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Effects of different schedules of oxaliplatin treatment on the peripheral nervous system of the rat

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Abstract

The aim of this study was to determine the influence of oxaliplatin scheduling on the onset of peripheral neurotoxicity and ototoxicity in a rat model. Animals were treated with four different schedules of oxaliplatin using two cumulative doses (36 and 48 mg/kg intraperitoneally (i.p.)). The neuropathological examination evidenced dorsal root ganglia (DRG) nucleolar, nuclear and somatic size reduction with nucleolar segregation in the treated rats. Sensory nerve conduction velocity (SNCV) was reduced after oxaliplatin treatment, while the auditory pathway was unaffected. After treatment, platinum was detected in the kidney, DRG and sciatic nerve. After a 5-week follow-up period, recovery of the pathological changes in the DRG and sciatic nerves occurred, although platinum was still detectable in these tissues. The following conclusions may be drawn: the main targets of oxaliplatin neurotoxicity were the DRG; the shorter the interval between the injections, the higher the severity of peripheral neuropathy and this was also related to the cumulative oxaliplatin dose; the peripheral neurotoxicity tended to be reversible; ototoxicity was absent even with high cumulative doses of oxaliplatin.

Keywords: Audiometry; Neuropathy; Oxaliplatin; Rat; Toxicity

1. Introduction

Oxaliplatin [(trans-l)1,2-diaminocyclohexaneoxalatoplatinum(II)] is a platinum-based chemotherapeutic agent with a 1,2-diaminocyclohexane carrier ligand that produces bulkier DNA conjugates due to the restricted freedom of motion of the platinum atom [1]. Several *in vitro* studies in cultured human cancer cell lines and *in vivo* studies in mice have demonstrated the superior anti-proliferative activity of oxaliplatin in comparison to cisplatin for different human malignancies including colon, ovarian and lung cancer [2,3]. Oxaliplatin is effective against metastatic colorectal cancer as a monotherapy and in combination with other agents [4–8] and against cisplatin-resistant ovarian cancer [9] and is not associated with nephrotoxicity [1].

Oxaliplatin, therefore, is a very promising antineoplastic drug, although neurotoxicity is a dose-limiting symptom [10]. In humans, two different types of neurological symptoms occur during oxaliplatin treatment. Acute neurotoxicity manifests as pareasthesias and dysesthesias in the extremities, triggered or enhanced by exposure to the cold. This neuropathy remains unexplained, is dose-limiting after low total cumulative doses of oxaliplatin, is always reversible, and does not require discontinuation of therapy. No relevant model has yet been determined for studying this type of neuropathy. Chronic and cumulative peripheral neurotoxicity presents as symptoms similar to those seen with cisplatin. It depends on the cumulative dose of oxaliplatin and generally occurs at total cumulative doses >600 mg/m^2 over four cycles or more of therapy [11]. This neurological toxicity is generally reversible, but may last for several months [1].

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The features of the peripheral neurotoxicity observed following the use of the platinum-based drugs can be studied with neurophysiological, pathological and analytical methods in several well-characterised animal models [12–15]. The neuropathy induced by the platinum derivatives in these models is characterised by a decrease in nerve conduction velocity induced by damage to neuronal cell bodies (decrease in somatic, nuclear and nucleolar area) and peripheral axonopathy. The major site of damage appears to be the dorsal root ganglia (DRG), a result which is consistent with the platinum accumulation studies in humans [16].

In preclinical studies, Holmes and colleagues [15] studied the neurotoxicity of oxaliplatin in a rat model. Up to now, however, no comparison of neurotoxicity between different schedules of oxaliplatin has been performed.

In our experiment, we used neurophysiological, pathological and analytical methods to compare the effect on the peripheral nervous system of different intervals between each single-dose administration and of increasing by more than 30% the cumulative dose of oxaliplatin with a fixed interval between administrations. Moreover, in the latter groups we also compared the effect of high-dose oxaliplatin treatment on the auditory pathway, which is damaged by treatment with cisplatin.

2. Materials and methods

2.1. Animal treatments

All animal procedures were performed according to the European Economic Community (EEC) Council directive 86/609. During the experiment, the animals were kept in individual cages, fed with commercial cubes and drinkable water *ad libitum* and maintained in an air-conditioned housing room with a 12 h light–dark cycle. A total of 50 female Wistar rats weighing 180–200 g on arrival at the housing room were used. By random selection they were divided into five groups (10 rats in each group) and treated as follows:

- group 1, oxaliplatin 4 mg/kg twice weekly (a total of nine intraperitoneal (i.p.) injections);
- group 2, oxaliplatin 2.4 mg/kg for 5 consecutive days every other week (15 i.p. injections);
- group 3, oxaliplatin 2.25 mg/kg every other day (16 i.p. injections);
- group 4, oxaliplatin 3 mg/kg every other day (16 i.p. injections);
- group 5: untreated controls.

The cumulative dose of oxaliplatin was 36 mg/kg in groups 1, 2 and 3, while it was 48 mg/kg in group 4.

The general condition of the animals was evaluated and recorded daily, and body weight was assessed twice a week. Oxaliplatin was dissolved in water to obtain a stock solution of 1 mg/ml in glucose solution (5 g/100 ml w/v) and this was aliquoted and kept frozen until use.

2.2. Neurophysiological examination of the tail nerve

At baseline, at the end of the treatment, and after a follow-up period of 5 weeks each animal underwent a determination of the antidromic sensory nerve conduction velocity (SNCV) in the tail. Details of the methods are reported elsewhere in Ref. [17]. Briefly, the antidromic nerve conduction in the tail nerve was assessed by placing recording ring electrodes distally in the tail, while the stimulating ring electrodes were placed 5 and 10 cm proximally with respect to the recording point. The latencies of the potentials recorded at the two sites after nerve stimulation were determined (peak-to-peak) and the nerve conduction velocity was calculated accordingly. All the neurophysiological determinations were performed under standard conditions in a temperature-controlled room adjacent to the animal housing room.

2.3. Pathological examination and morphometry

After the treatment period (i.e. after 5 weeks in each group), five animals from each group were sacrificed under xylazine/ketamine anaesthesia, while all the surviving animals in each group were sacrificed at the end of the follow-up period.

At each time point, left L4–L6 dorsal root ganglia (DRG) and left sciatic nerves of rats from each group were dissected out, fixed by immersion in glutaraldehyde solution (3 ml/100 ml v/v) in 0.12 M phosphate-buffered solution (PBS) [14] for 3 h, rinsed in 0.18 M PBS and osmicated according to previously reported protocols [14]. These specimens were resin embedded and used for light and electron microscope observations and for morphometry. Morphometric determinations of the cross-sectional area of the somata, nuclei and nucleoli of DRG neurones were performed on 1 μm thick semithin sections using an automatic image analyser (Tas Plus, Leica GmbH, Germany) [14]. The determination of the incidence of multinucleolated neurones and of eccentric nucleoli was also performed.

2.4. Determination of tissue platinum concentration

Ten DRG, the right sciatic nerve and kidney specimens were dissected out from each animal sacrificed, snap frozen in liquid nitrogen and stored at -80° C. These specimens were used for the analytical determination of tissue platinum concentration determined on pooled lumbar DRG specimens and on separate specimens of sciatic nerve and kidney with Inductively Coupled Mass Spectrometry (ICP-MS). Sample pre-treatment

consisted of acid digestion in a microwave oven followed by appropriate dilution [18].

2.5. Neurophysiological assessment of ototoxicity

The effect of oxaliplatin administration on the auditory system was assessed by means of electrocochleography (ECoG) performed in all the animals belonging to groups 3 and 4 before starting and immediately after oxaliplatin treatment. Animals were anaesthetised with xylazine/ketamine and ECoG recordings were performed after administration of an alternating click stimulus (intensity 80 dB SPL (decibel sound pressure level). AgCl electrodes were placed transtimpanically (active electrode), on the vertex (reference electrodes) and on the tail (ground) [19].

2.6. Statistical analysis

The differences in body weight, tail SNCV, morphometric data in DRG, tissue platinum concentrations and in ECoG results obtained in the five groups were statistically evaluated using the analysis of variance (ANOVA, Tukey–Kramer post-test) and a difference was considered significant if $P<0.05$.

3. Results

3.1. General toxicity

All the treated animals completed the treatment period of the study, while three animals died in the follow-up period (one in group 1, one in group 2 and one in group 4). All the surviving animals had only a mild reduction in motility during the treatment period with a rapid and complete recovery occurring in all the rats which did not develop any intraperitoneal fluid accumulation (see below) in the follow-up period.

Mean body weight changes in the treatment period are reported in Fig. 1. At the end of the treatment period, they evidenced the expected increase in control animals (group 5), while all the oxaliplatin-treated groups had a reduced increase (group 2) or even a decrease in the mean weight (groups 1, 3 and, in particular, group 4). The statistical comparison between groups evidenced significant differences between group 3 and controls ($P<0.05$) and between group 4 and controls ($P<0.001$). The difference between groups 2 and 4 was also significant ($P<0.01$). The sum of these data suggests that the schedule used in group 1 was the least toxic in terms of general side-effects, while that used in group 4 was the most toxic.

Abdominal bloating occurred in the follow-up period in several animals in all of the treated groups (see Table 1): this prevented a reliable measure of weight changes in the follow-up period in the oxaliplatin-treated rats. At necropsy, fibrino-purulent peritonitis was found, probably representing a local reaction to the oxaliplatin administration with a superimposed, secondary bacterial infection. Two rats in group 2 had alopecia after 7–8 oxaliplatin administrations, and this abnormal aspect was still present in the follow-up period.

3.2. Tail nerve neurophysiological evaluation

The determination of the tail SNCV (Fig. 2) was performed in all the treated animals and in controls at baseline, after treatment and at the follow-up examination. At baseline, no difference was found between the five groups of animals. On the contrary, after oxaliplatin administration, a significant difference was observed between the controls and all of the treated groups ($P<0.001$), although the most severe effect was observed in group 4. When the statistical intergroup comparison was performed between the oxaliplatin-treated groups, no significant difference was observed.

At the follow-up examination, initial recovery in the nerve conduction velocity was observed in all the treated animals, although the difference compared with the control group was still significant for all of the oxaliplatin-treated groups ($P<0.05$ for group 2, $P<0.01$ for groups 3 and 4, $P<0.001$ for group 1).

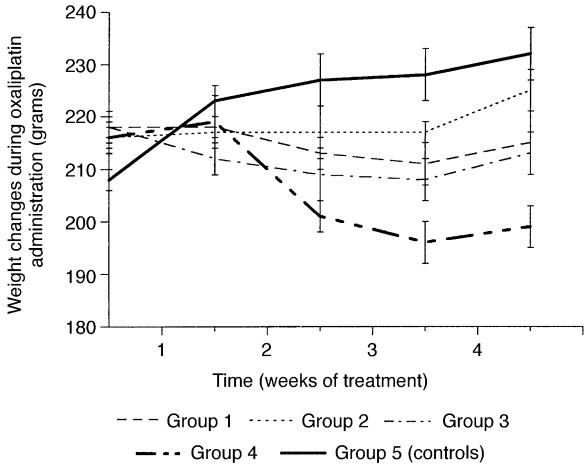

Fig. 1. Changes in body weight in the treatment period (mean ±standard deviation (S.D.)).

Table 1
General toxicity of oxaliplatin treatment

	Deaths	Abdominal bloating		Alopecia
		Mild	Severe	
Group 1	1/10	2/9	1/9	0/10
Group 2	1/10	1/9	2/9	2/10
Group 3	0/10	1/10	2/10	0/10
Group 4	1/10	1/9	1/9	0/10
Group 5 (controls)	0/10	0/10	0/10	0/10

3.3. Pathological examination

3.3.1. Morphometry under the light microscope

Under the light microscope, the DRG were smaller in size, but there was no other evidence of cell damage present. Similarly, the satellite cells had a normal appearance.

The morphometric determinations performed on the DRG neurones obtained from all of the animals in each group after treatment and at the follow-up examination are reported in Table 2.

At the morphometric examination performed after treatment, a significant difference in the somatic area between controls and all the oxaliplatin groups was observed. When we compared the oxaliplatin-treated groups, the only intergroup difference was observed between groups 2 and 4. A significant difference between controls and all the oxaliplatin groups was also present in the nuclear and nucleolar area. At this time of examination, the overall comparison between the oxaliplatin groups in the nuclear and nucleolar area showed a significant difference between groups 1 and 2 in comparison with groups 3 and 4. On the contrary, at the follow-up examination no significant differences were observed between the control and oxaliplatin-treated groups.

Table 2 also reports the incidence of eccentric nucleoli and of multinucleolated neurones in the five groups of animals after treatment and at the follow-up examination. Only a mild increase in the percentage of DRG neurones with eccentric nucleoli was observed after treatment, while the increase induced by oxaliplatin in the occurrence of multinucleolated neurones was marked in comparison to controls, particularly in groups 2 and 4 (Fig. 3). At the follow-up examination, no differences could be observed between the control and the oxaliplatin-treated groups in the incidence of eccentric nucleoli, while a tendency to an increased incidence of multinucleolated neurones was still present in the oxaliplatin-treated groups (particularly in groups 2 and 3).

3.3.2. Electron microscopy

The electron microscopic examination performed on the DRG specimens obtained from the oxaliplatin-treated rats immediately after treatment demonstrated the occurrence of nucleolar segregation in several neurone nucleoli, while cytoplasmic changes were absent. However, the overall severity of the pathological aspect observed was not severe and no clear-cut differences could be established between the various oxaliplatin-treated groups. In the satellite cells only very mild chromatin thinning was observed in all of the oxaliplatin groups.

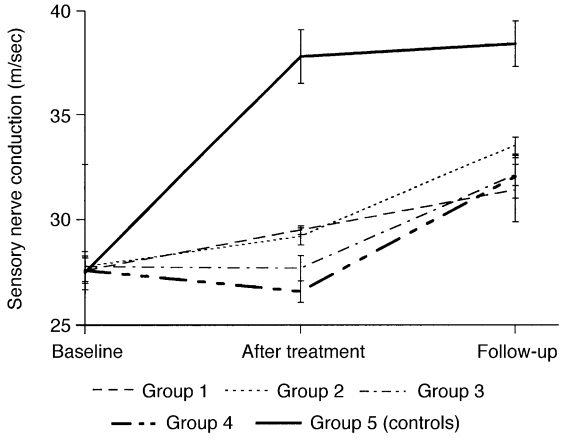

Fig. 2. Tail nerve conduction velocity along the entire experiment (mean ± standard deviation (S.D.)).

Table 2
Morphometric determinations performed on DRG ganglia neurones

	Soma[a] (μm²)	Nucleus[a] (μm²)	Nucleolus[a] (μm²)	Eccentric nucleoli (%)	Multinucleolated neurones (%)
After treatment					
Group 1	780.3* (19.1)	116.6 */‡/§ (2.4)	9.2 */¶ (0.2)	35.7	8.9
Group 2	813.0 **/† (22.2)	113.2 */†/‡ (2.7)	9.3 */¶ (0.2)	46.1	13.5
Group 3	735.5 * (18.4)	95.6 * (2.2)	7.2 * (0.2)	47.0	9.8
Group 4	699.6 * (16.7)	100.2 * (2.2)	7.0 * (0.1)	54.9	12.5
Group 5 (controls)	901.5 (26.0)	132.4 (3.0)	10.7 (0.3)	31.1	3.6
Follow-up					
Group 1	897.6 (27.1)	135.4 (3.2)	11.0 (0.3)	34.4	4.6
Group 2	878.5 (26.7)	131.1 (3.1)	10.9 (0.3)	35.8	6.5
Group 3	900.9 (27.5)	134.8 (3.1)	11.2 (0.3)	35.9	5.8
Group 4	850.8 (26.7)	130.0 (3.1)	10.8 (0.3)	34.1	4.9
Group 5 (controls)	913.3 (27.6)	135.1 (3.2)	11.5 (0.3)	30.7	3.9

DRG, dorsal root ganglia. *$P<$ 0.001 vs. controls; **$P<0.05$ vs. controls; †$P<0.01$ vs. group 4; ‡$P<0.001$ vs. group 3; §<0.05 vs group 4; ¶$P<0.001$ vs groups 3 and 4.

[a] Mean value (standard error of the mean (S.E.M.)).

The DRG neurones and satellite cells were normal in the specimens obtained at the follow-up examination.

The changes observed in the sciatic nerve after oxaliplatin treatment were even milder than in the DRG: rare axons undergoing axonal degeneration were observed in transverse sections in all of the treated groups, while the myelin had a normal aspect. No endoneural vessel changes were seen and the endoneural space had a normal appearance.

In the specimens obtained after the follow-up period, some degenerating axons were still observed in all of the oxaliplatin-treated groups. No other pathological aspects were present.

3.4. Determination of the tissue platinum concentration

Tissue platinum concentration was measured on pooled specimens of DRG and on individual specimens of sciatic nerve and kidney obtained immediately after treatment and after the follow-up period. The results of these determinations obtained in the oxaliplatin-treated rats are reported in Table 3. Platinum concentration was always below the detection limit of the ICP-MS method (i.e. <0.001 μg/gram of tissue) in the control specimens, while it was detectable in all of the specimens obtained from the oxaliplatin-treated rats.

Platinum levels were higher in all of the tissues examined immediately after treatment than at the examination performed after the follow-up period. The kidney was the site where platinum reached the highest concentration immediately after administration and also after the follow-up. No significant differences were observed at any of these observation times between the oxaliplatin-treated groups. The amount of platinum detected in the pooled DRG samples was also very similar in the treated groups, and the concentration decreased in the DRG after the follow-up period. The only tissue where a significant intergroup difference in the platinum concentration was observed after oxaliplatin treatment was the sciatic nerve, where the measurement of the highest levels of platinum gave further evidence of the fact that the schedule used in group 4 was more toxic than those used in groups 1 and 2. However, this significant difference between groups 1 and 4 ($P<0.05$) and between groups 2 and 4 ($P<0.05$) was evident only immediately after treatment and was no longer present after the follow-up period.

Table 3
Tissue platinum concentrations (μg/gram of tissue)

	DRG[a]	Sciatic nerve[b]	Kidney[b]
After treatment			
Group 1	2.63	1.36 (0.07)	10.79 (1.87)
Group 2	2.00	1.05 (0.08)	9.03 (0.47)
Group 3	3.16	1.52 (0.09)	12.03 (1.36)
Group 4	2.18	1.64 (0.18)	8.29 (0.71)
Group 5 (controls)	nd	nd	nd
Follow-up			
Group 1	0.65	86 (0.13)	5.9 (1.23)
Group 2	0.82	88 (0.09)	6.01 (1.23)
Group 3	0.66	88 (0.20)	6.01 (0.33)
Group 4	0.97	1.35 (1.8)	4.45 (0.57)
Group 5 (controls)	nd	nd	nd

nd, not determined; DRG, dorsal root ganglia.
[a] Pooled data.
[b] Mean value standard error of the mean (S.E.M.)).

3.5. Audiological evaluation

The results obtained in the ECoG determinations performed in groups 3 and 4 did not evidence any significant difference between the controls and oxaliplatin-treated rats in the latency of the N1 and N2 waves which represent the cochlear evoked potential. Accordingly, the morphology of the cochlear potentials was normal. Our results, therefore, indicate that oxaliplatin was not ototoxic in this experimental setting.

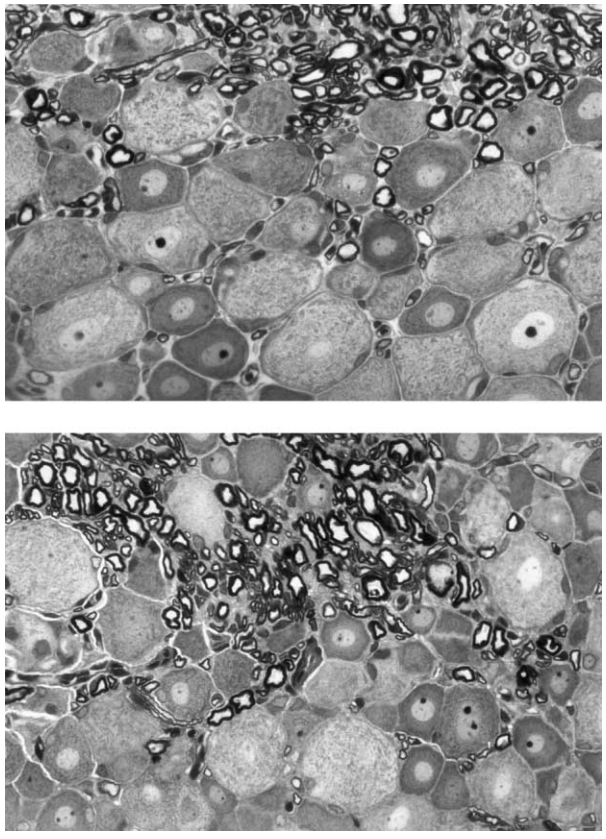

Fig. 3. Light micrographs of dorsal root ganglia (DRG) from a control (top) and from an oxaliplatin-treated rat (group 2, bottom) sacrificed immediately after treatment, showing neurones with eccentric nucleoli and multinucleolated neurones (original magnification 40×).

4. Discussion

Our experiment was designed to evaluate how the treatment schedule and the total dose of oxaliplatin influence the development of peripheral neurotoxicity in an animal model.

The general toxicity observed in the oxaliplatin-treated rats during the administration period was acceptable, although a decrease in weight gain was observed in all the animals belonging to the treated groups compared with controls. In the follow-up period, on the contrary, peritonitis with abdominal bloating frequently occurred in the treated rats. It was remarkable, however, that most of the animals which showed abdominal bloating did not evidence other signs of illness and behaved normally. Concerning the peripheral neurotoxicity of oxaliplatin, our results confirmed that this drug was neurotoxic, but indicated that the peripheral damage tended to be reversible with all the schedules used in our experimental setting. The temporal course observed in the peripheral neurotoxicity in our models, therefore, closely resembled the course observed in patients treated with oxaliplatin. Moreover, our study, which included for the first time a detailed pathological examination of the DRG and sciatic nerves after treatment with different schedules of oxaliplatin, demonstrated that the primary target of oxaliplatin was represented by the DRG ganglia, while the secondary changes in the sciatic nerve were rather mild and they likely occurred secondarily to the DRG damage. These changes were very similar to those already reported in similar animal models obtained after the administration of different platinum-derived drugs [12–15,20].

The overall evaluation of the pathological changes demonstrated a more severe involvement in groups 3 and 4, the groups treated with the shorter interval between each administration and with the highest cumulative dose (group 4). Although the pathological changes observed in the sciatic nerve were rather mild in all the treated groups, the oxaliplatin schedules induced a significant SNCV decrease when compared with the controls immediately after treatment. It is very unlikely that the obvious changes in weight gain which can be observed in our study are responsible for the concurrent neurophysiological modifications. In fact, the very careful paper by Cornblath and Brown [21] clearly demonstrated the lack of any relationship between reduced weight gain and peripheral nerve changes in developing animals. Moreover, in our experiment there is also no direct relationship between the weight changes and the neurophysiological modifications after treatment. This is clear, for instance, when evaluating the results obtained in group 2 versus group 4, where despite a significant ($P<0.01$) difference in weight there is no difference in the nerve conduction velocity.

At the follow-up morphometric examination, we did not observe any significant difference in the DRG morphometric parameters between controls and any of the oxaliplatin groups, a finding which is in agreement with the evolution of the neurophysiological SNCV data which showed initial recovery from drug-induced damage. However, a longer observation time would have been necessary in order to establish the actual extent of the recovery in the peripheral nerve conduction functioning. This observation was in keeping with the fact that, even at the follow-up examination, platinum was still detected in all of the DRG and sciatic nerve specimens obtained from the treated rats, a feature which might affect the possibility of a complete recovery.

Although our analytical data on DRG were very similar to those reported by Holmes and colleagues [15] in an experiment performed using only a schedule identical to that used in group 1 of our study, there was a discrepancy between the morphometric results obtained in the two experiments. The major difference was that Holmes and colleagues [5] reported that morphometric evidence of oxaliplatin-induced damage of the DRG was still present 8 weeks after drug discontinuation, while we observed complete recovery after a follow-up of 5 weeks. A possible explanation for this difference might be represented by different male-to-female susceptibility to the oxaliplatin toxic effect. In fact, in the study by Holmes and colleagues [15] the mortality was much higher than in any of our oxaliplatin-treated groups, and this was particularly so for our group 1 which was treated with the same schedule (6 out of 10 male animals treated versus 1 out of 10 females in group 1 in our study). Similarly, severe abdominal bloating also occurred in all the male animals, while it was observed in only 3 out of the 9 surviving females belonging to group 1 in our study. Therefore, their observations on DRG damage performed 8 weeks after drug treatment withdrawal might have been magnified by these general toxic side-effects.

The overall evaluation of the pathological and neurophysiological results obtained after the comparison of different schedules of administration indicated that the peripheral neurotoxicity of oxaliplatin depended primarily on the cumulative dose administered and on the recovery time allowed between the single doses (the shorter the recovery time, the higher the severity of the peripheral neurotoxicity). This was also confirmed by the observation of a significantly higher concentration of platinum in the sciatic nerve in the group 4 animals which were treated with the highest cumulative dose of oxaliplatin and which had the shortest recovery time between single administrations.

The auditory pathway of the rats belonging to the two groups (i.e. groups 3 and 4), which were the most severely affected as far as the peripheral nervous system

is concerned, was not impaired even at the highest cumulative dose of oxaliplatin administered. These results were obtained with a non-invasive method, i.e. ECoG, which is a reliable neurophysiological technique used to assess the occurrence of ototoxicity, and were in agreement with the clinical data in humans.

In conclusion, oxaliplatin primarily damaged the DRG, where significant concentrations of platinum could be detected immediately after treatment, but also after a follow-up period of several weeks, and secondarily in the peripheral nerves. The nucleolus was the subcellular structure which showed the most prominent pathological changes. However, these changes were reversible as demonstrated by the morphometric follow-up and confirmed by the neurophysiological evidence that the severity of the drug-induced impairment decreased after drug withdrawal. Finally, no clinically-evident impairment in motor activity was induced by oxaliplatin treatment. The severity of the neurotoxic effects of oxaliplatin depended directly on the cumulative dose of the drug and were inversely correlated to the time between each drug administration.

The results of our study may be useful in order to plan future studies with oxaliplatin and suggest that, a long-term follow-up period must be included in the experimental design of the schedule. Finally, the use of a different method of administration (i.e. intravenously) should be considered also for oxaliplatin models in order avoid the local toxic effect of this drug.

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