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RESEARCH ARTICLE

Ameliorative effects of dexpanthenol and/or melatonin application in experimental spinal cord injury

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Background and Aims: Potential ameliorative effects of melatonin and dexpanthenol alone or in combination were investigated by neurological, histopathological, biochemical, and molecular means in an experimental spinal cord injury model in rats.
Methods and Results: Forty-two Sprague Dawley female rats were equally divided into six groups as control (C), sham (S), spinal cord injury (SCI), spinal cord injury with dexpanthenol application (SCI+Dex), spinal cord injury with melatonin application (SCI +Mel), and spinal cord injury with dexpanthenol and melatonin application (SCI+Dex +Mel). In neurochemical analysis, mean Modified Tarlov Scale measurement was significantly better in the SCI+Dex and SCI+Dex+Mel groups as compared to the SCI group at the last measurement point. In biochemical analysis, tissue levels of GSH, SOD, MDA, and XO increased significantly in the SCI group compared to the control. However, no changes were detected among the groups in gene expression levels of *XO, SOD, NF-κB,* and *CASP3*. Histopathological examination revealed severe neuronal degeneration in the SCI group, while the severity of the lesions decreased in the Mel and/or Dex given groups.
Conclusion: Overall, the results indicated that Dex+Mel application may have an ameliorative effect on neuroprotection especially at the later stages of spinal cord injury.

Keywords
Spinal cord injury; dexpanthenol; melatonin; gene expression

Introduction

Spinal cord injury (SCI) is an important medical condition affecting an estimate of more than 900,000 people annually worldwide (1). It can cause acute and chronic long-term secondary complications with end results of serious physical and socioeconomic consequences (2). Acute SCI may cause hypertension and reduced cardiac output as systemic effects and loss of autoregulation at the site of injury and reduction of the microcirculation both in white and gray matter, most prominently in the hemorrhagic loci as well as the nearby regions as local effects (3). The pathophysiology of SCI is complex and comprised of the events occurring in primary and secondary injuries. Acute SCI most commonly occurs as a result of sudden trauma to the spine. The destruction of neuroparenchyma, hemorrhage, and disruption of axonal networks and glial membranes take place over the course of injury. Important biochemical and pathological events including ischemia, oxidative stress, inflammation, apoptotic cell death, and locomotor dysfunctions play significant roles in progression of the disease (4). Following the traumatic injury, reactive oxygen species are generated most often through the actions of phospholipases, and other cellular enzymes such as xanthine oxidase play significant roles. In cases when the cellular defense mechanisms overcome the excessive production of reactive oxygen species, cellular injury takes place due to the interactions of these substances with cellular lipids, proteins, and nucleic acids (5). In SCI, cell death via apoptosis and/or necrosis may occur as a result of cellular dysfunction or inflammatory processes (6). In secondary damage of the SCI, apoptosis is thought to play a

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significant role in cell death (7). Caspases, a series of cellular enzymes mediating apoptotic cell death, involved throughout the cellular destruction through the activation of other caspases, which eventually results in the destruction of important cellular molecules. Caspase 3, activated by both extrinsic and intrinsic pathways of apoptosis, is often used to show apoptotic cells in an injured site (8). NF-κB is an important transcription factor and regulates the activities of various genes that are important in cellular responses, innate immunity, inflammation, cell growth and cell death, and is highly sensitive to oxidative stress (9). It has been shown that NF-κB is involved in inflammatory responses (10). It was also shown to be activated in cell death during the secondary injury phase after SCI (11). There is no consensus on the most appropriate method for the treatment of SCI. Anabolic steroids, polyethylene glycol, magnesium sulphate, and acetyl salicylic acid have been used as neuroprotective agents (12, 13). Various therapeutic strategies including non-steroidal anti-inflammatory agents, steroids, and growth factors have been suggested to treat SCI, mostly targeting to reduce the effects of secondary neuronal damage (14, 15). Attempts to accomplish neuro-regenerative therapies are also in try (16). Currently, corticosteroids are widely used to treat SCI. However, their effects on secondary damage are still uncertain (17). Due to the common side effects of corticosteroids, other drugs with neuroprotective effects against secondary damage are being sought (18). Although some changing successes have been accomplished, safer and better treatment regimens are still in need. Owing to the pathogenesis of cellular degeneration due to oxidative stress, antioxidant agents are of great importance for their potential ameliorative effects. Melatonin, a strong antioxidant agent secreted mainly from the pineal gland but to lesser extent from retina, lymphocytes, guts, bone marrow, and thymus (19). It is still widely under investigation in various experimental setups for its beneficial effects (20). Melatonin mediates its action through receptor mediated and non-receptor mediated mechanisms (21). Its strong free radical scavenging capacity potentiates its use in oxidative stress. Melatonin may exert neuroprotective effects on the secondary damage of SCI. It can regulate the levels of biological molecules such as malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), and myeloperoxidase (MPO), which show abnormal changes after SCI. Additionally, it may play a role in alleviating edema, inhibiting apoptosis, and preventing inflammation in protecting tissues from SCI induced secondary damage (22). Dexpanthenol (Dex), an alcoholic analogue of pantothenic acid also known as provitamin B5, has been reported to have antioxidant, anti-inflammatory, anti-apoptotic, and neuroprotective properties (23). Dex increases the levels of some important cellular molecules such as glutathione, coenzyme A, and adenosine-5′-triphosphate (24). These actions are responsible for its anti-inflammatory and antioxidant activities. However, its exact mechanism of action is still controversial. There is still no consensus in the treatment method of SCI. Although some successes are achieved, complexity of the neural tissue and healing process make it difficult to choose a proper treatment method. Dex and Mel both having strong anti-inflammatory, antioxidant, and anti-apoptotic properties have been aimed to be investigated for their ameliorative potential in an experimental SCI model in rats.

Materials and methods

Experimental design

Forty-two female Sprague Dawley rats weighing 250–300 g were used in this study. Ethical approval was obtained from Animal Experiments Local Ethics Committee with the decision number 2021/44. Rats were kept in a temperature and humidity-controlled environment under 12-hour light/dark cycle. They were fed with standard species-specific pellet feed and water was provided *ad libitum*. The number of animals in each group was calculated by Power Analysis. When the highest SOD difference between the groups was considered, the minimum sample size required to find a significant difference using this test was determined as 7 for each group, while the Type I error (alpha) was 0.05, the power of the test (1-beta) was 0.9, and the effect size was 0.88. In the study, animals were divided into six groups by the simple randomization method. Control (C): Left intact, Sham (S): only laminectomy was performed between the spinal cord T7–T10, SCI: only spinal cord injury was induced, SCI+Dex: spinal cord injury was created, and 3 weeks later daily 500 mg/kg dexpanthenol was given intraperitoneally (ip) for a period of 3 weeks, SCI+Mel: spinal cord injury was created and 3 weeks later daily 5 mg/kg melatonin was given ip for a period of 3 weeks, and SCI+Dex+Mel: spinal cord injury was created and 3 weeks later daily a combination of 500 mg/kg dexpanthenol and 5 mg/kg melatonin was given ip for a period of 3 weeks.

Induction of SCI

All surgical procedures were performed under general anesthesia with 75 mg/kg ketamine, 5 mg/kg xylazine. Anaesthetized rats were fixed in the sternal position. The spinal cord was exposed by total laminectomy at the T7–T10 level without disrupting the integrity of the dura mater. SCI was induced by dropping a 3-mm-diameter metal rod weighing 18 g from a height of 10 cm. Following hemostasis, the paravertebral muscles and skin were sutured in accordance with the anatomical layers, and the rats were allowed to wake up at constant room temperature after the operative intervention.

Radiology

Magnetic resonance imaging (MRI) was performed to detect spinal cord damage. General anesthesia was performed to all rats by ip, injection of 10 mg/kg xylazine hydrochloride and 50 mg/kg ketamine hydrochloride. After the anesthesia was accomplished, rats were fixed in the sternal position and then the images were taken with the help of 1.5T MRI device (Siemens, Avanto, Erlangen, Germany) and 8-channel coils were used. All rats underwent FSE T2A-weighted MR sequences in sagittal and coronal planes on the 21st and 42nd days of the study. T2A-weighted images were obtained using 14450 ms/84 ms TR/TE, 240 × 320 image matrix, and 2 mm slice thickness parameters. On the 21st and 42nd days of the study, MR images were obtained in the sagittal plane using the T2-weighted sequence in all groups. In these images, the level of the affected spinal cord segment, abnormal signal increase corresponding to the edema-contusion region in the spinal cord, volume change in the cord, and accompanying hemorrhagic signal were evaluated and signal changes detected in the spinal cord on T2 sequence were classified as absent = 0, mild = 1, moderate = 2, and significant = 3.

Neurological examinations

All rats were subjected to three neurochemical tests to evaluate the motor function capacity. Finger Opening Test (FOT), Modified Tarlov Scale (MTS), and Inclined Plane Test (IPT) were used. The tests were performed on 0, 7th, 14th, 21st, 28th, 35th, and 42nd days of the treatments. The results of all tests were given as mean ± SD and percentile median, and the differences among the groups were evaluated for each measurement period. The Tarlov mobility assessment scale was as follows: 0: no movement, no weight bearing and total paralysis in hindlimbs, 1: no weight bearing, weak movements in hindlimbs, 2: hindlimb movements with no weight bearing or locomotion, frequent and/or strong hindlimb movement, 3: weight bearing on hindlimbs, 1 or 2 footsteps, 4: slight loss in walking, and 5: normal walking. Finger opening test used a three-scale measure as 0: no opening, 1: partial opening, and 2: full opening (25, 26).

Tissue collection

At the end of the experimental period, all rats were sacrificed under xylazine–ketamine anesthesia, and spinal cord tissues were spared. Samples for molecular, biochemical, and histopathological examinations were taken. For mRNA analysis, samples taken from the rats were cut into small pieces under sterile conditions on ice and stored in RNA storage solution in −80°C deep freezer until the day of analysis. Tissue samples for biochemical analysis were kept in freezer until analysis while they were fixed in neutral buffered formalin for histopathological examination.

qRT-PCR for XO, SOD, NF-κB, and CASP3 *mRNA analysis*

mRNA expression levels of *xanthine oxidase (XO), SOD, NF-κB*, and *CASP3* were determined as reference to *β-Actin*. RNA was isolated from tissue samples taken from animals using 151 Hibrigen General RNA Isolation kit (Catalogue no: MG-RNA-01-100). In one-step qRT-PCR analysis, BioRad CFX96 Real Time Thermal Cycler device, "iTaq™ Universal SYBR® Green One-Step Kit" (BioRad, Catalogue no: 172-5151) was used. RNA isolated from tissue samples was amplified by one-step qRT-PCR using primers specific for *XO, SOD, NF-κB*, *CASP3,* and *β-Actin* genes (Table 1) and the ratios of gene levels to *β-Actin* gene level were determined.

Table 1. Primer pairs used in qRT-PCR analysis.

Genes	Primers	Size (bp)
β-Aktin-F	5′-CTGAAGTACCCCATTGAACA-3′	178
β-Aktin-R	5′-GTCTCAAACATGATCTGGGT-3′	
XO-F	5′-TTCACCACCCTGTGTGT-3′	77
XO-R	5′-TGGAGACCTTCTCTTCAGAT-3′	
SOD-F	5′-GAGAACCCAAAGGAGAGTTG-3′	146
SOD-R	5′-ACCTTGCTCCTTATTGAAGC-3′	
NFKB-F	5′-CTGTCAACAGATGGCC-3′	200
NFKB-R	5′-CCATTTGTGACCAACTGAAC-3′	
CASP3-F	5′-GGAAGCCGAACTCTTCAT-3′	163
CASP3-R	5′-TCCAGGAATAGTAACCGG-3′	

F: Forward primer, R: Reverse primer.

Biochemical analysis

Spinal cord tissue samples were homogenized in 100 mM phosphate buffer (pH 7.4) for 6 min at a frequency of 25/s with a tissue homogenizer. Homogenates were centrifuged at 9500×*g* for 15 min at 4°C and then supernatants were separated. The supernatants obtained from the tissues were analyzed by the Bradford method using bovine serum albumin as the standard (27). GSH (mg/L) [Bioassay Technology Laboratory, CAT No EA0113Ra], SOD (ng/L) [Bioassay Technology Laboratory, CAT No: E2269Ra], MDA (nmol/mL) [Bioassay Technology Laboratory, CAT No: E0156Ra] and XO (ng/mL) [Bioassay Technology Laboratory, CAT No: E1263Ra] levels were measured by ELISA (Multiskan FC Microplate Photometer; Thermo Scientific, United States).

Histopathology

Tissue samples of spinal cords fixed in formalin were routinely processed to obtain paraffin blocks and then 5 µm sections were cut. Tissue sections were stained with hematoxylin and eosin and observed under a light microscope. Histopathological changes including hemorrhage, edema, neuronal and axonal degeneration, and inflammatory cellular infiltration were evaluated. A scale of 0 = none, 1 = mild, 2 = moderate, and 3 = severe was used to determine the presence and severity of the histopathological changes. The pathology score for each spinal cord was measured by calculating the average of the scores obtained for these six parameters.

Statistical analysis

Descriptive statistics of the obtained data were calculated as mean, standard deviation (SD), and quartiles. Kruskal–Wallis test was used to compare the experimental groups in each measurement period, and post-hoc Dunn test was used to determine different groups. Friedman test was used to examine the difference between measurement periods in each group, and post-hoc Dunn test was used to determine the different groups. The relationship between categorical variables and groups was analyzed by using the Fisher–Freeman–Halton exact test. Statistical significance level $P < 0.05$ was accepted and SPSS (version 23) program was used for calculations.

Results

Radiological findings

MR images were taken in the 21st and 42nd days of the study. All findings were summarized in Table 2. On the 21st day measurements, T2A MR finding was not observed in group C, but was seen in all the other groups at a similar rate (100% in all). This finding was significantly compared to the control group ($P < 0.05$). Hemorrhage was not observed in groups C and S, but was seen at a similar rate in the other groups (100% in all). The frequency of hemorrhage in all SCI induced groups was significantly higher than that of groups C and S ($P < 0.05$). T2 signal enhancement was not observed in the group C. In the group S, a mild enhancement was observed in all animals. In the SCI+Dex, SCI+Mel, and SCI+Dex+Mel groups, T2 signal enhancement was moderate in all animals. In the SCI group, high level of enhancement was

Table 2. MRI findings.

			Groups (each $n = 7$)						
			C	S	SCI	SCI+Dex	SCI+Mel	SCI+Dex+Mel	P value
21st day	T2A MR Finding		0	7	7	7	7	7	<0.001
	Hemorrhage		0	0	7	7	7	0	<0.001
	T2 Signal enhancement	None	7	0	0	0	0	0	<0.001
		Mild	0	7	0	0	0	0	<0.001
		Moderate	0	0	0	7	7	7	<0.001
		High	0	0	7	0	0	0	<0.001
	Volume increase in spinal cord	None	7	7	0	0	0	0	<0.001
		Mild	0	0	0	0	0	0	
		Moderate	0	0	0	0	0	7	<0.001
		High	0	0	7	7	7	0	<0.001
42nd day	T2A MR Finding		0	0	7	7	7	7	<0.001
	Hemorrhage		0	0	7	7	7	7	<0.001
	T2 Signal enhancement	None	0	0	0	0	0	0	<0.001
		Mild	7	7	0	0	0	7	<0.001
		Moderate	0	0	0	7	7	0	<0.001
		High	0	0	7	0	0	0	<0.001
	Volume increase in spinal cord	None	0	7	0	0	0	0	<0.001
		Mild	7	0	0	0	0	7	<0.001
		Moderate	0	0	0	7	7	0	<0.001
		High	0	0	7	0	0	0	<0.001

Fisher–Freeman–Halton exact test.

observed in all animals in this group and it was significantly different from all the other groups. Spinal cord volume increase was not observed in all animals in the C and S groups. Moderate volume increase was observed in the SCI+Dex+Mel group. A significant volume increase was seen in the SCI, SCI+Dex, and SCI +Mel groups. No mild volume increase was observed in any of the groups.

On the 42nd day measurements, no measurements were taken from the group C since there was no intervention requiring a change in the spinal cord during the 3-week period, day 21 values were considered valid. While T2A MR finding was not observed in the group S, it was observed at a similar rate in all the other groups ($P < 0.05$). Hemorrhage not observed in the groups C and S, but was present in all the other SCI induced groups ($P < 0.05$). T2 signal enhancement was high in the SCI group and none in the group C. Mild enhancement was detected in the S and SCI+Dex+Mel groups, and it was moderate in the SCI +Dex and SCI+Mel groups. Volume increase in spinal cord was not observed in the group S and it was mild in the group C. While volume increase was high in the SCI group, it was moderate in the SCI+Dex and SCI+Mel groups and mild in the SCI+Dex+Mel group.

Neurological examination findings

Finger Opening Test (FOT)

The results of the FOT were given in Table 3. In the first measurement period, all animals in all the six groups of the study had a score of 2 with fully opening fingers, and no differences were observed among the groups. In all other measurement periods, no significant difference between the groups C and S was detected. On the other hand, FOT scores in these groups were better than those of other SCI induced groups ($P < 0.05$). However, no difference was noted among the SCI, SCI+Dex, SCI+Mel, and SCI+Dex+Mel groups. While the median value of FOT measurements in the SCI, SCI+Dex, and SCI+Mel groups was 0, it was 1 in the SCI +Dex+Mel group.

Modified Tarlov Scale (MTS)

The results of the MTS were given in Table 4. In the first measurement period, all animals in all the six groups of the study had the highest mean score of 5, with no difference among the groups. On all the other measurement periods, no significant difference between the groups C and S was also observed. On the other hand, significant decreases were detected in all the SCI induced groups and these differences were statistically significant compared to the groups C and S. On the last measurement period, slight differences among the SCI induced groups were noted showing better results with the SCI+Dex and SCI+Dex+Mel as compared to the SCI and SCI+Mel groups ($P < 0.05$); however, they were all still significantly lower than those of the groups C and S, indicating limited improvement.

Table 3. Finger opening test (FOT). Measurements were done in seven periods with 1-week intervals. FOT uses a three-scale measure as 0: no opening, 1: partial opening, and 2: full opening.

Periods	Groups (n = 7)	Mean	SD	25th	Median**	75th	P*
1st period	C	2.00	0	2	2	2	1.000
	S	2.00	0	2	2	2	
	SCI	2.00	0	2	2	2	
	SCI+Dex	2.00	0	2	2	2	
	SCI+Mel	2.00	0	2	2	2	
	SCI+Dex+Mel	2.00	0	2	2	2	
2nd period	C	2.00	0	2	2[a]	2	<0.001
	S	1.57	.787	1	2[a]	2	
	SCI	0	0	0	0[b]	0	
	SCI+Dex	0	0	0	0[b]	0	
	SCI+Mel	0	0	0	0[b]	0	
	SCI+Dex+Mel	0	0	0	0[b]	0	
3rd period	C	2.00	0	2	2[a]	2	<0.001
	S	1.57	.787	1	2[a]	2	
	SCI	0	0	0	0[b]	0	
	SCI+Dex	0	0	0	0[b]	0	
	SCI+Mel	0	0	0	0[b]	0	
	SCI+Dex+Mel	0	0	0	0[b]	0	
4th period	C	2.00	0	2	2[a]	2	<0.001
	S	1.57	.787	1	2[a]	2	
	SCI	.14	.378	0	0[b]	0	
	SCI+Dex	.29	.488	0	0[b]	1	
	SCI+Mel	.29	.488	0	0[b]	1	
	SCI+Dex+Mel	.29	0	0	0[b]	1	
5th period	C	2.00	0	2	2[a]	2	<0.001
	S	1.57	.787	1	2[a]	2	
	SCI	.29	.756	0	0[b]	0	
	SCI+Dex	.43	.535	0	0[b]	1	
	SCI+Mel	.43	.535	0	0[b]	1	
	SCI+Dex+Mel	.43	.535	0	0[b]	1	
6th period	C	2.00	0	2	2[a]	2	<0.001
	S	1.57	.787	1	2[a]	2	
	SCI	.29	.756	0	0[b]	0	
	SCI+Dex	.43	.535	0	0[b]	1	
	SCI+Mel	.43	.535	0	0[b]	1	
	SCI+Dex+Mel	.57	.535	0	1[ab]	1	
7th period	C	2.00	0	2	2[a]	2	0.003
	S	1.57	.787	1	2[a]	2	
	SCI	.29	.756	0	0[b]	0	
	SCI+Dex	.43	.535	0	0[b]	1	
	SCI+Mel	.43	.535	0	0[b]	1	
	SCI+Dex+Mel	.86	.900	0	1[ab]	2	

*Kruskal–Wallis test and post-hoc Dunn test. **Median values with different superscripts in a column for a given period indicate statistically significant difference.

Inclined Plane Test (IPT)

The results of the IPT were given in Table 5. In the first measurement period, no significant differences were observed among all the groups. However, in all other measurement periods, IPT values of all the SCI induced groups were significantly lower than those of the groups C and S. In all measurement periods, no significant differences among the SCI, SCI+Dex, SCI+Mel, and SCI+Dex+Mel groups were detected.

mRNA expression findings

mRNA expression levels of *XO, NF-κB*, *CASP3,* and *SOD* genes as reference to the housekeeping gene *β-Actin* were detected by the qRT-PCR, and the results were given in Table 6. Single and desired size of PCR products were observed for each investigated mRNA (Figure 1). The Ct values of *CASP3, NF-κB, SOD, and XO* genes

Table 4. Modified Tarlov Scale (MTS). Measurements were done in seven periods with 1-week intervals. MTS uses a five-scale measure as 1: no weight bearing, weak movements in hindlimbs, 2: hindlimbs movements with no weight bearing or locomotion, frequent and/or strong hindlimb movement, 3: weight bearing on hindlimbs, 1 or 2 footsteps, 4: slight loss in walking, and 5: normal walking.

Periods	Groups (n = 7)	Mean	SD	25th	Median**	75th	P*
1st period	C	5.00	0	5	5	5	1.000
	S	5.00	0	5	5	5	
	SCI	5.00	0	5	5	5	
	SCI+Dex	5.00	0	5	5	5	
	SCI+Mel	5.00	0	5	5	5	
	SCI+Dex+Mel	5.00	0	5	5	5	
2nd period	C	5.00	0	5	5^{a}	5	<0.001
	S	4.43	.976	3	5^{a}	5	
	SCI	0	0	0	0^{b}	0	
	SCI+Dex	0	0	0	0^{b}	0	
	SCI+Mel	0	0	0	0^{b}	0	
	SCI+Dex+Mel	0	0	0	0^{b}	0	
3rd period	C	5.00	0	5	5^{a}	5	<0.001
	S	4.43	.976	3	5^{a}	5	
	SCI	0	0	0	0^{b}	0	
	SCI+Dex	0	0	0	0^{b}	0	
	SCI+Mel	0	0	0	0^{b}	0	
	SCI+Dex+Mel	0	0	0	0^{b}	0	
4th period	C	5.00	0	5	5^{a}	5	<0.001
	S	4.43	.976	3	5^{a}	5	
	SCI	.43	1.134	0	0^{b}	0	
	SCI+Dex	.43	.787	0	0^{b}	1	
	SCI+Mel	.57	.976	0	0^{b}	2	
	SCI+Dex+Mel	.57	.787	0	0^{b}	1	
5th period	C	5.00	0	5	5^{a}	5	<0.001
	S	4.71	.488	4	5^{a}	5	
	SCI	.71	1.496	0	0^{b}	1	
	SCI+Dex	.43	.787	0	0^{b}	1	
	SCI+Mel	.86	.900	0	1^{b}	2	
	SCI+Dex+Mel	.71	.951	0	0^{b}	2	
6th period	C	5.00	0	5	5^{a}	5	<0.001
	S	4.86	.378	5	5^{a}	5	
	SCI	1.14	1.864	0	0^{b}	2	
	SCI+Dex	1.00	.816	0	1^{b}	2	
	SCI+Mel	1.00	.816	0	1^{b}	2	
	SCI+Dex+Mel	1.57	1.134	1	1^{b}	3	
7th period	C	5.00	0	5	5^{a}	5	<0.001
	S	4.86	.378	5	5^{a}	5	
	SCI	1.29	1.799	0	1^{c}	2	
	SCI+Dex	2.00	1.155	1	2^{b}	3	
	SCI+Mel	1.00	.816	0	1^{c}	2	
	SCI+Dex+Mel	2.00	1.414	1	2^{b}	3	

*Kruskal–Wallis test and post-hoc Dunn test; **Median values with different superscripts in a column for a given period indicates statistically significant difference.

were calculated in their groups according to *β-Actin* values. The ratio of the gene in the control group to *β-Actin* gene was evaluated by comparing with different groups. As *XO, NF-κB*, *CASP3*, and *SOD* gene expressions of nerve tissues were compared, no significant changes were seen among the groups as well as the control and sham groups (P > 0.05).

Biochemical findings

Tissue levels of GSH, SOD, MDA, and XO were detected, and the results were given in Table 7. GSH level in spinal tissue of the SCI group increased significantly compared to the control group, but this increase was

Table 5. Inclined Plane Test (IPT). Measurements were done in seven periods with 1-week intervals.

Periods	Groups (n = 7)	Mean	SD	25th	Median**	75th	P*
1st period	C	40.14	.900	39	40[a]	41	0.025
	S	40.00	.816	39	40[a]	41	
	SCI	41.14	.690	41	41[b]	42	
	SCI+Dex	41.00	1.000	40	41[b]	42	
	SCI+Mel	40.57	.535	40	41[b]	41	
	SCI+Dex+Mel	41.43	.787	41	42[ab]	42	
2nd period	C	40.14	.900	39	40[a]	41	<0.001
	S	37.14	4.598	31	39[a]	40	
	SCI	27.00	.816	26	27[b]	28	
	SCI+Dex	26.29	1.113	25	26[b]	27	
	SCI+Mel	27.14	.900	26	27[b]	28	
	SCI+Dex+Mel	27.00	.816	26	27[b]	28	
3rd period	C	40.14	.900	39	40[a]	41	<0.001
	S	37.14	4.598	31	39[a]	40	
	SCI	27.14	1.069	26	27[b]	28	
	SCI+Dex	26.43	.976	26	26[b]	27	
	SCI+Mel	27.29	1.113	26	27[b]	28	
	SCI+Dex+Mel	27.14	.900	26	27[b]	28	
4th period	C	40.14	.900	39	40[a]	41	<0.001
	S	37.43	4.117	32	39[a]	40	
	SCI	27.86	1.952	26	27[b]	30	
	SCI+Dex	26.86	1.215	26	26[b]	28	
	SCI+Mel	28.14	1.574	27	28[b]	30	
	SCI+Dex+Mel	27.71	1.380	27	27[b]	29	
5th period	C	40.14	.900	39	40[a]	41	<0.001
	S	37.43	4.117	32	39[a]	40	
	SCI	28.71	2.812	27	28[b]	31	
	SCI+Dex	27.57	1.512	26	27[b]	29	
	SCI+Mel	28.86	1.864	27	29[b]	31	
	SCI+Dex+Mel	28.86	1.574	27	29[b]	30	
6th period	C	40.14	.900	39	40[a]	41	<0.001
	S	37.57	3.910	33	39[a]	40	
	SCI	29.43	2.992	27	28[b]	32	
	SCI+Dex	28.00	1.414	27	28[b]	29	
	SCI+Mel	29.14	2.268	27	29[b]	32	
	SCI+Dex+Mel	30.43	1.718	29	30[b]	32	
7th period	C	40.14	.900	39	40[a]	41	<0.001
	S	37.71	3.638	33	39[a]	40	
	SCI	30.14	3.976	28	28[b]	33	
	SCI+Dex	28.29	1.976	27	28[b]	29	
	SCI+Mel	29.57	2.573	27	29[b]	33	
	SCI+Dex+Mel	31.29	2.138	29	32[b]	33	

*Kruskal–Wallis test and post-hoc Dunn test. **Median values with different superscripts in a column for a given period indicates statistically significant difference.

Table 6. Spinal cord tissue mRNA expression levels of *CASP3, NF-κB, SOD,* and *XO* as reference to *β-actin.*

	C	S	SCI	SCI+Dex	SCI+Mel	SCI+Dex+Mel
CASP3	19.6 ± 0.6	19.5 ±0.7	18.0 ±0.3	19.6 ±0.7	19.9 ±1.2	18.7 ±1.0
NF-κB	7.7 ±0.5	7.6 ±0.5	7.2 ±0.3	7.6 ±0.4	8.1 ±0.5	8.4 ±1.0
SOD	14.9 ±0.9	14.8 ±0.7	14.9 ±0.4	14.5 ±0.6	14.8 ±0.9	13.6 ±1.0
XO	9.3 ±0.5	9.4 ±0.9	6.9 ±1.1	8.8 ±0.9	9.2 ±1.3	9.1 ±1.5

Data are presented as mean ± SD.

not prominent as compared to the sham group. GSH level of SCI+Dex was similar to that of the SCI group, and it was highest in the SCI+Mel group. GSH level of the SCI+Dex+Mel group showed similar level to that of the control group and it was significantly lower than the SCI group ($P < 0.05$). A similar pattern of levels was observed for both SOD and MDA, in that significantly higher levels of SOD and MDA were detected for the

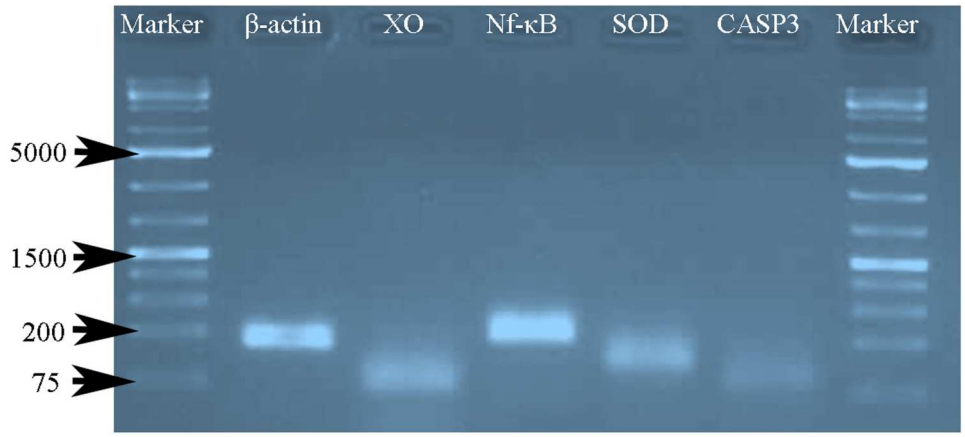

Figure 1. Agarose gel electrophoresis analysis showing the expected sizes of XO, NF-κB, CASP3, SOD, and β-actin mRNA expressions

Table 7. Tissue levels of biochemical parameters (GSH, SOD, MDA, and XO) detected in spinal cord tissue.

Groups	GSH (mg/L)	SOD (ng/L)	MDA (nmol/mL)	XO (ng/mL)
C	0.05 ± 0.01[a]	0.40 ± 0.12[a]	2.32 ± 0.20[a]	9.56 ± 1.50[a]
S	0.15 ± 0.02[b]	0.87 ± 0.05[b]	2.75 ± 0.22[a]	11.65 ± 0.86[a,b]
SCI	0.18 ± 0.03[b]	0.98 ± 0.28[b]	4.04 ± 0.23[b]	30.83 ± 1.98[d]
SCI+Dex	0.18 ± 0.04[b]	0.96 ± 0.04[b]	4.05 ± 0.56[b]	24.58 ± 3.14[c,d]
SCI+Mel	0.40 ± 0.04[c]	1.20 ± 0.03[c]	7.85 ± 0.02[c]	82.17 ± 10.93[e]
SCI+Dex+Mel	0.07 ± 0.01[a]	0.53 ± 0.09[a]	2.67 ± 0.18[a]	18.47 ± 4.85[b,c]

Data are presented as mean ± SD. Groups with different superscripts are significantly different in a column for a given parameter.

SCI group as compared to the control group. SOD and MDA levels of the SCI+Dex group was similar to the SCI group, and the highest SOD and MDA levels were seen in the Dex+Mel group. In the SCI+Dex+Mel group, levels of SOD and MDA were significantly lower than that of the SCI group ($P < 0.05$) and similar to that of the control group ($P > 0.05$). Significantly higher level of XO was also observed for the SCI group as compared to the control group ($P < 0.05$). These increases were also noted in other SCI induced groups. XO levels among all the SCI induced groups were lowest in the SCI+Dex+Mel group, and they were significantly lower than those of the SCI group. This level was still significantly higher than that of the control group ($P < 0.05$) but not higher than that of the sham group ($P > 0.05$).

Histopathological findings

The results of histopathological observations were shown in Figures 2(a–f) and summarized in Table 8. In histopathological examination, C, S, and SCI+Dex+Mel groups had similar morphological features and no pathological findings were observed except mild edema. The most prominent changes were observed in the SCI group. In this group, severe edema, hemorrhage, and inflammation were present. Lymphocytes and macrophages constituted the majority of the inflammation. In the SCI +Mel group, severe edema and moderate hemorrhage were accompanied by mild inflammation. In the SCI+Dex group, moderate edema and hemorrhage were observed but no inflammation was observed. When the findings were analyzed statistically, moderate hemorrhage in the SCI+Dex and SCI+Mel groups was more than the other three groups ($P < 0.001$). Severe hemorrhage was observed in the SCI group compared to the other five groups ($P < 0.001$). The incidence of moderate edema was significantly higher in the SCI+Dex group than in the other five groups, while the incidence of severe edema was higher in the SCI and SCI+Mel groups than in the other four groups ($P < 0.001$). The incidence of neuronal/axonal degeneration was significantly higher in the SCI group than in the other five groups ($P < 0.001$). Mild inflammation was higher in the SCI+MEL group compared to the other five groups. Severe inflammation was detected in the SCI group compared to the other five groups ($P < 0.001$).

Table 8. Histopathological evaluation of spinal cord tissues and comparation among the groups.

		Groups						
Histopathological changes	Severity	C	S	SCI	SCI+Dex	SCI+Mel	SCI+Dex+Mel	P*
Hemorrhage	0	7_a	7_a	0_b	0_b	0_b	7_a	<0.001
	2	0_a	0_a	0_a	7_b	7_b	0_a	
	3	0_a	0_a	7_b	0_a	0_a	0_a	
Edema	1	7_a	7_a	0_b	0_b	0_b	7_a	<0.001
	2	0_a	0_a	0_a	7_b	0_a	0_a	
	3	0_a	0_a	7_b	0_a	7_b	0_a	
Neuronal and axonal degeneration	0	7_a	7_a	0_b	7_a	7_a	7_a	<0.001
	1	0_a	0_a	7_b	0_a	0_a	0_a	
Inflammation	0	7_a	7_a	0_b	7_a	0_b	7_a	<0.001
	1	0_a	0_a	0_a	0_a	7_b	0_a	
	3	0_a	0_a	7_b	0_a	0_a	0_a	

*Fisher–Freeman–Halton exact test, groups with different subscripts are significantly different in a row for a given parameter. None: 0, Mild: 1, Moderate: 2, Severe: 3.

Discussion

Cerebrospinal injuries are quite common and may disturb the life quality of the patient or even be life-threatening. SCI also mostly results in long-lasting physical impairment, and the patients may even lead a bedridden life that lasts for decades and may never return to their normal lives. Rather than the primary damage, which is caused by the physical force and impact of the traumatic event resulting compression, cutting or tearing of the tissue, secondary damages may be more important in the pathogenesis of SCI. Despite limitations in our understanding of the underlying mechanisms causing secondary injury, it has been established that this injury can be attributed to a variety of factors. These include ischemia-reperfusion injury, inflammatory processes, edema, generation of reactive oxygen and nitrogen species, glutamate-mediated excitotoxicity, intracellular calcium accumulation, and activation of proteases and caspases. In addition, necrosis and apoptosis of the cell have also been identified as contributing factors (4). This complex and poorly known process makes the treatment strategies more problematic and difficult. Free radical damage is in the core of many tissue degenerations. Reactive oxygen and nitrogen species such as superoxide, hydroxyl radical, singlet oxygen, hydrogen peroxide, and peroxynitrite can be formed after SCI (28). Under physiological conditions, cellular defense mechanisms using enzymatic and nonenzymatic pathways can provide a state of homeostasis protecting the cell from damaging effects of these substances. GSH, GSH-Px, and SOD, which are endogenous antioxidants, are at the top of these defense mechanisms (29). SOD is the first detoxification enzyme and the most powerful antioxidant in the cell acting as the first-line of defense against reactive oxygen species. In cases where these defense mechanisms cannot overcome these radicals, oxidative and/or nitrosative injuries take place. This basic cellular, and hence tissue, degeneration is also plausible for SCI. Therefore, treatment strategies for SCI are focused mostly on secondary injuries (30). Reactive oxygen species generated by phagocytic inflammatory cells during tissue degeneration cause oxidative damage on lipid membranes, cellular proteins, and DNA. Lipid peroxidation occurs as a result of the reactivation of polyunsaturated fatty acids with free oxygen radicals. This leads to the production of toxic phospholipid by-products oxidized phosphatidylcholine, acrolein and 4-hydroxynonenal (31). Hydroxyl radicals are highly reactive and can react with membrane polyunsaturated fatty acids while superoxide anions can react with nitric oxide and hence form peroxynitrite (32). Peroxynitrite is one of the radical molecules that initiate lipid peroxidation. Thus cellular membrane fluidity and permeability are disturbed, and metabolic processes are interrupted and ion transport systems are altered causing cellular degeneration and death. Bioactive compounds such as Dex and Mel have been shown to suppress lipid peroxidation due to their antioxidant properties by scavenging ROS, chelating redox-active metal ions, and inhibiting xanthine oxidase (33, 34). We have also investigated their antioxidant potentials in ameliorating damage as a result of SCI. Increased tissue levels of GSH, SOD, MDA, and XO in the SCI group as compared to the control group clearly indicated the occurrence of oxidative damage and cellular reaction. Applications of dexpanthenol and melatonin alone in SCI induced rats could not balance the levels of these parameters to that of the control group, yet significantly changed as compared to the SCI only group. In the SCI+Dex+Mel group, significant changes were observed to the benefit of cellular homeostasis indicating better combinatory effects. Ameliorative effect of melatonin on SOD, MDA, and GSH levels in spinal cord ischemia model was also

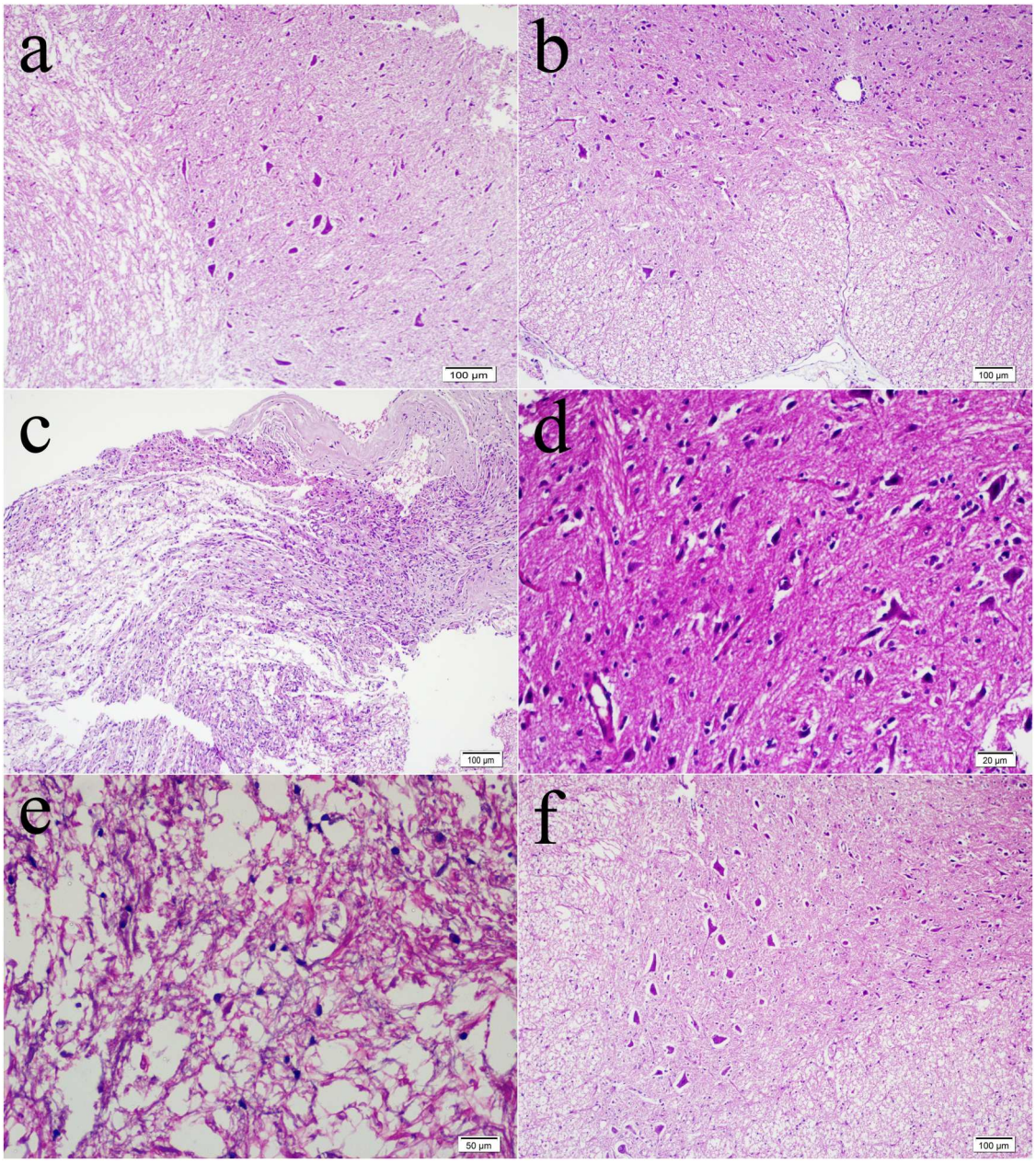

Figure 2. Histopathological evaluation of the samples of spinal cord tissues: (a) control, (b) sham, (c) SCI, (d) SCI+Dex, (e) SCI +Mel, and (f) SCI+Dex+Mel. Significant hemorrhage, edema, neuronal and axonal degeneration, and inflammatory cellular reaction are evident in the SCI group. Severity of these lesions attenuated by dexpenthanol and melatonin application that was most evident in the SCI+Dex+Mel group. Hematoxylin–eosin stain

previously shown (35–38). Dexpanthenol, which is converted to pantothenic acid intracellularly, has long been known for its anti-inflammatory and anti-apoptotic effects. Although the physiological mechanisms related to the antioxidant properties of dexpanthenol are not clear, endogenous antioxidant mechanisms have been shown to be involved (39). Strong antioxidant capacity of dexpanthenol and melatonin was indicated in many studies, with the effects on decreasing MDA and XO levels while increasing SOD and GSH levels (33, 40–42). Melatonin was indicated as a good treatment alternative in neurodegenerative diseases especially due to its easy crossing of the blood–brain barrier (22, 43). In a study, 50 mg/kg melatonin was reported to be sufficient to show beneficial effects in an experimental SCI model in rats by ameliorating oxidative damage (44). In addition to being a strong antioxidant, melatonin also shows strong anti-apoptotic effects due to preventing free radical formation from mitochondria (45). In an ischemia-reperfusion injury

model, melatonin was reported to prevent apoptosis (46). In another study, through affecting Bax and Bcl-2 melatonin was reported to inhibit apoptosis in testicular tissues (47). Similarly, in a renal ischemia-reperfusion injury model in rats, melatonin prevented apoptosis by blocking caspase-3 activation (48). Melatonin can directly detoxify reactive oxygen species and reactive nitrogen species through non-receptor mediated pathways. In addition, melatonin can reduce molecular damage caused by free radicals. Major antioxidant enzymes stimulated by melatonin under basal conditions include intracellular superoxide dismutase, selenium-containing glutathione peroxidase and catalase. Melatonin can also increase the activities of enzymes that increase the level of GSH (49). Dexpanthenol was previously reported to have protective effect on ischemia-reperfusion injury, which was evaluated by the decreased GSH level and also shown to prevent lipid peroxidation, which was detected by the decreased MDA level (50). Dexpanthenol can be used as an adjuvant in corneal or conjunctival lesions as well as in lesions located in mucosal surfaces (51). Topical use of dexpanthenol could upregulate *IL-6, IL-1β, CYP1B1, CXCL1, CCL18,* and *KAP 4-2* gene expression while downregulating psorasin mRNA and protein expression indicating that it might affect important cellular pathways (52). Although dexpanthenol as well as melatonin use in the current study did not cause any significant changes in gene expression levels of the investigated parameters, biochemical parameters and histopathological and neurochemical analysis clearly indicated ameliorating effects on SCI. NF-κB is a transcription factor that plays a critical role in various cellular processes and it is also highly sensitive to oxidative stress (53). Activation of NF-κB can be an early marker of cellular stress in tissues including the neural tissues. Melatonin was shown to attenuate inflammatory reaction by inhibiting NF-κB activation in various experimental models (54, 55). These studies indicate the role of melatonin as a proteasome inhibitor. Melatonin can also be a natural NF-κB inhibitor. These inhibitory effects of Mel on NF-κB may contribute to its anti-inflammatory and pro-apoptotic effect in tissue. However, we did not observe any changes in the gene expressions of *NF-κB* among the groups. Apoptosis may take place as a result of various exogenous or endogenous activators. Caspases, effector enzymes of apoptosis, are commonly investigated to show the presence of apoptotic cell death. Caspase activities can be shown by different methods including immunohistochemistry and gene expression analysis. Increased activities of caspase-3 and -9 were reported in a brain ischemia model in rats, and also shown that melatonin could decrease their levels (56). As in *NF-κB*, no difference on *caspase-3* gene expression among the groups was detected in the present study. In different studies, it was observed that there was no linear relationship between gene expression and enzyme activities and protein levels, which are the end products of genes, and gene expression remained the same despite the change in enzyme activities or protein levels compared to the control group (57, 58). This may be due to the fact that mRNAs produced after mRNA expression are degraded in a very short time. In addition, the fact that the translation step controls protein production by different mechanisms independent of the mRNA level may also explain the change in enzyme/protein levels. In addition to conventional MR sequences, diffusion MR and Diffusion Tensor Imaging can be used to determine the extent of axonal injury and ischemic changes in spinal cord injury by determining ADC and diffusion anisotropy values to determine the severity of damage, stages and responses to treatment in more detail (59, 60). Conventional MRI sequences are not sensitive enough to show axonal changes caused by spinal cord injury. Ellingson *et al.* (60) found an increase in diffusion in both grey and white matter, marked rostral caudal asymmetry and a significant decrease in fractional anisotropy values in the damage region. Both MRI and histopathological findings of this study indicated significant degenerative changes in the SCI group as compared to the control and sham groups. The levels of inflammatory cellular infiltrations, edema, and hemorrhages as well as neuronal and axonal degenerations reduced with dexpanthenol and melatonin applications. The most significant change was detected by the combinatory use of them. These findings were also assessed by neurochemical tests. When two drugs are given together, if the amount of the combined effect they produce is more than the expected effect in the sum of their individual effects, it is called supra-additive interaction or potentiation. We think that the combined use of dexpanthenol and melatonin plays a more effective role in preventing tissue damage in SCI rather than their use alone, and that they do this by inducing the effect of each other.

Disclaimer statements

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