ORIGINAL SCIENTIFIC ARTICLE

ANTIOXIDANT SUPPLEMENTATION WITH N-ACETYLCYSTEINE AS A PROTECTION AGAINST CISPLATIN-INDUCED MOTOR IMPAIRMENT IN RATS

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ABSTRACT

The aim of this study was to estimate a potential beneficial influence of N-acetylcysteine (NAC) in the treatment of cisplatin-induced motor impairment. We included 32 male Wistar albino rats, divided into 4 equal groups: control (received saline on the 1st and 5th day), cisplatin – CIS (received saline on the 1st and cisplatin 7.5 mg/kg i.p. on the 5th day), NAC (received NAC on the 1st and 5th day, 500 mg/kg i.p.), and CIS+NAC (received NAC on the 1st and 5th day, 500 mg/kg i.p. and cisplatin 7.5 mg/kg i.p. on the 5th day) group. Motor performance was estimated by rotarod, grip wire, open field (OF), elevated plus maze (EPM) and beam-walking (BW) tests on the 10th day. Cisplatin administration resulted in decreased motor performance in all tests, except for BW test, compared to the control group. NAC supplementation on its own had no significant effect on motor performance parameters. However, simultaneous administration of NAC along with cisplatin reversed negative impact of cisplatin in rotarod, OF and EPM tests, with no significant effect on the results obtained in grip wire test. The results of this study confirmed numerous motoric manifestations of cisplatin-induced neurotoxicity in rats. However, the decline in most of the estimated parameters was successfully prevented by antioxidant supplementation with NAC.

Keywords: *Cisplatin, N-acetylcysteine, motor performance, rats.*

INTRODUCTION

Since the almost accidental discovery of its biological effects in 1965 (1), and the first results of clinical trials in 1978 followed by FDA approval, cisplatin (cis-diamminetetrachloroplatinum(IV)) has been pronounced as "the drug of the 20th century" and became the frontline therapy in the treatment of various malignancies (2). Unfortunately, the therapy with platinum-based compounds has very serious limitations including numerous adverse effects.

Although they have not been evaluated as some other adverse effects of cisplatin therapy such as nephrotoxicity (3) and hepatotoxicity (4), clinical manifestations of cisplatin-induced neurotoxicity are very common and represent a serious limitation in therapeutic protocols that include cisplatin. Platinum-based therapy induces neurotoxicity that usually appears in clinical features that are manifested both in the form of peripheral and central neuropathy. The peripheral neuropathy induced by platinum compounds is believed to be the consequence of the initial accumulation of platinum compounds and their metabolites in the dorsal root ganglia after their systemic administration. Formation of platinum-DNA adducts are considered to be a key step in neurotoxicity development (5). The most pronounced effects are observed on large-diameter sensory nerve fibers that appear to be the most affected by platinum drugs. That condition is usually leading to symmetrical glove and stocking type of sensory loss, numbness, tingling, pain and burning sensation (6). Besides, platinum-based therapy also significantly affects central nervous system. The most frequent manifestations of this specific adverse effect are classified as ototoxicity (frequently with permanent hearing loss), nausea and vomiting (7). Also, there are numerous reports about various mood disorders accompanied with platinum-based therapy (8). Many of those clinical symptoms that are characteristic for platinum-based therapy have been evaluated using animal experimental models. Although considered rare, clinical manifestations of motor impairment induced by platinum-based therapy are very intriguing since they may appear as the symptoms that resemble the combined consequences of both peripheral and central neurotoxicity.

Despite the fact that adverse effects of cisplatin toxicity are diverse, it is well-known that underlying mechanisms that are common for cisplatin-induced toxicities are of the same origin. The most commonly described pathophysiological base for adverse effects of cisplatin can be categorized in a few interconnected processes that include oxidative damage, mitochondrial dysfunction, apoptosis and inflammation. Each of those processes may be the target that should be solved in order to reduce cisplatin-induced toxicities and, therefore, achieve the breakthrough in the principle limitations of cisplatin therapy.

The aim of this study was to estimate the potential beneficial influence of N-acetylcysteine (NAC) in the treatment of cisplatin-induced motor impairment, since NAC is established as safe for clinical use (9).

MATERIAL AND METHODS

A total of 32 male Wistar albino rats, 12-14 weeks old (250–300 g), purchased from the Military Medical Academy (Serbia), were randomly divided into four equal groups. The animals were housed in transparent cages (four per cage) under standard environmental conditions, which include maintaining the constant temperature at 23 ± 1 °C and humidity at 50 ± 5%, with a light/dark cycle (12/12h). All animals were allowed food and tap water intake ad libitum.

The animals were treated in previously defined conditions, according to predefined groups, as follows: control, cisplatin (CIS), NAC and CIS+NAC group. Rats in the control and cisplatin groups received saline (approximately 2 mL i.p.) at the start of the experimental protocol (day 1). At the same time, at the start of trials (day 1), NAC and CIS+NAC group rats were administered with NAC (500 mg/kg i.p., Sigma-Aldrich, Germany). On the fifth day of the trials, the animals in the control group were treated with saline (approximately 2 mL i.p.), while the rats in the CIS group received a single dose of cisplatin (7.5 mg/kg i.p., Merck, France). Also, the animals in the CIS+NAC group, besides the single dose of cisplatin (7.5 mg/kg i.p.), were administered with NAC (500 mg/kg i.p.). Finally, the animals in NAC group were treated with NAC again in a dose of 500 mg/kg i.p.

All animals were exposed to the training protocols for rotarod and beam-walking tests. Training protocols for those tests consisted of 5-7 attempts for each animal, on an adequate apparatus, on the 4th and 8th day of the trial.

Behavioral testing was performed after completing pretreatment trials, on the 10th day. In order to allow accommodation, the animals spent 1-2 hours in the testing room (at approximately 9 a.m.), prior to the testing.

Rotarod test

Rotarod test is one of the most commonly used tests for the evaluation of motor coordination and balance in the experimental behavioral animal models, especially in rodents (10, 11). The apparatus for this test consisted of rubber coated rotating rod elevated 50 cm above surface. The space below the rod was appropriately equipped (for the prevention of injury following animals' falls) with soft pad. Starting rotation speed was 5 rounds per minute, and then it was gradually increased for 5 rpm each 30 seconds, up to the maximal speed of 60 rpm. On the final testing day, the rats had three attempts and the best score was taken into consideration. The time spent on the rod was expressed in seconds.

Open field (OF) test

The equipment and basic methodology for OF test, as one of the principle tests for mood disorders testing in animal experimental models, is very common and minutely described in literature (12). The apparatus consisted of the black painted wooden square arena (60×60×30 cm). The testing, that lasted 5 minutes, had begun by placing the animal in the

center of the square arena. Spontaneous activity was allowed throughout the test. As an indicator of the overall motor activity, we estimated the mean velocity. This parameter was calculated by dividing the total distance and test duration, expressed in cm/s.

Elevated plus maze (EPM) test

The elevated plus maze test is usually considered the most sensitive behavioral test for the estimation of anxiety state level (13). Like in OF test, spontaneous activity of the animals was evaluated in the apparatus that consisted of two open (50×20 cm) and two enclosed (50×20×30 cm) opposite arms elevated 100 cm above the floor. Again, the test was initiated by placing the animal in the center of the maze (facing the open arm), and each animal was individually allowed 5 minutes for free exploration. As an indicator of the overall motor activity, we estimated the mean velocity. This parameter was calculated by dividing the total distance and test duration, expressed in cm/s.

Grip wire test

Grip wire test is used for the estimation of muscle strength and balance in rodents (14). In this test, we used square metal frame (15x15 cm) with wire thickness of 5 mm. The rats were allowed to grasp the wire of metal frame (elevated 30 cm above the floor) with their forepaws. The space below the apparatus was adequately equipped to prevent the fall of animals. On the testing day, the rats were allowed three attempts, and the best score was evaluated for further analysis. In this test, we quantified the time on the wire (expressed in seconds).

Beam-walking (BW) test

Beam-walking test is used for the estimation of motor coordination, integration, balance and motor skills (15, 16). The apparatus consisted of the stainless steel beam (100x3x2 cm). The beam had rubber pad in order to reduce animals' slip down, fixed between two vertical steel columns heightened 60 (start point) and 100 cm. Escape wooden box (20x20x20 cm), with the hole (the opening is adapted to thesize of the animal) was located on the highest side of the beam, and represented the safe place for the escape of rats. The cushions were placed below the whole apparatus in order to prevent the possible injury.

In order to remove possible interfering scents, the equipment for behavioral testing was thoroughly cleaned with the water solution of ethanol after each animal testing.

All tests were recorded using a digital video camera mounted above the mazes. Interpretation of video files was conducted by Ethovision software XT 12 (Noldus Information Technology, the Netherlands).

All research procedures were carried out in accordance with the European Directive for the welfare of laboratory animals No 86/609/EEC, the principles of Good Laboratory Practice (GLP), and in accordance with the ARRIVE guidelines. All experiments were approved by the Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

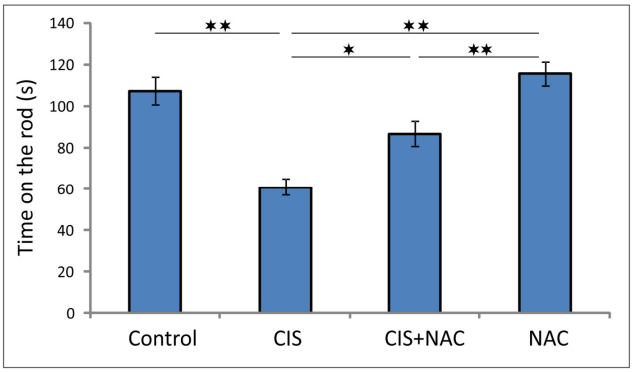
Statistical Analysis

The data were presented as means ± S.E.M. After completing the tests for homogeneity (Levene's) and normality (Shapiro-Wilk), comparisons between groups were performed using One-way ANOVA, followed by Bonferroni post hoc analysis. Significance was determined at $p<0.05$. Statistical analysis was performed with SPSS version 20.0 statistical package (IBM SPSS Statistics 20).

RESULTS

As shown in Figure 1, the applied protocols significantly altered time on the rod estimated in rotarod test (df=3, F=19.127). Cisplatin administration in a single dose (7.5 mg/kg i.p.) markedly reduced this parameter often used as an indicator of endurance performance when compared to control values ($p<0.01$). This effect of cisplatin was successfully attenuated with NAC supplementation ($p<0.05$) reversing the values of this parameter back to control values. NAC itself did not significantly alter the time on the rod when compared to control values.

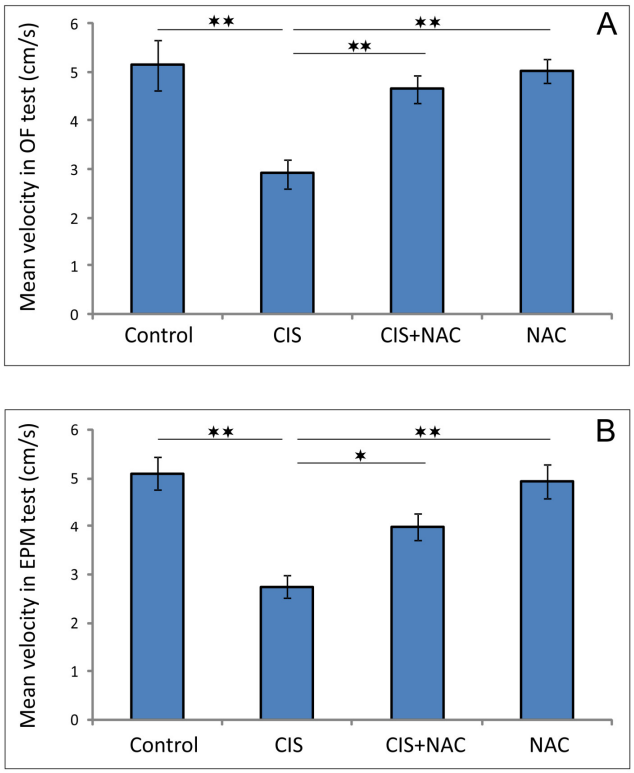
Figure 1. The effects of cisplatin and NAC in the rotarod test. The values are mean ± standard error of the mean (SEM), *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$.



The motor performance was also evaluated in this study by means of two behavioral tests, originally used for the estimation of mood disorders (OF and EPM test, Figure 2A and B, respectively). The same parameter, a mean velocity during five-minute testing, was determined in both tests, in order to analyze the influence of the applied protocols. Both indicators of motor activity in OF and EPM test were significantly altered in this experimental design (df=3, F=8.552 and 12.827, respectively). Cisplatin application resulted in significant increase in MV in both OF and EPM test ($p<0.01$) compared to control. However, although NAC administration on its own showed no significant effect when compared to control values, simultaneous administration with cisplatin

resulted in diminishing effect on cisplatin-induced decline of overall motor activity in OF ($p<0.01$) and EPM test ($p<0.05$). The attenuation of cisplatin-induced motor impairment by simultaneous administration of NAC was sufficient to increase the mean velocity observed in both tests back to control values.

Figure 2. The effects of cisplatin and NAC on the mean velocity in the open field (A) and the elevated plus maze (B) test. The values are mean ± standard error of the mean (SEM), *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$.



The evaluation of motor performance by means of muscle strength estimation in this study was performed in grip wire test (Figure 3). The applied protocols significantly influenced the time on the wire ($df=3$, $F=14.893$). A single dose of cisplatin significantly lowered this parameter of muscle strength ($p<0.01$). Unlike previously described tests, the administration of NAC along with cisplatin treatment failed to reverse the diminishing effect of cisplatin on muscle strength. The time on the wire, in this (combined) group remained below the control values ($p<0.05$). On the other hand, when applied solely, NAC produced no significant effect on this parameter when compared to control group.

The testing of motor abilities by means of motor coordination in this study was performed in beam-walking test (Figure 4). However, neither of applied protocols had shown significant impact on the time to cross the beam ($df=3$, $F=2.372$).

Figure 3. The effects of cisplatin and NAC in the grip wire test. The values are mean ± standard error of the mean (SEM), *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$.

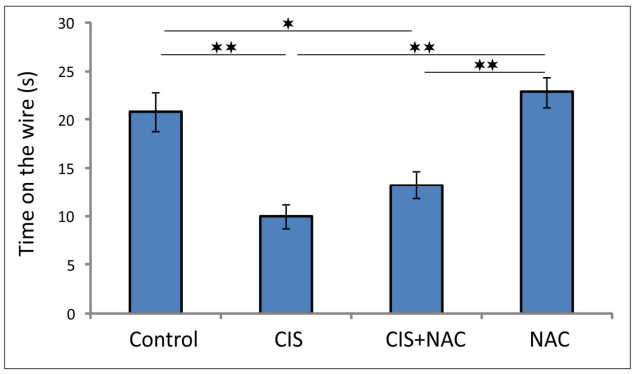
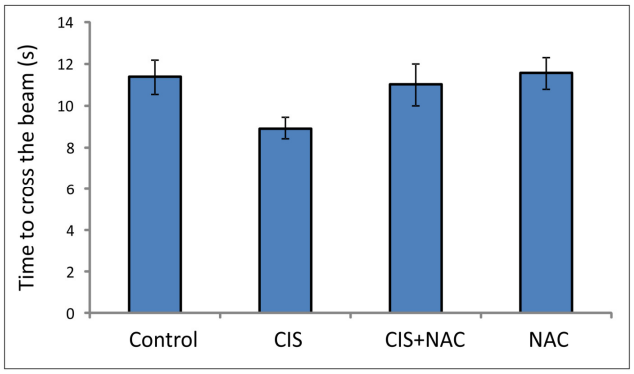


Figure 4. The effects of cisplatin and NAC in the beam-walking test. The values are mean ± standard error of the mean (SEM).



DISCUSSION

Apart from the unquestionable benefits of cisplatin usage in the treatment of various malignancies, we are still facing some major difficulties in this therapeutic approach. Predominantly, they are connected with numerous adverse effects that are often reported during cisplatin therapy. However, the numerous side effects of cisplatin usually have similar pathophysiological background that includes oxidative damage, mitochondrial dysfunction, apoptosis and inflammation. Therefore, it seems worth to investigate the potential therapeutic approaches that can reduce the processes that usually limit platinum-based therapy.

According to the results obtained in this study, it seems that even initial administration of cisplatin produces respectable effects on motor abilities in rats. However, the response to cisplatin in an early phase of treatment is very complex, with strong variations depending on the specific aspects of motor performance. The highest (negative) impact of cisplatin was observed in tests used for estimation of continuous, prolonged motor activity. Namely, a single dose of cisplatin in just a few days was sufficient for significant reduction of

motor performance observed in rotarod test (Figure 1). Our results are in line with previously reported data regarding the reduction of the time on the rod following chronic treatment with cisplatin (17). This test is usually considered a good indicator of endurance performance. Therefore, this kind of exercise, in order to achieve results at the physiological level, aside of optimal functioning of neuromuscular units with an adequate reflex coordination at the spinal cord level, requires undisturbed function of supporting systems, such as cardiovascular and respiratory system. So, the basic mechanism of adverse effect of cisplatin should be addressed both to affection of basic neuromuscular units involved in this characteristic motor pattern continually repeated in this test, and, at the same time, to systemic action of cisplatin that includes the adverse effects on cardiovascular system. Indeed, it has been reported that platinum-based compounds (oxaliplatin) produced significant motor impairment by means of endurance performance that was accompanied with verified signs of peripheral neuropathy (18). Nevertheless, it has been also previously noticed that cisplatin administration produced deleterious effects on cardiovascular system, even after the treatment in a single dose (19, 20).

The similar effects of cisplatin in this study were also observed in other tests for continual motor activity, only this was spontaneous (explorative) activity in OF and EPM test, instead of forced (endurance) performance in rotarod test. Again, a single dose of cisplatin significantly diminished overall motor activity, expressed by means of decline in mean velocity in both tests (Figure 2). Besides the potential explanation for negative impact of cisplatin on motor performance presented for rotarod test, the influence of cisplatin on motor action in OF and EPM tests may require additional analysis based on the specific, original nature of those two tests. As a matter of a fact, both tests are considered sensitive for estimation of mood disorders, such as increased anxiety level. Since our previous report (21) confirmed clear and strong anxiogenic effects following a single dose of cisplatin (the same dose as applied in this study), and due to the fact that increased anxiety has been proposed as a reason for decreased motor performance in both OF and EPM tests (22), it seems that, at least, the part of observed reduction in mean velocity of moving in OF and EPM tests should be addressed to anxiogenic effect of cisplatin that takes place concomitantly to affection of motor units. Even more, as those two tests do not exert forced (extreme) motor performance (like rotarod test) that is sufficient to reveal the decreased abilities of cardiovascular system following cisplatin treatment, it is likely that cisplatin-induced motor impairment observed in OF and EPM tests may appear as a result of combined motor-disturbing and anxiogenic effects of cisplatin. The results for an overall motor activity in primary behavioral tests following cisplatin treatment are in accordance with previously published reports (17, 23).

The results obtained in grip-wire test (Figure 3) offer the conformation that cisplatin also had a negative impact on other specific feature of physical performance, such as strength and/or power. Besides the previously described adverse effect on neuromuscular units and the regulation of their function, the observed decline in principal strength performance following cisplatin administration could be also accompanied by previously described disturbances in body composition. Namely, it has been reported that cisplatin treatment resulted in significant decrease in body weight by 27.5% after the three-week administration protocol with a total dose of 15 mg/kg (24). The reduction of body weight as a result of cisplatin treatment is very likely to be accompanied by simultaneous reduction of skeletal muscle mass, which can lead to proportional decline of motor performance by means of strength and/or power. This is in accordance with previously published study with a single dose of cisplatin (16 mg/kg), which resulted in significant muscle atrophy in female rats of the same age as in this study (25). The proposed explanation can even be augmented by the fact that all animals evaluated in this study were male, so the reduction of skeletal muscle mass following body weight decline should be potentiated to a higher level. This conclusion is in line with reported muscle mass wasting characterized by decline in myofiber diameter and cross-sectional fiber areas in male rats (24).

Unlike the other test that estimated other features of physical performance, the results obtained in the beam-walking test revealed that a single dose of cisplatin failed to significantly affect specific motoric elements (Figure 4), such as balance and coordination. On the other hand, the literature data describes the motor impairment following cisplatin treatment that was accompanied with specific cerebellar injury manifested by degenerative changes including shrunken nuclei and eosinophilic cytoplasm in Purkinje cells (23). The observed differences can be addressed to different experimental protocols, including the duration (5 weeks *vs*. single dose) and the total applied dose (25 *vs*.7.5 mg/kg), when compared to experimental protocol performed in this study. It seems that adverse impact of cisplatin on principally cerebellar-controlled functions may appear later when compared to spinal cord level and systemic responses to cisplatin administration.

The results of this study also showed the beneficial effects of antioxidant supplementation with NAC in the treatment of cisplatin-induced motor impairment. This protective effect of NAC was significantly manifested in several tests. For example, NAC supplementation along with cisplatin treatment attenuated cisplatin-induced decline in endurance performance estimated in rotarod test (Figure 1). This is in line with previously reported protective effect of new NAC-based product (N-acetylcysteine amide) that also improved performance in rotarod test following iatrogenic-induced neurotoxicity (26). Also, it should be mentioned that NAC administration showed beneficial effects in pathophysiological events characterized by neurodegeneration (27) that is similar to manifestations of cisplatin-induced neurotoxicity.

Furthermore, according to the results of this study, NAC supplementation showed beneficial effects on the impairment of strength/power feature of physical performance. Our

results obtained in grip wire test are in accordance with the results of the studies where the cisplatin-induced (20 mg/kg in two doses and 25 mg/kg in five doses, respectively) adverse effects in grip wire test were prevented by antioxidant supplementation with natural products from tomato (28) and antioxidant rich nuts (17). The results obtained in OF and EPM test also confirmed the protective effect of NAC supplementation by means of correcting of cisplatin-induced decline in overall motor activity. This manifestation of beneficial effect of NAC, besides previously commented action of NAC, should be also analyzed on the basis of corrective role of NAC in specific mood disorders that could be estimated in those two tests. In general, it has been confirmed that antioxidant supplementation can diminish anxiogenic effect of cisplatin that, in turn, may lead to decreased motor performance in OF and EPM tests (21). Obviously, NAC supplementation had no impact on the motor performance observed in BW test. This can be explained by the fact that applied experimental protocols were not sufficient to produce initial motor impairment that could be estimated in BW test. Therefore, the trials performed in this study do not seem suitable for the evaluation of the NAC impact on cisplatin-induced cerebellar dysfunction.

CONCLUSION

The results of this study confirmed numerous motoric manifestations of cisplatin-induced neurotoxicity in rats. However, the decline in most of the estimated parameters was successfully prevented by antioxidant supplementation with NAC.

ACKNOWLEDGMENTS

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ETHICS APPROVAL

All research procedures were carried out in accordance with the European Directive for the welfare of laboratory animals No 86/609/EEC, the principles of Good Laboratory Practice (GLP), and in accordance with the ARRIVE guidelines. All experiments were approved by the Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia

CONFLICT OF INTEREST

There are no conflicts of interest.

FUNDING

None.

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