Cognitive impairment induced by methotrexate is not long-lasting in rats

Riejanne Seigers, Sanne B. Schagen, Frits S.A.M. van Dam, Jaap M. Koolhaas, Bauke Buwalda

1 Department of Behavioral Physiology, University of Groningen
2 Department of Psychosocial Research and Epidemiology, Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, Amsterdam
Abstract

Methotrexate (MTX) is a cytostatic drug applied in chemotherapy that has been associated with cognitive impairment as a long-term side effect. Since we have shown in previous studies that methotrexate induces cognitive impairment till one month after treatment, we now explored the hypothesis that this deficit can also be noticed on even longer terms. Male Wistar rats were treated with either saline or MTX and cognitive behavior was tested six months after treatment. No differences in learning behavior were seen in the novel object recognition task, Morris water maze, or shock avoidance. In the elevated plus maze, animals treated with MTX spent less time in the open arms compared to control animals, indicating that the animals treated with MTX were more anxious.

These findings indicate that rats, in contrast to human patients, show functional recovery from methotrexate induced cognitive deficits after longer time intervals. The results in this paper do not suggest that rodent models are not suitable to test cognitive behavior after cytostatic treatment, but that more attention should be paid to physiological differences between rodents and humans when designing experiments.

Introduction

Cognitive impairment is a long-term side effect of adjuvant chemotherapy noticed in a subgroup of cancer patients. The deficits range from subtle to more severe and are mostly noticed in memory, processing speed, and more complex aspects of attention (Correa and Ahles, 2008). This cognitive impairment can be noticed within months after the treatment and last up to ten years (Ahles et al., 2002). Several clinical studies have been performed to study the impairment after chemotherapy (Bender et al., 2006; Correa and Ahles, 2008; Wefel et al., 2008), but the underlying mechanism is not clear.

Methotrexate (MTX) is a cytostatic agent used in chemotherapy cocktails for breast cancer and is associated with cognitive impairment (Bender et al., 2006; Correa and Ahles, 2008; Wefel et al., 2008). A number of animal studies have been carried out to explore the effect of MTX on learning and memory (Foley et al., 2008; Madhyastha et al., 2002; Seigers et al., 2008; Seigers et al., 2009; Siecklucka-Dziuba et al., 1998; Stock et al., 1995; Winocur et al., 2006; Yanovski et al., 1989). In these studies, animals were treated with MTX and subsequently behaviorally tested with time periods ranging from 30 minutes up till 12 weeks after administration.

Since cognitive impairment can be noticed in cancer patients up to years after treatment with adjuvant chemotherapy (Bender et al., 2006; Correa and Ahles, 2008; Wefel et al., 2008), we hypothesized that cognitive impairment is still visible or might even be increased after longer time intervals following cytostatic treatment in a rodent model. Therefore, in this experiment, rats were treated with a single, high dose of MTX and behavioral tasks were performed six months after treatment. Three behavioral tasks, the novel object recognition task, the Morris water maze, and contextual fear conditioning, were chosen since previous studies have shown that animals treated with MTX have impaired learning behavior in these tasks (Seigers et al., 2008; Seigers et al., 2009). An additional task, the elevated plus maze, was added to the protocol to see if MTX affected explorative and anxiety behavior. As a control experiment, a subgroup of the animals received a novel object recognition task two weeks after treatment since we have already shown that cognition is affected at this time point after treatment with MTX (Seigers et al., 2008).
Methods

General method and procedure
Adult (3 months of age, n = 32) male Wistar rats (Harlan, Zeist, the Netherlands, average body weight at the start of the experiment 346 gram ± 3.0 SEM) were individually housed in clear Plexiglas cages (25 x 25 x 30 cm) on a layer of wood shavings with a fixed 12:12h light:dark cycle (with lights on at 08.00 a.m.) and food and water ad libitum. The experiment started 2 weeks after arrival of the animals according to the protocol described below. The experiment was approved by the Animal Experimentation Committee of the University of Groningen.

Adjuvant chemotherapy in the CMF cocktail (cyclophosphamide 100 mg/m² orally on days 1 and 14, methotrexate 40 mg/m² intravenously on days 1 and 8, and 5-fluorouracil 600 mg/m² intravenously on days 1 and 8) induces a body weight loss of approximately 10% and mild diarrhea in patients (Flombaum and Meyers, 1999). In order to achieve similar effects in animals and based on previous studies performed in our lab (Seigers et al, 2008; Seigers et al, 2009), rats were injected with 250 mg/kg MTX (100 mg/ml, Pharmachemie BV, Haarlem, the Netherlands) via the tail vein under a short-lasting (< 3 minutes) mild O₂-isoflurane anesthesia. Control animals were injected with saline according to the same procedure. After injection with MTX, animals received intraperitoneal injections of calcium leucovorin (10 mg/ml, Pharmachemie BV, Haarlem, the Netherlands), which is clinically used as a so-called rescue therapy in combination with the cytotoxic agent. Pilot studies showed that high-dose MTX without leucovorin is lethal, due to severe diarrhea and weight loss. This rescue therapy is based on the fact that leucovorin is a tetrahydrofolate (THFA) that does not require activation by THFA reductase. Tetrahydrofolate is a cofactor in DNA synthesis; MTX is an inhibitor of the enzyme THFA reductase and depletes the pool of tetrahydrofolates (Genestier et al, 2000; Huennekens, 1994). The rescue therapy of leucovorin was administered in a protocol similar to the application in patients. Eighteen hours after the injection of MTX, leucovorin was administered in a concentration that was 8% of the injected MTX dosage (20 mg/kg); at 26, 42, and 50 hours the administered concentration was reduced to 4% (10 mg/kg).

Behavioral tasks
Sixteen animals received saline and 16 animals received MTX as previously described. Eight animals per group were exposed to a novel object recognition task 2 weeks after treatment, as a control experiment in order to repeat previously found data (Seigers et al, 2008). Long-term changes in cognitive behavior were tested in all animals and started 6 months after treatment with tasks in the following order: novel object recognition, the Morris water maze, elevated plus maze, and contextual fear conditioning. Each new behavioral task started 2 weeks after the previous one to give the animals time to recover.

Novel object recognition
The novel object recognition task was performed in a wooden box (40 x 50 x 80 cm) without bedding. The animals received a habituation session on day 1 to explore the box for 3 minutes without objects. Day 2 consisted of 2 trials, each lasting 3 minutes, with an inter trial time of 1 hour. In the first trial, the acquisition phase, 2 identical objects (Duplo Lego toys, cubes, 6.5 x 6.5 x 7 cm) were placed in opposite corners of the box in such a way that the animals could walk around them. In the second trial, the recollection phase, one object was replaced by a novel object, with a different shape and color (rectangle, 3 x 13 x 8 cm). The objects were securely fixed to the floor of the box using tape, so the animals could not move them around. The objects and the box were cleaned after each session with 70% alcohol. Exploration of the different objects (sniffing or touching the objects) was analyzed using Eline 0.9. The discrimination index (in percentage) was defined as the time spent exploring the novel object divided by the time spent exploring the familiar object.
**Morris water maze learning**

The Morris water maze was performed in a circular black pool (Ø 140 cm) with a black platform. The pool was filled with water of 26 ± 1 °C, so that the platform was about 1 cm below the water surface. The pool was surrounded with external, constant cues and the observer always sat in the same position. The task consisted of 5 training days with 2 trials per day with an inter-trial time of 1 hour. One trial lasted for 3 minutes or until the rat found the platform and sat on it for 10 seconds. If a rat did not find the platform within 3 minutes, it was guided by hand. The platform was removed before the last trial on day 5 and the rat was placed in the pool for a probe trial which lasted 1 minute. The pool was divided into four quadrants with the platform located in the center of one of these quadrants. For each trial, the rat was placed in the center of a randomly selected quadrant with its nose facing the edge of the pool.

Behavior of the animal was tracked by using Ethovision 3.0 and analyzed for escape latency in the learning phase, being the time from the beginning of the trial until the rat sat on the platform. During the probe trial the time the animal spent in the right quadrant, average swim speed and total distance moved was analyzed.

**Elevated plus maze**

The elevated plus maze consisted of a black wooden plus-shaped construction, 50 cm above the floor, with 2 open arms (50 x 10 cm) opposite to each other and 2 closed arms (50 x 10 x 40 cm, with an open roof). Both open arms were divided into 2 equal zones. The animal was placed in the center of the maze, facing a closed arm and was allowed to explore the maze freely for 5 minutes. Explorative behavior was scored using Ethovision 3.0 and analyzed for time spent in the open or closed arms, the number of entries, latency to enter an open arm, latency to reach the second half of the open arm, velocity, and total distance moved.

**Contextual fear conditioning**

The contextual fear conditioning test was performed in a passive avoidance box composed of black Plexiglas (42 x 42 x 42 cm), with a grid floor. The animal was placed into the box for 5 minutes after which a foot shock was given of 0.8 mA for 3 seconds. After the shock the animals remained in the box for 30 seconds before they were placed back into their home cage. Recollection was tested 24 hours later by placing the animal into the box for 5 minutes. The last session was recorded and analyzed for immobility behavior using Eline 0.9.

**Statistics**

The discrimination index of the NOR was analyzed with a one-sample t-test, compared to 100%; the probe trial of the Morris water maze was analyzed with a one-sample t-test, compared to 25%. The learning phase of the Morris water maze was analyzed using a repeated measure ANOVA. The elevated plus maze and contextual fear conditioning were analyzed using one-way ANOVA with treatment as between-subject variable. LSD post-hoc tests were performed when the ANOVA test was significant. For all statistical tests, a probability value less than 0.05 was considered to be statistically significant.
Results

Novel object recognition

Two weeks after treatment, half of the animals were tested in a novel object recognition task and the discrimination index was calculated as time spent exploring the novel object/ time spent exploring the familiar object * 100 for the recollection phase. A discrimination index of more than 100% means that the animals spent more time exploring the novel object than the familiar one. Animals treated with MTX spent an equal amount of time exploring both objects, which suggests cognitive impairment (119.7% ± 20.8 SEM). The control animals did learn the task adequately (240.9 ± 74.8 SEM, P < 0.001, one-sample t-test) (figure 1A). Figure 1B shows the discrimination index of the novel object recognition task performed 6 months after treatment. Even though control animals and animals treated with MTX appeared to spent more time exploring the novel object than the familiar one (with a discrimination index of 151.4% ± 32.69 SEM and 139.2 ± 21.2 SEM respectively), this observation failed to reach significance.

Figure 1: Discrimination index of the novel object recognition test, data are represented as average with the standard error of the mean. Figure 1A shows the effect of MTX on novel object recognition 2 weeks after treatment with saline (white bar, n = 8) or MTX (closed bar, n = 8). The control animals spent significantly more time explore the novel object than the familiar one (P < 0.001), whereas animals treated with MTX spent an equal amount of time exploring both objects. Figure 1B shows the effect of MTX on novel object recognition 6 months after treatment with saline (white bar, n = 16) or MTX (closed bar, n = 16). Both control animals and animals treated with MTX spent more time exploring the novel object then the familiar one.

Morris water maze learning

Figure 2A shows the daily average escape latency during the training period of the Morris water maze. Both control animals and animals treated with MTX showed a significant learning curve with $F_{4, 12} = 11.791$, $P < 0.001$, and $F_{4, 12} = 5.442$, $P = 0.01$ respectively. Control animals and animals treated with MTX spent significantly more time in the correct quadrant compared to 25% (chance level) with $P < 0.005$ and $P < 0.001$ respectively, and no difference was seen between the two groups (time spent in the correct quadrant is 30.4% ± 1.5 SEM for control animals and 33.4% ± 1.8 SEM for animals treated with MTX) (figure 2B). Also, no differences were seen in total distance traveled or average swim speed (data not shown).
Figure 2: Results from the Morris water maze for control animals (white bar, n = 16) and animals treated with MTX (closed bar, n = 16). Figure 2A shows the mean escape latency and figure 2B the results from the probe trial. The data are represented as averages of the 2 trials per day with the standard error of the mean. Both control animals and animals treated with MTX showed a significant learning curve with $F_{4, 12} = 11.791$, $P < 0.001$, and $F_{4, 12} = 5.442$, $P = 0.01$ respectively. Control animals and animals treated with MTX spent significantly more time in the correct quadrant compared 25% (chance level) with $P < 0.005$ and $P < 0.001$ respectively, and no difference was seen between the two groups.

**Elevated plus maze**

Figure 3 shows the results from the elevated plus maze. The animals treated with MTX showed a longer latency time (94.4 seconds ± 34.6 SEM) to enter the second half of an open arm compared to control animals (17.0 seconds ± 6.8 SEM), with $F_{1, 23} = 6.627$, $P < 0.02$ (figure 3A). The animals treated with MTX also spent significantly less time (16.1% ± 2.9 SEM) on the open arms compared to the control animals (26.3% ± 2.3 SEM), with $F_{1, 23} = 8.647$, $P < 0.01$ (figure 3B).

Figure 3. Latency time to enter the second half of the open arm (figure 3A) and time spent on the open arm (figure 3B) of the elevated plus maze of control animals (open bar, n = 16) and animals treated with MTX (closed arm, n = 16). Compared to control animals, animals treated with MTX showed a longer latency time to enter the second half of the open arm ($F_{1, 23} = 6.627$, $P < 0.02$) and spent less time on the open arms ($F_{1, 23} = 8.647$, $P < 0.01$).
Contextual fear conditioning
Figure 4 shows the percentage of time spent on immobility behavior during the recollection trial of the contextual fear conditioning. Even though it appears that the animals treated with MTX show less immobility behavior (56.7% ± 4.8 SEM) compared to control animals (66.7% ± 4.9 SEM), this observation failed to reach significance.

Discussion
Cognitive impairment is a long-term side effect noticed in some patients after adjuvant chemotherapy which can last for years after treatment (Correa and Ahles, 2008). The majority of studies using animal models to study possible mechanisms behind this cognitive deterioration explore cognitive behavior relatively shortly after treatment, ranging from 30 minutes up to several weeks after injection. In this paper we explored the effects of MTX on cognition on much longer term.

Cognitive behavior was tested between six and eight months after treatment with MTX in a novel object recognition task, Morris water maze, and contextual fear conditioning. All animals learned the tasks adequately with no differences between the control animals and the animals treated with MTX. Our previously performed studies have shown that animals treated with MTX were not able to learn the novel object recognition task and the Morris water maze shortly (2/3 weeks) after treatment (Seigers et al., 2008). Seven months after treatment, animals treated with MTX were more anxious in the elevated plus maze. However, shortly after treatment, animals treated with MTX were not more anxious compared to control animals when tested in the same tasks (unpublished data).

This is not the first paper that does not show cognitive impairment long-term after chemotherapy treatment. In a study of Lee and colleagues middle aged (7 months of age) and old (18 months of age) female Fischer-344 rats received five intraperitoneal injection of cyclophosphamide or 5-fluorouracil. A Morris water maze was performed only with the middle aged animals and all animals were subjected to a Stone 14-unit T-maze task. In this task the animals first received a foot shock after which they had to walk through the maze making correct left-right turns to avoid receiving another foot shock. Seven weeks after treatment, the middle aged animals treated with cyclophosphamide or 5-fluorouracil performed better in the Morris water maze compared to control animals. However, seven months after treatment with cyclophosphamide or 5-fluorouracil, treated animals and control animals performed equally well in the Morris water maze. Similar learning effects were seen in the Stone 14-unit T-maze.
Seven weeks after treatment with cyclophosphamide or 5-fluorouracil, the treated animals performed better than the control animals, and no differences in learning behavior were seen seven months after any treatment regimen. The authors suggest that this unexpected beneficiary effect of cyclophosphamide and 5-FU on learning behavior can be explained by the estrogen cycle. Cytostatic treatment causes pre-mature menopause, and whereas lowered estrogen levels have a negative effect on cognition in humans, it has a positive effect on learning in rats (Lee et al., 2006). Obviously, the effect of cytostatics on the estrogen cycle can not explain our results, since we used male rats, but it does suggest that the cognitive impairment in animals after chemotherapy is subtle and appears to depend on the protocol used.

The subtlety of the cognitive impairment after chemotherapy in animals is also shown in a study of Stock and colleagues where no cognitive impairment was found when animals were tested long-term after cytostatic treatment. In this paper, male and female Sprague-Dawley rats received a single intraperitoneal injection of MTX and cognitive behavior was tested nine weeks after treatment in two different Pavlovian training paradigms and a conditioned taste aversion task. No significant differences were found in any task between control animals and animals treated with MTX. The authors suggest they did not find a learning impairment after MTX treatment due to strain differences compared to other studies (Stock et al., 1995). However, since this paper, more studies have been performed using Sprague-Dawley rats with different cytostatics and cognitive impairment was found in all these studies (Konat et al., 2008; Li et al., 2008; MacLeod et al., 2007), which also supports the idea that cognitive impairment in animals is very subtle.

In general, we can conclude that animals treated with MTX do not suffer from long-lasting cognitive impairment, which suggests that rats, in contrast to human patients; functionally fully recover from MTX induced cognitive deficits after longer time intervals. The results in this paper do not suggest that rodent models are not suitable to test cognitive behavior after cytostatic treatment, but that more attention should be paid to physiological differences between rodents and humans when designing experiments.

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**Reference list**


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Li CQ, Liu D, Huang L, Wang H, Zhang JY, Luo XG. Cytosine arabinoside treatment impairs the remote spatial memory function and induces dendritic retraction in the anterior cingulate cortex of rats. Brain Res Bull, 2008;Epub ahead of print-


