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Full Length Research Paper

The effects of tongdujianbu particle treatment on matrix metallopeptidase 9 (MMP-9), tissue inhibitor of metalloproteinase (TIMP)-1, and transformation growth factor (TGF)-β1 expression in a rat model of spinal core injury

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This work investigated the effect of tongdujianbu particle (TP) on the expression patterns of matrix metallopeptidase 9 (MMP-9), tissue inhibitor of metalloproteinase (TIMP)-1 and transformation growth factor (TGF)-β1 and the behavioural changes in a rat model of spinal cord injured (SCI). A total of 105 adult Sprague-Dawley rats were used for the spinal cord injury as well as different groups of TP treatments. The expression level of MMP-9, TIMP-1, TGF-β1 and Nissl bodies' distribution in the injured spinal cord were examined. Basso-Beattie-Bresnahan (BBB) behavioural score was used to evaluate the recovery of the motor ability. The results suggested that the TP improved the recovery of the behavioural functionality post-SCI. The TP treatment could inhibit the expression of MMP-9 and enhance the expression of TIMP-1, and alter the MMP-9/TIMP-1 ratio whilst maintaining the MMP-9 and TIMP-1 equilibrium. The TP treatment could enhance the expression of TIMP-1 by stimulation of TGF-β1 expression. We concluded that the TP treatment provides a potential approach in integrative medicine for improved SCI recovery.

Key words: Tongdujianbu particle, spinal cord injury, matrix metallopeptidase 9 (MMP-9), tissue inhibitor of metalloproteinase (TIMP)-1, transformation growth factor (TGF)-β1 , blood spine barrier, BBB behavioural function score, immunohistology.

INTRODUCTION

Spinal cord injury (SCI) is referred to the severe symptoms of the sensory block, loss of motor, reflex, and autonomic nerve dysfunction caused by damage of spinal cord. Instant damage to primary SCI is an irreversible process; current worldwide research has been focused on secondary SCI. In clinical treatment, corticosteroids such as methylprednisolone are most commonly applied apart from physical operations (Ahn and Fehlings, 2008; Kube and Olby, 2008; Miller, 2008; Tederko et al., 2009). However, due to significant side effects and treatment induced complications, corticosteroids are with limited applications in many clinical instances. The MMP family factors are gaining increased attention from researchers on their role in SCI. The MMP protein family is a group of proteinases effective to multiple intercellular elements. MMP family proteins are widely expressed in human tissues, degrading extra cellular matrix elements. MMP proteins play important role in tissue shaping, angiogenesis and wound healing (Platt et al., 2003;

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Table 1. Animal grouping and observation time course list.

Group	Number of rats	Time points (days)				
		1	3	5	7	14
TP	25	5	5	5	5	5
MP	25	5	5	5	5	5
MO	25	5	5	5	5	5
SO	25	5	5	5	5	5
NO	5	5				

Zhang et al., 2011).

The involvement of MMP proteins in SCI pathogenesis includes the MMP mediated extracellular matrix (ECM) degradation for blood-spinal cord barrier opening, facilitating the entry of pro-inflammatory cells into neural system and the subsequent damage of spinal cord by antigen peptides released by immunological cells. As a result, inhibition of MMP protein activities in order to maintain the blood-spinal cord barrier may reduce the consequential injury in SCI. Among MMP proteins, MMP-9 has been shown to be strongly relevant to SCI (Zhang et al., 2011). A number of cell types and growth factors such as TGF-β1 can regulate MMP-9 transcription and enzyme activity (Buss et al., 2008; Kohta et al., 2009). Previous data suggested that MMP-9 is involved in tissue restructure, angiogenesis, and vasogenic edema. The TIMP family members can inhibit MMP proteins, and subsequently protect the damaged spinal bone marrow from vasogenic edema (Anik et al., 2011; Sandhir et al., 2011). MMP-9 expression level is low in normal spinal tissues, whilst significantly higher in neuron cells, astrocytes, vascular endothelial cells, and inflammatory cells in damaged neural tissues.

The expression difference of MMP-9 in normal and damaged tissues suggests that MMP-9 widely participates the pathogenic process in neural system damage, inflammation and degenerative symptoms. TIMP proteins are endogenous, specific MMP family protein inhibitors. TIMP proteins and MMP proteins are expressed simultaneously, and the equilibrium between MMP and TIMP proteins is critical to sustainable ECM metabolism. Abnormal shift of the equilibrium leads to malignant matrix degradation and the related pathogenesis. TGF is a multifunctional growth factor. TGF is involved in cell growth, differentiation, immunology, anti-inflammatory, the growth of intercellular matrix protein and the tissue reparation. In later SCI stages, TGF-β1 prevents neuron apoptosis, and promotes neuron growth via alleviation of the physical barrier caused by scar formation (Buss et al., 2008; Lagord et al., 2002). The current study examined the effects of a type of herbal medicine product, TP on SCI recovery in rat model of SCI. This work attempted to investigate the expression pattern of MMP-9, TIMP-1 and TGF-β1 in post SCI rat model under TP treatment to understand the potential mechanism of TP's beneficial effects.

MATERIALS AND METHODS

Animal model and surgery

105 three-month old Sprague-Dawley rats were provided by Laboratory Animal Test Department of Centre for Disease Control (CDC) of Hunan Province. Sprague-Dawley rats are half male, half female with net body weight ranging from 250 to 300 g. The animals were raised in CL clean level, Culture ambient temperature 22 to 25°C, humidity 50% for 1 week with free water and food access before the surgery. Rats were assigned into TP group, methylprednisolone (MP) group, MO group and SO group with 25 rats in each group. Animals were sacrificed at the time points of interest (1, 3, 5, 7 and 14 days) after SCI operation with 5 rats in each time spots. Normal control (NO) group was assigned with 5 rats (Table 1). Rat animal model was constructed by WD bone marrow percussion method which is a modified Allen's method. Rats were intraperitoneally injected with 1% sodium pentobarbital. Anesthetised animal was fixed at prone position on operation table, iodised alcohol disinfected. A 3 cm incision was made at T9 area with T8~T10 exposed. The SCI treatment area was on 5 mm bone marrow tissue.

The spinal dural sac was left intact in operation, and a soft cushion with the identical curvature as dural sac was introduced upon the treated area. Animal was subsequently mounted onto the percussion positioning tool and the calibrated glass tube was vertically placed and fixed upon the intended spinal cord injury area. A 15 g Kirschner wire was allowed to fall from 10 cm above the soft cushion along the calibrated glass tube, generating 15×10 g·cf impact force, hitting on the bone marrow indirectly. Medium SCI treatment was considered successful when intensive convulsion on both posterior limbs and animal tail flicking before complete relaxation was observed. The soft cushion was then removed and cotton slice was used for haemostasis.

Then the wound was sutured up, and covered by sterile gauze. Sham Operation group was opened but without Kirschner wire percussion treatment. Normal control group was left without operation. Posterior limb motor functionality assessment was carried out 1 h after animal's recovery from paralysis. The America spinal injury association motor score (ASIA-MS) was adopted as the standard for the motor functionality assessment. Rats experienced irregular death during and after the operation were replaced and remarked. 2 times/day 100 kU penicillin muscle injection was performed for all rats after the operation for 1 week to prevent infection. Rat groups with SCI were given manual urination twice per day at 8 am to 5 pm until micturition reflex was re-established. The study was approved by ethic committee of live animal research in Department of Spine surgery, Xiang Ya Hospital, Central South University.

Drugs

TP

TP was constituted by 6 g haemophagic leech (Cat. No. 0705156, 1.5 g particle equivalent of 3 g tablets), 9 g Eupolyphaga sinensis (Cat.N o. 0704038, 0.7 g particle equivalent of 6 g tablets), 10 g Ligusticum wallichii (Cat. No. 0711081, 1.3 g particle equivalent of 6 g tablets), 10 g Prunus persica fruit core (Cat. No. 0710005, 2.0 g particle equivalent of 10 g tablets), 10 g Angelica acutiloba (Cat.No. 0712145, 3.0 g particle equivalent of 10 g tablets). 10 g Rehmannia glutinosa (Cat. No. 0711044, 2.5 g particle equivalent of 10 g tablets), 10 g Herbaceous peony root (Cat. No. 0712021, 1.0 g

particle equivalent of 10 g tablets). TP article was heat melted in distilled water at 70 to 80°C to make 2.0 g/ml TP particle liquid before each feeding.

MP

Sodium methylprednisolone succinate injection 40 mg/bottle was from Pfizer Belgium NV (Import. No. H20060054).

Treatments

TP, MO, and SO group animals were given the oral medicine dosage (D) according to the body surface area ratio (R) up to the human adult equivalence by the formula [$D_{rat} = D_{human} \times R_{rat} / R_{human}$], which was 1.6 g/day. The TP group rats were gastric lavage dosed twice per day with TP solution at 0.4 g/ml and 0.8 g each time, starting from 1 h after the successful SCI modeling. The MO and SO group were dosed the same way with distilled water. The MP group were dosed by injection in caudal vein at 30 mg/kg for 15 min, and then injected once every 4.5 h in *cavum abdominis* to achieve the equivalent of 5.4 mg/kg·h^{-1} for 23 h caudal vein injection. The group was gastric lavage dosed with distilled water in day-2 under the same way as of the other groups. The NO group was cultured without intervention.

BBB score

The BBB evaluation method was adopted to assess the behaviour of the SCI rat (Cho et al., 2011; Cho et al., 2009). The assessment was performed by three persons familiar with the BBB method independently in a double-blind manner from the surgery team. The final assessment result is an average of the three assessments. Briefly, SCI model rats were placed on a flat and non-slippery working bench with ample light. The posterior limbs were subsequently observed continuously for 5 min and assessed. All observations and assessments were carried out in the same time. The evaluation is constituted by 3 parts: the evaluation of joint movement of posterior limbs (0 to 7 marks); the evaluation of pace movement of posterior limbs (8 to 13 marks); the evaluation of paw movement details of posterior limbs (14 to 21 marks). The full mark is 21.

Histology

The rats were sacrificed on the days of interest and the blood was drained from thorax and right auricle. The rats were then perfused with heparin saline and then 4% paraformaldehyde (PFA) in 0.1M phosphate buffer saline (PBS). 2 cm bone marrow tissue was retrieved from around T9 damaged area including 0.5 cm of T8 and T10. The samples were further fixed in 4% paraformaldehyde for 24 h before paraffin section treatments. 5 µm paraffin sections were cut, and 3 random slices were taken from each bone marrow tissue for the following immunohistology and Nissl staining (Kohta et al., 2009). MMP-9, TIMP-1 and TGF-β1 were measured by SABC immunohistochemistry method. Slices mounted to glass slides were pre-coated with 0.01% polylysine. After rehydration, the slices were treated with 3% H_2O_2 to quench endogenous enzymes. Antigen was retrieved by citric acid saline (pH=6) treatment before 5% bovine serum albumin (BSA) solution blocking treatment. The MMP-9 rabbit anti-rat IgG (BA2202), TIMP-1 rabbit anti-rat IgG (BA0575), and TGF-β1 rabbit anti-rat IgG (BA0290) (Boster, Wuhan) antibodies were used at 1:100 for primary antibody incubation overnight at 4 degree, respectively. Then the slices were washed and incubated with Biotinylated goat anti-rabbit IgG (Boster, Wuhan) before visualized with 3,3–diaminobenzidine tetrahydrochloride (DAB) treatment. The negative controls were performed without adding primary antibodies.

Stereology

The stained slices were photographed and digitised with MoticB5 microscopic system. Images were analysed by MIAS medical image analysis system. Semi-Qualitative analysis was performed by comparing the SABC signal. 5 high resolution (400X) view clips of the damaged area were randomly selected, and the average value of in these clips was taken as the sample value. Nissl staining measures the cross-section area of the stained neural cells and the grey-scale level.

Statistical analysis

The data analysis was performed with SPSS16.0 software. Data were recorded by two individuals. Normalised data are shown as mean ± s. For difference comparison, α=0.05. For two-group comparison, independent T test was used. For multiple-group comparison, ANOVA was used. When $p \geq 0.05$, the groups were compared with least significant difference (LSD) PostHoc test. Correlationship was tested by Pearson linearity analysis with $p<0.05$ as significant and $p<0.01$ as very significant.

RESULTS

The BBB score and behaviour recovery in treated groups

We found that there was no BBB score comparison significance before SCI ($p>0.05$) with all groups. At day 1 all SCI groups showed significant BBB score loss in compared to SO and NO groups ($p<0.05$) (Table 2), and the MO group was lowest. In the following days the SCI groups began to show the recovery, and the TP group showed significant improvement than MP and MO groups ($p<0.05$).

The tissue changes and cell loss in treated groups

With Nissl staining, we found that in the SO and NO group that neuron cells are rich and normally aligned. The Nissl bodies are populated clearly and thickly in purple staining, and evenly distributed in cytoplasm. 1 day after the SCI induction, the tissue showed haemorrhage, cellular swelling, karyopyknosis, and intracytoplasmic Nissl body conglomeration, disintegration or disappearance. Bone marrow tissue showed vacuolation. The following days, the TP and MP group showed increased Nissl bodies in the soma is compared to the MO group (Figure 1). The Nissl body concentration and positive cell greyscale before and after SCI treatment is shown in Tables 3 to 5. These data all suggested of an improved histological re-construction of the TP group in compared to MP and MO groups.

Table 2. The BBB stores before and after SCI.

Group	Time course (days)					
	0	1	3	5	7	14
TP	21.00±0.00	1.14±0.06*	3.98±0.28△	7.39±0.62△	12.64±1.78△	17.78±2.38△*
MP	21.00±0.00	2.87±0.68*	3.64±0.75△	5.26±0.95△	8.23±1.18△	12.37±2.26△*
MO	21.00±0.00	0.53±0.06*	1.42±0.37*	2.67±0.45*	4.59±1.27*	8.62±1.68*
SO	21.00±0.00	19.78±0.73	20.32±0.85	20.86±0.94	21.00±0.00	21.00±0.00
NO	21.00±0.00	21.00±0.00				

[*]: p<0.05 against SO, NO. [Δ]: p<0.05 against MO. [**]: p<0.05 against MP.

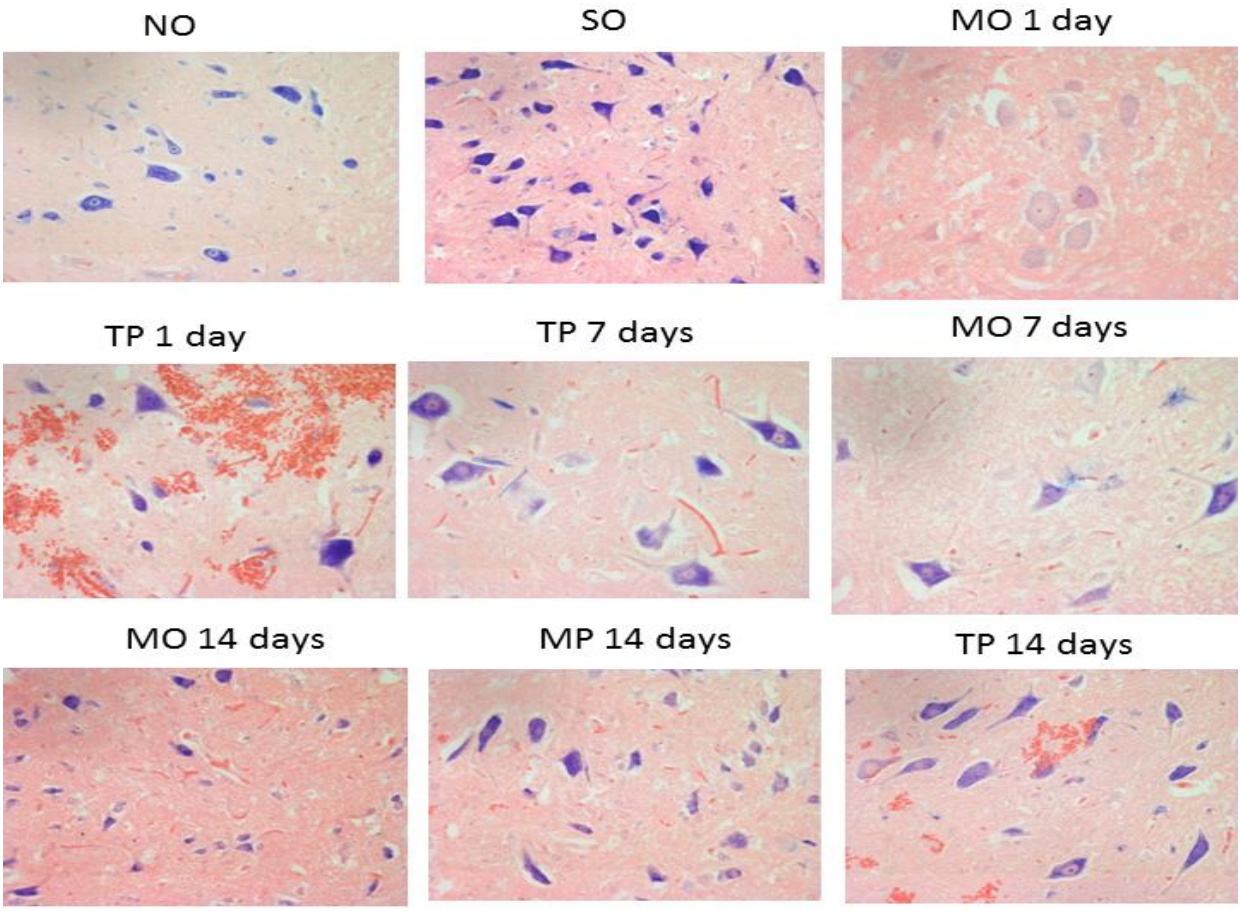

Figure 1. Nissl staining (400X).

The MMP-9, TIMP-1 and TGF-β1 expression

The representative histological pictures of the MMP-9, TIMP-1, and TGF-β1 staining were shown in Figures 2, 3, and 4, respectively. We then measured the MMP-9 expression by area and followed the changes at different time points. We found that the MMP-9 expression area increased in MO, MP and TP groups at day 1, and began to decrease afterwards. TP group constantly showed faster decrease in compared to MO group (Table 6). Post-SCI expression of TIMP1 showed increase in MO, MP and TP groups at day 1, with continued increase and peaked at 1 week time point. By 2 weeks, it decreased a little bit. The TP group showed highest TIMP1 expression level at day 7, and was significantly different from all other groups (Table 7). The post-SCI expression level of TGF-β1 was similar to the case of TIMP-1: increase after SCI induction, peaked at day 7 and began to decrease

Table 3. Spinal cord neuron cell cross-section area (±s, μm^2).

Group	N	Time course (days)				
		1	3	5	7	14
TP	5	208.69±3.91*	235.57±4.28	276.38±5.62	302.97±3.86△	338.78±2.78**
MP	5	222.88±4.92*	247.39±3.82	268.26±3.78	282.54±8.25△	310.13±6.59*△
MO	5	98.27±3.32*	147.26±4.98	179.28±6.42	223.74±5.64	288.37±4.69
SO	5	342.69±8.36	337.86±2.36	345.29±6.37	336.58±7.29△	347.69±7.86**
NO	5	343.45±7.62**				

*: p<0.05 against SO, NO. △: p<0.05 against MO. **: p<0.01 against MP and MO; *△: p<0.05 against MP.

Table 4. Nissl body density in spinal cord neuron post-SCI (±s, pexel).

Group	N	Time course (days)				
		1	3	5	7	14
TP	5	0.47±0.05*	0.52±0.03	0.59±0.03	0.64±0.04△	0.74±0.05**
MP	5	0.48±0.04*	0.54±0.02	0.56±0.04	0.60±0.03△	0.69±0.05*△
MO	5	0.38±0.04*	0.40±0.03	0.44±0.03	0.48±0.04	0.59±0.05
SO	5	0.74±0.06	0.74±0.08	0.75±0.05	0.75±0.07△	0.76±0.08**
NO	5	0.76±0.06**				

*: p<0.05 against SO, NO. Δ: p<0.05 against MO. **: p<0.01 against MP and MO; *Δ p<0.05 against MP.

Table 5. Nissl body average greyscale comparison post-SCI (±s).

Group	N	Time course (days)				
		1	3	5	7	14
TP	5	108.4±11.5*	141.7±15.6	169.3±14.5	187.2±11.7△	238.5±14.6**
MP	5	123.7±16.5*	143.2±12.7	156.4±13.8	167.4±15.2△	197.6±12.8*△
MO	5	96.2±11.3*	112.3±20.6	133.5±13.7	142.6±8.7	173.4±12.5
SO	5	189.2±15.3	199.3±12.6	181.7±13.5	182.6±11.8△	212.3±19.2
NO	5	222.13±12.6				

*: p<0.05 against SO, NO. △: P<0.05 against MO. **: p<0.01 against MP and MO; *△: p<0.05 against MP.

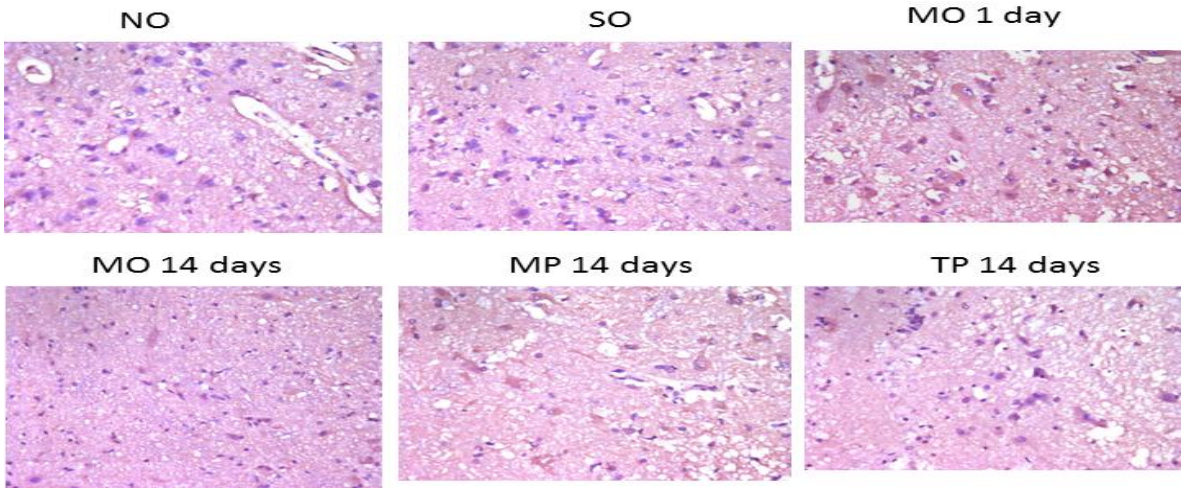

Figure 2. MMP-9 immunohistochemistry stanning (400 X).

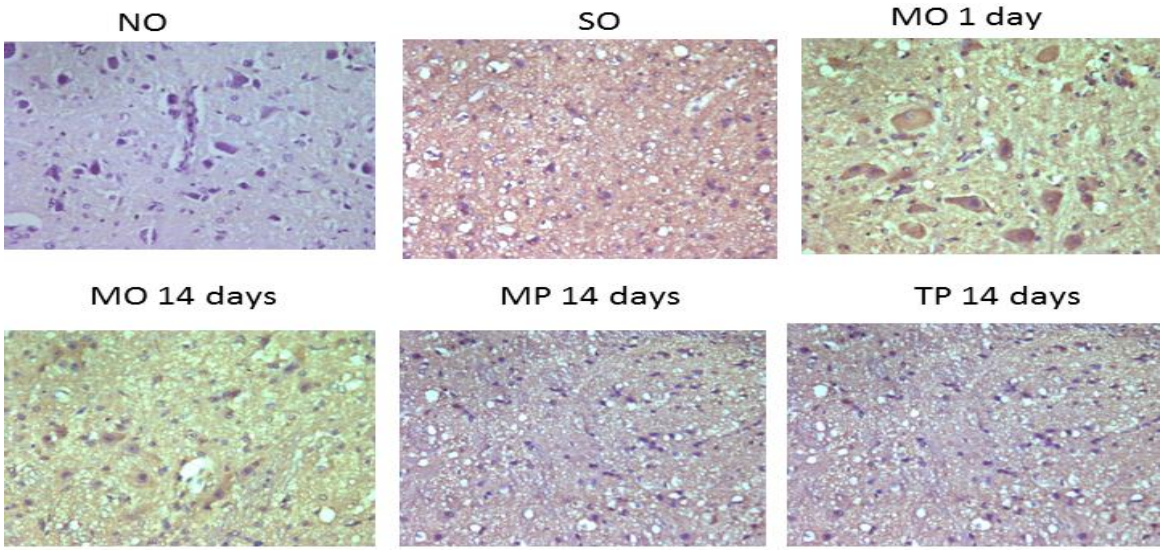

Figure 3. TIMP-9 immunohistochemistry stanning (400 X).

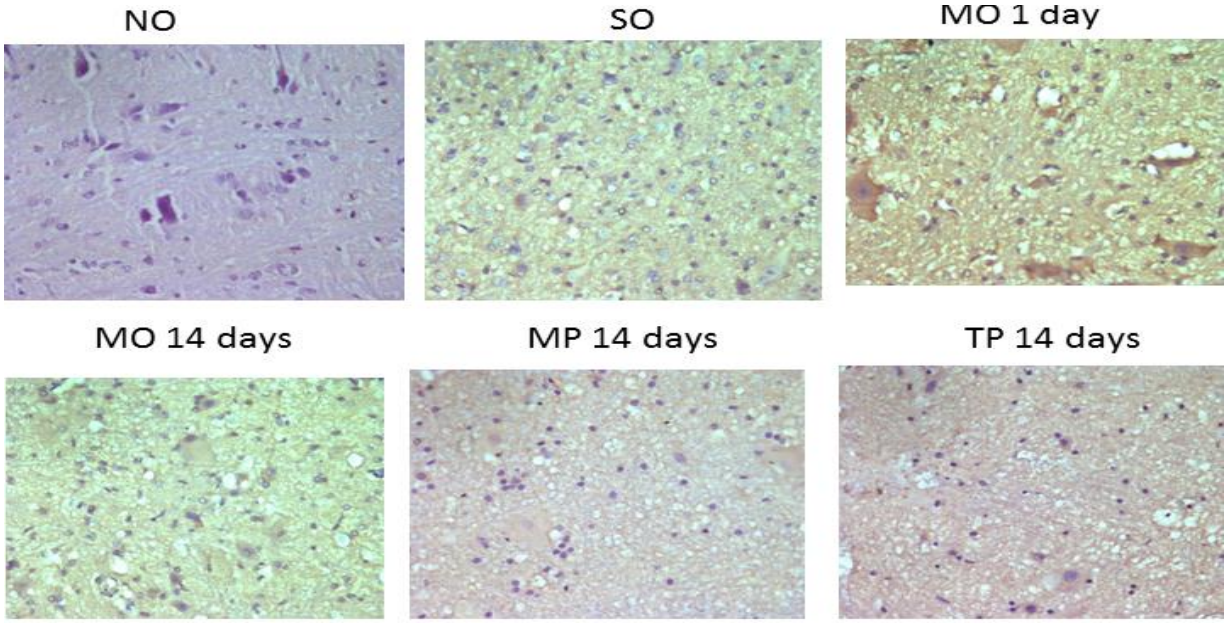

Figure 4. TGF-β1 immunohistochemistry stanning (400 X).

Table 6. MMP-9 expression area post-SCI. (±s, μm^2).

Group	N	Time course (days)				
		1	3	5	7	14
TP	5	73.37±13.68*$^{\Delta}$	65.25±14.78$^{\Delta}$	54.67±14.58$^{\Delta}$	33.79±14.56$^{\Delta}$	15.25±2.67*$^{\Delta\Delta\Delta}$
MP	5	50.32±14.37*$^{\Delta}$	47.37±12.98$^{\Delta}$	38.65±14.12$^{\Delta}$	35.58±13.67$^{\Delta}$	28.28±6.54$^{\Delta}$
MO	5	96.62±11.37**	89.48±12.64**	87.32±14.25**	82.08±15.32**	76.38±16.42**
SO	5	5.83±0.42	6.35±0.64	6.62±0.37	5.84±0.45	5.29±0.49
NO	5	0.00±0.00				

*: p<0.05 against SO, NO. $^{\Delta}$: p<0.05 against MO. **: p<0.01 against MP and MO; *$^{\Delta}$ p<0.05 against MP.

Table 7. Comparison of Post-SCI expression area of TIMP-1 (±s, μm^2).

Group	N	Time course (days)				
		1	3	5	7	14
TP	5	36.54±11.17**	58.67±12.59**	62.59±13.47**	118.34±14.64△**	82.38±11.37**
MP	5	85.17±12.38**	88.69±12.78**	94.32±13.26**	99.48±12.97 **	78.29±11.30**
MO	5	52.36±10.27**	63.45±13.29**	70.68±11.65**	88.75±16.67**	62.54±12.32**
SO	5	4.26±0.35	4.75±0.84	5.32±0.46	5.84±0.39	4.38±0.27
NO	5	0.00±0.00				

*: ^{P}<0.05 against SO, NO. $^{\Delta}$: p<0.05 against MO. **: p<0.01; *$^{\Delta}$: p<0.05 against MP.

Table 8. Post-SCI TGF-β1 expression area comparison (±s, μm^2).

Group	N	Time course (days)				
		1	3	5	7	14
TP	5	24.56±12.36△	50.38±12.48△	54.64±11.39	59.62±12.49△	44.62±11.58**
MP	5	12.26±2.47*	32.69±11.56**	38.24±10.42**	42.59±11.37**	34.29±10.21*
MO	5	22.54±3.28*	38.35±11.34**	42.56±12.35**	45.48±11.23**	36.28±10.52*
SO	5	5.17±0.29	4.38±0.68	5.49±0.62	6.48±0.45	5.26±0.49
NO	5	0.00±0.00				

*: p<0.05 against SO, NO. $^{\Delta}$: p<0.05 against MO. **: p<0.01; *Δ p<0.05 against MP.

Table 9. Correlation of the expression level of MMP-9, TIMP-1, TGF-β1, and R(M/T) among SCI treated groups (n=25).

Group	MMP-9 and TIMP-1		TIMP-1 and TGF-β1		TIMP-1and $R_{(M/T)}$	
	r	*P*	*r*	*P*	*r*	*P*
TP	-0.928	P<0.001	0.563	P<0.01	-0.985	*P*<0.001
MP	-0.527	P<0.01	0.552	P<0.01	-0.764	*P*<0.01
MO	0.682	P<0.01	0.534	P<0.01	0.425	*P*<0.05

afterwards. We found that in TP group the expression of TGF-β1 showed significant differences from MO groups, and at most time points different from MP group (Table 8).

The correlationships among the expression of MMP-9, TIMP-1, and TGF-β1

Assessed by Pearson linearity analysis, the expression changes of TGF-β1, MMP-9 and the ratio MMP-9/TIMP-1 (R(M/T)) are not intercorrelated, evidenced by the r=0.127, r=0.153, respectively, where p >0.05 in both case. In TP group, MMP-9 expression was negatively correlated with TIMP-1 expression (r=-0.928, p<0.001), and TIMP-1 was negatively correlated with R(M/T) (r=-0.985, p<0.001). From day 1 to 7, TGF-β1 and TIMP-1 were correlated (r=0.563, p<0.01). The MP and MO group demonstrated similar correlation post-SCI (Table 9).

DISCUSSION

The present study demonstrated that the TP treatment to a rat SCI model could lead to improved behavioural recovery, correlating with altered molecular changes of MMP-9, TIMP-1, and TGF-β1 expression. Further, the TP treatment improved the neuronal and tissue restoration. TIMP-1 is the endogenous inhibitor of MMP-9, and is upregulated when MMP-9 was increased (Buss et al., 2007; Sandhir et al., 2011). The balance of two molecules confers important information of cellular etiology. In the present case the MMP-9 increased after the induction of SCI, accompanied with the increase of TIMP-1. The changes were more evident in MP and TP groups in compared to MO group, suggesting that these changes might be underlying the neurorehabilitation mechanism of behavioural recovery. Moreover, TP showed stronger efficiency than MP treatment, suggesting that the efficiency

of integrative medicine. It is highly possible that the upstream regulation of MMP-9 expression was controlled by TGF-β1, the expression of which increased after SCI, and could be regulated by TP treatment, suggesting for one potential signalling pathway involved in TP treatment. Interestingly, MP treatment showed decreased TGF-β1 expression than MO control until day-7, in accordance with previous studies. The mechanism underlying methylprednisolone inhibition effect on TGF-β1 expression requires further investigation. Both TP and MP could regulate the post-SCI expression of MMP-9 and TIMP-1 in preventing the secondary SCI. Our studies showed that in earlier time window (days 1 to 3), MP was more effective (Akhtar et al., 2009), whilst in later stage (day 3 to 13), the TP was more effective. This finding suggests that the steroid and the herbal treatment should be combined for clinical application at different time phases of the SCI cases. The active molecules in TP are to be further isolated, purified and studied for their pharmacological applications.

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