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Publisher: Routledge

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Nutrition and Cancer

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/hnuc20>

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To cite this article: E. Hervouet, O. Staehlin, D. Pouliquen, E. Debien, P-F. Cartron, J. Menanteau, F. M. Vallette & C. Olivier (2013) Antioxidants Delay Clinical Signs and Systemic Effects of ENU Induced Brain Tumors in Rats, *Nutrition and Cancer*, 65:5, 686-694, DOI: [10.1080/01635581.2013.789541](https://doi.org/10.1080/01635581.2013.789541)

To link to this article: <http://dx.doi.org/10.1080/01635581.2013.789541>

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Antioxidants Delay Clinical Signs and Systemic Effects of ENU Induced Brain Tumors in Rats

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According to our previous study suggesting that antioxidant properties of phytochemicals in the diet decrease glioma aggressiveness, we used a SUVIMAX-like diet (“Supplémentation en Vitamines et Minéraux Antioxydants”) (enriched with alpha-tocopherol, beta carotene, vitamin C, zinc, and sodium selenite), adapted to rats. The present results showed that each of the antioxidants inhibited growth of glioma cells *in vitro*. When used in combination for *in vivo* studies, we showed a highly significant delay in the clinical signs of the disease, but not a statistical significant difference in the incidence of glioma in an Ethyl-nitrosourea (ENU)-model. The SUVIMAX-like diet decreased candidate markers of tumoral aggressiveness and gliomagenesis progression. The mRNA expressions of 2 common markers in human glioma: Mn-SOD (Manganese Superoxide Dismutase) and IGFBP5 (insulin growth factor binding protein) were reduced in the tumors of rats fed the antioxidant diet. In addition, the transcripts of two markers linked to brain tumor proliferation, PDGFR β (platelet-derived growth factor receptor beta) and Ki-67, were also significantly decreased. On the whole, our results suggest a protective role for antioxidants to limit aggressiveness and to some extent, progression of gliomas, in a rat model.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most frequent primary malignant brain tumor in adults. Malignant brain tumors

have a very high mortality rate, because of their insidious invasion and extensive neovascularization. Glioma cells are highly resistant to apoptosis normally induced by drugs and malignant gliomas react poorly to available therapies (1). In these conditions, it is essential to develop alternative strategies to limit occurrence and/or aggressiveness of this tumor.

The etiology of brain tumors is poorly understood. Epidemiological data are somewhat conflicting as concerns the role of food components. Some reports show an inverse association of antioxidants or carotene alone intake with glioma (2–4), whereas others do not (5, 6). Some studies have shown a correlation between the presence of GBM and oxidative stress (7), or lipid peroxidation (8). The brain is one of the tissues the most sensitive to oxidative stress because of the high contents of oxidizable substrates and the low antioxidant levels. Indeed, tumor cells as compared to normal cells, present high levels of reactive oxygen species (ROS) and oxidative damage markers (9). Despite this feature, tumor cells are able to survive and proliferate, indicating that a high level of ROS may play an important role in stimulating cell proliferation (10). Several intracellular signaling pathways that stimulate cell proliferation, including tyrosine kinase receptors, mitogen-activated protein kinases (MAPKs), or redox sensitive transcription factors could be activated by ROS (11).

In a previous study we explored the influence of a phytochemical-enriched diet on gliomagenesis and the associated systemic effects in rats (12). In this model of ethylnitrosourea-induced glioma in pregnant females, we showed that the degree of aggressiveness and systemic effects (weight loss, survival, reduced liver weight), were decreased by a dietary intervention and we suggested that phytochemicals with antioxidant

Submitted 26 April 2012; accepted in final form 21 March 2013.

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properties participated in this mechanism. The object of the present study was to test this hypothesis. For this purpose, we used the results of the "Supplémentation en Vitamines et Minéraux Antioxydants" (SUVIMAX) interventional study. This randomized double-blind study was designed to test whether a combination of antioxidant nutrients (alpha-tocopherol, beta carotene, vitamin C, zinc, and sodium selenite), at nutritional doses could reduce the incidence of cancer in the middle-aged general French population (13). The results of this trial showed a beneficial effect in cancer prevention but only in men. In the present work, the SUVIMAX diet was adapted for dose levels in rats (SUVIMAX-like diet) and used in the ENU-model. The strategy was to mimic the SUVIMAX study to determine the impact of a dietary supplement on brain tumorigenesis. Our results showed a highly significant delay in the clinical signs of the disease, in the group fed the SUVIMAX-like diet, as compared to the group fed a standard diet, as well as a small reduction in the incidence of glioma in males.

To understand the mechanisms involved, we measured the expression of genes dealing with proliferation (Ki-67) versus apoptosis (*bax* and *bcl-2*). Markers of glioma progression and the oxidative pathways were also investigated at the mRNA level in the tumors. SOD-2, which is a key regulator of oxidative stress, is also a possible prognostic marker for glioblastoma (14, 15). Insulin growth factor (IGF)-binding protein (IGFBP) isoforms have been implicated in the pathogenesis of human neoplasms including glioma. In view of this, we evaluated the expression of the *IGFBP5* isoform (16), which was higher in GBM relative to anaplastic astrocytoma or control brain tissue (17). PDGFR (platelet-derived growth factor receptor) was also measured because its modulation is a key event in gliomagenesis involved in the survival of glioma cells. A high active PDGF pathway in gliomas results from the amplification-dependent overexpression of receptors or ligands. This signaling axis plays a central role in the events underlying gliomagenesis. PDGF signaling is relevant for both tumor expansion and survival, stimulating proliferation and indirectly promoting nutrient supply to the tumoral mass (18).

MATERIALS AND METHODS

Materials

Cell Culture

Ntva cells were obtained from Dr. E. C. Holland (Memorial Sloan Kettering Cancer Center, New York, NY). Ntva-PDGFR mimics a grade II glioma and Ntva-Ras/Akt mimics a grade IV glioma. Cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin, glutamine at 37°C with 5% CO₂.

Tumor Induction and SUVIMAX Treatment

All experiments reported in this article comply with the guidelines of the European Union for case and use of animals

in research protocols. The protocol of treatment was similar as those previously described in Pouliquen et al. (12). The animals were given free access to tap water and pellet standard diet (StD) (RM1, Special Diets Services, Witham, Essex, UK), or SUVIMAX-like diet. SUVIMAX-like basic composition was identical to the StD diet enriched with a combination of 78 mg vitamin C (Sigma Aldrich, Saint-Quentin Fallavier, France), 19.5 mg vitamin E (Fluka, Saint-Quentin Fallavier, France), 13 mg zinc (Sigma Aldrich, Saint-Quentin Fallavier, France), 65 µg sodium selenite (Sigma Aldrich, Saint-Quentin Fallavier, France), and 3.6 mg beta-carotene/kg food (Sigma Aldrich, Saint-Quentin Fallavier, France). The different SUVIMAX compounds were calculated in reference to the Human SUVIMAX study (13).

Tumors of the central nervous system were induced according to the procedure of Koestner (19), which produces malignant gliomas in the progeny of rats between 4 and 8 mo of age. At 12 wk of age, females ($n = 18$) were paired with males ($n = 27$). The day on which the presence of a vaginal plug was confirmed was defined as Day 0 of gestation. On the 19th day of gestation, the females received an intravenous administration of 50 mg/kg ethylnitrosourea (ENU; Isopac[®], Sigma, St Louis, MO) dissolved in saline. Offspring were weaned on Day 21 postnatal and individually marked on the tail. The total number of offspring induced by ENU was 55 in StD group (32 males and 23 females), 45 in SUVIMAX-like diet group (27 males and 18 females). Rats were anaesthetized with ketamin and Rompun[®] (Bayer, Puteau, France) and then killed by decapitation. Animals were obtained from Charles River Laboratories and were housed in polycarbonate rat breeding cages in a room controlled temperature (19–21°C) with 60–70% humidity, renewed air, a 12-h light/dark cycle light and with StD or SUVIMAX diet ad libitum.

Quantitative PCR Analysis

RNA extraction and reverse transcription were done as described above. The PCR reaction was performed in a Mx 4000 apparatus (Stratagene) using the SYBR[®] green qPCR core reagent kit (Stratagene, Massy, France). DNA (1 µl) was mixed with a buffer containing 3.5 mM MgCl₂, 0.4 mM dNTPs, 0.2 µM forward and reverse primers, 1/13 200 ROX as reference dye, 1/30 000 SYBR green I dye, 1 U Taq DNA polymerase, and appropriate 1× final buffer in a final volume of 25 µl. Expression of *gapdh* as housekeeping gene, *sod-2*, *bax*, *bcl-2*, Ki-67, *PDGFRb*, *IGFBP-5* was estimated using primers in Table 1. The PCR reaction was performed as follows: initial denaturation at 94°C for 5 min, and 40 cycles at 95°C for 30 s, annealing temperature for 1 min and 72°C for 30 s. All results presented were the mean of 5 to 15 different animals. All experiments were repeated 3 times.

Western Blotting

Frozen brain tumor tissues were cut in small pieces and incubated in RIPA buffer for 30 min on ice. Lysates were

TABLE 1
Primers used in quantitative and semi-quantitative PCR analyses

| | primers sequences |
|---------|--|
| igfbp-5 | 5'-TGAGACAGGAATCCGAACAAG-3' 5'-CACAGTTGGGCAGGTACACAG-3' |
| ki-67 | 5'-AGACGTGACTGGTCCCAAC-3' 5'-ACTGCTCCCGAGAACTGAA-3' |
| pdgfrb | 5'-AATGACCACGGCGATGAGA-3' 5'-TCTTCCAGTGTTCAGCAGC-3' |
| sod-2 | 5'-GGCTTGGCTTCAATAAGGAG-3' 5'-TAGTAAGCGTGCTCCCACAC-3' |
| bax | 5'-ACTAAAGTGCCGAGCTGAT-3' 5'-ATGGTCACTGTCTGCCATGT-3' |
| bcl2 | 5'-GAGTACCTGAACCGGCATCT-3' 5'-CAAATCAAACAGAGGTCGCA-3' |
| gapdh | 5'-ATGACTTACCCACGGCAAG-3' 5'-TGATGGGTTTCCCATGATGA-3' |

centrifugated for 5 min at 2000 g and supernatant was used for SDS-PAGE separation. Forty μ g proteins were size fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, after estimation of protein concentration using the Bradford technique (Biorad, Marnes-La-Coquette, France). Proteins were transferred onto nitrocellulose or PVDF membrane. Saturation and blotting were realized by using SNAP i.d Protein Detection System (Millipore, Molsheim, France). The detection of proteins was carried out using ECL (Amersham Biosciences, Pantin, France) and/or SuperSignal west femto maximum sensitivity (Pierce, Thermo Fisher Scientific, Brebières, France) chemiluminescence reagents. Bands were quantified using Quantity One quantification software (BioRad, Marnes-La-Coquette, France). Anti-PDGFRb (sc-432 Santa-Cruz Biotechnology (1/200) for Western blotting, and goat antirabbit [75011 (Biorad) (1/2000), anti-actin (1/3000) (Chemicon, Hants, Great Britain, MAB1501R)] and goat antimouse (1/3000).

Horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence reagents were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Fluorescent secondary antibodies were obtained from Invitrogen (Cergy Pontoise, France)

ROS Production

Fluorescence of DCF (2',7'-dichlorofluorescein) (Invitrogen, Cergy Pontoise, France) was measured, as described previously in Hervouet et al. (19) at t0 and t2 h following incubation in saline buffer (135 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4 , 1 mM MgSO_4 , 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4, 5.55 mM glucose, and 1 mM CaCl_2) with 1 μ M 5- (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester), in presence

or absence of compounds (ascorbic acid, sodium selenite, β -carotene, tocopherol). This buffer was used to preserve cell integrity and contrary to cell medium is devoid of autofluorescence.

Proliferation Test

Effects of compounds on cell proliferation were estimated by crystal violet (Sigma Aldrich, Saint-Quentin Fallavier, France) staining as previously described in Hervouet et al. (20). Briefly, Ntva-PDGF cells were cultured for 7 days in presence or absence of the different compounds and then cells were washed with PBS. Control cells proliferated normally (exponentially), during the 7-days experiment. Then 2500 cells/well were seeded and compounds were added 16 h later. Cells were stained for 30 min with 50 μ L of a solution containing 0.5% crystal violet in 20% methanol. Staining solution was then removed and wells were rinsed twice with water and dried at room temperature. To solubilize the stained cells, 100 μ L per well of a solution of 0.1 M citrate sodium (Sigma Aldrich, Saint-Quentin Fallavier, France) pH 4.2 and 50% methanol were added and plates were kept for 30 min at room temperature. Absorbance of each well was measured at 550 nm using a plate reader.

Statistical Analysis

Results were presented as mean \pm SD. Statistical analyses were estimated using Graphpad Prim Software Inc. (La Jolla, CA). Statistical significance of the differences between groups was assessed by use of Student's *t*- and χ^2 test.

RESULTS

Antioxidants of the SUVIMAX-Like Diet Decreased Proliferation and Noninduced Oxidative Stress of Ntva-PGDF Glioma Cell Line

To assess the ability of the antioxidants contained in the SUVIMAX-like diet to control non-induced oxidative stress, we measured ROS produced by the glioma cell line Ntva-PGDF representative of grade II glioma (21). ROS production was determined after 2 h incubation of cells with tocopherol (20 μ M), ascorbic acid (200 μ M), sodium selenite (0.05 μ M), or beta-carotene (20 μ M). The variation in ROS production was calculated from DCF fluorescence variations, which all lowered significantly ROS production, beta-carotene and ascorbic acid being the most potent compounds (Fig. 1A). It is known that ROS play an important role in stimulating cell proliferation. Several intracellular signaling pathways that stimulate cell proliferation, including TRKs, MAPKs, or redox sensitive transcription factors could be activated by ROS (11). Proliferation of Ntva-PGDF cells was significantly reduced after 7 days exposure to beta-carotene, ascorbic acid, tocopherol, and selenium, the 2 latter compounds being the most efficient (Fig. 1B). Similar results were obtained with Ntva-Ras Akt, a glioma cell line representative of grade IV glioblastoma (21) (data not shown).

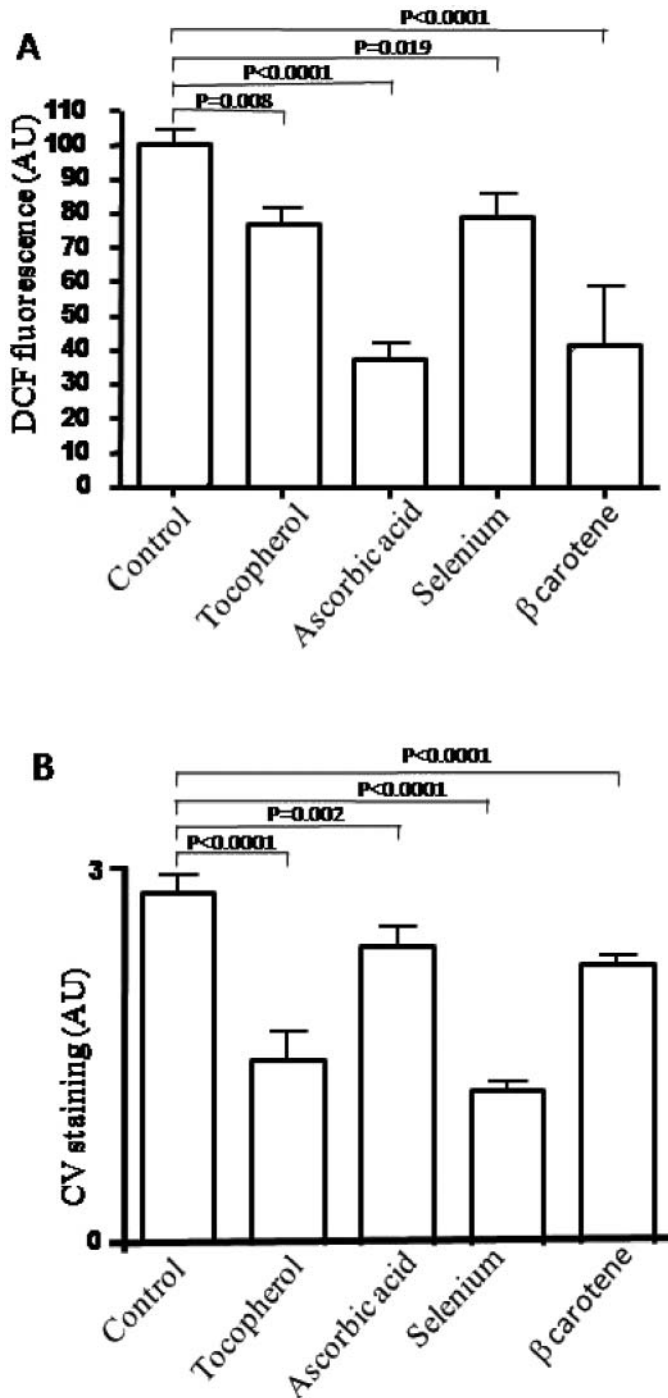


FIG. 1. A: Antioxidants decreased endogenous oxidative stress on Ntva-PGDGF glioma cell line. Reactive oxygen species production was measured by fluorescence of DCF (2',7'-dichlorofluorescein) 2 h after the incubation in presence or absence of alpha tocopherol (20 μ M) or ascorbic acid (200 μ M) or sodium selenite (0.05 μ M) or beta-carotene (20 μ M). B: Antioxidants reduced glioma cell proliferation. Effects of compounds on cell proliferation were estimated by crystal violet staining. Ntva-PGDGF cells were cultured for 7 days in presence or absence of the different antioxidants.

The SUVIMAX-Like Diet Reduced Moderately the Occurrence of ENU-Induced Tumors and Brain Tumors Incidence in Male Rats

We previously showed that a phytochemical-enriched diet reduced ENU-induced gliomagenesis in rats (12). We suggested that a major part of this effect could be explained by the antioxidant activity of the diet. In the present study, ENU-induced rats were fed a diet containing antioxidants (tocopherol, ascorbic acid, sodium selenite, beta-carotene and zinc). The concentrations of micronutrients used were adapted from human doses in SUVIMax study (one to three times the recommended dietary intake), taking into account the weight, amount of food intake per day and physiologic needs of the rats. On this basis, it would represent 2 times the recommended rat dietary intake. The body weight of the offsprings was determined weekly after 4 mo (after birth) as this corresponds to the earliest time the rats developed tumors in the control group. The end of the experiment was defined as 10 mo of age, as most of the brain tumors usually develop before that date. The histological analysis of brain tumors induced in our previous study with the same model showed characteristic features of glioma.

The rats with evident signs of illness (weight loss, nose bleed or eye bleed, fur standing on end) were isolated in separate cages and weighed daily until sacrifice. Twelve percent of the rats fed the StD diet died before the end of the experiment, compared to only 6% in the group fed the SUVIMAX-like diet ($P = 0.31$) (Fig. 2A). The proportion of healthy animals (no weight loss, no evident sign of illness) was not significantly higher ($P = 0.45$), in rats fed the SUVIMAX-like diet (35.6% vs. 27.3%). Statistical analysis showed a nonsignificant reduction of total number of tumor neither for brain tumor incidence (37.8% vs. 41.8%) (Fig. 2A) ($P = 0.6$). The incidence of brain tumors was also reduced by 18% with the SUVIMAX-like diet in males (Fig. 2B) but still not significantly ($P = 0.3$).

The SUVIMAX-Like Diet Delays the Outbreak of Signs of Illness Associated with Gliomagenesis

Because of interindividual weight heterogeneity or sex differences, the ratio between tumor weight and rat weight, allowed us to reduce this bias. More, because we used the ENU-induced model, the heterogeneity of the tumor was greater than in a grafted tumor model. With SUVIMAX-like diet, tumor occurrence was delayed and the size reduced, resulting in a decrease of 40% as compared to the StD diet, the difference was close to significant ($P = 0.057$).

In contrast, the positive effect on tumor growth was associated with a profit of survey very significant, observed over the whole course of the period study, even for old rats. Mean survival was significantly higher ($P = 0.004$) with the SUVIMAX-like diet (265 days \pm 43) than for the StD diet (205 days \pm 42) (Fig. 3A). Separating the males and females, the mean survival for males: 199 days \pm 38 (StD diet) vs. 266 days \pm 36 (SUVIMAX-like diet) was highly significant ($P = 0.0002$). For females the difference in survival was 219 days \pm 68 (StD

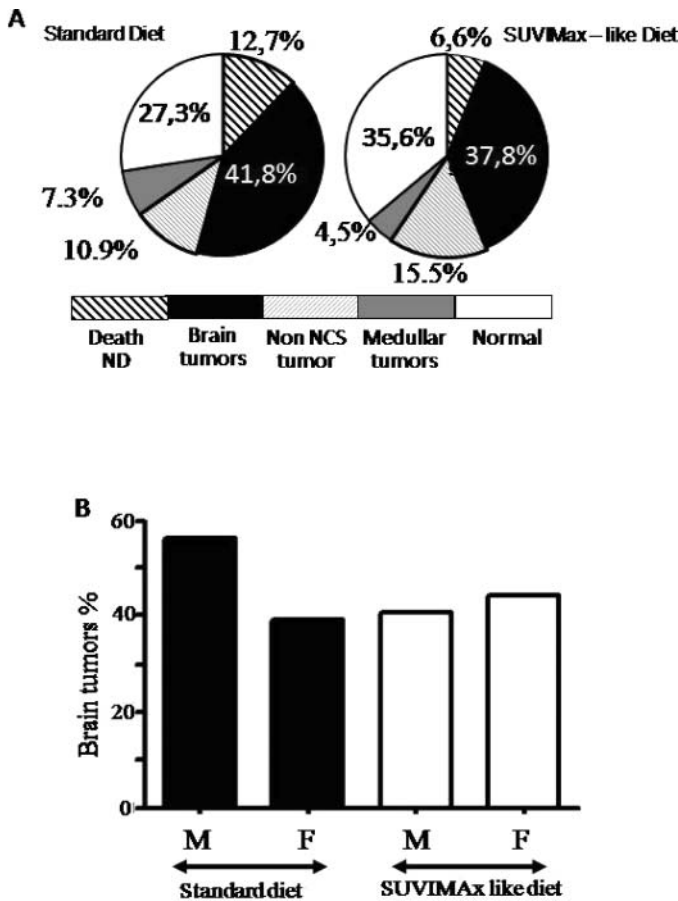


FIG. 2. A: Picture of tumor incidence in ethylnitrosourea (ENU)-induced rats fed either the standard diet (left) or the SUVIMAX-like diet (right). The differences observed were not statistically significant (incidence of death, brain, medullar or non-central nervous system tumors or percentage of healthy rats). B: SUVIMAX-like diet reduced, but not significantly ($P = 0.31$), brain tumor occurrence in males (M) rats.

diet) vs. 275 days \pm 46 (SUVIMAX-like diet), was close to significant ($P = 0.071$) (Fig. 3B).

Because this difference could be linked to the hormonal status (22, 23) (potential factor of bias), and few females develop gliomas, further experiments were limited to males only. As previously observed with our phytochemical-enriched diet (Pouliquen et al.) (12), systemic effects, as observed by total and liver weights, were reduced in the group of rats fed the SUVIMAX-like diet, suggesting a link between antioxidant activity and systemic effects. 30% of animals fed the SUVIMAX-like diet and bearing brain tumors, did not present body or liver weight decrease (Fig. 3C).

SUVIMAX-Like Diet Decreases Markers of Tumor Aggressiveness and Gliomagenesis Progression

As antioxidant SUVIMAX-like diet decreased the proliferation of glioma cells, both in vitro and in vivo, we searched for a molecular signature. MnSOD is an antioxidant enzyme located in the mitochondrial matrix; suggested to be a marker in 2 independent studies, which is inversely linked to long-term survival

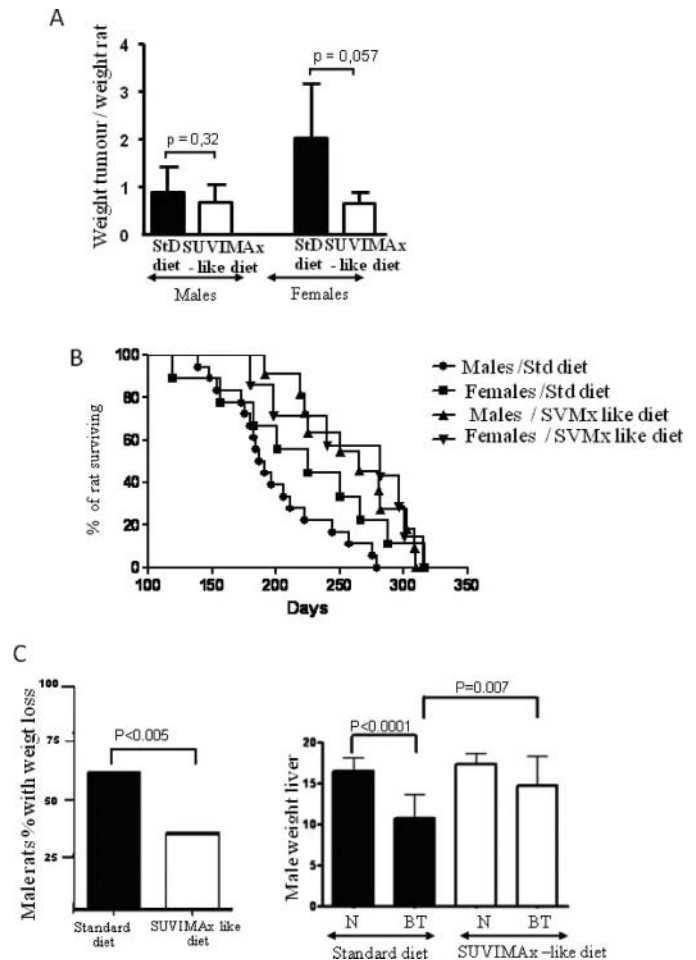


FIG. 3. SUVIMAX-like diet modulates gliomagenesis and signs of illness on ethylnitrosourea (ENU)-induced rats. A: SUVIMAX-like diet delays the emergence of brain tumors. Severity of brain tumors was estimated by spotting ratio of tumor mass/body mass depending on the age. B: SUVIMAX-like diet delayed gliomagenesis in male rats and increased their survival. Survival curves were analyzed using Log-rank Test and are significantly different ($P = 0.019$). Median survival of tumor bearing rats was respectively 208 days and 265 days with standard diet (StD) diet and SUVIMAX-like diet. C: SUVIMAX-like diet reduced systemic effects associated with brain tumors. Right: influence of diet on systemic effect reflected by body weight loss in male rats. Left: influence of diet on weight liver, another marker of illness. Comparison between healthy tissue (N) and brain tumor tissue (BT) was performed.

of glioma patients (14, 15). The SUVIMAX-like diet lowered MnSOD mRNA expression when compared with tumors of rats fed the StD diet (Fig. 4A). IGFs present in normal fetal/neonatal developing brain are absent in mature brain and reappear in neoplastic developing neuroglial derived tissues, including the most malignant brain tumor, GBM (24). Thus, IGFBP isoforms have been implicated in the pathogenesis of human neoplasms including glioma. Santosh et al. showed the association of IGFBP-2, -3, and -5 expression with an increasing grade of malignancy in astrocytomas. IGFBP-5 mRNA was higher in GBM relative to anaplastic astrocytoma or controls (17). Antioxidants induce a significant decrease in IGFBP-5 mRNA expression (Fig. 4B).

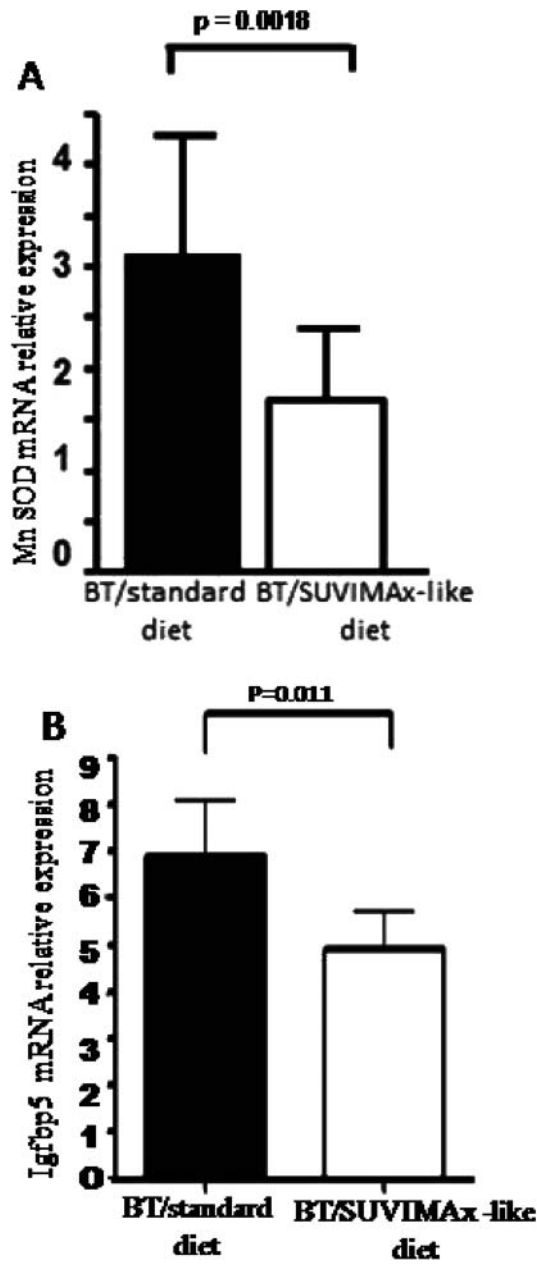


FIG. 4. Analysis of putative markers of glioma progression by RT-PCR. A: MnSOD mRNA relative expression in brain tumor (BT) was evaluated and compared between StD Diet and SUVIMAX-like diet, in males. B: IGFBP-5 mRNA relative expression was evaluated in the same way.

Furthermore, we did not detect any differences in the Bax/Bcl2 balance between the tumors of the 2 diet groups (data not shown). So we focused on Ki-67, a classical marker of cell proliferation (25) and PDGFRb, which is specifically associated with proliferation and aggressiveness in glioma (26). Ki-67 expression was significantly decreased (35%) in brain tumor of rats fed the SUVIMAX-like diet (Fig. 5A). Because PDGFR modulation is a key event in gliomagenesis involved in the survival of glioma cells, the expression of PDGFRb was assessed and

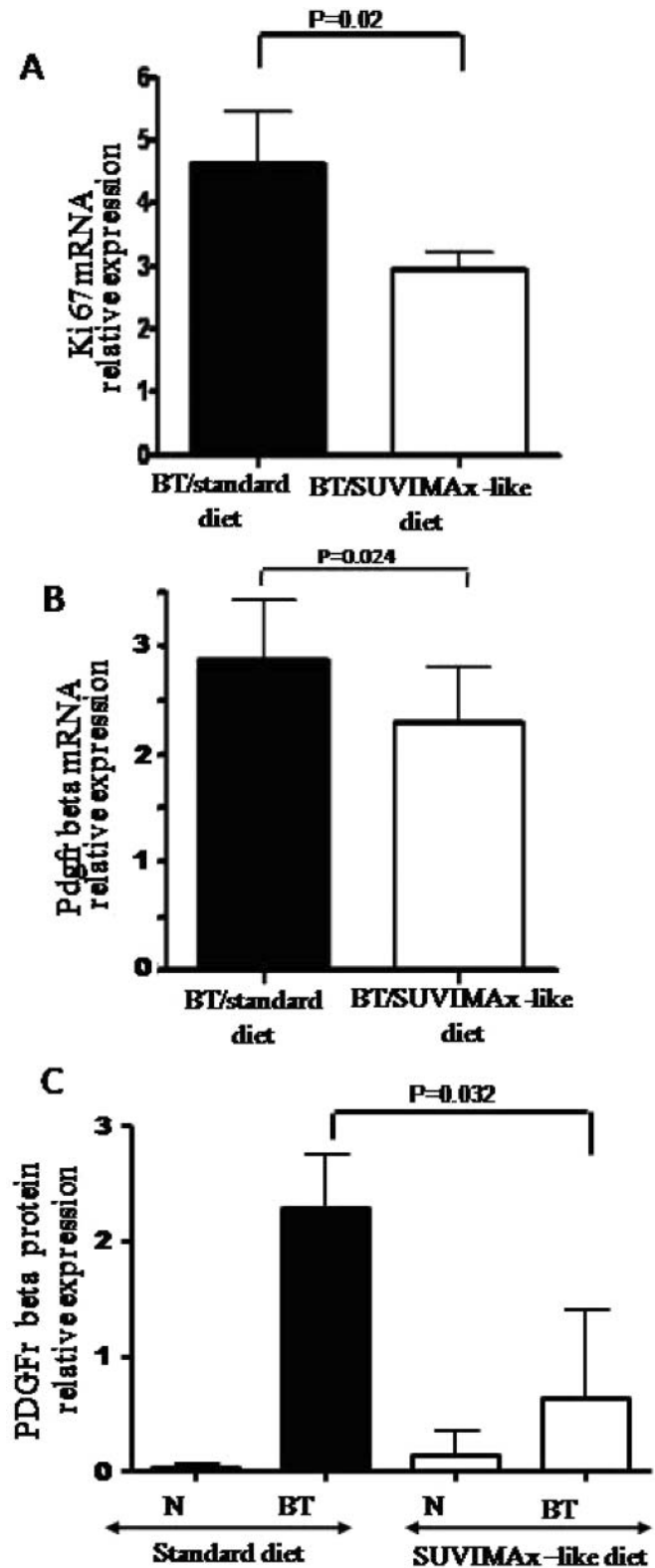


FIG. 5. SUVIMAX-like diet decreased brain tumor proliferation associated parameters (RT-PCR). A: Ki-67 relative expression. B: PDGFRb relative expression. C: PDGFRb protein relative expression evaluation by Western-blot, each column is the mean of values from 3 tumors.

showed a significant decrease ($P = 0.024$) at the gene expression level in the brain tumors of rats fed the SUVIMAX-like diet (Fig. 5B). This was confirmed at the protein level ($P = 0.032$) (Fig. 5C).

DISCUSSION

Brain tissue is vulnerable to the damaging effects of free radicals (27). A relationship between dietary intake and adult glioma has been suspected (28, 29). However, epidemiological studies (3, 4, 6, 34, 35) have provided conflicting results. Some of them suggested a protective role for antioxidants from fruits and vegetables while others not. These discrepancies could be related to differences in the preinclusion antioxidant status of the studied population, to high variability in the regimen habits of the studied populations, and/or to the size of the cross-section. In contrast, a case-control study of primary tumors of the brain and cranial meninges showed a significant protective effect among glioma pairs with the use of vitamin supplements, which increased with an increasing frequency of use (30). Moreover, high intake of vitamin E is correlated with greater survival for all patients diagnosed with Grade III malignant glioma (31). Dietary supplementation with antioxidants (e.g., vitamins C and E) was found to reduce the incidence of brain tumors in children whose mothers took these vitamins throughout pregnancy (32, 33). Decreases in antioxidant levels were correlated with the severity of the malignancy of brain tumors and also with the accumulation of considerable amounts of oxidative stress products including free radicals, which damage this tissue.

Some *in vitro* studies have shown a role of antioxidants in the control of glial cell proliferation. It was established that selenium not only induces tumor cell specific apoptosis but has also an anti-invasive potential in biopsies from gliomas (36). Similarly, tocopherols exhibited an antiproliferative effect on murine glioma C6 cells (37). In agreement with these studies, we show that alpha-tocopherol, ascorbic acid, beta-carotene and sodium selenite, all of which have antioxidant properties, control the constitutive oxidative stress status of mouse glioma cells, representative of grade II and grade IV gliomas and also inhibit tumor cell proliferation. Indeed, although ROS are predominantly implicated in cell damage, they also play a major physiological role in several aspects of intracellular signaling and regulation (38). It has been clearly demonstrated that ROS, which are frequently increased in cancer cells including glioma cells, interfere with the expression of a number of genes and signal transduction pathways and modulate the control of proliferation (39, 40, 41).

First, we demonstrated *in vitro* that each component of the diet was able separately, to decrease glial tumor cell proliferation. We observed that proliferation decreased more with treatments that reduced DCF-DA fluorescence. Uncontrolled cell proliferation requires the upregulation of multiple intracellular signaling pathways, (the components of which have been shown to be shared by redox-dependent stimuli) including cascades involved in survival, proliferation and cell cycle progression (42). The action on tumor proliferation can also exist through

various mechanisms in addition to the antioxidant action. Selenium, immunity modulator, is also able to act through its metabolites. The selenotrisulfides and methylselenol, for example, are known to have antitumorigenic properties (43). Alpha-tocopherol has properties independent of its antioxidant/radical scavenging ability such as the inhibition of protein kinase C (PKC), which is an important factor in the evolution and proliferation of malignant gliomas (44). The mechanism involved in the latter function is not related to the radical scavenging properties (45). All of the above could explain that individual antioxidant activities do not correlate with the severity of the antiproliferative effect observed here.

Secondly, we looked at the effect of a diet enriched with dietary supplement with these 5 antioxidants and systemic effect associated with developing tumors in the ENU-induced glioma rat model. We noticed brain tumor incidence reduction, which was not statistically significant. Interestingly, a larger number of brain tumors was generated in males, correlating with the higher rates observed in men as compared to women. Indeed, the incidence of glioma is twofold higher in men (46). This suggests a protective role of female hormones and/or a harmful role of male hormones (47).

Our main result, was a greatly delayed brain tumor emergence and growth in ENU-induced glioma model. When fed the SUVIMAX-like diet, tumor occurrence was significantly delayed and the size reduced as compared to that of the StD diet. When the results are separated by sex, the analysis of tumor size show significant difference only in female rats probably due to the limit of number in the groups. The most important aspect is that the positive effect against tumor growth was associated with a very significant profit of survey, mainly in males. Our observation is consistent with a number of published data on glioma, suggesting that females are already protected by their antioxidants/female hormones, This could explain why the benefits of an antioxidant treatment were difficult to interpret in females rats. Indeed, glioma-female rats with ovariectomy present a survey similar to males (48). A low level of oxidative stress can stimulate cell division in the promotion stage (49). This implies that production of ROS during this stage of carcinogenesis is a major factor in ROS-related tumor promotion.

We observed that a high proportion of rats with brain tumors fed the StD diet exhibited large systemic effects characterized by weight loss and reduction in liver weight. These systemic effects were prevented by the antioxidant dietary intervention. We observed that the Bax/Bcl2 ratio was not significantly different with supplementation (data not shown), but many examples exist where the mitotic index was not directly correlated with the apoptotic index (50) and the antiproliferative effect of antioxidants may not be due only to apoptosis or necrosis in glioblastoma but could also implicate an inhibition of cell cycle progression (51). We concentrated our work on markers of proliferation and aggressiveness. Altered levels of antioxidant enzymes and nonenzymatic antioxidants as well as changes in the related signaling pathways are common in many human cancers (52, 53). We show an increase in Mn-SOD transcripts in

brain tumors, which were largely reduced by the SUVIMAX-like diet. It has been shown that MnSOD expression level in tumor tissue is a candidate marker for prognosis of glioblastoma patients and high Mn-SOD expression was associated with short survival for patient. MnSOD was markedly increased in grade IV astrocytoma (15) and a decrease in its expression correlate with a less severe grade of brain tumor. Moreover Li et al. (2011) demonstrated that high MnSOD content correlated with poor prognosis in glioma patients and could be explained by H₂O₂-dependant MAPKs, PI3Ks, and MPPs increase (54). Compared with normal brain tissue, malignant gliomas express an increased number of insulin-like growth factor (IGF) receptors (55). IGFBPs comprise a family of proteins that bind and regulate the functions of IGFs. IGFBP-2, -3, and -5 expression correlates with grades of malignancy in astrocytomas, and IGFBP-5 mRNA is higher in GBM relative to anaplastic astrocytoma and controls (17). Rahman and Thomas also reported that IGFBPs (including IGFBP-5) were increased following ROS production (56). Expression of IGFBP-5 also correlates significantly with glioma histologic grade and increases with glioma anaplastic progression (57) and IGFBP-5 was shown to be a marker of recurrence of brain tumor (58) and correlated with a shorter overall survival in breast cancer (59). In agreement, we show that the antioxidant-enriched diet decreased the expression of IGFBP-5 in brain tumors. The SUVIMAX-like diet reduced the expression of Ki-67 mRNA, which is considered to be a classical cancer cell marker of proliferation. We also looked at PDGFRb expression because one of the most consistent cellular signaling defect observed in malignant gliomas is the establishment of a PDGF autocrine loop attributable to the coexpression of PDGF-A and -B and their cognate receptors. Glioblastoma cell lines and primary tumor tissue frequently overexpress PDGFRa and b (alpha and beta). Because PDGF autocrine signaling is an initiating event, additional defects in cell signaling are probably required for progression to GBM (60). PDGFRs activation by their agonists induces a moderate production of ROS involved in the mitogenic effect of the activation and survival pathways (Akt, NFkB) (61), which can be controlled by antioxidants. Expression of PDGFRb transcripts was significantly decreased with the SUVIMAX-like diet, a phenomenon confirmed at the protein level. H₂O₂ can activate the PDGFR via inhibition of protein tyrosine phosphatases. This might have an effect on tumor vessels and as such could explain the modulation of tumor growth, in part because the vessels are stimulated via PDGFR-b (62).

On the whole, in addition to the classical Ki-67 marker of proliferation, other markers linked to severity and tumor grade were decreased with the antioxidant diet. It appears that the antioxidant-enriched diet could control gliomagenesis. It operates less on tumor incidence than on the delay of their clinical emergence, aggressiveness, and associated systemic effects. The control of brain tumor incidence is delayed by exposure for several years, to new risks, such as radio frequencies. This increases the interest in the study of preventive factors likely to oppose the growth of tumors in central nervous system.

ACKNOWLEDGMENTS

We thank Dr. Lisa Oliver for the critical reading of this manuscript and its comments; Dr S. Hercberg for permitting us to use SUVIMAX-like diet for our experiment and helpful discussion. Dr. Eric C. Holland from Memorial Sloan Kettering Cancer Center, Departments of Surgery (Neurosurgery), Neurology, and Cancer Biology and Genetics (New York, NY) for providing the Ntva-PDGF and Ntva-Ras/Akt cells. E. Hervouet was supported by a grant from INCa (France).

REFERENCES

- Huse JT and Holland EC: Targeting brain cancer: advances in the molecular pathology of malignant glioma and medulloblastoma. *Nat Rev Cancer* **10**, 319–331, 2010.
- Tedeschi-Blok N, Lee M, Sison JD, Miike R, and Wrensch M: Inverse association of antioxidant and phytoestrogen nutrient intake with adult glioma in the San Francisco Bay Area: a case-control study. *BMC Cancer* **6**, 148–160, 2006.
- Lee M, Wrensch M, and Miike R: Dietary and tobacco risk factors for adult onset glioma in the San Francisco Bay Area (California, USA). *Cancer Causes Control* **1**, 13–24, 1997.
- Chen H, Ward MH, Tucker KL, Graubard BI, McComb RD, et al.: Diet and risk of adult glioma in eastern Nebraska, United States. *Cancer Causes Control* **7**, 647–655, 2002.
- Jacob RA, Aiello GM, Stephensen CB, Blumberg JB, Milbury PE, et al.: Moderate antioxidant supplementation has no effect on biomarkers of oxidant damage in healthy men with low fruit and vegetable intakes. *J Nutr* **133**, 740–743, 2003.
- Holick CN, Giovannucci EL, Rosner B, Stampfer MJ, and Michaud DS: Prospective study of intake of fruit, vegetables, and carotenoids and the risk of adult glioma. *Am J Clin Nutr* **85**, 877–886, 2007.
- Schwartzbaum JA and Cornwell DG: Oxidant stress and glioblastoma multiforme risk: serum antioxidants, gamma-glutamyl transpeptidase, and ferritin. *Nutr Cancer* **38**, 40–49, 2000.
- Yilmaz N, Dulger H, Kiyamaz N, Yilmaz C, Bayram I, et al.: Lipid peroxidation in patients with brain tumor. *Int J Neurosci* **116**, 937–943, 2000.
- Szatrowski TP and Nathan CF: Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* **51**, 794–798, 2000.
- Khan AU and Wilson T: Reactive oxygen species as cellular messengers. *Chem Biol* **2**, 437–445, 1995.
- Valko M, Rhodes CJ, Moncol J, Izakovic M, and Mazur M: Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* **160**, 1–40, 2006.
- Pouliquen D, Olivier C, Hervouet E, Pedelaborde F, Debien E, et al.: Dietary prevention of malignant glioma aggressiveness, implications in oxidant stress and apoptosis. *Int J Cancer* **123**, 288–295, 2008.
- Hercberg S, Czernichow S, and Galan P: Antioxidant vitamins and minerals in prevention of cancers: lessons from the SU.VI.MAX study. *Br J Nutr* **96**(Suppl 1), S28–S30, 2008.
- Park CK, Jung JH, Moon MJ, Kim YY, Kim JH, et al.: Tissue expression of manganese superoxide dismutase is a candidate prognostic marker for glioblastoma. *Oncology* **77**, 178–181, 2008.
- Ria F, Landriscina M, Remiddi F, Rosselli R, Iacoangeli M, et al.: The level of manganese superoxide dismutase content is an independent prognostic factor for glioblastoma. Biological mechanisms and clinical implications. *Br J Cancer* **84**, 529–534, 2001.
- Beattie J, Allan GJ, Lochrie JD, and Flint DJ: Insulin-like growth factor-binding protein-5 (IGFBP-5): a critical member of the IGF axis. *Biochem J* **395**, 1–19, 2006.
- Santosh V, Arivazhagan A, Sreekanthreddy P, Srinivasan H, Thota B, et al.: Grade-specific expression of insulin-like growth factor-binding proteins-2, -3, and -5 in astrocytomas: IGFBP-3 emerges as a strong predictor of survival in patients with newly diagnosed glioblastoma. *Cancer Epidemiol Biomarkers Prev* **19**, 1399–1408, 2010.

18. Calzolari F and Malatesta P: Recent insights into PDGF-induced gliomagenesis. *Brain Pathol* **20**, 527–538, 2010.
19. Koestner A, Swenberg JA, and Wechsler W: Transplacental production with ethylnitrosourea of neoplasms of the nervous system in Sprague-Dawley rats. *Am J Pathol* **63**, 37–56, 1971.
20. Hervouet E, Cízková A, Demont J, Vojtková A, Pecina P, et al.: HIF and reactive oxygen species regulate oxidative phosphorylation in cancer. *Carcinogenesis* **29**, 1528–1537, 2008.
21. Dai C and Holland EC: Glioma models. *Biochim Biophys Acta* **1551**, M19–M27, 2001.
22. Kabat GC, Etgen AM, and Rohan TE: Do steroid hormones play a role in the etiology of glioma? *Cancer Epidemiol Biomarkers Prev* **19**, 2421–2427, 2010.
23. Altioek N, Ersoz M, and Koyuturk M: Estradiol induces JNK-dependent apoptosis in glioblastoma cells. *Oncol Lett* **2**, 1281–1285, 2011.
24. Trojan J, Cloix JF, Ardourel MY, Chatel M, and Anthony DD: Insulin-like growth factor type I biology and targeting in malignant gliomas. *Neuroscience* **145**, 795–811, 2007.
25. Senft C, Polacin M, Priester M, Seifert V, Kögel D, et al.: The nontoxic natural compound Curcumin exerts anti-proliferative, anti-migratory, and anti-invasive properties against malignant gliomas. *BMC Cancer* **10**, 491, 2010.
26. Claesson-Welsh L: Platelet-derived growth factor receptor signals. *J Biol Chem* **269**, 32023–32026, 1994.
27. Sheweita SA and Sheikh BY: Can dietary antioxidants reduce the incidence of brain tumors? *Curr Drug Metab* **12**, 587–593, 2011.
28. Ross DA, Kish P, Muraszko KM, Blaivas M, and Strawderman M: Effect of dietary vitamin A or N-acetylcysteine on ethylnitrosourea-induced rat gliomas. *J Neurooncol* **40**, 29–38, 1998.
29. Gagliano N, Aldini G, Colombo G, Rossi R, Colombo R, et al.: The potential of resveratrol against human gliomas. *Anticancer Drugs* **21**, 140–150, 2010.
30. Preston-Martin S and Mack W: Gliomas and meningiomas in men in Los Angeles County: investigation of exposures to N-nitroso compounds. *IARC Sci Publ* **105**, 197–203, 1991.
31. Sheweita SA and Sheikh BY: Can dietary antioxidants reduce the incidence of brain tumors? *Curr Drug Metab* **12**, 587–593, 2011.
32. Goh YI and Koren G: Prenatal supplementation with multivitamins and the incidence of pediatric cancers: clinical and methodological considerations. *Pediatr Blood Cancer* **50**, 487–489, 2008.
33. Bunin GR, Gallagher PR, Rorke-Adams LB, Robison LL, and Cnaan A: Maternal supplement, micronutrient, and cured meat intake during pregnancy and risk of medulloblastoma during childhood: a children's oncology group study. *Cancer Epidemiol Biomarkers Prev* **15**, 1660–1667, 2006.
34. Il'yasova D, Marcello JE, McCoy L, Rice T, and Wrensch M: Total dietary antioxidant index and survival in patients with glioblastoma multiforme. *Cancer Causes Control* **20**, 1255–1260, 2009.
35. DeLorenze GN, McCoy L, Tsai AL, Quesenberry CP Jr, Rice T, et al.: Daily intake of antioxidants in relation to survival among adult patients diagnosed with malignant glioma. *BMC Cancer* **10**, 215, 2010.
36. Rooprai HK, Kyriazis I, Nuttall RK, Edwards DR, Zicha D, et al.: Inhibition of invasion and induction of apoptosis by selenium in human malignant brain tumour cells in vitro. *Int J Oncol* **30**, 1263–1271, 2007.
37. Betti M, Minelli A, Canonico B, Castaldo P, Magi S, et al.: Antiproliferative effects of tocopherols (vitamin E) on murine glioma C6 cells: homologue-specific control of PKC/ERK and cyclin signaling. *Free Radic Biol Med* **41**, 464–472, 2006.
38. Palmer HJ and Paulson KE: Reactive oxygen species and antioxidants in signal transduction and gene expression. *Nutr Rev* **55**, 353–361, 1997.
39. Owuor ED and Kong AN: Antioxidants and oxidants regulated signal transduction pathways. *Biochem Pharmacol* **6**, 765–770, 2002.
40. Thannickal VJ and Fanburg BL: Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* **279**, L1005–L1128, 2000.
41. Poli G, Leonarduzzi F, Biasi E, and Chiarpotto E: Oxidative stress and cell signaling. *Curr Med Chem* **11**, 1163–1182, 2004.
42. Muller JM, Cahill MA, Rupec RA, Baeuerle PA, and Nordheim A: Antioxidants as well as oxidants activate c-fos via Ras-dependent activation of extracellular signal-regulated kinase 2 and Elk-1. *Eur J Biochem* **244**, 45–52, 1997.
43. Zeng H and Combs GF Jr: Selenium as an anticancer nutrient: roles in cell proliferation and tumor cell invasion. *J Nutr Biochem*, **19**, 1–7, 2008.
44. Bredel M and Pollack IF: The role of protein kinase C (PKC) in the evolution and proliferation of malignant gliomas, and the application of PKC inhibition as a novel approach to anti-glioma therapy. *Acta Neurochir* **139**, 1000–1101, 1997.
45. Ricciarelli R, Zingg JM, and Azzi A: Vitamin E: protective role of a Janus molecule. *FASEB J* **15**, 2314–2325, 2001.
46. Inskip PD, Linet MS, and Heineman EF: Etiology of brain tumors in adults. *Epidemiol Rev* **17**, 382–414, 1995.
47. McKinley BP, Michalek AM, Fenstermaker RA, and Plunkett RJ: The impact of age and sex on the incidence of glial tumors in New York state from 1976 to 1995. *J Neurosurg* **93**, 932–939, 2000.
48. Plunkett RJ, Lis A, Barone TA, Fronckowiak MD, and Greenberg SJ: Hormonal effects on glioblastoma multiforme in the nude rat model. *J Neurosurg* **90**, 1072–1077, 2000.
49. Dreher D and Junod AF: Role of oxygen free radicals in cancer development. *Eur J Cancer* **32A**, 30–38, 1996.
50. Mattern J and Volm M: Imbalance of cell proliferation and apoptosis during progression of lung carcinomas. *Anticancer Res* **24**, 4243–4246, 2004.
51. Michaud-Levesque J, Bousquet-Gagnon N, and Béliveau R: Quercetin abrogates IL-6/STAT3 signaling and inhibits glioblastoma cell line growth and migration. *Exp Cell Res* **318**, 925–935, 2012.
52. McEligot AJ, Yang S, and Meyskens FL: Redox regulation by intrinsic species and extrinsic nutrients in normal and cancer cells. *Ann Rev Nutr* **25**, 261–295, 2005.
53. Kern JC and Kehrer JP: Free radicals and apoptosis: relationships with glutathione, thioredoxin, and the BCL family of proteins. *Front Biosci* **10**, 1727–1738, 2005.
54. Li F, Wang H, Huang C, Lin J, Zhu G, et al.: Hydrogen peroxide contributes to the manganese superoxide dismutase promotion of migration and invasion in glioma cells. *Free Radic Res* **45**, 1154–1161, 2011.
55. Merrill MJ and Edwards NA: Insulin-like growth factor-I receptors in human glial tumors. *J Clin Endocrinol Metab* **71**, 199–209, 1990.
56. Rahman MS and Thomas P: Characterization of three IGFBP mRNAs in Atlantic croaker and their regulation during hypoxic stress: potential mechanisms of their upregulation by hypoxia. *Am J Physiol Endocrinol Metab* **301**, E637–E648, 2011.
57. Wang H, Wang H, Zhang W, and Fuller GN: Overexpression of IGFBP5, but not IGFBP3, correlates with the histologic grade of human diffuse glioma: a tissue microarray and immunohistochemical study. *Technol Cancer Res Treat* **5**, 195–199, 2006.
58. Chinnaiyan P, Chowdhary S, Potthast L, Prabhu A, Tsai YY, et al.: Phase I trial of vorinostat combined with bevacizumab and CPT-11 in recurrent glioblastoma. *Neuro Oncol* **14**, 93–100, 2012.
59. Ahn BY, Elwi AN, Lee B, Trinh DL, Klimowicz AC, et al.: Genetic screen identifies insulin-like growth factor binding protein 5 as a modulator of tamoxifen resistance in breast cancer. *Cancer Res* **70**, 3013–3019, 2010.
60. Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA, and Giese NA: Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. *Cancer Res* **62**, 3729–3735, 2002.
61. Hensley K, Robinson KA, Gabbita SP, Salsman S, and Floyd RA: Reactive oxygen species, cell signaling, and cell injury. *Free Radic Biol Med*; **28**, 1456–1462, 2000.
62. Nazarenko I, Hede SM, He X, Hedrén A, Thompson J, et al.: PDGF and PDGF receptors in glioma. *Ups J Med Sci*; **117**, 99–112, 2012.