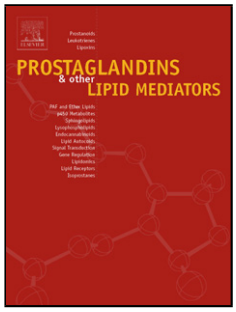

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GAMMA-LINOLENIC ACID ALTERS MIGRATION, PROLIFERATION AND APOPTOSIS IN HUMAN AND RAT GLIOBLASTOMA CELLS

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HIGHLIGHTS

- Gamma-linolenic acid alters glioblastoma multiforme cell behavior
- Gamma-linolenic acid shows to inhibit GBM cells (human and rat) proliferation and migration.
- Gamma-linolenic acid increased apoptosis in GBM cells (human and rat).
- Gamma-linolenic acid has promising therapeutic potential in GBM altering PGE synthesis.

ABSTRACT

Glioblastoma multiforme (GBM) is the most malignant astrocytoma, the main treatments consist of surgical resection followed by radiotherapy and chemotherapy. Patients, after diagnosed, have a survival rate of one year. GBM cells have an invasive, proliferative and migratory characteristic, also they do not respond properly for usual cancer treatment (radiotherapy, chemotherapy). Fatty acids have been studied as an adjuvant cancer treatment in breast, colorectal and GBM. The fatty acid can alter

tumoural cell metabolism causing a modification of eicosanoids production. This study has observed some cellular aspects modified by fatty acid treatment *in vitro*, using GBM cells (human and rat). Modifications in cell behaviour were analyzed like cell proliferation, apoptosis, migration and invasion cell capacity after treatment with fatty acid (gamma-linolenic acid). The treatment suggested in this study showed an increased number of apoptotic cells and a decreased number of proliferative and migratory cells. These data recognize that gamma-linolenic acid could be used as an alternative treatment for glioblastoma.

ABBREVIATION

AA- arachidonic acid

BSA- Bovine serum albumin

COX-2- ciclooxigenase-2

CYP- cytochrome P450

ECM- Extracellular Matrix

GBM- glioblastoma multiforme

GLA- gamma-linolenic acid

LOX- lipoxygenase

MMP- Metalloproteinase

PG- prostaglandin

PGE_2-prostaglandin E_2

PI3K- phosphatidylinositol 3-kinase

PUFA- polyunsaturated fatty acid

Key-words: Glioblastoma multiforme; Gamma-linolenic acid; Prostaglandin; Apoptosis; Migration.

1. INTRODUCTION

Glioblastoma multiforme (GBM) is the most malignant astrocytoma, the treatment consists of surgical resection followed by radiotherapy and chemotherapy. However, despite of multimodal therapies, the average survival of patients with GBM is between

9-12 months after surgical resection, due to post-surgical reincidence of the tumour of almost 100% [1,2]. The main characteristics of GBMs are high rates of cell proliferation; ability to migrate and to invade the surrounding cerebral structures (mainly along vessels and myelinated fibres); areas of necrosis and accentuated angiogenesis (which is reflected by an increased endothelial cell proliferation). Glioma cell invasiveness maybe is one cause of reincidence tumour. These cells not eradicated after surgery, proliferate and raise a tumour again [3].

Polyunsaturated fatty acids (PUFAs), including gamma-linolenic acid (GLA-ω-6), have been reported to exert anti-tumour effects in several tumour types [4,5]. Nowadays, some studies revealing how GLA acts in GBM cells [6]. GLA alters cyclooxygenase metabolism, stimulates reactive oxygen species and caspases activation, cytochrome c liberation and finally, apoptosis [7]. Also, GLA acts in C6 energetic metabolism [8], angiogenesis and cell cycle [9]. But the mechanisms of how this fatty acid act in an anti-migratory pattern is unclear.

Cells that absorb increased amounts of GLA, incorporate this fatty acid in the phospholipid membrane, later this fatty acid will be metabolized by phospholipase A, which turns fatty acid in eicosanoids by enzyme cyclooxygenase-2 (COX-2). COX-2 form prostaglandins (PG), leukotrienes and prostacyclins. Unremarkable COX-2 metabolizes arachidonic acid (AA) and produces eicosanoids from series 2 (e.g. PGE_2). The free AA is metabolized into prostaglandins, hydroperoxy eicosatetraenoic acids, and epoxy eicosatrienoic acids through the action of cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 (CYP) epoxygenases, respectively [10]. PUFAs compete with AA and when COX-2 metabolizes PUFAs the eicosanoid produced is from series 3 (e.g. PGE_3), which has lower effects than PGE_2, also is not mitogenic and decrease phospholipase A and COX-2 action. Because of these effects, GLA can modify the production of the eicosanoids in the cell [11], and these lipids have lower inflammatory and aggressive characteristics than PGE_2. Omega-6 (ω-6) might be involved in other cellular mechanisms like signal transduction and gene expression [10,12].

The migratory process occurs when (I) cell develops directional polarity forming a protrusion of the cell's leading edge, (II) these protrusions (pseudopodia, filopodia, or lamellipodia) adhere the surface anchoring on Extracellular Matrix (ECM) and (III) body cell is tracked and the process is repeated. All these steps have cytoskeleton involvement [13,14]. Moreover, the invasiveness of tumoural cells was observed when

the cell synthesizes some enzymes that degrade ECM. Metalloproteinases (MMP) is an enzyme family that is very focused on cancer because many different types of tumoural cells use this enzyme to degrade ECM and facilitating them migratory process [15]. It was observed in C6 in vivo and ex-vivo GBM model treated with GLA, the migratory pattern of tumoural cells was reduced after the treatment, compared with the control. This work aims to elucidate the mechanisms on how acts GLA to reduce the migration of the tumoural cells.

2. MATERIAL AND METHODS

2.1. Cell culture

C6 cells (rat glioma) and T98G (human glioma) were purchased from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50U/ml penicillin, and 50µg/ml streptomycin. Cells in the exponential phase of growth were used, growing in 75cm^2 flasks in a humidified atmosphere of 5% CO_2, 95% air at 37°C. After growth to the desired density, cells were washed with PBS and trypsinized (trypsin 0.025%/ EDTA 0.02%) for subsequent plating. All experiments used cells grown in DMEM with FCS and antibiotics as stated above.

2.2. Migration assay

The cell migration assay was performed using a 24-well Boyden chamber plate with 8 µm pore size polycarbonate membrane filters. C6 cells were placed in the upper part of the Boyden chamber containing DMEM and 10% FCS, the lower chamber also contained DMEM and 10% FCS.

After a cell adhesion period (12h) the medium was changed and treatment GLA (100µM) was added. The concentrations of the treatments were previously established in our laboratory. The culture media with GLA treatment was changed daily for 48h. After incubation, the cells on the membrane filter were fixed with methanol and stained with 0.05% crystal violet for 30min. The cells on the upper surface of the filter were removed with a cotton swab. The membranes were then rinsed in PBS until the excess stain was removed. The membranes were then air-dried for 20min. The migratory cells were determined by counting the cells that migrated to the lower side of the filter by

bright field microscopy at 200× magnification. Five random fields were counted for each filter, and each treatment was assayed in triplicate.

2.3. Spheroids and brain slice (*ex vivo*)

When the C6 cell cultured flask reached 80% of confluence, they were scraped and counted in the Neubauer chain. Cells were cultured in Petri dishes with 5 in 5 x 104 cells/10µl medium, like a drop. After 24h in the incubator, the weight of the cells formed a round shape cluster cells (spheroids). Wistar rats (180-200g) were anaesthetized with ketamine:xylazine (10mg:1,5mg/100g) intraperitoneal, the brain was removed and sliced from a dorsum-ventral direction in air flux to avoid contamination. These slices were cultured on a millicell membrane of 0,4µm 30mm in a 6-well plate with 1ml medium. After 1 hour the spheroids were added on the brain slice. After 24 hours the medium was changed for GLA enriched medium (150µM) or BSA (10%) as a control. Two days after the treatment the brain slices were fixated with formaldehyde 4% P(K) 0,1M (pH 7,2) overnight, dehydrated and embedded in paraffin. Slices were made in Leica (RM2145) and stained with hematoxylin-eosin (H&E). Slices were analyzed in light microscope Nikon Optiphot-2, images were collected randomly and mitoses were counted. Also, the distance between the cells on the borderline from the spheroids migrating to the brain tissue was analyzed. This procedure was approved by the Ethical Commission for Animal Experimentation of the Biomedical Institute (University of São Paulo) – protocol number 190/02.

2.4. Hematoxylin-Eosin Stain (H&E)

Slices were immersed in xylol for 30 min 60ºC, and 20 min RT to remove paraffin. Slices were hydrated in an ethanol gradient, stained for 2min in hematoxylin, washed in water for 2min. and in an alcohol-acid solution. Washed 5min in water and stained in eosin for 2min more. Slices were washed in absolute ethanol 3x after in xylol for 3x and mounted with Permount.

2.5. TUNEL assay

TUNEL assay kit by Calbiochem TdT-fragEL DNA fragmentation detection. We used slices from ex-vivo with C6 spheroids, removed paraffin (as H&E stain). Histones were digested in proteinase K 20µg/ml Tris buffer 10mM for 20min. Endogenous peroxidase

blocked in H_2O_2 3%: methanol (1:10) for 5min. Washed in TBS and incubated with TdT+enzyme for 90min 37ºC. Washed and conjugated with streptavidin-HRP and revealed with DAB and H_2O_2/urea 10min and counter-stained with methyl green, dehydrated and mounted with permount. Slices were analyzed and counted the number of apoptotic cells in Nikon Optiphot-2 light microscopy.

2.6. ELISA PGE2 (enzyme-linked immunosorbent assay)

ELISA assay kit by Cayman Biochemicals PGE_2 monoclonal. We used medium from C6 cells ($2x10^4$ cells $25cm^2$ flasks) with 10%BSA (control), treated medium with GLA (75μm and 150μm) and one group added 1μm calcium ionophore (a23187). Calcium can stimulate phospholipase activity and consequently prostaglandin production. This assay is based on competition of PGE_2 acetylcholinesterase (AchE) conjugated. PGE_2 compete with AchE to link with anti-PGE_2. After rinsing the medium, in the wells were added the Ellman solution (AchE substrate). The colourimetric reaction is measured by absorbance. We can determine the PGE_2 concentration according to a standard curve.

2.7. Statistical analysis

All data are presented as the mean ± SEM. Statistical analysis was carried out with the GraphPad InStat software. Statistical analyses were conducted by Student's unpaired t-test for independent samples. The difference at the level of $p < 0.05$ was considered to be significant.

3. RESULTS

3.1. Migration assay

Control has a migration average distance of 147,56μm from the spheroid edge. Cells treated with GLA have an average distance of 100,4μm. There was a 32% p<0,001 reductions between Control (BSA) and treated cells (Figure 1-A). The number of migratory cells was equivalent with no significance (BSA 63,6 ±11; GLA 55,8 ± 4,4).

3.2. Proliferation assay

H&E stained slices were analyzed to count the number of mitotic cells, we considered mitotic cells all the mitosis stages (prophase, metaphase, anaphase, and telophase). Results were: Control (BSA 16,4 cell ± 0,6) and GLA (9,9 cell ± 0,5) there was a 39,5% $p< 0,0005$ reduction in number of mitotic cells after treatment (Figure 1-B).

3.3. Apoptosis assay

TUNEL reaction was analyzed in microscopy and counted stained cells. Control cells (BSA 104 cells ±1,1) and GLA (151 cells ±2,1) results showed a 44,7% $p=0,001$ increasing apoptotic cells after treatment (Figure 1-C).

3.4. Transwell assay

As shown in Figure 1-D, migration capacity was reduced by 68% $p< 0,0001$ in cells C6. Control cells (BSA 23,6 ±2,01) and treated with 100 μM GLA (GLA 16,1 ± 1,91).

3.5. ELISA PGE_2

ELISA PGE_2 assay detected PGE_2 in all groups, so C6 cells can produce this prostaglandin. Control cells (BSA 1,9pg/μl±0,34), treated group GLA 75μm (18,95pg/μl±0,29) and 150μm (35,15pg/μl±0,70). Group calcium ionophore (20,51pg/μl±1,19) of PGE_2. According to these data, GLA increased PGE production after treatment with 75μm and 150μm, but this kit has a cross-reactivity. This kit did not distinguish PGE_1, PGE_2 or PGE_3.

4. DISCUSSION

This study showed that GLA modifies some cellular characteristics involved in fatty acid metabolization. GLA is incorporated by glioma cell and change the phospholipids pattern and composition in the cell membrane (including organelles). We observed changes in cell behaviour after treatment with GLA *in vitro* and using *ex vivo* model, these modifications were related to diminished migration/invasion cells and increased apoptotic cells. Similar results were observed in previous studies [9] in the group. When GLA is incorporated by glioma cells, this fatty acid might be metabolized and incorporated by the cells. GLA can modify the production of eicosanoids in glioma cells, including prostanoids. PGE_2 is the most abundant prostaglandin found in various

human malignancies cancer like breast, colon [5,16] and glioma [17]. PGE_2 has been shown to be a possible mediator of cell migration and might be involved in other cellular mechanisms. Recently treatments with exogenous PGE_2 have demonstrated significantly increased migration in the T98G human glioma cell line [17] and increased production of PGE_2 related to high-grade glioma in human samples [18].

One of the hypotheses for the possible influence of PGE_2 on migration is through the EP2 and EP4 receptors. The receptors EP2 and EP4 are coupled with G-stimulatory (Gs), stimulating adenylate cyclase (AC) followed by PKA activity; EP4, unlike EP2, can also activate an additional phosphatidylinositol 3-kinase (PI3K) pathway leading to Akt activation [19,20]. Following the activation of the PI3K/Akt pathway, β–catenin translocated into the nucleus and triggered COX-2-promoting proteins, LEF-1 and TCF-4 [16], this mechanism increased COX-2 expression and PGE_2 production subsequently. Singh et al [21], observed that PGE_2 acting via EP4 receptors stimulated the migration of human melanoma cells. In colorectal carcinoma human cells, PGE_2 stimulated spreading, migration, and invasion of these cells and acts predominantly through the EP4 receptor [22]. The activation of these pathways, PKA and PI3K/Akt play an important role in the migratory and survival signalling pathway.

In breast cancer, PGE_2 stimulated cell migration via EP4 receptors linked to PKA and Akt signalling [23]. In another study, HeLa or NIH3T3 cells were allowed to adhere to ECM and it was observed that elevations in COX and PKA stimulated the formation of actin bundles that contain myosin II, associated with small focal adhesions and increased cell motility [24]. In murine mammary carcinoma cells treated with exogenous PGE_2, a selective inhibitor of PKA (Rp-cAMPS) reduced migration [25]. Recently, Tai et al [26], showed that using celecoxib (COX-2 inhibitor) in hepatocellular cells caused an up-regulation of E-cadherin via inhibition of COX-2-PGE2-EP2-p-Akt/p-ERK expression pathway. Increased expression of E-cadherin caused inhibition of migration and invasion of these cells because cells cannot be detached from each other.

Studies in gliomas demonstrated that PI3K/Akt signalling pathway inhibits glioma invasion by reducing the migratory activity of glioma cells by inactivating Rho GTPases [11]. Another study showed that the pathway RhoA-ROCK pathway contributes to COX-2 expression. Increased COX-2 expression is correlated with increased production of PGE_2, and COX-2/PGE_2 signaling contributes to cancer cell migration [27].

COX-2 and PGE_2 are related to increased MMP expression. This enzyme is responsible for extracellular matrix degradation compounds, allowing cells to migrate and invade through the matrix and normal tissue. MMP-2 is related to the invasiveness phenotype in tumoural cells [9,28]. Studies using pancreatic cancer cells were observed a relation between expression of MMP, COX-2, and PGE_2. Using COX-2 inhibitor, Bu et al, [29] detected decreased PGE_2 levels, reduced MMP production, and inhibited cell invasiveness. Treatment with ω-3 (docosahexaenoic acid), suppressing MMP-2 and MMP-9 secretion through inhibition of NFκB and Cox-2 signalling pathway in breast cancer cells [30].

High GLA concentration in the cell causes modification on the mitochondrial membrane, modifying the mitochondrial membrane potential. Studies showed that modification in mitochondria caused by GLA increased cytochrome-c releasing and caspase-3 activation, both involved in the apoptotic process [7,31-32].

5. CONCLUSION

In conclusion, our findings demonstrate evidence that PGE_2 is involved in the regulation of cell migration, invasion, and apoptosis in glioma cells. Further exploration of the PGE_2 signalling pathway may provide new targets for glioma prognosis or therapeutics.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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REFERENCES

1. Giese A, Bjerkvig R, Berens ME. Westphal, M. Cost of migration: invasion of malignant gliomas and implications for treatment. J Clin Oncol. 2003; 21: 1624-36.

2. Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Cavenee WK, DePinho RA. Malignant glioma: genetics and biology of a grave matter. Genes Dev. 2001; 15: 1311-33.

3. Bush NA, Chang SM, Berger MS. Current and future strategies for treatment of glioma. Neurosurg Rev. 2017; Jan;40(1):1-14.

4. Das UN. Essential fatty acids, lipid peroxidation and apoptosis. Prostaglandins Leukot Essent Fatty Acids 1999; Sep;61(3):157-63.

5. Newell M, Goruk S, Mazurak V, Postovit L, Field CJ. Role of docosahexaenoic acid in enhancement of docetaxel action in patient-derived breast cancer xenografts. Breast Cancer Res Treat. 2019; Jun 24.

6. Das UN. From bench to the clinic: gamma-linolenic acid therapy of human gliomas. Prostaglandins Leukot Essent Fatty Acids 2004; Jun;70(6):539-52.

7. Colquhoun A, Schumacher RI. gGamma-Linolenic acid and eicosapentaenoic acid induce modifications in mitochondrial metabolism, reactive oxygen species generation, lipid peroxidation and apoptosis in Walker 256 rat carcinosarcoma cells. Biochim Biophys Acta. 2001 a; Oct 31;1533(3):207-19.

8. Ramos KL, Colquhoun A. Protective role of glucose-6-phosphate dehydrogenase activity in the metabolic response of C6 rat glioma cells to polyunsaturated fatty acid exposure. Glia 2003; Aug;43(2):149-66.

9. Miyake JA, Benadiba M, Colquhoun A. Gamma-linolenic acid inhibits both tumour cell cycle progression and angiogenesis in the orthotopic C6 glioma model through changes in VEGF, Flt1, ERK1/2, MMP2, cyclin D1, pRb, p53 and p27 protein expression. Lipids Health Dis. 2009; Mar 17;8:8; 2009.

10. Colquhoun A, Miyake JA, Benadiba M. Invited Review: Fatty acids, eicosanoids and cancer. Nutrit Ther Metabol. 2009;27(3):105-12.

11. Fortin ESP, Mathews IT, Symons MH, Berens ME, Tran NL. Implications of Rho GTPase Signaling in Glioma Cell Invasion and Tumor Progression. Front Oncol. 2013; Oct 4;3:241.

12. Hawcroft G, Volpato M, Marston G, Ingram N, Perry S, Cockbain A, Race A, Munarini A, Belluzzi A, Loadman P, Coletta P, Hull M. The omega-3 polyunsaturated fatty acid eicosapentaenoic acid inhibits mouse MC-26 colorectal cancer cell liver metastasis via inhibition of PGE(2) -dependent cell motility. Br J Pharmacol. 2012; Jul;166(5):1724-37.

13. Hagihara K, Miura R, Kosaki R, Berglund E, Ransht B, Yamaguchi Y. Imunohistochemical evidence for the brevican-tenascin-R interaction: colonization in perineuronal nets suggests a physiological role for the interaction in the adult rat brain. J Comp Neurol. 1999; 410:256-264.

14. Baker EL and Zaman MH. The Biomechanical Integrin. J Biomech. 2010; January 5; 43(1): 38.

15. Chintala SK, Tonn JC, Rao JS. Matrix metalloproteinases and their biological function in human gliomas. Int J Devl Neuros. 1999; 17(5-6):495-502.

16. Hsu HH, Lin YM, Shen CY, Shibu MA, Li SY, Chang SH, Lin CC, Chen RJ, Viswanadha VP, Shih HN, Huang CY. Prostaglandin E2-Induced COX-2 Expressions via EP2 and EP4 Signaling Pathways in Human LoVo Colon Cancer Cells. Int J Mol Sci. 2017; May 25;18(6). pii: E1132.

17. Gomes RN, Souza FC, Colquhoun A. . Eicosanoids and cancer. CLINICS, 2018; v. 73, p. e530s.

18. Panagopoulos AT, Gomes RN, Almeida FG, Da Costa SF, Veiga JCE, Nicolaou A, Colquhoun A. The prostanoid pathway contains potential prognostic markers for glioblastoma. Prostaglandins & other lipid mediators, 2018; v. 137, p. 52-62.

19. Breyer RM, Bagdassarian CK, Myers SA, Breyer MD. Prostanoid receptors: subtypes and signaling. Annu Rev Pharmacol Toxicol. 2001;41:661-90.

20. Fujino H, XuW, Regas JW. ProstaglandinE2 induced functional expression of early growth response factor-1 by EP4, but not EP2, prostanoid receptors via the phosphatidylinositol 3-kinase and extracellular signal-regulated kinases. J Biol Chem. 2003; Apr 4;278(14):12151-6.

21. Singh T, Vaid M, Katiyar N, Sharma S, Katiyar SK. Berberine, an isoquinoline alkaloid, inhibits melanoma cancer cell migration by reducing the expressions of cyclooxygenase-2, prostaglandin E(2) and prostaglandin E(2) receptors. Carcinogenesis. 2011;32:86–92.

22. Sheng H, Shao J, Washington MK, DuBois RN.: Prostaglandin E2 increases growth and motility of colorectal carcinoma cells. J Biol Chem. 2001; May 25;276(21):18075-81.

23. Timoshenko AV, Xu G, Chakrabarti S, Lala PK, Chakraborty C. Role of prostaglandin E2 receptors in migration of murine and human breast cancer cells. Exp Cell Res. 2003; Oct 1;289(2):265-74.

24. Glenn HL, Jacobson BS. Cyclooxygenase and cAMP dependent protein kinase reorganize the actin cytoskeleton or motility in HeLa cells. Cell Motil Cytoskeleton. 200;3 Aug; 55(4):265-77.

25. Timoshenko AV, Lala PK, Chakraborty C. PGE2 mediated upregulation of NOS in murine breast cancer cells through the activation of EP4receptors. Int J Cancer. 2004; Jan 20;108(3):384-9.

26. Tai Y, Zhang LH, Gao JH, Zhao C, Tong H, Ye C, Huang ZY, Liu R, Tang CW. Suppressing growth and invasion of human hepatocellular carcinoma cells by celecoxib through inhibition of cyclooxygenase-2. Cancer Manag Res. 2019; Apr 9;11:2831-2848.

27. Patel M, Kawano T, Suzuki N, Hamakubo T, Karginov AV, Kozasa T.: Gα13/PDZ-RhoGEF/RhoA signaling is essential for gastrin-releasing peptide receptor-mediated colon cancer cell migration. Mol Pharmacol. 2014; Sep; 86(3):252-62.

28. Panagopoulos AT, Lancellotti CL, Veiga JC, de Aguiar PH, Colquhoun A. Expression of cell adhesion proteins and proteins related to angiogenesis and fatty acid metabolism in benign, atypical, and anaplastic meningiomas. J Neurooncol. 2008; Aug;89(1):73-87.

29. Bu X, Zhao C, Dai X. Involvement of COX-2/PGE(2) Pathway in the Upregulation of MMP-9 Expression in Pancreatic Cancer. Gastroenterol Res Pract. 2011; 2011:214269.

30- Yun EJ, Song KS, Shin S, Kim S, Heo JY, Kweon GR, Wu T, Park JI, Lim K.Docosahexaenoic acid suppresses breast cancer cell metastasis by targeting matrix-metalloproteinases. Oncotarget. 2016; Aug 2;7(31):49961-49971.

31. Colquhoun A, Schumacher, RI. Modifications in mitochondrial metabolism and ultrastructure and their relationship to tumour growth inhibition by gamma-linolenic acid. Molecular and Cellular Biochemistry 2001 b; v. 218, p. 13-20.

32. Colquhoun A. Invited Review: Lipids, mitochondria and cell death:implications for neuro-oncology. Molecular Neurobiology, 2010; v. 42, p. 76-88.

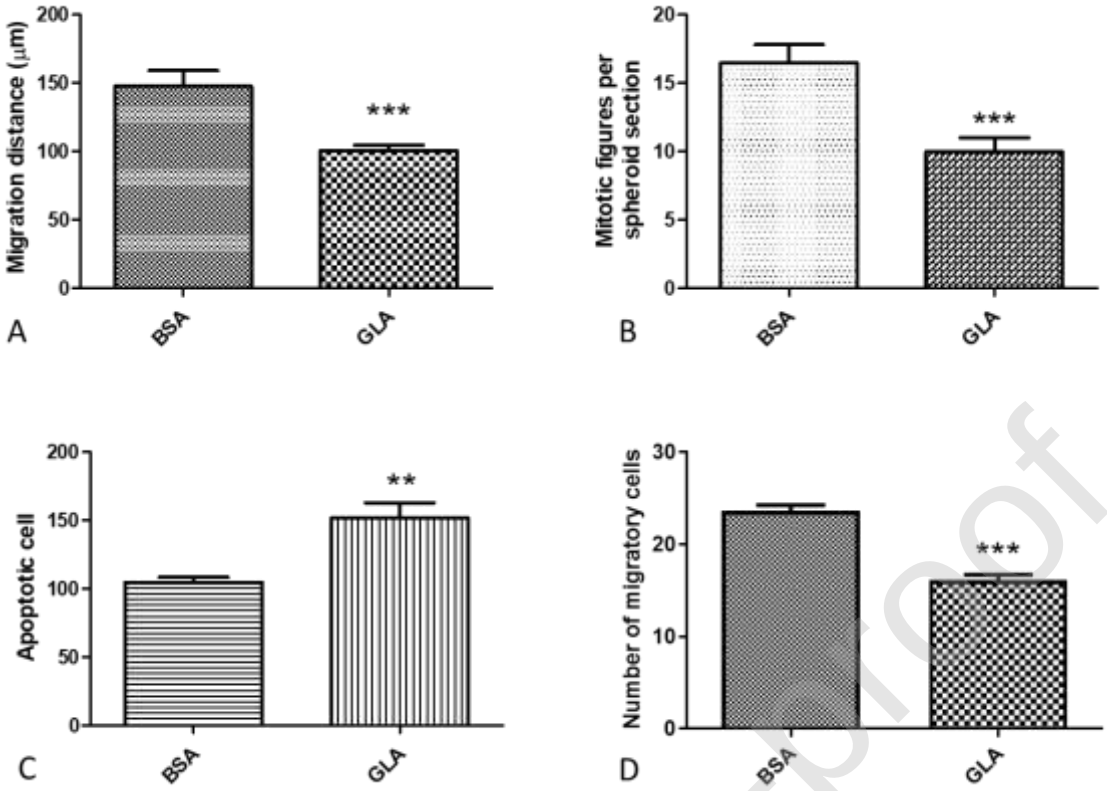

Figure 1- (A) Migration distance of C6 cells: BSA (control) 147,56µm, GLA (Cells treated) 100,4µm ($p<0,001$). (B) Number of mitotic cells per spheroid: BSA (control) 16,4 cells, GLA (Cells treated) 9,9 cells ($p< 0,0005$). (C) Number of apoptotic cells: BSA (control) 104 cells, GLA (Cells treated) 151 cells ($p=0,001$) Stained cells. (D) Number of migratory cells in Transwell assay: BSA (control) 23,6µm GLA (Cells treated) 16,1µm ($p<0,0001$).

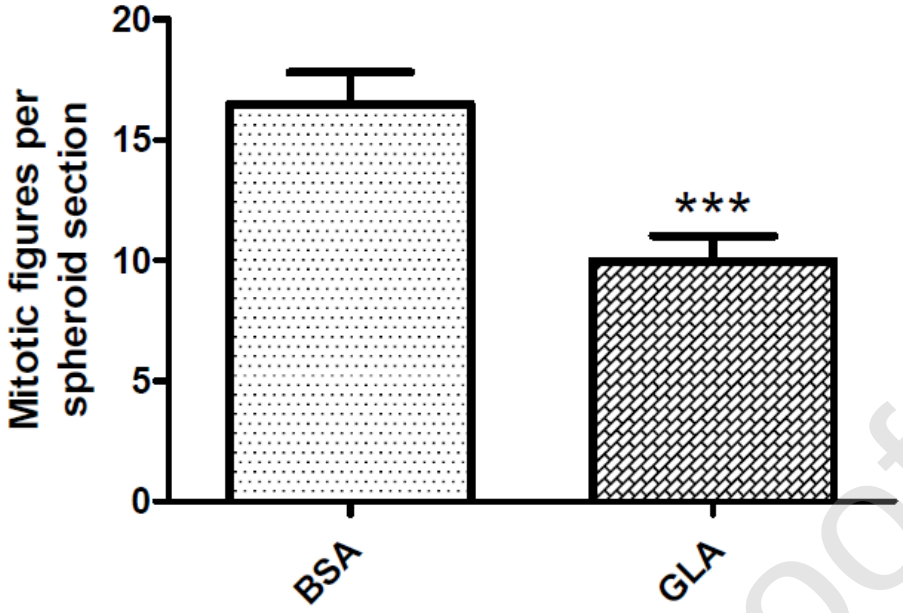

Figure 2- Number of mitotic cells per spheroid: BSA (control)16,4 cells, GLA (Cells treated) 9,9 cells (p< 0,0005).

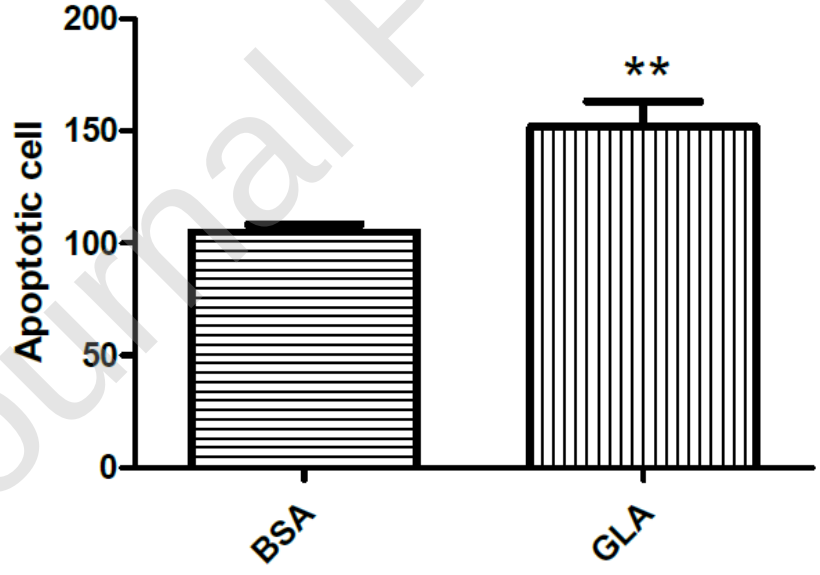

Figure 3- Number of apoptotic cells: BSA (control) 104 cells, GLA (Cells treated) 151 cells (p=0,001) Stained cells.

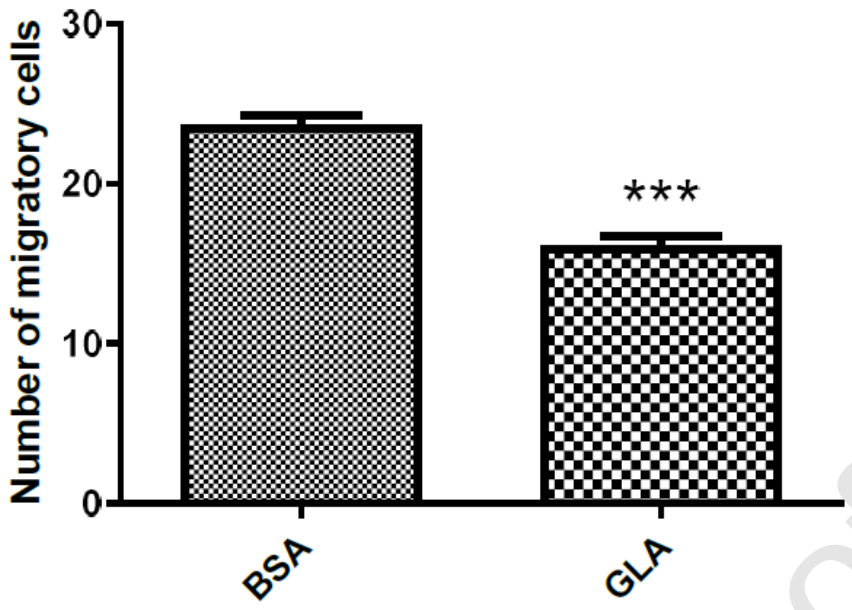

Figure 4- Number of migratory cells in Transwell assay: BSA (control) 23,6□m GLA (Cells treated) 16,1□m ($p<0,0001$).