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The possible effects of sodium fluoroscein to primary cell culture sampling in glioblastoma surgery

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1. Introduction

Primary cell cultures more accurately reflect the characteristics of cancer cells in vivo, and established cell lines have increasingly been phased out of cancer research. Finding growing circumstances that limit changes in the biological state of the cells is a significant challenge with primary culture, though. Therefore, the culture conditions should be chosen so that the cell population in culture most closely resembles tumor cells in vivo in order to assure reproducibility and translational potentials for research findings.

Sodium fluorescein (FL) dye is used to delineate the tumor and normal tissue, especially in ophthalmological surgeries (Rabb et al., 1978). Owing to FL ability to pass blood-brain barrier and the advent of new technological developments, it is also preferred in the neurosurgical field lately (Schebesch et al., 2016). During tumor surgery, FL-stained regions are regarded as tumor tissue, and they are resected.

Glioblastoma (GBM) represents the most common and aggressive primary brain tumor with a poor prognosis. Advances in genetic studies have revealed GBM as a highly heterogeneous disease. GBM is among the cancers most resistant to radiotherapy and chemotherapy, so it is known as an incurable disease with an average survival of 15 months.

Primary GBM cell (GC) cultures have become a crucial tool in the research of brain tumors, with the potential of revealing patient-specific variations in therapeutic response. Unfortunately, quantitative data on the stability of these cells over the first 20–30 passes of culture are few. All areas stained with sodium fluorescein are regarded as tumors and eliminated during the excision of a GBM tumor. Consequently, those regions used for primary cell culture are also stained with FL. However, it is not known that the effects of FL on GBM cell cultures.

Our study aims to understand and report whether FL affects GBM cell cultures.

2. Materials and methods

In our study, human GBM A172, LN229 and T98G cell lines were used. GBM cell lines were obtained from cell line stocks of Koç University, Faculty of Medicine, Brain Cancer Research and Treatment Laboratory which were taken from American Type Culture Collection (ATCC, USA). All cell lines were cultured at 37 °C and 5% CO_2 containing incubators inside 10% fetal bovine serum (Invitrogen, USA) and 1% penicillin-streptomycin containing DMEM medium (Gibco, USA).

GBM cell lines that were grown with standard cell culturing procedures were divided into control and FL applied groups. These groups were compared with regard to cell counts, cell viability, cellular aging, and beta-galactosidase analyses. In addition, microscopic images of cells were evaluated.

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2.1. Cell count

Cell counting was done with the trypan blue exclusion method. These methods separated viable and non-viable cells, and viable cells were counted.

2.2. Cell viability analyses

Cell Titer-Glo (CTG) Luminescence Cell Viability Assay Kit (Promega, USA) was used for cell viability analyses. This kit identifies viable cells and counts them by quantifying the amount of Adenosine Triphosphate, an indicator of metabolically active cells, using luciferase reaction. CTG chemical destructs the cell membrane and inhibits the endogenous ATPases simultaneously. It also contains luciferin, luciferase, and other chemicals to allow the bioluminescent response. For the economical use of our chemicals, CTG analyses were conducted following the protocol provided by the manufacturer.

2.3. Preparation of FL doses

While adjusting FL doses, doses that were described in surgical literature were evaluated, and it was adjusted to 3 mg/kg. The amounts were experimentally increased and decreased to measure the effects of cell culture.

2.4. Actin staining and evaluation of FL-stained glioblastoma cell series with confocal microscopy

Actin is one of the most found proteins in the intracellular environment; when it forms a microfilament type called F-actin, it can easily be labeled with a fluorophore. An actin-binding protein, phalloidin, becomes a practical tool for cell biologists using fluorescent microscopy to analyze cells. F-actin helps to understand the cell shape and structure and provides a binding site for other fluorescent labels. In our study, actin staining was done for FL applied cells to evaluate the intracellular skeletal system.

2.5. Cell aging of FL strained glioblastoma cell lines

Increased activity of β-galactosidase (β-Gal) has been shown to be related to cellular senescence. FL applied GBM cell lines are stained and counted after using the β-Gal StainingKit (Invitrogen, San Diego, CA, USA). Aged cells are positive for β-galactosidase activity.

3. Results

3.1. Invert microscopic images of cells

In our study, A172, LN229 and T89G were used as control cell lineages. After being incubated at 37^{O}C with 5% CO_2 inside DMEM medium, they were viewed under inverted microscopy.

3.2. Effect of FL dye on glioblastoma cell lines

FL dyes were applied to A172, LN229 and T98G cell lines at doses determined based on the literature. Effects of FL dye on cell viability was evaluated with CTG method after 48 h of FL application. Dose-dependent results are shown in Fig. 1A with microscopic images and cell viability results in Fig. 1B. On A172, LN229, and T98G GBM cell lines, FL application in lower or higher doses did not affect cell viability.

3.3. Evaluation of cells in FL applied medium

A172, LN229 and T98G FL applied GBM cell lines were grown in DMEM media. The initial amount of seeded cell lines were equal for the FL applied and control groups. Cells were passaged and counted every three days and were compared with the control group. Morphologic changes were also followed-up with microscopic imaging.

Based on microscopic evaluations, no morphologic difference between control and FL applied groups was found (Fig. 1A).

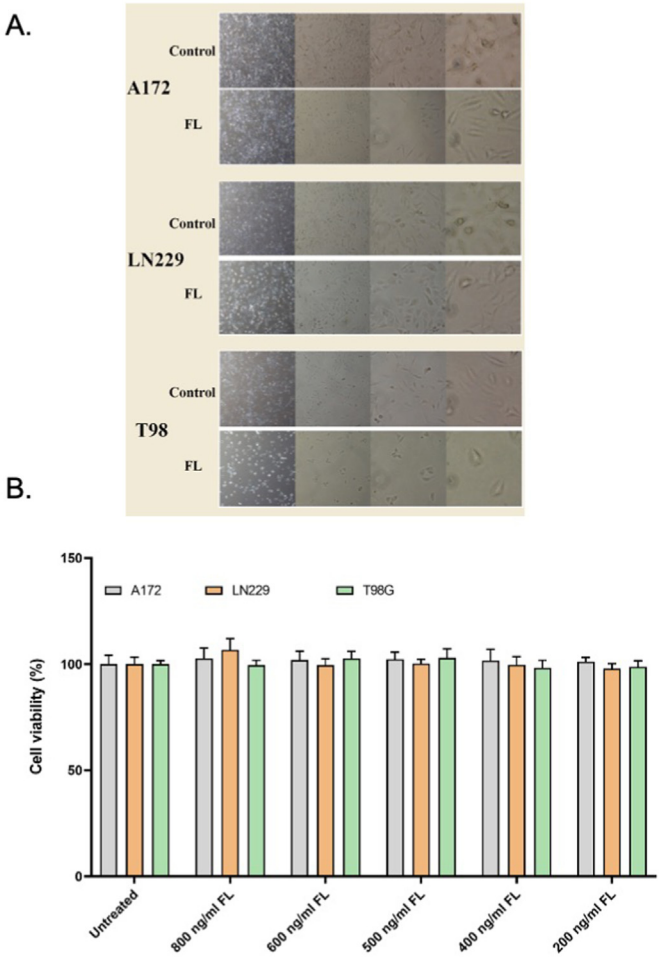

Fig. 1. A. Microscopic views show morphologic differences between control and FL applied GBM cell groups. **B.** The graphic shows the results of the CTG analyses of A172, LN229 and T98G GBM cells.

3.4. Cell viability

The doses determined according to the literature were applied to the A172, LN229 and T98G glioblastoma cell lines. The effect of FL on cell viability was demonstrated at the end of the 48th hour with the CTG method (Fig. 1B). In A172, LN229 and T98G cell lines, it was observed that FL did not have any effect on cell viability at low or higher doses.

3.5. Cell count

FL applied and non-applied cell lines were seeded in petri dishes in equal amounts and were incubated in similar conditions. On days three and seven, average cell counts of cell lines in 1 ml medium were calculated. Compared to the control group, it was found that the FL applied group had lower cell counts.

3.6. Evaluation of growth rate in FL applied glioblastoma cell lines

After three and seven days of incubation, a daily recording of cell count was done to observe the difference in growth rates between FL applied and control groups. A172, LN229 and T98G cell lines were grown

in petri dishes separately, control and FL groups were then compared. Initially, 50.000 cells were seeded in separate petri dishes. Cell plates were observed under the microscope daily and cell counts were determined with trypan blue. Increase in cell counts and changes in morphology were evaluated under 4X, 10X, 20X, and 40× magnification between days 1 and 5.

Under the microscope, A172 cell lines were observed sparsely, whereas their number increased by day 5 (Fig. 2A**).** FL applied group was observed to have fewer cells (Fig. 2B).

A graphic of cell counts between days 1 and 5 for FL applied and control LN229 cell lines is shown (Fig. 3A). The cell count of FL applied group was lower (Fig. 3B).

Under the microscope, T98G cell lines were observed sparsely, whereas their number increased by day 5 (Fig. 4A). A graphic of cell counts between days 1 and 5 for FL applied and control T98G cell lines is shown. Although to a lesser extent, when compared to other cell lines, cell counts were lower in the FL applied group (Fig. 4B).

3.7. Effect of FL on actin organization of glioblastoma cell lines

Intracellular skeletal structure of FL applied T98G GBM cell lines was demonstrated with actin staining. No difference with regards to actin structure was detected between control and FL groups under confocal microscopy (Fig. 5A).

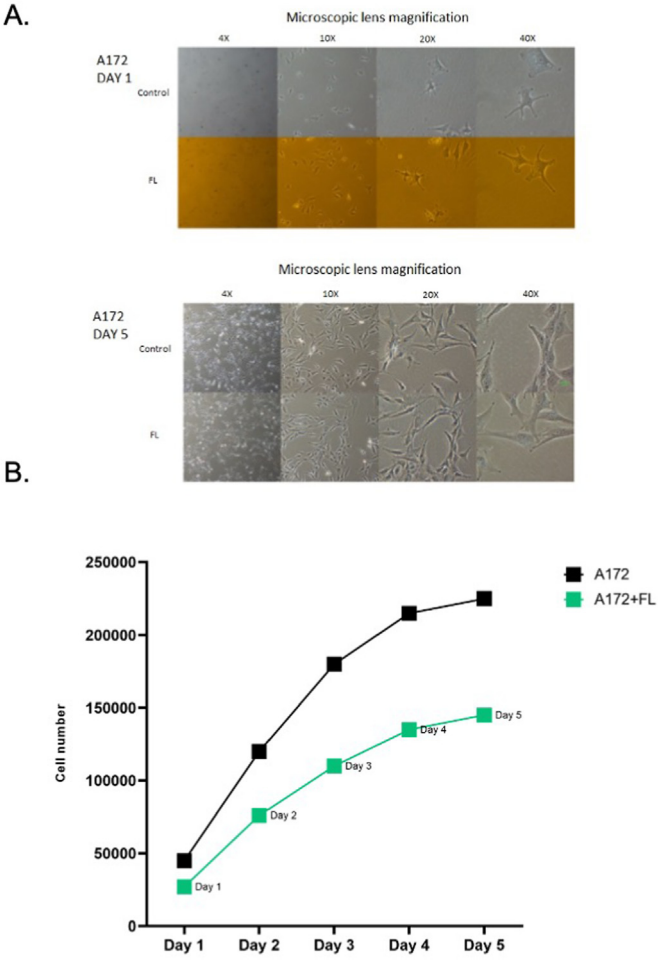

Fig. 2. A. Microscope images of control and FL added A172 cell culture on day 1 and day 5. **B.** The graphic shows the daily cell counts of control and FL added A172 cells from day 1 to day 5.

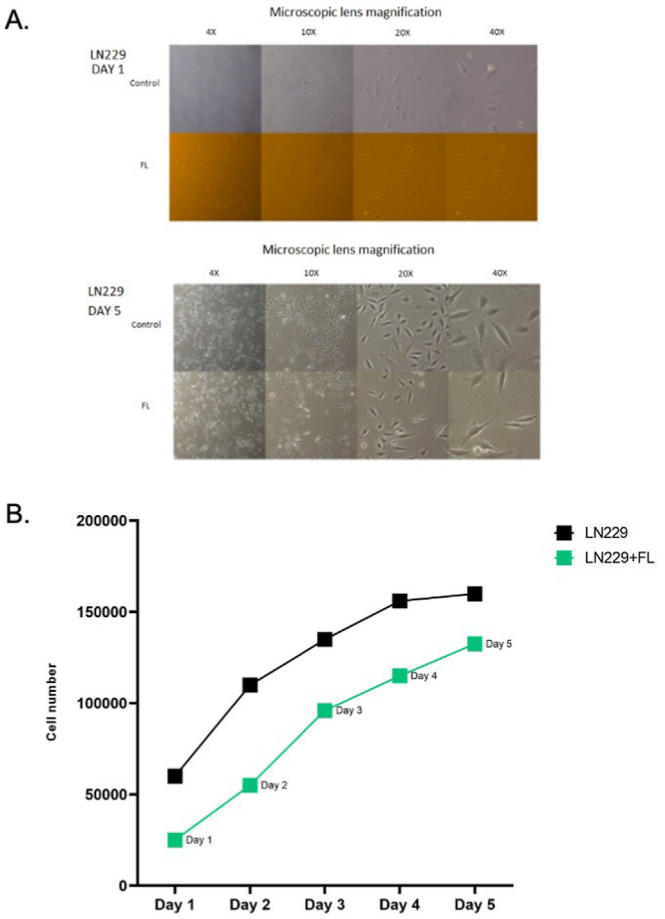

Fig. 3. A. Microscope images of control and FL added LN229 cell cultures on day 1 and day 5. **B.** Daily cell counts of control and FL added LN229 cell cultures from day 1 to day 5.

3.8. Effect of FL on cell aging of glioblastoma cell lines

Although aging cells lose their ability to replicate, they remain viable, metabolically active and resist to apoptosis. It is shown that increased β-gal activity is associated with cellular aging. Therefore, aged cells are accepted as positive for β-gal activity. It has been shown that FL applied cells show increased senescence (Fig. 5B).

4. Discussion

GBMs are grade 4 astrocytomas and are the most malignant astrocytic tumors. They are separated into two groups as IDH mutant and non-mutant (Louis et al., 2021). GBMs form nearly more than half of all primary brain tumors in the adult age group. With GBM treatment, associated survival is between 9 and 14 months (Davis, 2016). The search for effective means of treatment is still ongoing.

Surgery occupies an essential place in GBM treatment. The main goal is the resection of the tumor without neurologic damage. Depending on the location of the tumor, partial or total removal of the tumor can be done. The extent of resection is an important prognostic factor increasing overall survival, and efficacy of the adjuvant therapy (Young et al., 2015). Even though the extent of resection is an important prognostic factor, it is almost always impossible to resect the tumor totally due to the tumor's infiltrative nature. Even after a successful surgery with extensive surgical resection, a microscopic residual can cause recurrence of the tumor (Urbanska et al., 2014).

In the light of recent research, intraoperative use of FL is increased. When the blood-brain barrier is disrupted due to tumors, FL can pass

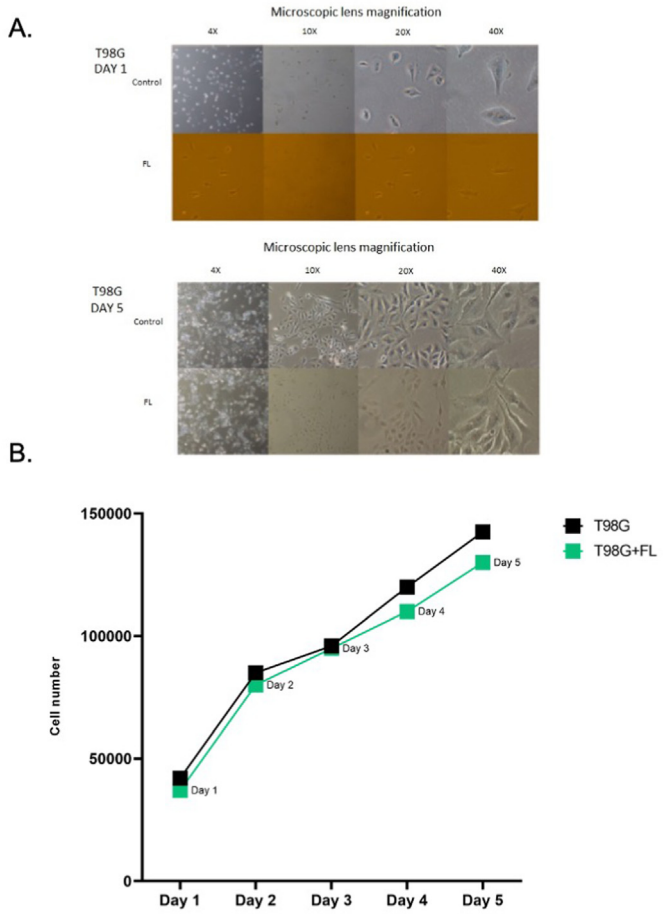

Fig. 4. A. Microscope images of control and FL added T98G cell cultures on days1 and day 5. **B.** Daily cell counts of control and FL added T98G cell cultures from day 1 to day 5.

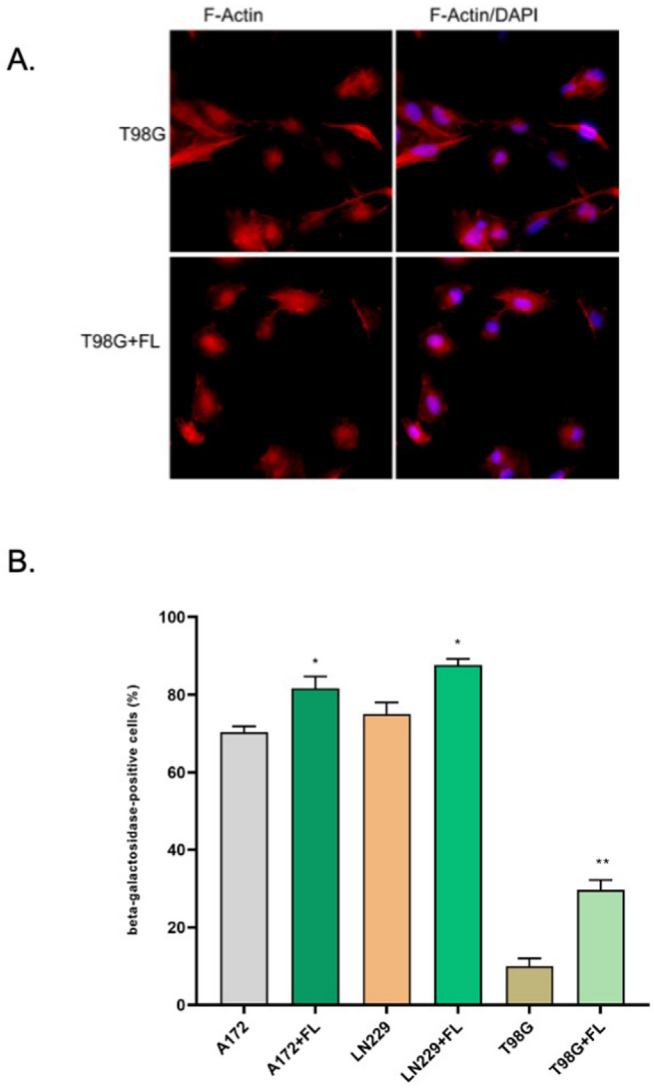

Fig. 5. A. The images after actin staining of control and FL dyed T98G cell culture under confocal microscopy. **B.** Beta-galactosidase positive cell counts after Beta-gal staining of A172, LN229 and T98G GBM cell cultures.

freely after IV application and show tumor infiltration. It was reported that contrast-enhancing regions in contrasted MRI and FL-stained regions show high compatibility. High sensitivity and specificity for tumor tissues at FL-stained areas were also reported (Schebesch et al., 2016; Babu and Adamson, 2012; Stummer et al., 2003). In addition, FL staining-guided tumor resection has been shown to increase total resection rates (Schebesch et al., 2016; Rey-Dios and Cohen-Gadol, 2013; Martirosyan et al., 2014).

After tumor samples are taken intraoperatively. The tumor tissue is physically and enzymatically degraded and purified into single cells. These cells are later grown in an appropriate cell medium and environment. This whole process of growing tumor cells obtained from patients is called primary cell culturing (Fukaya et al., 2010). Appropriate conditions should be maintained to transfer cells to culturing medium and preserve their viability (Caragher et al., 2019).

Primary GBM cell cultures provide experimental means to develop more efficient treatment methods and understand the physiopathology of GBMs (Robertson et al., 2019). Due to biologic variables of GBMs, acquisition of tumor cells from all patients is important (Perez-Garcia et al., 2012). Neurosurgical teams should be aware of how important these samples are for research purposes and should be informed about sampling procedures (Ledur et al., 2017).

Although it was described as being applied intravenously after durotomy, no standard and routine procedure has been described (Stummer and Suero Molina, 2017). In surgeries where FL staining is used as a surgical guide, tumor samples taken during that surgery are also exposed to FL. It is not known whether FL has any effects on GBM cell lines (Diaz et al., 2015). In their study on FL application on GBM cells and its impact on those cells, Diaz et al. reported that FL does not enter the intracellular environment and stains the extracellular matrix with confocal microscopy (Diaz et al., 2015). Our experiment on cellular viability shows that FL does not affect cellular viability. In our opinion, this can be explained by the fact that it can't enter the intracellular environment.

After seeding tissue samples for primary cell cultures may take a long time for cells to settle inside the medium. This is a sensitive procedure and may cause delays before forming primary cell cultures in FL applied cases due to contamination. Mechanisms behind biochemical changes occurring in FL applied cell cultures can be a topic of another study.

FL does not enter cells and does not affect cell viability (Diaz et al., 2015). Decreased cell counts in FL applied media can be explained by alteration of sensitive environmental conditions in which the cells grow and develop. The exact mechanism is yet to be explained. Histologically, the region with marked fluorescence was linked to dense cellular clusters, while areas with sparse cell growth did not exhibit fluorescence. Increased blood brain barrier permeability and extracellular FL leakage within the infiltrative tumor determine the tumor area to be removed.

According to Diaz et al., the effects of FL staining pursue for an hour in surgical applications. Their study applied FL to cell lines and observed changes in the first hour after application (Diaz et al., 2015). FL was applied for one week to test the effects of FL on GBM cell lines, and it was

seen that FL could not pass into the intracellular environment. Therefore, it was found that the intracellular passage properties of FL were time-independent.

5. Conclusion

Effects of FL on GBM cells have been examined in our study and in the light of our results, we think that using FL in regular doses does not cause any barrier to cell culture procedures. Most studies on FL applications involve comparisons of rates of total resection with or without intra-operative use of FL. Our study is the first one that examines the effects of FL on primary cell cultures. Many other substances that are used for diagnostic purposes should be evaluated for their impact on primary cell cultures and application protocols may be formed.

In our study, we reported that FL applied cultures showed a decreased number of cells compared to control groups and increased senescence. However, the mechanism behind decreased cell counts and increased β-gal activity is unknown. Therefore, our primary findings on using FL and its effects on cell count raise the possibility that FL may affect the enviroment of GBM cell cultures. In our opinion, this situation should be further evaluated in another study.

During surgeries, neurosurgeons should consider using FL after sampling the tumor tissue to avoid negative effects on primary cell culture formation.

Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

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