Potential Therapeutic Effect of Allogenic Mesenchymal Stem Cells on Chronic Cerebral Murine Toxoplasmosis

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Background and Aim: *Toxoplasma* infection is mainly latent and cause severe disease only if reactivation occurs especially for brain cysts. This study aimed to evaluate the therapeutic effect of BM-MSCs on murine chronic toxoplasmosis experimental model.

Methods: Female Swiss albino mice (n=100) were divided into 5 groups (20 mice each). Group I (infected, injected with BM-MSCs); Group II (infected, treated with BM-MSCs and Spiramycin-Metronidazole); Group III (infected, treated with Spiramycin-Metronidazole); Group IV (infection control) and Group V (non infected, injected with BM-MSCs).

Results: In Regarding the mean *Toxoplasma* brain cyst count, after 7 and 14 days, group I was significantly lower than group II, higher than group III and non-statistically different from group IV. Group II was significantly higher than in groups III and IV. Group III showed a significant decrease in brain cyst count versus group IV. As regards the histopathological examination of brain sections, after 7 and 14 days, group I showed the least histopathological inflammatory changes which was significantly lower than that of group IV. Group II revealed the most profound histopathological inflammatory changes. Group III showed mild to moderate inflammatory changes with a non-significant difference from group IV. As regards the survival rate, the group I showed the highest, group II showed the lowest mean survival time, which was statistically significant versus group IV. Group III showed a non-significant difference versus group IV.

Conclusion: MSCs have an anti-inflammatory effect and prolong the survival of *T. gondii* infected mice; however, they have a non-significant effect on brain cyst count.

INTRODUCTION

Toxoplasmosis is an infectious disease which affects around > 60% of some populations worldwide **[1]**. The most common form of infection in humans is latent but in immuno-suppressed patients, it may cause serious disease mainly through reactivation of cerebral toxoplasmosis which has severe neurological outcomes that may cause death **[2]**. Recent findings indicated that latent toxoplasmosis may play various roles in the etiology of different mental disorders such as schizophrenia, Alzheimer's and Parkinson's diseases **[3]**.

The outcome of an infection with toxoplasmosis depends on a balance between pro-inflammatory (IL-12, IFN-γ and TNF-α) and anti-inflammatory (IL-10, IL-27,TGF-1β)) signals that inhibit parasite proliferation and also control the inflammatory response. Absence of any one of these pro-inflammatory mediators leads to higher mortality during infection due to uncontrolled tachyzoite growth **[4]**. The brains of infected mice showed evidence of inflammatory infiltration, gliosis together with *T. gondii* cysts and proinflammatory cytokines **[5]**.

The treatment of toxoplasmosis involves a combination of pyrimethamine and sulfadiazine, but their efficacy is limited due to significant toxicity or development of drug-resistances in parasites. Unfortunately, the available therapies affect only tachyzoites in acute infection but can't affect tissue cystic stage in chronic infection [6]. Spiramycin, when co-administered with metronidazole showed significant effect on the treatment of chronic toxoplasmosis **[7]**.

Mesenchymal stem cells (MSCs) are self-renewing, clonal precursors of non-haematopoietic tissues, have the capacity to differentiate to the major specialized cell types **[8]**. The transplantation of MSCs has emerged as a promising tool for the repair of several tissues, including the CNS **[9]**. Stem cells can cross the blood brain barrier (BBB) and reverse neuronal damage **[10]**. MSC treatment modulates the immune response and decrease the expression of pro-inflammatory cytokines, directing the immune response from a predominantly pro-inflammatory to an anti-inflammatory profile **[11, 12]**. MSCs secret certain cytokines that are helpful for anti-apoptosis and antifibrosis, including vascular endothelial, insulin-like, and hepatocyte growth factors **[13]**. Recently, stem cell therapy was evaluated in the treatment of parasitic infections. MSCs were found to relieve liver injury and fibrosis caused by *S. japonicum* and prolongs the survival of infected mice **[14]**. There was a trial to use MSCs in *Leishmania* major infection and cerebral malaria **[15] [16]**.

The aim of this study is to evaluate the potential therapeutic effect of allogenic mesenchymal stem cells on chronic cerebral toxoplasmosis in experimentally infected mice.

MATERIAL AND METHOD

Isolation and Culture of BM-MSCs

Sterile phosphate-buffered saline (PBS) was thoroughly used to wash bone marrow that was obtained aseptically from tibiae and femurs of 7-wk-old male Albino donor rats. The cavities of the bone marrow were flushed into 15 ml sterile falcon tubes, by complete medium containing; 100 ml Dulbecco's Modified Eagle's Medium (DMEM), 12 ml fetal bovine serum, 1 ml penicillin/streptomycin mixture (5000 units/mL of penicillin and 5000 µg/mL of streptomycin) and 50 µl of 250 µg/ml Amphotericin B (Lonza, Switzerland). We harvested and re-suspended the nucleated cells in the complete medium inside sterile flask 25 cm^2 (NUNC Company, Denmark). Cells were incubated at 37 ^{O}C in 5% humidified CO2 with daily examination by Axiovert -inverted microscope- 100-ZEISS. When cultures become confluent, the cultures were treated with 2 ml of 0.25 % trypsin/0.02 % EDTA for 2 min at 37 ^{O}C to detach cells from the flasks. Trypan blue was used to test the viability of cells, and then a hemocytometer was used to count the cells and adjusted to 10^6 cells /ml, then used immediately for animal treatment **[17]**.

Characterization of the cultured BM-MSCs

A. Fixation and Staining of BM-MSCs [18]:

a. A sterile pipette was used to obtain the medium, and then sterile PBS was used to wash the adherent cells twice.

b. On day 9 of culture, fixation step was performed for the adherent MSCs by using freshly prepared pre-cooled mixture of acetone/methanol that was added to the culture dish with incubation at room temperature for 10 minutes.

c. Giemsa stain was applied to the fixed adherent MSCs.

d. The culture dishes were incubated with the staining solution for 30 minutes and then washed with running H_2O. By using the inverted microscopy, we examined the dishes then took photographs. The cells appeared as fibroblast-like shaped cells (fig.1).

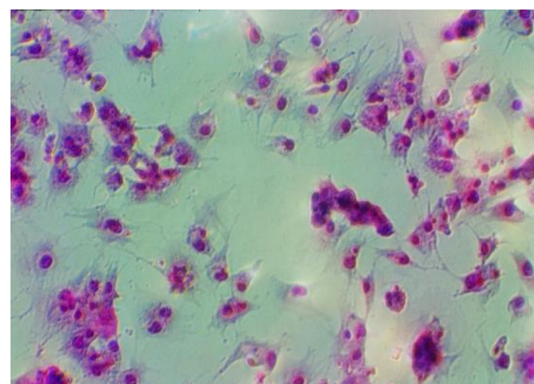


Fig. (1): Showing Giemsa stained BM-MSCs with bluish cytoplasm and vesicular nucleus (Axiovert -inverted microscope X200)

B. Immunocytochemical characterization [19].

The cultured BM-MSCs were characterized by using the streptavidin-biotin immune-peroxidase

technique for CD44 and CD 34 in the cultured cells.

a. The Petri dish was fixed on day 12 of culture by Acetone: Methanol (1:1).
b. The fixative was removed, and then the PBS was used to wash the cells twice.
c. The dish was covered by H_2O_2 (10%) for 15 minutes to block endogenous peroxidase.
d. Then the 1ry antibody was added. Antibodies were diluted in PBS.
e. The Petri-dish was incubated with the primary antibody for 1 hour at 37°C. The Petri-dish was washed three times with PBS.
f. The Petri-dish was incubated with the secondary antibody diluted in PBS for another 1 hour at 37◦c.
g. The Petri-dish was washed 3 times with PBS.
h. The Petri-dish was covered completely by 80-100 ml of Streptavidin horseradish peroxidase conjugate for 15 minutes, then washing twice with PBS.
i. The color was developed using 1-2 ml of Diaminobenzidine for 10 minutes. Then, Petri-dishes were washed well with distilled water.
j. Positive nuclear reactions appeared brown in color (fig.2).

Negative control was performed using the 2nd antibody only to check for any cross reaction (fig.3).

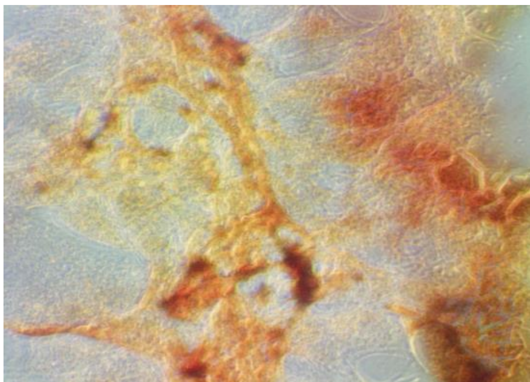


Fig. (2): Immuno-stained BM-MSCs with positive brownish immune reaction for anti-CD 44 antibody (Axiovert -inverted microscope X200).

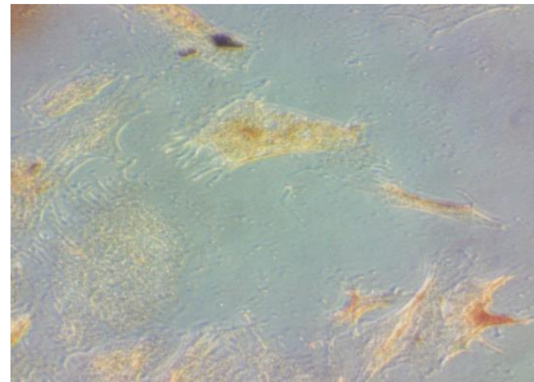


Fig. (3): Immuno-stained BM-MSCs with negative immune reaction for anti-CD 34 antibody (Axiovert -inverted microscope X200).

Parasite

Me49 non-virulent strain of *T. gondii* was kindly provided by Zoonotic Diseases Department, Division of Veterinary Research, National Research Centre, Giza, Egypt. The parasite strain was regularly maintained by repeated oral inoculation of Swiss albino mice with 20 brain cysts/0.2 ml/mouse of brain homogenate of previously infected mice every 2-3 months to establish chronic toxoplasmosis **[20]**.

Experimental animal grouping and study design.

Female Swiss Albino mice (n= 100), 7 weeks old and weighing about 25-30 gm were maintained under conventional conditions (fed a standard commercial diet, housed in 12-hour dark cycle at 22±2^{O}C and continuous air renovation) at Medical Research Center, Faculty of Medicine, Ain Shams University.

Mice (n=80) were infected orally, using a nasogastric feeding tube, by brain homogenate of the Me49 strain of *T. gondii* with a dose of 20 cysts/0.2ml/mouse **[21]**. Infection was guaranteed by finding *T. gondii* brain cysts 2 months post-infection in Giemsa stained brain homogenate. Chronic toxoplasmosis infected mice were divided into 4 groups of 20 mice each, as follows:

Group I (infected-MSCs treated) (n=20)**:** Chronically infected mice, intravenously injected in the tail vein with a single dose of 5 x 10^5 allogenic MSCs/0.5ml/mouse **[17]**, 2 months post-infection.

Group II (infected-MSCs and drug treated) (n=20)**:** Chronically infected mice, treated with both; a single dose of 5 x 10^5 allogenic MSCs/0.5ml/mouse, IV injection in the tail vein

and Spiramycin (400 mg/kg)- Metronidazole (500 mg/kg), orally as liquid suspensions, using a nasogastric feeding tube, daily for 7 days, 2 months post-infection.

Group III (infected-drug treated) (n=20)**:** Chronically infected mice, treated with 400 mg/kg of Spiramycin (Medical Union Pharmaceuticals) and 500 mg/kg of Metronidazole (Sanofi Aventis). The drugs were orally administered as liquid suspensions, using a nasogastric feeding tube, daily for 7 days; 2 months post-infection **[7]**.

Group IV (infection control group) (n=20)**:** Chronically infected mice with a dose of 20 cysts/0.2ml/mouse orally, using a nasogastric feeding tube.

Group V (MSCs-control group) (n=20)**:** Non-infected mice, IV injection in the tail vein with a single dose of 5 x 10^5 allogenic MSCs/0.5ml/mouse **[17]**.

Mice (n=5) from each group were euthanized by cervical dislocation, at the 7th, 14th days after starting treatment administration (Spiramycin-Metronidazole and MSCs), according to the previously mentioned animal grouping **[22, 7]** (Fig 4).

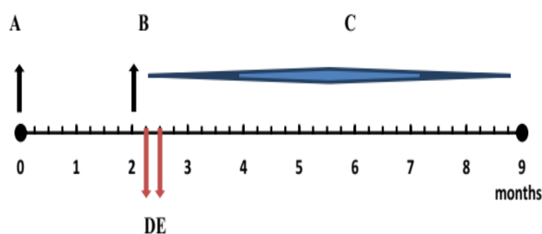


Fig. (4): A scaled line into 9 months showed the study period, **A**: The start of mice infection with Me49 non-virulent strain of *T. gondii.* **B:** Infection was established in mice 2 months after infection, also treatment administration (Spiramycin-Metronidazole and or MSCs) was started, according to the previously mentioned animal grouping. C: Deaths of mice (10 mice in each group) were recorded daily for 7 months, 2 months post-infection. D: 5 mice from every group were euthanized 7 days after starting treatment administration. E: 5 mice from every group were euthanized 14 days after starting treatment administration.

PCR detection of male-derived MSCs

Genomic DNA was extracted from the mice brain tissue homogenate in each test group, using Wizardt Genomic DNA purification kit (Promega, Madison, Wisconsin). The presence or absence of the sex determination region on male Y chromosome (sry) gene in recipient female mice was assessed by PCR to amplify a product of 104 vase pairs (bp). Separation of PCR products were done by 2% agarose gel electrophoresis and stained with ethidium bromide. Positive and negative controls were included in each assay **[23]**.

Evaluation of MSCs and drug treatment:

1- Brain cyst count

Mice (n=5) from each group were euthanized by cervical dislocation, on the 7th, 14th days after starting treatment administration. Each brain sample was rinsed with sterile saline solution, followed by homogenization. Then, glass slide was made by putting 25ul of brain homogenate on it (4 slides for each brain sample), allowed to dry in the air, then fixed and stained with methanol and Giemsa stain respectively. Using a compound microscope, the number of cysts were counted and calculated in each brain **[7]**.

2- Histopathological examination

Paraffin blocks were made after fixation of brain samples in 10% formalin. The thickness of the sections was 4-µm. The sections were then rehydrated and stained with hematoxylin and eosin (H&E). All the slides were examined by pathologist who was blinded to the experimental design. scoring of brain inflammation (0-4) was determined as follows; **score 0**: no lesion, **score 1**: minimal lesion restricted to localized perivascular cuffs with slight meningeal mononuclear cell infiltration **score 2**: mild lesion involving perivascular cuffs, local glial cell infiltration, and meningitis **score 3**: moderate lesion involving perivascular cuffs, glial cell activation, meningitis, focal necrosis, and rarefaction of the neuropil with occasional macrophage infiltration **score 4**: severe lesion involving perivascular cuffs, glial cell activation, meningitis, rarefaction of the neuropil, and focally extensive necrosis **[24]**.

Image analysis for brain cyst diameter was performed by two pathologists independently, using computerized Image Analyzing Software (Special SIS starter. version 3.2, Olympus, Germany) which is connected to an Olympus microscope (model BX51, Olympus, Japan).

3- Mice survival

Mice (n=10) from each group were monitored daily and deaths were recorded for 7 months, 2 months post infection.

Statistical analysis

SPSS software package version 17.0 was used for data analysis. Quantitative data was expressed using mean and standard deviation. Differences were considered significant if P values were equal to or less than 0.05 by Student “t” test.

One way ANOVA test, after 7 and 14 days post treatment was performed.

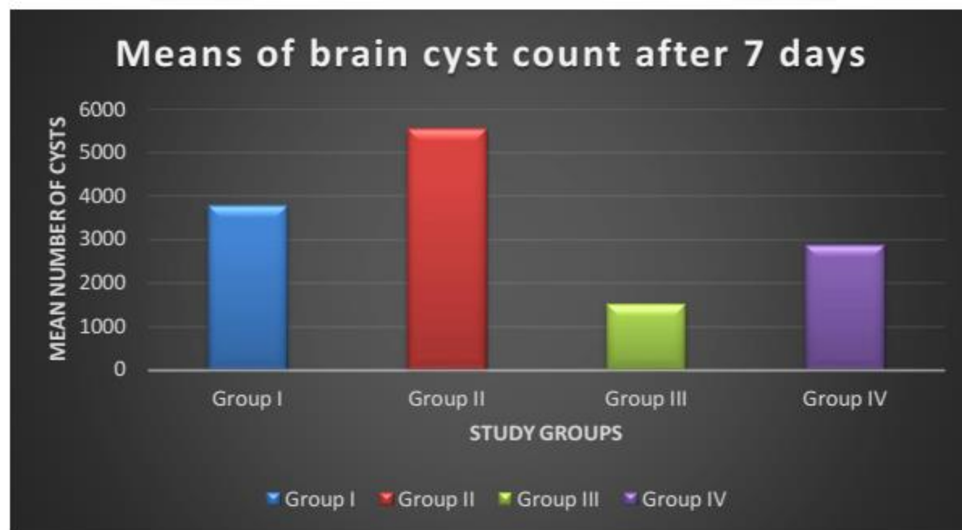
Survival of mice on different drug regimens was evaluated by the Kaplan-Meier product limit method, also the difference between the curves obtained was analyzed by Log Rank test.

RESULTS

1- Brain cyst count

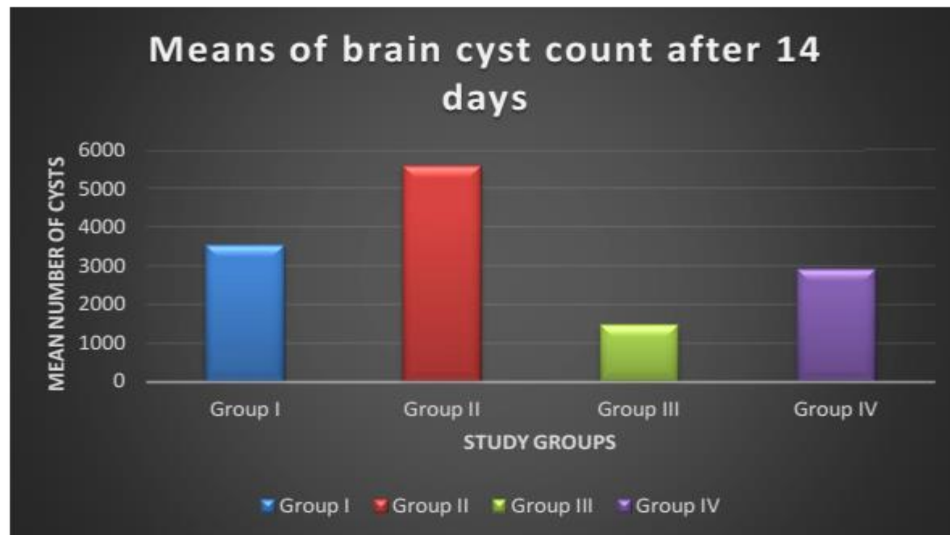
Counting of brain cyst 7 days after treatment revealed that, **group I** (3784 ± 534) was non-statistically ($p > 0.05$) different from group IV (2878 ± 277). **Group II** (5559 ± 656) was significantly ($p < 0.05$) higher than group I (3784 ± 534) and group IV (2878 ± 277). **Group III**, showed the lowest mean cysts count (1527 ± 119) which was considerably ($p < 0.05$) less than that of group IV (2878 ± 277) Fig. (5).

Counting of brain cyst 14 days after treatment revealed that, **group I** (3533 ± 727) was non-statistically ($p > 0.05$) different from group IV (2908 ± 260). **Group II** (5569 ± 667) was significantly ($p < 0.05$) higher than group I (3533 ± 727) and group IV (2908 ± 260). **Group III** showed the lowest mean cysts count (1479 ± 94.1) which was considerably ($p < 0.05$) less than that of group IV (2908 ± 260) Fig. (6).



Bar chart illustrating mean count of brain cysts, in all infected groups 7 days after the start of treatment administration.

Fig (5): Mean count of brain cysts in all infected groups, 7 days after the start of treatment administration.



Bar chart illustrating mean count of brain cysts, in all infected groups 14 days after the start of treatment administration.

Fig. (6): Mean count of brain cysts in all infected groups, 14 days after the start of treatment administration

2- Histopathological examination

Histopathological examination of brain sections revealed tissue cysts in all infected groups (Fig. 7). Scoring of brain inflammation (1-3) was determined [25]. Group I showed mild histopathological changes (score 1). The cerebral architecture was slightly preserved with apparently less dark nuclei with multiple normal pyramidal cells and less inflammatory reaction (Fig. 8). Group II revealed profound histopathological changes (score 3). Multiple deformed neurons with dark shrunken nuclei together with acidophilic masses of inflammatory cells were detected. Foci showed gliosis, numerous lymphoid cells and macrophages infiltration of perivascular and leptomeningeal areas (Fig. 9). Group III showed minimal to moderate inflammatory changes (score from 1 to 2). Few neurons were deformed and shrunken with acidophilic cytoplasm and dark nuclei. Focal mononuclear inflammatory infiltrate and focal necrosis were noticed (Fig. 10). Group IV showed moderate inflammatory changes (score 2). Perivascular cuffing by lymphocytes, focal mononucleated cell infiltrates and focal areas of gliosis were noticed (Fig. 11). Group V showed normal cerebral architecture (Fig. 12).

Histopathological examination showed the same picture at the 7th and the 14th day after starting treatment administration.

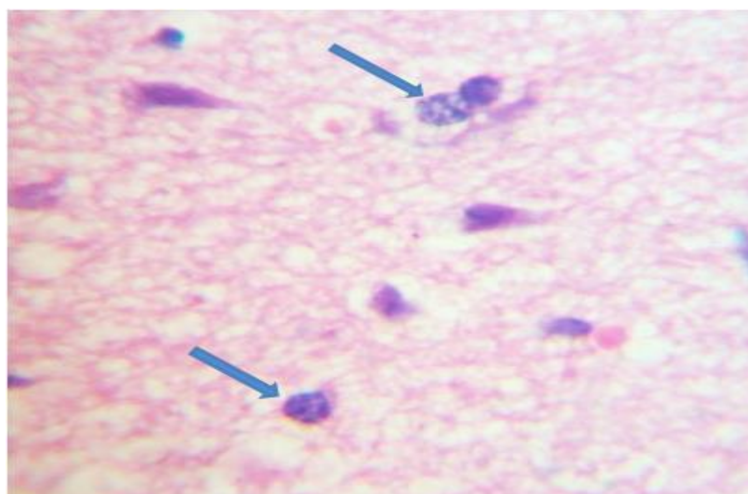


Fig. (7): Brain section of group IV showing *T. gondii* cysts containing bradyzoites "blue arrow" (H&E stain x1000).

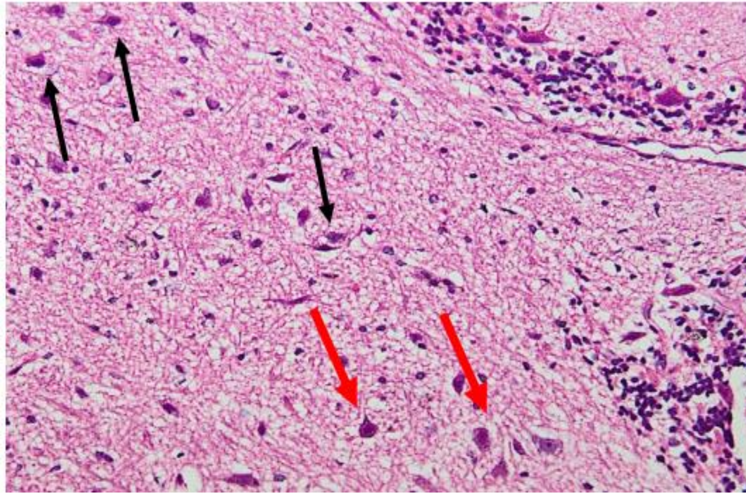


Fig. (8): Brain section of group I showing slightly preserved with apparently less dark nuclei "black arrow" with multiple normal pyramidal cells "red arrow" (H&E stain x 400)

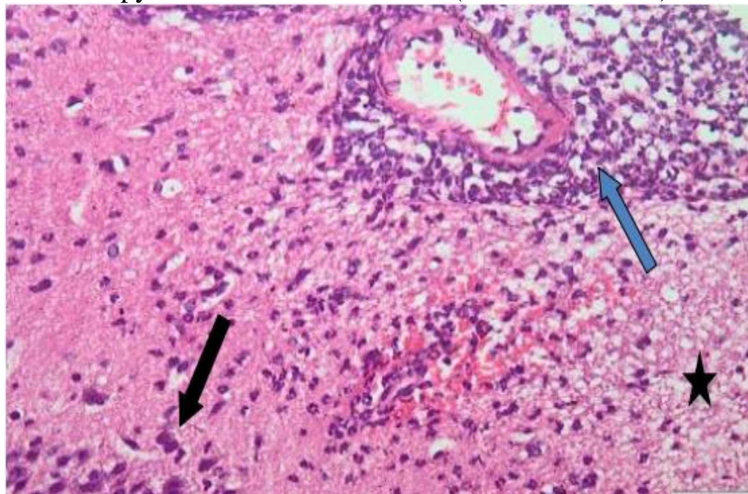


Fig. (9): Brain section of group II showing multiple deformed neurons with dark shrunken nuclei "black arrow" with perivascular cuffing by lymphocytes "blue arrows" and gliosis "black star" (H& E stain x 400).

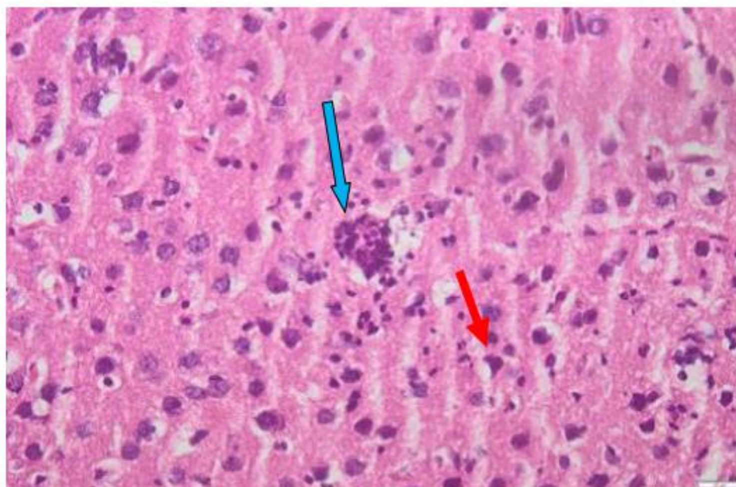


Fig. (10): Brain section of group III showing focal mononuclear inflammatory infiltrate "blue arrow" and scattered normal pyramidal cells "red arrow" (H&E stain x 400)

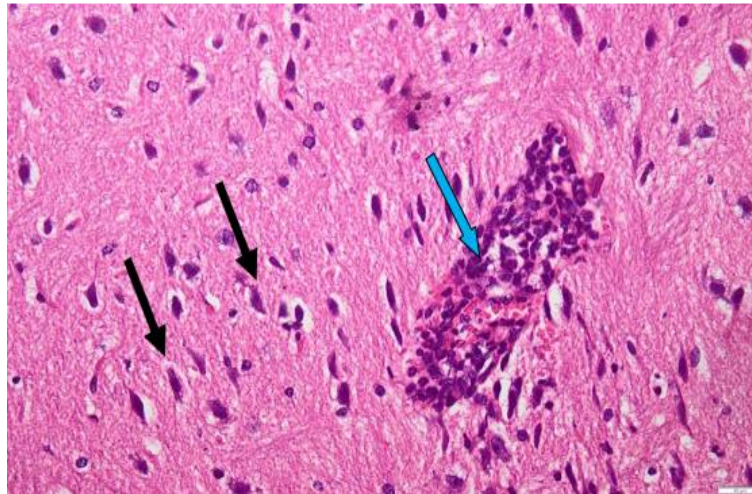


Fig. (11): Brain section of group IV showing cerebral cortex demonstrated few neurons exhibiting acidophilic cytoplasm and dark nuclei, few deformed and shrunken neurons "black arrow" and perivascular cuffing by lymphocytes "blue arrow" (H&E stain x 400).

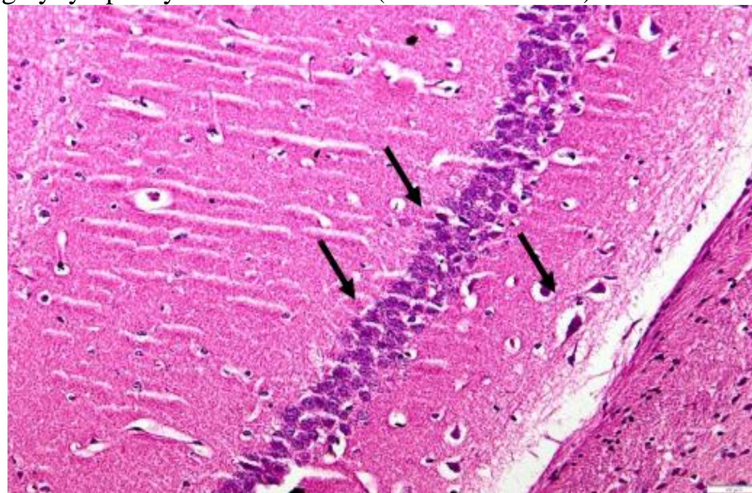


Fig. (12): Brain section of group V showing normal cerebral architecture with multiple neurons containing large pale vesicular nuclei "black arrow" (H&E stain x 400)

There was no statistical significance between different groups regarding the size of brain cysts in euthanized mice 7 days and 14 days post treatment (table1).

Table (1): Size of brain cysts in euthanized mice groups 7 days and 14 days post-treatment.

	Euthanized mice (n=3) after 7 days	**Euthanized mice (n=3) after 14days**
Group	**Mean brain Cyst diameter ± SD**	**Mean brain Cyst diameter ± SD**
I	5.16 ± 0.96	5.16 ± .25
II	4.9 ± 1.3	5.03 ± .25
III	5.3 ± 0.76	5.03 ± .5
IV	4.9 ± 1.2	4.80 ± 1.04

One way ANOVA test, after 7 days, p value= 0.068
One way ANOVA test, after 14 days, p value= 0.5

3. Mice survival:

Mice (n=10) from each group were monitored daily and deaths were recorded for 7 months, 2 months post infection. Kaplan-Meier method was used to assess the cumulative survival of mice during the whole experimental period with the results of survival being 100% and 50% of group V and group I respectively (Fig. 14). Group I showed the highest mean survival time 154.3 days, with significant difference from group II (18.2 days) the least survival, group III (93,9 days) and group IV (68.3 days) Fig (13).

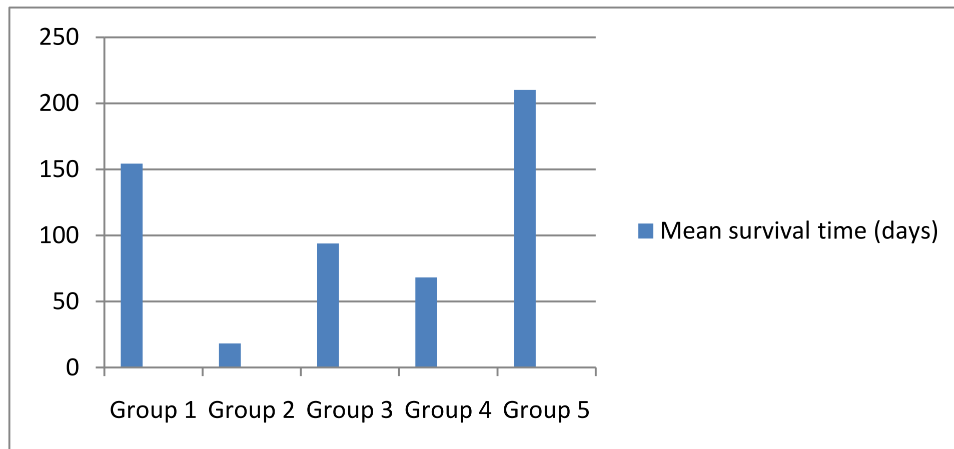


Fig. (13): Mean survival time in all groups for 7 months, 2 months post infection.

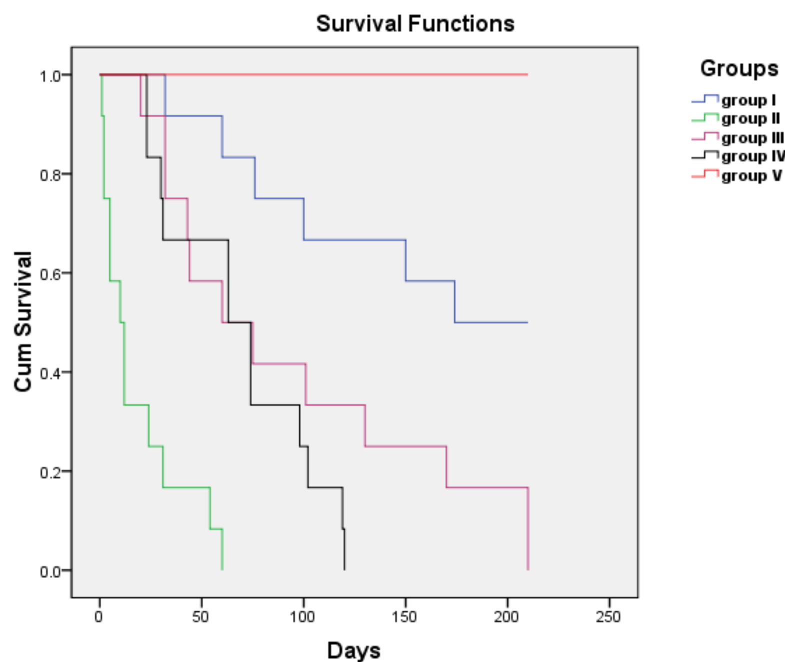


Fig. (14): Kaplan-Meier curve for survival in mice of in all the five groups (10 mice in each group).

DISCUSSION

T. gondii infection causes important cerebral and behavioural changes in humans. These changes are mainly due to the host immune response against the parasite **[26, 27]**. The tachyzoites rapidly infect a wide range of host cells. By the effect of the immune system, tachyzoites changed into slowly multiplying bradyzoites. The bradyzoites remains quiescent inside brain cysts for the host`s life. Dormant tissue cysts requires a good local immunity in the CNS with the assist of peripheral immune cells that infiltrate the CNS to prevent cyst reactivation and encephalitis **[28, 29]**.

MSCs are used in treatment of brain diseases including tumors, due to their ability in homing in areas with tissue pathology then healing and regeneration occur **[30]**.

This study aimed to assess the potential therapeutic effect of MSCs on chronic murine toxoplasmosis by brain cyst count, histopathological examination and survival analysis.

In the current study, xenogenic transplantation of rat BM-MSCs into infected mice with *Toxoplasma* was done. This was because rat stem cells could be obtained with more yields of cells

in shorter time to avoid contamination of culture dishes. MSCs are hypoimmunogenic and can evade the host immune elimination. It also enhances the cost benefit effectiveness and support the idea of xenogenic MSCs therapy trials in humans. This xenogenic transplantation was previously done with success by different authors who reported successful survival of xenogenic MSCs **[17, 31]**.

In this study, mice (n=5) from each group were euthanized by cervical dislocation, on the 7th, 14th days after starting treatment administration. Such times were proven to be effective for successful testing of the therapeutic MSCs effect on the infected mice **[22]**.

Regarding the mean *Toxoplasma* cysts count, group I showed non-significant effect (p value >0.05) when compared to group IV. MSCs may not have anti-*Toxoplasma* activity. The cyst structure itself is an effective mean of immune evasion by which the parasite protects itself from immune system and drugs **[5]**. Group II, showed considerable rise in the mean *Toxoplasma* cysts count (p value< 0.05) compared to group IV and I. This may be due to an imbalance between pro and anti-inflammatory effects of MSCs, when combined with Spiramycin-Metronidazole. Moreover, synergistic immunosuppressive effect may happen, as both MSCs and Spiramycin caused increased level of IL-10 which reduced IFN-γ and IL-12 leading to reactivation and fulminant *Toxoplasma* encephalitis **[32, 33]**. On the other hand, group III showed important decrease in the mean *Toxoplasma* cysts count (p value< 0.05) compared to group IV and group I. Nevertheless, poor penetration of Spiramycin through the BBB because of the existence of the efflux transporters protein 2 and P-glycoprotein, Metronidazole can prevent the efflux of Spiramycin by inhibiting the efflux transporters present at the BBB, leading to Spiramycin-increased brain uptake **[34]**. This agreed with authors who used the treatment of Spiramycin-Metronidazole, and showed an exceptional effectiveness against chronic cerebral toxoplasmosis. There was a 10-fold and a 15-fold decrease in brain cysts compared to the Spiramycin-treated group, and the untreated control group respectively **[9]**. The anti-*Toxoplasma* activity of Spiramycin was also evaluated and showed that in chronic infection, there was a significant decrease in brain cyst burdens **[35]**.

Regarding the histopathological examination in the present study, brain sections of group I showed mild inflammatory changes (score 1) (Fig. 3), while group II, showed marked inflammation, fibrosis (score 3) (Fig. 4)**.** This agreed with authors who reported reduced microglia/macrophages, decreased the peripheral infiltrating leukocytes, reducing proinflammatory cytokines in experimental traumatic brain injury model treated with MSCs **[36, 37]**. The MSCs are not always immunosuppressive but their effects are determined by the conditions of the microenvironment. Action of certain receptors expressed by MSCs can ascertain their pro or anti-inflammatory effects. Sever inflammation caused by MSCs and Spiramycin-Metronidazole combination, may be due to the previously mentioned synergistic immunosuppressive effect that also caused significant increase in the mean *Toxoplasma* cysts count **[33]**.

Regarding the survival analysis in the present study, mice in all groups were followed for 7months after starting treatment administration. 50% of mice treated with MSCs, survived after 7 months**.** Group I showed the highest mean survival time (154.3 days) which was significantly higher than the mean survival time of group IV (68.3 days) and group III (93.9 days). This was in accordance with the report about MSCs prolonged survival in Huntington's disease mouse by increasing the laminin, vWf and stromal cell-derived factor-1 **[38]**. Some authors reported decreased levels of caspase-3 enzyme in adult rats leading to protection against spinal cord ischemia/reperfusion injury **[39]**.

Group II showed the lowest mean survival time (18.2 days) which is significantly (p value <0.05) different from group IV and group III. This high mortality rate in group II goes well with the above results of the increased brain cyst count and severs inflammation in histopathological examination.

In conclusion MSCs exert anti-inflammatory effect on mice chronically infected with *T. gondii,* and prolong their survival, although they can't reduce *Toxoplasma*- brain cyst count.

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Ethical consideration: The study was approved by the Research Ethics Committee, Faculty of Medicine, Ain Shams University. All the animal experiments were performed according to the

national regulations for the Animal Ethics rules, Ain-Shams University, Cairo, Egypt.

Conflict of interest: We wish to confirm that there are no known conflicts of interest associated with this publication. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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