrows) and others with undamaged neurons (X400) and (e) Ischemic rats receiving heparin injection showed some well-preserved neurons and some damaged neurons with eosinophilic cytoplasm (white arrows). Note that Ischemia/reperfusion injury induce a visibly congested blood capillary (black arrows) (X400). ML: Molecular layer, GCL: Granular cell layer, CA3 and CA4 (CA: Cornu Ammonis)

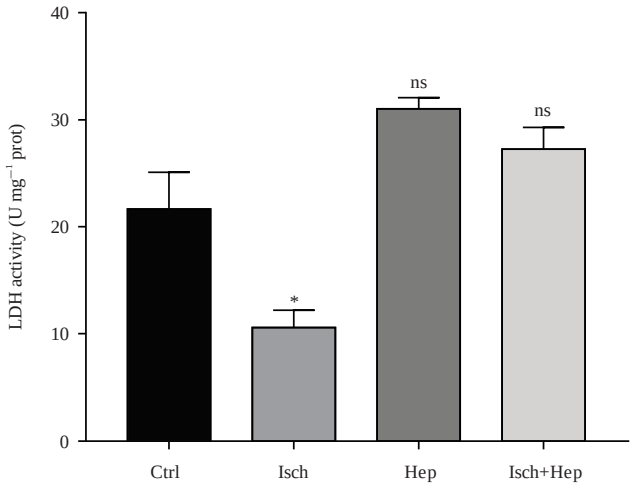

Fig. 2: Effect of LMWH on LDH activity following ischemia/reperfusion

Control group (Sham), Isch: Ischemia/reperfusion group, Hep: Heparin pre-treatment group, Isch+Hep: Group receiving a pre-treatment with heparin and exposed to bilateral common carotid artery occlusion. *p<0.05 and ns not statistically different from the control

Fig. 1b, the CA3 region of the hippocampus demonstrate a normal structure of different cells in non-treated rats. In Fig. 1c, neurons were almost preserved in the heparin treated groups. In Fig. 1d, most of the morphologically damaged neurons present eosinophilic aspect with shrunken, dark cytoplasm. The nucleus is polygonal with dense chromatin and no nucleolus. In Fig. 1e, the histological analysis demonstrated that heparin partially attenuated neuronal damage after ischemia.

LDH activity: The results presented in Fig. 2 revealed that LDH levels decreased following ischemia/reperfusion by 10.64 U mg^{-1} protein ($p = 0.0182$). Diminished LDH levels indicate that ischemia/reperfusion injury induces the deterioration of energy metabolism. Enoxaparin treatment did not induce any change in LDH level ($p = 0.0686$) compared to the sham group. Besides no significant difference was observed in LDH level between the control and ischemic group treated with 200 IU enoxaparin ($p = 0.4646$). The restoration of LDH to normal levels highlights that LMWH injection re-establishes energy metabolism balance.

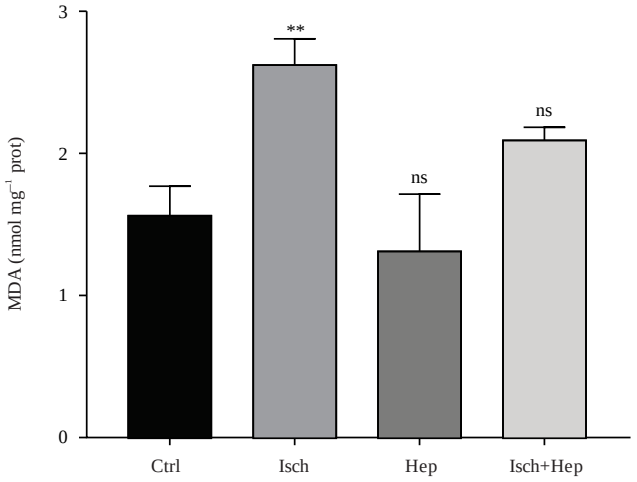

Fig. 3: Effect of enoxaparin on brain lipoperoxidation

Control group (Sham), Isch: Ischemia/reperfusion group, Hep: Heparin pre-treatment group, Isch+Hep: Group receiving a pre-treatment with heparin and exposed to bilateral common carotid artery occlusion. **p<0.01 and ns not statistically different from the control

Brain lipoperoxidation: The evaluation of MDA content is an indicator of brain oxidation (Fig. 3). Ischemia/reperfusion injury-induced an increase in MDA level by 2.636 nmol mg^{-1} protein (p = 0.0050). This result shows that ischemic injury induces an increase in MDA level which refers to a state of lipid peroxidation. The injection of enoxaparin to ischemic rats decreased MDA level in brain tissue by 2.104 nmol mg^{-1} protein (p = 0.2577). These results indicate that LMWH induces the decrease of MDA levels and affords protection to brain tissue from lipid peroxidation.

Antioxidant enzyme activities: To investigate the antioxidative defence systems, SOD, catalase and peroxidases activity were evaluated (Fig. 4). In Fig. 4a, SOD activity significantly decreased following ischemia/reperfusion by 7.286 U min^{-1} mg^{-1} protein (p = 0.0077) compared with the control group. Pre-treatment with heparin re-established normal cellular levels of SOD (p = 0.9791). In Fig. 4b, catalase activity increased by 0.1645 mM min^{-1} mg^{-1} protein (p = 0.0014) following ischemia/reperfusion injury compared with the control group. Pre-treatment with heparin backed I/R-induced elevation in catalase (p = 0.7767) to near control level. In Fig. 4c, POD activity decreased by 1.119 (10^{-5} mmol min^{-1} mg^{-1} protein) (p = 0.0144) compared with the control group following ischemic injury. Enoxaparin injection backed I/R-induced decrease in POD (p = 0.1430) to near control level. The ischemic injury affects the antioxidant enzymes balance with a decrease of SOD and peroxidases levels and an

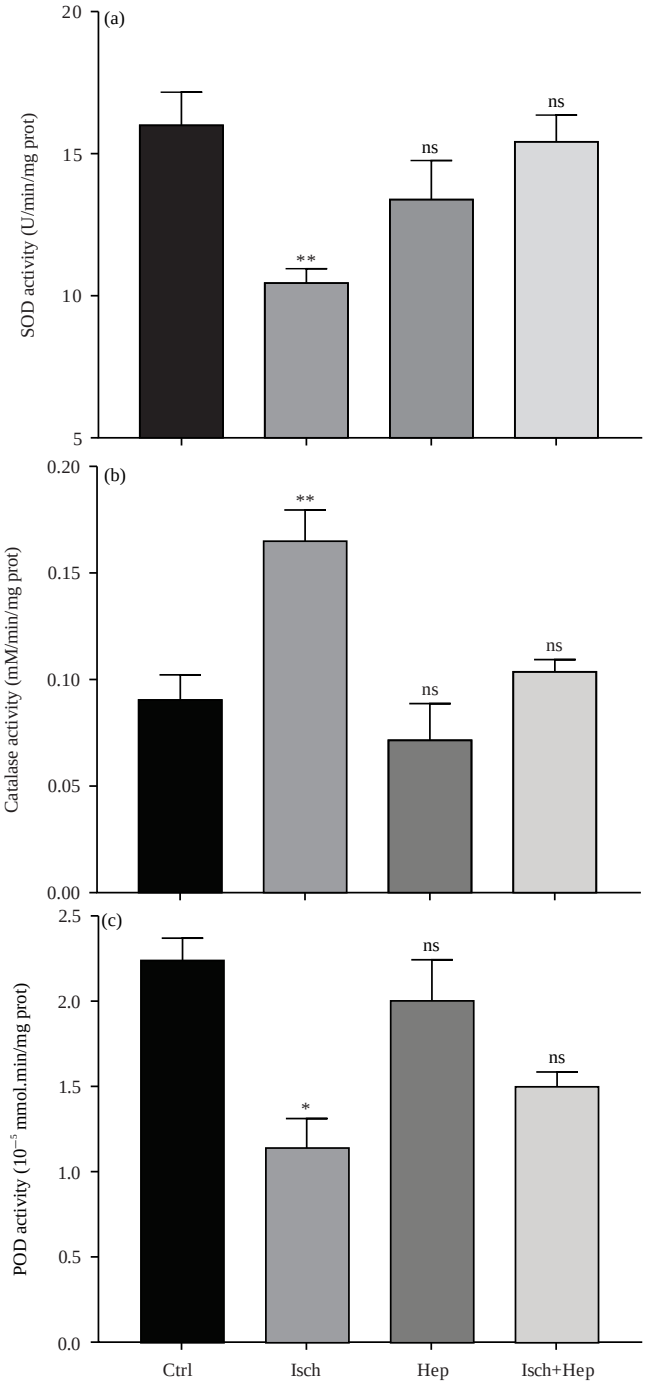

Fig. 4(a-c): Effect of enoxaparin on antioxidant enzymes SOD, Catalase and peroxidases following ischemia/reperfusion

(a) Effect of enoxaparin on SOD levels in each group, (b) Effect of enoxaparin on catalase levels in each group and (c) Effect of enoxaparin on peroxidases levels in each group. Control group (Sham), Isch: Ischemia/reperfusion group, Hep: Heparin pre-treatment group, Isch+Hep: Group receiving a pre-treatment with heparin and exposed to bilateral common carotid artery occlusion. *p<0.05, **p<0.01 and ns not statistically different from the control

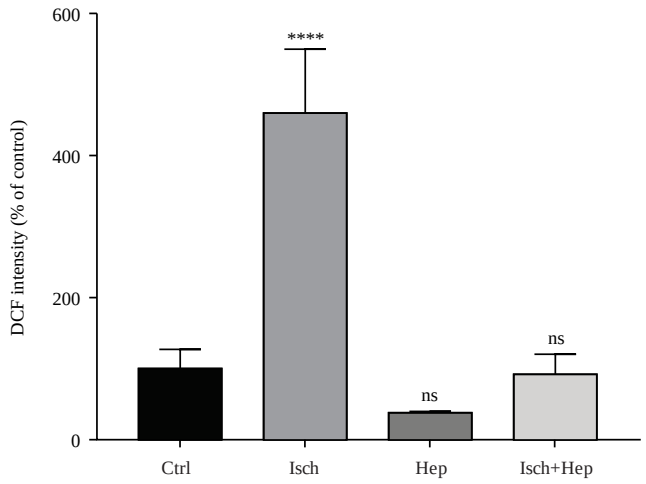

Fig. 5: Effects of enoxaparin on ischemia-induced ROS accumulation

Control group (Sham), Isch: Ischemia/reperfusion group, Hep: Heparin pre-treatment group, Isch+Hep: Group receiving a pre-treatment with heparin and exposed to bilateral common carotid artery occlusion. The results are expressed as percentages of control. ****p<0.0001 and ns not statistically different from the control

elevation of catalase level. The restoration of antioxidant enzymes levels indicates that LMWH treatment provides cellular protection against oxidative injury.

ROS measurement: We further investigated the level of ROS involved in ischemia-reperfusion injury and whether enoxaparin can reduce its accumulation (Fig. 5). Compared with the control group ROS level was higher in the ischemic group by 457.1% (p<0.0001). Enoxaparin injection significantly counteracted ischemia/reperfusion-induced damage.

Intracellular mediators: We studied the effect of LMWH on the intracellular mediators implicated in ischemic stress (Fig. 6). In Fig. 6a, ischemia/reperfusion injury elevated calcium levels by 0.0366 nM mg^{-1} protein (p = 0.0015). Rats exposed to ischemia and pre-treated with enoxaparin did not show any significant changes in calcium level (p = 0.8997) compared to control. Calcium accumulation in the group exposed to ischemia was prevented following treatment with enoxaparin.

In Fig. 6b, ischemia/reperfusion injury-induced the increase of NO level by 160.3% (p = 0.0059) while heparin pre-treatment backed I/R-induced increase to near control level (p = 0.1009). LMWH injection prevented the increase of NO levels which remained at levels less than the control.

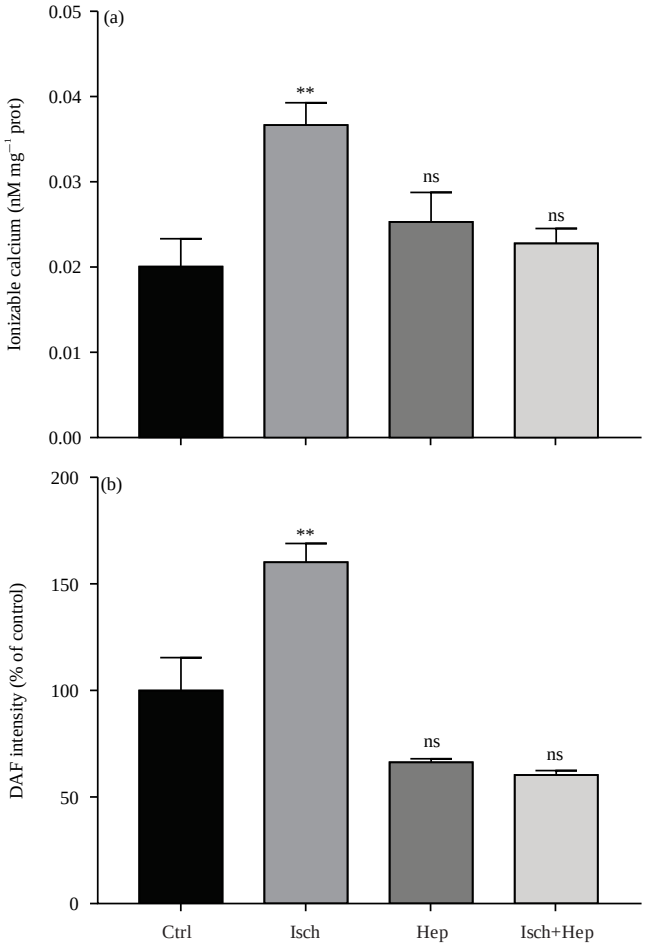

Fig. 6(a-b): Effect of heparin on intracellular Ca^{2+} and nitric oxide levels of BCCAO rats

(a) Effect of LMWH on intracellular calcium level and (b) Effect of heparin on nitric oxide levels. Control group (Sham), Isch: Ischemia/reperfusion group, Hep: Heparin pre-treatment group, Isch+Hep: Group receiving a pre-treatment with heparin and exposed to bilateral common carotid artery occlusion. **p<0.01 and ns not statistically different from the control

DISCUSSION

Our research study indicates that the neuroprotective effects of enoxaparin against cerebral ischemia are intimately associated with the maintenance of antioxidant activities (SOD, catalase and POD). Our results also show that the modulation of cellular defence systems will restore the balance of calcium homeostasis, will attenuate lipid peroxidation and will prevent the accumulation of ROS (Reactive Oxygen Species) and NO (nitrogen monoxide).

The deficiency of oxygen induces disruption of redox homeostasis and results in different degrees of tissue damage which can lead to apoptotic, autophagic, necroptotic and necrotic neuronal cell death[29-31]. Brain cells are extremely sensitive to oxygen deprivation, in particular hippocampal CA1 pyramidal neurons and the Purkinje neurons of the cerebellum[32]. The extreme vulnerability of cerebellar Purkinje cells to ischemia is due to the high metabolic rate of these neurons[33]. Brain tissue sensitivity is explained by the high metabolic rate, high oxygen consumption rate, high concentrations of iron, high content of lipids and low levels of antioxidants[34].

Ischemic brain injury and oxygen deprivation-induced the activation of anaerobic metabolism and the excessive production of lactate, leading to the decrease of intracellular pH and the depletion of ATP[18]. Thus, lactic acidosis can cause oedema, blood-brain barrier impairment and enhanced ROS production, promoting cell death through the inhibition of excitatory amino acids re-uptake and the excessive cellular accumulation of calcium[18,35]. LDH which converts pyruvate into lactate and generates NAD under anaerobic conditions is involved in glucose metabolism and energy production[36,37]. Cell injury induces the release of Lactate Dehydrogenase (LDH) from the cell into the extracellular environment due to cell integrity disruption, which causes the release of molecules and enzymes into the interstitial space and this might be an explanation for the decrease in intracellular LDH activity levels observed after BCCAO. LMWH administration increased LDH levels in brain tissue showing that enoxaparin reduces the degree of energy failure.

Ischemia-reperfusion insult induces an increase in ROS levels leading to oxidative damage of cellular membranes, which consists of polyunsaturated fatty acids, thus resulting in a loss of structural integrity and tissue lipid peroxidation. The current study showed an elevation of ROS and MDA levels following ischemia-reperfusion injury. Enoxaparin administration prevented the cerebral ischemia-induced increase of lipid peroxidation and attenuated ROS accumulation.

Cells dispose of non-enzymatic and enzymatic antioxidant defensive systems including Glutathione Peroxidase (GPx), superoxide dismutase, catalase and glutathione[38,39]. In our experiment, bilateral cerebral carotid artery occlusion induced a decrease in the activity of the SOD and peroxidases and an increase in catalase level.

These results prove the presence of cortical oxidative stress due to the excessive production of nitrogen- and oxygen-free radicals and the alteration of antioxidant defence systems. These highly reactive radicals lead to the damage of intracellular biofilm lipids, proteins and nucleic acids and may promote cell death via apoptosis by intracellular calcium overload and mitochondrial metabolic impairment[40,41]. In the enoxaparin-treated group, we noted a significant increase in the activity of SOD and a decrease in catalase level compared to the ischemic group. The results fully agree with another study by Zhang *et al.*[42], who showed that LMWH protects brain cells through increasing SOD levels and decreasing MDA levels. Several studies have proposed that heparin and its analogues, act as free radical scavengers, inhibit the expression of pro-apoptotic genes and stimulate the expression of anti-apoptotic genes, providing cell protection against intestinal ischemia/reperfusion lesions[19,43].

The extracellular SOD isozyme (EC-SOD) has a high affinity for heparin. It has been showing that in brain tissue EC-SOD can bind heparin through its heparin-binding domain, which can explain the ability of heparin to inhibit tissue injury induced by free radicals[44].

According to our results, LMWH pre-treatment induced a remarkable decrease in calcium and nitric oxide levels. The release of high levels of calcium induces the activation of NOS and oxidative metabolism of arachidonate, during reperfusion, thus causing NO and peroxynitrite generation, enhanced free radicals production, inhibition of mitochondrial respiration and subsequently leads to cell death[14,16]. The action of enoxaparin opens up a new therapeutic perspective for effectively attenuating ischemia/reperfusion lesions. Other studies highlighted the great potential of LMWH since its applications can include other diseases like cancer and asthma[45,46]. In addition, this new treatment alternative for ischemic effects offers the advantage of triggering fewer side effects if we compare it to unfractionated heparin. It is highly recommended that more studies should be carried out to understand the biochemical signalling of the neuroprotective and/or glioprotective effects of low molecular weight heparin.

CONCLUSION

The data, which confirm all the bibliographic results related to the anti-inflammatory and anti-oxidant properties of enoxaparin-provides a better understanding of the implicated action of the enoxaparin which declines the inflammatory response and reduces the excessive extent of oxidative stress produced during ischemia/reperfusion.

SIGNIFICANCE STATEMENT

This study discovers more details about the protective effect of Low Molecular Weight Heparin on brain cells that can be beneficial for treating rats following ischemic injury. This study will help the researcher to uncover the critical areas of LMWH effect on various parameters that many researchers were not able to explore. Thus a new theory on the neuroprotective effect of LMWH and its applications as a new adjuvant treatment strategy for therapeutic purposes may be arrived at.

REFERENCES

1. Laremore, T.N., F. Zhang, J.S. Dordick, J. Liu and R.J. Linhardt, 2009. Recent progress and applications in glycosaminoglycan and heparin research. Curr. Opin. Chem. Biol., 13: 633-640.
2. Young, E., 2008. The anti-inflammatory effects of heparin and related compounds. Thrombosis Res., 122: 743-752.
3. Shriver, Z., I. Capila, G. Venkataraman and R. Sasisekharan, 2012. Heparin and Heparan Sulfate: Analyzing Structure and Microheterogeneity. In: Heparin - A Century of Progress, Lever, R., B. Mulloy and C.P. Page (Eds.)., Springer, Berlin, Heidelberg, pp: 159-176.
4. Carlsson, P. and L. Kjellén, 2012. Heparin Biosynthesis. In: Heparin - A Century of Progress, Lever, R., B. Mulloy and C.P. Page (Eds.)., Springer, Berlin, Heidelberg, pp: 23-41.
5. Marszalek, P.E., A.F. Oberhauser, H. Li and J.M. Fernandez, 2003. The force-driven conformations of heparin studied with single molecule force microscopy. Biophys. J., 85: 2696-2704.
6. Mulloy, B., R. Lever and C.P. Page, 2017. Mast cell glycosaminoglycans. Glycoconjugate J., 34: 351-361.
7. Spiess, B.D., 2017. Heparin: Effects upon the glycocalyx and endothelial cells. J. Extra Corpor Technol., 49: 192-197.
8. Cassinelli, G. and A. Naggi, 2016. Old and new applications of non-anticoagulant heparin. Int. J. Cardiol., 212: S14-S21.
9. Lima, M., T. Rudd and E. Yates, 2017. New applications of heparin and other glycosaminoglycans. Molecules, Vol. 22. 10.3390/molecules22050749.
10. Mary, V., F. Wahl, A. Uzan and J.M. Stutzmann, 2001. Enoxaparin in experimental stroke: Neuroprotection and therapeutic window of opportunity. Stroke, 32: 993-999.
11. Doré, S and A.S. Ahmad, 2015. Cytoprotective Role of Prostaglandin D_2 DP1 Receptor against Neuronal Injury Following Acute Excitotoxicity and Cerebral Ischemia. In: Brain Neurotrauma: Molecular, Neuropsychological and Rehabilitation Aspects, Kobeissy, F.H. (Ed.)., CRC Press/Taylor & Francis, pp: 97-116.
12. Ribe, E.M., E. Serrano-Saiz, N. Akpan and C.M. Troy, 2008. Mechanisms of neuronal death in disease: Defining the models and the players. Biochem. J., 415: 165-182.
13. Szydlowska, K. and M. Tymianski, 2010. Calcium, ischemia and excitotoxicity. Cell Calcium, 47: 122-129.
14. Li, V., X. Bi, P. Szelemej and J. Kong, 2012. Delayed Neuronal Death in Ischemic Stroke: Molecular Pathways. In: Advances in the Preclinical Study of Ischemic Stroke, Balestrino, M. (Ed.)., In Tech, New York, pp: 117-144.
15. Sutherland, B.A., J. Minnerup, J.S. Balami, F. Arba, A.M. Buchan and C. Kleinschnitz, 2012. Neuroprotection for ischaemic stroke: Translation from the bench to the bedside. Int. J. Stroke, 7: 407-418.
16. White, B.C., J.M. Sullivan, D.J. DeGracia, B.J. O'Neil and R.W. Neumar *et al*., 2000. Brain ischemia and reperfusion: Molecular mechanisms of neuronal injury. J. Neurol. Sci., 179: 1-33.
17. Smith, W.S., 2004. Pathophysiology of focal cerebral ischemia: A therapeutic perspective. J. Vascular Interventional Radiol., 15: S3-S12.
18. Kalogeris, T., C.P. Baines, M. Krenz and R.J. Korthuis, 2012. Cell biology of ischemia/reperfusion injury. Int. Rev. Cell Mol. Biol., 298: 229-317.
19. Ghadie, M.M., R. Miranda-Ferreira, N.S.A. Taha, A.S. Maroso and R.J.Z. Moreti *et al*., 2012. Study of heparin in intestinal ischemia and reperfusion in rats: Morphologic and functional evaluation. Trans. Proceed., 44: 2300-2303.
20. Ueda, M., H. Muramatsu, T. Kamiya, A. Muramatsu, T. Mori, A. Terashi and Y. Katayama, 2000. Pyruvate dehydrogenase activity and energy metabolite levels following bilateral common carotid artery occlusion in rat brain. Life Sci., 67: 821-826.
21. Safwen, K., S. Selima, E. Mohamed, L. Ferid and C. Pascal *et al*., 2015. Protective effect of grape seed and skin extract on cerebral ischemia in rat: Implication of transition metals. Int. J. Stroke, 10: 415-424.
22. Bergmeyer, H.U., 1975. New values for the molar extinction coefficients of NADH and NADPH for the use in routine laboratories (author's transl). Z Klin Chem. Klin Biochem., 13: 507-508.
23. Rebai, O., M. Belkhir, A. Boujelben, S. Fattouch and M. Amri, 2017. Morus alba leaf extract mediates neuroprotection against glyphosate-induced toxicity and biochemical alterations in the brain. Environ. Sci. Pollut. Res., 24: 9605-9613.
24. Draper, H.H. and M. Hadley, 1990. Malondialdehyde determination as index of lipid peroxidation. Meth. Enzymol., 186: 421-431.
25. Misra, H.P. and I. Fridovich, 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem., 247: 3170-3175.
26. Aebi, H., 1974. Catalase. In: Methods of Enzymatic Analysis, Bergneyer H. (Ed.)., Academic Press, Weinham, New York, pp: 673-684.

27. Hamdi, Y., O. Masmoudi-Kouki, H. Kaddour, F. Belhadj and P. Gandolfo *et al*., 2011. Protective effect of the octadecaneuropeptide on hydrogen peroxide-induced oxidative stress and cell death in cultured rat astrocytes. J. Neurochem., 118: 416-428.
28. Stern, J. and W.H.P. Lewis, 1957. The colorimetric estimation of calcium in serum with o-cresolphthalein complexone. Clin. Chim. Acta, 2: 576-580.
29. Erin, N., R.A.W. Lehman, P.J. Boyer and M.L. Billingsley, 2003. *In vitro* hypoxia and excitotoxicity in human brain induce calcineurin–BCL-2 interactions. Neuroscience, 117: 557-565.
30. Xu, M. and H.L. Zhang, 2011. Death and survival of neuronal and astrocytic cells in ischemic brain injury: A role of autophagy. Acta Pharmacol. Sinica, 32: 1089-1099.
31. Yang, X.S., T.L. Yi, S. Zhang, Z.W. Xu and Z.Q. Yu *et al*., 2017. Hypoxia-inducible factor-1 alpha is involved in RIP-induced necroptosis caused by *in vitro* and *in vivo* ischemic brain injury. Sci. Rep., Vol. 7. 10.1038/s41598-017-06088-0.
32. Rahaman, P. and M.R.D. Bigio, 2018. Histology of brain trauma and hypoxia-ischemia. Acad. Forensic Pathol., 8: 539-554.
33. Au, A.K., Y. Chen, L. Du, C.M. Smith and M.D. Manole *et al*., 2015. Ischemia-induced autophagy contributes to neurodegeneration in cerebellar purkinje cells in the developing rat brain and in primary cortical neurons *in vitro*. Biochim. Biophys. Acta (BBA) Mol. Basis Dis., 1852: 1902-1911.
34. Hu, D.Y., Q. Li, B. Li, R.J. Dai, L.N. Geng and Y.L. Deng, 2009. Normobaric hypoxia-induced brain damage in wistar rat. J. Biomed. Sci. Eng., 2: 632-636.
35. Ma, W., S. Wang, X. Liu, F. Tang and P. Zhao *et al*., 2019. Protective effect of troxerutin and cerebroprotein hydrolysate injection on cerebral ischemia through inhibition of oxidative stress and promotion of angiogenesis in rats. Mol. Med. Rep., 19: 3148-3158.
36. Zager, R.A., A.C.M. Johnson and K. Becker, 2014. Renal cortical pyruvate depletion during AKI. J. Am. Soc. Nephrol., 25: 998-1012.
37. Rossignol, F., M. Solares, E. Balanza, J. Coudert and E. Clottes, 2003. Expression of lactate dehydrogenase A and B genes in different tissues of rats adapted to chronic hypobaric hypoxia. J. Cell. Biochem., 89: 67-79.
38. Li, L., J. Tan, Y. Miao, P. Lei and Q. Zhang, 2015. Ros and autophagy: Interactions and molecular regulatory mechanisms. Cell. Mol. Neurobiol., 35: 615-621.
39. Pizzino, G., N. Irrera, M. Cucinotta, G. Pallio and F. Mannino *et al*., 2017. Oxidative stress: Harms and benefits for human health. Oxid. Med. Cell. Longevity, Vol. 2017. 10.1155/2017/8416763.
40. Birben, E., U.M. Sahiner, C. Sackesen, S. Erzurum and O. Kalayci, 2012. Oxidative stress and antioxidant defense. World Allergy Organiz. J., 5: 9-19.
41. Fiskum, G., C.A. Danilov, Z. Mehrabian, L.L. Bambrick and T. Kristian *et al*., 2008. Postischemic oxidative stress promotes mitochondrial metabolic failure in neurons and astrocytes. Ann. N. Y. Acad. Sci., 1147: 129-138.
42. Zhang, Z., Q. Zhang, Y. Cheng, S. Ji and G. Du, 2007. Antagonistic effects of ultra-low-molecular-weight heparin against cerebral ischemia/reperfusion injury in rats. Pharmacol. Res., 56: 350-355.
43. Taha, M.O., R.M. Ferreira, N.S.A. Taha, H.P. Monteiro, A. Caricati-Neto and D.J. Fagundes, 2014. Heparin modulates the expression of genes encoding pro and anti-apoptotic proteins in endothelial cells exposed to intestinal ischemia and reperfusion in rats. Acta Cirurgica Bras., 29: 445-449.
44. Adachi, T., M. Yamamoto and H. Hara, 2001. Heparin-affinity of human extracellular-superoxide dismutase in the brain.. Biol. Pharm. Bull., 24: 191-193.
45. Castelli, R., F. Porro and P. Tarsia, 2004. The heparins and cancer: Review of clinical trials and biological properties. Vasc. Med., 9: 205-213.
46. Shute, J.K., E. Puxeddu and L. Calzetta, 2018. Therapeutic use of heparin and derivatives beyond anticoagulation in patients with bronchial asthma or COPD. Curr. Opin. Pharmacol., 40: 39-45.