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BASIC RESEARCH

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Effects of methylprednisolone and dextromethorphan on lipid peroxidation in an experimental model of spinal cord injury

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Abstract *Objective*. This study examines the effects of agents purported to improve recovery following spinal cord trauma, methylprednisolone sodium succinate, dextromethorphan, and the combination of both, on the post-traumatic alterations of membrane lipid metabolism. *Methods*. After sparing ten rats for a control group (G1) and performing T3–T6 laminectomies in 150 rats, spinal cord injuries were accomplished in 120 of 150 Wistar rats with an aneurysmal clip compression at the level of T4-5 for 30 sec. Hence the G2 group (*n* 30) included the "only laminectomy/sham" group. The 120 injured animals were subdivided into four equal groups (*n* 30 each). Group G3 underwent no therapy, G4 methylprednisolone (MP), G5 dextromethorphan (DM), and G6 MP+DM therapies. Groups G2–G6 were killed ten by ten at 10 min, 30 min, and 120 min after the operation. We measured tissue (MDA) and blood malonyldialdehyde (MDAb), (a product of lipid peroxidation) levels as an indicator of oxidative damage by thiobarbituric acid method and activity levels of antioxidant enzymes superoxide dismutase and glutathione peroxidase in erythrocytes. Intergroup and intragroup results were compared statistically. *Results*. Methylprednisolone was able to keep the levels for all parameters close to baseline except for 30-min MDA, MDA_b, and SOD values. But their results were all different from those of G3. Dextromethorphan was successful in this respect at 30-min GSH-Px and 120-min SOD and GSH-Px, and all values were also different from G3 values except for 10-min MDA, SOD, and GSH-Px. Combined therapy was not able to keep levels close to baseline for all parameters, but they were different from G3's except for the GSH-Px values. Methylprednisolone values displayed minimal alterations according to baseline at 120 min. Dextromethorphan was relatively unsuccessful at 10 min. Combined therapy did not show benefit superior to MP/DM single therapies.

Keywords Dextromethorphan · Malonyldialdehyde · Methylprednisolone · Lipid peroxidation · Spinal cord injury

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Introduction

Traumatic spinal cord injury (SCI) leads to serious biochemical, pathological events that result in tissue necrosis and functional deficit. Among the earliest biochemical reactions are hydrolysis of fatty acids from membrane phospholipids, production of biologically active eicosanoids, and peroxidation of lipids with formation of reactive oxygen species (ROS). These latter are the main agents responsible for cellular damage [1]. Superoxide, ferryl, and hydroxyl anions are the common reactive compounds that cause lipid peroxidation [2]. Under normal conditions, superoxide (O2-) anions are generated during mitochondrial electron transport. There is a balance between antioxidants and oxidants produced by aerobic cellular systems. One of the antioxidant defense systems is superoxide dismutase (SOD) which eliminates superoxides by converting them to hydrogen peroxide (H_2O_2). H_2O_2 is reduced to water by cytosolic antioxidants, catalase, and glutathione peroxidase (GSH-Px) [3]. Products of lipid peroxidation, e.g., malonyldialdehyde (MDA) levels, together with SOD and GSH-Px activity levels, can be measured in monitoring the degree of lipid peroxidation.

With the development of experimental spinal cord injury models and recent advances in spinal cord injury research, many treatment regimens such as receptor blockers, physiologic antagonists, inhibitors of biosynthetic pathways, and membrane-stabilizing drugs have come

into use with time. Still, there is no effective treatment to remedy the detrimental effects of SCI. Methylprednisolone has been found to be useful for its radical scavenging, antilipid peroxidation, and neuroprotective effects. Also, NMDA antagonist drugs have recently gained much credit in the treatment of SCI. But so far no combination therapy of MP and NMDA antagonist drug has been tried and published in the literature. Therefore we decided to investigate the blocking effects of MP, DM (a dextrorotatory morphinan and NMDA antagonist), and their combination on lipid peroxidation and compared with each other by the method of thiobarbituric acid reaction (TBA) with MDA and by simultaneous measurement of levels of erythrocyte SOD and GSH-Px, which are expected to be consumed proportionally to the degree of peroxidation.

Material and methods

One hundred sixty male albino Wistar rats were included in the prospective randomized study program. The rats, weighing 250–340 g, were handled according to "Principles of Laboratory Animal Care" [4] and monitored in terms of blood pressure, heart rate, and body temperature during the study. Induction anesthesia with 3–4% halothane accompanied with a flow of about 1–2 l/min of oxygen was given in a small induction chamber until the rats remained immobile. Then a face mask was used to deliver 1–2% halothane to maintain the anesthesia. The rats were divided into six groups.

In group 1 (G1) (n 10), the control group, neither laminectomy nor medication was performed. Spinal cord tissues were provided by biopsy right after they were killed. The MDA values provided the basal values for the other groups. Group G2 (n 30) was the only laminectomized/sham group; no medication. Group G3 (n 30) received laminectomy and injury; no medication. Group G4 (n 30) received laminectomy + injury + 30 mg/kg methylprednisolone sodium succinate intraperitoneally (MP-Prednol-L, Mustafa Nevzat, Istanbul, Turkey). Group G5 (n 30) had laminectomy + injury + 30 mg/kg DM intraperitoneally (d-3-metoxy-N-methyl morphinan hydrobromide monohydrate) (Sigma, Steinheim, Germany). Group G6 (n 30) received laminectomy + injury + combined therapy (MP and DM at the same doses).

In all animals of groups 2–6, the spinal cords were exposed with T3–T6 total laminectomies. Spinal cord injuries (G3–G6) as described by Rivlin and Tator [5] were accomplished by extradural compression of the exposed spinal cords at the T4-5 level for 30 sec using a Yasargil aneurysmal clip (FE 760) with a closing force of 180 g on the cord. Except in G2 and G3, medications were performed at the time of injury. After removal of the clip, spinal cords were provided at 10 min, 30 min, and 120 min after killing the animals by decapitation. The cord samples were provided in 1-cm lengths at the lesion level and stored at –20°C for assays for MDA. Simultaneously, 3-ml blood samples were drawn into tubes containing EDTA. The blood samples were processed and plasma and serum samples were separated and assayed immediately.

Measurement of tissue MDA levels

Lipid peroxidation in injured spinal cord was estimated by the thiobarbituric acid reaction method for MDA (MDA defined as the product of lipid peroxidation) described by Ohkawa et al. [6] to give a red species absorbing at 535 nm. The MDA results were expressed as nmol/g wet tissue.

0.2 ml of 10% (weight/volume) tissue homogenate was added to 0.2 ml of 8.1% sodium dodecyl sulfate and a 1:5 aqueous solution of thiobarbituric acid. The mixture was diluted to 4.0 ml with distilled water heated in an oil bath at 95°C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of a mixture of N-butanol and pyridine (15:1 volume:volume) were added and the mixture was shaken. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured spectrophotometrically. Tetramethoxy propane was measured as an external standard, and the level of lipid peroxides was expressed as nanomoles of MDA per gram wet weight [6, 7].

Measurement of blood MDA levels (MDA_b)

Serum lipid peroxide levels were measured colorimetrically by the thiobarbituric acid method, which was modified from the methods of Satoh [8] and Yagi [9] as reported recently. MDA levels were expressed as nanomoles per milliliter.

Measurement of erythrocyte SOD activity levels

The role of SOD is to accelerate the dismutation of the toxic superoxide radical (0_2^-) produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity is then measured by the degree of inhibition of this reaction. This was accomplished with a RANSOD kit (Randox, Crumlin, UK). Levels of hemoglobin were measured according to the method of Drabkin [10]. Results were expressed as units per gram of hemoglobin.

Measurement of GSH-Px activity levels

GSH-Px activity levels were measured using a RANSEL kit (Randox) using the method of Paglia and Valentine [11] in which GSH-Px activity is coupled with the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) by glutathione reductase. The oxidation of NADPH was followed spectrophotometrically at 340 nm and at 37°C. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 mM glutathione and 1U/ml of glutathione reductase. The absorbance at 340 nm was recorded for 5 min. The activity was calculated from the slope of the lines as micromoles of NADPH oxidized per minute. Results were expressed as units/g hemoglobin (Hb).

Statistical analysis

SPSS 9.0 software was used in statistical calculations and graphics. For intergroup comparison, Kruskal-Wallis variance analysis was applied for six independent groups each consisting of ≤30 subjects. In case the P value was <0.05, the groups were compared two by two by Mann-Whitney U test (MWU). In order to prevent significance inflation, $P<0.02$ was accepted as significant. Intragroup comparison was made initially with Friedman variance analysis; the P value was calculated as <0.05. Therefore Wilcoxon's rank test (WRT) was used for comparison two by two and to prevent significance inflation, $P<0.03$ was accepted as significant. Results were expressed as mean ± standard error of the mean (Table 1).

Results

Intergroup comparison was done. G1–G3, G1–G6, G2–G3, G2–G6, and G3–G4 were differed from each other for all parameters at all times ($P<0.02$, MWU). G1–G4 30-min MDA, MDA_b, and SOD values differed ($P=0.001$, 0.002, and 0.00, respectively). For G1–G5, all parameters were different except for 120-min SOD and

Table 1 The mean tissue MDA levels (nmol/g wet tissue), blood MDA_b levels (nmol/ml) and SOD (U/gHb) and GSH-Px levels (U/gHb) of each group were identified as below. Values are given as mean ± SEM. *Lam* laminectomy, *I* injury, *MP* methylprednisolone, *DM* dextromethorphan

Parameter		G1 (control)	G2 (lam)	G3 (lam+I)	G4 (lam+I+MP)	G5 (lam+I+DM)	G6 (lam+I+MP+DM)
0 min	MDA	35.4±0.59					
	MDA_b	1.80±0.004					
	GSH-Px	6.80±0.24					
	SOD	2500±55.7					
10 min	MDA		36.2±0.57	47.8±0.8	37±0.57	46.6±0.68	42.6±0.8
	MDA_b		1.94±0.003	2.77±0.002	1.96±0.004	2.42±0.003	2.45±0.007
	GSH-Px		6.76±0.25	4.69±0.21	6.14±0.21	5.36±0.24	5.52±0.21
	SOD		2494±56.5	1707±52.5	2410±58.1	1763±43.9	2020±44.2
30 min	MDA		36.1±0.60	45.7±0.44	39±0.57	39.90±0.45	42±0.93
	MDA_b		1.87±0.004	2.71±0.004	2.16±0.005	2.29±0.005	2.34±0.007
	GSH-Px		6.77±0.25	4.88±0.20	5.95±0.19	6.20±.0.23	5.57±0.21
	SOD		2487.5±59.3	1751±47.7	2085±55.8	2278±57.3	2045±45
120 min	MDA		36.40±0.49	45.1±0.5	36.3±0.55	39.1±0.37	41.8±.71
	MDA_b		1.92±0.005	2.51±0.003	1.83±0.004	2.19±0.007	2.29±0.007
	GSH-Px		6.72±0.25	5.01±0.2	6.75±0.25	6.39±0.23	5.8±0.20
	SOD		2488±55.3	1829±55.1	2491±55.8	2350±53.2	2115±47.1

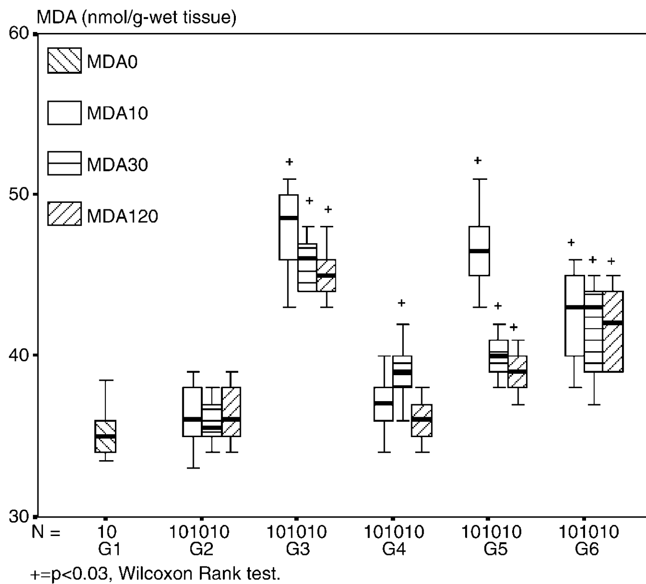

Fig. 1 Basal (G1) and 10, 30, and 120 min tissue MDA levels for each study group (G2–G6) represented by box plot graphic

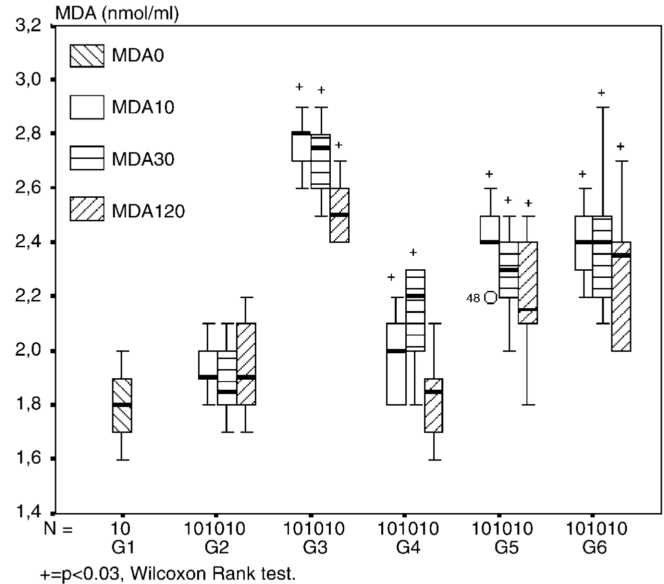

Fig. 2 Basal (G1) and 10, 30, and 120 min blood MDA_b levels for each study group (G2–G6) represented by box plot graphic

30- and 120-min GSH-Px (P>0.02). For G2–G4, 30-min MDA, MDA_b, and SOD values were different (P=0.005, 0.002, and 0.001, respectively). For G2–G5, all were significantly different (P<0.02) except for 120-min SOD and 30- and 120-min GSH-Px values (P>0.02). For G3–G5, all were different except for 10-min MDA, SOD, and GSH-Px values (P=0.2, 0.3, and 0.06 respectively). For G3–G6, only GSH-Px values were similar at all times (P>0.02). For G4–G5, 10- and 120-min MDA (P=0.00 and P=0.003) and MDA_b (P=0.00 and P=0.013), and 10-min SOD values (P=0.00) were different. For G4–G6, 10- and 120-min MDA (P=0.001 and P=0.00), MDA_b (P=0.001 and P=0.00), SOD (P=0.001 for both) and 120-min GSH-Px (P=0.01) were significantly different. For G5–G6, 10- and 120-min MDA (P=0.003 and P=0.01), all SOD values (P<0.012) were different from each other.

Intragroup comparison was performed. For G2, all values were similar to baseline (P>0.03, WRT). For G3, all values were different from the baseline at all times (P=0.005). For G4, 10-min MDA together with 120-min MDA, MDA_b, SOD, and GSH-Px were similar to baseline levels (P>0.03). For G5 and G6, all were different from the basal levels at all times (P<0.03) (Figs. 1, 2, 3, 4).

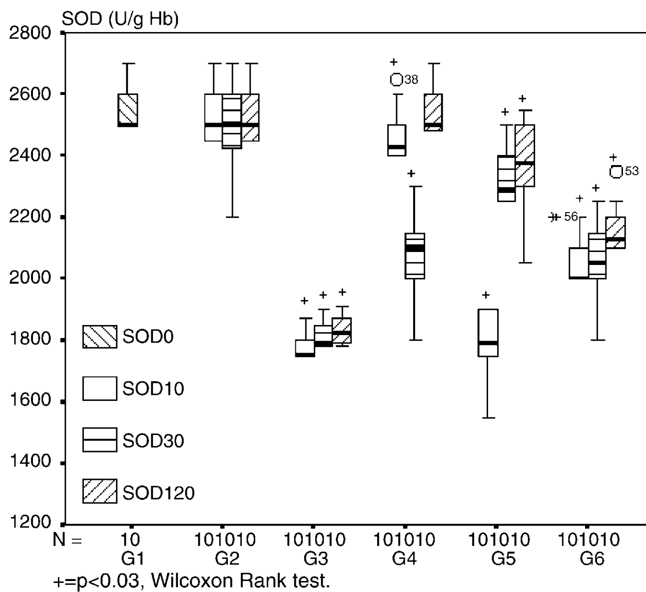

Fig. 3 Basal (G1) and 10, 30, and 120 min SOD levels for each study group (G2–G6) represented by box plot graphic

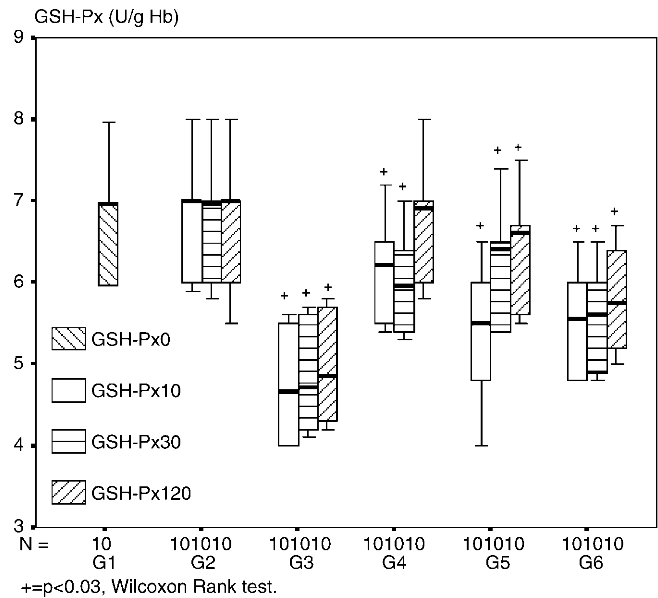

Fig. 4 Basal (G1) and 10, 30, and 120 min GSH-Px levels for each study group (G2–G6) represented by box plot graphic

Intragroup comparison revealed that laminectomy did not alter the values, whereas the alterations were the greatest with the injury group. Methylprednisolone was effective in keeping the values close to baseline, particularly at 120 min. The DM and MP+DM values deviated from basal levels significantly.

Discussion

Traumatic and ischemic injuries to the central nervous system including the spinal cord cause tissue damage through both direct (primary) and indirect (secondary) mechanisms. Secondary injury is caused by the activation of endogenous substances. *Acute inflammatory response* at the site of injury and *spinal cord hemorrhage* with release of Fe and hemoproteins yield the production of reactive oxygen species (ROS) and cytotoxic edema which in turn contribute to lipid peroxidation and ischemia [12, 13, 14, 15, 16]. *Neurogenic shock* with the increase of monoamines promotes ischemia which increases the amount of extracellular glutamate [17, 18, 19, 20]. Glutamate activates an NMDA receptor which promotes Ca ion influx (and Na). Also, voltage-gated Ca influx contributes to a decrease in extracellular Ca levels [21]. Subsequently, the release of lysosomal phospholipases A act upon the cell membrane. Free fatty acids – mainly arachidonic acid (AA) – are released. The AA metabolizes to eicosanoids, (by lipo-oxygenase to leukotrienes and by cyclo-oxygenase to prostaglandin D, E, F, tromboxane, and prostacyclin) [25, 27, 28, 29]. This leads to tissue edema and inhibits membrane-dependant Na-K-ATPase, which is responsible for blood flow, clotting mechanisms, and radical reactions [13, 14, 25, 26, 30]. This, in turn, with the help of the Ca influx, induces vasospasm and ischemia [31]. Likewise, protein kinase C activation promotes neurofilament degradation which contributes lipid peroxidation that can be assessed by MDA levels [22, 23, 24]. Besides uncoupling of oxidative phosphorylation, anaerobic glycolysis, the ATP stores' depletion, and hypoxia facilitate the formation of ischemia [32, 33, 34] (Fig. 5).

The spinal cord and brain are particularly vulnerable to free radical oxidation following hypoxic or traumatic insults because of their high lipid content [13] and poor iron-binding capacity. Accumulating knowledge about triggering mechanisms and the contributing factors and biochemicals on each step of the algorithm in SCI promoted the use of many substances in this field. Anti-inflammatory [12, 35] and/or immunosuppressive drugs [36], phospholipase inhibitors, cyclo-oxygenase/lipoxygenase/mixed lipoxygenase-cyclo-oxygenase inhibitors, thromboxane synthetase, thromboxane, and leucotriene receptor antagonists [27, 28], ROS scavengers/antioxidants [32, 33], the biological enzymes [13, 24, 28, 32, 33, 34, 35], vitamins [24, 34, 37, 38], selenium cation [24], ubiquinole [38], glucose depletion [39], spinal cord blood flow restoration [40, 41], hyperbaric oxygen therapies [42], hypothermia [43, 44], epidural cord cooling [43, 45], metal chelators, protein synthesis inhibitor [46], Mg [47], melatonin [48] or α-lipoic acid [49], calpain inhibitor [50], adenosine [51], opiate antagonists [28, 52, 53, 54], and calcium channel antagonists [55, 56, 57] have been tried before and were found to be promising. Despite the high concentrations of monoamines on SCI [18], the use of α- and β-catecholamine antagonists has not gained much credit in clinical practice [58].

Finally, *NMDA receptor antagonists* (-glicin site [59, 60] -competitive [20, 60, 61, 62] or -noncompetitive [62, 63]*)* have been widely accepted in clinical use to prevent

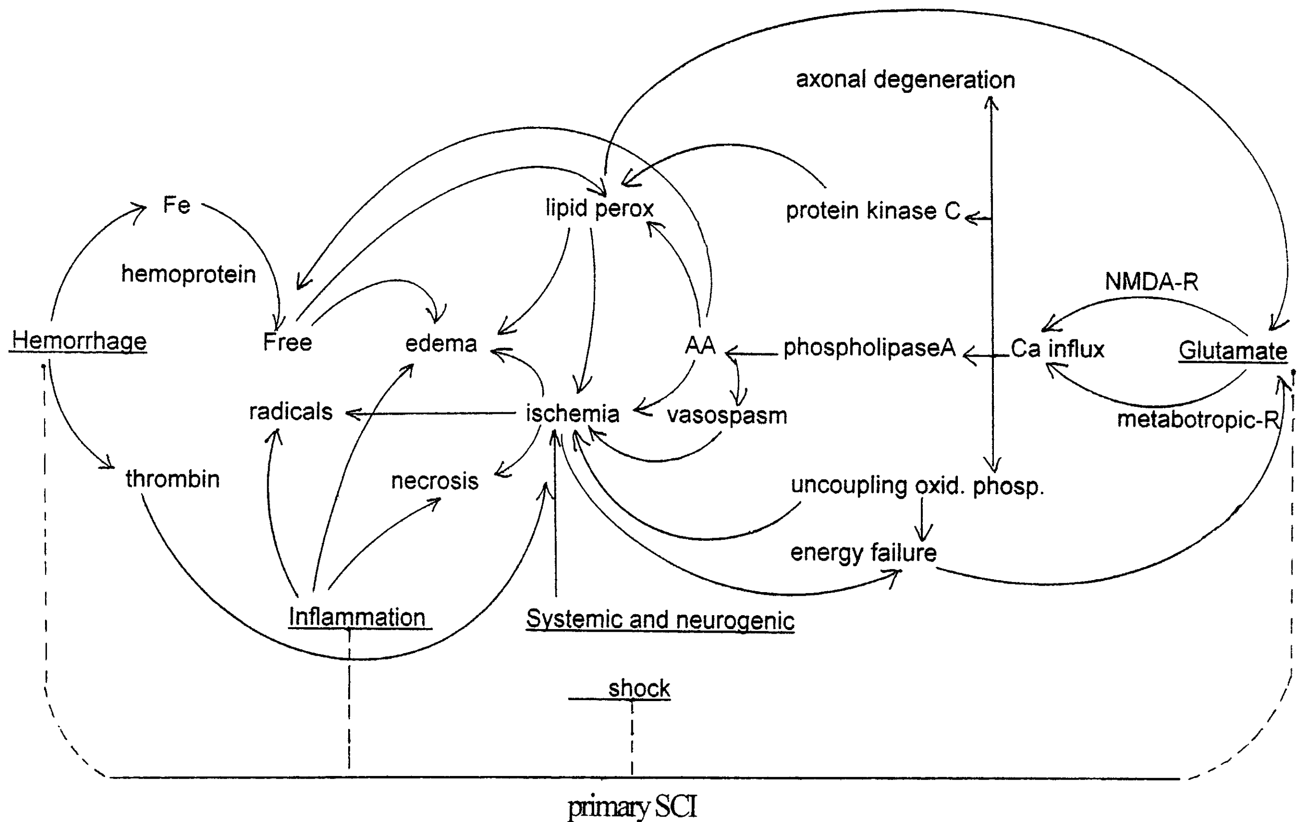

Fig. 5 Secondary injury mechanisms in SCI

the detrimental effects of excitatory amino acids. They are known to have anticonvulsant [60], muscle relaxant and anxiolytic [61], neuroprotective [62, 63], and cytotoxic antiedema effects [15, 64]. The morphinan derivatives [65, 66, 67], particularly dextrorotatory morphinans DM (D-3-methoxy-N-methyl morphinan) and its active metabolite dextrorphan [65, 68, 69], have the unique ability to block multiple major routes of Ca entry into neurons due to both NDMA-antagonist [46, 65, 66, 68, 70, 71] and voltage-gated Ca influx-inhibiting effects. They bind at the PCP/sigma-opiate receptor site and may underlie the similar subjective effects of the dissociative anesthetics and psychomimetic opiates [65, 66, 72]. Dextromethorphan is a water-soluble semisynthetic morphinan derivative and possesses antitussive, antiepileptic, antineurotoxic effects against p-chloroamphetamine but is devoid of opioid action [67, 68, 73]. It protects the brain tissue from edema and ischemia and provides neurologic recovery [34]. Kato used the active metabolite D to protect spinal cord [46]. Therefore, with the use of the NMDA antagonist dextromethorphan, we aimed to prevent the initiation of many pathophysiological processes of the central nervous system or lipid peroxidation by providing the blockade of Ca-2 channels, and we compared its effects with MP, a very well-known neuroprotective agent in SCI.

The use of corticosteroids has been based on a number of theoretical justifications such as their antioxidant and antiedemic properties or their ability to stabilize lysosomal membranes [19]. Hydrocortisone is ineffective as an inhibitor of lipid peroxidation even at exceedingly high concentrations [74]. Dexamethasone, a steroid widely used in neurosurgery, also possesses lipid antioxidation activity [19, 75], but it is slightly less effective than MP and P [19, 52, 74, 76, 77]. Methylprednisolone obviates the early rise of spinal cord blood flow [78, 79, 80], prevents post-traumatic spinal cord ischemia [78, 81] and neurofilament degeneration, improves energy metabolism [33], restores extracellular calcium [80], improves nerve impulse conduction (reducing excitatory amino acid release) [80, 82, 83] and enhances the activity of NaK-ATPase [82], blocks the synthesis of prostaglandin – $F2\alpha$, thromboxane – A2, ROS and the release of free fatty acids [14, 72, 74], and in turn inhibits tissue lipid peroxidation [34, 37, 74, 84] (at 30 min to 1 h [24, 78, 84]). It reduces lesion volumes in contused cord and suppresses vasogenic edema with inflammatory response [35, 85]. The recent development of the 21-aminosteroids (lazaroids) – newer methylprednisolone esters – may attenuate ischemic endothelial cell injury or activation of leukocytes and reduce MDA levels without any glucocorticoid activity [33, 37, 74, 76, 77, 86].

The route and the dose

In focal cerebral ischemia, an i.p. dose of 30 mg/kg of DM reduced infarct volume by 65% [87]. The bioavailability of DM is 1.3-fold lower and the formation of

dextrorphan and other metabolites are threefold greater after i.p. injection of 30 mg/kg of DM. Neurologic recovery was observed when compared to the subcutaneous (s.c.) route [88]. Moreover, s.c. 30 mg/kg DM has PCP-like effects [72, 88]. If given i.p., the PCP-like discriminative effect of dextromethorphan is most likely controlled by its metabolic conversion to dextrorphan [88]. Therefore we chose the intraperitoneal route to give DM at a dose of 30 mg/kg. The study of Fossati et al. [68] on DM pharmacokinetics has shown DM to have the highest concentrations at 60 min and 120 min, which then diminished gradually when given at 30 mg/kg to rabbits. Our study supports their finding, since DM was found to be relatively effective at 120 min after SCI.

Neuroprotective [19, 74, 85] doses of MP greatly exceed those required for glucocorticoid receptor activation and are close to doses that inhibit lipid peroxidation in SCI [74, 82, 84]. Therefore, the effects are probably unrelated to its glucocorticoid receptor-mediated activity [85]. The optimal i.v. dose of MP to achieve these actions is found to be 30 mg/kg [81, 82, 85]. It has been found less effective at the dose of 15 mg/kg or even ineffective at 60 mg/kg [33, 74, 78]. We administered MP at 30 mg/kg.

Different experimental trauma models are employed for the study of disorders in acute SCI [89]. A standard trauma may not be accomplished on the cord since the area of the weight contact is quite variable in the weight-drop method and compression with a contrast weight for a definite time. We used the compression method by horizontally applied clips as described by Rivlin and Tator to achieve more standardized trauma [5]. In gray matter, 5 min of compression injury resulted in a sixfold increase in total free fatty acids (threefold in white matter) and a 20-fold increase in arachidonic acid (AA) levels over controls or laminectomized animals [25]. The reason for our choice of rats is that they are easily available, resistant to long-standing anesthesia, and have vascularization similar to that of humans [90, 91].

Among the outcome measures used in previous studies, e.g., blood flow [58], somatosensory evoked responses [47, 56], histology [46, 50, 63], and function [28, 55], we used the TBA reaction with MDA [6, 92] and erythrocyte SOD and GSH-Px activity level measurements [1, 10, 11, 93, 94, 95, 96] to monitor metabolic changes [32, 33, 49]. Malonyldialdehyde, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of the peroxidation reaction [7, 23, 92]. An antioxidant defense system (enzymatic, nonenzymatic) prevents the damage caused by ROS by reducing the local O_2^- concentrations, sweeping away the catalytic metallic ions Fe and Cu and ROS such as O_2^- and H_2O_2, scavenging the reaction initiators (OH, LO, LOO) and single O_1, and breaking down the chain reactions. Among the enzymatic antioxidant systems are mitochondrial cytochrome oxidase, SOD, catalase, and GSH-Px [97]. Superoxide dismutase is available in every O_2^- metabolizing cell. It prevents O_2^- toxicity by catalyzing the superoxide anion (O_2^-) to H_2O_2 and molecular oxygen. The concentration is higher in the intracellular compartment than the extracellular part [3, 98, 99]. The second intracellular enzyme, GSH-Px, oxidizes glutathione to glutathione disulfide and reduces the hydroperoxides. It catalyzes the turnover of peroxides to alcohols and prevents the oxidation of membrane lipids and hemoglobin by peroxidases. This reaction reduces the ratio of oxidative conversion of hemoglobin to methemoglobin and prolongs the survival of erythrocytes [3, 100]. Since these antioxidant enzymes play an important role in the antioxidant defense mechanisms, many studies relating to their activity levels have been done to monitor the extent of peroxidation and tissue insult. Theoretically, when MDA levels are increased, the blood levels of these enzymes may be expected to decrease. In experimental studies, when rats were exposed to thinner inhalation, it was reported that tissue MDA levels were increased whereas there was a decrease in SOD activity levels [1]. This was valid in our study for SOD levels. However, GSH-Px consumption was rather indolent. Furthermore, a decrease in enzyme levels in relation to the increase of MDA levels may not be the case in every ischemic medium. A compensatory mechanism may induce the enzyme elevations in chronic cases [93, 94]. But at least it can be expected in acute ischemic events, as was noted in our experimental study. In this study, MP was found to be the most effective in keeping MDA, SOD, and GSH-Px levels close to basal levels. It was rather insufficient at 30 min, since MDA and MDA_b levels were elevated, whereas SOD and GSH-Px levels were lower at this time. At this critical point, supporting the effect of antilipid peroxidation with another drug or increasing the MP dose may well be considered. Likewise, DM was not effective at 10 min as with 30 min or 120 min. It was rather effective at 120 min but not as much as with MP. This might have stemmed from the high plasma concentrations of DM at 60–120 min as reported previously [68]. Surprisingly, combined therapy was not superior to MP or DM single therapies at any time point. This might have resulted from a different type of competition between the drugs.

Acknowledgements The experiments comply with the current laws on the protection of animals.

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