University of Groningen

Chemotherapy

Seigers, Riejanne

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

[Link to publication in University of Groningen/UMCG research database](#)

Chapter 7

General discussion

Summary of the results

The objective of this thesis was to gain more insight in the potential neurobiological processes that underlie the cognitive impairment seen after adjuvant chemotherapy in some patients. Therefore, rats were treated with methotrexate (MTX), a cytostatic agent that has been frequently applied in chemotherapy cocktails for breast cancer and is associated with cognitive impairment in a number of animal studies.

In **chapter 2** we studied the effect of MTX on cognition and hippocampal cell proliferation. A single high dose of MTX induced a learning impairment as measured in a Morris water maze and a novel object recognition task, two and three weeks after treatment respectively. MTX also affected hippocampal cell proliferation in a dose-dependent manner. Because hippocampal cell proliferation is thought to play an important role in learning and memory, our findings are suggestive of a lasting decrease in cell proliferation that may contribute to the learning deficit after MTX treatment.

Chapter 3 explored the effect of MTX on the consolidation of a previously learned memory and the short-term effect on hippocampal cell proliferation and white matter density. Animals were treated with a single high dose of MTX directly after Morris water maze learning or contextual fear conditioning. Treated animals were less able to remember the location of the platform or the foot shock when they were retested which suggests that MTX impairs the ability to consolidate memory. To test the hypothesis that this cognitive impairment may also be mediated by effects of MTX on hippocampal cell proliferation, Ki-67 immunohistochemistry was performed one day and one week after treatment. One day after treatment, no significant decrease in Ki-67 positive cells was present although the data showed a large individual variation. One week after treatment, a significant decrease in hippocampal cell proliferation was seen. MTX also significantly decreased the density of white matter measured in the lateral corpus callosum shortly after treatment (one day) as well as on the long-term (three weeks).

In **chapter 4** we explored the effect of MTX on cognition several months after administration. Animals treated with MTX did not show impaired learning behaviour six months after treatment as observed in the Morris water maze, novel object recognition task, and contextual fear conditioning. However, MTX did affect anxiety as measured in an elevated plus maze, but this observation was not seen shortly after treatment. These findings indicate that rats functionally fully recover from MTX induced cognitive deficits after longer time intervals.

Chapter 5 was aimed at a further improvement of the animal model by exploring the effect of MTX on hippocampal cell proliferation in tumor-bearing animals. This is an important control in animal models studying cognitive deterioration following administration of cytostatic compounds since in the clinical setting MTX is generally given to patients suffering from cancer. Furthermore, cognitive impairment is also noticed in some patients after the diagnosis and before any systemic treatment is initiated. MTX significantly decreased the number of proliferating cells in the hippocampus, regardless of the presence of a tumor. In tumor-bearing animals, a decrease in hippocampal cell proliferation was seen although this observation failed to reach significance, and the tumor furthermore failed to potentiate the effect of MTX. The absence of an interaction effect supports the validity of our previous results in healthy animals treated with MTX.

In **chapter 6** we explored the hypothesis that the cognitive deficits observed after MTX treatment may be mediated by changes in brain vascularization, central glucose metabolism, microglia activation, and neuroinflammation. MTX significantly decreased blood vessel density one week and three weeks after treatment as measured with EBA immunohistochemistry. Since decreased blood vessel density may relate to decreased central glucose metabolism, [^{18}F]FDG PET was performed. Methotrexate reduced tracer uptake in the hippocampal region one week after treatment, which was not seen three weeks after treatment. MTX activated microglia one week and three weeks after treatment as measured with IBA-1 immunohistochemistry. Since microglia activation may be indicative of neuroinflammation, [^{11}C]PK11195 PET was performed one week and three weeks after treatment. No difference in tracer

uptake was seen between control animals and animals treated with MTX. To further explore the possible role of (neuro)inflammation in the cognitive effects of MTX, cytokine levels were measured in plasma and homogenized hippocampal tissue five and twenty days after treatment. No differences in cytokine levels were found in the hippocampus between control animals and animals treated with MTX. However, MTX caused a striking reduction in plasma cytokine levels five and twenty days after treatment indicating a lasting suppression of peripheral immune functioning. This suggests that the microglial activation in this study is not a marker for neuroinflammation and that the cognitive impairment seen after adjuvant chemotherapy is not caused by an increase in central cytokine release or neuroinflammation.

Which neurobiological processes underlie cognitive impairment?

In this thesis, we aimed to find objective evidence in an animal model that cytostatic treatment can lastingly affect brain structures that are involved in cognitive processes. In patients it is difficult to dissociate cognitive deterioration caused by the chemotherapy, the cancer itself, and the emotional stress from being diagnosed with a potentially lethal disease. Furthermore, the risk of cognitive impairment may simply be a reflection of a genetic vulnerability to cancer (Ahles and Saykin, 2007). We tried to find objective evidence for the cognitive impairment after adjuvant chemotherapy at the behavioral level using learning and memory tasks and neurobiologically by exploring a number of potential underlying mechanisms including hippocampal cell proliferation, microglia activation, (neuro)inflammation, blood vessel density, central glucose metabolism, and white matter density.

Our experiments show that MTX treatment clearly has negative effects on learning and memory processes. Moreover, it reduces hippocampal neurogenesis and affects brain vascularization, glucose metabolism, white matter density, and microglia activation as well. The experiments do not allow a conclusion to what extent these changes at the level of the brain may explain the behavioral changes. To provide evidence that mechanisms are involved in the cognitive impairment following chemotherapy, one should experimentally prevent or restore the neurobiological changes caused by the cytostatic agents and show that cognitive performance is retained. This strategy is scarce in the literature; most papers describe the effects of cytostatics on either cognition or neurobiological processes. However, Konat and colleagues did study a potential mechanism that can explain cognitive impairment and how this mechanism can be restored. Female Sprague-Dawley rats were treated with doxorubicin and cyclophosphamide in combination with the antioxidant N-acetyl cysteine. The authors showed in this paper that the antioxidant treatment did prevent oxidative stress and the occurrence of cognitive impairment (Konat *et al*, 2008). The data described in this thesis will help to further specify hypotheses on the causal brain mechanisms involved in cognitive impairment after chemotherapy. Clearly, more mechanistic experiments have to be performed aimed at causation before current chemo-therapeutic strategies can be improved.

In this thesis, we explored the effect of MTX on hippocampal cell proliferation, a process thought to play an important role in learning and memory (Gould *et al*, 2000; Kemperman *et al*, 2002; Kemperman *et al*, 2004). However, neurogenesis is probably not the only process involved in the development of cognitive impairment after adjuvant chemotherapy. This is supported by clinical data showing that chemotherapy mostly affects cortical functions, and in particular executive functions (Ahles and Saykin, 2007). Furthermore, the exact role and function of neurogenesis in cognitive performance is highly debated.

We showed in chapter 5 that MTX decreases blood vessel density and that this effect can already be noticed one day after treatment. In the introduction of this thesis the close relation between brain vascularization and neurogenesis was already indicated (Palmer *et al*, 2000). This reduction in blood

vessel density with the accompanying reduction in energy supply and proliferative signals may play a major role in the decreased hippocampal cell proliferation as seen in chapter 2 and 3.

In chapter 6 we explored the hypothesis that MTX treatment induces (neuro)inflammation and activates microglia. Microglia are the immune cells of the brain and are activated in response to homeostatic changes (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009). When activated, microglia cells secrete cytokines and chemokines to attract more microglia to the activation site (Garden and Möller, 2006). Increased activation of microglia could lead to neuroinflammation, which is associated with cognitive impairment (Wilson *et al*, 2002). Furthermore, neuroinflammation and microglia activation are also known to have a negative effect on neurogenesis (Das and Basu, 2008; Ekdahl *et al*, 2003). Neuroinflammation can be a direct, central effect, but may also be caused by an indirect, peripheral effect. Chemotherapy treatment can induce a peripheral inflammatory cytokine response, which can lead to inflammation and central cytokine release (Seruga *et al*, 2008; Wilson *et al*, 2002). In our experiment, plasma cytokine levels were lastingly decreased after MTX treatment. This probably is due to the strong inhibitory effect of MTX on stem cells in the bone marrow (Pannacciulli *et al*, 1982). In fact, MTX is prescribed to people that suffer from chronic inflammatory diseases and MTX normalizes the high levels of T-cells and cytokine levels in these patients (Gerards *et al*, 2003; Johnston *et al*, 2005; Morita *et al*, 2006). However, one should be cautious in excluding the possibility of a local peripheral inflammatory response as mucositis since the inhibitory effect of MTX on bone marrow might overrule an increase caused by local infection.

MTX treatment activated microglia without increasing hippocampal cytokine levels. Microglia activation is not an indication of neuroinflammation per se, but more a change in phenotype. The result of this activation is dependent on the signals presented to and secreted by the microglia and therefore not per definition a marker for neuroinflammation (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009).

In summary, we can conclude from our data that MTX decreases hippocampal cell proliferation, blood flow, central glucose metabolism, and white matter density. Furthermore, microglial cells were activated, without the presence of neuroinflammation. Even though all these processes can individually impair cognition, they can also have additive effects on other neurobiological processes involved in cognitive performance. There is presumably not one causal factor in the development of cognitive impairment after treatment with MTX, but MTX rather induces diffuse and diverse changes in the brain that jointly induce cognitive impairment.

Validation of the animal model

There are a number of differences between clinical studies and the animal studies performed in this thesis or in the literature in general. These differences include differences in cognitive impairment between animals and humans, individual vulnerability for cognitive impairment, age and gender, treatment strategy, but also the cognitive tasks used in the different studies, and the presence (or absence) of a tumor. By describing these differences I will try to validate my animal model with the focus on face validity.

Differences in cognitive impairment between animals and humans

A large difference between clinical and animal studies is the time period in which cognitive impairment can be seen. In cancer survivors the cognitive impairment after adjuvant chemotherapy can be noticed up to years after treatment (Bender *et al*, 2006; Correa and Ahles, 2008; Wefel *et al*, 2008). However, in animals most studies are performed shortly after treatment, with time periods ranging from minutes (Boyette-Davis *et al*, 2009; Foley *et al*, 2008; Phillips *et al*, 1986) to days or weeks (Eijkenboom *et al*, 1999; Gandal *et al*, 2008; Konat *et al*, 2008; Li *et al*, 2008; MacLeod *et al*, 2007; Madhyastha *et al*, 2002; Reiriz *et al*, 2006; Seigers *et al*, 2008; Seigers *et al*, 2009; Sieklucka-Dziuba *et al*, 1998; Winocur *et al*, 2006) and in some studies even up to months (Lee *et al*, 2006; Stock *et al*, 1995;

Yanovski *et al*, 1989). When we tested cognitive performance six months after treatment, no significant differences in learning behavior were seen (chapter 4). Furthermore, cognitive impairment was also not found in other long-term animal studies (Lee *et al*, 2006; Stock *et al*, 1995). This suggests that rats are either more resilient to the damage or are more capable to restore the damage caused by the cytostatic treatment and attention to this difference between humans and animals should be paid when designing experiments.

Individual vulnerability for cognitive impairment
In the clinic, only a subgroup of patients seems to suffer from cognitive impairment. This could mean that also in animals only a subgroup shows an effect of cytostatic treatment on cognition and neurobiological processes, which could lead to false positive or false negative results. However, most animal studies including ours used a genetically rather homogeneous rodent species in which individual difference is minimal. Humans are less homogeneous and differences in gene expression, IQ, co-morbidity, etc. could lead to differences in cognitive impairment. Ahles and colleagues explored the relationship between the presence of the ε4 allele of the apolipoprotein E (APOE) gen and cognitive impairment following adjuvant chemotherapy. The APOE gen is a polymorphic gene that occurs in three common alleles, ε2, ε3, and ε4. The presence of the ε4 allele of APOE is associated with an increased risk for the development of Alzheimer's disease. Cancer patients treated with adjuvant chemotherapy carrying at least one ε4 allele scored lower in visual memory and spatial ability domains then patients carrying the other alleles, suggesting that the ε4 allele may be a potential marker for the development of cognitive impairment after adjuvant chemotherapy (Ahles *et al*, 2003). However, it cannot be excluded that cancer patients carrying the ε4 allele of the APOE gen already have a higher risk of cognitive impairment independent of chemotherapy treatment since this study did not include healthy controls or cancer patients who had not received chemotherapy.

Other individual differences amongst humans can include genes encoding for multi-drug resistance pumps, DNA repair mechanisms, telomere length/ activity, cytokine regulation, neurotransmitter activity, and hormone production. It is known that the functionality of multi-drug resistance pumps differs among the human population. A more functional pump can clear the cytostatic agent faster out of the brain which could result in less damage and a decreased risk of developing cognitive impairment, whereas a less functional pump will take more time to pump the cytostatic out of the brain which can lead to more damage and an increased risk of developing cognitive impairment. Furthermore, DNA and DNA repair systems are affected by chemotherapy, and in people with more (pre-existing) damage or impaired repair systems this could lead to a higher chance of developing cognitive impairment (Ahles and Saykin, 2007).

From an experimental point of view, it is most appropriate to first explore the effects of chemotherapy on neurobiological processes involved in cognition in a homogenous set of animals. When the affected processes are fully explored, they should also be studied in a less homogenous group of animals as well, to examine the basis of the individual variation as seen in patients. These studies could be conducted in a wild-type population or in animals that have been genetically manipulated and lack, for example, certain multi-drug resistance pumps.

Age and gender differences
A large difference between clinical and animal studies is the age of the subjects. Most clinical studies showing cognitive impairment after chemotherapy were performed in middle aged people, since this age group is most at risk to develop cancer, hence forming a large study population. The majority of animal studies are performed in young, healthy animals even though age and age-related disorders can also influence cognitive behavior. However, the young adult animals used in this thesis did suffer from cognitive impairment after treatment with MTX, which suggests that the process of aging is not prerequisite for this impairment. However, to fully examine the role of age on chemotherapy-induced

cognitive impairment, aged animals should be treated with cytostatic agents followed by exploration of cognitive behavior.

In all chapters of this thesis, male rats were used to study the effect of chemotherapy on cognition. However, the cytostatic agent used in these experiments was MTX, which is generally given to women in a chemotherapy cocktail for breast cancer. Furthermore, the majority of clinical studies have been performed in women suffering from breast cancer. The rational behind this gender preference is that breast cancer is a common type of cancer leading to a large study population. This might suggest that female rodents are more suitable to study the effects of chemotherapy on cognition than male rodents. However, a (small) number of studies have also been performed to explore the effects of chemotherapy on cognition in men and it appears that men are just as vulnerable as women to develop cognitive impairment (Ahles *et al*, 2002; Ahles *et al*, 2003; Ahles *et al*, 2005). The fact that present animal studies in male individuals show cognitive deficits in combination with changes in neurocognitive substrates after cytostatic treatments suggests that using male rodents is as appropriate to study cognitive impairment as using females.

Generally, male rodents are most frequently used to study the effect of chemotherapy and only a small number of studies used female rodents or both genders. There are large physiological differences between male and female rodents. Males have constant hormone levels, whereas in females the levels of the different hormones change every day according to the estrogen cycle. This variation in estrogen levels is associated with changes in cognitive performance in humans (Hampson, 1990) as well as in rodents (Bimonte-Nelson *et al*, 2003). Hampson studied the effect of variations in hormone levels across the menstrual cycle in women. It was shown that these fluctuations affect cognition when tested at two different moments in their menstrual cycle, representing maximal (preovulatory phase) and minimal (menstrual phase) estrogen levels. Women during their preovulatory phase performed better in tests of speeded manual, fine motor, and verbal articulatory skills and worse on spatial ability and abstract reasoning compared to performance during menstruation (Hampson, 1990). Furthermore, chemotherapy is known to induce premature menopause (Ganz *et al*, 2005; Van Dam *et al*, 1998) which is associated with decreased estrogen levels (Buckler, 2005). A confusing issue in relation to studying cognitive aspects of reproductive hormones in animal models is that low estrogen levels are associated with impairment in tasks in which women as a group excel, such as verbal memory and verbal fluency (Sherwin, 2000), whereas in rodents low estrogen levels are associated with increased learning ability (Bimonte-Nelson *et al*, 2004; Healy *et al*, 1999). This suggests that it is less complex to use male rodents to explore the neurobiological processes underlying cognitive impairment than using female animals.

Differences in treatment strategy

In this thesis, animals were treated with a single high dose MTX to explore the effects on cognition. However, in the clinic, cytostatic agents are always given in a chemotherapy cocktail which is repeatedly administered for a number of days/ weeks since this treatment strategy increases the efficacy of the agents. The rationale of using a cocktail of cytostatics is that multiple pathways of the cell division are attacked. This suggests that a single treatment with only one cytostatic can yield an underestimation of the damage caused by chemotherapeutic cocktails. However, from an experimental point of view, treating animals with multiple cytostatics or multiple injections with one cytostatic has a number of downsides. First, when more than one cytostatic is given, it is impossible to say which cytostatic agent is responsible for which effect. Second, multiple injections or multiple cytostatics and the timing of the administrations increase the risk of side effects such as sickness, which in itself can also have an effect on cognition (Lee *et al*, 2004). Third, it is stressful for an animal to receive multiple injections, and stress is also known to have a negative effect on learning and memory (Alzoubi *et al*, 2009; Bowman, 2005; Kasar *et al*, 2009; Wang *et al*, 2009). To fully explore the effects of a cytostatic

drug on neurobiological processes, the agent should be given in a single high dose, before combination studies are performed.

MTX is considered to be an "older" cytostatic, meaning that it has been on the market for a long-time and is not often prescribed anymore in cancer treatment. It used to be very frequently applied in the CMF (cyclophosphamide, MTX, and 5-fluorouracil) cocktail for breast cancer, but this treatment strategy is nowadays generally replaced by other cocktails. This means that the newer cytostatics have to be tested to see if they exhibit the same effects as MTX. However, we have indicated in the introduction of this thesis that, in general, the different cytostatics give rise to the similar complaints and disturb similar neurobiological processes, such as impaired neurogenesis, oxidative stress, white matter damage, blood flow impairment, and neurotoxicity. This suggests that many cytostatic agents act in a similar way on neurobiological substrates. However, this suggestion needs to be substantiated in a systematic approach where individual cytostatics will be tested in their effects on cognitive performance and multiple neurobiological processes.

Which cognitive tasks to use?

The cognitive tasks used in this paper were of hippocampal nature, meaning that we studied the effect of MTX on hippocampal learning capacity such as spatial learning in the Morris water maze. These tasks were chosen since they are well described in the literature and are relatively easy to execute. However, the cognitive functions that are affected by chemotherapy are not restricted to hippocampal tasks only. Also other functions and brain regions, many of them being cortical areas, are affected by chemotherapy e.g. working/ episodic/ remote memory (bilateral prefrontal and parietal regions, frontal and (medial) temporal lobes, and the prefrontal cortex), verbal/ visual memory (left and right hemisphere), executive function (bilateral dorsal lateral prefrontal cortex), processing speed (distributed frontal subcortical network), visual/ spatial/ constructional ability (right parietal and bilateral frontal regions), attention/ concentration (distributed frontal subcortical network), reaction time (distributed frontal subcortical network), and motor speed/ dexterity (bilateral frontal and pyramidal/ extrapyramidal motor systems) (Ahles and Saykin, 2007).

The majority of the cognitive tasks used for clinical studies are not applicable to rodent studies. Most cognitive tasks in the clinical setting involve word/ color recollection which is impossible to perform with rodents. However, since in animals spatial memory is affected after cytostatic treatment, as seen in a Morris water maze paradigm (Eijkenboom *et al*, 1999; Li *et al*, 2008; Seigers *et al*, 2008; Seigers *et al*, 2009; Winocur *et al*, 2006), this suggests that testing spatial memory in animals is representative for cognitive impairment as seen in patients. But more attention should be paid to also explore cortical learning tasks with, for example, a delayed reinforcement paradigm. In this paradigm, animals are trained to press a lever for a delayed food reward. This task is cortical dependent since animals that had received a lesion in the orbital region of the prefrontal cortex were not able to learn this task adequately (Cardinal *et al*, 2004; Mobini *et al*, 2002). However, exploring the cognitive domains affected was not the main aim of the thesis, but simply served as a read-out to see if the cytostatic treatment induced damage. And as mentioned previously, spatial memory is affected by cytostatic treatment in animals, suggesting that testing this type of cognition in rats treated with cytostatic agents is sufficient to explore cognitive impairment.

What is the role of cancer in the development of cognitive impairment?

Only one study in this thesis was performed in which rats were given a tumor, to study the combined effect of MTX and cancer on hippocampal cell proliferation. We showed in this study that there is no interaction effect between MTX and the presence of a tumor, meaning that the cancer did not have an extra negative effect on hippocampal cell proliferation when combined with MTX. This suggests that our animal model in which we treat healthy male rats with MTX is a good model to explore the potential neurobiological processes involved in cognitive impairment. However, no other studies are

known in the literature in which chemotherapy was given to a tumor-bearing animal to study cognitive impairment or the neurobiological mechanisms involved. Therefore, more studies have to be performed to explore the effect of cancer and chemotherapy on cognition and cognition-associated neurobiological processes.

Another factor contributing to the individual differences in cognitive impairment could be the role of cancer-associated stress. This so-called distress is very frequently described in the literature and the National Comprehensive Cancer Network states that distress is 'a multi-determined unpleasant emotional experience of a psychosocial (cognitive, behavioral, emotional), social, and/ or spiritual nature that may interfere with the ability to cope effectively with cancer, its physical symptoms, and its treatment'. The number of patients that suffer from this distress differs amongst the clinical studies with percentages varying between 6 to 62% (Desaive and Ronson, 2008). This distress can possibly influence cognition and neurobiological processes via the permeability of the blood-brain barrier. A recent paper from De Klerk and colleagues show that chronic mild stress decreases the functionality of P-glycoprotein, an efflux transporter highly important for drug entry into the brain, which may lead to enhanced vulnerability to the influx of harmful substances (De Klerk *et al*, 2009). This suggests that the stress of the diagnosis and treatment may decrease the functionality of the P-glycoprotein as well, leading to enhanced influx of the cytostatic agents in the brain, causing more damage.

The role of cancer or cancer-related stress on cognitive impairment can not be excluded, but was not the main aim of this research line. Furthermore, combining cancer and chemotherapy makes it more difficult to dissociate which effects are responsible for the cognitive impairment.

Concluding remarks

In the previous paragraphs I have given evidence that the animal model used in this thesis is a valid model to explore potential neurobiological processes underlying cognitive impairment after adjuvant chemotherapy, in spite of the differences between clinical and animal studies. I have shown that the cognitive impairment should be tested shortly after chemotherapy treatment, even though in patients these deficits can be noticed up to years after treatment. With the current state of knowledge, it seems appropriate to use a homogeneous set of young, male animals, even though the majority of the clinical cognitive impairment studies have been performed in middle-aged, female patients which are not homogeneous in their genetic background. It does not seem to matter which cytostatic agent is tested, even though MTX is an older drug not frequently used anymore. Treating animals with a single dose is suitable to explore the underlying mechanisms of the cognitive impairment, even though in the clinic cytostatic agents are given in a chemotherapy cocktail with multiple injections. Finally, using a spatial cognitive task is suitable as read-out, even though cognitive impairment in humans is generally noticed in (sub)cortical tasks. It is important to notice that this validation mainly concerns face validity. The construct and predictive validity cannot be determined because of a lack of data of the underlying neurobiology in humans. In that sense, the current animal model clearly may contribute to the formulation of evidence based hypotheses to be tested in humans.

Cognitive impairment is a long-lasting side effect in some patients treated with adjuvant chemotherapy and can have a large impact on the quality of life. This calls for large scale research investigating not only the effects of cytostatic agents on cognition or on mechanisms involved, but also on genetic variation that may play a contributory role in the development of cognitive impairment. With this research it may be possible, in the future, to predict which people are more at risk to develop this impairment and as a consequence design more personalized treatment strategies. Since not only the incidence rate of cancer is increasing but also the survival rate, it is important to further elucidate the mechanisms underlying the cognitive impairment and improve the quality of life of cancer survivors.

The animal model presented in this thesis is highly suitable to test not only the underlying mechanisms of cognitive impairment following adjuvant chemotherapy but also potential new treatment and/ or protection strategies.

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