An evaluation of the effectiveness of pre-ischemic hyperbaric oxygen and post-ischemic aminoguanidine in experimental cerebral ischemia

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ABSTRACT

الأهداف: دراسة تأثير الأوكسجين مفرط الضغط بعد حدوث قصور الإمداد الدموي، وتأثير أمينوغوانيدين بعد حدوث القصور وذلك على حجم الاحتشاء لدى الحالات المصابة بالانسداد الدائم للشريان الدماغي الأوسط.

الطريقة: لقد تمت الموافقة على هذه الدراسة من قبل مجلس أخلاقيات التجارب على الحيوانات والتابع لأكاديمية غولهان الطبية العسكرية، أنقرة، تركيا، وقد أُجريت خلال الفترة من مارس إلى أغسطس 2006. شملت الدراسة 28 جرذي من النوع سبراغو دوللي (200–320 غرام)، وقد قُسموا إلى المجموعات التالية: مجموعة الشاهد K (العدد=7)، ومجموعة الأوكسجين مفرط الضغط بعد حدوث قصور الإمداد الدموي HBO (العدد=7)، ومجموعة الأوكسجين مفرط الضغط بعد قصور الإمداد الدموي وأمينوغوانيدين بعد حدوث القصور HBO+AG (العدد=7)، ومجموعة أمينوغوانيدين بعد حدوث القصور AG (العدد=7). لقد قمنا بعمل انسداد للشريان الدماغي الأوسط من خلال شق القحف تحت منطقة الصدغ، وهكذا قمنا بتكوين قصور دائم للإمداد الدموي. عُرضت المجموعة HBO والمجموعة HBO+AG لما يبلغ 2.8 من الضغط الجوي للأوكسيجين مفرط الضغط وذلك لمدة 45 دقيقة، وبعد مرور ساعتين تم تكوين الانسداد. ولقد قمنا ببدء إعطاء أمينوغوانيدين (100 ملغ/كلغ) للمجموعة HBO+AG داخل الصفاق بعد عمل الانسداد بحوالي 6 ساعات، وواصلنا بإعطاء ذلك لمرتين يومياً وعلى مدى 3 أيام.

النتائج: لقد وصل معدل الاحتشاء في الدراسة إلى 22.2±3.1% في مجموعة الشاهد، و16.1±2.7% في مجموعة HBO، و15.2±1.9% في مجموعة HBO+AG، و14.4±3.3% في مجموعة AG. وأشارت نتائج الدراسة إلى أن معدل الاحتشاء وبالتالي حجم الاحتشاء في المجموعات HBO، وHBO+AG، و AG قد انخفض بشكل واضح من الناحية الإحصائية وذلك بالمقارنة مع مجموعة الشاهد (p=0.002, p=0.001, p=0.001).

خاتمة: أظهرت هذه الدراسة بأن لدى الأوكسجين مفرط الضغط وأمينوغوانيدين تأثير فعال في تقليل حجم الاحتشاء وذلك لدى الجرذان التي حُفز لديها قصور الإمداد الدموي من خلال انسداد الشريان الدماغي الأوسط، غير أنه لم يكن لديهما أي تأثير إضافي. ويمكن شرح مثل هذا الوضع بالعديد من الميكانيكيات المختلفة.

Objective: To study the effects of pre-ischemic hyperbaric oxygen (HBO) and post-ischemic aminoguanidine (AG) on the infarct volume in permanent middle cerebral artery occlusion.

Methods: This study was approved by the Animal Experiments Ethics Committee of Gulhane Military Medical Academy, Ankara, Turkey and carried out from March 2006 to August 2006. A total of 28 Sprague-Dawley rats (200-320 g) were divided into 4 groups: control (K) group (n = 7); HBO group (n = 7); HBO + AG group (n = 7); and the AG group (n = 7). All rats underwent middle cerebral artery occlusion (MCAO) by subtemporal craniectomy, and permanent ischemia was created. A 2.8 atmospheric pressure of HBO was first applied to the HBO and HBO + AG groups for 45 minutes, and occlusion was created after 2 hours. In the HBO + AG group, intraperitoneal administration of AG hemisulfate (100 mg/kg) was started 6 hours after MCAO, and was continued twice a day for 3 days.

Results: The rate of infarction was found to be 22.2±3.1% in the control group, 16.1±2.7% in the HBO group, 15.2±1.9% in the HBO+AG group, and 14.4±3.3% in the AG groups. The rate of infarctions (therefore the volume of infarct) in the HBO, HBO + AG, and AG groups were found to be significantly decreased compared with the control group (p=0.002, p=0.001, and p=0.001).

Conclusion: In permanent MCAO-induced ischemia in rats, HBO and AG were observed to have a lowering effect on the infarct volume, but no additive effect was observed. This situation can be explained by different mechanisms of action.

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Partial or complete cerebral ischemia-induced stroke is the third most common cause of death in the world.[1-3] It is responsible for approximately 10% of all deaths in the world. Stroke may be due to hemorrhagic or ischemic origin. Ischemic strokes are responsible for 90% of all stroke cases. Cerebral infarction includes histopathological findings of the brain due to ischemic damage.[1,3,4] There are still a very limited number of approaches that can be attempted to fight the causes of stroke. For this reason, extensive experimental and clinical research is conducted, various approaches to treatment are being tried, and the pathophysiology of ischemia is currently under detailed examination all over the world. In this study, we evaluate the hypothesis that hyperbaric oxygen (HBO) given prior to the experimental focal cerebral ischemia, and aminoguanidine (AG) given after the ischemia can decrease the cerebral infarct volume in rats.

Methods. This study was approved by the Animal Experiments Ethics Committee of Gulhane Military Medical Academy, Ankara, Turkey and all experiments were carried out in accordance with established guidelines from March 2006 to August 2006. An HBO cabin was provided by the Department of Physiology.

A total of 28 male Sprague-Dawley (200-320 g) rats used in the study were divided into 4 groups. The control (K) group (7 rats) underwent only middle cerebral artery occlusion (MCAO), and the HBO group (7 rats) underwent 2.8 atmospheric pressure of HBO for 45 minutes, 2 hours prior to the occlusion. The HBO + AG group (7 rats) underwent MCAO, 2.8 atmospheric pressure of HBO for 45 minutes, 2 hours prior to the occlusion, and in which intraperitoneal administration of AG hemisulfate was performed for 72 hours, twice a day, 6 hours after the occlusion (SIGMA A 7009-25G, diluted with 100 mg/kg 1 mL saline). The AG group (7 rats) underwent MCAO, and each individual was given intraperitoneal AG hemisulfate for 72 hours, twice a day, 6 hours after the occlusion.

Operation. Anesthesia was provided by intramuscular injection of 10 mg/kg xylazine (Xylazyne, Bayern, Istanbul, Turkey), and 90 mg/kg ketamine hydrochloride (Ketalar, Parke-Davis, Pontypool, England). The method of Tamura et al[5] was used as the MCAO model. The rats were placed in the left lateral decubitus position. A 2 cm vertical incision was performed between the right orbit and tragus. The temporal muscle was dissected from the cranium and removed up to the zygomatic arch and the infratemporal fossa was revealed. Craniectomy was performed by drilling the infratemporal fossa starting immediately beside the zygomatic borders under the operation microscope (Zeiss OPMI-I, Warsaw, Poland). Irrigation was performed with 0.9% normal saline during drilling to prevent cortical damage. The dura mater was opened with a 30-gauge needle tip. After the cortex was revealed, the place where the MCA crossed the inferior cerebral vein and bifurcated in the rhinal fissure could be seen. Then, the cerebral cortex was dissected slightly with a microdissector and the place where the MCA crossed the olfactory nerve was observed (**Figure 1**). The MCA was coagulated before and immediately after it crossed the olfactory nerve with the help of a micro-tipped bipolar. It was cut to prevent recanalization with the help of micro-scissors. Then, the temporal muscle and skin were sutured and closed. The operation was considered finished, and the rat was placed back in its cage. In the HBO and HBO + AG groups, the operation was started after the rats were maintained under an atmospheric pressure of 2.8 for 45 minutes in the HBO cabinet, and MCA was cauterized 2 hours after the administration of HBO. In the HBO + AG group, the first dose of intraperitoneal AG hemisulfate was given to the rats 6 hours after the operation, and the administration of AG hemisulfate was continued at 12-hour intervals for a total of 72 hours.

Neurological examination. If normal healthy rats are picked up by holding their tails, they extend both front limbs forward. A rat that has undergone MCA occlusion cannot extend its contralateral upper extremity front wards. Strength loss was observed in all rats that underwent MCAO in the experiment.

Calculating the rate of the infarct. After all the groups of rats were sacrificed by high doses of xylazine and ketamine hydrochloride on the fourth day, they were decapitated and their brains were quickly removed. After the extracted brain tissue was maintained in frozen saline solution for 10 minutes, it was placed in a 2 mm rat brain cutting apparatus (Harvard Apparatus Ltd., Holliston, MA, USA) and divided into 6 coronal parts by a micro-wire. Each piece was immersed with 2% TTC (2,3,5 triphenyltetrazolium chloride) which was prepared in 0.9% saline solution and incubated in the dark environment of a drying oven set to 37^{0}C for 30 minutes. After incubation, the tissues were buffered in 10% formalin. As a result of this process, ischemic areas were seen as white and non-ischemic areas were seen as pink-to-red (**Figure 2**). Sections of the individuals of the 4 groups were photographed by a digital camera (Nikon Corporation Coolpix 4300, Tokyo, Japan) and transferred to a computer and the measurement of ischemic areas was performed section by section by using the UTHSCSA Image Tool for Windows version

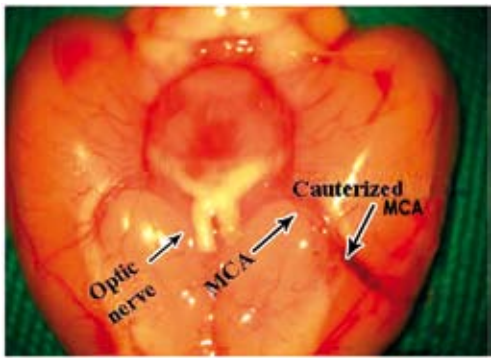

Figure 1 - Representative base view of a rat brain. Middle cerebral artery (MCA) and optic nerves are shown.

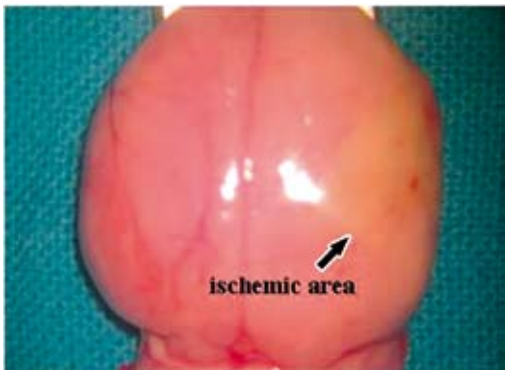

Figure 2 - Representative view of rat brain after decapitation. Ischemic area in the right lobe can be seen.

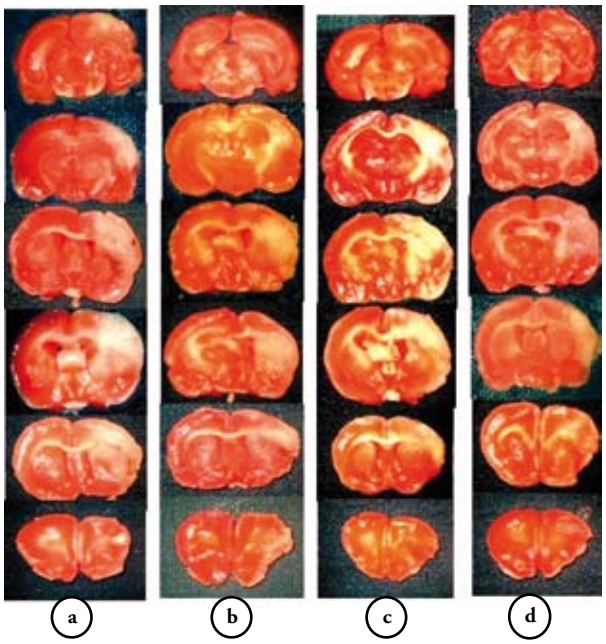

Figure 3 - Representative pictures of hemispheric slices of the study groups. **a)** K group; **b)** HBO group; **c)** HBO + AG group; **d)** AG group. K - control; HBO - hyperbaric oxygen, AG - aminoguanidine

3.0 (**Figure 3**). For the measurement of ischemic areas in each section, the contralateral hemispheric area was primarily measured to eliminate the error due to the effect of edema, and the true ischemic area was found by subtracting the ipsilateral non-ischemic area from the contralateral hemispheric area. After the ischemic areas in each section of the rat brains were measured one by one, these results were summed, and the result was multiplied by slice thickness (2 mm); the ischemic volume of that brain was calculated in mm^3. Then, the sum of the contralateral hemisphere areas were multiplied by 2 mm and the total volume of one half of the brain was calculated. As the weight of each rat used in this study varied, the percentage of the infarct was obtained by dividing the total ischemic volume (TIV) by the total hemispheric volume, to provide standardization (**Table 1**).[2,6]

Statistical analysis. The Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) Windows Version 10.01 was used for statistical analysis. Normal distribution of the groups and variance homogeneity were evaluated respectively with Kolmogorov-Smirnov and Levene tests, and it was found to be accordant with parametrical statistical assessment. Intergroup means were calculated with one-way ANOVA test and post-hoc Tukey HSD test. The statistically significant level was accepted to be $p<0.05$.

Results. A study of the mean infarct rates of each group revealed that the mean infarct area in the control group was the highest, and the mean infarct area in the AG group was observed to be the lowest (**Table 2**). Among all of the rats, the highest infarct rate was in

Table 1 - Formulas used to measure middle cerebral artery occlusion ischemic areas in rats.

Ischemic area (IA) (mm^2) = Contralateral hemispheric area (mm^2) - Ipsilateral non-ischemic area (mm^2)
Total ischemic volume (TIV) (mm^3) = Sum of IAs (mm^2) x 2 mm
Total hemispheric volume (THV) (mm^3) = Total of the contralateral hemispheric areas (mm^2) x 2 mm
Infarct rate = TIV x 100/THV

Table 2 - Infarct rates (%) of the K, HBO, HBO+AG, and AG groups after middle cerebral artery occlusion ischemic areas in rats.

K group (n=7)	HBO group (n=7)	HBO+AG group (n=7)	AG group (n=7)
22.2±3.1	16.1±2.7	15.2±1.9	14.4±3.3

Data were expressed as % arithmetical average ± SD, K- control, HBO - hyperbaric oxygen, AG - aminoguanidine, CI - confidence interval

Table 3 - Total hemispherical volumes, ischemic volumes, and infarct rates in all rats after the middle cerebral artery occlusion procedure.

Rats in the groups	Total hemispheric volume (mm^3)	Ischemic volume (mm^3)	Infarct rate (%)
K1	750.75	172.92	23.03
K2	749.71	120.37	16.06
K3	726.19	173.22	23.85
K4	743.16	178.54	24.02
K5	728.33	150.37	20.65
K6	653.78	165.94	25.38
K7	737.91	166.75	22.60
HBO1	735.12	141.76	19.28
HBO2	691.77	120.20	17.38
HBO3	779.07	107.46	13.79
HBO4	730.08	118.88	16.28
HBO5	769.26	99.02	12.87
HBO6	748.16	143.99	19.25
HBO7	737.25	99.86	13.55
HBOAG1	639.57	112.52	17.59
HBOAG2	690.82	97.40	14.10
HBOAG3	792.05	100.80	12.73
HBOAG4	695.78	117.51	16.89
HBOAG5	689.84	115.82	16.79
HBOAG6	738.20	97.73	13.24
HBOAG7	668.66	98.88	14.79
AG1	671.22	86.66	12.91
AG2	739.92	72.04	9.74
AG3	743.39	129.43	17.41
AG4	714.41	114.72	16.06
AG5	719.33	116.92	16.25
AG6	738.27	131.23	17.78
AG7	633.69	68.56	10.82

K1-K7 - rats in the control group, HBO1-HBO7, rats in the hyperbaric oxygen group, HBOAG1-HBOAG7 - rats in the hyperbaric oxygen + aminoguanidine group, AG1-AG7 - rats in the aminoguanidine group

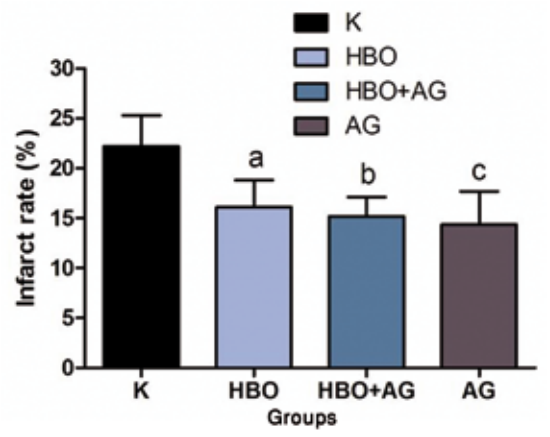

Figure 4 - Infarct rates after middle cerebral artery occlusion procedure in the study groups. Mean infarct rates of the study groups were compared by ANOVA with post hoc Tukey test. Data were expressed as mean±SD. [a]p=0.002 (95% CI; -10.3 - -2.1) versus K group, [b]p=0.001 (95% CI; -11.2 - -2.9) versus K group, [c]p=0.001 (95% CI; -11.9 - -3.7) versus K group. K - control group, HBO - hyperbaric oxygen group, HBO+AG - hyperbaric oxygen + aminoguanidine group, and AG - aminoguanidine group

the control group with 25.4%, and the lowest infarct rate was in the AG group with 9.7% (**Table 3**). When the control group was compared with the other groups, decreases in the infarct rates were found to be significant in the HBO group (p=0.002), in the HBO+AG group (p=0.001), and in the AG group (p=0.001). When the HBO, HBO+AG, and AG groups were compared between themselves, decreases in infarct rates were assessed to be comparable (p>0.05) (**Figure 4**).

Discussion. While blood flow is blocked at the center of the affected region, it declines in the penumbra in focal cerebral ischemia. Due to this occurrence, oxygen and glucose levels decrease below the critical value, and neuronal cell death and ischemia occur as a result of the impaired aerobic metabolism. Inflammatory reactions begin following these changes occurring in the acute stage.[1,3,4] Nitric oxide (NO), a cellular signal molecule, which plays a cytotoxic role in these reactions, begins to be released, particularly from the microglia and inflammatory cells, with its INOS (inducible nitric oxide synthase) enzymatic activity beginning by the sixth hour after the event, and is active for up to 3-4 days.[7-9] The presence of NO from this period onwards, has tended to exhibit increased ischemic damage and infarct volume. The presence of oxygen in the environment may trigger NO production.[7,9-11] It can be considered that HBO increases blood flow and tissue oxygenation. However, HBO could be useful in cases where it is administered just before the ischemia, or during the ischemia, because HBO administered from the sixth hour may be a disadvantage by increasing NO synthesis, and thus increasing free radicals.[12-14] Therefore, HBO should be administered outright and within the acute stage. The HBO administration immediately after the ischemia is possible in rats, but such an application in humans is highly difficult in practice due to the shortage of HBO units.[14-16] This was the main rationale for HBO treatment as prophylaxis prior to ischemia in this study.

In cases where AG, an iNOS inhibitor, is administered within 6-72 hours after the ischemia, it can decrease post-ischemic iNOS activity and limit secondary damage, and may result in a decrease in infarct volume.[17-19] As seen from the results of this study, Iadecola et al[10] reported that AG, which began to be administered within the sixth hour following ischemia and continued to be given twice a day for 4 days, inhibited iNOS and reduced the ischemic area, and this result prevented inflammatory damage. The HBO was applied immediately after the ischemia or during the ischemia, as carried out in this study, and

obtained positive results in decreasing ischemic volume. In our study, it can be suggested that HBO and AG applications have significant effectiveness in decreasing ischemic volume; but when applied in combination, although this combination has a significant effectiveness in decreasing the ischemic volume, there is no additive activity. Kawamura et al[20] and Veltkamp et al[21] reported that HBO applied during the ischemic period led to a decrease in infarct volume and also enabled development of a tolerance against any ischemic neuronal damage that could occur. In another study, HBO was applied just before and just after forming ischemia; and a more significant decrease in the infarct rate and a smaller increase in myeloperoxidase activity in the former group was observed.[2] In a study similar to this study, utilizing an MCAO ischemia/reperfusion model in rats by the intraluminal cord method, pre-ischemia HBO treatment was applied and the post-ischemia infarct volume and myeloperoxidase activity and accumulation of neutrophils were tested, and finally, it was revealed that there was a significant reduction in the infarct volume and a decrease in neutrophil accumulation.[7]

The damage as a result of the blood flow blockage, particularly in the ischemic core, is severe. Penumbral tissue surrounding the ischemic core was less damaged because this area is supported by collateral blood flow. The most critical function of blood flow is oxygenation, and a sudden decline in blood flow results in ischemia formation.[3,15,16] If intervention is not swift, the cells in the penumbral region cannot maintain the ionic homeostasis and will die, and hence the ischemic volume increases. The target of many investigators is to prevent this transformation.[8,11,22,23] Again, focal cerebral neurotransmitter response during HBO application was studied, and it was pointed out that striatal dopamine release increased after the ischemia created by MCAO, and HBO administration during ischemia reduced ischemic damage by decreasing the dopamine release from the striatum.[24] The HBO may reduce the injury by inhibiting the beta-2 integrin-dependent neutrophil adhesion.[25] In humans, beta-2 integrin inhibition was demonstrated to continue for 12 hours after a 45-minute HBO application under a 3 atmospheric pressure condition.[24,25] In a study conducted with rats and mice, HBO applied in the post-ischemic early period was demonstrated to decrease cerebral edema and ischemic volume in the early and late periods by reducing the blood-brain barrier.[21,26] Mink and Dutka[27] reported that HBO treatment increased tissue oxygen distribution (particularly within the area of reduced blood flow), strengthened the neuronal viability, and decreased brain edema. Presumably, HBO effects viable cells in the penumbra, and provides a certain reduction in the ischemic volume, and AG prevents the progression of the ischemic volume by its anti-inflammatory and antioxidant effects with its iNOS inhibition.[13,15,28,29]

In conclusion of this experimental study, it has been demonstrated that HBO applied in the pre-ischemic period, and AG administered after the ischemia ensured a statistically significant reduction in the infarct rate, and also in the ischemic volume. However, in cases where these 2 are administered in combination, no additive effect was observed. Experimental treatments administered at present aim at limiting only the ischemic damage, as much as possible, and it is clear that current methods do not provide a definite cure. Prophylactic HBO application may be tried or new generation medications, such as AG may be considered as the treatment of choice to be used after the ischemia, prior to the major neurosurgical operations performed due to the requirement of temporary cerebral artery clipping.

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ETHICAL CONSENT

All manuscripts reporting the results of experimental investigations involving human subjects should include a statement confirming that informed consent was obtained from each subject or subject's guardian, after receiving approval of the experimental protocol by a local human ethics committee, or institutional review board. When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed. Research papers not involving human or animal studies should also include a statement that approval/no objection for the study protocol was obtained from the institutional review board, or research ethics committee.