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The reversibility of neurofilaments decline induced by 2,5-hexanedione in rat nerve tissues

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ABSTRACT

To investigate the reversibility of the neuropathy induced by 2,5-HD, adult male rats were administered at a dosage of 400 mg/kg/day 2,5-HD (five times per week) for 2, 4, and 8 weeks, respectively. After stopping HD exposure, half of 8-week treated animals were allowed to naturally recover for 16 weeks. The relative levels of NF-H, NF-M, and NF-L in spinal cords and sciatic nerves of rats were determined by immunoblotting during the HD neuropathy. The results showed that NFs content in nerve tissues demonstrated a progressive decline as the intoxication continued. Furthermore, after a recovery of 16 weeks, the levels of three NF subunits in spinal cords of treated rats returned to normal while those in sciatic nerves displayed an inconsistent reversal. Among them, the level of NF-H in sciatic nerves returned to normal completely, and NF-L also showed a significant improvement, whereas NF-M did not demonstrate an obvious reversal. These findings suggest that HD-induced NFs decline is at least partially irreversible within the time frame of this study, which might be associated with the incomplete recovery of neurological dysfunctions of HD-treated rats.

1. Introduction

n-Hexane is a solvent widely used in the preparation of fabrics, adhesives, lacquers and other coatings. Exposure of humans and experimental rats to *n*-hexane produces a central–peripheral axonopathy, the longest and largest distal axons are preferentially targeted [1–3]. Metabolism studies have suggested that the neuropathy induced by *n*-hexane is mediated by its metabolite, 2,5-hexanedione (2,5-HD) [4]. The morphologic hallmark of HD neuropathy has been traditionally considered as giant axon swelling, which contains massive accumulations of neurofilaments (NFs). Recent researches, however, have suggested that the swellings are a non-specific effect related to subchronic exposure to HD, and, instead, that atrophied axons in PNS and CNS are the defining morphologic feature of HD neuropathy [5–7].

NFs are the most abundant cytoskeletal components in large neurones and myelinated axons, and an essential determinant of axon caliber [8–10]. Previous studies have demonstrated a significant decrease of NF contents in CNS and PNS from HD-intoxicated rats, and the evidence suggests that the decrements in axonal NF contents are partly responsible for HD-induced axon atrophy [11–15]. Since these data are limited to one time point at the end of HD exposure, the comprehensive data of HD's effect on NFs, especially the time course of NFs alterations following HD is still lacking. Hence, previous results are insufficient to evaluate the causative contribution of NFs alteration to HD-induced neuropathy. Furthermore, whether the decline of NFs induced by HD is reversible has not thoroughly been investigated, which is expected to provide some theoretic basis for the treatment and recovery of HD poisoning.

In the present study, we have investigated the time course of alterations of NFs proteins in spinal cord and sciatic nerve during HD neuropathy, and studied the reversibility of NFs decline induced by HD.

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2. Materials and methods

2.1. Materials

2,5-HD (purity > 97%) and Protease Inhibitor Cocktail set IIII were purchased from Merck biosciences, inc. (Darmstadt, GER). Monoclonal antibodies anti-NF-H (clone NE-14), anti-NF-M (clone NN-18), anti-NF-L (clone NR-4), anti-β-actin (clone AC-15), and HRP-conjugated goat-anti-mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BCA™ Protein assay Kit and SuperSignal® West Pico Chemiluminescent Subtrate Kit were purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). Film was obtained from Eastman Kodak Co. (Rochester, NY, USA). All other chemicals were of the highest grade commercially available.

2.2. Animal treatment and neurological testing

Adult male Wistar rats, weighing 180–220 g, obtained from Experimental Animal Center of Shandong University, were used in this study. Drinking water and a commercial animal feed were available ad libitum. The animal room was maintained at approximately 22 °C and 50% humidity with a 12 h light/12 h dark cycle. All experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and followed the principles in the ''Use of Animals in Toxicology''. After 7 days for acclimatization, the animals were randomly divided into nine groups, i.e. four experimental groups (2, 4, 8, and 24-week treated groups; $n = 10$ in each group) and five control groups (0, 2, 4, 8, and 24-week control groups; $n = 10$ in each group). The rats in four experimental groups were treated with HD by intraperitoneal injection at dosages of 400 mg/kg/day HD, respectively (5 days per week). HD was dissolved in 0.9% normal saline and administered at 3 ml/kg/day. Age-matched control rats received an equivalent volume of normal saline. The periods of 2, 4, 8, and 24-week treated groups exposed to HD were 2, 4, 8, and 8 weeks, respectively. To assess the recovery of HD-treated rats, the animals of 24-week treated group continued to be observed for 16 weeks after 8-week exposure ended. The onset and development of neurotoxicity were determined by neurological testing two times per week, the changes of body weight also recorded. Experimental animals together with their age-matched controls were sacrificed by decapitation at time points of 0, 2, 4, 8, and 24 weeks of the experiment, respectively. The nerve samples were quickly dissected and frozen in liquid nitrogen before storing them at −80 °C.

HD-induced neurological defects were detected and quantified using gait score, which was chosen because it represents sensitive and reliable indices of toxicant-induced changes in neurological status. Neurological evaluation was performed by a blinded observer who was not involved in animal care and administration. To measure gait abnormalities, rats were placed in an open field, and were observed for 3 min [16]. Following observation, a gait score was assigned from 1 to 4, where 1 = a normal, unaffected gait; 2 = a slightly abnormal gait (tiptoe walking, hindlimb adduction); 3 = moderately abnormal gait (obvious movement abnormalities characterized by dropped hocks and tail dragging); 4 = severely abnormal gait (dragging hindlimbs and complete absence of rearing).

2.3. Tissue preparation, electrophoresis and immunoblotting

Sciatic nerves were broken into powder with pestle in liquid nitrogen, then spinal cords and sciatic nerves were homogenized in ice-cold buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 25 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM dithiothreitol, Protease Inhibitor Cocktail (50 μl/g tissue) and phosphatase inhibitors (5 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, and 1 mM iodoacetic acid). Nerve homogenates were centrifuged at 100,000 × g for 1 h to yield a high-speed pellet (P) and a high-speed supernatant (S) fraction [13,14,17,18]. Protein concentration of two fractions was determined by using BCA™ Protein assay Kit.

To assess relative changes in protein content of high-speed nerve fractions (P and S), corresponding protein samples from both control and experimental animals were subjected to SDS-PAGE on 4% stacking and 7.5% or 10% resolving gel. Regardless of the comparison, NF-M and NF-H proteins were separated on 7.5% gels, whereas NF-L and β-actin were separated on 10% gels. To insure analysis within a linear range, we conducted initial studies to establish the proper electrophoresis protein loads, primary antibody dilutions, incubation duration, and band intensity levels for densitometric measurements. The protein amounts for per lane of NF-H, NF-M, NF-L, and β-actin were 15, 15, 15, 15 μg for the pellet fraction and 30, 30, 30, 30 μg for the supernatant fraction of spinal cords, 10, 15, 10, 10 μg for the pellet fraction and 10, 15, 10, 10 μg for the supernatant fraction of sciatic nerves, respectively. Following electrophoresis, proteins were transferred electrophoretically to nitrocellulose membranes. Then the membranes were blocked with 4% fat-free milk for 45 min and incubated with primary antibody diluted in 0.1% BSA for 3 h. Following primary antibody, membranes were washed in TBS and incubated with horseradish peroxidase-conjugated secondary antibody for 3 h at the room temperature. After being washed again, the membranes were incubated by using the SuperSignal® West Pico Chemiluminescent Subtrate reagents for 5 min, and then exposed to film for 15 s. Immunoreactive bands of proteins were scanned with Agfa Duoscan T1200 scanner and digitized data were quantified as integrated optical density (IOD) using Kodak Imaging Program and Image-Pro Plus software.

2.4. Statistical analysis

Gait scores from HD-treated animals and age-matched controls were compared using Mann–Whitney U-test. The data of body weight and IOD value of immunoreactive bands were expressed as mean ± S.D., statistical analysis was performed with one-way analysis of variance (ANOVA), followed by LSD's post hoc tests, which was provided by SPSS 10.0 statistical software. The differences were significant at $P < 0.05$.

3. Results

3.1. Clinical signs and body weight changes

Exposure to HD produced obvious reductions of weight gain and progressive gait abnormalities. On week 1 following HD,

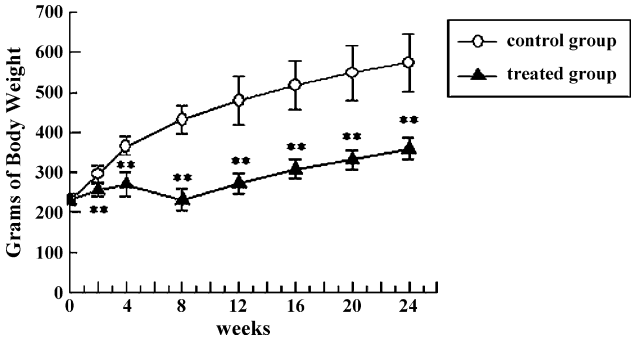

Fig. 1 – Temporal effect of HD on body weight gain of rats. The data of body weight are presented as mean ± S.D. Statistical significance is determined by using one-way ANOVA. The asterisk indicates statistical difference ($^{*}P < 0.05$, $^{**}P < 0.01$).

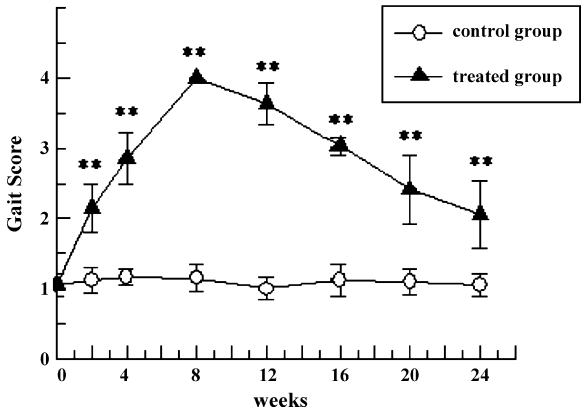

Fig. 2 – HD-induced changes of gait score in rats. The data of gait scores are presented as mean ± S.D. Statistical significance is determined by using Mann–Whitney *U*-test. The asterisk indicates statistical difference ($^{*}P < 0.05$, $^{**}P < 0.01$).

the rats began to show a slow growth. As the intoxication continued, the mean weight gain slowed down gradually. After 4 weeks of exposure, the body weight of treated animals no longer increased, but began to decrease. At the end of week 4 or 8, the mean body weight of treated animals was 73.9% and 53.5% of their age-matched controls, respectively. After stopping HD treatment, the body weight of treated animals began to increase again, and returned to 62.6% of their age-matched controls by the end of week 24 (shown in Fig. 1).

At the beginning of HD exposure, rats exhibited a normal, unaffected gait. On about week 2, the rats of treated groups showed slightly gait abnormality, i.e. tiptoe walking, hindlimb adduction. As intoxication went on, the symptoms aggregated progressively. On about week 4, animals showed moderately abnormal gait characterized by dropped hocks and tail dragging. By the end of 8 weeks exposure, all animals showed severely abnormal gait (crawling, unable to support weight completely). After stopping the treatment of HD, the neurological dysfunctions of treated animals displayed obvious recovery. At the end of 24 weeks, the average gait score of treated animals returned to 2, which indicated a slightly abnormal gait. In contrast, no clinical signs were observed in the control rats throughout the experiment (shown in Fig. 2).

3.2. *Alterations of NF subunits in the pellet and supernatant fractions of spinal cord*

To investigate the time course of NFs alterations in spinal cord of rats treated with HD, we used the monoclonal antibodies that recognize NF-H, NF-M, and NF-L to examine the corresponding protein levels by immunoblotting. Throughout the experiment, no significant difference of NFs contents was observed among five control groups (data not shown), so 0-week control group was chosen as the control of following studies.

As shown in Fig. 3, NFs content in spinal cords of treated animals demonstrated a progressive decrease as the intoxication of HD continued. In the supernatant fraction, NF-H content, relative to 0-week control, decreased by 16%, 57%, and 58%, respectively after 2, 4, and 8-week treatment with HD ($P < 0.01$, Fig. 3A); accordingly, NF-M decreased by 36%, 61%, and 65%, respectively ($P < 0.01$, Fig. 3B); NF-L decreased by 21%, 44%, and 45%, respectively ($P < 0.01$, Fig. 3C). In the pellet fraction of spinal cords, all the contents of NFs showed significant decline except those of NF-H and NF-L in group of 2-week exposure. Compared to the control, the contents of NF-H in groups of 4 and 8 weeks' exposure to HD decreased by 33% and 43%, respectively ($P < 0.01$, Fig. 3A), and those of NF-L decreased by 26% and 42%, respectively ($P < 0.01$, Fig. 3C). As far as NF-M was concerned, an obvious decline was detected as early as 2 weeks following the beginning of exposure to HD. The contents of NF-M in groups of 2, 4, and 8 weeks' exposure decreased by 23%, 34%, and 64%, respectively ($P < 0.01$, Fig. 3B), compared to the control. Moreover, after treated animals were allowed to recover naturally for 16 weeks, the level of three NF subunits in both fractions returned to normal.

3.3. *Alterations of NFs in the pellet and supernatant fractions of sciatic nerves*

Throughout the 24-week experiment, no significant difference of NF-H contents in sciatic nerves was observed among five control groups (data not shown), so 0-week control group was chosen as control for following studies. As shown in Fig. 4, NF-H content in sciatic nerves of treated animals demonstrated a trend of progressive decline significantly as the intoxication of HD continued. In the pellet fraction, in comparison with 0-week control, NF-H content decreased by 19%, 22%, and 43%, respectively after 2, 4, and 8-week treatment of HD ($P < 0.01$). Accordingly, in the corresponding supernatant fraction, a similar pattern of NF-H alteration was observed. Relative to the control, the contents of NF-H in groups of 4 and 8 weeks' exposure decreased by 12% and 52% ($P < 0.01$). However, after a 16-week recovery of treated animals, the relative contents of NF-H in supernatant and pellet fractions of sciatic nerves were 102% and 104% of age-matched controls, respectively, which suggested that the level of NF-H in sciatic nerves returned to normal after 16 weeks' recovery.

As showed in Fig. 5A, NF-M content in sciatic nerves of control groups displayed obvious changes throughout the

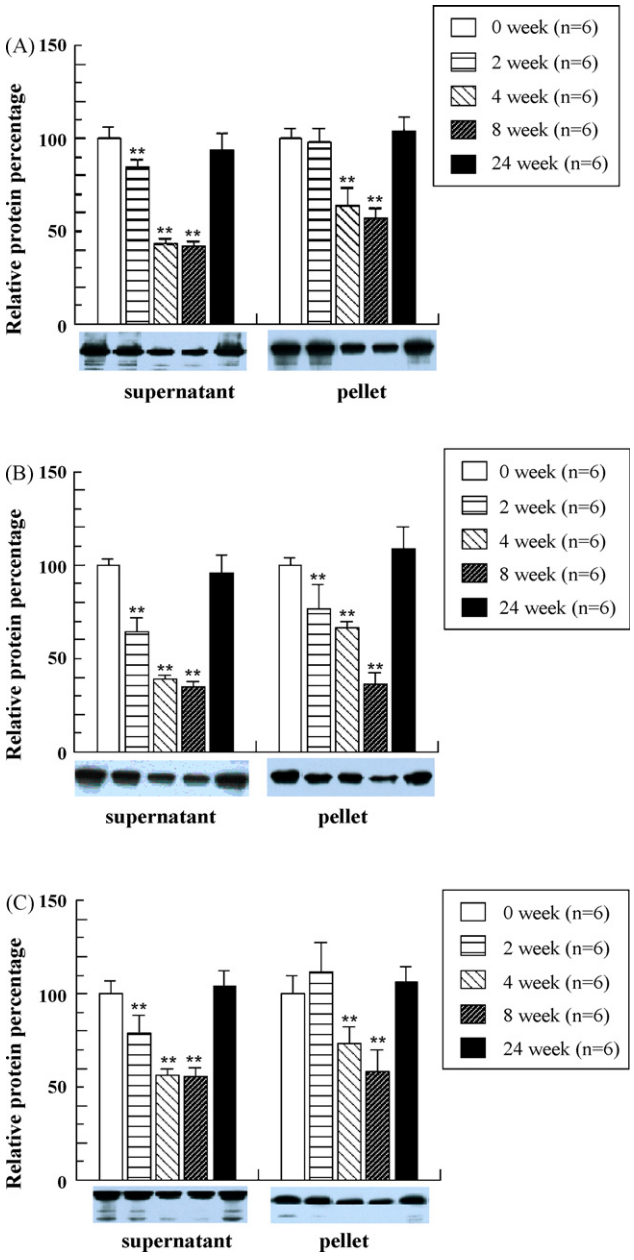

Fig. 3 – The time course of NFs alterations in spinal cords following HD. A, B, and C represent expression of NF-H, NF-M and NF-L, respectively. Representative immunoblots of NFs are also shown below the graph. The 0-week control is presented with 100%, the one of the treated groups is described with the percentage of 0-week control. The results are presented as a mean percentage of 0-week control ± S.D. Statistical significance is determined by using one-way analysis of variance (ANOVA). The asterisk indicates statistical difference ($^{*}P < 0.05$, $^{**}P < 0.01$).

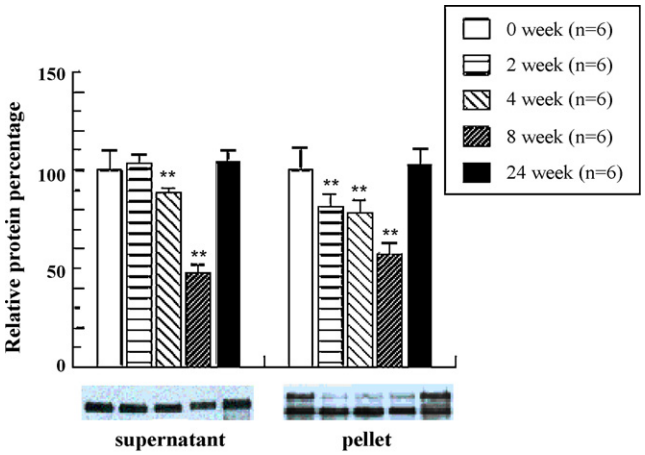

Fig. 4 – The time course of NF-H alterations in sciatic nerves following HD. Representative immunoblots of NF-H are also shown below the graph. The 0-week control is presented with 100%, the one of the treated groups is described with the percentage of 0-week control. The results are presented as a mean percentage of 0-week control ± S.D. Statistical significance is determined by using one-way analysis of variance (ANOVA). The asterisk indicates statistical difference ($^{*}P < 0.05$, $^{**}P < 0.01$).

period of experiment. In the supernatant fraction, NF-M increased by 141%, 330%, 322%, and 392% at the time-point of 2, 4, 8, and 24 week compared to 0-week control. Accordingly, in the pellet fraction, NF-M increased by 151%, 331%, 347%, and 426%. Following HD, the alteration of NF-M in sciatic nerves of rats was illustrated in Fig. 5B. In comparison with 0-week control, the level of NF-M in the pellet increased by 120%, 251%, 105%, and 211% at week 2, 4, 8, and 24 of the experiment, whereas that of NF-M in the supernatant increased by 105% and 229% at weeks 2 and 4, and decreased by 49% and 33% at weeks 8 and 24 of experiment. When compared to the age-matched control, the level of NF-M in both fractions of treated group displayed a consistent decline. NF-M decreased by 15%, 24%, 88%, and 83% at weeks 2, 4, 8, and 24 of the experiment in the supernatant, and accordingly decreased by 12%, 19%, 54%, and 41% in the pellet (shown in Fig. 5C). Generally, HD treatment resulted in a progressive decline of NF-M in both fractions of sciatic nerve, which did not yet improved at the end of 24-week test (18 weeks after exposure ended).

As showed in Fig. 6A, NF-L content in both fractions of sciatic nerve of control groups displayed some different changes throughout the period of experiment. In the supernatant fraction, no significant difference of NF-L contents was observed among five control groups. However, in the pellet fraction, NF-L content in 24-week control group increased by 42%. Following HD, the alteration of NF-L in sciatic nerve was illustrated in Fig. 6B. In comparison with 0-week control, the level of NF-L in the supernatant decreased by 19%, 45%, 59%, and 38% at week 2, 4, 8, and 24 of the experiment, whereas NF-L in the pellet decreased by 34% and 50% at week 4, 8 of the experiment, and increased by 37% at week 24. When compared to the age-matched control, the alteration of NF-L in both fraction of sciatic nerve was as follows. In the supernatant, the levels of NF-L in HD-treated animals decreased by 15%, 40%, 58%, and 34% at week 2, 4, 8, and 24 of the experiment (shown in Fig. 6C). However, in the pellet, a

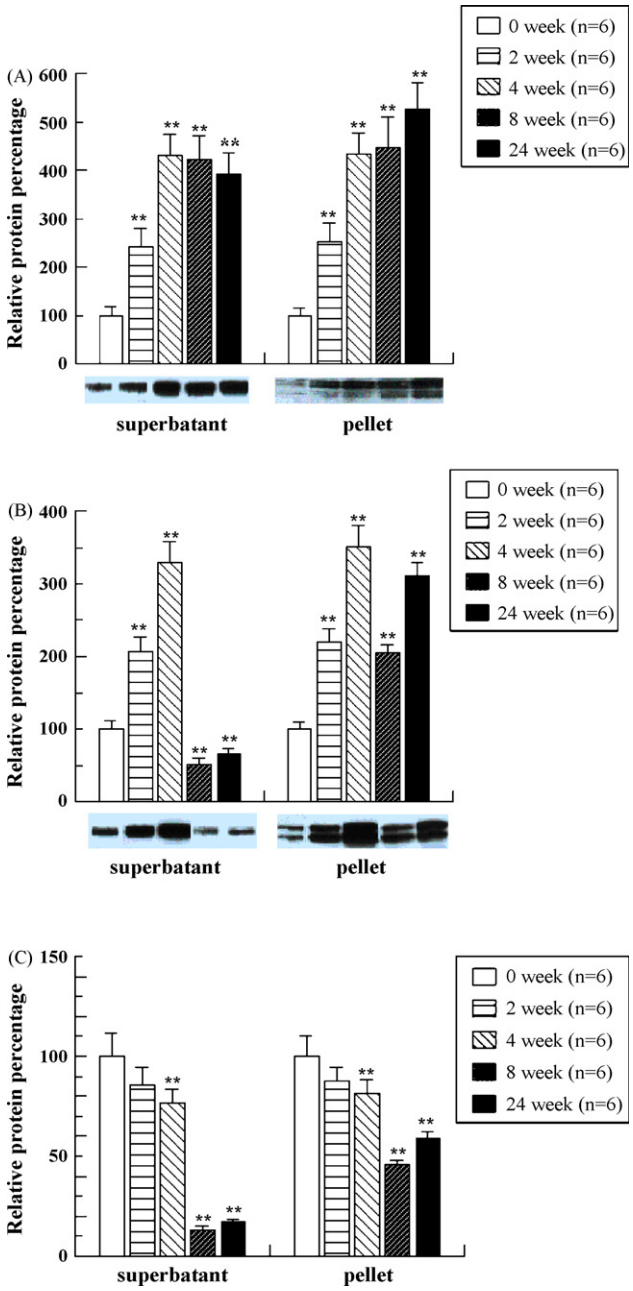

Fig. 5 – The time course of NF-M alterations in sciatic nerves following HD. A and B represent the expression of NF-M in control groups and HD-treated groups, respectively. Representative immunoblots of NF-M are also shown below the graph. The 0-week control is presented with 100%, the one of other control groups and treated groups is described with the percentage of 0-week control. The results are presented as the mean ± S.D. C represents the time course of NF-M in sciatic nerves of treated animals when normalized to their age-matched controls. Statistical significance is determined by using one-way analysis of variance (ANOVA). The asterisk indicates statistical difference ($^{*}P < 0.05$, $^{**}P < 0.01$).

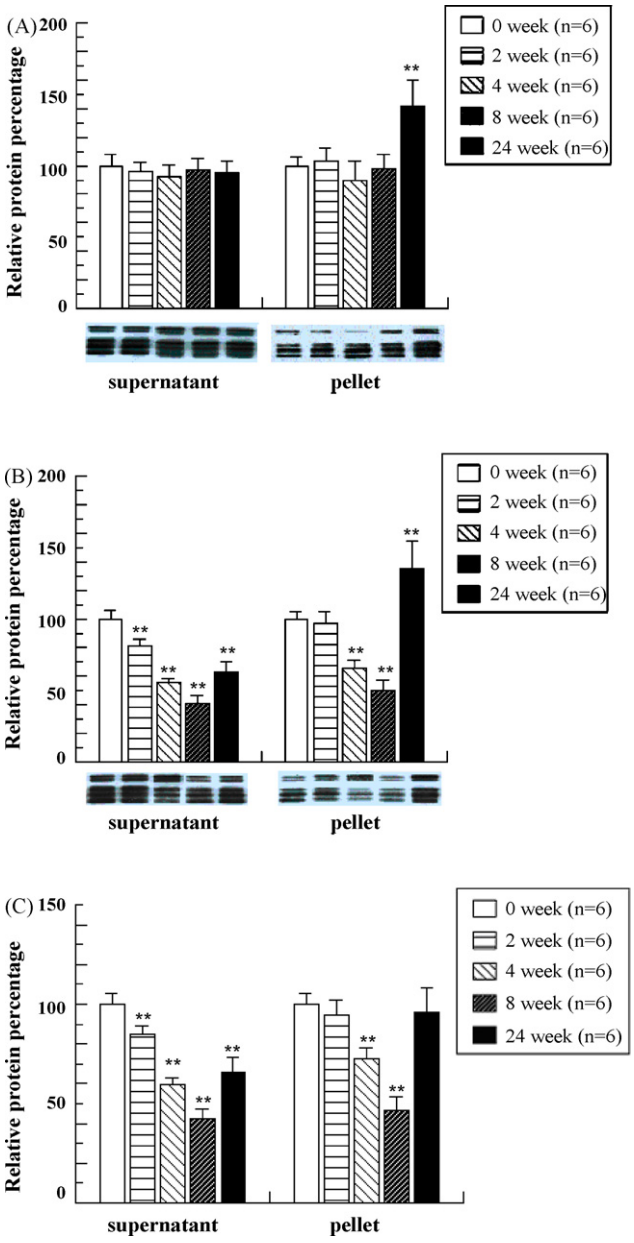

Fig. 6 – The time course of NF-L alterations in sciatic nerves following HD. A and B represent the expression of NF-L in control groups and HD-treated groups, respectively. Representative immunoblots of NF-L are also shown below the graph. The 0-week control is presented with 100%, the one of other control groups and treated groups is described with the percentage of 0-week control. The results are presented as the mean ± S.D. C represents the time course of NF-L in sciatic nerves of treated animals when normalized to their age-matched controls. Statistical significance is determined by using one-way analysis of variance (ANOVA). The asterisk indicates statistical difference ($^{*}P < 0.05$, $^{**}P < 0.01$).

significant decrease of NF-L was observed only in 4 and 8-week treated group (by 27% and 53%, respectively, in Fig. 6C). After 16-week recovery, the level of NF-L in the pellet returned to normal.

4. Discussion

HD can induce central–peripheral axonopathy in sensitive animals, but the underlying mechanism is not clear as yet. Several lines of evidence now implicate axon atrophy as the primary neuropathic change in nerve tissue of HD-intoxicated rats. This atrophy appears to be a specific, neurotoxicologically relevant effect that could mediate or contribute to development of nerve dysfunction and behavioral defects [5–7]. In considering possible molecular mechanisms, we noted that NFs played an essential role in establishing and maintaining axon caliber.

NFs, belonging to the type IV IFs, are the most abundant cytoskeletal components in large neurones and myelinated axons. In mammals, the NF triplet comprises proteins with molecular weights of about 68, 160, and 200 kDa known as light (NF-L), medium (NF-M) and high (NF-H) subunit, respectively. NFs in vivo are obligate heteropolymers requiring NF-L with either NF-M or NF-H for polymer formation [19]. An appropriate ratio among three NF proteins is critical to maintain the normal assembly of NFs, the disturbance of the normal ratio by decreasing or increasing any individual subunit would compromises the ability of NF to stimulate growth.

In this study, we examined the time course of NF subunits alterations in spinal cords and sciatic nerves of rats following HD. Our results showed that the exposure to HD resulted in a significant decline of NF subunits in both fractions, which was consistent with the results of previous studies [13,14]. Based on previous researches, the high-speed pellet fraction (P) is composed of polymerized triplet proteins and represents the triton-insoluble filamentous cytoskeletal network [17,20,21]; the high-speed supernatant (S) likely contains triton-soluble NF proteins in the form of intermediate heterotetramers and monomers that represent the mobile population of exchangeable NF proteins [13,21,22]. In the present study, the contents of NFs in rats intoxicated with HD decreased significantly. It suggested that the intoxication of HD disturbed the balance between the assembly and the disassembly of NFs, and interfered with the dynamic interaction of the polymeric and mobile monomeric pools. The loss of mobile NF proteins and disruption of polymer turnover could lead to eventual dissolution of the cytoskeleton in nerve tissue, and consequently to a reduction of axonal caliber. Since axonal diameter is the principal determinant of the conduction velocity of nerve impulses along the axons, the loss of axon caliber certainly resulted in altered cable properties and slowed nerve conduction velocity [23]. In this study, as the intoxication continued, the animals manifested progressive neurological abnormalities. These changes were consistent with the decrease of NFs proteins in spinal cords and sciatic nerves. It indicated that the changes of NFs protein contents could be attributed to the specific effect of HD, and might be involved in the neuropathy induced by HD.

With regard to the recovery of HD neuropathy, a complete reversibility of neurobehavioral changes induced by HD (ambulation and rearing in open field, balance on the accelerating rotarod) was observed in Ladefoged's study [24]. Furthermore, Rebert's paper also showed that the effects of HD on the brainstem auditory-evoked response and cortical auditory-evoked response could be well restored after a 6-week recovery period although there was little recovery of somatosensory evoked responses [25]. These studies mentioned above were mainly focused on the HD-induced neurobehavioral and neurophysiological effects, however, whether the NFs alteration induced by HD is reversible has not thoroughly been investigated. The only observation on the reversibility of NFs alteration in HD neuropathy has been reported by Decaprio [26]. In Decaprio's study, rats received 0.5% (v/v) HD in the drinking water for 8 weeks followed by 9 weeks of recovery. Apparent time-related decrease in the relative concentration of the three NF subunit proteins in brain stem preparation was seen, and this decrease was partially reversed after cessation of exposure. However, no significant alteration of NF subunit proteins in spinal cord was observed throughout that experiment. Considering that decline of NFs in rat's spinal cord has been repeatedly observed in previous studies, and a time-dependant decline of NF induced by HD has been described in this study, hence the lack of marked changes in NFs protein concentrations of spinal cord in Decaprio's study may represent a lack of detection sensitivity rather than a specific tissue difference.

In order to investigate the reversibility of NFs alteration, we examined the recovery of HD-induced neuropathy. After the cessation of HD exposure, neurobehavioral dysfunctions of HD-treated animals have ameliorated gradually. By the end of 24-week experiment, treated animals retuned to a slightly abnormal gait again, which was assigned to score 2. Accompanied with the recovery of gait abnormality of animals, NFs level in spinal cord returned to the normal while those in sciatic nerve demonstrated recovery of different extents. Among them, the level of NF-H in both fractions returned to normal completely; NF-M did not show any significant recovery in both fractions; furthermore, the improvement of NF-L lay between NF-H and NF-M. The irreversibility of NF-M decline in sciatic nerve induced by HD might have important functional implication. Recent gene disruption studies revealed different roles of each NF subunit in the formation of the NF network in axons. Targeted deletion of the NF-L gene resulted in the loss of NFs, indicating the absolute requirement for NF-L in filament assembly [27]. NF-M also has an important role in maintaining the filamentous organization; the number of NFs is significantly reduced in the NF-M gene-disrupted mouse [28]. However, the NF-H null mouse does not exhibit marked changes in the number and organization of axonal NFs [29,30]. The irreversibility of NF-M reductions in sciatic nerves might be responsible for the incomplete recovery of neurobehavioral deficient observed in HD-treated rats.

Interestingly, a time-dependent elevation of NF-M expression in sciatic nerve has been observed among control groups throughout our study. Considering that immunoreactivity of NF-H and β-actin in the same samples did not increase correspondingly (data not shown), the increase in NF-M isoforms in two fractions of sciatic nerve was not a general effect on cytoskeletal proteins. During development, the expression of NF proteins is suggested to be unique for each

NF subunit. In the embryonic rat brain, NF-L and NF-M proteins are coexpressed, while the appearance of NF-H is delayed to a very late embryogenetic or postnatal period [31,32]. In a rat cerebellum's study, there was an increase in NF subunits content with age, and lasted to P90 [33]. According to the published works, NF-M has some unique biological features relative to NF-H and NF-L. Firstly, NF-M is the first of NF subunits to be synthesized during nerve system development of animals, which indicates that NF-M may have other functions in neurons, particularly at the early stage of it [34-36]. Secondly, the expression of NF-M fluctuates more remarkably than its isoforms during the development and maturity of nerve system. Shea's study indicated that NF-M expression in the Triton-insoluble fraction of mice brain at p120 was more than that at p60, which was consistent with our results of NF-M in sciatic nerves [20]. These characteristic features of NF-M might account for its susceptibility to toxicant injury. In the present study, HD intoxication was associated with significant reductions in NF subunits contents in sciatic nerve, among which the declines of NF-M appeared to be more noticeable than NF-H and NF-L, and did not display obvious reversibility.

The present report describes the evidence for a progressive decline of NFs in nerve tissues of rats exposed to HD and a partial reversibility of this decrease after cessation of exposure. The possible mechanisms for NFs alterations following HD exposure might be related to the declining of NFs synthesize and /or increasing of NFs degradation. Since previous studies indicate that the decrements in NF gene expression are not solely responsible for the loss of NF contents in sciatic nerve [7], it is conceivable that the disturbance of NFs degradation might be involved in the HD-induced alterations of NFs. NFs are normally degraded by proteases at the nerve terminal, and degraded material is transported back to the cell body for further processing. Among them, Ca^{2+}-activated neutral proteases or calpains are known to degrade NFs rapidly. Furthermore, recent studies have provided compelling evidence for ubiquitin-proteasome system (UPS) involvement in the degradation of NFs, and the impairment of the UPS has been postulated to be at the root of neurodegenerative disease [37–40]. In this regard, to find the links between the calpains or UPS-mediated NFs degradation and HD neuropathy can expectedly reveal new insight into toxicant-induced neuropathy.

In summary, in the present study we have delineated the time-course of NFs alterations following HD intoxication. The intoxication of HD could induce a progressive decline of NFs content in rat nerve tissues, which was related with the development of HD-induced neuropathy. Furthermore, HD-induced NFs decline was at least partially irreversible within the time frame of this study, which might be associated with the incomplete recovery of neurological dysfunctions of HD-treated rats.

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REFERENCES

[1] Altenkirch H, Mager J, Stoltenburg G, Helmbrecht J. Toxic polyneuropathies after sniffing a glue thinner. J Neurol 1977;214:137–52.
[2] Spencer PS, Schaumburg HH, Sabri MI, Veronesi B. The enlarging view of hexacarbon neurotoxicity. Crit Rev Toxicol 1980;7:279–356.
[3] Cavanagh JB, Bennetts RJ. On the pattern of changes in the rat nervous system produced by 2,5-hexanediol. A topographical study by light microscopy. Brain 1981;104:297–318.
[4] Couri D, Milks M. Toxicity and metabolism of the neurotoxic hexacarbons *n*-hexane, 2-hexanone, and 2,5-hexanedione. Annu Rev Pharmacol Toxicol 1982;22:145–66.
[5] Lehning EJ, Dyer KS, Jortner BS, LoPachin RM. Axonal atrophy is a specific component of 2,5-hexanedione peripheral neuropathy. Toxicol Appl Pharmacol 1995;135:58–66.
[6] Lehning EJ, Jortner BS, Fox JH, Arezzo JC, Kitano T, LoPachin RM. Gamma-diketone peripheral neuropathy. I. Quality morphometric analyses of axonal atrophy and swelling. Toxicol Appl Pharmacol 2000;165:127–40.
[7] Opanashuk LA, He DK, Lehning EJ, Lopachin RM. Diketone peripheral Neuropathy. III. Neurofilament gene expression. Neurotoxicology 2001;22:215–20.
[8] Cleveland DW, Monteiro MJ, Wong PC, Gill SR, Gearhart JD, Hoffman PN. Involvement of neurofilaments in the radial growth of axons. J Cell Sci Suppl 1991;15:85–95.
[9] Hoffman PN, Cleveland DW, Griffin JW, Landes PW, Cowan NJ, Price DL. Neurofilament gene expression: a major determinant of axonal calibre. Proc Natl Acad Sci USA 1987;84:3472–6.
[10] Lee MK, Cleveland DW. Neuronal intermediate filaments. Annu Rev Neurosci 1996;19:187–217.
[11] Chiu FC, Opanashuk LA, He DK, Lehning EJ, LoPachin RM. Gamma-diketone peripheral neuropathy. II. Neurofilament subunit content. Toxicol Appl Pharmacol 2000;165:141–7.
[12] Karlsson JE, Rosengren LE, Haglid KG. Quantitative and qualitative alterations of neuronal and glial intermediate filaments in rat nervous system after exposure to 2,5-hexanedione. J Neurochem 1991;57:1437–44.
[13] LoPachin RM, He D, Reid ML, Opanashuk LA. 2,5-Hexanedione-induced changes in monomeric neurofilament protein content of rat spinal cord fractions. Toxicol Appl Pharmacol 2004;198:61–73.
[14] LoPachin RM, He D, Reid ML. 2,5-Hexanedione-induced changes in the neurofilament subunit pools of rat peripheral nerve. Neurotoxicology 2005;26:229–40.
[15] Watson DF, Fittro KP, Hoffman PN, Griffin JW. Phosphorylation-related immunoreactivity and the rate of transport of neurofilaments in chronic 2,5-hexanedione intoxication. Brain Res 1991;539:103–9.
[16] LoPachin RM, Ross JF, Reid ML, Das S, Mansukhani S, Lehning EJ. Neurological evaluation of toxic axonopathies in rats: acrylamide and 2,5-hexanedione. Neurotoxicology 2002;23:95–110.
[17] Chiu FC, Norton WT. Bulk preparation of CNS cytoskeleton and the separation of individual neurofilament proteins by gel filtration: dye-binding characteristics and amino acid compositions. J Neurochem 1982;39:1252–60.
[18] Tsuda M, Tashiro T, Komiya Y. Increased solubility of highmolecular-mass neurofilament subunit by suppression of dephosphorylation: its relation to axonal transport. J Neurochem 1997;68:2558–65.
[19] Lee MK, Xu Z, Wong PC, Cleveland DW. Neurofilaments are obligate heteropolymers in vivo. J Cell Biol 1993;122:1337–50.

[20] Shea TB, Dahl DC, Nixon RA, Fischer I. Triton-soluble phosphovariants of the heavy neurofilament subunit in developing and mature mouse central nervous system. J Neurosci Res 1997;48:515–23.
[21] Shea TB, Sihag RK, Nixon RA. Dynamics of phosphorylation and assembly of the high molecular weight neurofilament subunit in NB2a/d1 neuroblastoma. J Neurochem 1990;55:1784–92.
[22] Cohlberg JA, Hajarian H, Tran T, Alipourjeddi P, Noveen A. Neurofilament protein heterotetramers as assembly intermediates. J Biol Chem 1995;270:9334–9.
[23] Sakaguchi T, Okada M, Kitamura T, Kawasaki K. Reduced diameter and conduction velocity of myelinated fibers in the sciatic nerve of a neurofilament-dificient mutant quail. Neurosci Lett 1993;153:65–8.
[24] Ladefoged O, Roswall K, Larsen JJ. Acetone potentiation and influence on the reversibility of 2,5-hexanedione-induced neurotoxicity studied with behavioural and morphometric methods in rats. Pharmacol Toxicol 1994;74:294–9.
[25] Rebert CS, Sorenson SS. Concentration-related effects of *n*-hexane on evoked responses from brain and peripheral nerve of the rat. Neurobehav Toxicol Teratol 1983;5:69–76.
[26] DeCaprio AP, O'Neill EA. Alterations in rat axonal cytoskeletal proteins induced by in vitro and in vivo 2,5-hexanedione exposure. Toxicol Appl Pharmacol 1985;78:235–47.
[27] Zhu Q, Couillard-Després S, Julien JP. Delayed maturation of regenerating myelinated axons in mice lacking neurofilaments. Exp Neurol 1997;148:299–316.
[28] Elder GA, Friedrich VL, Bosco P, Kang C, Gourov A, Tu PH, et al. Absence of the mid-sized neurofilament subunit decreases axonal calibers, levels of light neurofilament (NF-L), and neurofilament content. J Cell Biol 1998;141:727–39.
[29] Rao MV, Houseweart MK, Williamson TL, Crawford TO, Folmer J, Cleveland DW. Neurofilament-dependent radial growth of motor axons and axonal organization of neurofilaments does not require the neurofilament heavy subunit (NF-H) or its phosphorylation. J Cell Biol 1998;143:171–81.
[30] Zhu Q, Lindenbaum M, Levavasseur F, Jacomy H, Julien JP. Disruption of the NF-H gene increases axonal microtubule content and velocity of neurofilament transport: relief of axonopathy resulting from the toxin beta, beta′-iminodipropionitrile. J Cell Biol 1998;143: 183–93.
[31] Shaw G, Weber K. Differential expression of neurofilament triplet proteins in brain development. Nature 1982;298: 276–99.
[32] Schlaepfer WW. Neurofilaments: structure, metabolism and implications in disease. J Neuropathol Exp Neurol 1987;46:117–29.
[33] Sawant LA, Hasgekar NN, Vyasarayani LS. Developmental expression of neurofilament and glial filament proteins in rat cerebellum. Int J Dev Biol 1994;38:429–37.
[34] Pachter JS, Liem RK. The differential appearance of neurofilament triplet polypeptides in the developing rat optic nerve. Dev Biol 1984;103:200–10.
[35] Giasson BI, Mushynski WE. Developmentally regulated stabilization of neuronal intermediate filaments in rat cerebral cortex. Neurosci Lett 1997;229:77–80.
[36] Tohyama T, Lee VM, Rorke LB, Trojanowski JQ. Molecular milestones that signal axonal maturation and the commitment of human spinal cord precursor cells to the neuronal or glial phenotype in development. J Comp Neurol 1991;310:285–99.
[37] Gou JP, Leterrier JF. Possible involvement of ubiquitination in neurofilament degradation. Biochem Biophys Res Commun 1995;217:529–38.
[38] Zhai Q, Wang J, Kim A, Liu Q, Watts R, Hoopfer E, et al. Involvement of the ubiquitin-proteasome system in the early stages of wallerian degeneration. Neuron 2003;39: 217–25.
[39] Hernandez F, Diaz-Hernandez M, Avila J, Lucas JJ. Testing the ubiquitin-proteasome hypothesis of neurodegeneration in vivo. Trends Neurosci 2004;27:66–9.
[40] Ehlers MD. Deconstructing the axon: Wallerian degeneration and the ubiquitin-proteasome system. Trends Neurosci 2004;27:3–6.