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Culture Insights of Cellular Mechanisms of Glioblastomas Growth

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ABSTRACT

At present, glioblastomas of the brain are incurable tumours and the new developments of clinical neurosurgery, radiation therapy and chemotherapy do not solve the issue. Studying the cellular and subcellular growth mechanisms of the initial glioblastoma cells taken from tumours during neurosurgery and cultivating said cells for 3-6 months, allows us to put forward a relatively new hypothesis about the amitotic division of the glioblastoma cells. It is important to note that the tumour growth occurs *via* the transfection and expression of fragments of proteins and nucleic acids produced by the glioblastomas into the healthy contractile glial cells. This hypothesis is indirectly confirmed by conducing experimental studies with the introduction into the cell culture of polymerase, adenovirus of the AstraZeneca vaccine, as well the visual data obtain during magnetic resonance imaging (MRI) to observe the dynamic and character of the growth of glioblastomas in the brain.

Keywords: Glial cell; Glioblastoma; Mitosis; DNA; mRNA; Transfection; Biochemical resonance; PCR; MRI; Infiltrative growth of glioblastoma.

INTRODUCTION

Glioblastomas of the brain are incurable tumours with a one hundred % mortality rate within 1.5 years-2 years after the full macroscopic removal by using all of the modern techniques, alongside with radio and chemotherapy. When talking about a partial tumour removal, this period is reduced to one year [1-3].

One of the reasons of such modest success over the course of decades is the incomplete understanding of the cellular mechanism of growth of glioblastomas. It's commonly assumed that the malignancy of tumours is determined by acceleration of the cell division rates. Moreover, the root cause of the genetically modified clone is a single cell that is continuously dividing, resulting in a tumour [4]. This is caused by various DNA defects remaining unrepaired when the cell enters the next cycle of the G1-G2-M division; meaning that at key phases (check points), G1/S the integrity of the DNA double helix are not checked. When the correctness of the DNA replication is not checked at the G2/M point and finally at the M point, the proper attachment of the spindle to kinetochores and centromeres is also not verified.

The fact of cells going through these checkpoints without undergoing DNA repairs is caused by the deactivation of suppressor genes, such as P53 and RS1, as well as the activation of proto-oncogenes; leading to the accumulation of gene mutations without triggering apoptosis and stimulation of the rate and number of mitoses [5]. Both of these processes result in a malignant transformation of cells. Epigenetic factors, particularly the hypo–methylation of the DNA cancer cells, enrich the more modern concepts of oncogenesis. Alongside mitosis the hyper-methylation of individual suppressor genes will sharply reduce the control on having artefact–free DNA and encourage the accumulation of mutations [6,7].

There are multiple studies dedicated to the polyploidy and the amitotic cell division during the process of carcinogenesis. Given the unstable nature and genetic variability, polyploidy is

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viewed as a characteristic feature of cancer [8]. Certain metabolic aspects of aneuploidy clearly contribute to the resistance and recurrence of tumors with a high degree of malignancy [9-11]. Cells that divide amitotically are not highly clonogenic; however, the giant polyploidy cells have a high survivability rate and are typical not only for the late stages of cancer but can also be seen during the precancerous stage, a fact which suggests the possibility of them having another metabolic role besides genetic selection [12-14] Given that, one must evaluate the amitosis as an important prognostic and curative factor [15,16].

On a system level, the insufficient limitation of the intensive proliferation of malignant cells is caused by the failure of humoral and cellular mechanisms of immunity. It is considered that the membrane of the cancer cell carries very few antigen bodies that are foreign to the human organism and since usually they are monovalent, they trigger a wear precipitation reaction with the immunoglobulins that are produced by B lymphocytes, as well as a weak cytotoxic reaction of T lymphocytes.

Our experimental studies on the long term *in vitro* cultivation of glioblastomas combined with an analysis of MRI imaging of the cells and their intraoperative morphology allow us to propose a hypothesis of a non-mitotic division of glioblastoma cells and an increase in tumour volume caused by the transfection of healthy glial cells with glioblasts.

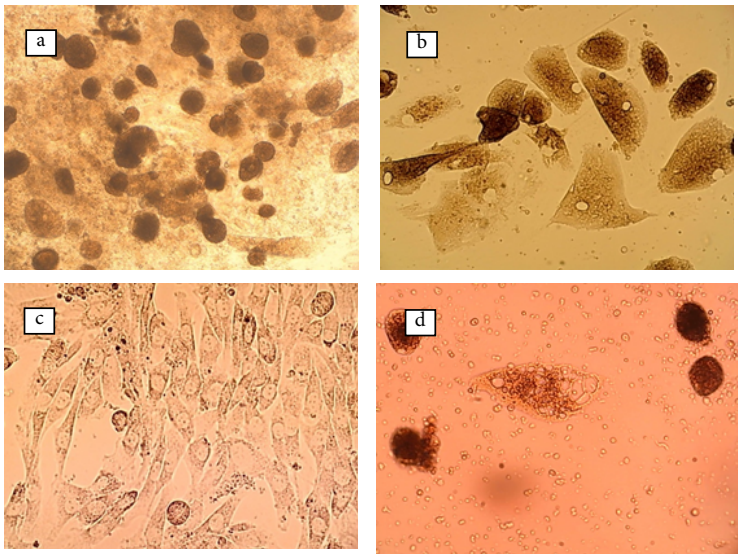
METHODS AND MATERIALS

Small glioblastoma fragments around 5-6 cm^3 in volume, taken directly during the tumour removal, were placed in sterile dishes filled with 20 ml of the patient's own plasma. No less than six hours later, the tumour tissue would be grinded and dispersed by filtering through sieves with a mesh size from 1.5 mm to 0.5 mm. This was then planted in sterile flasks filled with DMEM medium with a high glucose concentration (4.5 grams per litre) and an added 20% of a serum obtained from the patient's blood and fluconazole (100 milligrams per litre). The culture was then placed in a CO_2 incubator in which the following conditions were maintained: A temperature of 37°, a CO_2 concentration of 3.5% and a humidity of 95%. Over a period of three weeks, the culture would be re-suspended and re-centrifuged. On the fourth week the amount of cells would be sufficient to allow checking their individual sensitivity to various chemotherapy drugs, the cell tropism of lymphocytes to glioblastoma cells as well as allowing video monitoring of globalists' division. In the search for the growth factors of glioblastomas after two/three months of cell cultivation, repeated removal form the culture media and for overcoming the crisis of cell division in every 15 ml. of the culture media a DNA polymerase I (20 to 40 UA activity units) and 70 microgram of all 4 deoxyribonucleotides was added. In addition, in order to evaluate the growth of glioblastoma cells in the presence of a recombinant vaccine, 0.1 ml of standard Oxford/AstraZeneca (ChAdOx1-S [recombinant] vaccine) COVID-19 vaccine was supplemented to each 15 ml of culture media.

RESULTS

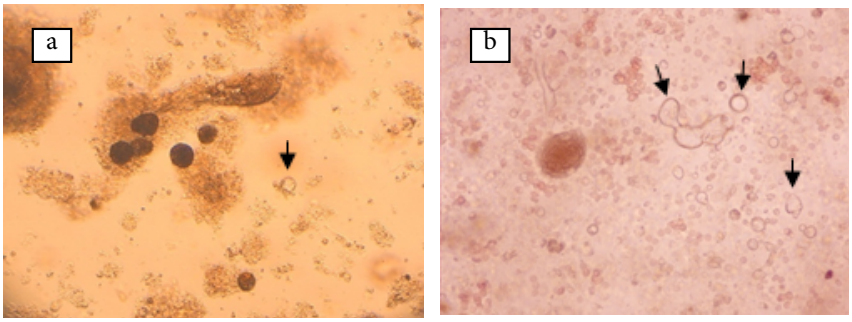
Our research on the cultivation and monitoring of primary glioblastoma cultures obtained from post-operative patients, over a period of three-six months, allows us to state that the first two weeks of incubation, the cellular make-up of the culture is made up by glioblastoma cells that are round in shape, have a diameter of 20 mm-30 mm and have intensely hyper chromic nuclei (×200) (**Figure 1a**).

The vast majority of these cells have a significant higher mass than the medium in which they are placed and they settle on the bottom of the dish, where they spread out in a fibrous like fashion (**Figure 1b**). Prolonged observation of the steady increase in number of these cells gown in a monolayer allows us to conclude that the cells that have condensed nuclei spread out evenly, reaching the shape of an irregular oval elongated along one axis. Once they reach the bottom of the dish, they clump into clearly separated cell formations, which do not move (**Figure 1c**). These cells are not capable to undergo multiple rounds of mitosis in the medium and within three-four months of cultivation, they die due to karyorrhexis and karyopyknosis, leaving behind but a shadow (**Figure 1d**).



Figures 1: The dynamic of hyper chromic glioblast cell cultivation (X200). (a) hyper chromic glioblastoma cells in suspension, (b) the initial stages of the monolayer of hyper chromic glioblasts, (c) the stage of the subtotal filling of the monolayer of hyper chromic glioblasts, (d) the death phase of the hyper chromic glioblasts - karyorrhexis and karyopyknosis.

That division capacity is retained by a very small number of cells (in the single digits percentage wise from the full sample), characterized by a transparent cytoplasm and increased volume. They can be seen clearly see only on the 4th-8th week of cultivation, when the medium is less cloudy. Toward the 4th-8th month of cultivation, these cells make-up the majority of the cell population (**Figures 2a and 2b**).



Figures 2: The dynamic of cultivation of glioblastomas with a transparent cytoplasm. (a) single cell with a transparent cytoplasm – 1-2 months within cultivation, (b) multiple cells with transparent cytoplasm – 4-6 months within cultivation.

Their cytoplasm remains relatively transparent, there aren't any nuclei, there are no condensed chromosomes and often, there isn't even a state of hyper chromium cytoplasm. If the culture medium contains a serum concentration of 20%-30%, then the glioblasts don't migrate to the bottom and get cultivated in suspension while retaining their round shape (**Figures 3a and 3b**). The process of a new cell creation is more reminiscent of gemmation and can be recorded as it happens *via* video monitoring under the microscope. In the meanwhile the cells that have a transparent cytoplasm one can observe movement of that cytoplasm without any nucleus edges, which suggests the movement of the replicated DNA and protein in the synthetic phase of the interphase. This seemingly chaotic movement is quite intense and the outside cellular membrane is constantly changing shape and creating cytoplasmic peaks, which appear/disappear at various times (**Figures 4a-4d**).

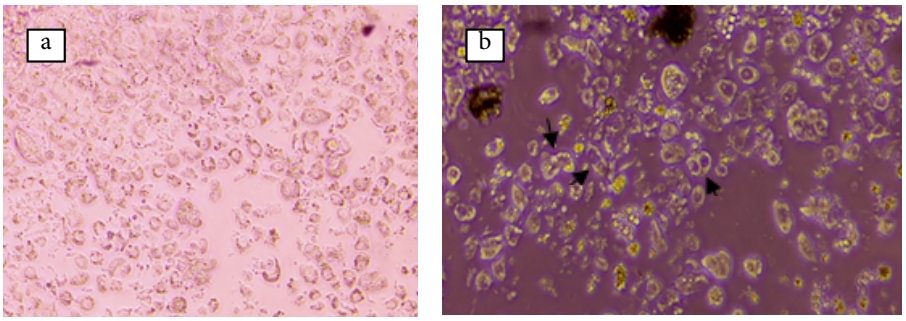
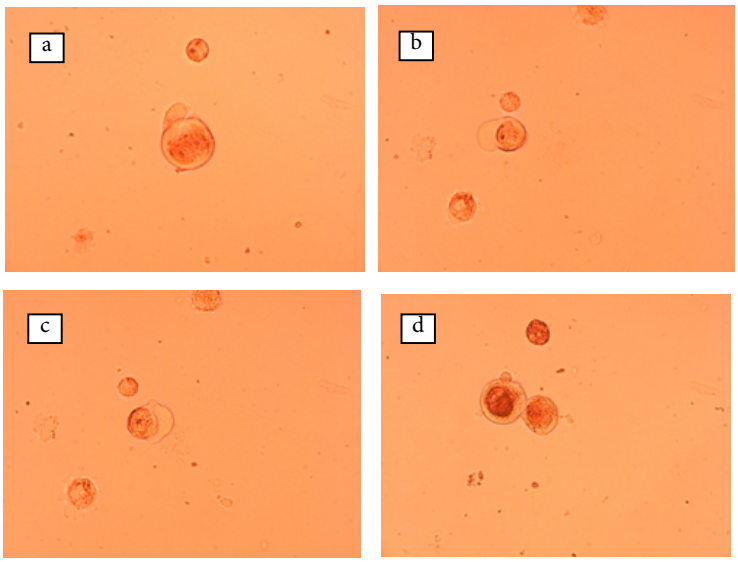
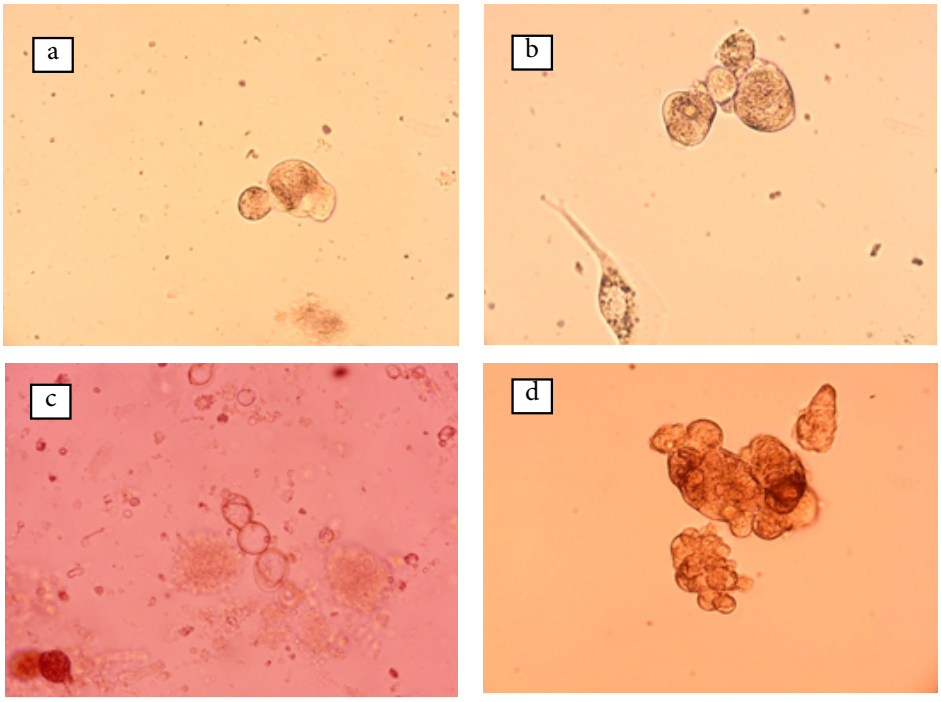


Figure 3: A culture of metastasized lung adenocarcinoma cells in the brain. (a) The mitotic division of the adenocarcinoma in the monolayer observed under the microscope in a bright field, (b) The mitotic division of the adenocarcinoma – phased contrast microscopy (the mitoses are indicated with arrows)under.



Figures 4: The process of cytoplasm's active movement with protrusions of the outer membrane and the gemmation of the daughter glioblast cell. (a-c) the circular motion of the protruding membrane of the glioblastoma cell, (d) The formation and sub-total division of the mother-daughter glioblastoma cell.

Within a couple of minutes, we can observe one-two protrusions on the membrane, which are increasing to a size that is comparable to the mother's cell. After that, this protrusion is pulled to a point that there is only a narrow attachment between this pseudo cell and the original one. In the process of lacing, you can see how the contents of the cytoplasm are chaotically transferred from the mother to the daughter's cell. Upon continuous observation of the gemmation process one can clearly see that there isn't any mitosis but rather the contents of the mother's cytoplasm are haphazardly transferred into the daughter's cell, creating a multi-cell conglomerate (**Figures 5a-5d**).



Figures 5: Variants of glioblastomas' division via gemmation. (a) Subtotal separation of the daughter's cell from the mother's, (b) Multiple buds with total and subtotal separation of the mother/daughter cells, (c)Gemmation with complete separation of the daughter cells, (d) Gemmation without a complete separation of cells, resulting in "monster" cells.

A fraction of the cells with this transparent cytoplast spread quite significantly, resulting in the creation of spindles. It is important to note that one cellular spindle is continued by another one and collectively they form long chains where you can't really see the membranes that separate one spindle from the other (**Figure 6**). In essence, the sequential nature of these spindles mirrors the aforementioned gemmation of daughter cells from one mother cells, same are cultivation in suspension; however as the third coordinate of the cell freedom is missing, i.e. the fixation at the bottom of the dish, these spindles is formed instead. The contents of the cytoplasm of the daughter cells is transferred just as haphazardly from one cell to the other, without any indication of chromosome condensation, typical for mitosis.

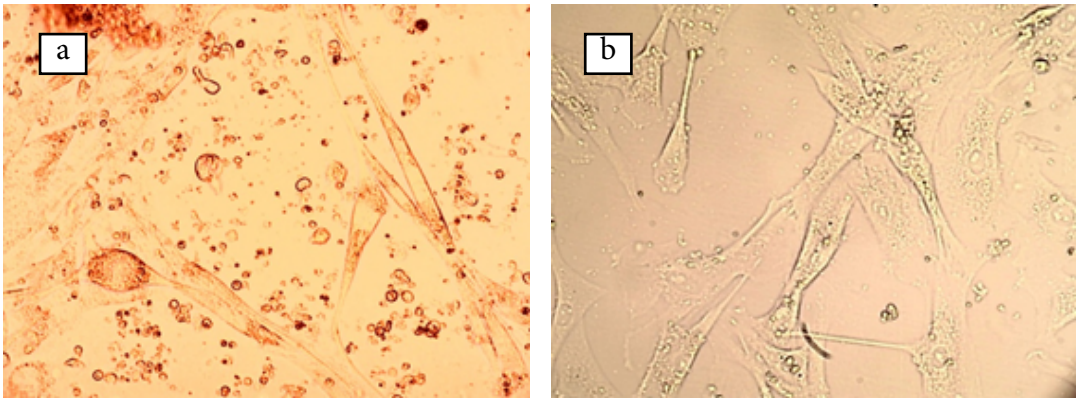
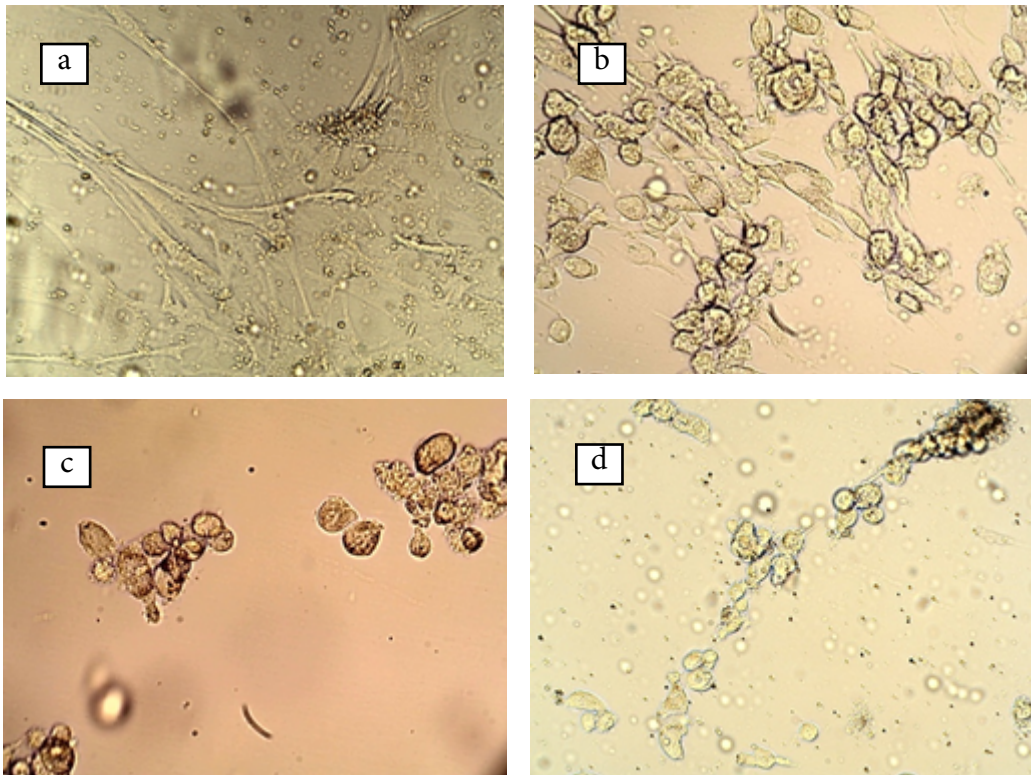


Figure 6: The creation of spindles of glioblasts in the monolayer. (a) Beginning stage of the formation of the glioblast spindles' grid in the monolayer, (b) Subtotal filling of the glioblast spindles' grid in the monolayer.

A clear confirmation of the gemmation process in the monolayer is the trypsinization of glioblasts, during which the process of cell separation from the bottom of the dish is accompanied by dragging several other cells *via* a thin continuous membrane, one after the other. During a complete separation from the bottom of such cells and their transition to a suspended state,

daughter cell groups are formed, with a high number of bulbous protrusions (Figures 7a-7d).



Figures 7: Trypsinization of the monolayer of glioblasts. (a) Subtotal monolayer of glioblatst prior to trypsinization, (b) The beginning of the separation of the layer from the bottom of the dish with the formation of daughter cells, (c) Subsequent separation and rounding of cell groups, (d) Complete separation of a group of cells with their transition into suspension.

Taking that into consideration, when primary glioblastoma cells are being cultivated in suspension or in a monolayer, it's practically impossible to observe a mitotic cell division with hyperchromic condenced nuclei. Those cells are present at the beginning stages of incubation in the absolute majority of cases; however towards the 3rd-4th month of the process almost all of those cells die. In contrast, the cells that have a decondenced and potentially replicated DNA, and are present in very small numbers at the beginning, make up the majority of the population at the later stages of incubation. Nevertheless, in the process of sifting the culture, if the cells are not frozen during the preliminary division stages to pseudo-immortalize the culture; the cell mass is constantly declining and ultimately all of the cells are dead by the 4th-6th month.

At the later stages of the incubation, the cell population is almost exclusively made up by the cells with a transparent cytoplasm and the transformation process gets triggered, resulting in an increase in cells' volume, a significant shape distortion and the appration of "monster" cells (Figure 8)

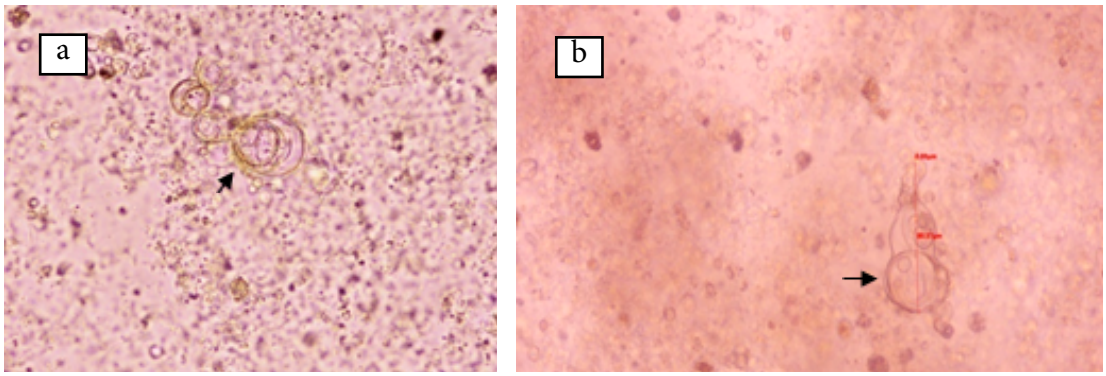


Figure 8: "Monster" cells – late incubation stages.

Adding into this environment of the culture media with a DNA polymerase I (20 to 40 UA activity units) and 70 microgram of all 4 deoxyribonucleotides contributed to the cell gemmation process in a drastic fashion, as well as increasing in their size. These "monster" cells have a linear size between 40-100 micro meters and they become a key component of the entire cell mass. There are multiple inclusions that are visible in the cell's cytoplasm as well as multiple barriers. Besides that, we can observe the emergence of small (7-10 micro meters in diameter), oval cells with transparent cytoplasm, that following multiple

gemmations become similar to bunches of grapes (**Figure 9**)

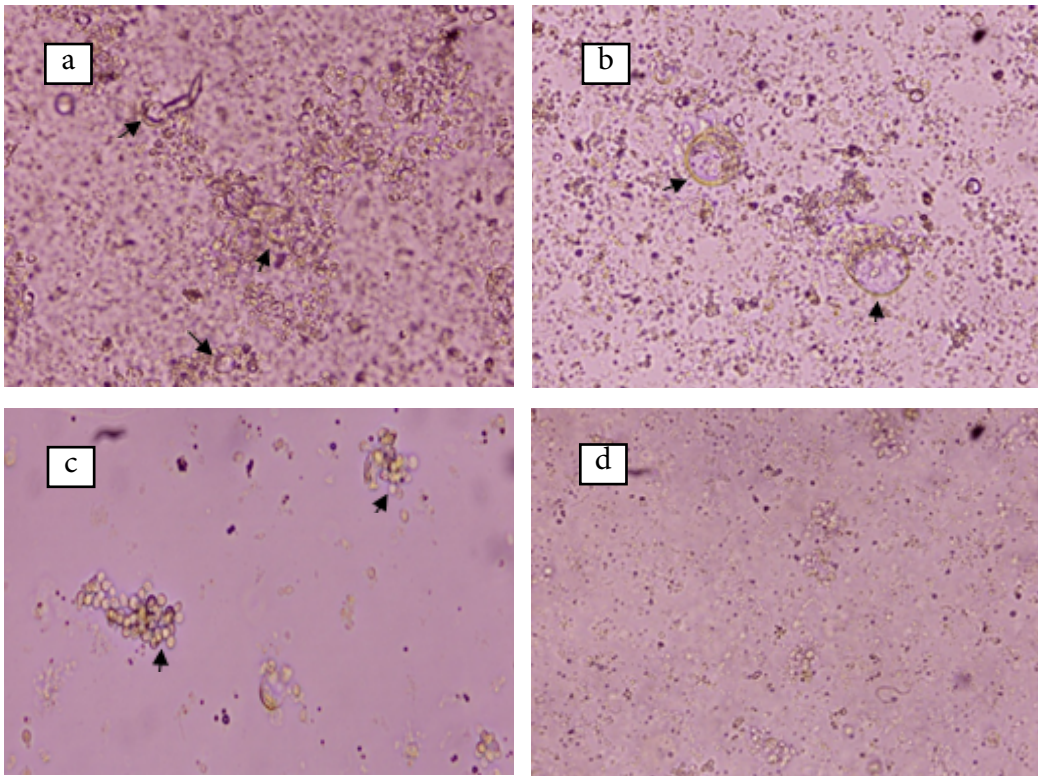


Figure 9: The introduction of polymerase 1 into a glioblastoma culture. (a) Significant increase of the number of glioblasts under the influence of polymerase 1, (b) The emergence of a large number of "monster" cells under the influence of polymerase 1, (c) The emergnece of colonies of small cell under the influence of polymerase 1, (d) Control sample of the same culture without the influence of polymerase 1.

It is important to note that the increase in numbers is caused exclusively by gemmation and not mitosis; during which the cytomplasm is always transparent or semi-transparent and there isn't any chromosome concensation.

An almost identical effect of a significant acceleration of glioblastomas' division as well as their increase in size is observed when introducing into the culture the Oxford/AstraZeneca (ChAdOx1-S [recombinant] vaccine), with a concentration of 0.1 ml to 15-40 ml of culture. Both the Oxford/AstraZeneca and Pfizer vaccines also supported the division of glioblasts in the later stages of incubation, after the control sample has already died (**Figure 10**)

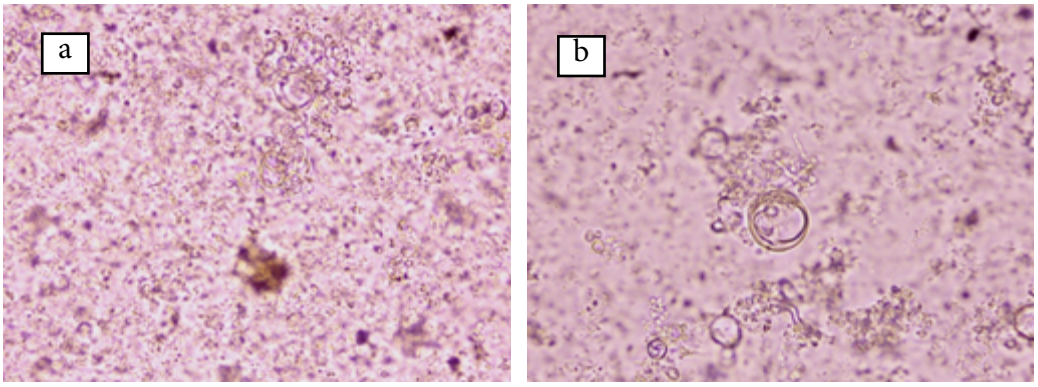


Figure 10: The influence of AstraZeneka on the glioblasts' growth. (a) Signification increase in the number of glioblasts after the introduction of the vaccine (control sample on Fig. 9), (b) Increase in the number of "monster" cells after the introduction of the vaccine.

DISCUSSION

By observing the division process of the glioblastoma cells *in vitro*, we are allowed to look at that process from a different angle and give logically indisputable interpretations of imagistic and intraoperative characteristics of glioblastomas.

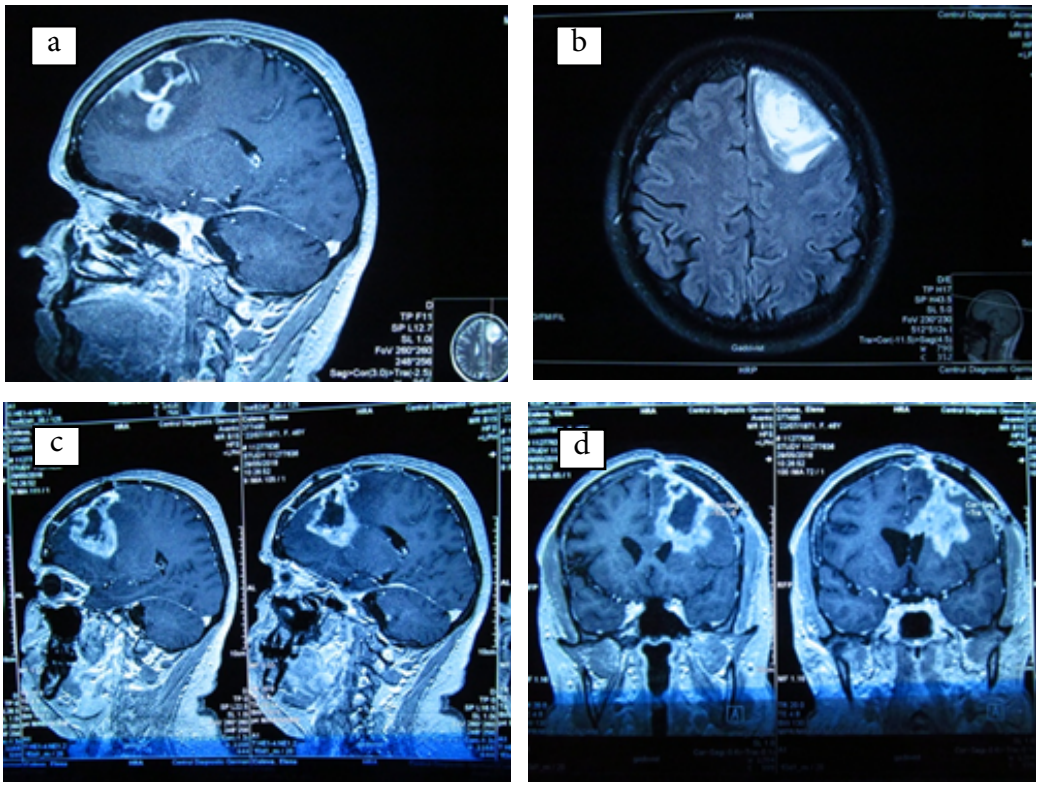
During an MRI examination of glioblastomas' defining characteristics, as a rule we can observe the following:

- Infiltrative growth
- Significantly irregular shape of the new formation
- Irregular edges of the tumour

- The tumour crossing the boundaries of brain lobes
- Infiltration into the corpus callosum and the ventricular ependyma
- Often, the presence of multifocal nodes that are closely positioned but not directly linked to each other
- A significantly lower mass effect compared to other brain tumours, especially meningiomas of the same size
- Presence of necrosis at the base of the tumour

An accumulation of gadolinium and infiltration by small capillaries of the irregular edge of the tumour (**Figures 11a and 11b**)

What stands out in the post-operation follow-up MRI where there were indications of a relapse is not the overall tumour growth in the yet unformed post-op cavity, but rather the tumour infiltration *via* a narrow slit on the edge of the cavity deep into the healthy brain tissue and the significantly higher area and steep of the infiltrative growth, compared to the pre-operation state. You can see the appearance of a couple of unlinked nodules of the tumour not far from the main process (**Figures 11c and 11d**)



Figures 11: MRI of a patience with a glioblastoma of the right frontal lobe. (a-b) MRI prior to the first surgery, (c-d) MRI four months after the surgecy – continual tumour growth.

The observations made during the surgery itself indicate the heteromorphic nature of the glioblastoma and the state of the brain tissue surrounding it. Areas of the tumour that got involved in the infiltration process into the white matter at later stages do so without any borders and are virtually indistinguishable from the brain, even when operating on an area that has been expanded by 5-7 times. The areas of the original tumour growth are much easier to distinguish that area has significantly less blood vessels and the density of the tumour can reach that of a fibroblastic meningioma. The newly formed small vessels and capillaries are concentrated on the outer edges of the tumour and the surrounding brain tissue whereas inside the tumour they are present in much smaller numbers or not at all. The tumour core that is considered on an MRI as necrosis, shows up as a as a colloid, protein. Opalescent, yellow-grey, non-vascular mass; that often transforms into a light brown cyst.

Comparison of data coming from experimental cultivation of glioblastomas *in vitro*, imagery results from MRI alongside with the observations made during the surgical process of removing glioblastomas, allow to postulate a non-traditional model of amitotic growth of glioblastomas in the brain. This hypothesis differs from the traditional understanding of the glioblastoma cell clones that underline the actual tumour theory after multiple uncontrolled mitotic divisions.

In essence, the healthy glial cells, under the influence of unknown catalysts, go from the G0 phase to the synthetic G1 phase. The single–stranded DNA of these cells gets replicated and starts amplifying uncontrollably gets transcribed into the mRNA and synthetises a large amount of protein with the formation of a bulla and gemmation of new cells. Said cells significantly increase in size, swell up and then proceed to gemmate again. The outer membrane of these cells is very thin and penetrable for DNA fragments-small expresser proteins for DNA amplification. These components go through the membrane of the healthy glial cell and this process spreads on its own, creating a picture of the infiltrative nature of the tumour growth. This way, the volume of the tumour increases not only due to the amitotic increase of the number of cloned cells but also due to

the involvement of the healthy glial cells into the self-sufficient process of them becoming now glioblastomas and genome of the gemmated and transfected cells does not correspond at all with that of a healthy glial cell. They are in perpetual synthetic phase and remain transparent and without a nucleus. At the same time, the process of copying remains self-stimulating and it should be viewed as a biochemical process, rather than biological; which can be replicated *in vitro* such as polymerase chain reaction (PCR). The cell goes into a state of biochemical resonance that is not controlled by the homeostatic cell mechanisms. It is important to note that for the maintenance of this process only the following components are needed-all nucleosides, ribose, triphosphates and polymerase. It's not excluded that the proteins synthesized in these cells are expressers for the DNA amplification, so one can talk about there being positive biochemical feedback between the proteins and the DNA of these cells.

An indirect confirmation of this hypothesis is the experimentally confirmed fact of a significant increase in the rate of cell gemmation, and the appearance of monster cells when polymerase and nucleotides or the vaccine, in our case is Oxford/AstraZeneca (ChAdO×1-S [recombinant] are added to the glioblastoma cells medium), which transform the cell division process into a self-sustained biochemical resonance process.

This concept allows a reasonable explanation of all the main signs of glioblastomas *via* MRI examination and intraoperative morphology.

CONCLUSIONS

1. The cellular proliferation of glioblastomas is polymorphic in size and shape, with hyperchromic nuclei and condensed chromosomes; and insignificant number of glial cells with a transparent cytoplasm and invisible nuclei. The latter type of cells is difficult to observe in histological samples and culture dishes at the early stages of incubation.
2. Cells with condensed chromosomes divide *via* mitosis, however the percentage of mitoses is insignificant. Cells that have a transparent cytoplasm and de-condensed chromosomes divide amitotically, without entering the mitosis phase, creating a daughter cell *via* gemmation.
3. Infiltrative growth of glioblastoma in the brain is mainly due to amitotic division, "budding" or gemmation of glioblastic cells and transfection of pathological fragments of DNA (RNA) into neighbouring healthy glial cells of the brain.
4. Chemotherapeutic drugs that affect various mechanisms of blocking mitosis are not effective, since the process of glioblast growth is amitotic, subcellular, biochemical in nature.

The protocol of this study was approved by the Research Ethics Committee of the "Nicolae Testemitanu" State University of Medicine and Pharmacy (nr.81 of 19.09.2020). All participants gave their informed consent, both orally and in writing, in accordance with the principles of the Helsinki Treaty adopted in June 1964 with subsequent revisions and additions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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