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Vitamin D_3 Up-Regulated Protein 1 (VDUP1) Gene Expression in Spinal Cord Injury

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Vitamin D_3 up-regulated protein 1 (VDUP1) gene is known to be a novel member of early response genes as an oxidative stress mediator. To elucidate role of VDUP1 expression in neuronal injury, VDUP1 gene expression and histological change were tested in the spinal cords after traumatic spinal cord injury (SCI) in young and adult rats. VDUP1 transcript was detected weakly in a few cells in the spinal cords of control young and adult rats. VDUP1 transcript was increased in the contused spinal cords 1 day after SCI in both young and adult rats. VDUP1 transcript was decreased in the spinal cords 7 days after SCI in young rats. However, VDUP1 transcript was not decrease significantly 7 days in the spinal cords after SCI in adult rats. Cell damage in the spinal cords and hind limb dysfunction were more prominent 7 days after SCI in adult rats compared with that in young rats. These data show that VDUP1 may be involved in neurodegeneration after traumatic SCI.

Key Words: Spinal cord injury, VDUP1, Neurodegeneration

INTRODUCTION

Vitamin D_3 upregulated protein 1 (VDUP1) is originally reported as a gene upregulated by vitamin D_3 in HL-60 cells[1]. VDUP1 is an interacting factor with thioredoxin (TRX). It locates inthe cytoplasm and is strongly induced by stress responses. These characters of VDUP1 render cell more vulnerable to oxidative stress. Its anti-TRX function and expression pattern imply that VDUP1 is a key modulator for stress responses to modify the redox status in the cells[2]. VDUP1 gene is a novel member of early response genes in neuronal apoptosis whose expression is directly regulated with the transcriptional factor c-jun in cultured cerebellar granular cells[3].

Spinal cord injury (SCI) results in significant necrotic reaction characterized by extensive cell death. Additionally, it is well established that a series of secondary degenerative processes takes place that lead to the further loss of tissue[4,5]. This secondary reaction involves a variety of neurochemical changes that initiate an excitotoxic cascade leading to changes in the physiological state of spinal neurons[6~8]. This excitotoxic cascade includes a series of cytoplasmic events that direct changes in gene expression. Traumatic injury to the spinal cord triggers several secondary effects, including oxidative stress and compromised energy metabolism, which play a major role in biochemical and pathological changes in spinal cord tissue[9,10]. Here we tested the hypothesis that VDUP1 is involved in neural injury after traumatic SCI. We have reported first that VDUP1 gene expression is upregulated in the spinal cord, and can be involved in neurodegeneration after traumatic SCI.

MATERIALS AND METHODS

1. Spinal cord injury model

Sprague-Dawley rats (5-week old and 16-week old, male) were anesthetized with under gaseous anesthesia with a mixture of halothane and 1:2 flow ratio of NO_2/O_2, the back was shaved and the skin disinfected with betadine solution. The rat was placed in a prone position on a warmed surgical surface to maintain body temperature throughout the procedure (37±0.5℃). A midline longitudinal incision was made at the T12 and L2 level to expose the dorsal laminae

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and spinous process under the light microscope visualization. Laminectomy was performed at the L1 spinal cord level. Using a New York University (NYU) Impactor, spinal cord injury was induced by dropping a 10 g weight at a height of 20 mm. The muscle and skin were sutured in layers. Following surgery, lesioned rats were warmed and returned to their cages. The bladder was emptied once a day by manual pressure everyday. In the control (shame-operated) groups of both young and adult rats, only L1 laminectomy was performed.

2. In situ hybridization for VDUP1

VDUP1 antisense and sense riboprobes were prepared form the 561 bp human cDNA fragment cloned pGEM-T vector flanked by Sp6 and T7 promotors. For the antisense riboprobe, the plasmid was linealized with NcoI and transcribed with Sp6 RNA polymerase; for the sense probe, the plasmid was linearized with SalI and transcribed with T7 RNA polymerase.

The animals were subjected to pentothal sodium anesthesia (50 mg/kg, i.p.), 1 and 7 days after spinal cord injury. Subsequently, they underwent transcardiac perfusion fixation with saline followed by 4% phosphated-buffered paraformaldehyde. The tissues of spinal cord were obtained, post-fixed in cold 4% paraformaldehyde solution, and changed into 30% sucrose solution at 4℃ overnight. Frozen sections (30 μm in thickness) of each tissue were made and collected in PBS in 24-well plate. The tissues were incubated in 0.4% Triton X-100, treated with proteinase K (25 μg/ml, Roche) for 20 min at room temperature, and acetylated in the solution containing 0.25% acetic anhydride, 0.1 M triethanolamine, and 0.9% NaCl. The tissues were prehybridized in 0.3 M NaCl, 50% deionized formamide, 20 mM Tris-HCl, pH 8.0, and 1x Denhardt's solution for 1 hour. The tissues were then incubated with hybridization solution containing 0.5 mg/ml tRNA, 20 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 1x Denhardt's solution, 0.3 M NaCl, 50% deionized formamide, 0.1% Tween 20, and 0.5 μg/ml digoxigenin-labeled VDUP1 antisense or sense riboprobes overnight at 55℃. The hybridized tissues were washed with 2 x SSC/50% formamide at 55℃ for 1 hour, treated with RNase A (20 μg/ml, Roche) at 37℃ for 30 min, and washed with 1 x SSC/50% formamide at 55℃ for 1 hour and 0.5 x SSC/50% formamide 60℃ for 1 hour. The tissues were incubated with anti-digoxigenin alkaline phosphate conjugated serum (diluted 1:500, Roche) overnight at 4℃. Final coloring reaction was done by nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) solution. The each sample section was mounted on the gelatin-coated slide, dehydrated, and coverslipped with the permount for viewing.

3. General histopathology

The fixed spinal cords were dehydrated in graded ethanol solution, cleared in xylene, embedding in paraffin, and horizontal sections (5 μm) were cut. The deparaffinized sections were stained with hematoxylin-eosin.

4. Behavioral test

Behavioral test were performed using Basso-Beatti-Bresnahan (BBB) scale[11,12]. Testing was done 1, 3 and 7 days after weight drop injury.

5. Data analysis

Quantatification of the VDUP1 transcript was performed by counting by measuring the number of positively labeled cell per section. Photomicrographs (original magnification, ×100) were scanned, digitally processes, and complied using a computer image software (IMT-VISUS). Occasional particles of dust and other obvious artifacts were digitally retouched. The number of VDUP1transcript positive cells and the BBB score after weight-drop injury in young and adult rats were expressed as mean ± SEM. The difference in the number of VDUP1 transcript positive cells and the BBB score between young and adult rats were analyzed by paired t-test with significance at $P<0.05$.

RESULT

1. VDUP1 gene expression in the spinal cord and general appearance after SCI

VDUP1 transcript was seen in a few cells in the white matter of young control rats (Fig. 1A). VDUP1 transcript was increased in white and gray matters 1 day after SCI (Fig. 1B, D), and then decreased in white and gray matters close to the young control level 7 days after SCI (Fig. 1C). Tissue destruction or damage was minimal after SCI in young rats (Fig. 1B-D, Fig. 3).

The area of tissue destruction or damage in adult rats was much more extensive than that in young rats after SCI

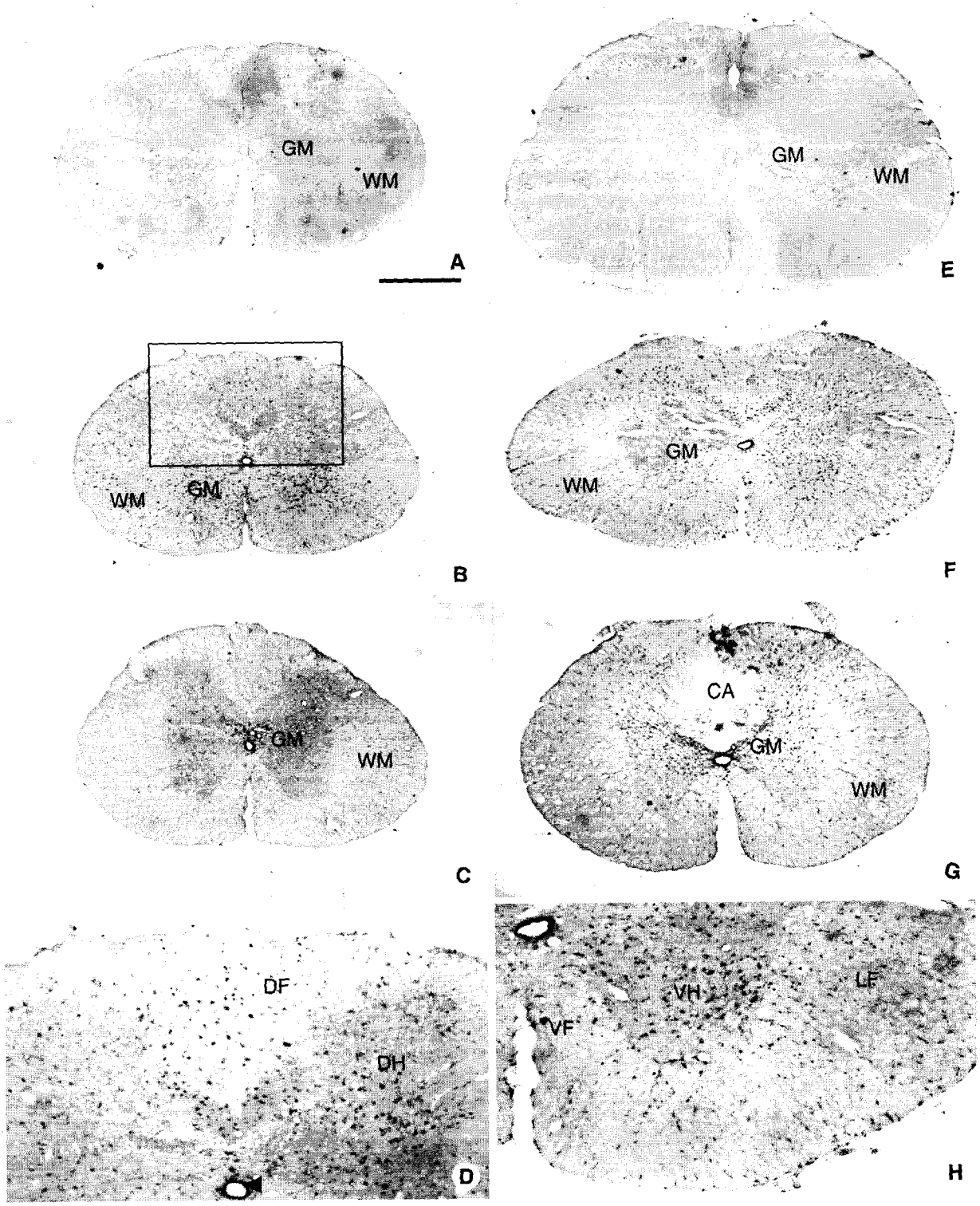

Fig. 1. Comparison of VDUP1 gene expression in the spinal cord between young and adult rats. A few weakly VDUP1 transcript positive cells exist in the white matter in young control rats (**A**) and adult control rats (**E**), but not seen distinctively in this photographs of low magnification (×40). The number of the VDUP1 transcript positive cells increase prominently in the spinal cord in young rats (**B**) and adult rats (**F**) 1 day after traumatic SCI. The number of the VDUP1 transcript positive cells decrease in the spinal cord close to that of the control in young rats (**C**), however, the number of the VDUP1 transcript positive cells do not decrease significantly in the spinal cord in adult rats 7 days after traumatic SCI (**G**). D, photomicrograph of rectangular area in B. H, photomicrograph of rectangular area in **F**. Scale bar: **A-C** and **E-G**, 500 μm; **D** and **H**, 200 μm. GM, gray matter; WM, white matter; DF, dorsal funiculus; DH, dorsal horn; arrowhead, central canal; CA, cavity, arrows, vacuolation, VH, ventral horn; VF, ventral funiculus; LF, lateral funiculus.

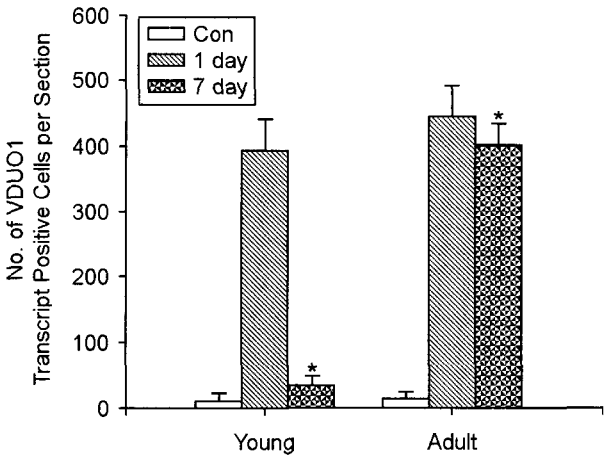

Fig. 2. The number of VDUP1 transcript postive cells in the spinal cords after SCI in young (n=5) and adult (n=5) rats (Con, control rats; 1 day, 1 day after SCI; 7 day, 7 days after SCI). Error bars indicate SEM; $^{*}P<0.01$

(Fig. 1F, 1G). A large cavity was formed in the gray matter, and small vacuoles were seen in the white matter 7 days after SCI in adult rats (Fig. 1G). No intact neuron was seen in the gray matter 7 days after SCI (Fig. 3). VDUP1 transcript was also seen weakly in a few cells in the white matter of adult control rats (Fig. 1E). VDUP1 transcript was increased prominently in white and gray matters 1 day after SCI (Fig. 1F, 1H). However, VDUP1 transcript was maintained in gray matter around the cavity 7 days after SCI similar to the level of 1 day post-injury in adult rats (Fig. 1G). Quantitative comparison of VDUP1 gene expression between young and adult rats after SCI was shown in Fig. 2.

2. Behavior analysis

Open field motor testing using the BBB Locomotor Rating showed hindlimb function compared between young and adult rats (Fig. 4). The adult rats showed low BBB score 1 day (5.4±1.8), the BBB score gradually increase approximately to half of the control score 3 days (8.9±2.5), and 7 days (11.5±2.1) after injury. The young rats showed low BBB score at 1 day (7.3±1.2) after injury, but showed marked improvement in the BBB score at 3 day (16.8± 1.5) and 7 days (19.1±1.8) after injury.

DISCUSSION

Data presented here showed that adult rats (16-week old) appear more severe behavioral dysfunction of hindlimb and neurodegeneration in the spinal cord compared with young rats (5-week old) rats 1 and 7 days after weight-drop spinal cord injury. VDUP1 gene expression increased in the gray matter and white matter of the injured spinal cord 1 day after SCI in both young and adult rats. VDUP1 gene expression gradually decreased in the spinal cord 7 days after SCI in young rats. However, VDUP1 gene expression was maintained 7 days after SCI in adult rats.

Traumatic spinal cord injury is a consequence of a primary mechanical insult and a sequence of progressive secondary pathophysiological events that confound efforts to mitigate neurological deficits. Ischemia, release of toxic chemicals from disrupted neural membranes, and electrolytes shifts trigger a secondary cascade that substantially compounds initial mechanical damage by harming or killing neighbouring cells. Glutamate plays a key part in a highly disruptive process known as excitotoxicity in secondary events after traumatic SCI[13,14]. Other molecules, such as voltage-sensitive sodium channel[15], voltage-gated potassium channels[16], cytotoxic cytokines[17,18], caspase-3[19], neurotrophic factors[20] are involved in neural changes after traumatic SCI. These studies show that multiple mechanisms and interactions contribute to secondary injury after SCI. We found that VDUP1 gene expression was upregulated in the spinal cord after traumatic SCI, which shows that VDUP1 is new molecule related to neural injury after traumatic SCI.

Injury to the developing spinal cord results in a greater amount of axonal growth compared to similar injury in the adult spinal cords[21,22]. However, considerable disagreements exist concerning the degree to which sparing and/or recovery of function occurs following CNS damage at birth rater than in adulthood[22,23]. We chose 5-week old (young) and 16-week old (adults) rats as experimental animal. For, there were prominent differences in motor function, histopathology, and VDUP1 gene expression in the spinal cord between two groups after traumatic SCI. VDUP1 gene expression was correlated with cell damage in this model, which reflect VDUP1 can be involved in neurodegeneration in traumatic SCI.

VDUP1 is an interacting factor with TRX[2,24,25]. It locates in the cytosol and is strongly induced by stress responses. VDUP1 render cells more vulnerable to oxidative stress, is a key modulator for stress responses to modify the redox status in the cells. There was the only one report about

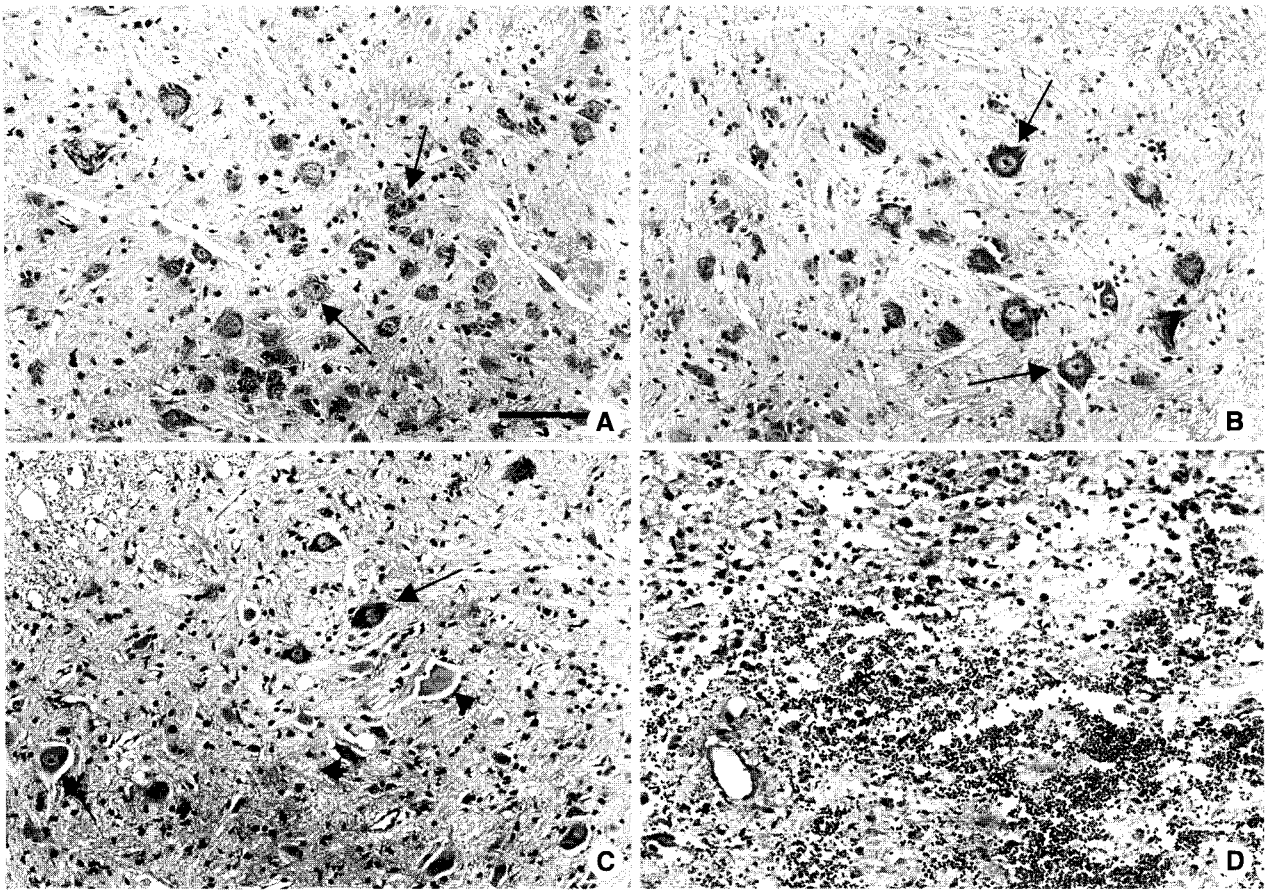

Fig. 3. General histopathology in the spinal cords after SCI, H-E stain. Intact neurons (arrows) exist in the gray matter in young control rats **(A)** and adult control rats **(B)**. Intact neurons (arrows) and dead neurons (arrowheads) present in the gray matter 7 days after traumatic SCI in young rats **(C)**. No intact neuron was seen in the gray matter 7 days after traumatic SCI in adult rats (asterisks, red blood cells in hemorrhagic site) **(D)**. Scale bar, 50 μm.

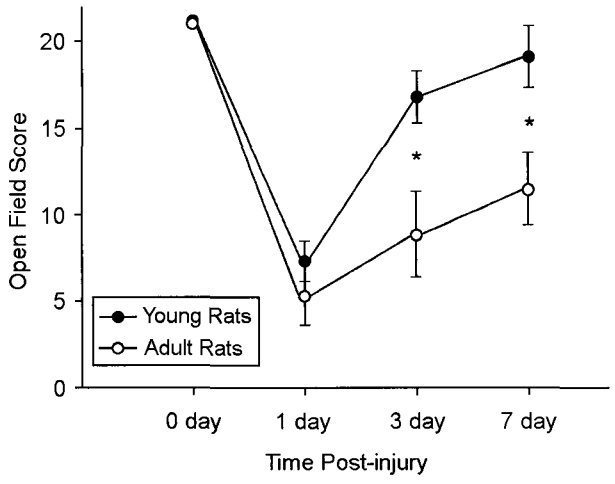

Fig. 4. Neurological function after SCI between young (n=5) and adult (n=5) rats, assessed by the BBB Locomotor Rating Scale. An open field score of 0 means no obserbable hind limb movement; an open field score 21 means normal hind lim movement. Error bars indicate SEM; $^{*}P<0.01$.

function of VDUP1 in the nervous system up to now[3]. VDUP1 transcript was rapidly up-regulated in cerebellar granular cells undergoing apoptosis following high K^+ withdrawal. VDUP1 mRNA expression was also enhanced in various paradigms of neuronal apoptosis in vitro. The VDUP1 transcript was upregulated coordinately with c-jun mRNA up-regulation and specific phosphorylation of c-jun. Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channels suppress the up-regulation of VDUP1 mRNA through a post-translational modification. We made the following primary observation on VDUP1 gene expression in traumatic SCI in vivo: VDUP1 transcript was induced in the spinal cord by stress, weight drop injury, response.

In summary, VDUP1 gene expression was correlated with cell damage in the spinal cord related to oxidative stress after SCI, which show that VDUP1 can be involved in neurodegeneration after traumatic SCI.

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