Sex-Related Differences in Cisplatin-Induced Neuropathy in Rats

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Background: *Cisplatin is used as an anti-neoplastic agent against several cancers. Neuropathy is one of its major side effects that contributes to patients' intolerance to the standard regimen. Sex-related differences have been reported in nerve injury and neuropathies. However, there has been no study on cisplatin regarding this issue.*
Objective: *Compare various abnormalities in cisplatin neuropathy between sexes.*
Material and method: *Two mg/kg of cisplatin was administered intraperitoneally twice a week for five consecutive weeks. Body weight, heat latency of hind paw and sciatic motor nerve conduction velocity (MNCV), pathological alterations in the sciatic nerve and dorsal root ganglion (DRG) including the level of NGF in the sciatic nerve were examined. Untreated rats of both sexes were used as controls.*
Results: *Weight loss, prolonged heat latency, and slow MNCV in the treated rats of both sexes with higher severity in males were showed. Furthermore, reduction in myelinated fiber diameter, myelin thickness, and myelinated fiber density was more severe in females, whereas, atrophy of neuronal cell body, nucleus, and nucleolus was more striking in males. The decreased level of NGF was similar between sexes.*
Conclusion: *These data suggest the differences in various aspects of cisplatin neurotoxicity between sexes. However, future studies are needed to verify this issue in a clinical condition and clarify the underlying mechanisms.*

Keywords: *Cisplatin, Neuropathy, Sex*

J Med Assoc Thai 2009; 92 (11): 1485-91
Full text. e-Journal: *http://www.mat.or.th/journal*

Cisplatin has been used as an anti-neoplastic agent against cancers of the stomach, lung, urinary bladder, and ovary[1-3]. However, cisplatin also causes severe side effects including nephrotoxicity and neurotoxicity that could result in cessation of treatment. Neurotoxicity is dose dependent and distal symmetrical polyneuropathy is the most common form[4,5]. Sensory perception deficits and sensory nerve conduction defects with motor sparing have been reported in patients treated with cisplatin[6]. Evaluation of sural nerve biopsies has found loss of large myelinated fibers[5]. In an animal model, sensory abnormalities similar to those observed in humans have been consistently shown[7-12]. In addition, morphometric analysis of dorsal root ganglion (DRG) in the animals has revealed atrophy of neuronal soma, nucleus, and nucleolus[7,8,12-15].

Existing data suggest the sexual dimorphism in peripheral nerve abnormalities. Vincristine, another anti-cancer drug, induced neuropathy with greater mechanical hyperalgesia in female than in male rats[16]. In case of nerve injury, the rate of regeneration was different between sexes[17-19]. Moreover, in cancer patients treated with cisplatin-based chemotherapy, the worse neurological outcome was found with higher frequency in males than in females[20]. However, an extensive comparison in the cisplatin-induced neuropathy between sexes has not been performed. Therefore, the aim of the present study was to elucidate the sex-related differences in cisplatin-induced neuropathy. The sensory test, nerve conduction study, morphometric analysis of sciatic nerve and DRG

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were done in male and female rats. Since it has been reported that the circulating nerve growth factor (NGF) was reduced in the cisplatin-treated rats[21,22], the level of NGF in the sciatic nerve, which is more closely related to the neuropathic condition, was also measured.

Material and Method

Thirty male and female Wistar rats weighing 200-250 g (National Laboratory Animal Center, Mahidol University, Thailand) were housed in aluminum cages on a 12 h light-dark cycle with free access to food and water. The room temperature was maintained at 25 ± 2°C. This experiment was approved by the institutional ethics committee and was carried out in accordance with the guidelines of the National Research Council of Thailand. All efforts were done to minimize pain or discomfort.

Drug administration

For each sex, the animals were divided into two groups (15 rats per group) which were control (normal saline-treated) and cisplatin-treated groups. Therefore, there were four groups altogether: male + normal saline (MN), male + cisplatin (MC), female + normal saline (FN), and female + cisplatin (FC). The MC and FC groups received cisplatin 2 mg/kg intraperitoneally twice a week for five consecutive weeks (20 mg/kg accumulative dose). Cisplatin was diluted in normal saline to the final concentration of 0.5 mg/ml. The dilution method was used to give excess fluid to prevent nephrotoxicity. Dose of cisplatin used in the present study had been shown to induce both physiological and structural abnormalities of peripheral nerve[10]. The control groups received only normal saline with the same method and schedule as in the cisplatin-treated groups.

General toxicity

To study the effect of cisplatin on general health, body weight of the animals was measured twice a week before drug administration.

Hind-paw heat latency

The hot plate analgesia meter (Harvard Apparatus, UK) was used to measure the hind-paw heat latency. Prior to any treatment, the rats were allowed to familiarize with the test procedure and apparatus and baseline values were obtained. Then, the test was done twice a week before each injection. The rat was placed on the hot plate maintained at 55°C and a timer was started. When the rat licked its hind paw on either side, the timer was stopped and the animal was removed from the apparatus. Elapsed time was recorded as latency. The cut-off duration of 35 seconds was used to avoid skin injury. If the latency was over this limit, 35 s was used for further analysis. The test was repeated at least 3 times with an interval of 10 min and an average latency was calculated for each rat. Mean latency was determined for each group at each time point.

Motor nerve conduction velocity

The motor nerve conduction velocity (MNCV) was determined prior to the start of any treatment as baseline and after the last injection in the fifth week. The rat was anesthetized using halothane. Rectal temperature was maintained at 37 ± 0.3°C using a heating pad and digital rectal thermometer. The stimulating and recording needle electrodes were placed at the sciatic notch and the second interosseous muscle of the hind foot, respectively. All these electrodes were connected to the oscilloscope (Neurostar, Oxford Instrument). The sciatic nerve was stimulated with a supramaximal stimulus and latency was measured from the stimulation artifact to the positive peak of compound muscle action potential. An average latency was derived from at least five stimulations. Then, the stimulating electrode was moved to the side of Achilles' tendon and the procedure was repeated. Difference between the latencies from two stimulation points was determined. The MNCV was calculated by dividing the distance between the two stimulation points by the latency difference. Average MNCV was determined for each group at each time point.

Specimen collection

At the end of the fifth week, eight rats per group were sacrificed using overdose halothane. Soleus muscle and sciatic nerve were freshly removed and kept at -70^{O}C for determination of NGF by ELISA. The other seven rats in each group were transcardially perfused by a pump with 100 ml of normal saline, followed by 500 ml of 4% paraformaldehyde. The sciatic nerve, the fourth lumbar dorsal root ganglion (L4 DRG) were removed and post-fixed in 3% glutaraldehyde for six hours. The tissues were then processed and embedded in epoxy resin.

Nerve morphometry

Transverse 1 μm thick sections of the sciatic nerve were cut, mounted on slides, and stained with paraphenylenediamine. The slides were examined

under a microscope and the cross-sectional areas were chosen using the three-window sampling method[23,24]. Images of these areas were imported into the microcomputer via a CCD camera. Morphometric analysis was done using Image-Pro Plus software. The number of myelinated fibers (MF), diameters of axons and MF, density of MF, myelin thickness, and g ratio were determined and averaged for each group.

Morphometry of DRG neurons

The DRG were serially cut into 2 μm thick sections, mounted on slides and stained with toluidine blue. Every 20th section was selected and the number of neurons with prominent nucleus and nucleolus was counted. Then, this number was extrapolated to the total number for the whole DRG. Moreover, at least 300 DRG neurons were randomly chosen for morphometric analysis. With the Image-Pro Plus, areas of the cell body, nucleus and nucleolus were measured and averaged for each group.

Level of nerve growth factor

Level of NGF in the sciatic nerve was determined by ELISA according to the manufacturer's instruction (NGF Emax ELISA kit, Promega). Briefly, the tissues were weighed, homogenized, and centrifuged at 14,000 x g for 30 min. The supernatant and NGF standard were transferred to the 96-well plate coated with the NGF antibody and kept at 4°C overnight. Then, the plate was washed and incubated with the secondary antibody conjugated with horse radish peroxidase (HRP) for 2.5 hr at room temperature. After washing, tetramethyl benzidiene (TMB) was added in each well and, then, the reaction was stopped by adding 1N HCL. The OD was determined at 450 nm by a microplate reader. The standard curve of NGF was plotted and used for calculating the concentration of NGF in the samples. The mean value of each group was obtained.

Statistical analysis

Student's unpaired t-test was used to compare means ± standard error of mean (SEM) of experimental groups with the same sex. This was done using SPSS for Windows version 11. Statistically significant difference was noted when $p < 0.05$.

Results

General toxicity

The mean body weight (BW) of the control and treated groups in each sex was similar at the beginning with that of males higher than that of females (Fig. 1). One week after the start of cisplatin administration, the BW of the cisplatin-treated groups became significantly lower than that of the control groups in both sexes ($p < 0.001$). This decrease was increasingly prominent at later time points with higher severity in males. At the end of the experiment, the average weight loss of males and females was 41% and 23%, respectively. It is worth noting that four rats in the MC group, but none in the FC group, died in the fifth week.

Hind-paw heat latency

The average heat latencies were not significantly different among the groups at baseline (Fig. 2). The latencies started to be significantly prolonged in the cisplatin-treated groups since the second week with the higher difference in males ($p <$

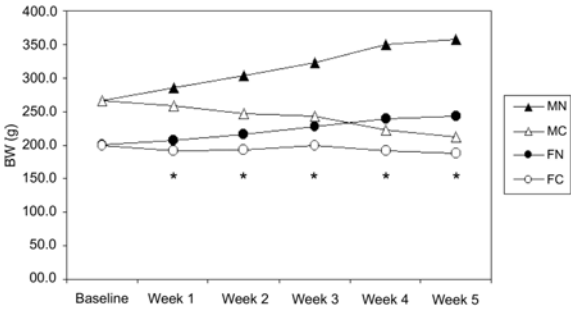

Fig. 1 Changes in the average body weight of rats in each group. * $p < 0.001$ MC vs. MN and FC vs. FN

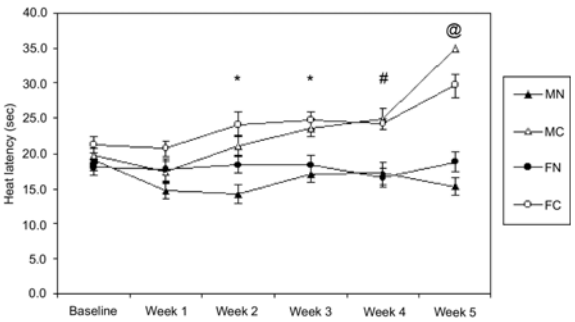

Fig. 2 Changes in the heat latency of rats in each group. Data are means ± SEM, * $p < 0.01$ MC vs. MN and $p < 0.05$ FC vs. FN, # $p < 0.01$ MC vs. MN and FC vs. FN, @ All rats in the MC group and 9 of 15 rats in the FC group had heat latencies more than 35 s; therefore, 35 s was used for calculation of means, SEM = standard error of mean

0.01) than in females ($p < 0.05$). The maximal change was observed in the fifth week with the latencies over the cut-off value (35 s) in all rats in the MC group and nine of 15 rats in the FC group. Therefore, 35 s was used as the representative latency of the MC group in the last week.

Motor nerve conduction velocity

The sciatic MNCV were similar among the groups at the beginning (Fig. 3). However, at the end of the present study, decreased MNCV were found in the MC and FC groups compared with the corresponding controls. The change was more striking in males.

Nerve morphometry

The morphometric data of sciatic nerve are summarized in Table 1. The MF and myelinated axon diameters including the MF density were significantly decreased after five weeks of cisplatin treatment relative to the untreated controls in both sexes. However, there was only a trend toward reduced myelin thickness in the cisplatin-treated compared with the control groups with no significant change in the number of total fiber or g ratio. The sex differences varied depending on the parameters with higher severity in females in the fiber and axon diameters (13.3% vs. 8.9% and 16.2% vs. 8.6%, respectively) but similar changes in the myelin thickness and fiber density.

Morphometry of DRG neurons

The morphometric data of DRG neurons are summarized in Table 2. The values of all parameters studied were decreased in the cisplatin-treated compared with the control groups with significant differences in the somatic area, nuclear area (only in female) and nucleolar area. The degree of reduction in these parameters was higher in males than in females.

Level of NGF

The NGF levels were decreased in the sciatic nerves of the MC and FC groups relative to those of the MN and FN groups, respectively (Fig. 4). However, these changes were not statistically significant and were not different between sexes

Discussion

The results showed that cisplatin caused significant weight loss and prolonged heat latency in both sexes. These findings were similar to the previous studies[7-12]. However, two studies have reported that

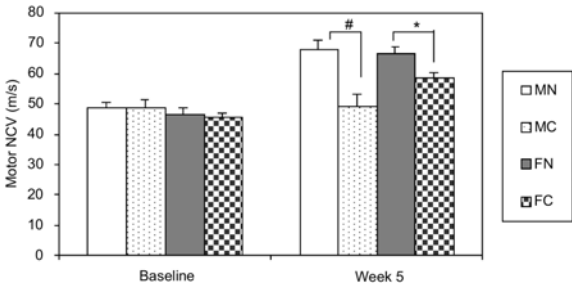

Fig. 3 Sciatic motor nerve conduction velocity of rats in each group at baseline and in the fifth week. Data are means ± SEM, # $p < 0.001$ MC vs. MN, * $p < 0.01$ FC vs. FN, SEM = standard error of mean

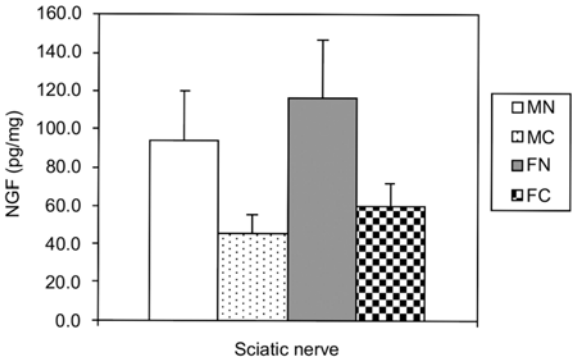

Fig. 4 Level of NGF in the sciatic nerve in each group in the fifth week. Data are means ± SEM

MNCV was unaffected by cisplatin[11,25] which was inconsistent with the present finding of reduced MNCV. This discrepancy was likely due to the lower doses used in those studies since Authier et al have shown that the NCV abnormality was dose-dependent[10].

Atrophy of cell body, nucleus, and nucleolus of DRG neurons observed in the present study was consistent with other reports[7,8,12-15]. This and another study[15] also found the trend toward reduced number of DRG neurons in the cisplatin-treated rats compared with the controls. The cause of this reduction is still unknown as TUNEL staining failed to detect apoptotic neurons[12]. As for the nerve, the present results demonstrated a significant decrease in MF and myelinated axon diameters including lower MF density with preserved g ratio. Similar findings have been previously reported[7,13,15]. It is noteworthy that the authors also found reduced myelin thickness despite no significant difference. This may explain the unaffected g ratio found in most studies. However, two studies

Table 1. Morphometric data of sciatic nerve

Group	Fiber diameter (μm)	Axon diameter (μm)	Myelin thickness (μm)	g ratio	Fiber density (/mm^2)	Total fiber
FN	7.00 ± 0.11	4.21 ± 0.13	1.40 ± 0.04	0.59	13,657.4 ± 523.2	8,455 ± 1,058
FC	6.07 ± 0.10^c	3.53 ± 0.06^b	1.27 ± 0.03	0.57	18,200.0 ± 798.1^b	7,578 ± 549
MN	6.62 ± 0.19	3.73 ± 0.10	1.45 ± 0.05	0.56	13,804.2 ± 800.9	8,815 ± 237
MC	6.03 ± 0.10^a	3.41 ± 0.04^a	1.31 ± 0.04	0.55	18,516.7 ± 465.4^b	9,242 ± 205

Data are means ± SEM, n = 8 per group
a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$ FC vs. FN or MC vs. MN
SEM = standard error of mean

Table 2. Morphometric data of DRG neurons

Group	Total number of neuron	Somatic area (μm^2)	Nuclear area (μm^2)	Nucleolar area (μm^2)
FN	17,343 ± 1,692	1,142.8 ± 19.5	181.3 ± 1.9	13.4 ± 0.9
FC	13,474 ± 1,397	906.1 ± 35.9^c	151.4 ± 5.2^c	10.2 ± 0.4^b
MN	15,693 ± 1,602	1,258.9 ± 109.0	188.6 ± 28.3	14.1 ± 1.7
MC	11,966 ± 1,941	806.2 ± 68.3^b	144.5 ± 8.8	7.9 ± 0.2^a

Data are means ± SEM, n = 8 per group
a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$ FC vs. FN or MC vs. MN

reported no myelin degeneration in the sciatic nerve of cisplatin-treated rats[10,13]. The involvement of myelin sheath in cisplatin neuropathy, therefore, remains to be clarified.

NGF is essential for normal functions of the peripheral nervous system. NGF is synthesized in the target organs of sensory neurons such as skin[26]. Then, it is retrogradely transported along the axon to the cell body to exert its actions[27,28]. Therefore, decreased level of NGF could impair the functions of sensory neurons as demonstrated in diabetic neuropathy[29]. Previously, the level of circulating NGF in plasma was found reduced in rats receiving cisplatin[21,22]. The authors found that the level of NGF tended to decrease in the sciatic nerve of the cisplatin-treated compared with the control rats.

Comparing between sexes, the general toxicity of cisplatin was more severe in males than in females as indicated by the higher degree of weight loss and mortality. The death found in the MC group but not the MN group suggests that this was likely related to cisplatin treatment. Similarly, a higher incidence of prolonged heat latency over 35 s was found in males (11/11) than in females (9/15) in the last week. In case of MNCV, reduction was more prominent in males. Furthermore, the morphological changes of nerve appeared to be more severe in females, whereas, those of DRG neurons were more striking in males. Regarding the level of NGF in sciatic nerve, the degree of reduction was not different between males and females. The influence of sex on the development of neuropathy and nerve injury is controversial. Joseph and Levine, 2003 reported the higher degree of hyperalgesia in vincristine-induced and diabetic neuropathies in female rats[16,30]. They have suggested that these sex-related differences were estrogen-dependent and related to certain PKC isoforms. Similarly, female patients tended to develop more severe neurological deficits induced by cisplatin[20]. In contrast, for nerve injury, two studies observed the faster regeneration and functional recovery in female animals[18,19]. The more effective cell support of peripheral nerve has been proposed as the responsible mechanism in this case[19]. However, more studies are needed to elucidate the exact underlying mechanism.

In conclusion, cisplatin treatment was associated with general toxicity, prolonged heat latency, and slow MNCV, which were more severe in male rats. However, sex-related differences in the structural alterations in the myelinated fibers and DRG

neurons were variable. Equal reduction of NGF in the sciatic nerve was observed in both sexes. This sexual dimorphism should be verified in clinical cisplatin-associated neuropathy and its underlying mechanism remains to be clarified.

Acknowledgement

This work was supported by Rachadapisek-sompoj Fund (Pilot project) 2006 provided by Faculty of Medicine, Chulalongkorn University.

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ความแตกต่างระหว่างเพศของภาวะ neuropathy ที่เกิดจาก cisplatin ในหนู

ตุลาภรณ์ ว่องธวัชชัย, สิทธิพร แอกทอง, อทิตยา แก้วเสมา, วิไล ชินธเนศ

Cisplatin ใช้เป็นยาต้านมะเร็งในมะเร็งหลายชนิด โรคเส้นประสาทเป็นผลข้างเคียงที่สำคัญหนึ่งที่ทำให้ผู้ป่วยไม่สามารถทนต่อขนาดยาตามมาตรฐานได้ มีข้อมูลจากการศึกษาก่อนหน้านี้บ่งชี้ถึงความแตกต่างที่เกี่ยวกับเพศในการบาดเจ็บของเส้นประสาท และโรคเส้นประสาทอื่น ๆ แต่อย่างไรก็ตามยังไม่มีการศึกษาเกี่ยวกับเรื่องนี้ในกรณีของ cisplatin ดังนั้นการศึกษานี้จึงมีจุดประสงค์เพื่อเปรียบเทียบความผิดปกติต่าง ๆ ของโรคเส้นประสาทจาก cisplatin ระหว่างหนูสองเพศ cisplatin ขนาด 2 mg/kg ถูกฉีดเข้าช่องท้องสองครั้งต่อสัปดาห์ เป็นเวลา 5 สัปดาห์ติดต่อกัน มีการประเมินน้ำหนักตัว การรับรู้ความร้อนที่เท้า และ motor nerve conduction velocity (MNCV) ของเส้นประสาท sciatic ในระหว่างการทดลอง ขณะที่ประเมินการเปลี่ยนแปลงทางโครงสร้างของเส้นประสาทและปมประสาทไขสันหลัง และระดับ NGF ในเส้นประสาทเมื่อสิ้นสุดการทดลอง โดยใช้หนูเพศเดียวกัน ที่ไม่ได้ cisplatin เป็นกลุ่มควบคุม ผลพบว่าน้ำหนักตัวลดลง การรับรู้ความร้อนที่เท้าลดลง MNCV ช้าลง ในหนูทั้งสองเพศที่ได้รับ cisplatin โดยที่หนูเพศผู้เกิดรุนแรงกว่า ส่วนการลดลงของ myelinated fiber diameter, myelin thickness และ myelinated fiber density ในเส้นประสาทของเพศเมียจะรุนแรงกว่า ขณะที่การหดตัวของส่วนต่าง ๆ ของเซลล์ประสาทในเพศผู้จะเด่นกว่า นอกจากนี้ระดับที่ลดลงของ NGF ใกล้เคียงกันระหว่างเพศ การศึกษานี้แสดงให้เห็นความแตกต่างในด้านต่าง ๆ ของโรคเส้นประสาทจาก cisplatin ระหว่างเพศ อย่างไรก็ตามการศึกษาในอนาคตมีความจำเป็นเพื่อยืนยันข้อสังเกตนี้ทางคลินิกรวมทั้งอธิบายกลไกที่เกี่ยวข้องต่อไป