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Research report

Methotrexate reduces hippocampal blood vessel density and activates microglia in rats but does not elevate central cytokine release

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ABSTRACT

Methotrexate is a cytostatic drug applied in adjuvant chemotherapy and associated with cognitive impairment in part of the cancer patients. In this paper we studied in rats whether a reduction in blood supply to the brain or neuroinflammation are possible mediators of this cognitive dysfunctionality.

Methotrexate reduced hippocampal blood vessel density 1 week and 3 weeks after treatment as measured immunohistochemically with an endothelial barrier antigen. Since reduced brain vascularization may relate to lowered central glucose metabolism [^{18}F]FDG PET was performed. Methotrexate reduced tracer uptake in the hippocampal region 1 week after treatment, which was not seen 3 weeks after treatment.

Neuroinflammatory processes were explored via a number of methods: a microglia immunohistochemical marker was applied to hippocampal sections, [^{11}C]PK11195 PET was performed, and cytokine levels in plasma and homogenized hippocampal tissue were measured. Methotrexate activated microglia in the hippocampus 1 week and 3 weeks after treatment. PET analysis, however, did not show an increase in hippocampal tracer uptake and the multiplex analysis of various cytokines showed that hippocampal cytokine levels were not increased after methotrexate administration. Methotrexate did reduce plasma cytokine levels indicating a suppression of peripheral immune functioning.

Methotrexate reduces hippocampal blood vessel density, indicative of a reduced brain glucose metabolism, which may contribute to the cognitive impairment following methotrexate administration. Although methotrexate activates microglia activation in the hippocampus, no effects were seen in [^{11}C]PK11195 tracer uptake or hippocampal cytokine levels. This suggests that the microglial activation in this study is not a marker for neuroinflammation.

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1. Introduction

Chemotherapy is a commonly used adjuvant treatment strategy for breast cancer; however, it is associated with many side effects. Cognitive impairment is one of these negative effects, and can be noticed up to years after treatment in a subgroup of patients [1]. The predominantly affected cognitive functions are memory, processing speed, and more complex aspects of attention [6]. A number of cytostatics are associated with cognitive impairment, but methotrexate (MTX), a cytostatic drug that frequently has been used in regimens of adjuvant chemotherapy for breast cancer, appears to be potentially more harmful then others. This has been shown in a number of clinical [30–32] and animal studies [12,35,41]. Therefore, in this paper, we study the effect of MTX on a number of neurobiological processes which may be involved in the cognitive impairment seen after treatment with chemotherapy.

An impaired blood supply to the brain may play an important role in the development of cognitive impairment following chemotherapeutic compounds. Women treated with chemotherapy showed a significantly altered blood flow in several brain areas and abnormal activation in the inferior frontal cortex dur-

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ing a short-term memory recall task compared to healthy control women [38]. MTX is also known to cause structural damage to blood vessels, as measured with elevated levels of circulating endothelial cells in mice after intraperitoneal injections of MTX [43], and elevated apoptosis markers in cultured bovine pulmonary artery endothelial cells after exposure to therapeutic levels of MTX [22].

Another possible process that may contribute to the development of cognitive impairment is the effect of chemotherapy on cytokines as described by Ahles and Saykin [1]. Chemotherapy is known to be capable of inducing an inflammatory cytokine response, which can lead to cytokine-induced sickness [27,42]. High dose MTX can induce mucositis, which is characterized by damage to the gastrointestinal mucosa [7]. This leads to a decreased barrier function and an enhanced risk of developing infections caused by micro-organisms originating from the intestines. Mucositis is associated with elevated cytokine release [7] which in turn can induce inflammation and cytokine release in the central nervous system [36,40]. This cytokine release in the central nervous system caused by peripheral sickness behavior can lead to central sickness behavior which is associated with cognitive impairment [3]. However, it is also known that chemotherapeutic compounds such as MTX have a strong inhibitory effect on stem cells in bone marrow which is the reason that these substances are used as potent immunosuppressors [14,18,24]. In the situation that central cytokine release is induced by MTX it may trigger the activation of microglia which are the immune cells of the brain. Microglia continuously scavenge their immediate environment and are activated in response to challenges of central nervous system homeostasis. Activated microglia can perform a number of functions, such as phagocytosis of debris, tissue repair, and neural regeneration. Fully activated neurons are known to have neurotoxic effects, leading to neuroinflammation [15,28].

To study these neurobiological processes in rats, we examined the effect of MTX on blood vessel density, central glucose metabolism, microglia activation, neuroinflammation, and cytokine levels using different techniques: immunohistochemistry, positron emission tomography (PET), and multiplex bead-based immunoassay. Brain vascularization was studied by applying an endothelial barrier antigen (EBA) to hippocampal sections to visualize and quantify blood vessels in this brain structure [2,21]. Since a reduced brain vascularization may relate to a lowered glucose metabolism a tracer for regional glucose metabolism, [^{18}F]FDG (2-[^{18}F]-fluoro-2-deoxy-D-glucose) [8,39] was used for PET. Furthermore, hippocampal sections were immunohistochemically stained with an antibody visualizing ionized calcium binding adapter molecule 1 (IBA-1) which is upregulated in activated microglia [9,17]. Next to this immunohistochemical approach a ligand for peripheral benzodiazepine receptors, [^{11}C]PK11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide) was also administered and used as a tracer for PET [5,29]. In normal brain tissue binding of PK11195 is minimal, whereas in areas with activated microglia, in vivo binding is significantly increased [29]. Inflammatory processes in the periphery and in the brain were also studied by measuring cytokine levels in plasma and homogenized hippocampal tissue.

2. Methods

2.1. General method and procedure

Adult (3 months of age, $n = 84$) male Wistar rats (Harlan, Zeist, the Netherlands, average body weight at the start of the experiment 320 g ± 6.1 SEM) were housed in groups in clear Plexiglas cages (58 cm × 38 cm × 20 cm, 4 animals per cage) 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. The experiments started 2 weeks after arrival of the animals according to the protocol described below. All experiments were approved by the Animal Experimentation Committee of the University of Groningen.

Adjuvant chemotherapy in the CMF cocktail (cyclophosphamide 100 mg/m^2 orally on days 1 and 14, methotrexate 40 mg/m^2 intravenously on days 1 and 8, and 5-fluorouracil 600 mg/m^2 intravenously on days 1 and 8) induces a body weight loss of approximately 10% and mild diarrhea in patients [11]. In order to achieve similar effects in animals and based on previous studies performed in our lab [34,35], rats were injected with 250 mg/kg MTX (100 mg/ml, Pharmachemie BV, Haarlem, the Netherlands) in the tail vein under a short-lasting (<3 min) mild O$_2$-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 [13,16]. 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; at 26, 42, 50 h the administered concentration was reduced to 4%.

2.2. Blood vessel density

The animals were sacrificed 1 week or 3 weeks after treatment with either saline ($n = 6$ per group) or MTX ($n = 8$ per group) 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 0.1% azide until immunohistochemical staining.

Every twelfth serial section from each animal was selected and immunohistochemically stained with an endothelial barrier antigen. In brief, free-floating sections were pre-treated with 0.4% H$_2$O$_2$ for 30 min, to stop endogenous peroxidase activity. Non-specific binding of immunoreagents was blocked with 3% normal horse serum (Zymed, San Francisco, CA, USA). Subsequently, sections were incubated with mouse-anti-EBA (1:1500, Covance, SMI 71R), for 72 h at 4 °C. After a second blocking step, sections were incubated with a biotinylated secondary antibody (horse-anti-mouse, 1:400, Vector, Burlingame, CA, USA) for 1 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 0.075 mg/ml diaminobenzidine (DAB) and 0.003% H$_2$O$_2$ solution, after which the sections were mounted onto glass slides.

Total optical density (OD) was measured in 3 sections between bregma −3.14 and −3.80 [26]. Four regions of interest were selected: inner and outer blade of the dentate gyrus, CA1, and CA3 region. The OD was measured with a Quantimet 550 image analysis system (Leica, Cambridge, UK) with a magnification of 100×. Counts in both blades of all sections were summed and the total OD was calculated.

2.3. [^{18}F]FDG distribution

The animals were transported from the animal facility to the PET centre and back by car which took approximately 30 min. The animals were allowed 90 min recovery from transportation before the experiment started. Glucose metabolism was measured 1 week and 3 weeks after treatment with saline ($n = 5$) or MTX ($n = 7$) using PET with [^{18}F]FDG as the tracer (MicroPET Focus 220, Concorde Microsystems Incorporated). The tracer (70.4 MBq ± 5.6 SEM, 0.5–0.8 ml per animal) was injected intraperitoneally [33], after which the animals were placed back into their home cage. Twenty-five minutes after the injection, the animals were anesthetized with a mixture of medical air and isoflurane. After 45 min of tracer distribution, a static emission scan of 30 min and a transmission scan of 515 s were taken. After the scans, the animals were placed back in their home cage to recover from the anesthesia.

2.4. Microglia activation

Animals were sacrificed 1 week or 3 weeks after treatment with either saline ($n = 5$ per group) or MTX ($n = 8$ per group) 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 0.1% azide until immunohistochemical staining.

Every twelfth serial section from each animal was selected and immunohistochemically stained for IBA-1. In brief, free-floating sections were pre-treated with 0.4% H$_2$O$_2$ for 30 min, to stop endogenous peroxidase activity. Non-specific binding of immunoreagents was blocked with 3% normal goat serum (Zymed, San Francisco, CA, USA). Subsequently, sections were incubated with rabbit-anti-IBA-1 (1:2500, Wako, 019-19741), for 72 h at 4 °C. After a second blocking step, sections were incubated with a biotinylated secondary antibody (goat-anti-rabbit, 1:400, Jackson, Wet Grove, PA, USA) for 1 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 0.075 mg/ml diaminobenzidine (DAB) and 0.003% H$_2$O$_2$ solution.

After mounting of the sections onto glass slides for microscopic analysis, characterization and counting of IBA-1 positive microglia in the dentate gyrus was

performed under a light microscope with a magnification of 400×. Counting was performed in 3 sections between bregma −3.14 and −3.80 [26]. A grid with a surface of 0.0025 mm^2 was placed at the end of the dentate gyrus between the inner and outer blade; all IBA-1 positive microglia in this grid were counted by two different observers blind to the treatment and counts in both blades and 3 slices were summed. Based on morphology, a subdivision for the different stages of the microglia was made for stage 1, stages 2/3, and stages 3/4 [4,20].

2.5. [^{11}C]PK11195 distribution

Animals were transported from the animal facility to the PET centre and back by car. After a 90 min recovery the experiment started. Neuro-inflammation was measured 1 week and 3 weeks after treatment with saline (n = 5) or MTX (n = 7) using PET with [^{11}C]PK11195 as the tracer (MicroPET Focus 220, Concorde Microsystems Incorporated). [^{11}C]PK11195 was prepared according to the protocol described in a paper of Shah et al. [37]. Animals were anesthetized with a mixture of medical air and isoflurane after which the tracer was injected in the penile vein (86.2 MBq ± 10.2 SEM, 0.5–0.8 ml per animal). After 30 min of tracer distribution, a static emission scan of 30 min and a transmission scan of 515 s were taken. After the scans, the animals were placed back in their home cage to recover from the anesthesia.

2.6. Cytokine levels

Animals were sacrificed 5 days or 20 days after treatment with either saline (n = 8 per group) or MTX (n = 8 per group) to study the short- and long-term effect on central nervous and peripheral cytokine levels. The time period of 5 days was chosen since at this moment the effects of MTX are most severe as seen in a decrease in body weight and the presence of diarrhea. The animals were given an intraperitoneal injection of pentobarbital in overdose and a blood sample was taken and placed on ice with 9 μl EDTA per 100 ml blood. Whole blood was centrifuged for 15 min at 4 °C and 2600 g, after which plasma was collected and stored at −80 °C until further analysis. Immediately after the blood sample was taken, the animals were shortly transcardially perfused with saline (4 °C). The brain was rapidly removed and left and right hippocampus were taken out, separated, and stored at −80 °C. A randomly picked left or right side of the hippocampus was homogenized with a pellet pestle motor (Sigma, Z359971-1EA) in Tris–HCl buffer containing 150 mM NaCl, 0.002% Tween-20, and 1 protease inhibitor cocktail tablet per 100 ml solution (Roche, lot 14132300, Manheim, Germany). The homogenate was centrifuged for 15 min at 4 °C and 12000 rpm. The supernatant was collected and stored at −80 °C until further analysis.

Plasma was analyzed using a Bio-Plex rat cytokine 6-plex panel (X8000000E0, Bio-Rad, Veenendaal, the Netherlands) for IL-1β, IL-2, IL-6, IL-10, IFN-γ and TNF-α. Homogenized hippocampus tissue was analyzed using a Bio-Plex rat cytokine 9-plex panel (171-K11070, Bio-Rad, Veenendaal, the Netherlands) for IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ and TNF-α. The entire procedure, as described in the Bio-Rad assay protocol, was conducted at room temperature with the assay solutions provided by Bio-Rad. Standards and samples in duplex were incubated in a 96-well plate with the bead solution for 30 min. The wells were washed with washing solution and subsequently the detection antibody was incubated for 30 min. After washing the wells, streptavidin solution was added for 10 min, followed by washing steps. Assay buffer was added to the wells and the plate was analyzed using a Luminex 100 IS system (Luminex Corporation, Austin, TX, USA).

2.7. PET data analysis

The PET scans were reconstructed with MicroPET Manager 2.3.3.6 (OSEM2D) with a Gaussian smoothing filter of 1.3. Analysis of the PET scans was performed with MicroPET ASIPro VM 6.3.3.0 (CTI Concorde Microsystems, LLC). Regions of interest were drawn in nine horizontal images of the hippocampus [26], and the volume of interest was calculated in Bq/cc. The standardized uptake value (SUV) was calculated according to the following equation:

$$SUV = \frac{[\text{tissue activity concentration (MBq/cc)}]}{[\text{injected dose (MBq)} \times \text{body weight (g)}]}$$

It was assumed that 1 cc of brain tissue equals 1 g.

2.8. Statistics

The data are shown as average with standard error of the mean. The OD of EBA-positive vessels and the cytokine levels were analyzed using a one-way ANOVA. The different stages of IBA-1 positive microglia were analyzed using a multivariate ANOVA. SUV values of the PET images were analyzed using repeated measures ANOVA. Post-hoc tests or contrasts 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.
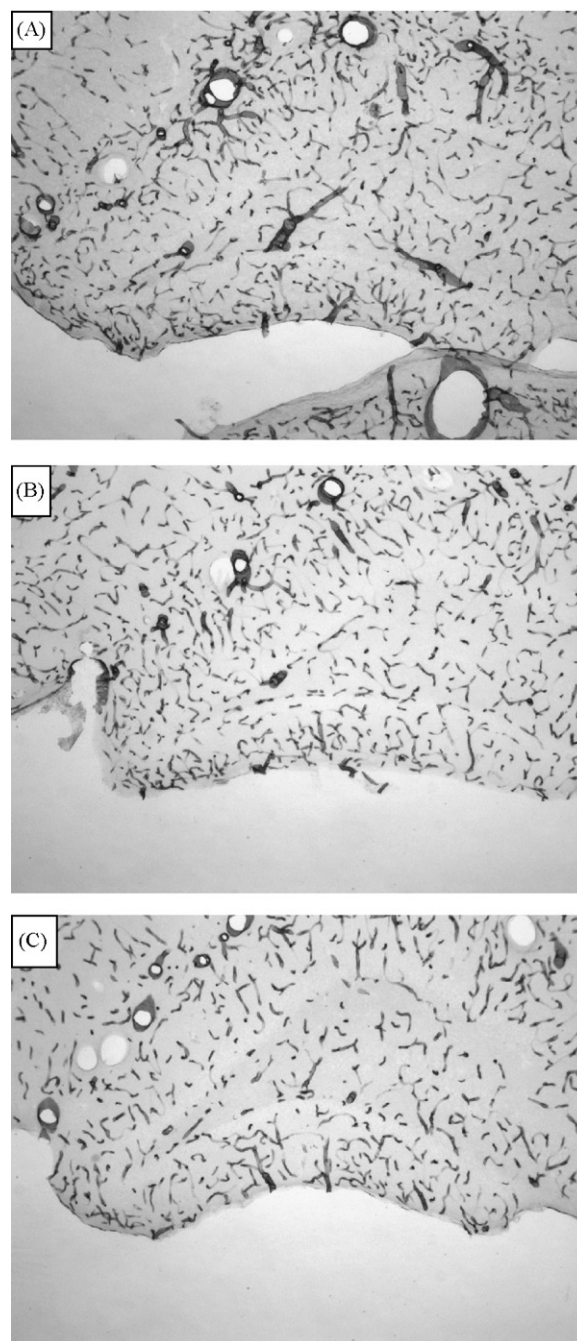

Fig. 1. Overview of the dentate gyrus of the hippocampus stained for EBA of a representative (A) control animal; (B) animal sacrificed 1 week after treatment with MTX; (C) animal sacrificed 3 weeks after treatment with MTX (25×).

3. Results

3.1. Blood vessel density

Control animals and animals treated with MTX were sacrificed 1 week or 3 weeks after treatment. Blood vessels in the hippocampus were immunohistochemically visualized with EBA (Fig. 1) and the optical density measured is presented in Fig. 2. Since there was no significant difference between the control groups (sacrificed 1 week or 3 weeks after treatment), the control data were combined and are shown as one group. The optical density is 195213 ± 7811.7 SEM for control animals, 156530 ± 12558.2 SEM for animals sacrificed 1 week after treatment with MTX, and 157191 ± 19451.3
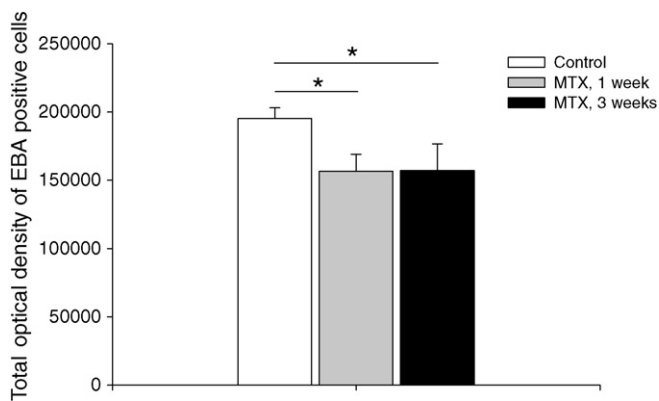
Fig. 2. Optical density of EBA-positive blood vessels in the dentate gyrus of the hippocampus of control rats (open bar, $n = 12$); animals treated with MTX, sacrificed 1 week after treatment (grey bar, $n = 8$); and animals treated with MTX, sacrificed 3 weeks after treatment (black bar, $n = 8$). One-way ANOVA revealed a significant group effect ($F_{2,26} = 3.747$, $P < 0.05$). Post-hoc test revealed that blood vessel density was significantly decreased in both MTX-treated groups (sacrificed 1 week or 3 weeks after treatment, $P < 0.05$).

SEM for animals sacrificed 3 weeks after treatment with MTX. One-way ANOVA revealed a significant group effect ($F_{2,26} = 3.747$, $P < 0.05$) in blood vessel density in the hippocampus. Post-hoc test revealed that blood vessel density was significantly decreased in both MTX-treated groups (sacrificed 1 week or 3 weeks after treatment) compared to the control group ($P < 0.05$).

3.2. [^{18}F]FDG distribution

The effect of MTX on [^{18}F]FDG distribution was measured 1 week and 3 weeks after treatment and Fig. 3 shows representative images of a control animal and animals treated with MTX 1 week and 3 weeks after treatment. The standardized uptake values of the hippocampus are shown in Fig. 4. The standardized uptake value for control animals 1 week and 3 weeks after treatment is 2.089 ± 0.122 SEM and 1.972 ± 0.142 SEM respectively. The standardized uptake value in animals 1 week and 3 weeks after treatment with MTX is 1.362 ± 0.365 SEM and 1.893 ± 0.258 SEM respectively. Repeated measures ANOVA revealed a significant difference between the groups ($F_{1,8} = 6.740$, $P < 0.05$). Post hoc test revealed that animals treated with MTX had significantly lower [^{18}F]FDG uptake during the first scan as compared to the control animals ($P < 0.05$). Three weeks after treatment with MTX [^{18}F]FDG uptake normalized and was similar to the level of control animals.
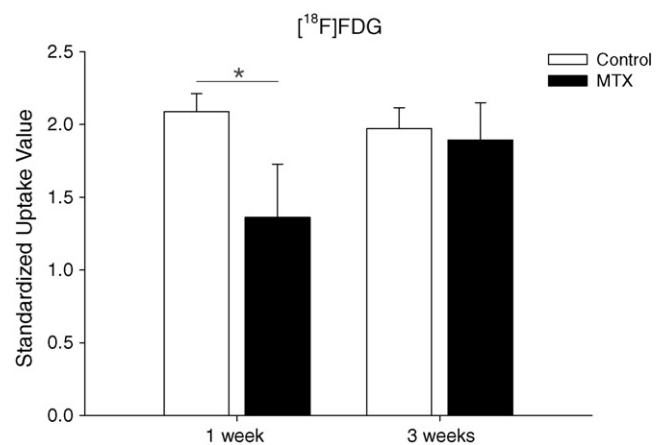

Fig. 4. [^{18}F]FDG distribution in animals treated with saline (open bar, $n = 5$) or MTX (closed bar, $n = 7$) 1 week and 3 weeks after treatment. Data are represented as mean with standard error or the mean. The standardized uptake value significantly differed between the groups, $F_{1,8} = 6.740$, $P < 0.05$ (repeated measures ANOVA). The animals treated with MTX had significantly less [^{18}F]FDG uptake compared with control animals 1 week after treatment, $P < 0.05$. Three weeks after treatment with MTX, [^{18}F]FDG had normalized to the level of control animals.

3.3. Microglia activation

Control animals and animals treated with MTX were sacrificed 1 week or 3 weeks after treatment. IBA-1 positive microglia in microtone sections of the hippocampus were visualized with immunohistochemistry and were categorized into different stages based in their morphology (Fig. 5A–C). Fig. 6 represents the total number of microglia counted; counts of the two observers were not significant different. Since there was no significant difference between the control groups sacrificed 1 week or 3 weeks after treatment, the control data were combined and are shown as 1 group. The total number of IBA-1 positive microglia significantly differed between the groups, $F_{2,22} = 2.715$, $P < 0.05$ (multivariate ANOVA). Tests for contrast revealed that the animals treated with MTX have significantly less resting and more activated microglia than the control animals ($P < 0.05$ for all different phases for animals sacrificed 1 week after treatment with MTX; $P < 0.005$ for phase 1 and phase 2/3 phases and $P < 0.05$ for phase 3/4 for animals sacrificed 3 weeks after treatment with MTX).

3.4. [^{11}C]PK11195 distribution

The effect of MTX on hippocampal [^{11}C]PK11195 uptake was measured 1 week and 3 weeks after treatment and Fig. 7 shows
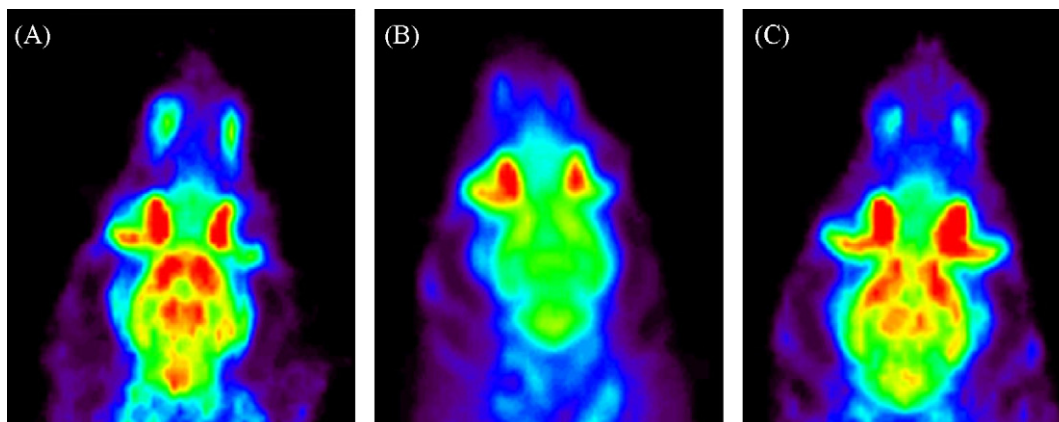

Fig. 3. [^{18}F]FDG distribution in one horizontal plane of a representative (A) control animal; (B) animal scanned 1 week after treatment with MTX; (C) animal scanned 3 weeks after treatment with MTX.
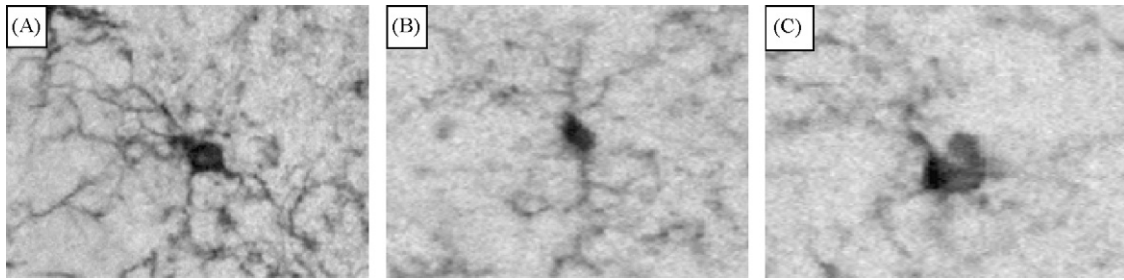
Fig. 5. Different phases of representative IBA-1 microglia (200×). (A) Phase 1; (B) Phase 2/3; (C) Phase 3/4.
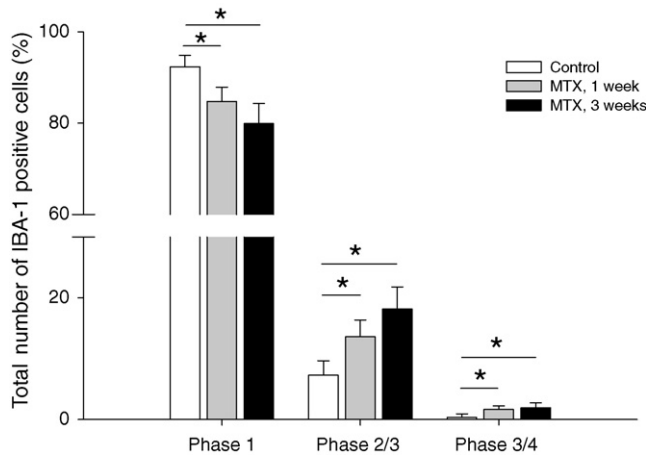

Fig. 6. Total number of IBA-1 positive microglia in the hippocampus of control rats (open bar, $n = 10$); animals treated with MTX, sacrificed 1 week after treatment (grey bar, $n = 8$); and animals treated with MTX, sacrificed 3 weeks after treatment (black bar, $n = 7$). The different stages of microglia are represented as percentage of total number of IBA-1 positive microglia with standard error or the mean. The total number of IBA-1 positive microglia significantly differed between the groups, $F_{2,22} = 2.715$, $P < 0.05$ (multivariate ANOVA). Tests for contrast revealed that the animals treated with MTX have significantly less resting and more activated microglia than the control animals ($P < 0.05$ for all different phases for animals sacrificed 1 week after treatment with MTX; $P < 0.005$ for phase 1 and phase 2/3 phases and $P < 0.05$ for phase 3/4 for animals sacrificed 3 weeks after treatment with MTX).
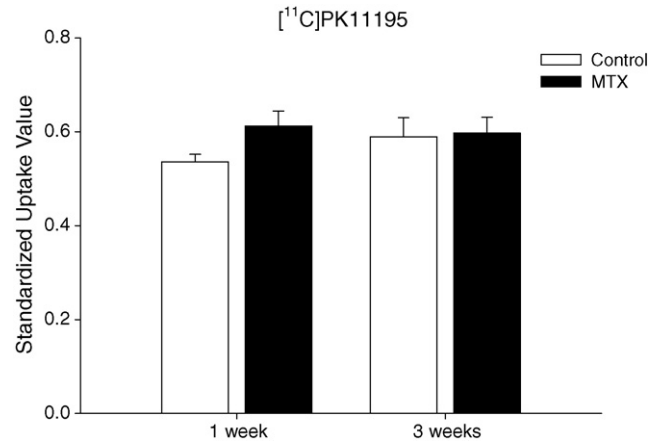

Fig. 8. [^{11}C]PK11195 distribution in animals treated with saline (open bar, $n = 5$) or MTX (closed bar, $n = 7$) 1 week and 3 weeks after treatment. Data are represented as mean with standard error or the mean. No difference was seen in the standardized uptake value between the two groups.

respectively. No significant effects were seen in [^{11}C]PK11195 distribution between the two groups or time points.

3.5. Cytokine levels

Table 1 shows the cytokine levels in plasma 5 days and 20 days following saline or MTX administration. The plasma cytokine levels in the control animals were significantly lower 5 days after treatment compared to 20 days after treatment. The cytokine levels in animals treated with MTX are visualized as percentage of the control levels in Fig. 9. MTX significantly suppressed the plasma levels of all cytokines as compared to control animals 5 days and 20 days after treatment.

representative images of a control animal and animals treated with MTX 1 week and 3 weeks after treatment. The standardized uptake value (Fig. 8) for control animals 1 week and 3 weeks after treatment is 0.536 ± 0.016 SEM and 0.589 ± 0.041 SEM respectively. The standardized uptake value in animals 1 week and 3 weeks after treatment with MTX is 0.613 ± 0.032 SEM and 0.598 ± 0.033 SEM
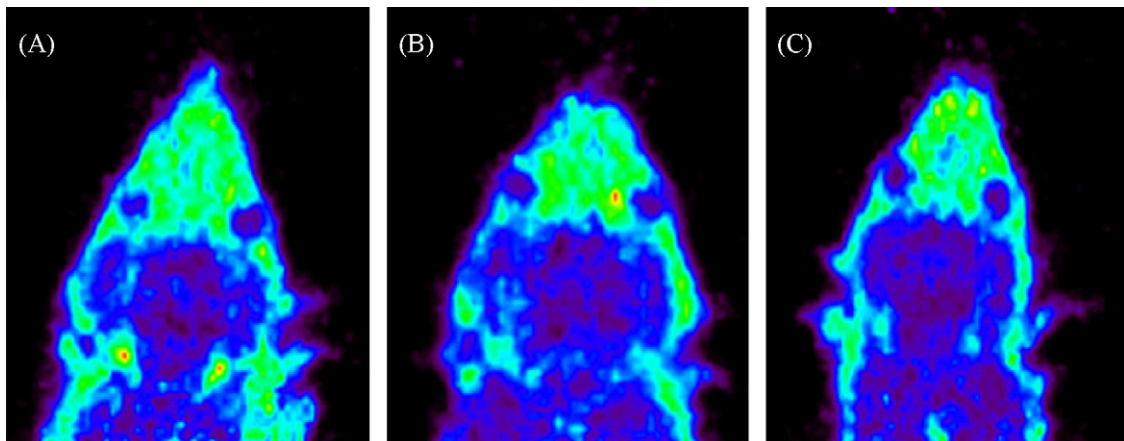

Fig. 7. [^{11}C]PK11195 distribution in one horizontal plane of a representative (A) control animal; (B) animal scanned 1 week after treatment with MTX; (C) animal scanned 3 weeks after treatment with MTX.

Table 1

The absolute values of cytokine levels (pg/ml) in plasma of animals sacrificed 5 days or 20 days after treatment with either saline or MTX ± the standard error of the mean. MTX significantly suppressed all cytokines levels at both time points (degrees of freedom for all F values are 3, 32) with the F and P value shown in the most right columns. The asterisks represent a significant difference between the MTX-treated animals and their corresponding control group, and all asterisks represent a P value smaller than 0.005.

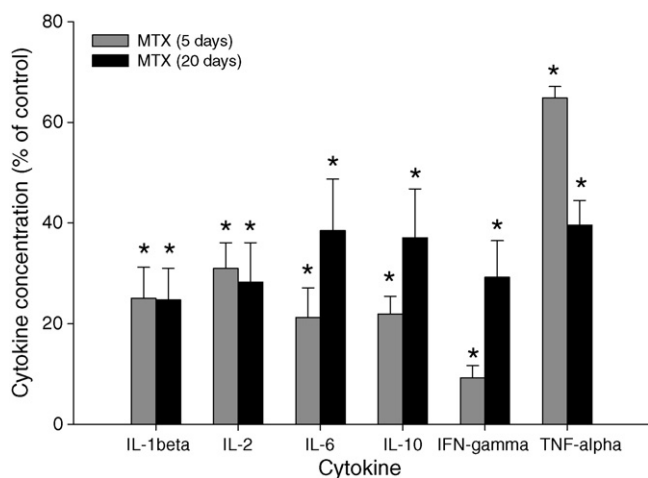|	Control, 5 days	MTX, 5 days	Control, 20 days	MTX, 20 days	F	P
IL-1β	17.4 ± 5.1	43.6 ± 1.1*	100.7 ± 38.8	24.9 ± 6.3*	5.395	0.004
IL-2	79.4 ± 23.1	24.7 ± 3.9*	429.4 ± 143.9	121.6 ± 33.3*	6.631	0.001
IL-6	96.5 ± 30.1	20.5 ± 5.7*	454.4 ± 141.7	174.8 ± 46.6*	6.133	0.002
IL-10	106.2 ± 4.7	23.3 ± 3.7*	491.7 ± 151.1	182.0 ± 47.9*	7.142	0.001
IFN-γ	55.6 ± 32.7	5.1 ± 1.4*	174.8 ± 58.7	51.2 ± 12.6*	4.110	0.015
TNF-α	16.4 ± 2.5	10.6 ± 0.4*	48.6 ± 12.8	19.2 ± 2.4*	7.398	0.001

Fig. 9. Cytokine levels in plasma from animals sacrificed 5 days after treatment with MTX (dark grey bar, $n = 8$), and 20 days after treatment with MTX (closed bar, $n = 8$). The cytokine levels of animals treated with MTX are represented as percentage of controls. MTX significantly suppressed the levels of all cytokines measured 5 days or 20 days after treatment compared to the levels in control animals.

Table 2 shows the cytokine levels in hippocampus homogenates 5 days or 20 days following saline or MTX administration. The cytokine levels in animals treated with MTX are visualized as percentage of the control levels in Fig. 10. Although there is a tendency that the levels of a number of cytokines are suppressed 5 days after treatment with MTX this observation failed to reach significance. Also no effect of MTX administration was seen on any of the cytokine levels measured 20 days after treatment.
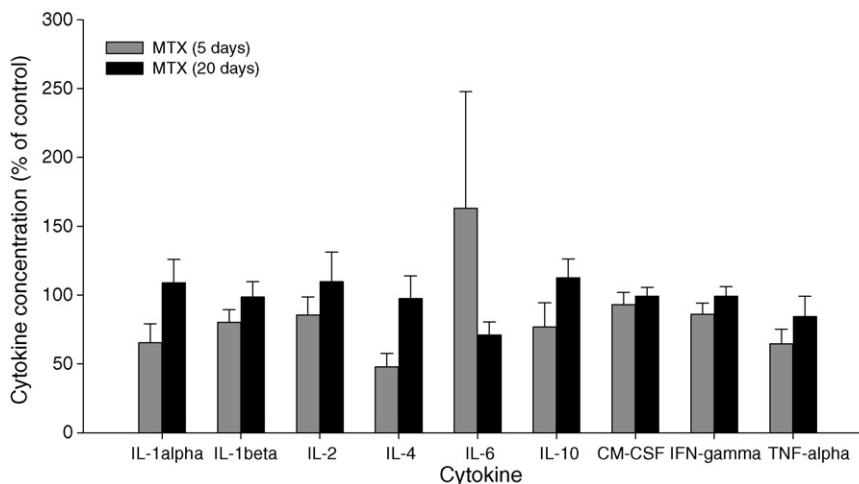
4. Discussion

Cognitive impairment is a long-term side effect in a subgroup of cancer patients that can have a significant impact on daily life functioning and the quality of life of patients [1]. Since the underlying mechanisms of this cognitive impairment are largely unknown we studied the effect of methotrexate (MTX) on potential mechanisms involved: blood vessel density, central glucose metabolism, microglia activation, and neuroinflammation. The immunohistochemical data show that 1 week and 3 weeks after MTX treatment significantly fewer blood vessels at both time points were present as compared to control animals. The PET analysis partially supported this finding showing that central glucose metabolism was decreased 1 week after treatment with MTX. In contrast to the immunohistochemical study, this effect was transient at this level of the PET analysis since it was not present any more 3 weeks after treatment.

Table 2

The absolute values of cytokine levels (pg/mg) in the hippocampus of animals sacrificed 5 or 20 days after treatment with either saline or MTX ± the standard error of the mean. No significant differences were seen between the different treatments or time points.

	Control, 5 days	MTX, 5 days	Control, 20 days	MTX, 20 days	P
IL-1α	0.70 ± 0.11	0.46 ± 0.10	0.43 ± 0.09	0.47 ± 0.07	n.s.
IL-1β	8.32 ± 0.60	6.67 ± 0.76	6.52 ± 0.72	6.42 ± 0.73	n.s.
IL-2	0.35 ± 0.05	0.30 ± 0.05	0.22 ± 0.06	0.29 ± 0.05	n.s.
IL-4	0.07 ± 0.01	0.03 ± 0.01	0.08 ± 0.02	0.06 ± 0.02	n.s.
IL-6		Not detectable			
IL-10	3.58 ± 0.48	2.75 ± 0.62	3.21 ± 0.79	3.61 ± 0.44	n.s.
GM-CSF	0.73 ± 0.05	0.67 ± 0.07	0.64 ± 0.06	0.63 ± 0.04	n.s.
TNF-α	0.83 ± 0.06	0.53 ± 0.89	0.75 ± 0.18	0.63 ± 0.11	n.s.
IFN-γ	4.84 ± 0.22	4.16 ± 0.39	4.22 ± 0.34	4.18 ± 0.30	n.s.

Fig. 10. Cytokine levels in hippocampal tissue from animals sacrificed 5 days after treatment with MTX (dark grey bar, $n = 8$), and 20 days after treatment with MTX (closed bar, $n = 8$). The cytokine levels of animals treated with MTX are represented as percentage of controls. MTX did not suppress the levels of any cytokine measured 5 days or 20 days after treatment compared to the levels in control animals.

Decreased cerebral blood flow or a lower blood vessel density caused by cytostatic compounds can lead to impaired brain functioning. In a study of Silverman and colleagues breast cancer survivors treated with adjuvant chemotherapy were compared to healthy controls for resting metabolism and blood flow in the brain. PET scans were made with [^{15}O] water during control and memory-related tasks; resting metabolism was measured with [^{18}F]FDG. Altered cerebral blood flow was measured in specific regions such as the frontal cortical areas and cerebellum in chemotherapy-treated subjects and compared to healthy subjects. Resting metabolism was significantly lower in the inferior frontal gyrus in chemotherapy-treated patients as compared to control subjects who had never received chemotherapy. This lowering correlated with the performance on a short-term memory task that was found to be particularly impaired in chemotherapy-treated subjects [38]. A study by Mizusawa and colleagues showed that MTX has an acute effect on local cerebral blood flow in rats. MTX was infused into the femoral vein of conscious male Sprague-Dawley rats for 2 h and cerebral blood flow was measured with [^{14}C]iodoantipyrine. Compared to control conditions, MTX reduced cerebral blood flow in a number of regions, including the hippocampus and frontal cortex [23]. Our PET results show a transient decrease in FDG uptake in the hippocampal region. Glial cells and neurons both participate in the uptake of glucose from the blood [19]. It is not possible to conclude on the basis of the present experiments what the contribution of neuronal and glial FDG uptake is.

MTX is not only associated with decreased blood flow, but also with damage to endothelial cells [22,43]. In the latter study, female Balb/c mice were given intraperitoneal injections of methotrexate on day 1, 3, 6, and 11 and blood samples were collected every other day. MTX induced elevation of circulating endothelial cells, which lasted for 3 weeks after the first injection [43] suggesting lasting endothelial damage. In our study, 3 weeks after treatment there were still less blood vessels in animals treated with MTX compared to control animals. This also indicates that the effect of MTX on endothelial cells is long lasting. The negative effect of MTX on endothelial cells was also seen in cultured bovine pulmonary artery endothelial cells. MTX induced apoptosis in the cells causing the number of cells to decline after exposure to therapeutic levels of MTX. Also an altered morphology was observed in MTX-treated cells with large gaps between the affected endothelial cells. This effect was unexpected because the cells used in this experiment were mitotically quiescent. Since there are many similarities between the pulmonary artery endothelial cells as used in this study and brain endothelial cells, the authors propose that the effect of MTX on nondividing endothelial cells may play a role in the cognitive impairment seen in children treated for acute lymphoblastic leukemia. The authors hypothesize that damage to endothelial cells might cause platelet aggregation which interferes with brain blood flow and can cause neuronal injury and death [22]. Our results suggest that next to this, damage to endothelial cells also may result in a reduction of vascularization of the brain. Brain endothelial cells are mitotically quiescent similar to the cells used in the study of Merkle et al. However, these cells are also affected by MTX and go into apoptosis. Since these cells do not divide frequently [22], this means that damage may last long before it is repaired which possibly explains why blood vessel density is still decreased 3 weeks after treatment with MTX.

The other question we aimed to address was whether MTX can induce neuroinflammation contributing to the cognitive deficits. Our immunohistochemical data showed that animals treated with MTX clearly had more activated microglia in the hippocampus than control animals, on the short-term (1 week after treatment) as well as on the long-term (3 weeks after treatment). Since microglia can be activated by elevated cytokine levels indicative of neuroinflammatory processes which are associated with cognitive impairment as well [3,40] animals were also scanned for the uptake of [^{11}C]PK11195. Increased binding of this marker for peripheral benzodiazepine receptors in the brain is regarded as an indication of neuroinflammation [5,29]. However, the PET study revealed no difference in tracer uptake between control animals and animals treated with MTX. This suggests that PET might be a tool that is not sensitive enough to detect the relatively small changes in the rat brain elicited by MTX treatment. Therefore, we also measured the levels of several cytokines in plasma and in homogenized hippocampal tissue in rats. No effect was seen in the cytokine levels in the hippocampus 5 days or 20 days after treatment. This supports the idea that the effects of MTX on microglia activation are not mediated by induction of neuroinflammation and it also indicates that the cognitive impairment following MTX treatment is not due to neuroinflammation.

MTX significantly suppressed the level of several cytokines in plasma both on the short- as well as the long-term. It has long been known that MTX in high dosages has a long-lasting suppressive action on hemopoietic stem cells [25]. Low dose MTX is prescribed to people with chronic inflammatory diseases such as rheumatoid arthritis and psoriasis. Both diseases are associated with high levels of T-cells, especially CD4$^+$ T cells, and high cytokine levels. MTX treatment normalizes these levels and the balance of T lymphocytes [14,18,24]. The MTX dosage prescribed to people suffering from rheumatoid arthritis and psoriasis is lower than the dosage given in adjuvant chemotherapy. Since low dosages already suppress cytokine levels in people with inflammatory diseases, it is likely that a high dosage of MTX has a much more profound lowering effect on cytokine levels than a low dosage, even on normal cytokine levels, which might explain the results found in this paper.

The cytokine levels in control animals were significantly lower 5 days after treatment compared to 20 days after treatment. This decrease is possibly caused by the four intraperitoneal injections that the animals received as a control for the leucovorin treatment. Repeated i.p. injections are stressful for the animals and stress is known to increase glucocorticoid release, which in turn exhibits an anti-inflammatory response by suppressing cytokine release [10].

The effects found in this study suggest that the activation of microglia and the cognitive impairment seen after treatment with MTX in previous studies [34,35] is not caused by inflammation or an increase of central cytokines. Activation of microglia occurs in response to local homeostatic challenges in the brain. The challenges can have a very diverse starting point. Small damage to the CNS, such as a declining neuron or astrocyte, might also be able to activate microglia to repair the damage. When fully activated, microglia is potentially neurotoxic, although activated microglia can also exert a neuroprotective response depending on the activating stimuli [15,28]. Our results clearly showed that MTX activates microglia; the role and function of this activation has to be further elucidated.

Increasing numbers of newly diagnosed cancer patients are receiving chemotherapy and every year there are more long-term cancer survivors as the mortality rate has fallen substantially for this disease. With the more widespread use of chemotherapy and patients' increasing survival rates there is also an increasing incidence of cognitive problems during and after exposure to chemotherapy. Studies that try to pinpoint the biological principles underlying these cognitive symptoms are relevant from both a descriptive as well as a preventive point of view.

Previous studies performed in our lab have shown that MTX induces cognitive impairment in rats. Animals treated with MTX did not adequately learn a cognitive task, as shown in a Morris water maze and a novel object recognition task. Furthermore, MTX also impaired the ability to consolidate a previous learned task, when treatment was given directly after the learning phase of a

Morris water maze and contextual fear conditioning [34,35]. The results from this paper suggest that cognitive impairment may be related to negative effects of MTX on blood supply to the brain by a decreasing blood vessel density. Since no effect was observed on [^{11}C]PK11195 tracer uptake or on hippocampal cytokine levels, the cognitive impairment seen after adjuvant chemotherapy is not likely to be caused by neuroinflammation.

Acknowledgments

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