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Exogenous Amylase Reverses Cerebral Ischemia Induced by Selective Intraarterial Injection of Degradable Starch Microspheres: An Angiographic and Histological Study in a Novel in Vivo Animal Model

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ABSTRACT

AIM: To validate a new particulate embolization method using degradable starch microspheres (DSM) and intraarterial exogenous amylase administration, which allow for regulated temporary cerebral arterial embolization without compromising tissue perfusion.

MATERIAL and METHODS: Twenty-four male New Zealand rabbits were randomly divided into three groups. All animals underwent routine angiography. The control group received no additional intervention. In the ischemia group, 0.2ml DSM was administered to the animals via the right carotid artery with pulsed, gentle injections to induce ischemia in the cerebral microcirculation. Animals in the reperfusion group received 0.05 ml of exogenous amylase along with DSM administration. Six hours after the procedure, the animals were sacrificed and histopathological analysis was performed.

RESULTS: The ischemia group was the most adversely affected group by embolization, with the highest number of pyknotic neurons. The reperfusion group, which received exogenous amylase, had lower pyknotic neurons than the ischemia group. The pyknotic neuron count was similar in some regions between reperfusion and control groups.

CONCLUSION: Exogenous amylase can rapidly attenuate cerebral ischemia caused by microembolization with DSM.

KEYWORDS: Exogenous amylase, Cerebral ischemia, Degradable starch microsphere, Angiography, Rabbit model

ABBREVIATIONS: DSM: Degradable starch microspheres, **H&E:** Hematoxylin & eosin, **ICA:** Internal carotid artery

INTRODUCTION

Transarterial embolization has been used to control hemorrhage in various vascular and neoplastic conditions (2,4,9,12). In neurosurgery, embolization is used as a standalone treatment or as an adjuvant treatment before surgery (4,8).

Chemoembolization, which involves selectively administering a chemotherapeutic chemical to the target tissue to cause a

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temporary arterial blockage, has been used to treat visceral organ tumors, particularly hepatic neoplasms (12,20). This procedure leads to the entrapment of the intraarterially administered drug at the target tissue, maintaining a high drug concentration in the tumor for a longer period while reducing blood flow to the neoplasm. Due to the susceptibility of neural tissues to ischemia, chemoembolization for cranial neoplasms has remained limited. Biodegradable embolic materials may be used to predict the outcome of embolizations that will be performed using permanent materials (similar to a balloon occlusion test) and protect adjacent, non-target vulnerable regions during chemoembolization (11).

Degradable starch microspheres (DSM) are one of the most frequently used degradable materials for embolization. DSM is composed of glucose polymers, particularly amylose and pectin. They are three-dimensional, cross-linked, hydrophilic granular molecules, which the alpha-amylase enzyme cleaves into 20–200 micron particles (13,15,17,21). Biodegradable particles are effective for cerebral microembolization (22). Though DSM is the fastest degrading particulate embolic agent, the natural degradation process is slow to protect neural tissue against DSM-induced ischemia (21).

We developed a new particulate embolization method using DSM and intraarterial exogenous amylase administration, which allows for regulated temporary cerebral arterial embolization without compromising tissue perfusion. Finally, we compared the histopathological changes in the brain tissue of rabbits in an ischemic model, reperfusion model, and the control group.

MATERIAL and METHODS

The study was approved by the Animal Experimentations Local Ethics Board of Hacettepe University (approval date: 26/5/2015, approval number: 2015/45-06).

In this study, 24 male New Zealand rabbits weighing between 2500 and 3500 grams were used. During the experiment, the animals were kept under standard laboratory conditions (in polycarbonate cages, 50%–60% humidity, 17°C-23°C, 12-hour light-dark cycle) and fed with standard feed (ad labitum water and pellet food).

All rabbits were randomly assigned to one of three groups. All animals underwent cerebral angiography via the right femoral artery, and microcatheters were advanced to the right internal carotid artery (ICA). Group 1 (control group, n=8) animals were administered only saline and contrast medium. Group 2 (Ischemia group, n=8) animals were injected saline, contrast medium, and 0.2 ml (12 mg) of DSM. Group 3 (Reperfusion group, n=8) animals received saline, contrast medium, DSM, and amylase. In the latter group, 0.05 ml amylase was administered as a bolus just before DSM infusion.

Six hours after the procedure, the rabbits were sacrificed to collect brain tissue samples for histopathological examination. Samples were obtained as 5-micron serial sections and were stained with hematoxylin & eosin (H&E) stain and examined under a Leica DM6000B light microscope.

DSM Properties

DSM are granular molecules that vary in size from 20–200 μm (17). Because they are primarily composed of amylase and amylopectin, the alpha-amylase enzyme completely cleaves them, yielding 100–106 dalton particles (21). The plasma half-life of DSM molecules is approximately 35 minutes (14). EmboCept®S (Serumwerk AG, Bernburg, Germany) is a DSM with an average particle size of 50 ± 7 μm (95% in the range of 20–90 μm). It has a half-life of 30–40 minutes (16).

Angiographic Procedure

All the animals were fasted overnight. Before the procedure, subjects received 50 mg/kg ketamine hydrochloride (Alfamine®, Ege Vet, Turkey) and 10 mg/kg xylazine (Alfazyne®, Ege Vet, Turkey) intramuscularly for anesthesia and analgesia. After anesthesia, the subjects were left to breathe on their own. The rabbits were placed in the angiography unit at the interventional neuroradiology unit (Siemens Artis Zee, Erlangen, Germany) for selective transarterial microcatheterization through their femoral arteries. Following local anesthesia with 0.2 mg/kg lidocaine (Lidoksep®, Anadolu İlaç, Turkey) and a 2 cm vertical skin incision, the femoral artery was isolated by dissection (Figure 1A). After the puncture of the femoral artery under direct vision, a microcatheter (SL10, Stryker Neurovascular, Michigan, USA) was advanced over a microguidewire and the right common carotid artery was catheterized. We injected iopromide (iodinated contrast media - Ultravist®300 mg/ml, Bayer/Germany) to obtain cerebral angiograms for all animals (Figure 2A).

Group 1 animals underwent routine angiography only.

In group 2, 0.2 ml DSM (EmboCept®S 450 mg/7,5 ml, 50-micron particle size, in 25% contrast medium) was administered with pulsed, gentle injections over 30 to 60 seconds via the right carotid artery to create ischemia in the cerebral microcirculation (Figure 2B). This was performed under high-resolution continuous fluoroscopy using the roadmap function of the angiography device.

Group 3 animals received 0.05 ml of exogenous amylase (alpha-amylase from Bacillus licheniformis Type XII-A, Sigma-Aldrich, Taufgirschen, Germany) along with DSM administration.

Histopathological Assessment

The animals were euthanized six hours after the procedure by intravenous injection of an anesthetic overdose. Immediately after death, the circulation was perfused at a constant rate with 4% paraformaldehyde solution (0.1 mol/L in phosphate buffer, pH: 7.4) via the left ventricle of the heart for fixation. Following craniectomy, the brains of the sacrificed subjects were gently removed without damaging the brain tissue (Figure 1B). Brains were fixed in 10% paraformaldehyde at room temperature for 48 h. In the coronal plane, each brain was divided into four parts. The right halves belonging to the right hemispheres were separated for analysis and numbered sequentially from anterior to posterior (ie. P1: Most anterior part, P4: Rearmost part) (Figure 1C). We obtained serial sections of 5-micron thickness from each piece by a sliding microtome and stained

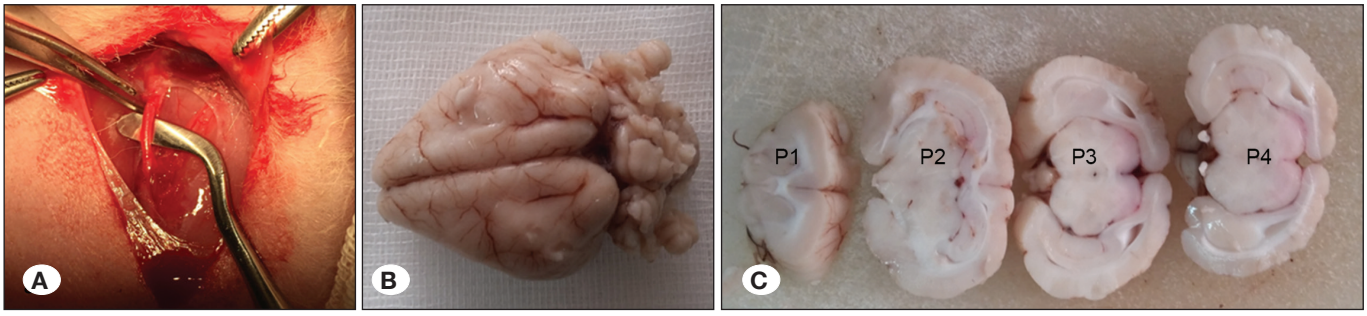

Figure 1: A) Femoral artery is isolated for the procedure. **B)** Brains are removed without minimum damage. **C)** All brains were divided into four initially.

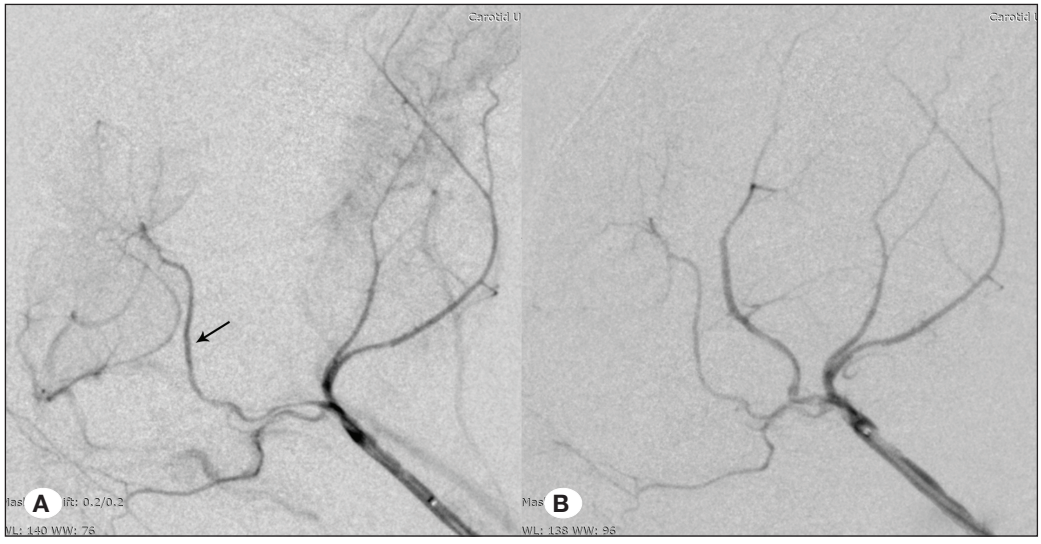

Figure 2: A) Selective common carotid artery injection shows the internal carotid artery (ICA) (arrow), which distally opacifies the middle cerebral artery branches. During microwire manipulations, non-occlusive vasospasm has developed at the origin of the ICA. The maxillary artery is not visible secondary to vasospasm at its origin. **B)** Selective common carotid artery injection after injection of microparticles reveals a prunated pattern of middle cerebral artery opacification. The vasospasm at the origins of the internal carotid and maxillary arteries has resolved. Despite this, there is a paucity of vascularization involving the distal branches of the external carotid circulation secondary to embolization with starch microparticles.

them with hematoxylin-eosin for histological examination. We examined all samples under a light microscope (Leica DM6000B) and transferred the images to a computer using a digital camera (Leica DC 500). Neurons that have basophilic cytoplasm and pyknotic appearance were counted in three different zones for each section at x40 magnification and their total values were obtained. Then, two histologists performed histological examinations.

Statistical Analysis

We performed all statistical analyses using SPSS v.22 (IBM, New York). The Kruskal-Wallis test was used to compare the groups. Furthermore, the Mann–Whitney U test was used to compare individual groups.

RESULTS

Histological Findings

In Group 1, most neurons in the cortex appeared normal, but there were a few condensed-looking pyknotic neurons with basophilic cytoplasm. Normal neurons were distinguished by their euchromatic nuclei and prominent nucleoli. There was no significant stasis in the blood vessels of the cortex and medulla, but minimal edema was observed around some vessels. Glial cell morphology was also normal (Figure 3A).

In the second group, pyknotic neurons were more profound in the outer regions of the cortex. Furthermore, we observed significant edema in the upper cortex, under the pia mater in all specimens. The amount of edema decreased toward the medulla and posterior regions (Figure 3B).

In the reperfusion group, similar to other groups, pyknotic neurons were scattered in the outer cortex in all regions. There was no pyknotic neuron in the deep parts of the cortex. Perivascular edema was also found, albeit at a low level. Edema was present in the upper cortex and under the pia mater, but unlike the ischemia group, there was no loosening of the neuropil. Edema under the pia mater was significant in

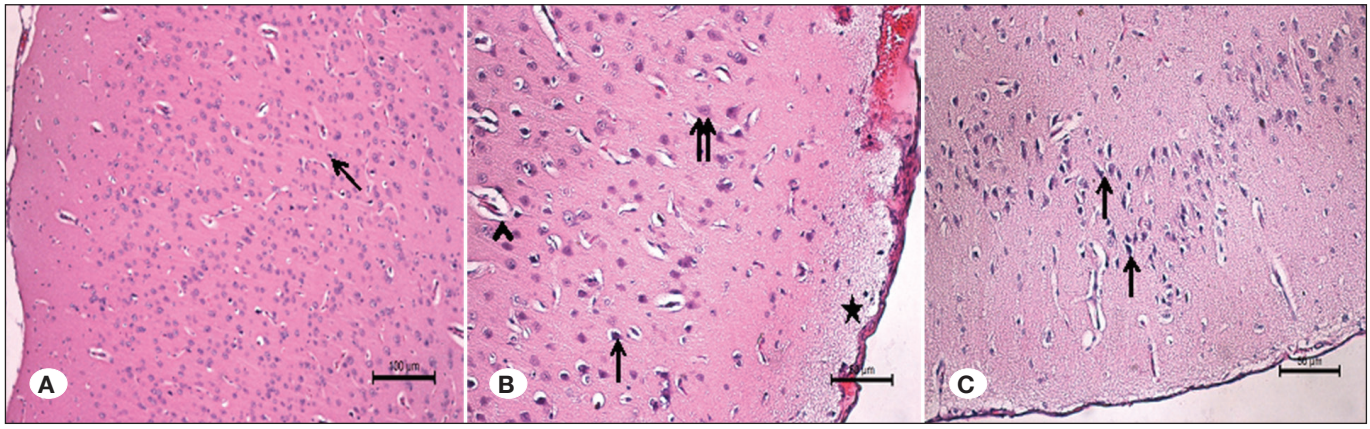

Figure 3: A) All sections stained with H&E and examined under x100 magnification with light microscopy. The control group had a healthy cortex except for occasional picnotic neurons (arrow). **B)** Ischemia group had several picnotic neurons (arrow) within normal neurons (double arrow) and subpial and perivascular edema (star and arrowhead, respectively). **C)** The reperfusion group had a minimum subpial and perivascular edema and few picnotic neurons (arrow) in the upper cortex.

Table I: Comparison of the Regions on the Right Side Regarding Pyknotic Neurons Between Groups

Regions	Control (n=8)	Ischemia (n=8)	Reperfusion (n=8)
P1	14.00 ± 1.3	29.00 ± 4.04*	21.25 ± 3.77*
P2	17.88 ± 3.23**	26.50 ± 4.90*	17.25 ± 4.46*,**
P3	9.38 ± 1.30	26.75 ± 8.86	18.88 ± 4.61
P4	15.88 ± 2.75**	29.00 ± 5.88*	16.88 ± 4.36*,**
Overall	14.28 ± 3.88***	27.81 ± 6.01***	18.56 ± 4.46***

** p<0.01, comparison between Ischemia and Reperfusion groups.*
*** p>0.05, comparison between Control and Reperfusion groups.*
**** p<0.001, comparison between all groups.*

the presence of pyknotic neurons and near absent when there were few pyknotic neurons. As in the control group, edema decreased toward the posterior and was least evident in the right P2 region (Figure 3C).

Intergroup Assessments

The number of pyknotic neurons in the right hemispheres showed significant variation (p<0.001) (Table I).

When groups were compared in pairs, compared to the ischemia and reperfusion groups, the control group had a significantly lower number of pyknotic neurons on the right side (both p<0.001).

The ischemia group had more pyknotic neurons compared to control and reperfusion groups on the right side (both p<0.001).

Compared with the ischemia group, the reperfusion group had a lower number of pyknotic neurons in the right P1, P2, and P4 regions (p=0.005, p=0.004, and p=0.003 respectively). However, pyknotic neuron count in right P2 and P4 was similar in the reperfusion and control groups.

Table I shows the data regarding intergroup comparisons.

DISCUSSION

In this study, with the highest number of pyknotic neurons, the ischemia group was the most adversely affected group from the embolization. The reperfusion group, which received exogenous amylase, had lower pyknotic neurons than the ischemia group. Moreover, the pyknotic neuron count of some regions in the reperfusion group was similar to the counterpart regions of the control group. These findings indicate that exogenous amylase administration limited neuronal damage by promoting DSM breakdown.

A successful embolization selectively occludes an abnormal vascular structure or nidus superselectively without hampering normal blood flow in the surrounding tissues (5,19). The choice of embolic agents depends on the type of pathology and desired duration of vascular occlusion. Because the primary aim of chemoembolization is to maintain higher concentrations of chemotherapeutic agents at target tissues for a prolonged period rather than total occlusion, nonpermanent embolic agents are usually preferred. The occlusion effects of embolic agents used in chemoembolization treatments vary from hours to several weeks. In the case of DSM, normal blood flow is restored within 1 hour (21). Because of this property, it

has been studied and used in liver and lung tumors, which are relatively ischemia-resistant (7,10,18,23).

One limiting factor for chemoembolization in brain tumors is the high sensitivity of neural structures to ischemia and the possibility of permanent neurological deficits. Ischemia caused by microembolization must be reversible at the desired time interval. Though DSM is the most suitable embolic agent, with a half-life of 35 minutes, restoring normal blood flow may take up an hour at the shortest (6,14). There are few experimental studies in the literature regarding transarterial cerebral microembolization with DSM products. Winding et al studied the clinical effects of cerebral embolization using Sephadex®, a DSM, which has structural differences from EmboCept®S and prolonged activity and discovered a relationship between neurological deficits and the number of particles administered. They did not perform any histological evaluation though (22). Similarly, Laccourreye et al demonstrated permanent neurological deficit following transarterial embolization with a higher Spherex® dosage, whose particle size ranges from 18–60 microns. Apart from the presence and location of infarctions, no histological data regarding pyknotic neurons or edema was presented (11).

Ischemic changes in the brain are generally reversible in the first one hour and the possibility of permanent damage increases progressively after 3–6 hours (24). Though blood flow is restored within one hour when DSM is used, it is still at the limit of irreversible brain damage. Because of this, we administered exogenous amylase to subjects to increase the breakdown rate of DSM particles. Those subjects were discovered to have less edema and pyknotic neurons compared to those that received DSM only. These findings reveal that DSM degradation is increased by exogenous amylase administration and suggest that this method can be used to prevent permanent brain damage for chemoembolization studies in the future. Also, it can be used as a model for cerebral transient arterial occlusions.

EmboCept®S used in this study differs from the DSM products studied in cerebral microembolization regarding its half-life and particle size. It has a shorter activity and a relatively constant particle size. Since our study showed that DSM degradation can be facilitated by alpha-amylase enzyme, an agent with a constant particle size can allow physicians to get consistent results for a given amount of alpha-amylase enzyme. To the best of our knowledge, this is the first study that used precisely sized DSM (EmboCept®S) as a transient cerebral transarterial embolic agent, it is also the only study that used alpha-amylase to limit the necrotic effects of DSM by manipulating its breakdown rate.

As an ischemia model, this method has some advantages over existing models. In the methods where foreign material is placed in the arterial lumen or where the artery is ligated mechanically, thrombotic processes do not contribute to the ischemic injury. Another method where ischemia is performed through craniectomy may cause increased intracranial pressure, which may affect the findings of a study (1,3). Experimental ischemia by transarterial DSM enables visualization of blood flow cessation during the procedure and confirmation of ischemia angiographically. Moreover, since a true embolization is achieved in the microvasculature, thrombotic events contribute to ischemic pathophysiology.

There are some shortcomings to this study. First, former DSM products are expressed in units, whereas EmboCept®S is expressed in millilters, making direct comparison of dosage with previous studies impossible. Therefore, the DSM amount, particularly EmboCept®S, required to induce ischemia in a certain area is yet to be standardized. Second, it is uncertain whether the pyknotic neurons, which are nerve cells affected by ischemia, are permanently injured apoptotic cells or neurons under ischemia. Additionally, since the animals were sacrificed at the 6th hour, we did not analyze the amount of neurological impairment between groups.

This study is significant because it introduced a novel experimental controllable ischemia method. This finding may pave the way of treating brain tumors via chemoembolization or achieving acceptable levels of antiangiogenic factors in the cerebral circulation for treating malignant cerebral neoplasms. This method can be employed for a regulated blood-brain barrier breakdown and as a model for controllable transient cerebrovascular occlusion.

CONCLUSION

In this study, we concluded that selective cerebral microembolization with DSM can be conducted successfully. Above that, transarterial amylase can rapidly attenuate ischemia caused by microembolization with DSM. These findings indicate the applicability of this method for treating cerebral tumors and stroke research.

AUTHORSHIP CONTRIBUTION

Study conception and design: IB, SH, BB, AA

Data collection: IB, SH, AA, BB, II, PA, SB

Analysis and interpretation of results: PA, SB

Draft manuscript preparation: SB, IB AA, SH, II

Critical revision of the article: AA, SB

Other (study supervision, fundings, materials, etc...): AA, BB, SH, II

All authors (IB, SH, SB, II, PA, BB, AA) reviewed the results and approved the final version of the manuscript.

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