The effects of inosine on clinical and histological findings after experimental spinal cord injury in rats

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ABSTRACT: Inosine is a naturally occurring purine nucleoside, the effect of which was discovered only in recent decades. It has potential to prevent neuronal and glial death and can stimulate axonal outgrowth. This study evaluated the effect of inosine (400 mg per rat) administered orally two hours after experimental spinal cord injury and continuously daily for 12 days. We observed the effect of inosine on clinical and histological changes by means of measurement of recovery of hind leg motor function and urinary bladder function, frequency of spasms, neuronal profile and spinal cord tissue sparing. The rats were randomly divided into three groups, SCI-Aqua and SCI-Inosine, with daily administration of aqua for injection or inosine, and SCI-Sham group without spinal injury. The motor function of hind legs and urinary bladder function were evaluated daily for 12 days after the spinal cord injury. In the SCI-Inosine group we recorded lower incidence of spasms due to spinal cord irritation in the early postoperative period when compared to the SCI-Aqua group. We used immunohistochemistry with specific neuronal antibodies to determine the neuronal profiles and the Luxol fast blue staining method to detect the white and grey matter tissue sparing. In our study we recorded significant differences in recovery between the SCI-Aqua and SCI-Inosine group from eigth days after surgery. Moreover, the *post mortem* investigation of transverse spinal cord sections revealed significantly higher numbers in the SCI-Inosine group (more neurons, greater tissue sparing). According to our findings inosine accelerates the recovery of neurological functions.

Keywords: spinal compression; secondary injury; locomotor function; urinary bladder function; axonal rewiring

Spinal cord injury (SCI) is a devastating clinical condition often associated with unfavourable prognosis for recovery of locomotor and sensitive function of the affected part of the body. In animals, damage to the spinal cord is usually manifested by sensory and motor deficits of the affected limb or/and tail, usually accompanied by ataxia, pain, paresis or complete paralysis.

Despite the growing number of therapeutic strategies used in experimental and clinical conditions, the prognosis and outcome after SCI are still unpredictable. The extent of damage to the spinal cord is, depending on the cause of trauma, directly linked to the prognosis (McKee 1990; Coates 2000; Webb et al. 2010; Park et al. 2012). It is known that cells of the central nervous system (CNS) and their axons in adult mammals have limited capability to regenerate, grow, and make functional synapses after injury (Fawcet and Asher 1999; Schwab 2004; Fawcet 2006). Initial mechanical disruption of the spinal cord structures is followed by secondary damage that injures the intact neighbouring tissue. Principally, a cascade of secondary biochemical and vascular events are the main cause of progressive cell damage within the grey matter (GM) which progresses to the white matter (Fehlings et al. 1989;

Tator and Koyanagi 1997; Mautes et al. 2000). Days or weeks after the initial trauma, apoptotic death of oligodendrocytes, located as far as four segments away from the trauma site, magnifies the original injury (Beattie et al. 2000).

Inosine is a dietary supplement which is taken often by athletes to handle strenuous exercise or intense training programs. It alleviates muscle fatigue, which may participate in reducing the spread of secondary injury after SCI (Gomez and Sitkovsky 2003; Liu et al. 2006; Bohnert et al. 2007; Conta and Stelzner 2008). The specific molecular mechanism of the action of inosine is not fully understood, but in some neurons, it activates an intracellular signalling pathway that regulates the expression of multiple genes involved in axon outgrowth (Petrausch et al. 2000). Axonal rewiring was confirmed following unilateral stroke, traumatic brain and spinal cord injury in rats (Zai et al. 2009; Zai et al. 2011). In addition, the neuroprotective effect of inosine against hypoxia/ischaemia was demonstrated in several studies (Jurkowitz et al. 1998; Litsky et al. 1999; Wu et al. 2008).

A normal dose of inosine in humans (50 mg/kg of body weight daily, up to a maximum of 4 g/day) is able to increase the metabolic activity and boosts the production of ATP. Although there are various ways of administration for inosine (Benowitz et al. 1999; Liu et al. 2009), we consider the safe oral gavage dosing method which delivers the dose of inosine directly into the stomach as the most appropriate. This method is suitable for clinical practice with the guarantee that the entire dose is correctly administered.

The goal of our study was to evaluate the efficacy of orally administered inosine. Although there are several studies evaluating the effects of inosine, we specifically wished to study oral administration and aimed to re-evaluate its effects in the first two weeks after spinal cord injury. We hypothesised that inosine would lead to the formation of only minor cavities at the injury site and to the higher survival of nerve cells. Further, we expected that inosine will accelerate the recovery of neurological functions after experimental spinal cord injury in rats. We evaluated the recovery after SCI by means of evaluation of clinical and histological signs within spinal cord transverse sections. The present data demonstrate the beneficial effect of inosine on the nerve pathways responsible for normal mobility and the physiological urinary bladder outlet.

MATERIAL AND METHODS

In this study, we focused on monitoring the impact of pathological processes in the tissue after spinal cord trauma and the effect of oral inosine administration. We used adult male Wistar rats, whose body weight was 290–310 g. The rats were randomly divided into three groups. Two experimental groups (16 rats) underwent traumatic spinal cord injury (SCI): SCI-Inosine group with daily administration of inosine and SCI-Aqua group with daily administration of aqua for injections. In the control group TM-Sham (eight rats), we performed laminectomy, but we did not fill the balloon of the epidural catheter with fluid, thus no spinal cord injury was caused. The rats in individual groups survived 12 days. During the whole period the rats were kept under standard conditions (ambient temperature 22–24 °C, natural light cycle). They were fed and provided access to water *ad libitum*.

This study was performed with the approval and according to the guidelines of the Institutional Animal Care and Use Committee of the Slovak Academy of Sciences and the European Communities Council Directive (2010/63/EU) regarding the use of animals in Research, Slovak Law for Animal Protection No. 377/2012 and 436/2012. All conditions of animal welfare were respected.

Spinal cord compression was performed in rats under general inhalation anaesthesia with a mixture of 1–2% isoflurane in oxygen. Surgery was preceded by the preparation of the catheter, which was used to produce a temporary compression of the spinal cord. We adapted the medical 2-French Fogarty catheter commonly used in embolectomy for use in this experimental model of spinal cord injury. In particular, we shortened the length to 18 cm. Then, we filled it with distilled water using a 50 µl Hamilton syringe fitted to the mechanical pump, which allows precise inflation of the catheter balloon with the desired fluid volume of 12.5 µl. After an accurate removal of all air bubbles from the system, we started the anaesthesia of experimental rats.

Induction of anaesthesia was performed by inserting the individual rat in the induction box with a mixture of 3% isoflurane in oxygen. After calming of the animal, preparation of the surgical site followed and the anaesthesia was maintained with a mixture of oxygen with 1–2% isoflurane via an inhalation mask. We tested the presence of a deep sensitivity in hind legs through pressing fingers

with Pean pliers. We considered the depth of anaesthesia as sufficient when we observed no reaction, no muscle contraction or alteration of respiratory or heart rate of the animal. The skin incision was in the midline at the level Th7 to Th11. After dissecting the muscles, spinous process of Th9 was fixed in the stand with Pean pliers so that it was possible to comfortably perform laminectomy on Th10 and thereby gain the access to the dorsal epidural space. The epidural catheter was inserted through the laminectomy site to the level of Th8 and quickly filled with a given volume of 12.5 µl liquid (aqua for injections) for 5 min (Vanicky et al. 2001). After this period of time we quickly emptied the balloon of the catheter and carefully pulled it from the epidural space. In the SCI-Sham group the same procedure was performed, but the balloon of the epidural catheter was not filled with liquid and did not cause any damage to the spinal cord. The surgical wound was closed in anatomical layers and anaesthesia was concluded.

The rats were kept in preheated boxes until awakening. The motor functions of rats in each group were tested 2 h after spinal cord injury, and then daily during the entire period of survival. Rats were tested using the BBB (Basso, Beattie, Bresnahan) score which represents 21 degrees of damage, where 0 represents paralysis – no spontaneous motor function of hind legs. The score increases with the improved movement of individual joints of the affected leg, coordination of joint movement and steps, supporting body weight, position and stability of the spine, finger position and the position of the tail, up to a maximum score of 21, which represents a physiological movement (Basso et al. 1995).

The spasms caused by irritation of the spinal cord due to injury were manifested as a short-duration episode of spasm of hind legs and hindquarters causing deviation of body position and inability to move. The frequency of spasms was evaluated depending on how many times during the daily BBB scoring examination they appeared in all rats in a representative group.

Two hours after awakening (Day 0) from anaesthesia all rats recorded a BBB score of 0 – complete paralysis of the hind legs in the groups with balloon inflated (SCI-Inosine, SCI-Aqua) and BBB score 21 – normal function of the hind legs in the SCI-Sham group. Each rat was tested daily, individually in an open area, for five minutes. For statistical analysis the values of the BBB scores at 0, 2, 4, 6,

8, 10, and 12 days after SCI were used, expressed as an average between the legs before calculation of the average of the groups.

Spinal cord injury in this model leads to damage to the motor and autonomic nerve pathways responsible for mobility, but also compromises urinary bladder function. Therefore, we had to perform manual emptying of the bladder in these rats two- to three-times daily until spontaneous return of its physiological function. Bladder function in experimental rats was recorded daily using the urinary bladder function score (UBFS), which we created especially for this study. For statistical analysis the score representing the bladder function at 0, 2, 4, 6, 8, 10, and 12 days after spinal cord injury was used, averaged within each group.

Urinary bladder function score:

0 = complete loss of urinary function, manual emptying of urine three times a day, in the urine macroscopically visible signs of blood

1 = partial recovery of urinary function, manual emptying two times a day, blood may still be present in urine

2 = partial restoration of urinary function, the sphincter spasm release (partial/complete), manual emptying two times a day, blood may still be in urine

3 = restoration of urinary function, manual emptying once daily, without blood in urine

4 = physiological urinary function, with no signs of blood and no residual urine volume

Given that inosine is available as a dietary supplement we decided on daily oral administration starting in the acute phase of the injury – two hours after experimental spinal cord injury. Given the unwillingness and inability of rats to receive inosine capsules with powder, we chose a method of dissolving the inosine powder in aqua for injections and administration through the oro-gastric tube. The tube was made of rubber, with a diameter of 2.3 mm, and was attached to a syringe, whereby the volumes were administered directly into the stomach. The length of the tube was determined by measuring the distance from the mouth to the last rib of a given animal.

Inosine (400 mg) was dissolved in 2 ml of aqua for injections and taken into the syringe. During administration the rats were fixed in a vertical position, with the head and neck in a single line with the oesophagus, allowing the free passage of the tube after the insertion through the oral cavity

directly into the stomach. The residual amount of inosine in the syringe was flushed with an additional 2 ml aqua for injections and was transferred in the same way into the stomach. The SCI-Aqua group received daily the relative volume of 2 + 2 ml of aqua for injections. After administration rats were observed for 10 min for signs of dyspnoea or signs of possible distress.

Rats were euthanised 12 days after surgery. After induction of deep general anaesthesia by intraperitoneal injection of thiopental (50 mg/kg) and performing the whole-body thoracotomy, rats were transcardially perfused with saline (500 ml) followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB, pH = 7.2). The spinal cords dissected from vertebral canals were post-fixed in 4% PFA at 4 °C for 24 h. After pouring into a gelatine-egg (albumin) protein matrix polymerised with glutaraldehyde (albumin from chicken egg white, grade II, Sigma, Aldrich) and subsequent fixation in 4% PFA, the spinal cords were left in 30% saccharose with 0.1M PB at 4 °C. Each spinal cord was subsequently cut into 1.5 cm long blocks containing the following three segments – lesion centre = L, rostral segment = R, caudal segment = C. The segments were serially sliced using a cryostat (Leica Instruments, Heidelberg, Germany) to 40 μm transversal sections collected in 0.1M PBS containing 0.1% sodium azide. Representative sections were randomly selected (10 from each block/30 from each spinal cord), half of which were selected for immunohistochemical (IHC) processing using NeuN primary antibodies to evaluate the number of surviving neurons and the other half of sections for histochemical staining using the Luxol Fast Blue method to evaluate the amount of preserved white and grey matter.

For immunohistochemistry monoclonal antibodies against NeuN were used, which is present in most types of CNS and PNS neurons. Non-specific reactions were blocked with 10% normal goat serum with 0.2% Triton in 0.1M PBS for 2 h at room temperature. Sections were incubated in the primary antibody: anti-mouse NeuN (1 : 500, Millipore, clone A60, MAB377) for 24 h at 4 °C. Then, free floating sections were washed in 0.1M PBS and incubated at room temperature for 2 h with a secondary fluorescent anti-mouse antibody, conjugated with Texas Red (Alexa Flour 594, Molecular Probes). Sections were further washed in 0.1M PBS, assembled on slides and overlaid with Vectashield mounting medium (Vector Laboratories).

For histochemical analysis a modified method of Luxol fast blue was used. This method is commonly used to identify the structures of the spinal cord, and helps to visually distinguish the grey and white matter of the spinal cord. The sections were incubated with Luxol fast blue dye for 10 min and then rinsed four times in 95% alcohol and finally in distilled water. Differentiation was performed in a lithium carbonate for 2 min followed by double rinsing in 95% alcohol and rinsing in distilled water. Then, the spinal cord sections were stained with cresyl violet for 1 min and washed in distilled water. Differentiation continued with dehydration in 95% alcohol for 3–5 min followed by brightening in xylene for 3 min. Finally, sections were assembled on slides with Enthelan mounting medium.

Histological samples were analysed using the Nikon-Ti inverted fluorescence microscope with Perfect Focus (LHRRB 113) at four- and ten-fold magnification and pictures were recorded with the iXON DU897 EMCDDA digital camera. The number of NeuN-positive neurons was evaluated in selected fields of 250 × 250 μm, located bilaterally in laminae I–IV, IV–V and VIII–IX, throughout the rostro-caudal extent, and all numbers were calculated in order to quantify the overall profile of NeuN cells within the grey matter in an area of 1 mm^2. Analysis of the spared area was conducted so that grey and white matter on transverse sections was traced using software for image analysis (Image J). Figures of grey and white matter were digitised and the area of each tissue component was calculated using Image J, while the two-dimensional area expressed in pixels was calculated as the percentage of the preserved areas in 1 mm^2 of tissue. Final values were expressed as mean values ± SD%.

Differences between experimental groups (BBB score, frequency of spasms, score of bladder function, quantification of neuronal profiles, and grey and white matter tissue sparing) were analysed using one-way analysis of variance (Anova) followed by the Tukey post-hock test. All data are presented as mean values ± SD. Differences between groups were considered statistically significant if ***P < 0.001, **P < 0.01, *P < 0.05.

RESULTS

The experimental model of spinal cord injury used in this study caused complete paralysis of

the hind legs in both experimental groups of rats, lasting for 24 h after SCI, which represents a BBB score = 0, followed by gradual improvement in motor function manifested as rare motion of one, two or three joints of the affected pelvic leg. With advanced time after SCI, we recorded a gradual improvement in motor function and increase in the BBB score in both groups. Significant improvement of motor function (broad movement in two or three joints, plantar position of paws, regular steps) was noted in the SCI-Inosine group much earlier, when compared to the SCI-Aqua, and these rats showed a significantly higher BBB score at 8, 10, and 12 days ($P < 0.05$) after SCI (Figure 1). The SCI-Sham group did not show any disturbance in motor function or urination following surgery and a BBB score of 21 was recorded throughout the entire period of survival.

In the SCI-Inosine group we recorded a total of only three spasms during the first four days of survival (all of them in a single rat) while in the SCI-Aqua group a total of seven spasms were recorded in two rats during six days of survival.

Both experimental groups required manual emptying of the urinary bladder two- to thre-times daily during the first week after spinal cord injury. The urine of the majority of these animals contained an admixture of blood, which disappeared over time in both groups. In the SCI-Inosine group, we recorded a significant improvement in bladder function with an earlier release of the initial sphincter spasm and only occasional presence of blood admixture in the urine at Days 8 ($P < 0.01$) to 12 ($P < 0.05$) after spinal cord injury (Figure 2).

NeuN immunoreactivity was used to quantify the neurons in the ventral and dorsal horns of the spinal cord grey matter in the transversal sections. In both experimental groups spinal cord injury was accompanied by a statistically significant reduction in the number of NeuN-positive neurons when compared to the SCI-Sham group ($P < 0.001$). Immunohistochemical analysis confirmed a significantly higher number of neurons in the SCI-Inosine group (SCI-Aqua = 217.8 ± 14.11, SCI-Inosine = 322.9 ± 24.0) ($P < 0.01$).

We evaluated the tissue sparing of white and grey matter in three segments of the spinal cord: R – rostral to the lesion, L – centre of injury/lesion site, C – caudal from lesions, in both experimental groups. The sections revealed a major loss of white and grey matter in all segments, but a more pronounced loss was visible at the central lesion site (L segment) in both experimental groups ($P < 0.05$). In addition, inosine significantly reduced the cavitation of spinal cord tissue so that the loss of total tissue area of white matter in the rostral spinal cord segment (R: WM-Aqua $40.16\% \pm 13.21\%$; WM-Inosine $49.43\% \pm 8.51\%$), and in the grey matter of the caudal segment (C: GM-Aqua $10.61\% \pm 1.07\%$; GM-Inosine $17.92\% \pm 1.29\%$; $P < 0.05$), was significantly lower in the SCI-Inosine group.
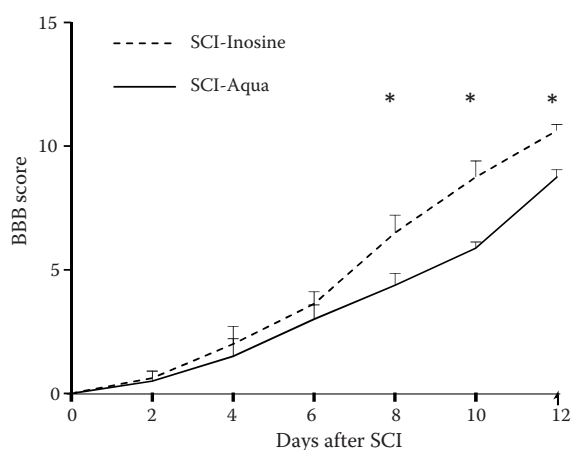

Figure 1. The progress of motor function recovery as measured by the BBB score over 12 days of survival. Inosine-treated rats recovered faster when compared to the control group SCI-Aqua (*$P < 0.05$)
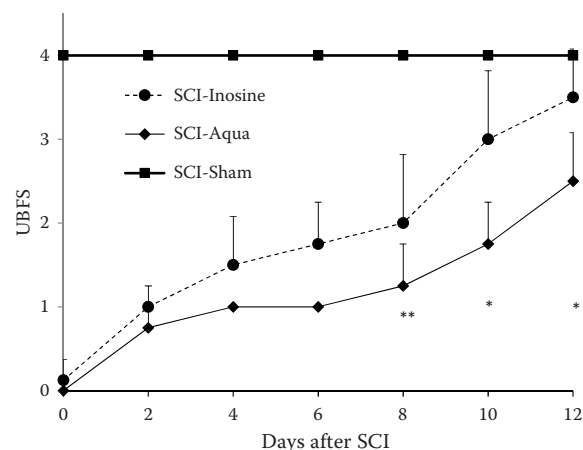

Figure 2. Evaluation of urinary bladder function by urinary bladder function score (UBFS). Note the significantly better recovery of urination in the SCI-Inosine group (**$P < 0.01$, *$P < 0.05$)

DISCUSSION

Inosine was confirmed to have a broad anti-inflammatory effect including inhibition of proinflammatory chemokines and cytokines and protection from endotoxin-induced inflammation and skeletal muscle reperfusion injury (Wakai et al. 2001). In addition, inosine is a known precursor of uric acid which is believed to block the effects of toxic free-radicals and protect the blood-brain barrier. Because of its potent anti-inflammatory and axon-promoting neuroplastic effects confirmed in experimental studies, inosine is currently tested in clinical trials for neurological diseases (Markowitz et al. 2009). Following spinal cord injury (SCI), the disruption of descending and ascending axonal pathways causes loss of motor, sensory and autonomic function. One of the most common complications associated with spinal cord injury are disorders of urinary bladder function. Urinary dysfunction is attributable to a lack of regeneration of supraspinal pathways that control the bladder after SCI (Lee et al. 2013). Traumatic spinal cord injury alters bladder function that may potentially lead to haematuria and cystitis which can be eliminated, based on its anti-inflammatory effects, by daily oral administration of inosine (Liu et al. 2009). In our study, the SCI-Inosine group showed faster recovery of urinary bladder function within the first week after injury, which is most likely due to inosine-mediated rewiring of lost bladder fibres with functionally relevant synaptic re-formations. The urinary bladder function score showed a significantly earlier improvement in the SCI-Inosine group. This improvement was concomitant with an alleviation of the impaired motor function of hind legs. Previous *in vitro* studies confirmed that inosine may play a dual role. It stimulates mast-cell degranulation resulting in pro-inflammatory effects, but on the other hand it is able to suppress macrophages and lymphocytes and reduce the production of pro-inflammatory cytokines (TNF-α, IL-12, MIP-α IFN-γ) (Hasko and Cronstein 2004; Hasko et al. 2008). All four adenosine receptors are expressed in the uroepithelium, where adenosine is produced (Yu et al. 2006). Because inosine is a naturally occurring derivative of adenosine, it may have a similar protective effect on the bladder as adenosine in the heart and other organs (Mubagwa and Flameng 2001).

Recent studies indicate that the loss of particular descending axonal pathways most likely results in the decreased activity of inhibitory inter neurons, which causes the overreaction of motor neurons to excitatory stimuli leading to periodic leg movement. If spasms become severe enough, they may require medical treatment (Sherwood et al. 2000; Pope et al. 2002). In our study we reported a low incidence of spasms, although twice the number occurred in the SCI-Aqua group which is associated with accelerated rehabilitation of relevant pathways after inosine administration in the SCI-Inosine group.

Recent studies have confirmed that inosine can improve axonal regeneration, prevent neuronal death, and induce significant axonal reorganisation (Benowitz et al. 1999), which is closely linked to the ability of inosine to enhance the recovery of locomotor function after central nervous system injury (Zai et al. 2011), in agreement with our results. Inosine increased the BBB score throughout the whole period of survival, which is most likely attributable to axonal-regrowth and plastic changes after SCI documented in experimental studies. Observations regarding the possible pathophysiological roles of axonal or dendritic sprouting or pruning and circuit reorganisation below the injury site and their contribution to the development of spastic symptoms and signs suggest, that after damage to the central nervous system, neuronal networks can spontaneously reorganise via the formation of new connections and sprouting of fibres, generally referred to as structural plasticity.

The current experimental study confirmed these possible connections through the clinically relevant effects of inosine on motor function and bladder, and our data also reveal the effect of inosine on histological signs related to the reorganisation of the injured spinal tissue. In addition to quantification of neurons in the spinal cord transversal sections we performed measurements of the percentage of preserved white and grey matter tissue. Liu et al. (2006) also reported a significant increase in the preserved spinal cord tissue. Our data confirm that inosine-treated rats (400 mg/animal/day) show significantly higher number of NeuN-positive cells in spinal cord sections which resulted in an improvement in behavioural function as recorded using the BBB score and also in an acceleration of urinary bladder function recovery, when compared to the SCI-Aqua group. The SCI-Inosine treated rats recovered faster and began to urinate almost normally already within the first week after injury.

The reported significant reduction in the number of neurons after SCI associated with alteration of motor function and occurrence of spasms may be attributed to the delayed degeneration of initially spared neurons. In a rat model of contusion injury, cell death peaks one week after the injury and continues throughout the first month (Li et al. 1999). The difference in urinary bladder scores between groups at the end of survival showed lower significance than at Day 8. The reason why inosine was effective predominantly during the second week after SCI is most likely attributable to its anti-inflammatory and immunomodulatory effects which influence the initial pathophysiological processes and cell death occurring in this period.

Due to very low oral toxicity of inosine and large therapeutic range it is preferable to administer inosine orally. These striking effects of inosine after spinal cord injury remain to be verified during a longer survival period and different dosing protocols should also be tested in further studies to reveal possible side effects.

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