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# Congenital Toxoplasmosis: *In Vivo* Impact of *Toxoplasma gondii* Infection on Myogenesis and Neurogenesis

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## Abstract

Congenital toxoplasmosis (TC) from *Toxoplasma gondii* positive mother to child transmission results in fetal death, abortion, or infantile neurologic and neurocognitive deficits as well as chorioretinitis. This study aims to analyze the morphological changes in brain and skeletal muscle cells of Swiss mouse embryos during experimental congenital toxoplasmosis. Swiss mice, before mating, were gavage inoculation infected with approximately 25 or 50 cysts of ME-49 strain *T. gondii*. Eighteen day postcoitus maternal and embryonic muscle and brain samples were collected and processed for histopathological analysis. The muscle tissue from embryos of infected mothers, in comparison with healthy muscle myofibers, exhibited discontinuous and shorter myofibrils, more interfibrillar space and immature cells with fewer stained and poorly defined striated profiles. These *in vivo* findings might be related to an adhesion protein decrease, observed *in vitro*, where myogenesis was completely affected during *Toxoplasma* infection. The neurogenesis was severely affected with irregularly arranged cells, reduced cell density, and a significant intercellular space increase. The brain tissue presented ischemia, cell death, necrosis, and thrombi, increasing according to the degree of the acute infection, which compromised the neurogenesis, thereby justifying brain size decrease in these embryos.

**Keywords:** *Toxoplasma gondii*, experimental toxoplasmosis, myogenesis, neurogenesis

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## 1. Introduction

*Toxoplasma gondii* is a parasite that persists during the chronic phase of the disease in the form of tissue cysts mainly in brain and skeletal muscle. *T. gondii* has been implicated in the pathogenesis of inflammatory myopathies and brain diseases, which are of great medical

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importance due to congenital toxoplasmosis (CT) [1, 2]. Prevention is an essential measure against *T. gondii* infection from mother to child [3, 4].

The ability to transmigrate through the placenta to replicate in various fetal tissues, evading the immune system of the fetus, makes *Toxoplasma* a major cause of prenatal complications. This infection can seriously interfere with the development of the fetus, possibly leading to abortion or serious pathologies after birth with severe consequences in childhood, adolescence, or adulthood [3–7]. Among the most common brain afflictions are encephalitis, altered mental status, seizures, weakness, cranial nerve disorders, sensory abnormalities, movement disorders, psychomotor or mental retardation, and behavioral alterations. Acute retinocorioidite is a severe condition, which may develop, involving vitreous hypervascularization and retinal necrosis with a total loss of vision [6, 8–12]. With regard to muscle tissue, the *T. gondii* infection can decrease and even inhibit the formation of new fibers by negatively modulating adhesion proteins such as cadherin [13–15]. Toxoplasmosis also causes myocarditis and polymyositis either by recent infection or by reactivation of tissue cysts in immunosuppressed, immunodeficient, and even immunocompetent individuals [1]. The study of conditions resulting from *Toxoplasma* infection in brain and muscle tissue has been developed from infected adult mice [7, 16–20]. The influence of *Toxoplasma* in these tissues during embryonic development in the murine model has yet not been explored despite the predilection of this parasite for these tissues in the chronic phase of the disease. The focus on skeletal muscle as a model for experimental toxoplasmosis opens new prospects for parasite biology understanding during interaction with one of the *T. gondii* target cells for parasite establishment in the chronic phase of the disease [13, 21–29]. Therefore, we decided to investigate the role of *T. gondii* in myogenesis and neurogenesis processes in murine embryos from adult females, that were infected before mating.

## 2. Experimental design

Three experimental protocols were developed in an attempt to establish trans-chromosome (TC) in mice, premating infection, imminently postmating, and during pregnancy. Swiss mice during the mating period (five females and one male per experiment,  $n = 5$ ) were divided into two groups and inoculated via gavage with approximately 25 (Group 1) or 50 cysts (Group 2) of ME-49 strain *T. gondii* (type II). After infection, the mice were placed in cages for 24 h with a suitable male of the same species and age. Thereafter, the animals were visually monitored daily (morning, afternoon, and evening) on the general aspects of lethargy, vaginal bleeding, and mortality. The dead animals were collected immediately and their embryos examined for macroscopic aspects of the peritoneum, spleen, liver, and lung. Pregnant females that sustained pregnancy underwent cesarean section approximately 18 days postcoitus, their embryos, alive or not, collected.

For the diagnosis of possible morphological changes in muscular and cerebral tissue caused by parasite presence in embryos from *T. gondii* cyst-infected mothers, embryonic thighs and brains were collected, sectioned longitudinally, and fixed in Millonig-Rosman solution

(10% formaldehyde in phosphate buffer). The tissues were then processed for impregnation in paraffin, and 3  $\mu\text{m}$  thick sections were stained as routine with hematoxylin and eosin (H&E) for the analysis of morpho-structural and parasitological parameters. Alternatively, part of the material was processed by freezing in liquid nitrogen ( $-196^{\circ}\text{C}$ ). Five-micrometer thick sections were obtained at  $-25^{\circ}\text{C}$  in Leica CM1800 cryostat (Germany), attached to slides treated in poly-L-lysine and fixed in 4% PFA in PBS.

### 3. Results

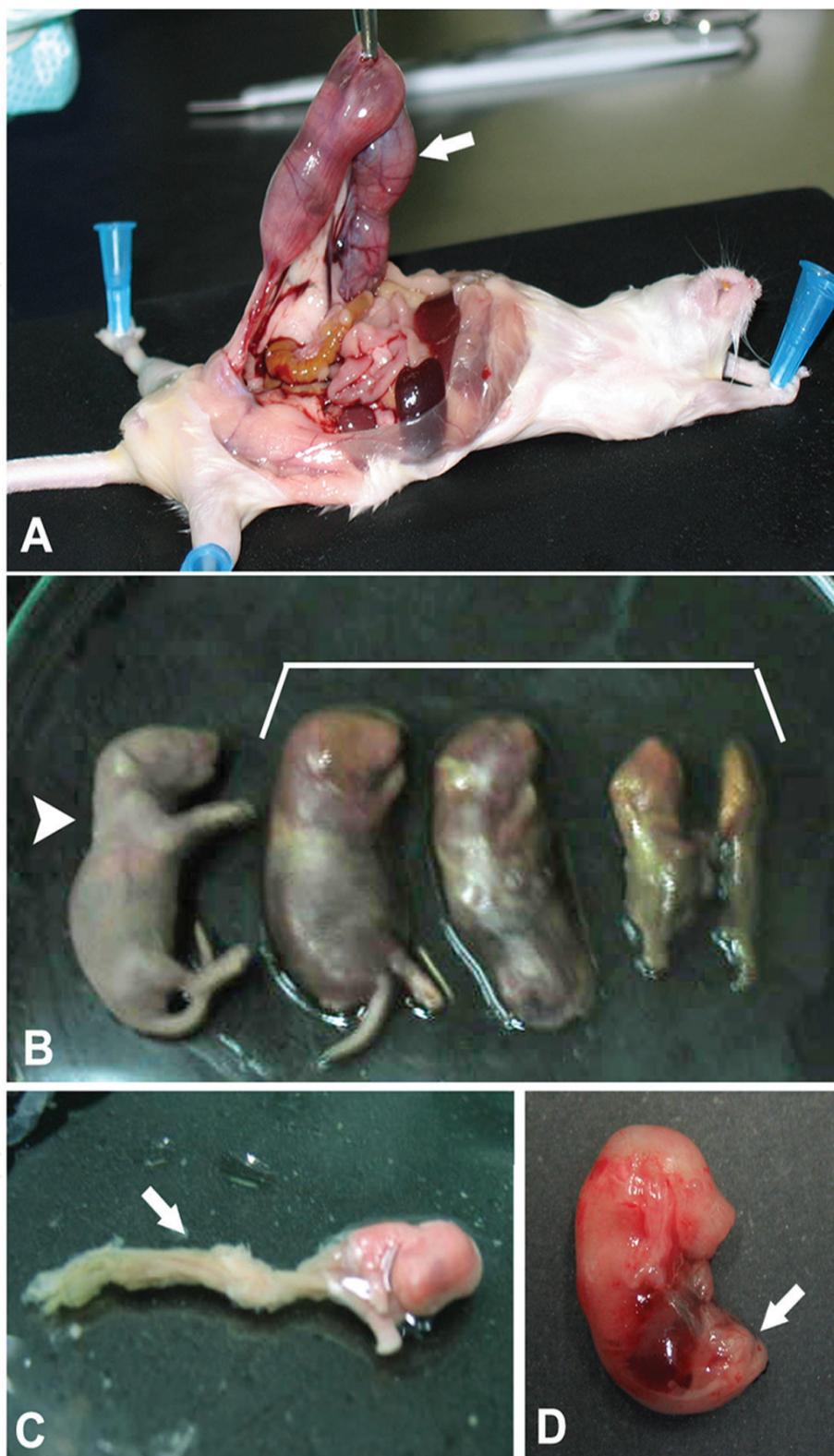
From three experimental protocols for *T. gondii* cyst mouse infection, postcoitus and during pregnancy promoted 100% in the number of abortions, embryo reabsorption, or adult female death. The most successful protocol was pre-mating adult female mouse infection. However, some animals did not carry the pregnancy to term and this complication arose soon after conception.

The severity of the *T. gondii* infection in adult females and embryos was directly proportional to the concentration of parasites (cysts) inoculated. Group 1 displayed fetal reabsorption, miscarriages, and stillbirths with a loss rate of over 50% (**Figure 1A**). This group was separated from animals with clinical signs of TC according to the increasing severity scale (**Figure 1B**). The stillborns presented different degrees of morphological impairment. The live embryos of this group were born underdeveloped and with little reaction to external stimuli (**Figure 1B**). They presented extensive liver disease characterized by necrosis, abdominal bloating with high fluid retention, and apparent swelling of the spleen as well as cutaneous and pulmonary hemorrhaging (data not shown).

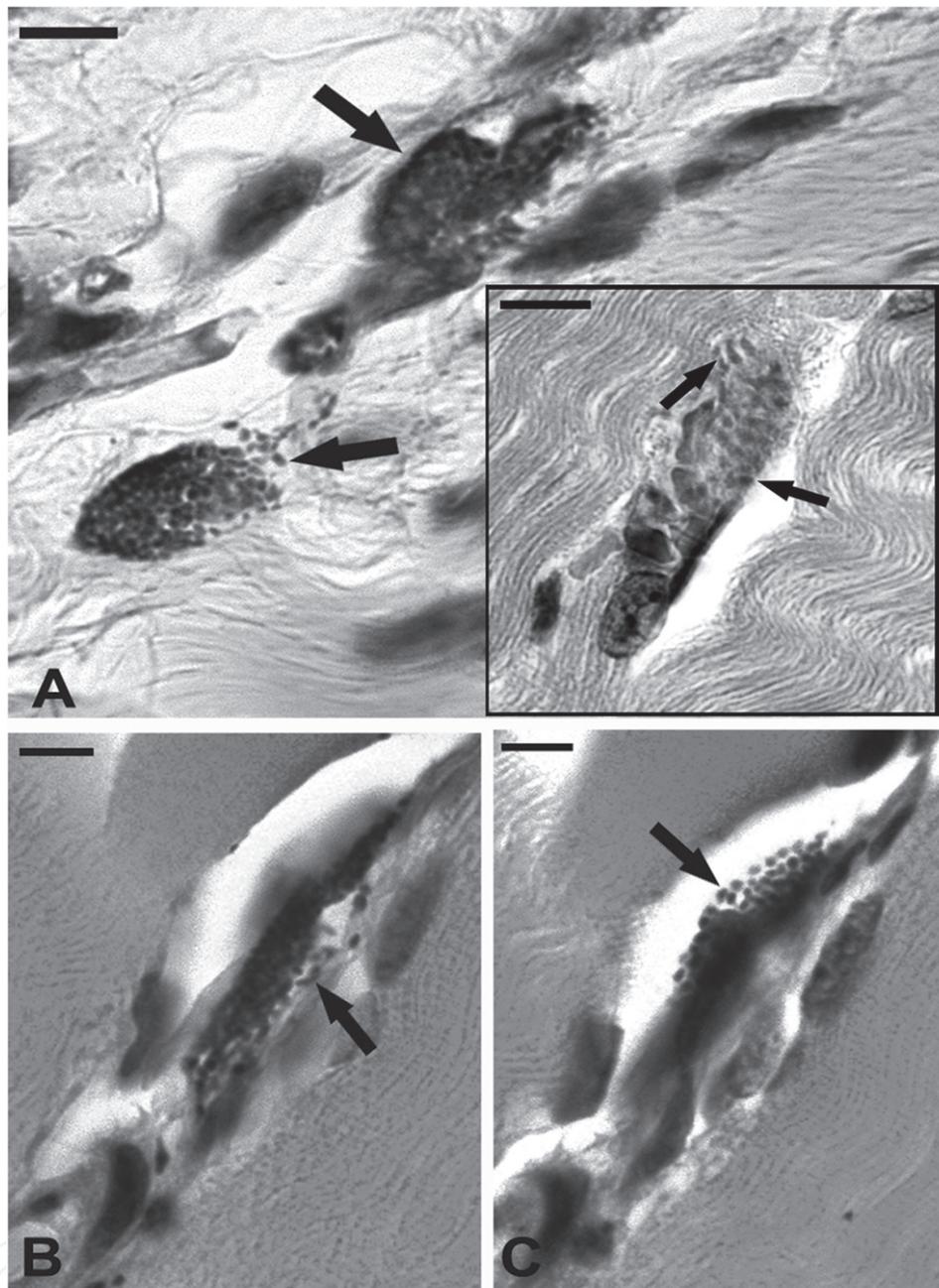
In addition, by macroscopic analysis, besides the swelling of some organs, there was malformation of the anterior and posterior limbs as well as significant decrease in skull size characterizing microcephalia in these animals (**Figure 1B**). These embryos from infected mothers were extremely swollen, both brain and muscle tissues retaining so much fluid that it was impossible to obtain samples for histological analysis (**Figure 1B**). Some of these embryos exhibited a very committed development such as malformation of the trunk (**Figure 1C**) and even total absence of hindlimb formation (**Figure 1C and D**).

Group 2 females inoculated with 50 cysts, although fertilized, did not terminate pregnancy, dying in few days. Many embryos were reabsorbed. In this experimental condition, there was nearly a total loss of females and their embryos.

Longitudinal sections of pregnant mouse muscle tissue infected with 25 cysts of *T. gondii* (Group 1), showed similar cyst structures between muscle fibers (**Figure 2A**). In various sections, the images suggested that parasites were evading the cysts and invading other cells (**Figure 2B and C**). The healthy mouse embryo leg muscle tissue demonstrated high density of mature myofibers arranged lengthwise, reduced interfibrillar space, and a well-organized striated profile (**Figure 3A, C, and E**). Even where these fibers were spaced, the amount of mononuclear cells from the connective tissue or inflammatory infiltrate was markedly small

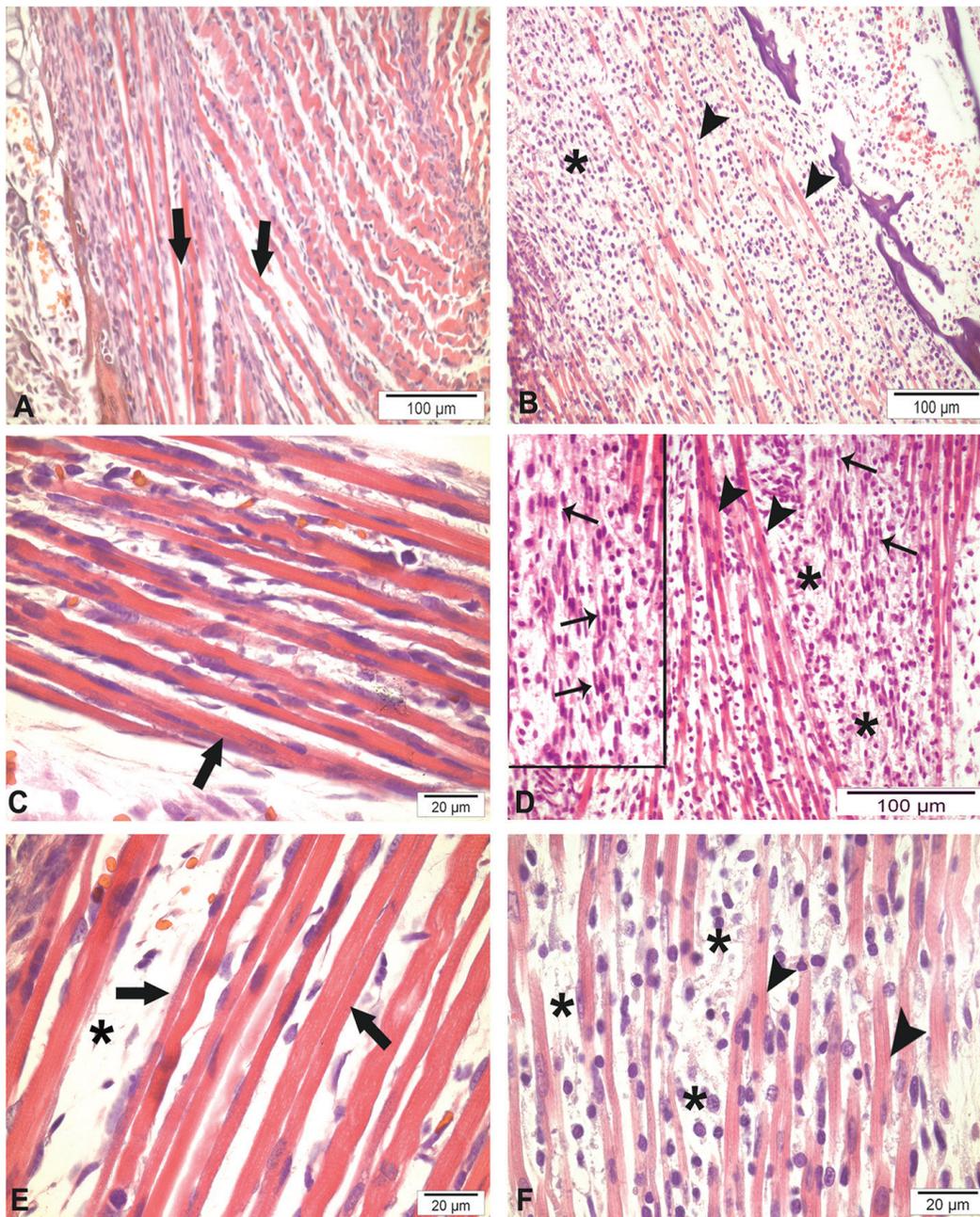


**Figure 1.** Swiss mouse embryos from experimentally infected mothers with *T. gondii*. (A) Harvesting of embryos by cesarean section (arrow). (B) The infection differentially affected pups in the same litter. In the extreme left of this picture is shown one pup apparently unaffected by the infection, whereas its littermate present different degrees of morphological alterations and decrease of skull size characterizing microcephaly in these animals. (C) Stillborn embryos show congenital malformation, including compromise of the trunk and hindlimbs (arrow). (D) Embryo present atrophy of the hindlimbs (arrow).



**Figure 2.** By light microscopy, longitudinal sections of adult muscle tissue of pregnant female infected with 25 cysts of *T. gondii* (Group 1). (A) Cyst-simile structures can be seen between myofibers (arrows), Bar: 15  $\mu\text{m}$ . Note that the parasites seem to evade these structures (inset), Bar: 20  $\mu\text{m}$ . (B and C) The same cell can be seen in two distinct focal planes showing small structures similar to *Toxoplasma* (arrows), Bars: 10  $\mu\text{m}$ .

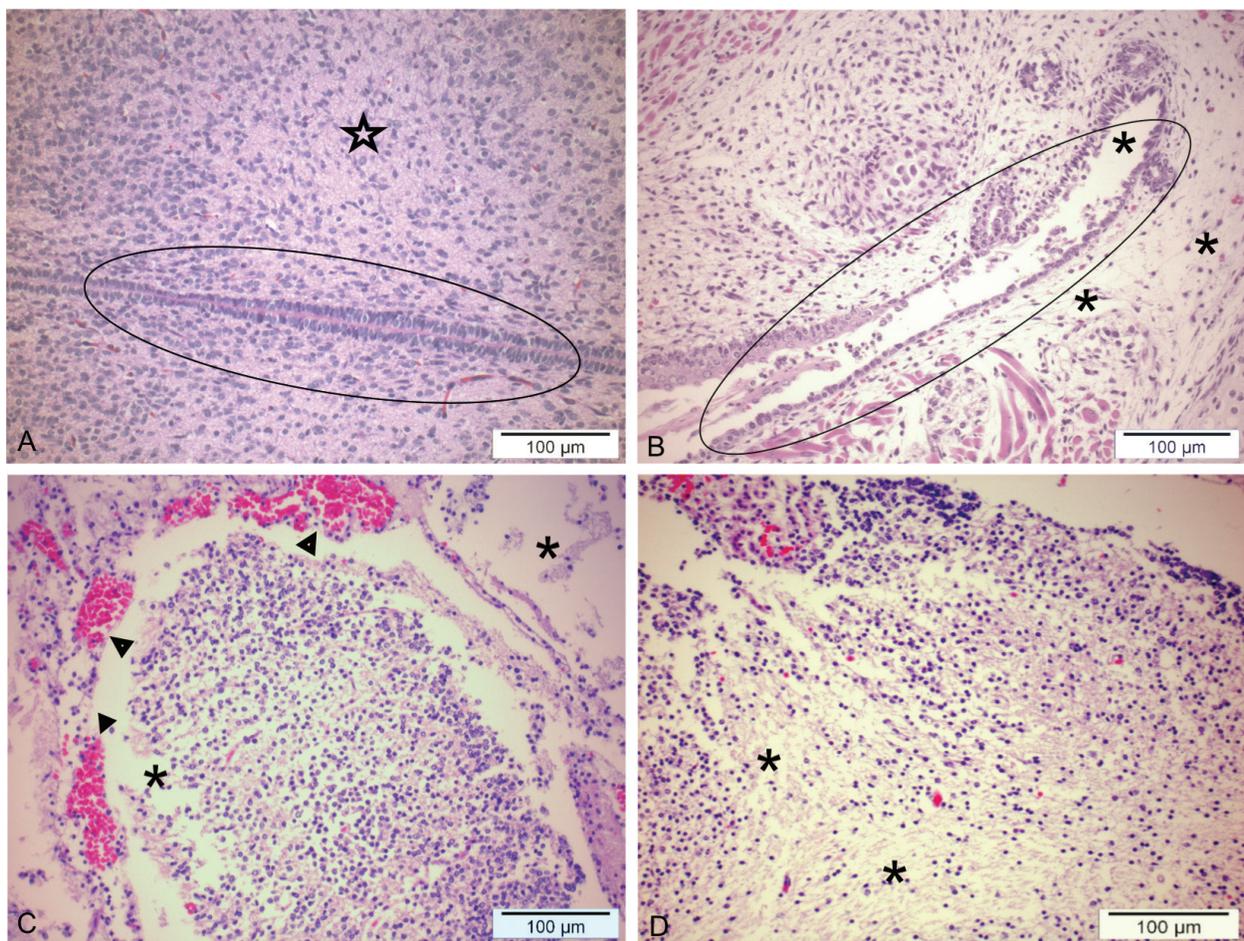
(Figure 3A, C, and E). However, the thigh muscles of embryos from *T. gondii* cyst-infected mothers, as compared to tissue from uninfected mice, contained a significant reduction in myofiber density and a great enlargement of the interfibrillar space (Figure 3B). Connective tissue cells filled this space, probably mononuclear cell inflammatory infiltrates such as macrophages, lymphocytes, mast cells, and eosinophils (Figure 3B, D, and F). Besides the shortening of mainly mature myofibers, muscle tissue analyzed of embryos born from infected mice presented several bipolar cells possessing small and elongated nuclei characterizing myoblasts



**Figure 3.** Longitudinal sections of the muscle tissue of thighs of murine embryos normal and from infected mother. (A, C, and E) Healthy embryo: note the high density of mature myofibers and small inter-fibrillar space (arrows). (B) Embryo muscle from infected mother: smaller density of myofibers (arrowhead) and greater spacing between them, probably filled by cells of connective tissue and cell inflammatory infiltrates such as: macrophages, lymphocytes, mast cells, and eosinophils (asterisks). (C) Healthy embryo: at higher magnification, dense and mature myofibers are observed with parallel arrangement and many nuclei. Note limited inter-fibrillar space and few mononuclear cells between myofibers. (D) Embryo from infected mother: muscle with scarcity of myofibers (arrowhead). Besides greater inter-fibrillar space, presence of a large number of mononuclear cells (asterisks). Note several bipolar cells with nuclei small and elongated (fine arrows). Inset: more detail these of aligned cells (myoblasts in the process of alignment and fusion). (E) Detail of healthy embryo myofibers. Higher cytoplasmic density of the myofibers with a striated profile and more organized. Inter-fiber space with number of cells, mononuclear cells markedly smaller (asterisk). (F) Embryo from infected mother: myofibers are smaller, less dense, less stained, and more spaced (asterisk). Apparently they are immature cells (less nuclei and cytoplasmic) and discontinuous in their extension (arrowhead).

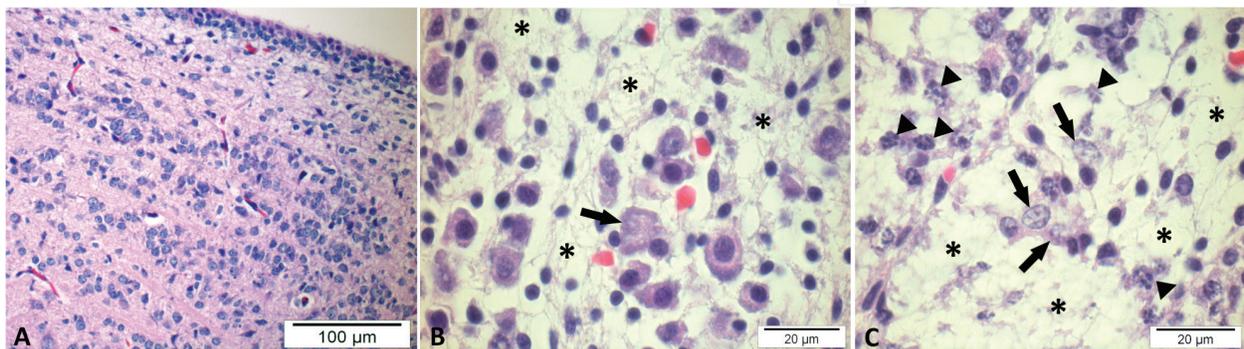
in the process of alignment and fusion (**Figure 3D**). These embryos presented discontinuous, shorter, less dense, and slightly colored myofibrils with more interfibrillar space and immature cells with fewer stained and poorly defined striated profiles (**Figure 3B, D, and F**).

Brain samples of control embryos and infected mothers were examined. All control embryo brain tissues did not show any changes, the brain tissue normal and cell density characteristics of healthy tissue. Tissue section showed arranged cells in a pink color with the cytoplasm unaltered in purple, the nuclei with no visible changes in the chromatin arrangement and almost no intercellular space (**Figure 4A**). In contrast, the brains of embryos from infected mothers exhibited irregularly arranged cells with higher concentrations at apparently less injured points, reduced cell density, and a significant increase of intercellular space, possibly due to the decrease of the adherent junctions (**Figure 4B–D**). In addition, there was weak



**Figure 4.** Using H&E staining, brain samples were examined for histopathological damages caused by *Toxoplasma* in embryos from infected mother. (A) Brain of an uninfected control, histology without any pathological changes. Regularly arranged cells of similar size (circle), cell density characteristic of healthy tissue, well stained, cells arranged in a pink color, with the cytoplasm unaltered in purple, the nuclei with no visible changes in the chromatin arrangement and almost no intercellular space (star). (B–D) The neurogenesis was severely affected and cerebral cortex of these embryos was compromised. Irregularly arranged cells, more concentrated at a point apparently less injured, reduced cell density, with a significant increase of intercellular space (asterisks). Note several points with recent thrombi of different sizes due to the massive presence of red blood cells (arrowhead).

cytoplasmic labeling and nuclear marking disparity, some larger nuclei almost transparent, others much smaller, marked. The presence of mononuclear cell inflammatory infiltrates was noted, such as macrophages, lymphocytes, mast cells, and eosinophils. There were several points with massive presence of red blood cells, implying recent thrombi in all analyzed sections (**Figure 4B** and **C**). In addition, different levels of brain tissue impairment were apparent (**Figure 4D**), such as presence of cells similar to astrocytes and neurons, which possibly suffered karyolysis (weak nuclear staining) (arrow) and eosinophilia (asterisk) (**Figure 5B** and **C**). Some of these cells presented nearly transparent cytoplasm resulting from the loss of basophilia and others an apoptotic process characteristic (**Figure 5C**). No analyzed sections revealed parasites.



**Figure 5.** Using H&E staining and light microscopy embryonic brain cell lesions with CT was analyzed. (A) General aspects of normal embryonic brain tissue. (B and C) Observe the lower density and cellular organization in the brains of embryos from *T. gondii*-infected mothers (asterisk). Some cells, similar to astrocytes, possibly suffered karyolysis (weak nuclear staining) (arrow) and eosinophilia (asterisk), with the almost transparent cytoplasm resulting from the loss of basophilia (asterisk). (C) Presence of cells with a nucleus characteristic of the apoptotic process (arrowhead).

## 4. Discussion

This study investigated the role of *T. gondii* infection in neurogenesis and myogenesis of skeletal muscle tissue of murine embryos from infected mothers. Our previous results *in vitro* disclosed significant parasite interference in the myogenesis process, hence the motivation for the development of this work.

Our first experimental strategy was gavage infection of the female with cysts aiming to mimic *T. gondii* primary transmission in nature through ingestion of raw or poorly cooked meat containing these cysts [30]. The developmental commitment of embryos from Toxoplasma-infected mothers was incontestable, and the *T. gondii* pathogenicity degree is determined by many factors such as susceptibility of host species, infection stage (acute or chronic), and parasite strain virulence [17, 30, 31]. Macroscopic images clearly expose the malformation or not of anterior and posterior limbs as well as other malformations during the development of ME-49 strain *T. gondii*-infected mice.

Our experimental model with Swiss mice involving infection of the female prior to mating demonstrated that during the first week of pregnancy, there was significant fertility reduction,

embryos with low birth weight, fetal reabsorption, miscarriages, and stillbirths, presenting a loss rate of over 50% for Group 1 (inoculated with 25 cysts) and almost 100% in Group 2 (inoculated with 50 cysts). However, since each cyst can vary in bradyzoite numbers (which may die or survive), it was not possible to define the parasite concentration that generated a certain vertical transmission rate. This would explain the loss of more than 70% of the embryos, considering that bradyzoites differentiate to tachyzoites that can damage the placenta, leading to loss of the embryo by spontaneous abortion or absorption, as proposed by Vargas-Villavicencio et al. [20]. These data confirm that the severity of *Toxoplasma* infection in females and embryos depends upon the concentration of parasites [18–20, 31]. TC is one of the most serious consequences of acute *Toxoplasma* infection, the greatest severity observed in early pregnancy as demonstrated experimentally in this study.

Embryos from infected mothers presented brain tissue with possible levels of ischemia and morphological aspects compatible with cell death mechanisms. Inflammatory reactions were apparent suggesting the focal presence of mononuclear cell infiltrates, necrosis, and thrombi, which increased according to the degree of infection development, compromising the neurogenesis. It is known that during apoptosis, the cells lose their adherent junctions [32], and lesions, due to ischemia or hypoxia together with other stimuli, also triggering cell death (necrosis and apoptosis) [33, 34]. Some cells, similar to astrocytes and neurons, underwent karyolysis (weak nuclear staining) and eosinophilia with the nearly transparent cytoplasm resulting from the loss of basophilia [35, 36]. The results described here during the cerebral histological analysis point to neuropathogenesis induced by the *T. gondii* infection. Sun et al. [37] demonstrated that this infection in early gestation might inhibit the proliferation, differentiation, and migration of neural stem cells in rats. It has been reported that *T. gondii* induces apoptosis of neural stem cells via the endoplasmic reticulum stress pathway [38]. Although we did not detect the presence of the parasite at the lesion site, some authors suggest that parasite proteins may directly interfere with neuronal function, either in infected or neighboring cells [18, 39–41]. These data corroborate our observations implying a *T. gondii* proapoptotic effect in cerebral cells and also justify the embryonic brain size decrease as described here.

The systemic inflammatory response induced by the parasite (toxoplasmosis sepsis) witnessed in our experiments may have caused multiple organ failure of these embryos, as proposed earlier [17]. Despite the already known involvement of muscle tissue in the development of the toxoplasmosis chronic phase [42], no studies involving embryos from *T. gondii*-infected mothers have ever investigated the influence of this infection in muscle tissue development. Our histological analysis demonstrated that muscle tissue from such embryos, in comparison with the healthy muscle myofibers, possessed lower density and higher interfibrillar space. Connective tissue cells and probably mononuclear cell inflammatory infiltrates such as macrophages, lymphocytes, mast cells, and eosinophils fill this space [1, 16]. Besides the shortening of myofibers and absence of mature myofibers, muscle tissue from *T. gondii*-infected embryos presented several bipolar cells with small, elongated nuclei characterized as myoblasts, still in the process of alignment and fusion (myogenesis). Myofibers from these embryos displayed, besides small size, lower density of cytoplasmic material (characterized by a slight staining, H&E), discrete striation, and a discontinuous profile. These apparently immature cells with few nuclei directly influenced the length of myofibrils thus interfering with the myogenesis

process. Healthy embryos have the muscle tissue with many satellite cells attached to the myofibers, myoblasts in the process of fusion, and mature myofibers parallelly aligned dense and continuous [43]. The histological staining technique of H&E showed well-defined myofibers with striated characteristics of this cell type, dense cytoplasmic material, and greater proximity between them. This high cytoplasmic density characterized by intense staining of myofibers must be associated with an increased myocyte fusion process in the early development of the muscle fiber. However, it has been well established that *T. gondii* can cause myositis either by recent infection or reactivation of tissue cysts, affecting the homeostasis of muscle tissue [1]. Regarding the perspective of this study, our results confirm that *T. gondii* primary infection of predated mouse females affects the embryonic development, inhibiting the process of in the skeletal muscle *in vitro* model myogenesis [13]. In this study was demonstrated that *T. gondii* infection in skeletal muscle cells downregulate the M-cadherin mRNA expression, leading to molecular modifications on the host cell surface that disarray at the contact sites between myoblasts and myoblasts-myotubes, promoting the instability of the junctions. This progressive process interferes with membrane fusion and consequently inhibits the myogenesis process. This set of data justifies the results by histology that point out significant changes in the formation of embryonic myofibers from infected females before mating, corroborated by macroscopic images, showing some embryos with no formation of anterior and posterior members. These changes could lead to the modulation of other molecules contributing to toxoplasmosis pathogenesis in the murine muscle tissue as described here in the *in vivo* system. Additionally, recent research has reported that a virulence factor secreted by *T. gondii* rhoptries (ROP18) may contribute to neuronal apoptosis through the ER stress-mediated apoptosis pathway, which result in neurological diseases by reduction of these cells [41]. In rat brain cells with the same *T. gondii* ME49 strain the apoptosis induction was described, but the cell type involved in this process was not specified [39]. Therefore, we believe that the disorders in muscle tissue during our assays *in vivo* may be also related to a program of cell death, for example, apoptosis with the decrease of the adherent junctions, as also observed in the brain cells of embryos from infected mothers. More detailed histopathological studies are in progress considering that our preliminary analyses clearly indicated that embryos from the same litter had a differentiated influence in the degree of embryo development commitment induced by Toxoplasma.

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