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Research report

# Long-lasting suppression of hippocampal cell proliferation and impaired cognitive performance by methotrexate in the rat

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**Abstract**

Methotrexate (MTX) is a cytostatic agent widely used in combination with other agents as adjuvant chemotherapy for breast cancer and is associated with cognitive impairment as a long-term side effect in some cancer patients. This paper aimed to identify a neurobiological mechanism possibly responsible for this cognitive impairment using an animal model.

The first study explored the hypothesis that MTX reduces neuronal cell proliferation. A dose-dependent long-lasting decrease in hippocampal cell proliferation was shown with Ki-67 immunocytochemistry, following a single intravenous injection of MTX (37.5–300 mg/kg). Animals treated with MTX also showed a dose-dependent transient decrease in body weight gain.

In the second study, the effect of MTX (250 mg/kg) on two spatial learning tasks was examined. Animals treated with MTX learned the Morris water maze task adequately; however, these animals showed a longer latency time to cross the platform location in the probe trial, reflecting an impairment of spatial memory function. In the novel object recognition task, animals treated with MTX failed to distinguish a novel object from a familiar one, indicating a decrease in the comparator function of the hippocampus.

Our studies indicated that, in the rat, MTX has a dose-dependent negative effect on hippocampal cell proliferation, and on cognitive behavior. These findings suggest that adverse effects of certain cytotoxic agents on hippocampal cell proliferation may have a potential contributory role in cognitive impairment observed in humans after chemotherapy.


*Keywords:* Methotrexate; Neurogenesis; Cognitive impairment; Rat; Neurotoxicity

## 1. Introduction

Cytostatic drugs are often applied in modern oncology; patients live longer and even survive. Hence, increasing attention is paid to negative side effects. In several neuropsychological and neurophysiological studies, cognitive impairment is consistently observed in a subgroup of patients treated with various regimens of adjuvant chemotherapy for breast cancer (CMF[1],

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[1] CMF regimen: cyclophosphamide, 100 mg/m$^2$ orally on days 1–14; methotrexate 40 mg/m$^2$ intravenously on days 1 and 8; 5-fluorouracil 600 mg/m$^2$ intravenously on days 1 and 8; repeated every 4 weeks.

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CTC[2], FEC[3]) [1,7,9,25,34–36,38,42,43]. These deficits range from very subtle to more severe and are observed in a wide range of brain functions, including memory, concentration, and speed of information processing [25], and can be noticed up to 10 years after completion of cytotoxic treatment [1]. Recently, a clinical study was performed in which the effect of CMF chemotherapy on grey and white matter volume was examined. People who had received adjuvant chemotherapy showed smaller grey and white matter volume in the prefrontal and parahippocampal gyrus 1 year after treatment, compared to those who had not received chemotherapy. These findings were significantly correlated with impairments in attention, concentration and/or visual memory. Effects of chemotherapy on brain volume were not noticed 3 years after treatment [21].

Despite these indications of negative effects on the central nervous system resulting in persistent cognitive dysfunction, our understanding of the nature of the cognitive impairment and the underlying mechanisms is fragmentary at best. Due to the compound nature of the chemotherapeutic regimens, in humans it is difficult to isolate specific etiological agents responsible for the cognitive impairment observed. Animal models of human cognitive function might help elucidate the neural mechanisms mediating cytostatic brain damage.

In the few animal studies performed, mainly methotrexate (MTX), a frequently used cytotoxic agent in the clinic, was examined. Philips et al. showed that, after high-dose administration of MTX into the femoral vein in rats, several behavioral changes occurred including reduced spontaneous activity and diminished startle response to loud noise or vibrissal stimulation [30]. Another study investigating high-dose intraperitoneal injections of MTX showed enhanced occurrence of seizures in mice (possibly due to a lowering of GABA content), and an impairment of long-term memory in a passive avoidance task [40].

A potential mechanism underlying the adverse effect on brain functioning but not yet studied, is the effect of cytostatic drugs on neurogenesis. In the 1960s it was established that the production of new cells in the adult brain is not restricted to glial cells, but there is also a persistent production of neurons [3]. This neurogenesis has recently received much attention. Studies have shown that stem cells continuously produce new neurons in several brain areas, the most prominent ones being the subventricular zone (SVZ), which lines the lateral ventricles, and the sub granular zone (SGZ) of the hippocampal dentate gyrus (DG) [17,24]. Since the hippocampal formation is well known for its involvement in learning and memory processes, it is suggested that neurogenesis in this brain structure plays a functional role in cognitive performance. Animals with lowered neurogenesis perform worse in hippocampal-dependent tasks and exhibit a learning impairment [17], whereas an enriched environment enhances neurogenesis, and increases learning capacity [23,28]. Since many cytotoxic compounds, like MTX, are aimed at the inhibition of the process of cell division, they will probably also affect cell proliferation in the brain if they are able to pass the blood–brain barrier. Although it is assumed that most cytotoxic agents do not pass the blood–brain barrier, MTX seems to be an exception [12]. Therefore, we hypothesize that due to MTX, hippocampal cell proliferation is negatively affected, which may play a contributory role in the cognitive decline observed in some cancer patients.

We tested this hypothesis in two different studies. The first study determined the dose–response relationship between MTX and hippocampal cell proliferation. Since Ki-67 is a nuclear protein antigen involved in cell proliferation regulation [33], Ki-67 positive cells were immunocytochemically visualized and quantified in the SGZ by light microscopic analysis of brain sections 3 weeks after administration of the cytostatic drug.

The second study explored the effects of a high-dose MTX on cognitive performance in hippocampal-dependent learning tasks. Three weeks after treatment with MTX, animals were trained in the Morris water maze (MWM). Another group of animals was subjected to the novel object recognition (NOR) task, performed 4 weeks after treatment with MTX.

## 2. Methods

### 2.1. General

Adult (3 months of age) male Wistar rats (Harlan, Zeist, The Netherlands, average bodyweight at the start of the experiment 297.1 g ± 11.67 S.E.M.) were housed individually in clear Plexiglas cages (25 cm × 25 cm × 30 cm) on a layer of wood shavings with a fixed 12:12 h light:dark cycle with lights on at 08.00 a.m., and food and water *ad libitum*.

Experiments started 2 weeks after arrival of the animals according to the protocol described below. After injection with MTX (100 mg/ml, Pharmachemie BV, Haarlem, The Netherlands), 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. Tetrahydrofolate (THFA) is a cofactor in DNA synthesis, MTX is an inhibitor of the enzyme THFA reductase and depletes the pool of tetrahydrofolates. Leucovorin is a tetrahydrofolate and does not require activation by THFA reductase [16,20]. The rescue therapy of leucovorin was administered in a protocol similar to that applied in patients. Eighteen hours after the injection of MTX, leucovorin was administered in a concentration that was 8% of the injected MTX dosage; at 26, 42, and 50 h the administered concentration was reduced to 4%. Pilot studies showed that leucovorin itself does not have an effect on neurogenesis and that high-dose MTX without leucovorin is lethal, due to severe diarrhea and weight loss.

All experiments were approved by the Animal Experimentation Committee of the University of Groningen.

### 2.2. MTX dose–response interaction for body weight gain and hippocampal cell proliferation

In the first study, rats were injected with various dosages of MTX (37.5, 75, 150 or 300 mg/kg) in the tail vein under a short-lasting (<3 min) mild $O_2$-$N_2O$-isoflurane anesthesia. Control animals were injected with saline. Body weight of all animals was measured on a daily basis. The animals were sacrificed 3 weeks after the injection through transcardial perfusion with saline followed by 4% paraformaldehyde. Brains were removed and placed in 30% sucrose solution at 4 °C. Microtome sections of the hippocampus (40 μm) were stored in 0.01 M PBS including azide until immunocytochemical staining.

[2] CTC regimen: cyclophosphamide, 6 g/m$^2$ intravenously; thiotepa, 480 mg/m$^2$ intravenously; carboplatin, 1.6 g/m$^2$ intravenously; divided over 4 days.

[3] FEC regimen: 5-fluorouracil, 500 mg/m$^2$ intravenously; epidoxorubicin, 90–120 mg/m$^2$ intravenously; cyclophosphamide, 500 mg/m$^2$ intravenously; repeated every 3 weeks.

From the serial sections, every twelfth section from each animal was selected and immunocytochemically stained for Ki-67 using a slightly adapted standard protocol [22]. In brief, free-floating sections were pre-treated with 0.4% $H_2O_2$ for 30 min, to stop endogenous peroxidase activity. Non-specific binding of immunoreagents was blocked with 3% normal goat serum (Zymed, San Francisco, USA). Subsequently, sections were incubated with mouse-anti-Ki-67 (1:200, Novocastra, Newcastle upon Tyne, UK), for 48 h at 4 °C. After a second blocking step, sections were incubated with a biotinylated secondary antibody (1:400, goat-anti-mouse, Jackson, Wet Grove, PA, USA) for 2 h at room temperature. This was followed by incubation in an avidin biotinylated peroxidase complex (1:400, ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Labeled cells were visualized with 0.15 mg/ml diaminobenzidine (DAB) and 0.003% $H_2O_2$ solution.

After mounting of the sections onto glass slides for microscopic analysis, sections were counterstained with a Mayer-haematox solution for 30 s. Counting of Ki-67 positive cells in both hemispheres of the dentate gyrus was performed under a light microscope with a magnification of 400×. Counting was performed in the subgranular layer of the dentate gyrus. Counts in both blades were summed. The borders of the area that was quantified was defined as the subgranular layer having a thickness of two cell diameters. All cells were counted in the subgranular layer of the dentate gyrus from top to bottom of the 40 μm thick section. Because every twelfth section of the brain was stained, the amount of positive cells was multiplied by 12 to get the estimated total amount of Ki-67 positive cells in the hippocampus.

### 2.3. Effects of high-dosage MTX on cognitive performance

In patients, MTX, in the dosage used as adjuvant chemotherapy in the CMF cocktail, induces a body weight loss of approximately 10% and mild diarrhea [13]. In order to achieve similar effects in animals and based on the effects of the dose–response interaction, we injected rats with 250 mg/kg MTX for the behavioral tasks, which were performed after the animals recovered from the sickness caused by MTX.

#### 2.3.1. Morris water maze learning

Animals were injected with either saline or MTX (250 mg/kg) as previously described. Three weeks after treatment, the MWM task was performed, which tests spatial cognitive performance. The task 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 five training days with two trials per day with an inter-trial time of 1 h. One trial lasted for 3 min or until the rat found the platform and sat on it for 10 s. If a rat did not find the platform within 3 min, it was guided by hand. On day 6, the platform was removed and the rat was placed in the pool for a probe trial which lasted 1 min. Behavior of the animal was tracked by using Ethovision 3.0 and analyzed for escape latency in the learning phase, meaning the time from the beginning of the trial until the rat sat on the platform. During the probe trial, the time spent before the animal crossed the platform location was recorded.

#### 2.3.2. Novel object recognition

Animals received an injection of either saline or MTX (250 mg/kg) as previously described. Four weeks after treatment, the comparator function of the hippocampus was tested in a NOR task. The task was performed in a wooden box (40 cm × 50 cm × 80 cm) without bedding. The animals received a habituation session on day 1 to explore the box without objects, lasting 3 min. Day 2 consisted of two trials, each lasting 3 min, with an inter trial time of 1 h. In the first trial, the acquisition phase, two identical objects (Duplo Lego toys, cubes, 6.5 cm × 6.5 cm × 7 cm) were placed in the center of the box, 13 cm apart. In the second trial, the recollection phase, one object was replaced by a novel object, with a different shape and color (rectangle, 3 cm × 13 cm × 8 cm). 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 behavior 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.

### 2.4. Statistics

Body weight was analyzed using repeated measure ANOVA. Light-microscopic counts of Ki-67 positive cells and the behavioral tasks were analyzed using one-way ANOVA with treatment as between-subject variable. LSD post-hoc test was performed when the ANOVA test was significant. For all statistical tests, a probability value less than 0.05 was considered to be statistically significant.

## 3. Results

### 3.1. MTX dose–response interaction for body weight and hippocampal cell proliferation

#### 3.1.1. Body weight gain

Body weight gain was calculated as absolute body weight/absolute body weight on the day of the injection, multiplied by 100. For day 0 (the day of the injection), this will give a relative body weight of 100%; body weight gain is determined as the relative body weight that is more than 100%.

All rats showed a decrease in body weight gain after the injection (Fig. 1). Whereas the control animals and animals that received 37.5 mg/kg MTX regained their body weight on day 2, the other animals (75, 150 and 300 mg/kg MTX) continued to lose weight and suffered from mild diarrhea. After day 4, all animals recovered and started to regain body weight. The difference in body weight gain between the different treatments was significant, $F_{4,60} = 3.057$, $P < 0.0001$. The difference between the control animals and the animals treated with 37.5 and 75 mg/kg MTX was significant with $P < 0.05$. The difference between the

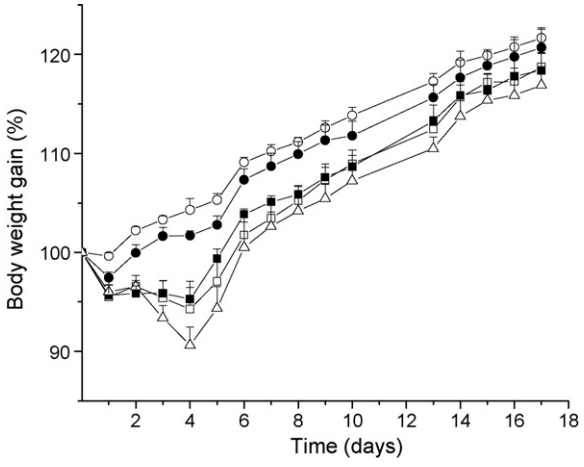


Fig. 1. Body weight gain after intravenous administration of saline (◯) or MTX ((●) 37.5 mg/kg; (□) 75 mg/kg; (■) 150 mg/kg; (△) 300 mg/kg; $n = 6$ per group). Body weight before the injection (given on day 0) is expressed as 100%, bars represent standard error of the mean. The difference in body weight gain between the different treatments was significant, $F_{4,60} = 3.057$, $P < 0.0001$. The difference between the control animals and the animals treated with 37.5 and 75 mg/kg MTX is significant with $P < 0.05$. The difference between the control animals and the animals treated with 150 and 300 mg/kg MTX is significant with $P < 0.0001$.

control animals and the animals treated with 150 and 300 mg/kg MTX was also significant with $P < 0.0001$.

### 3.1.2. Ki-67 immunocytochemistry

Ki-67 positive cells in microtome sections of the hippocampus were visualized and counted. The average number of sections counted per animal per group was $11.75 \pm 1.23$ S.E.M., $12.00 \pm 0.42$ S.E.M., $11.81 \pm 0.41$ S.E.M., $11.13 \pm 0.68$ S.E.M., and $12.90 \pm 0.62$ S.E.M. for the control animals and the animals treated with 37.5, 75, 150, and 300 mg/kg MTX, respectively.

The number of Ki-67 positive cells differed significantly between the groups, $F_{4,23} = 6.822$, $P = 0.0009$ (Fig. 2). Control animals had significantly more Ki-67 positive cells in the hippocampus than animals treated with 37.5, 75 (both $P < 0.005$), 150 ($P < 0.001$) or 300 mg/kg ($P < 0.0001$). Two other groups, 37.5 and 75 mg/kg MTX, differ significantly from 300 mg/kg MTX ($P < 0.05$).

## 3.2. Effects of high-dosage MTX on cognitive performance

### 3.2.1. Morris water maze learning

Three weeks after injection of 250 mg/kg MTX, a MWM task was performed. Fig. 3 shows the daily average escape latency during the training period. All animals learned the location of the platform, with no significant differences between the groups. Control animals and animals treated with MTX both significantly improved during the learning phase, with $F_{4,30} = 3.135$, $P < 0.05$; $F_{4,40} = 4.508$, $P < 0.05$ respectively. However, animals treated with MTX showed a longer latency time to cross the platform area during the probe trial compared to control animals, $F_{1,12} = 3.135$, $P < 0.05$ for both one-way ANOVA and post-hoc test (Fig. 4).

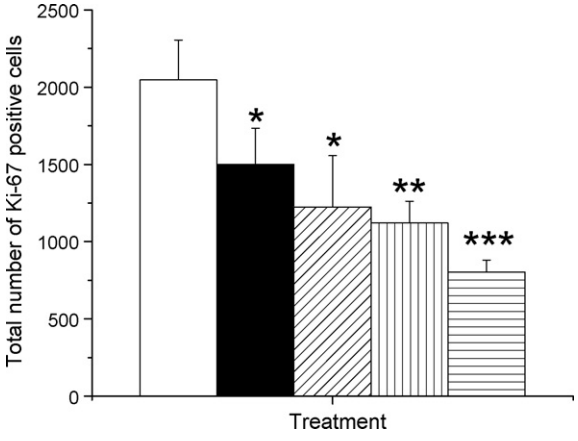


Fig. 2. Total number of Ki-67 positive cells in the hippocampus of control rats (open bar) and animals treated with MTX (solid bar 37.5 mg/kg; diagonal striped bar 75 mg/kg; vertical striped bar 150 mg/kg; horizontal striped bar 300 mg/kg; $n = 6$ per group). Data are represented as mean with standard error of the mean. The number of Ki-67 positive cells differ significantly between the groups, $F_{4,23} = 6.822$, $P = 0.0009$. Control animals have significantly more Ki-67 positive cells than animals treated with MTX (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$). Two other groups, 37.5 and 75 mg/kg MTX, differ significantly from 300 mg/kg MTX ($P < 0.05$).

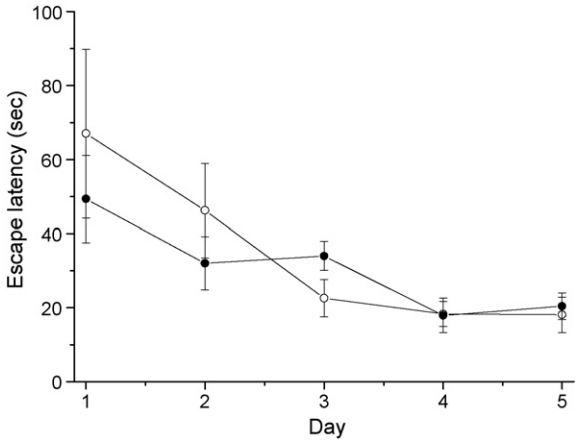


Fig. 3. The mean escape latency in the Morris water maze test for control animals (◯) and animals treated with MTX ((●) 250 mg/kg; $n = 8$ per group). The two trials per day are shown as average values with standard error of the mean. Control animals and animals treated with MTX both significantly improve during the learning phase, with $F_{4,30} = 3.135$, $P < 0.05$; $F_{4,40} = 4.508$, $P < 0.05$ respectively. No significant effects are seen between control animals and animals treated with MTX.

There was no difference in average swim speed or distance traveled between control animals and animals treated with MTX (data not shown).

### 3.2.2. Novel object recognition

Four weeks after treatment with 250 mg/kg MTX, a NOR task was performed. Fig. 5 shows the discrimination index (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. Control animals spent significantly more time exploring the novel object than the familiar one ($P < 0.05$), whereas animals treated with MTX spent an equal amount of time investigating both objects (Fig. 5). There was no difference in average total exploration time for the acquisition and recollection phase for both groups, and there was also no difference in average total exploration time

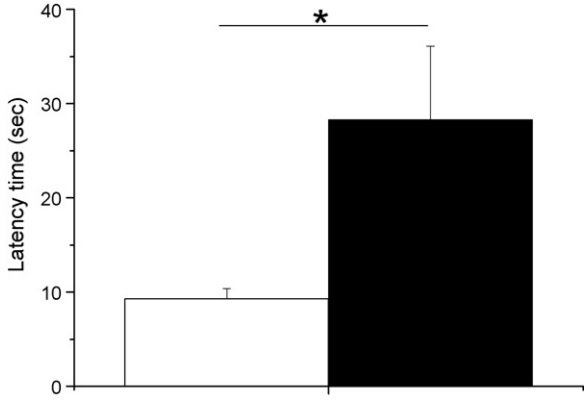


Fig. 4. Latency time to cross the platform location in the probe trial in the Morris water maze test ($n = 8$ per group). Data are represented as mean with standard error of the mean. Animals treated with MTX ((■) 250 mg/kg) show a longer latency time than control animals (□), $P < 0.05$.

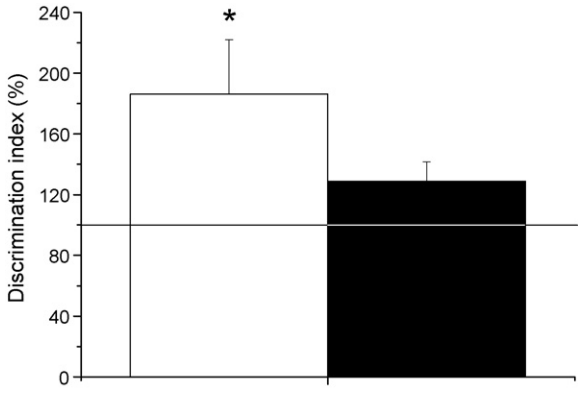


Fig. 5. Discrimination index in the novel object recognition task ($n = 8$ per group). The time spent exploring the novel object is expressed as percentage of the time spent exploring the familiar object (set as 100%) with the standard error of the mean. Control animals (□) spent more time exploring the novel object than the familiar one ($P < 0.05$), whereas animals treated with MTX ((■) 250 mg/kg) spent an equal amount of time exploring both objects.

between the control animals and the animals treated with MTX (data not shown).

## 4. Discussion

This study investigated the long-lasting effects of methotrexate (MTX) on hippocampal cell proliferation and cognitive behavior in rats. A distinct effect was seen on hippocampal cell proliferation in a dose-dependent manner 3 weeks after an intravenous injection of MTX. This was consistent with previous findings in our lab (unpublished data). Cytostatics, such as MTX, are designed to interfere with the process of cell division; therefore, the inhibition of hippocampal cell proliferation could be anticipated. The formation of new neurons out of stem cells in the dentate gyrus of hippocampus (neurogenesis) is thought to play an important role in learning and memory processes [11]. Studies have shown that aging [5], or stress [17] has a negative effect on neurogenesis and cognition, whereas an enriched environment [23], or running wheel activity [29] increases neurogenesis and cognitive performance.

In our study, the lowest dosage given to the animals (37.5 mg/kg) already resulted in a significant decrease in cell proliferation. This finding is of particular interest, since it is suggested that MTX does not pass the blood–brain barrier when administered in a dosage equivalent to the one used in the adjuvant regimen of chemotherapy for breast cancer [6]. Our findings may indicate that even a low dosage is able to pass the blood–brain barrier in rats, or that other factors play a role in the observed neurotoxicity in the current study. One of these factors may be the sickness that is observed in animals treated with MTX, although immunocytochemistry was performed after the animals recovered. All dosages used in this study induced sickness approximately 3 days after treatment, which was expressed in a decrease in body weight gain, fluffy fur, bad general appearance, and mild diarrhea. The effects are similar to the clinic, where patients treated with MTX also suffer from body weight loss and diarrhea [13]. Previous physiological studies in our laboratory indicate that directly after treatment with MTX body temperature slightly increased (suggesting a mild fever) although this observation failed to reach significance (unpublished data). Whether sickness itself can have long-lasting effects on hippocampal cell proliferation will be explored in a future study.

Dietrich et al. examined the effect of carmustine (BCNU) and cisplatin (two other frequently used cytotoxic agents) on cultured brain cells and found that the agents were more toxic to progenitor cells of the CNS and non-dividing oligodendrocytes than for multiple cancer cell lines. The effect of these cytotoxic agents on cell death and cell division was also studied in mice. Three intraperitoneal injections of BCNU significantly increased cell death for up to 10 days after treatment in the corpus callosum and the hippocampal dentate gyrus, and up to 6 weeks in the subventricular zone. Similar effects were seen after 3 intraperitoneal injections of cisplatin. BCNU also significantly decreased cell division up to 6 weeks after treatment in the subventricular zone, the corpus callosum, and in the hippocampal dentate gyrus. Cisplatin showed similar effects, but the number of dividing cells returned to normal levels in the subventricular zone and the dentate gyrus 6 weeks after treatment [10].

In breast cancer patients, CMF chemotherapy is associated with cognitive decline. This decline is mostly noticed 2–6 years after treatment [25,34], but can be noticed as early as 3 months after CMF chemotherapy [44]. The decline is observed in a wide range of brain functions, including memory, concentration, and speed of information processing. Of the cytotoxic agents that are represented in the CMF regimen, MTX is thought to be the agent most responsible for this cognitive impairment [25]. Since we showed that MTX induces a profound and long-lasting decline in hippocampal cell proliferation (which likely reflects an inhibition of the process of neurogenesis) we hypothesized that this decline in cell proliferation could also play a role in the cognitive impairment observed in cancer patients after CMF treatment. To test this hypothesis, we performed two hippocampal-dependent learning and memory tasks (MWM and the NOR task), after treatment with MTX. In a recent paper Ahles and Saykin reported that the most affected cognitive functions after treatment with chemotherapy are, amongst others, episodic, visual and spatial memory [2], which can be tested in a MWM and a NOR task.

In the MWM task, animals were trained to learn where a hidden platform was located allowing an escape from the water. Both control and treated animals learned the task adequately. However, the animals treated with MTX showed a longer latency time to cross the platform location in the probe trial, reflecting an impairment of spatial memory function. There was also a tendency for these animals to spend less time in the right quadrant compared to the control animals; however, this observation failed to reach significance (data not shown). In the NOR task, the animals treated with MTX failed to distinguish a novel object from a familiar one, indicating a decrease in the comparator function of the hippocampus. Cell proliferation in the olfactory bulb was also affected in the animals treated with MTX (data not shown), which could cause a change in the ability to

smell and the ability to make a distinction between the different objects. Since the objects in our test were made of the same material and cleaned after each session, it was not possible for the animals to recognize an object by smell. The hippocampal dependency of NOR task can be debated, however, Forwood et al. describe that when an NOR is performed with spatial cues present, which was the case in our study, the task becomes more hippocampal-dependent [14]. Furthermore, Gould et al. show that hippocampal lesions impair NOR memory performance in rats, when a 1 h delay was used between training and testing [18], a protocol that we also applied in our study.

The impairment in hippocampal-dependent tasks is in line with a number of studies. Madhyastha et al. found a learning and memory impairment, but no anxiolytic effects in the two-compartment conditioned avoidance task after multiple intracerebroventricular injections of MTX in Wistar rats [27]. Sieklucka-Dziuba et al. reported an impairment of long-term memory in the passive avoidance task 14 days after a single dosage of MTX injected intraperitoneal in Albino Swiss mice [40]. Moreover, Yanovksi et al. showed in Lewis-inbred rat pups impaired learning of environmental events in a conditioned emotional response task and conditioned taste aversion task after a single neonatal intraperitoneal MTX injection [46]. Shors et al. treated male Sprague–Dawley rats with the anti-mitotic MAM for 14 days and found a distinct decrease in BrdU positive cells in the dentate gyrus. This decrease, however, did not affect performance in the MWM or in the contextual fear conditioning, but did affect performance in the trace fear conditioning [39]. Winocur et al. treated BALB/C mice with a combination of MTX and 5-fluorouracil. Drug-treated mice showed impaired learning in a MWM, in a non-matching-to-sample test (NMTS), and in a delayed-NMTS of non-spatial memory, compared to control animals [45]. Since the latter tests are not hippocampal dependent, it is possible that the hippocampus is not the only brain area responsible for the cognitive decline seen in patients after treatment with chemotherapy. Other brain areas might also be involved, for example the volume of grey and white matter as described by Inagaki et al. [21].

Not all literature points to a learning impairment after chemotherapy. Lee et al. found that female Fischer-344 rats treated with cyclophosphamide or 5-fluorouracil (both commonly used cytostatics) learned to find the location of a platform in the MWM task even faster than control animals 7 weeks after recovery from the chemotherapy treatment; 29 weeks after the treatment, significant effects were no longer found between treated animals and controls [26]. In that study, animals received five intraperitoneal injections every 4 weeks, for a total of 18 weeks. The authors attribute the unexpected improvement of cognitive function to the fact that cytostatics lower estrogen production. Reduced plasma estradiol in rats is associated with improved learning in the MWM [19]; this is supported by Bimonte-Nelson et al. [4]. In humans, chemotherapy may lead to premature menopause [15,42], which reduces estrogen levels [8]. In contrast to rats, however, low estrogen levels in humans are associated with cognitive decline [37].

Not only chemotherapy is associated with cognitive decline. Other adjuvant treatments for cancer, such as radiation, are also associated with similar long-term effects. Raber et al. found that C57BL/J6 mice showed hippocampal-dependent spatial learning and memory impairments in the Barnes maze, but not in the MWM task 3 months after localized radiation to the bilateral hippocampus and cortex [31]. Rola et al. found a similar effect after whole brain radiation in C57BL/J6 mice, but here a spatial memory retention deficit was seen in the MWM task [32]. Comparable effects after radiation were seen in rats. For example, Madsen et al. found that Wister rats performed worse in an object recognition task, but performed well in the MWM task after multiple radiation treatments directed at the hippocampal area [28]. Yoneoka et al. showed that Fisher rats performed well in the MWM task and the passive avoidance task 6 and 9 months after multiple treatments with fractioned whole brain radiation, but worse in the same task 12 months after treatment [47].

In the current study, the observed decline in cell proliferation seen in the animals after treatment with MTX might not be the main or sole explanation for the observed cognitive impairment seen in patients after treatment with chemotherapy. Ahles and Saykin describe several potential mechanisms for this cognitive impairment, which could also contribute to the decrease in cell proliferation; these mechanisms include DNA damage, oxidative stress, encephalopathy, cytokine activation, telomere length and telomerase activity, neurotransmitter activity, and neuronal repair [2].

We want to emphasize that in our study, MTX was used as an animal model for adjuvant chemotherapy given to breast cancer patients. However, this does not mean that all cytostatics will produce the same effects on cell proliferation or behavior. In order to validate our animal model, we will test other cytostatics in a future study. Furthermore, it seems that the learning task and the time after treatment are crucial for finding a correlation between the decrease in hippocampal cell proliferation after cytostatics and the effect on cognitive behavior.

In conclusion, it can be said that MTX affects hippocampal cell proliferation in a dose-dependent fashion. This lasting decrease in hippocampal cell proliferation might be responsible for the negative effect seen in spatial and comparator memory after treatment with MTX.

It is expected that in 2015 the incidence of breast cancer will be around 17,000 in The Netherlands alone [41], and that in half of these patients chemotherapy will be part of the treatment strategy. Therefore, it is important to systematically investigate the underlying mechanisms of any potential late effect of chemotherapy that can seriously affect the quality of life of these patients. This may help elucidate the mechanisms that underlie the cognitive deficits found in a number of breast cancer patients treated with adjuvant chemotherapy.

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## References


[1] Ahles TA, Saykin AJ, Furstenberg CT, Cole B, Mott LA, Skalla K, et al. Neuropsychologic impact of standard-dose systemic chemotherapy in long-term survivors of breast cancer and lymphoma. J Clin Oncol 2002;20(January 15 (2)):485–93.

[2] Ahles TA, Saykin AJ. Candidate mechanisms for chemotherapy-induced cognitive changes. Nat Rev Cancer 2007;7(March (3)):192–201.

[3] Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 1965;124(June (3)):319–35.

[4] Bimonte-Nelson HA, Singleton RS, Williams BJ, Granholm AC. Ovarian hormones and cognition in the aged female rat. II. Progesterone supplementation reverses the cognitive enhancing effects of ovariectomy. Behav Neurosci 2004;118(August (4)):707–14.

[5] Bizon JL, Gallagher M. Production of new cells in the rat dentate gyrus over the lifespan: relation to cognitive decline. Eur J Neurosci 2003;18(July (1)):215–9.

[6] Boogerd W. Neurological complications of chemotherapy. Handbook clinical neurology, vol. 21 (65); 1995 [Intoxications of the nervous system, part II: 527–546].

[7] Brezden CB, Phillips KA, Abdolell M, Bunston T, Tannock IF. Cognitive function in breast cancer patients receiving adjuvant chemotherapy. J Clin Oncol 2000;18(July (14)):2695–701.

[8] Buckler H. The menopause transition: endocrine changes and clinical symptoms. J Br Menopause Soc 2005;11(June (2)):61–5.

[9] Castellon SA, Ganz PA, Bower JE, Petersen L, Abraham L, Greendale GA. Neurocognitive performance in breast cancer survivors exposed to adjuvant chemotherapy and tamoxifen. J Clin Exp Neuropsychol 2004;26(October (7)):955–69.

[10] Dietrich J, Han R, Yang Y, Mayer-Proschel M, Noble M. CNS progenitor cells and oligodendrocytes are targets of chemotherapeutic agents in vitro and in vivo. J Biol 2006;5(November 30 (7)):22.

[11] Doetsch F, Hen R. Young and excitable: the function of new neurons in the adult mammalian brain. Curr Opin Neurobiol 2005;15(February (1)):121–8.

[12] Dukic SF, Heurtaux T, Kaltenbach ML, Hoizey G, Lallemand A, Vistelle R. Influence of schedule of administration on methotrexate penetration in brain tumours. Eur J Cancer 2000;36(August (12)):1578–84.

[13] Flombaum CD, Meyers PA. High-dose leucovorin as sole therapy for methotrexate toxicity. J Clin Oncol 1999;17(May (5)):1589–94.

[14] Forwood SE, Winters BD, Bussey TJ. Hippocampal lesions that abolish spatial maze performance spare object recognition memory at delays of up to 48 h. Hippocampus 2005;15(3):347–55.

[15] Ganz PA. Breast cancer, menopause, and long-term survivorship: critical issues for the 21st century. Am J Med 2005;118(December 19 (Suppl 12B)):136–41.

[16] Genestier L, Paillot R, Quemeneur L, Izeradjene K, Revillard JP. Mechanisms of action of methotrexate. Immunopharmacology 2000;47(May (2–3)):247–57.

[17] Gould E, Tanapat P, Rydel T, Hastings N. Regulation of hippocampal neurogenesis in adulthood. Biol Psychiatry 2000;48(October 15 (8)):715–20.

[18] Gould TJ, Rowe WB, Heman KL, Mesches MH, Young DA, Rose GM, et al. Effects of hippocampal lesions on patterned motor learning in the rat. Brain Res Bull 2002;58(September 30 (6)):581–6.

[19] Healy SD, Braham SR, Braithwaite VA. Spatial working memory in rats: no differences between the sexes. Proc Biol Sci 1999;266(November 22 (1435)):2303–8.

[20] Huennekens FM. The methotrexate story: a paradigm for development of cancer chemotherapeutic agents. Adv Enzyme Regul 1994;34:397–419.

[21] Inagaki M, Yoshikawa E, Matsuoka Y, Sugawara Y, Nakano T, Akechi T, et al. Smaller regional volumes of brain gray and white matter demonstrated in breast cancer survivors exposed to adjuvant chemotherapy. Cancer 2007;109(January 1 (1)):146–56.

[22] Kee N, Sivalingam S, Boonstra R, Wojtowicz JM. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J Neurosci Methods 2002;115(March 30 (1)):97–105.

[23] Kempermann G. Why new neurons? Possible functions for adult hippocampal neurogenesis. J Neurosci 2002;22(February 1 (3)):635–8.

[24] Kempermann G, Wiskott L, Gage FH. Functional significance of adult neurogenesis. Curr Opin Neurobiol 2004;14(April (2)):186–91.

[25] Kreukels BP, Schagen SB, Ridderinkhof KR, Boogerd W, Hamburger HL, van Dam FS. Electrophysiological correlates of information processing in breast-cancer patients treated with adjuvant chemotherapy. Breast Cancer Res Treat 2005;94(Nov (1)):53–61.

[26] Lee GD, Longo DL, Wang Y, Rifkind JM, Abdul-Raman L, Mamczarz JA, et al. Transient improvement in cognitive function and synaptic plasticity in rats following cancer chemotherapy. Clin Cancer Res 2006;12(January 1 (1)):198–205.

[27] Madhyastha S, Somayaji SN, Rao MS, Nalini K, Bairy KL. Hippocampal brain amines in methotrexate-induced learning and memory deficit. Can J Physiol Pharmacol 2002;80(November (11)):1076–84.

[28] Madsen TM, Kristjansen PE, Bolwig TG, Wortwein G. Arrested neuronal proliferation and impaired hippocampal function following fractionated brain irradiation in the adult rat. Neuroscience 2003;119(3):635–42.

[29] Persson AI, Naylor AS, Jonsdottir IH, Nyberg F, Eriksson PS, Thorlin T. Differential regulation of hippocampal progenitor proliferation by opioid receptor antagonists in running and non-running spontaneously hypertensive rats. Eur J Neurosci 2004;19(April (7)):1847–55.

[30] Phillips PC, Thaler HT, Berger CA, Fleisher M, Wellner D, Allen JC, et al. Acute high-dose methotrexate neurotoxicity in the rat. Ann Neurol 1986;20(November (5)):583–9.

[31] Raber J, Rola R, LeFevour A, Morhardt D, Curley J, Mizumatsu S, et al. Radiation-induced cognitive impairments are associated with changes in indicators of hippocampal neurogenesis. Radiat Res 2004;162(July (1)):39–47.

[32] Rola R, Raber J, Rizk A, Otsuka S, VandenBerg SR, Morhardt DR, et al. Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. Exp Neurol 2004;188(August (2)):316–30.

[33] Rorstad O. Prognostic indicators for carcinoid neuroendocrine tumors of the gastrointestinal tract. J Surg Oncol 2005;89(March 1 (3)):151–60.

[34] Schagen SB, van Dam FS, Muller MJ, Boogerd W, Lindeboom J, Bruning PF. Cognitive deficits after postoperative adjuvant chemotherapy for breast carcinoma. Cancer 1999;85(February 1 (3)):640–50.

[35] Schagen SB, Hamburger HL, Muller MJ, Boogerd W, van Dam FS. Neurophysiological evaluation of late effects of adjuvant high-dose chemotherapy on cognitive function. J Neurooncol 2001;51(January (2)):159–65.

[36] Schagen SB, Muller MJ, Boogerd W, Mellenbergh GJ, van Dam FS. Change in cognitive function after chemotherapy: a prospective longitudinal study in breast cancer patients. J Natl Cancer Inst 2006;98(December 6 (23)):1742–5.

[37] Sherwin BB. Oestrogen and cognitive function throughout the female lifespan. Novartis Found Symp 2000;230:188–96, discussion 196–201.

[38] Shilling V, Jenkins V, Morris R, Deutsch G, Bloomfield D. The effects of adjuvant chemotherapy on cognition in women with breast cancer–preliminary results of an observational longitudinal study. Breast 2005;14(April (2)):142–50.

[39] Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E. Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 2002;12(5):578–84.

[40] Sieklucka-Dziuba M, Saczonek J, Dziuba J, Kleinrok Z. Central action of some cytostatics–methotrexate (MTX) and doxorubicin (DXR). II. The influence on the seizure activity and the learning and memory processes in mice. Ann Univ Mariae Curie Sklodowska [Med] 1998;53:81–8.

[41] Signaleringsrapport Kanker in Nederland: trends, prognoses en implicaties voor zorgvraag. Signaleringscommissie Kanker van KWF Kankerbestrijding, okt 2004, KWF Kankerbestrijding, p. 124, ISBN 90-71229-13-0.

[42] Van Dam FS, Schagen SB, Muller MJ, Boogerd W, vd Wall E, Droogleever Fortuyn ME, et al. Impairment of cognitive function in women receiving adjuvant treatment for high-risk breast cancer: high-dose versus standard-dose chemotherapy. J Natl Cancer Inst 1998;90(February 4 (3)) :210–8.

[43] Wefel JS, Lenzi R, Theriault RL, Davis RN, Meyers CA. The cognitive sequelae of standard-dose adjuvant chemotherapy in women with breast

carcinoma: results of a prospective, randomized, longitudinal trial. Cancer 2004;100(June 1 (11)):2292–9.

[44] Wieneke MH, Dienst ER. Neuropsychological assessment of cognitive functioning following breast cancer. Psycho-oncology 1995;4(1):61–6.

[45] Winocur G, Vardy J, Binns MA, Kerr L, Tannock I. The effects of the anti-cancer drugs, methotrexate and 5-fluorouracil, on cognitive function in mice. Pharmacol Biochem Behav 2006;85(September (1)):66–75.

[46] Yanovski JA, Packer RJ, Levine JD, Davidson TL, Micalizzi M, D'Angio G. An animal model to detect the neuropsychological toxicity of anticancer agents. Med Pediatr Oncol 1989;17(3):216–21.

[47] Yoneoka Y, Satoh M, Akiyama K, Sano K, Fujii Y, Tanaka R. An experimental study of radiation-induced cognitive dysfunction in an adult rat model. Br J Radiol 1999;72(December (864)):1196–201, 1999;72(864):1196–1201.