Effect of Humic Acid on Oxidative Stress and Neuroprotection in Traumatic Spinal Cord Injury: An Experimental Study

Background/aim: Traumatic spinal cord injury (TSCI) is an important health problem especially in developing countries with additional socio-economic loss. Humic acids (HA) usually have anti-oxidant, anti-inflammatory, blood-circulating and antiviral effects. We aimed to show effect of HA on neuroprotection in TSCI model.

Methods: We performed TSCI model in Twenty-four Wistar-Albino rats in four groups. Control group underwent only laminectomy. Sham group underwent laminectomy followed by TSCI. Low dose HA (5mg/kg) and high dose HA (10mg/kg) groups underwent laminectomy and TSCI followed by peritoneal administration of HA. Preoperative, postoperative 1st hour and postoperative 24th hour cardiac blood samples were obtained. Total Antioxidant Status (TAS), Total Oxidant Status (TOS) and Oxidative Index (OI) levels were evaluated in serum. The 24th hour motor functions were evaluated by Modified Tarlov Score.

Results: There were no significant changes in TAS values between sham- low dose and high dose humic acid groups (p:0.77/0.21). However there were a significant decrease of TOS levels in the 24th hour post operative blood samples comparing the sham group with low dose humic acid group (p=0.02). Pathological evaluation showed a significant decrease in the severity of edema, hemorrhage, Polymorphonuclear leucocytes (PNL) and Mononuclearleucocytes (MNL) /macrophage/microglia when we compare with the control group (p<0.05). There is a significant recovery in paraplegia level as we compared the HA groups with control groups (p<0.001).

Conclusion: In this study, we showed the effects of HA in the early stages of TSCI on oxidative stress, histopathological changes and neurological improvement. It is thought

to be a potential therapeutic agent in acute TSCI but needs to be further evaluated by showing proper effect on other neuroprotective pathways in larger series.

Keywords: Humic acid, spinal cord, trauma, oxidative stress, inflammation

1. Introduction

Traumatic spinal cord injury (TSCI) is an irreversible problem with increasing numbers. World-wide frequency of TSCI may be seen approximately with a frequency of 3.6-195.4/1.000.000. [1] There is still no consensus about medical treatment options. Despite the current treatment modalities, patients may need lifelong care and suffer serious physical and moral loss. This situation became a health problem that negatively affects the family and the countries economy. In addition to the less studied active agents such as naloxone, thyrotropin-releasing hormone and tirilazad, there are frequently used agents, such as GM-1 ganglioside (Sygen) and methylprednisolone, that is effective at various levels. None of these agents constitute treatment protocols based on level 1 and level 2 evidence. [2] Besides, various active agents have been studied in experimental spinal cord trauma models such as glutamate receptor and ion channel antagonists, cyclooxygenase inhibitors and erythropoietin. These agents are expected to be supported by further studies. [3]

Humic acid (HA) is a polyphenolic substance that is used effectively in veterinary and agriculture. HA contain various groups such as phenol, carboxyl acid and quinone groups which changes their effect. Their characteristic may vary depending on the source, age, climate and environmental factors. [4] Today, it is known that they accelerate and support plant growth, acts as a bactericidal in soil and fungicidal in plants. [5] In various studies, it has also been shown to be effective against pollution in soil and water and to have anti-inflammatory, anti-bacterial, anti-ulcerogenic and anti-allergic features. [6]

To date, the efficacy of HA in acute TSCI has not been shown in the literature. We aim to find out the efficacy of HA substance, which was previously never studied in the TSCI, by showing its antioxidant and oxidant effect comparing with the histopathological and neurological outcomes.

2. Material and Method

Ethical approval of the study was obtained from the Ege University Animal Experiments Local Ethics Committee in İzmir (Approval number 2017-113). Study was performed between august 2018 and september 2018. The first step of our study was carried out at Ege University Laboratory Animal Research and Application Center. Later, the biochemical evaluations were made in Biochemistry Department of Health Sciences University, Tepecik, Izmir. The last step, pathological evaluations, were made by Pathology Department of the University of Health Sciences, Tepecik, Izmir. The study was conducted in accordance with the Experimental Animals Local Committee guidelines.

There were 28 Wistar-Albino rats in total, equal numbers of male and female among each group. They were between 8-12 weeks old and weigh 250-450 gr. The rats were equally divided into 4 groups: laminectomy only (Control), laminectomy and TSCI (sham), laminectomy-TSCI-low-dose HA (5 mg/kg) and laminectomy-TSCI-high-dose HA (10 mg/kg). Reference for adaption of humic acid doses were taken from Ozkan et al. Study, but we also added another low dose 5 mg/kg humic acid group [15] All rats were placed in separate cages at an optimal temperature of 18-21 degrees Celcius with equal light and dark cycle with ad libitum food and water during the follow-up.

2.1. Experimental Protocol

28 Wistar-Albino rats were randomly divided into four groups as follows:

Group I (Control Group): number=7; Performance of only laminectomy (T8-T10 level) without additional spinal cord trauma or medical therapy.

Group II (Sham Group): number=7; Performance of laminectomy followed by spinal cord trauma (T8-T10) and administration of serum physiologic intraperitoneally.

Group III (Humic Acid 5 mg/kg): number=7; Performance of laminectomy followed by spinal cord trauma (T8-T10) and administration of HA 5mg/kg intraperitoneally.

Group IV (Humic Acid 10 mg/kg): number=7; Performance of laminectomy followed by spinal cord trauma (T8-T10) and administration of HA 10mg/kg intraperitoneally.

From each group , we aspirated 0.75 mm of blood sample three times.which were; before the surgery, in the 1st and 24th hour of surgery by intracardiac way. Humic acid injection was done immediately after spinal cord injury in HA groups (group 3 and group 4). For the sacrification procedure, thoracotomy was performed under high-dose anesthesia. Aorta was cannulated through the left ventricle and weclamped the descending aorta. Vascular system was perfused with 10% formaldehyde-PBS. After perfusion, the spinal cord was dissected and samples were taken regarding the level of laminectomy.

2.2. Operative Procedure

Rats were anesthetized with intraperitoneal xylazine hydrochloride (10mg/kg) (Rompon 2% Bayer Health Care AG, Germany) and ketamine hydrochloride (90-100 mg/kg) (Ketalar ; Pfizer, USA). We also injected 15 g/kg prophylactic cefazolin sodium subcutaneously one hour before the surgery. In prone position, the dorsal region was cleared with povidone-iodine after shaving. Under the microscope , following the dorsal midline skin incision, paravertebral muscles were dissected laterally and laminectomy was performed at the level of T8 – T10. The spinal cord was detected under the microscope. Control and HA groups were exposed to spinal cord injury by a dropping the

stainless steel bars weighing 5 gram from a 3 mm wide and 10 cm height tube vertically, spinal cord exposed to 50 g/cm of impact. Damage to the spinal cord was created approximately at the level of thoracic 9 vertebra. Then, the skin incision was closed step by step in anatomical layers.

2.3. Analysis of Blood Samples

Blood samples that are collected by intra-cardiac route from rats were centrifuged at 1500 g for 10 minutes and the serum was separated and stored at -20 °C until analyzed. Serum total antioxidant status (TAS) and total oxidative stress (TOS) levels were measured in the same auto-analyser (AU5800, Beckman Coulter Inc., CA, USA) using commercial test kits (Rel Assay Diagnostics, Gaziantep, Turkey). Serum TAS and TOS were determined with kits (Rel Assay Diagnostics kit; Mega Tıp, Gaziantep, Turkey) developed by Erel. Oxidative Stress Index (OSI) values were calculated.

2.4. Measurement of the TAS

Serum TAS levels were determined using a novel automated measurement method, developed by Erel. [7] In this method, the antioxidative effect of the sample against the potent free radical reactions, which is initiated by the produced hydroxyl radical, is measured. TAS results were given in mmol Trolox Eq/L.

2.5. Measurement of the TOS

Serum TOS values were determined using a novel automated measurement method developed. [8] The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide (H2O2) and the results are expressed in terms of micro-molar hydrogen peroxide equivalent per liter (μmol H2O2 Eq/L) [9].

2.6. Calculation of OSI

Oxidative stress index (OSI) values were calculated using the formula 100 x TOS (μmol H2O2 Eq/L) /TAS (mmol Trolox Eq/L). Results were given as arbitrary unit (AU).

2.7.Histopathological Assessment

Pathological analysis held at Tepecik Research and Training Hospital, Department of Pathology. Pathological specimens were collected after the scarification of the rats. Spinal cord samples extracted from the trauma applied region and put in a formalin solution of 10%. The tissues were removed from the formalin solution, embedded in paraffin, sectioned at 3-4 μm, and stained with hematoxylin and eosin (H&E). An experienced pathologist examined the histological preparations with a light microscope (Olympus BX51), and photographs were taken with an Olympus DP72 camera (Olympus Corporation, Japan). For the tissue evaluations, changes in Hemorrhage, Edema, Necrosis, PNL, MNL, Axonal Swelling, Chromatolysis were investigated and scored. Scores were no visible change (0), minimal or slight change (1), moderate change (2), and severe change (3). At least ten high-power fields (HPFs; magnification×400) per section were examined for each sample.

2.8. Immunohistochemical TUNEL Method

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method, which detects fragmentation of DNA in the nucleus during apoptotic cell death in situ, was employed using an apoptosis detection kit (In Situ Apoptosis Detection Kit, ApopTag, Millipore, USA). All reagents listed below are from the kit and were prepared

following the manufacturer's instructions. But, incubation times were increased. Xylene was used for deparaffinization of the sections and rehydrated through a graded ethanol series. They were then incubated with 20 μg/mL proteinase K for 30 min at room temperature and rinsed in dH2O. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide in Phosphate-buffered Saline at room temperature for 15 min. Sections were then incubated with equilibration buffer for 3-5 min. and then Terminal Deoxynucleotidyl Transferase enzyme, in a humidified atmosphere at 37 °C, for 120 min. They were subsequently put into pre-warmed working strength stop/wash buffer at room temperature for 10 min and incubated with anti-digoxigenin conjugate for 60 min. Each step was separated by thorough washes in Phosphate-buffered Saline. Labeling was revealed by applying peroxidase substrate, counter staining was performed using hematoxylin, and sections were dehydrated, cleared, and mounted. TUNEL positive cells/a high-power field cells ratio was used as the index of apoptosis. To count apoptotic cells, at least ten microscopic HPF in each section were evaluated and we count all the positive staining nuclei in the field. We graded the TUNEL positive nuclei as mild(1):0-1, moderate(2):2-9, severe(3):more than 10.

2.9.Neurological Assessment

Motor function was evaluated 24 hours after the surgery. Modified Tarlov Scoring system was applied for the motor function evaluation.

2.10. Statistical Analysis

SPSS 25.0 (IBM Corparation, Armonk, New York, United States) program was used to analyze the variables. Kruskal-Wallis H Test was used with the Monte Carlo Simulation

results for the comparison of ordinal variables; Hemorrhage, Edema, Necrosis, PNL, MNL, Axonal Swelling, Chromatolysis and Paraplegia. Dunn's test was used for Post Hoc analysis. Quantitative variables were grouped as Median, Minimum and Maximum in tables. The variables were examined at 95% confidence level and p-value was accepted as less than 0.05.

3. Results

3.1. Neurological outcomes

No movement was observed in the control group, an average of 0.25 and 1 point of motor function was observed in the HA groups receiving 5 mg/kg and 10 mg /kg, respectively (Table 1). There was a significant statistical difference between the 4 groups ($p < 0.001$) (Table 1).

3.2. Histopathological and TUNEL Staining

When the groups were compared, there were a significant change in hemorrhage, edema, PNL infiltration and MNL infiltration in HA groups related to the dose. P values were determined as 0.013-0.014-0.018-0.019, respectively ($p < 0.05$) (Figure 1 A-B-C). However, no change was observed in axonal swelling ($p = 0.39$). Although a decrease of 0.5 units in chromatolysis value was found, this was not statistically significant ($p = 0.08$) (Table 2). The number of neurons stained with TUNEL was lower in the sham group as there was no spinal cord trauma. Also, there was no significant change in nucleus of the neurons stained with TUNEL when we compare the control group with the HA injected groups ($p=0.92$) (Figure 2 A-B)

3.3. Biochemical analysis

Blood samples collected before the Spinal Cord injury or Laminectomy showed no difference in TAS, TOS and OI values. There was a significant decrease in TAS values

between the sham group and the 10 mg/kg HA group in the first-hour blood samples following the surgical procedure. Although there was no significant difference in TOS levels between the control and HA groups, there was a decrease in TOS values at the 1st-hour blood samples following the surgical procedure (p=0.06). OI was lower in the HA groups when compared to control and sham groups, but statistical analysis showed no significant result when they were analyzed together (p=0.14)

In 5 mg/kg HA group, there was a significant decrease in TOS values in 24th –hour compared to the control group. Although there was a decrease in TOS levels in HA 10 mg/kg group compared to the control group, the p-value was 0.06. (Table 1). The oxidative index value in 24th hour blood samples was lower in the group, injected with HA 5 mg/kg than HA 10 mg/kg compared to the control group. But this difference was not significant.

4. Discussion

In the pathophysiology of the Traumatic Spinal Cord Injury (TSCI), there are primary injury and secondary injury cascades. The disintegration of ion hemostasis, glutamate excitotoxicity, mitochondrial dysfunction and microvascular deterioration occurs within secondary damage mechanisms and lead to oxidative stress by causing free radical formation directly or indirectly. As a result of uncontrolled chain reactions, secondary damage cascade further causes ROS (Reactive Oxygen Species) production, inflammation, apoptosis and neuronal damage. Nowadays, there is no consensus on any agent to be used in spinal cord injury as there are harmful side effects.

One of the main concerns in traumatic spinal cord injury is the reactive oxygen species in the early stages of spinal cord injury. There were a sudden increase in superoxide and hydroxyl radicals (O2- and • OH) in spinal cord injury models and stated that it was high

up to 10 hours. [10, 11] Malondialdehyde increase in the first 5 hours in lumbar puncture samples taken after TSCI models was also shown. [11] Similarly, another study found an increase in MDA and cyclic guanosine monophosphate (cGMP) levels in about 1 hour. [11] In TSCI rat models, with microdialysis and high-pressure liquid chromatography method, MDA has been shown to increase as early as 2 hours. [11]

Studies mentioned above, studied each oxidant and anti-oxidants separately. We performed total antioxidant and total oxidant status analyses. Total antioxidant status shows the body's defense against oxidative stress. Antioxidants found in the circulating blood in our body help in the removal of ROS. Antioxidants are transported through the blood to the whole body to maintain this redox balance. These redox reactions carry out various antioxidants and it is not practical to measure all of them separately, so it is appropriate to measure total antioxidant capacity. Likewise, Xanthine oxidase in the body, glycolate oxidase, monoamine oxidase, such as the presence of endogenous oxidative enzymes such as the measurement of TOS was found to be appropriate. [12]

Humic acid is found to have antioxidant properties through phenol, carboxyl acid and quinone in its structure. [13, 14] Ozkan et al. examined the antioxidant and oxidant effects of HA in pathological examinations in the cerebral ischemia model. In the study, 10 mg/kg HA injection group, compared to the control group; Superoxide dismutase and Nuclear Respiratory factor-1 levels increased, while MDA levels were significantly decreased. [15] Histopathologic evaluations supported that ischemia-related damage was lesser. Another study by Akbas et al. showed a significant increase in TAS values in the renal ischemia model. TOS, OI and IMA (ischemia modified albumin) values were found to be significantly lower in HA groups compared with the control group. [8] Tubular dilatation, tubular cell degeneration and necrosis, bowman capsule dilatation, tubular

hyaline particles and tubular cell distribution in hematoxylin and eosin staining showed improvement compared to control group. Apoptosis evaluation by TUNEL technique indicated a significant decrease in apoptotic cells. Although there are examples of benefits in the literature, there are articles presenting opposing views. Cheng et al. showed an increase in superoxide anions and decrease in glutathione and other antioxidants with the administration of HA, giving rise to cause oxidative stress. [16]

In our study, there were no significant differences between the groups in terms of TAS values taken at the first hour postoperatively in HA injected groups. On the other hand, compared to the control group, the groups injected with 5mg/kg and 10mg/kg HA showed decrease in TOS values at the 1st hour, but the p value was found to be statistically insignificant ($p = 0.11$ and 0.06). Although there was a decrease in oxidative index value comparing the control group and HA groups at the first post-operative hour, it was not statistically significant ($p = 0.77$ and 0.62). There was a statistically significant decrease in TOS values in the group receiving 5 mg/kg HA in 24th-hour blood samples from rats ($p < 0.05$) (Table 2). According to these findings in variable doses of HA injection after TSCI, there was no correlation between the improvement of movement and oxidative stress.

Also, the anti-inflammatory effects of humic substances have been shown in previous studies either by blocking adhesion molecules or inhibiting the phagocytic stimulants. [17] It is found out that humic substances cause anti-inflammation by way of inhibiting the degranulation and adherence of neutrophils. [17, 18] In another study by Goel et al, it has been reported that they have a strong anti-inflammatory effect causing a decrease in paw edema, which was induced by protein injection in the legs of rats and used as a measure of inflammation, by intraperitoneally injected HA. [18, 19] It is shown a relation

between HA and blood flow stimulating effects, that may be related to anti-inflammatory features. [18, 19]

In a study conducted by the European Agency for Evaluation of Medicinal Products (EMEA), protective effects on the intestinal mucosa, antitoxic and antimicrobial effects were demonstrated. [20] Some long-term follow-up studies showed several effects of humic acids. In literature, the antiviral activity of HA is reported on HIV (human immunodeficiency virus) in in vitro studies. Vuckits et al, showed increased humoral immunity response in humic acid supplemented rats in 26 days follow-up. They also found out increased persistance of antibodies in the system. [21] Beside Joone et al. showed that oxyhumate increases interleukin-2 receptors and indirectly increases T-helper cell activity. [22] Similar to our study, but in a long term follow up (36 days), Weber et al, showed that HA may play a role in negating the effects of oxidative stress, with no effect on the Lipopolysacharide induced Interleukin- 6 response. [23] Çalışır et al, studied the long term effect of humic acid on wound healing. After 3 weeks of humic acid administration the found out statistically significant difference between the saline control and the chlorhexidine gluconate group at the end of three weeks.They observed reduced inflammation and the granulation tissue with constricted mucosal epithelial layer. [24]

When the groups were evaluated together in terms of pathological examinations, there was a statistically significant improvement in edema, hemorrhage, PNL, MNL / microglia / macrophage evaluations ($p < 0.05$). In the paraplegia evaluation with the modified Tarlov Scoring, there was a significant improvement in the HA group compared with the control group ($p < 0.001$) (Table 1). This may be related to anti-inflammatory or blood circulation stimulation effects of HAs.

There were some limitations regarding our study. First of all we just studied the early stages rather than long term effects. Secondly other inflamatory pathways may be studied to find the possible effective cascade of humic acid. Lastly, the groups have low number of rats due to ethical issues.

Although there was no significant difference in apoptotic cell count with TUNEL technique. There were significant histopathological changes such as decreased hemorrhage, edema, polymorhonuclear leucocytes and mononuclear leucocytes. As the motor function preserved significantly in HA groups depending on the dose, these findings may be supportive of HA in different doses are effective on spinal cord injury. In our study we conducted the early possible effects of humic acids in the spinal cord injury model. It must be further supported by larger case series not only in early stages but also in an extended period of time in chronic stages as there may be different effects regarding the time and continuous dosing of humic acids. We should also investigate the exact effect of HA on inflammatory substances and other possible mechanisms in TSCI to be used as an alternative therapy in the future.

References

1. Lee BB, Cripps RA, Fitzharris M, Wing PC. The global map for traumatic spinal cord injury epidemiology: update 2011, global incidence rate. Spinal Cord 2014; 52(2):110-116. https://doi.org/10.1038/sc.2012.158

2. Hurlbert RJ, Hadley MN, Walters BC, Aarabi B, Dhall SS et al. Pharmacological therapy for acute spinal cord injury. Neurosurgery 2013; 72 Suppl 2:93-105. https://doi.org/10.1227/NEU.0b013e31827765c6

307 3. Kwon BK, Tetzlaff W, Grauer JN, Beiner J, Vaccaro AR. Pathophysiology and
308 pharmacologic treatment of acute spinal cord injury. Spine J 2004; 4(4):451-464.
309 https://doi.org/10.1016/j.spinee.2003.07.007
310 4. Zhao SX, Yu XL, Li ZH, Yang Y, Zhang X, Wang XD, Zang AF. Effects of Biochar
311 Pyrolyzed at Varying Temperatures on Soil Organic Carbon and Its Components:
312 Influence on the Composition and Properties of Humic Substances. Huan Jing Ke Xue
313 2017; 38(2):769-782. https://doi.org/10.13227/j.hjkx.201604059
314 5. Wu M, Song M, Liu M, Jiang C, Li Z. Fungicidal activities of soil humic/fulvic acids
315 as related to their chemical structures in greenhouse vegetable fields with cultivation
316 chronosequence. Sci Rep 2016; 6:32858. https://doi.org/10.1038/srep32858
317 6. Andersson C, Abrahamson A, Brunstrom B, Orberg J. Impact of humic substances on
318 EROD activity in gill and liver of three-spined sticklebacks (Gasterosteus aculeatus).
319 Chemosphere 2010; 81(2):156-160. https://doi.org/10.1016/j.chemosphere.2010.06.073
320 7. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin
321 Biochem 2005; 38(12):1103-1111. https://doi.org/10.1016/j.clinbiochem.2005.08.008
322 8. Akbas A, Silan C, Gulpinar MT, Sancak EB, Ozkanli SS, Cakir DU. Renoprotective
323 Effect of Humic Acid on Renal Ischemia-Reperfusion Injury: An Experimental Study in
324 Rats. Inflammation 2015; 38(6):2042-2048. https://doi.org/10.1007/s10753-015-0185-2
325 9. Erel O. A novel automated direct measurement method for total antioxidant capacity
326 using a new generation, more stable ABTS radical cation. Clin Biochem 2004; 37(4):277-
327 285. https://doi.org/10.1016/j.clinbiochem.2003.11.015
328 10. Tarlov IM, Klinger H. Spinal cord compression studies. II. Time limits for recovery
329 after acute compression in dogs. AMA Arch Neurol Psychiatry 1954; 71(3):271-290.
330 https://doi.org /10.1001/archneurpsyc.1954.0232039000100

11. Qian H, Liu D. The time course of malondialdehyde production following impact injury to rat spinal cord as measured by microdialysis and high pressure liquid chromatography. Neurochem Res 1997; 22(10):1231-1236. https://doi.org/10.1023/a:1021980929422

12. Luo M, Yang S, Shen S, Li Y. Adsorption Characteristics of Oxytetracycline by Different Fractions of the Organic Matter from Humus Soil: Insight from Internal Structure and Composition. Int J Environ Res Public Health 2020; 17(3). https://doi.org/10.3390/ijerph17030914

13. Aeschbacher M, Graf C, Schwarzenbach RP, Sander M. Antioxidant properties of humic substances. Environ Sci Technol 2012; 46(9):4916-4925. https://doi.org/10.1021/es300039h

14. Ferrara G, Loffredo E, Senesi N, Marcos R. Humic acids reduce the genotoxicity of mitomycin C in the human lymphoblastoid cell line TK6. Mutat Res 2006; 603(1):27-32. https://doi.org/10.1016/j.mrgentox.2005.10.014

15. Ozkan A, Sen HM, Sehitoglu I, Alacam H, Guven M, Aras AB, Akman T, Silan C, Cosar M, Karaman HI. Neuroprotective effect of humic Acid on focal cerebral ischemia injury: an experimental study in rats. Inflammation 2015; 38(1):32-39. https://doi.org/10.1007/s10753-014-0005-0

16. Cheng ML, Ho HY, Huang YW, Lu FJ, Chiu DT. Humic acid induces oxidative DNA damage, growth retardation, and apoptosis in human primary fibroblasts. Exp Biol Med (Maywood) 2003; 228(4):413-423. https://doi.org/10.1177/153537020322800412

17. Joone GK, van Rensburg CE. An in vitro investigation of the anti-inflammatory properties of potassium humate. Inflammation 2004; 28(3):169-174. https://doi.org/10.1023/b:ifla.0000039563.90066.5d

355 18. Goel RK, Banerjee RS, Acharya SB. Antiulcerogenic and antiinflammatory studies
356 with shilajit. J Ethnopharmacol 1990; 29(1):95-103. https://doi.org/10.1016/0378-
357 8741(90)90102-y
358 19. Salz H. Salhumin-gel, ein lokaltherapeutikum mit hyperämisierender,
359 entzündungshemmender und analgetischer wirkung. Medizinische Monatsschrift 1974;
360 28(12):548-550.
361 20. Keuth U, Leinenbach A, Beck HP, Wagner H. Separation and characterization of
362 humic acids and metal humates by electrophoretic methods. Electrophoresis 1998;
363 19(7):1091-1096.
364 https://doi.org/10.1002/elps.1150190707
365 21. Vucskits AV, Hullar I, Bersenyi A, Andrasofszky E, Kulcsar M, Szabo J. Effect of
366 fulvic and humic acids on performance, immune response and thyroid function in rats. J
367 Anim Physiol Anim Nutr (Berl) 2010; 94(6):721-728. https://doi.org/10.1111/j.1439-
368 0396.2010.01023.x
369 22. Joone GK, Dekker J, van Rensburg CE. Investigation of the immunostimulatory
370 properties of oxihumate. Z Naturforsch C J Biosci 2003; 58(3-4):263-267.
371 https://doi.org/10.1515/znc-2003-3-421
372 23. Weber TE, van Sambeek DM, Gabler NK, Kerr BJ, Moreland S, Johal S, Edmonds
373 MS. Effects of dietary humic and butyric acid on growth performance and response to
374 lipopolysaccharide in young pigs. J Anim Sci. 2014 Sep;92(9):4172-9.
375 doi:10.2527/jas.2013-7402.
376 24. Çalışır M, Akpınar A, Talmaç AC, Lektemur Alpan A, Göze ÖF. Humic Acid
377 Enhances Wound Healing in the Rat Palate. Evid Based Complement Alternat Med.
378 2018 Aug 1;2018:1783513. doi: 10.1155/2018/1783513.

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380 Table 1: Statistical analysis of histopathological and neurological outcome

	Control group I (n=7) G.Med. (Min/Max)	Sham group II (n=7) G.Med. (Min/Max)	Humic acid 5mg/kg III (n=7) G.Med. (Min/Max)	Humic acid 10mg/kg IV (n=7) G.Med. (Min/Max)	P
Hemorrhage[a]	1.0 (0 / 2) [II,III]	3.0 (1 / 4)	3.0 (2 / 4)	2.0 (1 / 3)	**0.013**
Edema[a]	0.5 (0 / 1) [II,III]	1.3 (1 / 2)	1.1 (1 / 2)	1.0 (1 / 1)	**0.014**
Necrosis[a]	0.2 (0 / 1)	0.8 (0 / 1)	0.3 (0 / 2)	0.2 (0 / 1)	0.057
PNL[a]	0.0 (0 / 0)	0.8 (0 / 2) [I,IV]	0.5 (0 / 2)	0.0 (0 / 0)	**0.018**
MNL[a]	0.3 (0 / 1) [II,III]	1.3 (1 / 2)	1.3 (1 / 2)	1.0 (0 / 2)	**0.019**
Axonal Swelling[a]	0.5 (0 / 1)	0.8 (0 / 1)	1.0 (0 / 2)	0.8 (0 / 1)	0.390
Chromatolysis[a]	0.3 (0 / 1)	1.2 (1 / 2)	1.0 (0 / 2)	0.5 (0 / 1)	0.080
Paraplegia[b]	4.5 (4 / 5) [II,III]	0.2 (0 / 1) [IV]	0.3 (0 / 1)	1.2 (1 / 2)	**<0.001**

Classification for superscripts a: (0: No damage, 1: Very mild, 2: Mild, 3: Moderate, 4: Severe)

Classification for superscripts b: (0: Flasid, 1: Spastic, 2: Severe, 3: Moderate, 4: Mild, 5: Normal)

Kruskal Wallis Test(Monte Carlo), Post Hoc Test : Dunn's Test, n:number, G.Med.:Grouped Median, Min:Minimum, Max.:Maximum

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Table 2: **Statistical analysis of Biochemical findings. Comparement between each groups**

P I vs III	P I vs IV	P II vs III	P II vIV	P III vs IV	P I vs II

TAS Post-op 1st hour	0.22	0.007	0.77	0.21	0.07	0.21
TOS post-op 1st hour	0.096	0.07	0,11	**0.06**	**0.06**	0.77
OI post op-1.	0.44	0.12	0,77	0.62	0.34	0.22
TAS post-op 24th hour	0.71	0.09	0,88	0.74	0.61	0.93
TOS post-op 24th hour	0.20	0.48	0.02	**0.06**	0.33	0.47
OI post-op 24th hour	0.56	0.07	0.83	0.24	0.17	0.52

TAS/TOS/OI Post-op 1st hour and 24st hour: Biochemical analysis of TAS/TOS/OI levels in blood samples collected after 1 hour and 24 hour from the surgical procedure (either laminectomy or spinal cord trauma).

Figure 1:

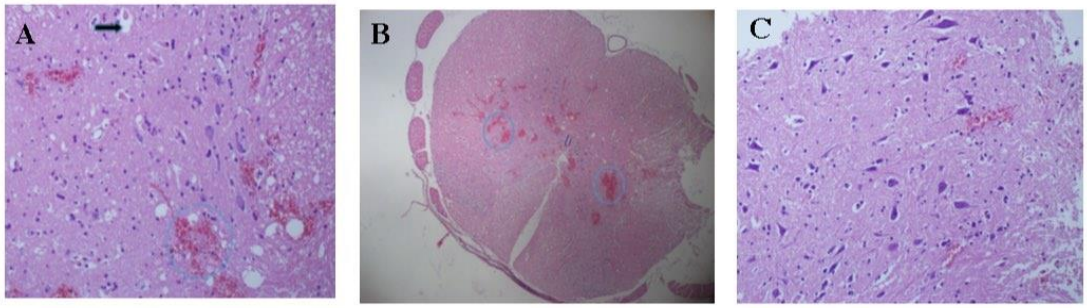

Figure 1: Hemotoxylene and Eosin staining showing: **(A)** Dense hemorrhage areas (in circle), chromatolysis and inflammation (arrow) (x200) (Control Group) , **(B)** Hemorrhage spots in low magnification (x40) (Control Group), **(C)** A sample only with minor areas of haemorrhage and low mononucleer lymphocytes (x200) (Humic acid 10 mg/kg)

Figure 2:

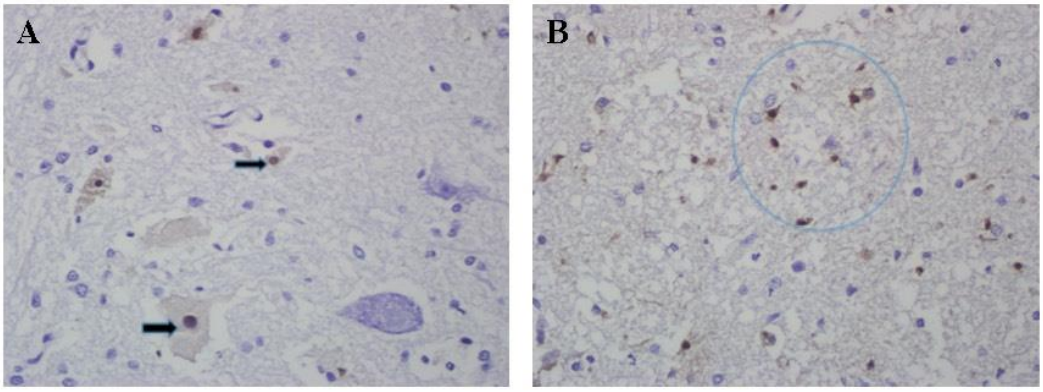

Figure 2: Positive staining (in brown color) with terminal deoxynucleotidyl transferase dUTP nick-end labeling in two different low-dose humic acid group specimens, showing the apoptotic activity in nucleus of neurons (A) and wide range of cells (B) .

Abbrevations: HA= Humic Acid; TAS= Total Antioxidant Status; TOS= Total Oxidant Status; OI= Oxidative Index; TSCI= Traumatic Spinal Cord Injury; PNL= Polymorphonuclear Leukocyte ; MNL= Mononuclear Leukocyte; GM1= Monosialotetrahexosyl ganglioside; MDA= Malondialdehyde; H&E= Hematoxylene and Eosin; TUNEL= The terminal deoxynucleotidyl transferase dUTP nick end labeling; ROS= Reactive Oxygen Species, Hpf= High Power Field, AU= Arbitrary Unit, ℃= Celcius Degree