RUNNING HEAD: GLIABLASTOME MULTIFORME
THE EFFECTIVENES OF HYPERBARIC OXYGEN THERAPY AND SOROFENIB IN AN ESTABLISHED INTRACEREBRAL TUMOR MODEL IN RATS USING THE C6 GLIAL CELL

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Abstract
Gliomas are the most common brain tumour in adults. Glioblastoma (GBM) is a type of tumor that is highly lethal despite maximal therapy. Tumor-related endothelial cells are of neoplastic nature and have malignant properties such as proliferation and invasion. Therefore, antiangiogenic approaches have several advantages compared with conventional chemotherapeutic approaches. This study was undertaken to investigate the preventive or therapeutic effect of hyperbaric oxygen therapy (HBOT) in an established intracerebral tumor model. In this regard, parameters such as the tumor size, vascularization rate, midkine (MK), nuclear factor kappa B (NF-kB), HIF-1 alpha, and caspase 3 were compared in the relevant groups.

INTRODUCTION
Gliomas are the most common brain tumour in adults, are observed among all age groups, and peak in the 5th and 6th decade of life. High-grade gliomas represent 60-75% of all gliomas. According to genetic and cytological findings, high-grade gliomas have a highly heterogeneous etiopathogenesis. Therefore, the factors contributing to the prognosis vary. The patient survival time and variety of responses to treatment is dependent on genetic and aetiological diversities (1). Despite current advanced diagnostic and treatment possibilities, the 5-year survival rate of patients with Glioblastoma Multiforme (GBM) is less than 5% (2, 3).

Tumour-related endothelial cells are of a neoplastic nature and have malignant properties such as proliferation and invasion (4). Anti-angiogenic treatment is focused on endothelial cells forming in the vascular walls. Therefore, anti-angiogenic approaches have several advantages when compared to conventional chemotherapeutic approaches (5). The goal of anti-angiogenic treatment is to ensure a decrease in systemic side effects because there are endothelial cells and other supportive cells belonging to the vessel walls (6).

Hypoxia plays a direct role in the embryogenesis period and the growth and development of the cancer tissue. Hypoxia causes a release of several growth factors that cause vascularization and therefore increases the tumour size. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are two of the secreted
factors. Hypoxia increases the level of these factors, and the chemotherapeutic agent sorafenib is the antagonist of these two factors.

The purpose of this study was to increase the oxygenation of the environment with Hyperbaric Oxygen Therapy (HBOT) and to prevent the angiogenesis effect of the factors being secreted by hypoxia. We also aimed to prevent angiogenesis by using sorafenib, which is a chemotherapeutic agent that blocks the receptors for these factors. In this study, we investigated the role of HBOT and Sorafenib (Nexavar®) in angiogenesis separately and tested their effects when used together. In this framework, tumour size, vascularization rate, and levels of midkine, transcription factors related to apoptosis and angiogenesis factors such as nuclear factor kappa B (NF-kB), HIF-1 alpha, and caspase 3 were compared among the related groups.

MATERIAL AND METHODS

Cell Culture

The C6 glioma cell line was obtained from the American Type Culture Collection and maintained in Dulbecco’s Modified Eagle’s Medium and Ham’s F12 media (DMEM-F12) containing L-glutamine, Hepes and sodium bicarbonate (Biological Industries, Haemek, Israel) (1:1). DMEM-F12 was supplemented with 10% heat-inactivated foetal calf serum (Sigma Chemical Co., St Louis, Missouri), 10,000-units/ml penicillin (Sigma Chemical Co., St Louis, Missouri) and 10 mg/ml streptomycin (Sigma Chemical Co., St Louis, Missouri). The 75 cm² culture flasks (TPP, Trasadingen, Switzerland) were kept in an incubator with a humidified atmosphere of 5% CO2 at 37°C, and after incubation, the medium was discarded. Prior to trypsinization, the cell layer was washed twice with Ca+2- and Mg+2 - free phosphate buffered saline (CMF-PBS) (pH7.4). Cells in semi-confluent flasks were harvested using 0.05% trypsin (Sigma Chemical Co., St Louis, Missouri) in CMF-PBS. DMEM-F12 was added for trypsin inactivation. The trypsinized cell suspension was centrifuged and resuspended in DMEM-F12. Cells were counted on a haemocytometer to achieve a concentration of 107 cells in 250 µl of the culture medium.

Animals and Intracranial Tumour Implantation

In Wistar-Furth rats, the C6 glioma tumour line drug interaction studies are extensively used worldwide (7). By the approval of the Istanbul University, Institute for Experimental Medical Research (DETAE) Animal Care Investigation Committee, the experiments were performed on Wistar-Albino 5-6 week old male rats, weighing 100-150 g. The male rats were obtained from the Animal Breeding and Research Center of Istanbul University of Medicine. The rats were anesthetized by intraperitoneal injection with 40 mg/kg pentobarbital. After sterile preparation with betadine and alcohol, 1 x 108 C6 glioma cells in 10 µl of the culture medium were injected intracranially into the left side of the rat’s frontal lobe by a burr-hole.

EXPERIMENTAL DESIGN

Radiological examinations with MR imaging methods at day 7 after inoculation confirmed tumour formation. Abnormal signal change in the left hemisphere was considered positive for inoculation. Groups were formed from rats meeting the MR criteria, and was started.

Rats were housed in groups of 4 in plastic cages in a temperature-controlled room with a 12 hour light/dark cycle and fed ad libitum with commercial feed (Korkut Ilim Yem Sanayi, Antalya, Turkey).

Four main groups were used for comparison in this study. Ten rats were included in each group. Group 1 - Control group: This group received a surgical procedure where tumour tissue was left to its own course after implantation.

Group 2 – Hyperbaric Oxygen Treatment: After day 7 following implantation, hyperbaric oxygen treatment was started. No other medical treatment was performed. The aim of this group was to evaluate effectiveness of hyperbaric oxygen treatment alone. Hyperbaric treatment was administered twice a day for 90 minutes at 2.5 ATA pressure. Group 3 - Sorafenib: At day 7 following implantation, oral sorafenib, a VEGFR and PDGFR blocker, was started with a daily dose of 10 mg/kg for 14 days. Group 4 - Both Hyperbaric Oxygen Treatment and Sorafenib: At day 7 following implantation, a 10 mg/kg/day dose of sorafenib and hyperbaric oxygen treatment were started. For
14 days, sorafenib was given orally and hyperbaric oxygen treatment was implemented for 90 minutes at 2.5 ATA pressure twice a day.

Medical Treatment

Sorafenib (SOR)

As described, the relevant groups were treated with sorafenib (Nexavar ®, a complimentary of Bayer, Leverkusen, Germany) via nasogastric lavage for 14 days with a dose of 10 mg/kg/day given at the same time daily.

Hyperbaric Oxygen Treatment

Groups were kept in special pressure rooms for animal experiments at the Istanbul University Faculty of Medicine, Hyperbaric and Underwater Medicine Department. They were treated for two weeks with 2.5 ATM hyperbaric oxygen treatments twice a day for 90 minutes.

After two weeks, the rats were euthanized with 100 mg/kg pentobarbital injection. Blood from the rats was drawn by the intracardiac puncture method to measure the MK levels in the serum using ELISA.(figure 1)

The brains were removed and examined for HIF-1α and NF-kappaB levels by western blotting, and tumour volume was evaluated using a light microscope and Haematoxylin-Eosin (H&E) staining.

Tumour Volume and Intratumoural Vascularization Rate by Light Microscopy

A microtome with a thickness of 3 μm was used to obtain sections from the paraffin blocks. These sections were transferred to poly-lysine lamina (microscope slides). The cell core was stained dark blue-violet, and the cytoplasm was stained pink-red. The images were taken with a camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and were evaluated with NIH Image software.

After the tumour area was calculated in mm2, it was multiplied by its section thickness to determine the tumour volume. The intratumoural vascularization rate was determined as a percentage using the formula intratumoural vessel count/total tumour area x100. Two different researchers confirmed the accuracy of these results (figure2).

HIF-1α, CASPASE-3, and NF-KAPPAB Levels by Western Blotting

The tumour tissue was ruptured with an extraction solution (Invitrogen, NY, USA) with the help of a homogenizer. To be able to measure the change in the protein amount, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), defined as a non-natural (denatured) gel electrophoresis, was used. This study used a vertical electrophoresis device with a discontinuous gel, sized 9.5 x 17 cm and 1 mm thickness. Standard protein markers were used. Three types of protein with different molecular weights named HIF-1α (115 kDa), caspase-3 (17 kDa), NF-kappaB p65 (65 kDa) were processed on a discontinuous gel. After controls were acquired, immunological painting was performed. Afterwards, images of the bands were taken using the ImageJ program (National Institutes of Health, Bethesda, MD) in the Raytest imagining device.

Serum Midkine Levels with ELISA

After the blood was separated into its sera, the serum midkine levels were measured using micro plates coated with commercial midkine antibody (PeproTech, NJ, USA). One hundred microlitres of serum were added to these plates and left for two hours. After this time, the micro plates were washed, polyclonal anti-rat MK antibody (Peprotech NJ,
USA) was added, and the micro plates were incubated in this antibody for one hour. Once this process was completed, the measurements were noted to be 450 nm in the ELISA reader (M2, Molecular devices, CA, USA).

**STATISTICS**

The SPSS 17.0 program was used for statistical analysis. Statistical analysis of all data was made performed using Student’s t test in accordance with variance analysis, and the results were shown as the mean± standard error (SD). Values of P<0.05 were considered to be statistically significant.

**RESULTS**

**TUMOR VOLUME AND VASCULARIZATION RATE**

In all groups, the tumour volume was reduced when compared to the control group (38±8.3 mm³). It was observed that tumour volume for the SOR, HBOT and combination groups were reduced by 5±3.2 mm³ (P<0.01), 30±6.1 mm³ (P<0.01) and 19±2.1 mm³ (P<0.001), respectively. When groups were compared, it was observed that the highest reduction rate in the tumour volume was detected in the combination group (PSOR<0.01, PHBOT<0.001).

The vascularization rate in the tumour for the control group was found to be 6.1±0.7%. The vascularization rate was reduced for all groups. The highest reduction rate was observed in the combination group (3.78±0.1%; PCONTROL<0.001; PHBOT<0.01; PSOR<0.01), followed by the SOR group (4.24±0.9%; PCONTROL<0.05; PHBOT<0.01; PCOMBINATION<0.01) and the HBOT group (4.9±0.2%; PCONTROL<0.05; PSOR<0.01; PCOMBINATION<0.01) (Figure 3).

**HIF-1α, CASPASE-3, and NF-KAPPA B LEVELS**

**HIF-1α Levels**

Compared to the control group, the HIF-1α levels increased slightly in the SOR group and (P<0.05) significantly in the HBOT group (P<0.001). For the combination group, it was observed that this reduced the HIF-1α levels (PCONTROL<0.05; PSOR<0.01; PHBOT<0.001). (Figure 4)

The lowest NF-kappaB level was found to be in the combination group. (PCONTROL<0.01; PSOR<0.05; PHBOT<0.01). When compared to the combination group, the NF-kappaB level of the SOR group showed a lower degree of increase (PCONTROL<0.01; PHBOT<0.05; PCOMBINATION<0.01). The increase was highest in the HBOT group (PCONTROL<0.05; PSOR<0.05; PCOMBINATION<0.01) (Figure 5).

**CASPASE-3 Levels**

Our results showed, compared to the control group, the caspase 3 levels increased in the other groups. The highest caspase-3 level was found to be in the combination group (P<0.01; P SOR<0.05; P HBOT<0.05). Second, in the SOR groups, the caspase-3 level was lower than in the combination group (P<0.05; P HBOT<0.05; P comb. <0.05). The increase was lowest in the HBOT group (P<0.01; P SOR<0.05; P comb. <0.05) (Figure 6).
MK Levels

Compared to the control group, the HBOT and SOR groups did see an increase in MK levels. Additionally, in the combination group, the value of the MK levels was close to the value of the control group. HBOT provided the highest increase in MK levels (PCONTROL<0.001; PSOR<0.01; PCOMBINATION<0.01). Similar to HBOT, SOR also increased these levels. However, this increase was not as high as with HBOT alone (PCONTROL p<0.05, PHBOT<0.01; PCOMBINATION<0.05). In the combination group, the value of the MK levels was close to the value of the control group (PCONTROL p>0.05, PHBOT<0.01; PSOR<0.01) (Figure 7).

DISCUSSION

It was previously reported that the creation of new vessels is required for oxygen transportation, which is necessary for progression of the tumour tissue, metastasis, invasion, meeting of growth factors and nutrients. If tumours cannot create new blood vessels, they are supplied by surrounding blood vessels via diffusion. Even if the tumour grows from 100 microns to 200 microns, this alone is not sufficient for oxygenation. After this phase, it has been discussed that micro-environmental hypoxia in tumour tissue occurs (7,8)

Hypoxia is the primary cause of neo-vascularization. This phenomenon contributes to the development of tissue in the foetal stage, thereby contributing to the development of the tumour, and it plays a negative role in terms of the reduction of the response of the tumour tissue to treatment. While HIF-1 is active in glial tumours, HIF-2 plays an active role in the glial tumour stem cells. (9,10)

There are two mechanisms that create blood vessel formation: vasculogenesis and angiogenesis. Vasculogenesis occurs in early embryogenesis and refers to the creation of the vascular mesh from the mesoderm (11).

Vasculogenesis starts with clustering as blood islets of pre-vascular cells or haemangioblasts. Angiogenesis is the creation of vessels in capillary structures with specific signals from vessels occurring with vasculogenesis (12,13).

Sorafenib is a receptor tyrosine kinase inhibitor that is given orally and reduces tumour cell proliferation. In this study, it was observed that sorafenib inhibits multiple intracellular (C-CRAF, BRAF and mutant BRAF) and cell surface kinases (KIT, FLT - 3, RET, VEGFR - 1, VEGFR - 2, VEGFR - 3, and PDGFR - b). Some of these kinases are considered to play a role in tumour cell signalling, angiogenesis, and apoptosis. By blocking intracellular or surface receptor kinases such as PDGFR, RAF kinase, VEGFR2, Flk - 1, and c-KIT, proliferation and cell growth are inhibited (14,15,16).

Transcription factor nuclear factor-kappa B (NF-kB) is a heterodimeric complex of Rel-family proteins . It controls the expression of many genes that are responsible for cell growth, differentiation, apoptosis regulation, cytokine production and neoplastic transformation . Comparing NF-kB activation in tumour cells to normal adjacent tissues, it was found to be significantly higher in tumour cells (17,18,19). Although the apoptotic process seems to be of a single type, the contribution of caspase sub-groups changes according to the cell type providing the apoptotic stimuli. Foremost, among them, the most important is that caspase-3 is directly related to the destruction of protein. Activities of caspase-3 play an active role in apoptosis. Therefore, caspase-3 levels are an important marker of apoptosis (20,21).

Midkine is a carbohydrate-binding protein, and it is also defined as a cytokine or growth factor. In various target cells, its role is growth, migration, and gene expression. The prognosis of patients with a high level of MK is worse than for those with low MK levels. Hypoxia increases MK levels. Increased MK levels lead to binding of HIF-1 α (hypoxia inducing factor) and forms a response to hypoxia. Studies show that MK is effective for tumour invasion, growth and angiogenesis and that in high-grade astrocytoma and GBM, the MDK and mRNA and protein expression levels are higher when compared to low level astrocytomas (oligodendroglioma, ependymoma, schwannoma, meningioma and pituitary adenoma). These studies showed that we must increase our focus on midkine levels (22,23).
Hyperbaric oxygen treatment involves the inhalation of 100% oxygen under high pressure (usually 2-3 atm) (24). During HBOT, oxygen is dissolved in the blood. Physiologically, as a short-term effect of HBOT, vasoconstriction increases oxygen in the presence of oxygen levels in the tissues and reduces oedema. An increase in phagocytic activation and anti-inflammatory activity occurs. Long-term effects include neovascularization, stimulation of osteogenesis and presence of fibroblasts of collagen products. To clarify the molecular mechanisms involved in this process, dose-dependent effects of HBOT have been shown to affect angiogenesis via VEGF levels. Today, HBOT is used for the regression of edema in the treatment of GBM, radiation therapy, and/or increases in the efficiency of antineoplastic agents used to minimize side effects (25-26). In this study, we used HBOT to reduce micro-environmental tumour hypoxia, causing growth of tumour tissue and neovascularization during tumour development.

According to this study, it was discovered that SOR individually reduces the tumour volume effectively. Though it is possible that SOR causes this result through various mechanisms such as multikinase inhibition, we have obtained supportive findings suggesting that it occurred in this study mainly through MK, HIF-1α, NF-kappaB and caspase-3. We found that HBOT potentiates the sorafenib efficacy. In this study, it was detected for the first time that HBOT shows synergistic action with SOR, HBOT increases serum MK levels, and despite decreasing the MK levels in human T98G GBM cell cultures, SOR levels increased similarly in this in vivo study.

In comparison, some studies showed increased angiogenesis of HBOT after ischaemic damage. Studies carried out by Ren et al. showed that pre-HBOT therapy increases the pro-angiogenesis effect similar to HIF expression and VEGF of HIF. Therefore, it can be concluded that HBOT may have an increasing effect on the growth of the cancer.

These results led to the conclusion that HIF-1α may increase MK levels by binding to the MK promoter in the tumour cells. The results demonstrated that the combination group reduces the levels of HIF-1α and, consequently, the levels of MK were less when compared to the HBOT and SOR group. It was shown in previous studies that by NF-kappaB binding to the HIF-1α promoter, an increase is observed in the molecule expression (19).

In this study, it was shown that the change in levels of HIF-1α are parallel to levels of NF-kappaB. The lowest levels of HIF-1α and NF-kappaB were observed in the combination group. While the increase of these two proteins was highest during HBOT, the increase was observed to be less in the SOR group. The fact that the apoptotic protein, caspase-3, levels in the combination group was at the highest level but was lower in the other groups also paralleled the levels of NF–kappaB, which is anti-apoptotic like HIF-1α. It was concluded that these protein levels in the combination group could be explained by the fact that in this group, the highest reduction in tumour mass and intratumoural vascularization was observed.

In this study, it was determined that SOR alone effectively reduces the volume of the tumour. This occurs even though SOR achieves this result through various mechanisms such as the inhibition of multiple kinases. In this study, it was investigated that this generally occurs via MK, HIF-1α, NF- kappaB and caspase-3. Liang et al. showed that hypoxia, resulting from the anti-angiogenic effect of SOR, creates a resistance against SOR, thereby reducing the effect of SOR in hepatocellular carcinoma (HCC) as a cytoprotective activity response. These researchers concluded that SOR resistance was due to an increase of intratumoural hypoxia in HCC patients already treated with SOR compared to untreated patients.

In the present study, we came to the conclusion that, compared to HBOT, less SOR reduces HIF-1α and NF-kappaB, and the reduction in MK levels may be lower. Regarding SOR, it was administered for two weeks at a 10 mg/kg/day dose. This dose was selected to avoid systemic toxicity because the half-life of SOR is 24-48 hours. It was concluded that a slight increase in HIF-1α, NF- kappaB and MK occurred as a result of the inability of SOR to enter the tumour due to the resistance caused by proteins following continuous implementation of the drug. With this result, maybe a decrease in caspase-3 levels could occur.

It has been concluded that a slight increase may occur in HIF-1α, NF-kappaB and MK due to inadequate entrance of SOR to the tumour with the resistance occurring over the drug pumping proteins as a result of subsequent administration. Additionally, it was concluded that the possibilities that HBOT administered 1½ hour per day for two weeks loses its effect between the two administrations and increases the hypoxic environment, and the
related/nonrelated resistance mechanisms, such as MK, may be an explanation for the high MK and NF-kappaB levels and the low caspase-3 levels. In addition, this may be the reason why HBOT is not as effective as SOR in reducing the tumour volume. It was concluded that closure of this gap by SOR in the combination group may be associated with high efficiency of the combination group.

It was understood that SOR covered this missing issue in the combination group and that this could be linked to the high efficiency of the combination group.

In our study, we showed that HBOT and sorafenib combination reduces HIF-1α levels and increases the efficacy of sorafenib. Although there are different characteristics of both administrations in this study, their similar characteristic is that they are not specific to the targets. Therefore, there is a need for more research studies in relation to the correct time of application of HBO therapy. Further studies are needed to evaluate maximal effect and optimal application regimen.

As our knowledge at the molecular level increases, it is observed that molecules contributing to the pathways can sometimes show even a counter effect in some cases, and this indicates that the sensitive balance is under the control of many control mechanisms, even if it is a pathologic process. To shed further light on these mechanisms, multi-centred studies are required.

REFERENCES

7. Folkman J. What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst.1990; 82: 4-6 PMID: 1688381

FIGURES

![Figure-1](image_url)
Figure-2
Image 2 Intratumoural Vascularization with Haematoxylin-Eosin (H&E) Staining.
A. Control B. SOR C. HBOT D. Combination Groups

<table>
<thead>
<tr>
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<th>Tumoral Volume</th>
<th>Vascularization Rate</th>
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<tbody>
<tr>
<td>CONTROL</td>
<td>38±8.3 mm³</td>
<td>6.1±0.7%</td>
</tr>
<tr>
<td>SOR</td>
<td>5±3.2 mm³</td>
<td>4.2±0.9%</td>
</tr>
<tr>
<td>HBOT</td>
<td>30±6.1 mm³</td>
<td>4.9±0.2%</td>
</tr>
<tr>
<td>SOR+HBOT</td>
<td>19±2.1 mm³</td>
<td>3.7±0.1%</td>
</tr>
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Figure-3

Table 4:
1. SOR Group, 2. Control Group, 3. HBOT Group, 4. Combination Group
Table 5:
1. Control Group, 2. Combination Group, 3. SOR Group, 4. HBOT Group

Figure-5

Table 6: 1. HBOT group, 2 Control group, 3. SOR group, 4. Combination group

Figure-6

Table 7: Representation of changes in MK levels using ELISA. Results have been shown as the result of 3 different experiments by average ± SD.

a: compared to Control,
b: compared to HBOT,
c: compared to SOR,
d: compared to combination

* p<0.01, ** p<0.001, ***p<0.05
## CONTRIBUTION

<table>
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<tr>
<th>Author Name</th>
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