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Systemic administration of doxorubicin impairs aversively motivated memory in rats

Pedro Emanuel Rubini Liedke [a,b,*], Gustavo Kellermann Reolon [c,d], Bruno Kilpp [c,d], Algemir Lunardi Brunetto [d,e,f], Rafael Roesler [c,d], Gilberto Schwartsmann [a,b,d,g]

[a] Programa de Pós-Graduação em Ciências Médicas, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcellos, 2400, 2° andar, 90035-903, Porto Alegre, Brazil
[b] Serviço de Oncologia do Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, zona 11, 90035-903, Porto Alegre, RS, Brazil
[c] Laboratório de Neurofarmacologia Molecular, Departamento de Farmacologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500, 90046-900, Porto Alegre, RS, Brazil
[d] Laboratório de Pesquisas em Câncer, Centro de Pesquisas, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2350, 90035-903, Porto Alegre, Brazil
[e] Instituto do Câncer Infantil do Rio Grande do Sul, Rua Ramiro Barcelos, 2350, 3° andar leste, 90035-903, Porto Alegre, RS, Brazil
[f] Serviço de Oncologia Pediátrica, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2350, 3° andar leste, 90035-903, Porto Alegre, Brazil
[g] Departamento de Medicina Interna, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2350, sala 700, 90035-903,Porto Alegre, Brazil

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ABSTRACT

There is growing clinical evidence of cognitive impairment in cancer patients treated with chemotherapy, especially in women treated with drug combinations for breast cancer. Clinical studies have a difficult task of defining which drugs individually are responsible for the cognitive changes and published papers evaluating single agents in experimental models are scanty. In the present study we have investigated the effect of single escalating doses of doxorubicin (DOX) on memory for inhibitory avoidance conditioning (IA) in rats. The doses used were comparable to those applied in the clinic. When given systemically before training, higher doses of DOX impaired IA memory retention measured 24 h and 7 days, but not 3 h after training. DOX did not affect IA retention when given either before or after training in a multiple-trial IA training protocol. Control experiments showed that DOX produced a decrease in exploratory behavior assessed by the number of rearings performed during exploration of an open field. The results indicate that a single systemic administration of DOX might impair long-term aversive learning.

1. Introduction

Cognitive impairment has been described in patients receiving combination chemotherapy, especially in women with breast cancer (Ahles et al., 2002; Brezden et al., 2000; Falleti et al., 2005; Fan et al., 2005; Schagen et al., 2001; Silverman et al., 2006; Tchen et al., 2003; Van Dam et al., 1998). Acute cognitive changes during chemotherapy are common and a subgroup of patients will remain with long term post-treatment cognitive impairment (Ahles and Saykin, 2007). These effects of chemotherapy are commonly referred to as "chemo-brain" or "chemo-fog" (Tannock et al., 2004).

Although the actual mechanisms of cognitive impairment are unknown, many hypothesis have been postulated, such as reductions in estrogen and testosterone levels, rupture of the blood-brain barrier, cytokine deregulation, DNA damage, telomere shortening and genetic susceptibility (Ahles and Saykin, 2007). Since these possible mechanisms are difficult to evaluate clinically, there has been a growing interest in investigation through animal models of cognitive performance (Lee et al., 2006; MacLeod et al., 2007; Reiriz et al., 2006; Seigers et al., 2008; Winocur et al., 2006). These experimental studies have used rodent models, in which animals were exposed to single or multiple administrations of cytotoxic drugs. Learning capacity of these animals after chemotherapy has been tested through the use of the Morris water maze (Lee et al., 2006; Seigers et al., 2008; Winocur et al., 2006), Stone 14-unit T-maze (Lee et al., 2006), inhibitory avoidance (IA) (Reiriz et al., 2006) and fear conditioning (MacLeod et al., 2007). Few drugs have been studied in these experiments, mostly combinations that included 5-fluorouracil (Lee et al., 2006; Winocur et al., 2006), methotrexate (Seigers et al., 2008; Winocur et al., 2006) and cyclophosphamide (MacLeod et al., 2007). Doxorubicin (DOX) was evaluated in only one study and in combination with cyclophosphamide (MacLeod et al., 2007). In our laboratory, a memory impairment was demonstrated in male rats with the administration of single-agent cyclophosphamide in rats (Reiriz et al., 2006).

* Corresponding author. Serviço de Oncologia do Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, zona 11, 90035-903, Porto Alegre, RS, Brazil. Tel.: +55 51 3359 8335; fax: +55 51 3388 2877.
E-mail address: pedroliedke@hotmail.com (P.E.R. Liedke).

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Since cancer patients are usually treated with combination chemotherapy, clinical studies are rarely capable to define the contribution of each individual drug in the induction of cognitive changes. In this study, we investigated the effect of DOX, one of the most commonly used chemotherapeutic agents in the clinic, in a rodent model of emotionally motivated memory.

2. Methods

2.1. Animals

One hundred and twelve adult male Wistar rats (2 to 3 months old; 180–350 g at the time of the experiment) obtained from the Department of Pharmacology of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil, were housed five to a cage with food and water available ad libitum, and maintained on a 12 h light/dark cycle. Behavioral procedures were performed during light hours. All experimental procedures were approved by the institutional animal care committee and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

2.2. Experimental groups

Four distinct experiments were done with four different groups of animals: In the first experiment, animals received DOX or vehicle systemically before single IA training and were tested 3 h, 24 h and 7 days after training. In order to control for permanent impairments induced by the drug treatment, animals were retrained immediately after the 7-day retention test trial and tested 3 h and 24 h later. In the second experiment, animals received DOX or vehicle systemically after single IA training and were tested 3 h, 24 h and 7 days after training. In the third experiment, animals received DOX or vehicle systemically before multi-trial IA training and tested 24 h later. Group 4, animals received DOX or vehicle systemically before open field and 24 h later were re-exposed to the same open field arena.

2.3. Behavioral procedures

We used the single-trial step-down inhibitory avoidance (IA) conditioning as an established model of fear-motivated, hippocampus-dependent memory (Izquierdo and Medina, 1997; Taubenfeld et al., 1999). In IA training, animals learn to associate a location in the training apparatus with an aversive stimulus (footshock). The IA behavioral training and retention test procedures were described in previous reports (Quevedo et al., 2004). The IA apparatus was a 50 cm × 25 cm × 25 cm acrylic box (Albarsch, Porto Alegre) whose floor consisted of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7 cm wide, 2.5 cm high platform was placed on the floor of the box against the left wall. On the training trial, rats were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, rats received a 0.5 mA, 2.0 s foot shock and were removed from the apparatus immediately after the foot shock. The retention test trials were procedurally identical to training, except that no foot shock was presented. Step-down latencies on the retention test trial (maximum 180 s) were used as a measure of IA retention.

Multi-trial repeated acquisition (Bianchin et al., 2000) was done in the same IA apparatus described above. Animals were placed in the platform and received 0.5 mA, 2.0 s foot shock immediately after stepping down. Animals were then once more placed on the platform and the foot shock was once again administered if the animal stepped down on the metal grid. This procedure was repeated until the animals remained in the platform for 180 s.

Open field behavior was performed as previously described (Picada et al., 2002) to examine possible drug-induced alterations in locomotor activity, exploration, and anxiety (Henderson et al., 2004). The open-field was a 20 × 30 cm arena, surrounded by 50 cm high walls, made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal squares by black lines. Rats were put in the apparatus, placed on its left rear quadrant, and left to freely explore the arena for 5 min. Crossings of the black lines, rearings performed, latency to start locomotion, and the number of fecal bolus during exploration were counted. The number of crossings was used as a measure of locomotor activity, the number of rearings as a measure of exploratory behavior, and the latency to initiate locomotion and the number of fecal bolus were used as measures of anxiety. Animals were re-exposed to the same environment 24 h after the first evaluation.

2.4. Drug treatment

Animals received DOX (Zodiac, Brazil; Bergamo, Brazil) by intraperitoneal route in one bolus dose. The reference dose of 8 mg/kg was chosen because it is roughly equivalent to the human dose of 60 mg/m^2 (Food and Drug Administration, 2007). Two other lower dosages were used: 0.5 mg/kg and 2 mg/kg to determine a possible dosage response curve. When used in pre-training IA experiment, the drug was administered between 20 and 30 min before training. When used in the pre-test trial, the drug was administered between 20 and 30 min before the 3 h memory retention trial. In the multi-trial repeated acquisition, DOX was administered before training, as in the pre-training experiment. In the open-field task, the animals also received the drug 20 min before exploratory behavior evaluation. Control animals received sodium chloride 0.9% in the same manner as DOX.

2.5. Statistical analysis

All data are expressed as means ± standard error (SE). Comparisons between multiple dosage groups and controls were done using Kruskal–Wallis analysis of variance, followed, when necessary, by two-tailed Mann–Whitney *U*-test. Comparisons between groups in the multi-trial repeated acquisition were done by two-tailed Mann–Whitney *U*-test. Open-field results were analyzed by Student's *T* test for independent groups. All results were considered statistically significant when $p < 0.05$.

3. Results

The results of the pre-training IA trial can be seen in Fig. 1. There was no difference between groups in the 3 h retention test ($p = 0.14$). In the 24 h retention test there was a statistically significant difference between controls and the 2.0 mg/kg and 8.0 mg/kg dosage groups ($p = 0.022$ and $p = 0.004$, respectively). This difference was also seen 7 days after training ($p = 0.01$ and $p = 0.004$, for 2.0 mg/kg and 8.0 mg/kg groups, respectively). No difference was observed for the 0.5 mg/kg group in these trials ($p = 0.08$ and $p = 0.16$, respectively). There was also no difference between groups during training and in the retraining trial (data not shown). In the pre-testing trial there were no differences between groups during training and in the 3 h, 24 h and 7 day tests (Fig. 2).

Multi-trial repeated acquisition was done only with the 8 mg/kg dosage ($n = 9$) and placebo ($n = 9$). There were no differences in comparison with controls in respect to latency of the first step-down from the platform ($p = 0.76$), the number of trainings ($p = 0.29$) or retention testing after 24 h ($p = 0.45$).

The open-field trial was also performed only with the 8 mg/kg dosage and placebo. There was no difference between this group and the control in the training with respect to latency to initiate locomotion, the number of crossings and rearings and the number

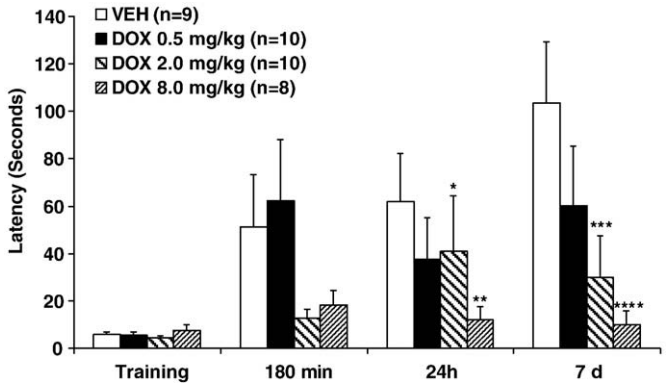

Fig. 1. Pre-training drug injection demonstrating differences in latency to descend from platform in the 24 h and 7 day posttraining IA retention tests. Differences were observed only for the higher dose concentrations when compared to controls. Vehicle (VEH); Doxorubicin (DOX). * $p = 0.022$; ** $p = 0.004$; *** $p = 0.01$; **** $p = 0.004$.

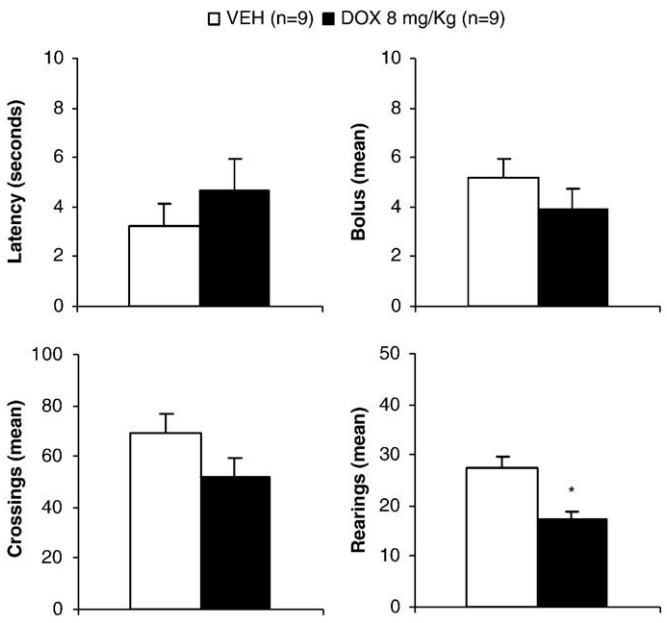

Fig. 3. Open field behavior 24 h after drug injection. There was no difference between groups in the latency to initiate locomotion, the number of crossings and the number of fecal bolus, only for the number of rearings. Vehicle (VEH); Doxorubicin (DOX). *$p = 0.004$ compared to control.

of fecal bolus (data not shown). Twenty four hours after the first exposure to the open-field environment there was a difference between the groups in respect to the number of rearings ($p = 0.004$) with the mean number of rearings being smaller in the DOX group (Fig. 3). There was no difference in respect to latency to initiate locomotion, crossings or fecal bolus.

There were no significant changes between groups in weight 7 days after injection, although animals that received 8 mg/kg of DOX had a trend of losing more weight (data not shown).

4. Discussion

We have conducted an experimental trial in a rodent model of IA, an emotionally motivated memory dependent on the hippocampus and amygdala. In this experiment administration of a single dose of DOX before training impaired the animals' learning capacity 1 and 7 days after training, while the results of testing 3 h after training were not altered in comparison to the controls. The long term memory effect was observed in the groups that received 2 mg/kg and 8 mg/kg, but not in the group that received 0.5 mg/kg of the drug suggesting a dose dependent response. When these animals were retrained 1 week after the administration of DOX they were able to learn not to descend from the platform. The ability of retaining memory 3 h but not 24 h and 7 days after training suggests that DOX interfered with long-term memory, sparing short term memory. This effect was reversible, since animals were able to learn one week after administration, indicating that a single dose of DOX does not cause permanent damage. When the animals were exposed to multiple training there was no effect of the drug on long-term memory, indicating that exposure to multiple stimuli overcame the inhibitory effect of a single dose of DOX in memory acquisition. No effect was observed when the drug was administered pre-testing, independently of the dosage used. This suggests that there is no interference of a single dose of DOX in short or long-term memory consolidation.

When the animals were evaluated in the open field 20 min after DOX, no difference in comparison to controls was observed in the first day in respect to latency to start locomotion, crossings, rearings and number of fecal bolus, which indicates no differences in anxiety, locomotive capacity and exploratory behavior. When tested 24 h after receiving DOX the only difference observed was in respect to the number of rearings, more common in the saline group, suggesting that DOX interfered with the exploratory behavior. This effect, however, corroborates the results of the pre-training IA experiment, since even having a decreased exploratory behavior the animals that received DOX still had a lower latency do descend from the platform onto the electrified grid in comparison to controls.

Other animal models have been used to investigate the effect of chemotherapeutics in memory and learning capacity. Winocur et al. (2006) have tested the combination of 5-fluorouracil and methotrexate administered in weekly doses, during three consecutive weeks. The results of this study demonstrated that the combination of 5-fluorouracil and methotrexate caused deficits in spatial memory, in conditional rule learning on the non-mach to sample test, and on the longest delay on the delayed non-mach to sample test. These results suggest alteration in hippocampal and frontal lobe function after using the combination of these drugs. Although in our experiment we used only a single dose of DOX, our results also suggest that there was impairment of hippocampal function, possibly by a similar mechanism of alteration of memory acquisition. In their study, Winocur et al. discuss that the possible effects of the drugs on the spatial memory test were due to performance-related variables. We controlled our experiment using the open-field for evaluation of anxiety and exploratory behavior and found a difference in exploratory behavior for the drug group 1 day after administration. In contrast to the use of the Morris water maze, where the animals have to actively find the

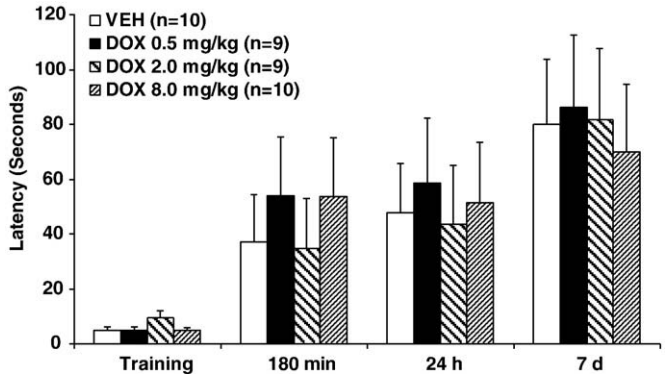

Fig. 2. Pre-testing drug injection. There was no difference between groups in latency do descend from platform in the IA retention test. Vehicle (VEH); Doxorubicin (DOX).

safety of the hidden platform, in IA the animals are placed directly on the safe platform and, driven by their natural behavior, tend to explore the environment. In this case, we would expect that animals with lower exploratory behavior would have a longer latency to descend onto the electrified grid. This, however, was not the case in our experiment, and even with lower exploratory behavior the latency of animals that received DOX was lower than that of controls, suggesting that these animals learned not to descend, while those of the drug group did not.

Methotrexate was also tested by Seigers et al. (2008). These authors used a high dose of methotrexate and found that the treated animals had a longer latency to cross the platform area in the Morris water maze test. Animals that received chemotherapy also showed a different response in the novel object recognition.

In another study by our group, Reiriz et al. (2006) performed a similar approach as in this study using cyclophosphamide. They also found memory impairment one day after training in the IA apparatus, although no effect was seen one week after. In this experiment no differences were seen on the open-field evaluation.

Lee et al. (2006) examined the effects of cyclophosphamide or 5-fluorouracil in 7 and 18 month old rats after administration every 4 weeks for 12 weeks, followed by a last administration after 6 weeks. Testing in this study was conducted 7 weeks after recovery from the last drug administration. Interestingly, and on the contrary of the above, there was an enhanced performance in the young female rats in the Stone 14-unit T-maze and in the Morris Water maze. This effect was no longer seen 42 weeks after recovery. Amongst the older rats no differences were observed. In this study synaptic plasticity of hippocampal slices of animals receiving cyclophosphamide was also analyzed and a decreased LTP was observed after four cyclophosphamide cycles in comparison to controls. In contrast, a higher LTP was seen 8 weeks after the last cycle and remained enhanced, although less in magnitude, 53 weeks after the last cycle. The contrasting results of this study might be explained by the moment in which the animals were tested, since Lee et al. (2006) waited 7 weeks to start testing, while our experiment and the other studies mentioned above started testing within a week after chemotherapy. This would be in accordance to Lee's finding of an initially decreased LTP, followed by an enhancement 8 weeks after chemotherapy.

In a study by MacLeod et al. (2007), a combination of DOX and cyclophosphamide was used to test the response to fear conditioning of female ovariectomized rats. In their experiment, rats treated with chemotherapy had a diminished response to contextual fear, but not in the cued memory test one week after the last of three doses of active drugs. Contextual fear resembles the testing of IA and their results are similar to ours and reinforce the occurrence of hippocampal dysfunction caused by chemotherapy. We also found no difference when animals were retrained one week after chemotherapy (Fig. 2).

Interestingly, our study did not show influence of DOX on memory after training, which indicates that this drug, on a single time point, does not influence memory consolidation, only acquisition. To our knowledge, no other studies investigating the effects of chemotherapeutics on memory consolidation have been published to date.

Although DOX is known not to penetrate the blood-brain barrier (BBB), part of its cytotoxic effects are mediated by free radical formation, lipid peroxidation, chelation of iron and formation of reactive oxygen species, resulting in oxidative stress (Danesi et al., 2002). Alteration in hippocampal oxidative stress has been related to memory impairment cased by other drugs such as haloperidol and clozapine (Schroder et al., 2005) and toxins such as microcystines (Maidana et al., 2006), thus reactive oxygen species and induction of oxidative stress might be an indirect mechanism through which DOX causes memory impairment. This will be subject of further studies by our group.

In conclusion, we have demonstrated in this experimental model that DOX in a single administration, in doses equivalent to those applied in the clinic, produces a reversible impairment in memory acquisition, but not in memory consolidation in Wistar rats.

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