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Establishment of Ectopic Xenografts Derived from Human Glioblastoma in Immunocompetent Mouse Model Treated with Pesticide

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Abstract

Background: In this study, we suggested an experimental procedure demonstrating the impact of pesticide on the development of ectopic xenografts of human glioblastomas in immuno-competent Balb/c mice.

Method: In this in-vivo study, the mice were treated with or without a mixture of pesticide (Glyphosate and Chlorpyrifos), using a concentration corresponding to 1/8 of LD50 of each pesticide. The pesticides were injected intraperitoneally every 72 hours. The human glioblastoma cell suspension was cultured with tumor cerebrospinal fluid and then injected subcutaneously into the treated and not treated mice with a mixture of pesticide (Glyphosate and Chlorpyrifos) following 18 days after the beginning of the experiment.

Results: The body mass index of the male and female mice treated with pesticide was statistically ($P = 0.0048$) higher than those not treated with pesticides. 66.6% of the mice treated with pesticides and xenografts of glioblastoma developed masses at the injection site. The histological analysis revealed that 41.66% of the masses were astrocytic tumors. The other found masses corresponded to inflammatory lymph nodes and fibroblastic tissue formations.

Conclusion: The treatment of mice with pesticide mixture was found to allow the development of glioblastoma xenografts in immunocompetent mice.

Keywords: Glioblastoma, Xenograft, Pesticides, Cerebrospinal fluid, Mouse model

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Introduction

Brain tumors represent 2% of all cancerous pathologies and rank 18th in terms of incidence and 12th in terms of mortality.[1,2] However, in developing countries, brain tumors appear to have a higher incidence and mortality.[1] This suggests that in developing countries, many exogenous biological factors (such as cytomegalovirus) or chemicals (such as plant protection products)

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not only play a pivotal role in the appearance and progression of brain tumors, but are also important in their degree of malignancy.[3,4] Gliomas, including glioblastomas subtype, are the most important histological type of intracranial primary tumors.[5,6] Additionally, glioblastomas are considered as the most aggressive histological subtypes, mainly due to their high angiogenic, proliferative, and invasive properties, but also their median low survival rate (less than 24 months).[6] Glioblastomas can be of two distinct origins; primary glioblastomas (de novo), whose main origin is glioblastoma stem cells and secondary glioblastomas originated mainly from the transformation of grade II and III gliomas. These two types discern from each other by different molecular profiles; they are also different in terms of evolution and treatment responses.[7] A better knowledge of the etiology of brain tumors and, more particularly, of glioblastomas, as well as the molecular mechanisms governing the progression of these tumors, will allow the establishment of appropriate therapies, thereby increasing survival. A more profound knowledge of cellular and molecular mechanisms requires the development of in vivo study tools. In oncology, the use of mouse models has improved our understanding of the processes governing tumor growth. Moreover, they have provided a better clinical model to screen and assess candidates for new anticancer drugs.[8,9] However, for brain tumors, apart from a few models of orthotopic xenografts,[10,11] a few models of ectopic xenografts has so far allowed easier study of brain tumors in general, glioblastomas in particular.[12,13] Thus, in this study, we evaluated the developmental capacity of ectopically grafted glioblastomas in immuno-competent mice treated with a mixture of pesticides.

Material and Methods

Glioblastoma tumor fragment

The tumor fragment of glioblastoma used in this in vivo study was collected after total excision in a 74-year-old male. The fragment weighed 6.5 grams and the histopathological analysis confirmed that it was a de novo grade IV glioblastoma.

Cerebrospinal fluid

10 mL of cerebrospinal fluid (CSF) was collected intra-operatively from a 24-year-old male patient with primary glioblastoma; for this study, we named it tumoral CSF. The two patients participating in this study provided informed consent to the use of their data and clinical samples for the present examination.

Mouse model

24 albino Balb/c mice (4-6 weeks), including 12 males and 12 females, were used in the present study. The animals were housed in a temperature- and humidity-controlled facility with 12-hour light/dark cycles. They had access to chow and water *ad libitum*.

Sample preparation

Cerebrospinal fluid

CSF was transferred to sterile tubes and centrifuged at 3000 rpm for 10 min and then filtered using a 0.45 µm filter (Merck-Millipore®). A cytological analysis was carried out using trypan blue to ensure the absence of cellular contaminants.

Tumor fragment

The tumor fragment was transported at 4ºC in a sterile bottle containing RPMI-1640 (GIBCO®). It underwent mechanical and enzymatic dissociation using scalpels and trypsin-EDTA (GIBCO®). This step was performed in triplicate. The tumor fragment was incubated for 10 min with a 20% RPMI trypsin solution, concomitantly with mechanical dissociation. The enzymatic reaction was stopped by adding a PBS-BSA solution of 200 mg/ml. The recovered suspension was centrifuged for 5 min at 2000 rpm. Following the end of the dissociation operation, the recovered suspension was washed with a PBS solution with centrifugation at 2000 rpm for 5 minutes. The suspension was filtered through the use of sterile gauze bands. The recovered filtrate was resuspended by the addition of 1 ml of PBS and 4 mL of tumoral CSF. Subsequently, the suspension was incubated for 2 hours in a CO_2 incubator (Figure 1A).

Mouse treatments

The mice were divided into two groups, one

receiving intraperitoneal injections of pesticides (Pesticides +) and the other receiving intraperitoneal injections of sterile physiological serum (0.9% NaCl) (Pesticides -). In the group of Pesticides + mice, each received a volume of 400 µl of the mixture of pesticides containing chlorpyriphos-ethyl (*O*, *O*-Diethyl *O*-3,5,6-trichloropyridin-2-ylphosphorothioate) at a sub-acute concentration (1/8 LD50 = 24 mg/Kg) and Glyphosate (*N*-(phosphonomethyl) glycine) at a sub-acute concentration (1/8 LD50 = 16.25mg/Kg). The mice were weighed and received injections in an interval of 72 hours until sacrifice.

Following the 6th injection, each mouse received a subcutaneous injection on the right flank, containing 200 µl of the glioblastoma cell suspension prepared previously (Figure 1B).

Histological sampling and analysis

The mice were sacrificed and their organs were removed. The brain and tissues were fixed using a 10% formalin solution. The histological sections of the paraffin blocks were 3 mm thick and colored with hematoxylin-eosin. Microscopic observations were carried out using an optical microscope (DM1000, Leica-microsystem®) with a camera (MC170-HD, Leica microsystem®). Image processing of histological sections was performed via LAS EZ3.0 image processing software (Leica-microsystem®).

Statistical analyses

Statistical analyses were performed utilizing GraphPad-Prism® software (Version 5.03). Student's test was used in the study.

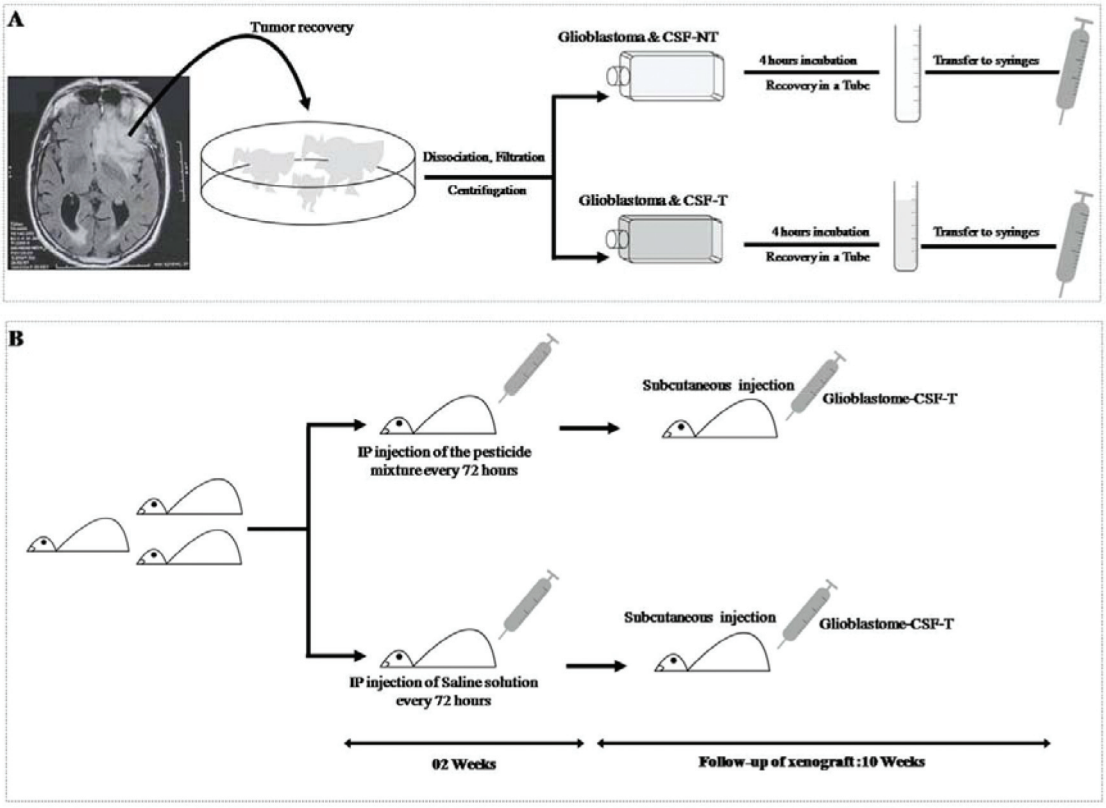

Figure 1. Experimental method for the development of glioblastoma xenografts in Balb/c mice. A: Preparation of glioblastoma cell suspension. Tumor fragments were recovered intra-operatively, in a 74-year-old male patient. Tumor fragments were mechanically and chemically dissociated and then filtered. Glioblastoma cell suspension was cultured with tumor cerebrospinal fluid (CSF-T); B: Experimental method for treating the mice and injecting cells subcutaneously. Balb/c mice aged 4 to 6 weeks were pretreated for 18 days (6 injections) with intraperitoneal (IP) injections of a mixture of pesticides (Pesticide +) or saline solution (0.9% NaCl) (Pesticide -). Xenografts were performed subcutaneously (SC) on the right flank of the mice. The injections of pesticides or saline solution were continued post-xenograft for eight weeks.

Results

All the mice in different lots of the study had a weight at the start of the experiment ranging from 23 to 25 grams. Whether treated or not with pesticides, both male and female mice showed a gradual increase in weight (Figure 2A). For note, males gained more weight than females. We also noticed that male mice treated with the mixture of pesticides had a more significant weight change than the untreated ones although this difference was not statistically significant (Figure 2A). Interestingly, in females, the weight of those treated with the pesticide mixture changed more significantly than that of the untreated female mice. In addition, this significant change in the weight of female mice treated with the pesticide mixture seemed to be similar to that of the untreated male mice (Figure 2A). Moreover, the analysis of the indices of body mass index (BMI) of the mice (Figure 2B) showed that unlike the male mice, the female mice treated with pesticides had a significantly higher BMI compared with those not treated with the mixture of pesticides ($P = 0.0048$) (Figure 2B).

In addition to the effects observed on the mice weight, the pesticides treatment seemed to affect the structure of the brain and the homeostasis of its tissue (Figure 3). The macroscopic study of the brains of the untreated mice represented a vascularized brain with a total conservation of the cerebral anatomy, but also the presence of fissures and a cerebellum with a physiologically normal size (Figure 3A). On the other hand, the brain of the mice treated with the mixture of pesticides exhibited a reduction in their vascular network (Figure 3A). Furthermore, our

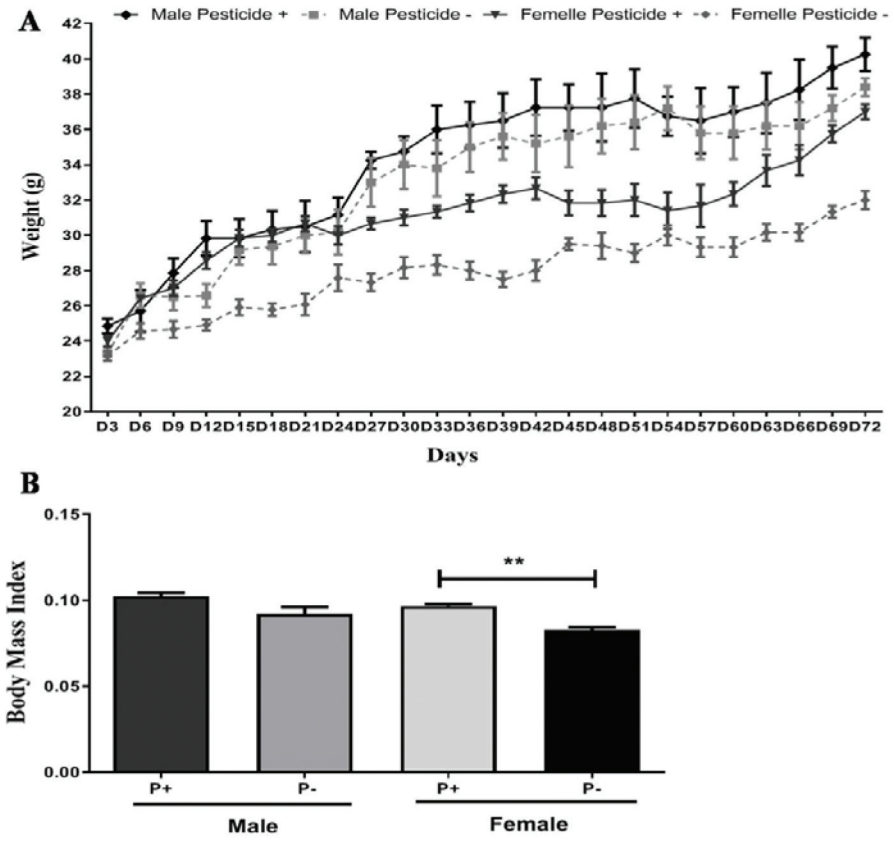

Figure 2. Effects of pesticide mixture injection on the evolution of weights and body masses of Balb/c mice. A: Graphical representation of Balb/c mice weights evolution: Animals receive intraperitoneal (IP) injections of a pesticides mixture (Pesticide +) or physiological serum (0.9% NaCl) (Pesticide -). The injections were performed every 72 hours. Animal weight was measured during each injection; B: Histograms of Balb/c mice body mass indices. Body mass index (BMI) was calculated after 72 days of experimentation.
BMI = mass (gram) / Height≤ (cm≤). *P*-value = 0.01

observations showed an increase in the size of the cerebellum, an erasure of the fissures, and the presence of areas corresponding probably to ischemic or gliosis processes (Figure 3A). The histological analysis also confirmed the macroscopic observations. In fact, histological sections carried out on the brains of those not treated with pesticides (Pesticides -) revealed a normal cell density with a homogeneous cell distribution with no mitotic or apoptotic signs or any regular vascularization (Figure 3B). Histological sections of the pesticides of the treated mice brains showed that 62.5% of these animals presented brain tissue with an abnormal cell density corresponding to a microcystic focus. They also indicated the presence of cells with imperfect cytoplasmic contours and condensed or fragmented nuclei (Figure 3B).

Following the right flank subcutaneous grafting of glioblastoma cells, the injection site was assessed daily, while the pesticides treatment was carried out every 72 hours (Figure 1B). Once the right flank of all the mice was shaved at the end of the 27th day post-subcutaneous injection (48th day following the beginning of the experiment), we observed the presence of masses in 66.6% (8/12) of the mice treated with pesticides (Figure 4). In addition, no mass was visible (0/12) in those not treated with the pesticide mixture (Figure 4).

Following the sacrifice of the mice and recovery of different masses, the histological analysis revealed that out of all the masses found in the Group I mice, 41.66% (5/12) corresponded to tumors of astrocytic types (Figure 5). The other found masses corresponded to inflammatory lymph nodes (28.57%) (Figure 5), as well as non-tumor, probably fibroblastic tissue formations (28.57%) (Figure 5A).

Discussion

This in vivo study demonstrated that the treatment of immunocompetent mice with

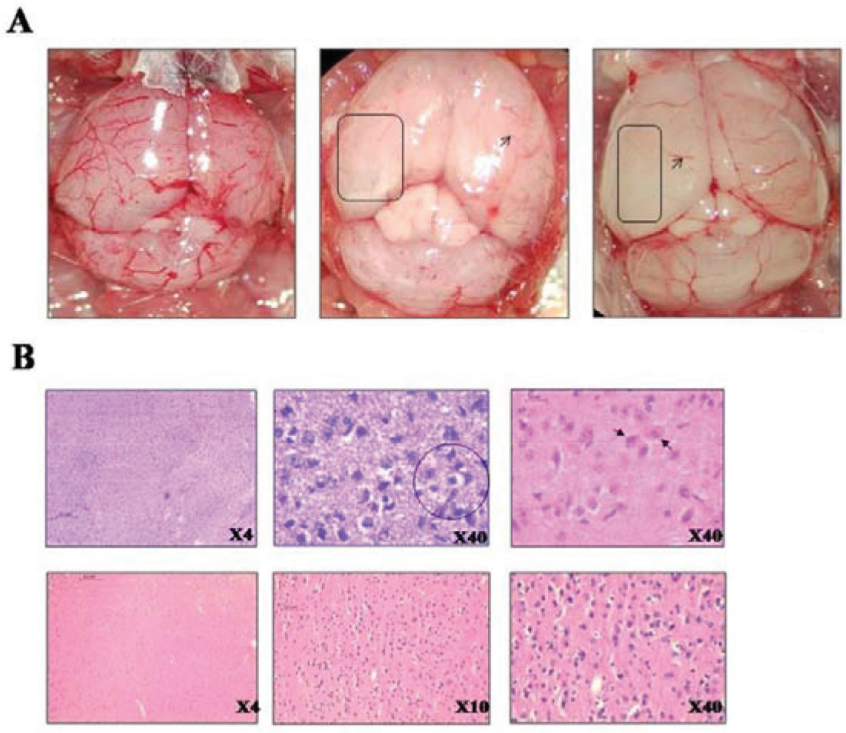

Figure 3. Impact of pesticide mixture on of Balb/c mice brain. A: Representative photographs of Balb/c mice brains macroscopy. Left: brain of a mouse injected with physiological saline (0.9% NaCl). Middle and right: mouse brains injected with pesticide mixture. The rectangles represent ischemic and gliosis areas. The arrows represent poor vascularity; B: Representative photographs of histological sections of Balb/c mice brains. Above: histological sections of mouse brains injected with the mixture of pesticides. The circle represents microcystic area. The arrows represent cells with irregular cytoplasmic contours. Below: histological sections of mouse brains injected with physiological saline (0.9% NaCl). (H & E: ×4, ×10, and ×40).

pesticide mixture allows the establishment and growth of glioblastoma xenograft.

The development of xenografts in the mice treated with pesticides seemed to indicate that treatment with the mixture of pesticide modifies mice biochemical status. These modifications provide favorable conditions for tumor xenografts growth. Additionally, the increase in the weight of the treated mice compared to that of the untreated ones, in particular in females, reinforced the hypothesis according to which pesticides have a disruptive effect on the endocrine system.[14,15]

In addition to the impact of the mixture of pesticides on the mouse model's organism, it seemed as though the brain cells were also sensitive to this mixture of pesticides.[4,16] The signs of ischemia and gliosis observed in the brains of pesticides+ mice indicated that the pesticide mixture crossed the blood-brain barrier and thus disturbed the homeostasis of brain tissue. Hence, our observations suggested that treatment with pesticides has a direct effect on xenografted tumor cells by increasing their oncogenic signaling,[17] in particular due to the fact that brain tissue cells, specifically astrocytic cells, are sensitive to mixtures of pesticides; it has been already reported by studies showing a link between the presence of pesticides and the development of brain tumors of astrocytic origin.[4,18]

However, the development of xenografts

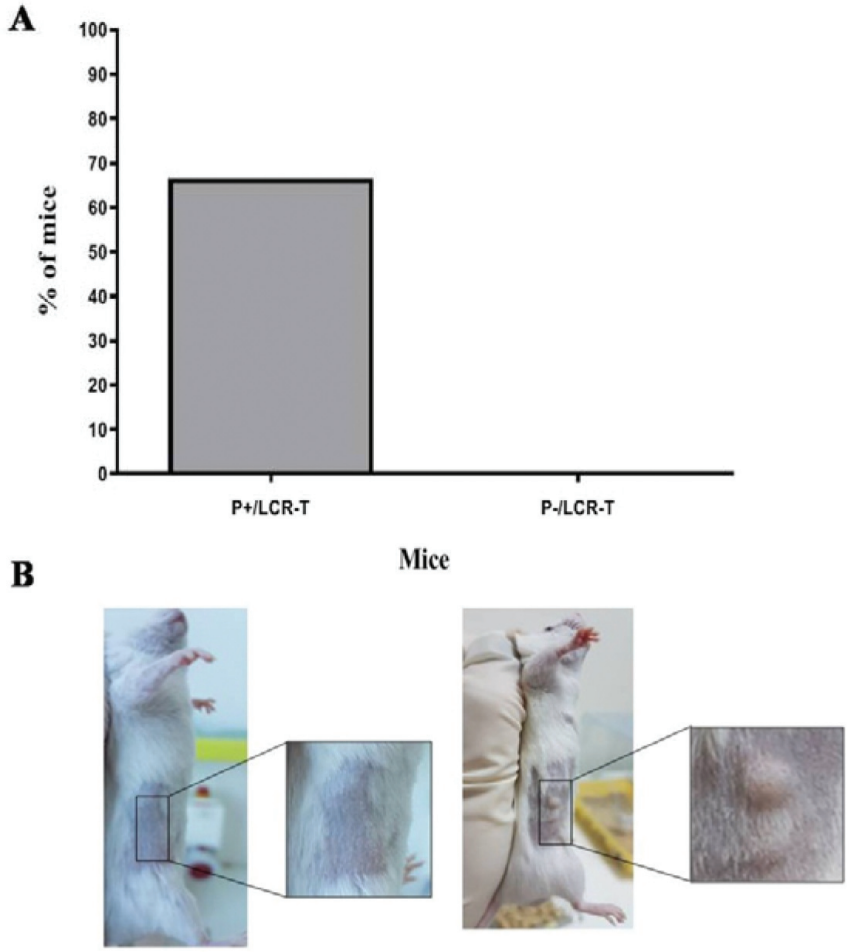

Figure 4. Effects of pesticide mixture injection on masses formation following xenografting. A: Histogram representing percentages of masses following xenograft. The mice treated with physiological saline (0.9% NaCl) did not develop any mass. B: Representative photographs of xenograft sites. Left: xenograft site showing no mass.

requires an immunosuppressed environment. In the current study, the immunosuppressed environment was probably created by the administration of the mixture of pesticides known to play a role as an immune system disturber.[15,19,20]

The experimental procedure presented in this study showed that the treatment of immune-competent mice with a mixture of pesticides and culture of human glioblastoma cells with a cerebrospinal tumor fluid allow the establishment and development of glioblastoma xenografts. Nonetheless, unlike other tumor types, where the use of immunodeficient mice allows the development of xenograft models, for cerebral tumors, there have been several unsuccessful attempts.[13] This could be explained by the particular microenvironment of the brain tissue, the interaction of which with cerebrospinal fluid is an essential element for its homeostasis.[21,22] Therefore, the culture of glioblastoma cells with tumoral CSF seems to allow the establishment of a favorable microenvironment, making tumor development possible.

Further studies are needed to further expand our knowledge of the effect of pesticides about the development of glioblastoma xenografts. The development of cell culture of glioblastoma and the establishment of neurospheres will also allow a better understanding of the impact of pesticides on normal and oncogenic cell signaling. The use

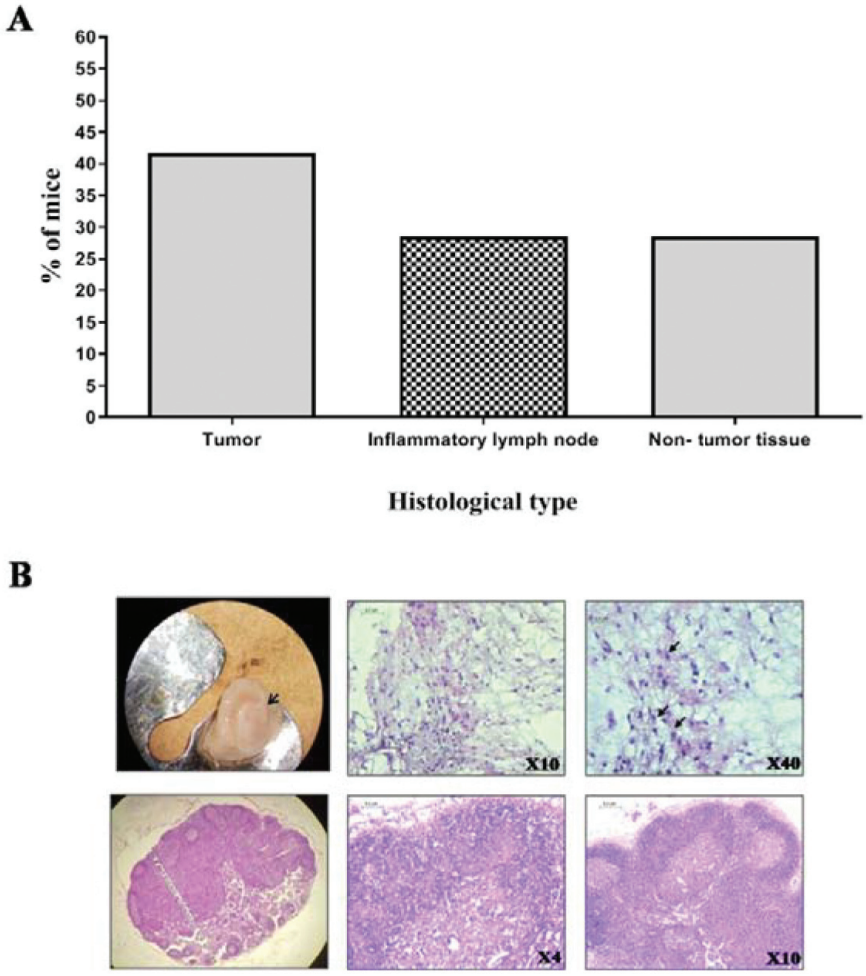

Figure 5. Development of xenografts in Balb/c mice. A: Histogram representing percentages of xenografts developed. The mice treated with physiological saline (0.9% NaCl) did not develop any mass (they are not represented on the graph). P+/CSF-T: mice treated with pesticide mixture and xenografted with glioblastoma cells cultured in tumor cerebrospinal fluid; B: Representative photographs of xenograft sites. Top left, macroscopic photograph of the tumor xenograft (designated by the arrow not full), in the middle and right: histological sections of the xenograft where astrocytic cells were observed (full arrows). (Below: inflammatory lymph nodes; H & E: ×10 and ×40)

of other types of animal models, but administrating lower doses of pesticides with longer time of treatment, will undoubtedly allow us to approach the physiological conditions observed in humans.

Conclusion

This study was one of the first works to demonstrate the involvement of pesticide mixtures composed of Glyphosate and Chlorpyrifos in the development/progression of glioblastoma in immunocompetent mouse xenograft. It thus allowed the development of an easy animal model to study the glioblastoma molecular processes. It also represented a promising tool for the development of new therapeutic molecules in the context of the fight against glioblastoma.

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Conflict of Interest

None declared.

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