Effect of Raloxifene on Cerebral Vasospasm Following Experimental Subarachnoid Hemorrhage in Rats

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Abstract

The effect of raloxifene on cerebral vasospasm following experimental subarachnoid hemorrhage (SAH) was investigated in a rat model. Seven groups of seven rats underwent no SAH, no treatment; SAH only; SAH plus vehicle; SAH plus 3 days intraperitoneal raloxifene treatment; SAH plus 4 days intraperitoneal raloxifene treatment; SAH plus 3 days intrathecal raloxifene treatment; and SAH plus 4 days intrathecal raloxifene treatment. The basilar artery cross-sectional areas were measured at 72 or 96 hours following SAH. The results showed raloxifene decreased SAH-induced cerebral vasospasm in all treatment groups, and suggested no difference between intraperitoneal and intrathecal application, or between 3 days and 4 days of raloxifene treatment. The present study demonstrates that raloxifene is a potential therapeutic agent against cerebral vasospasm after SAH.

Key words: cerebral vasospasm, raloxifene, subarachnoid hemorrhage

Introduction

Cerebral vasospasm occurs in 70% of patients with aneurysmal subarachnoid hemorrhage (SAH) and leads to symptomatic brain ischemia in 36% of patients. The occurrence of cerebral vasospasm is known to involve many factors including proliferation of the vascular cells, increased intracellular level of calcium in cerebral vascular smooth muscle cells in the early stages of cerebral vasospasm, oxidation of low density lipoproteins, increased activity of inducible nitric oxide synthase (iNOS), reduced activity of endothelial nitric oxide synthase (eNOS), increased levels of nitric oxide (NO), inflammation, and free radicals, and activation of the protein kinase system. 

SAH is associated with excessive production of reactive oxygen species (ROS), especially superoxide, that rapidly react with NO under oxidative stress. Consequently, endothelium-dependent relaxation is impaired, and this may be one of the mechanisms contributing to vasospasm. Therefore, therapeutic strategies focusing on the elimination of superoxide have been developed and tested in animal SAH models. Under physiological conditions, there is an equilibrium between the ROS and the endogenous scavenging system that removes the ROS. Overproduction of ROS during an ischemic event after SAH can overwhelm these endogenous antioxidative defense mechanisms and result in increased lipid peroxidation. Overproduction of NO under conditions of stress is associated with altered iNOS expression, which occurs in both endothelial and smooth muscle cells after SAH. Pretreatment with aminoguanidine, a selective inhibitor of iNOS, ameliorates vascular constriction after SAH. The beneficial effect of the estrogen family primarily depends on the inhibition of iNOS in SAH. The estrogen 17β-estradiol (E2) suppresses the activity of iNOS through selective estrogen receptor modulators that act as estrogen agonists in some tissues (bone and brain) and estrogen antagonists on others (breast and uterus).

Raloxifene is a non-steroidal bezothiopep derivative and selective estrogen receptor modulator which binds with high affinity to the nuclear estrogen receptors and demonstrates a pharmacological profile similar to that of E2. Raloxifene has demonstrated a vasoprotective effect in in vitro studies. Theoretically raloxifene could improve or
reverse the effects of vasospasm in many ways, particularly in the possible pathogenic cascade following SAH.

The present study investigated the effects of raloxifene on cerebral vasospasm after SAH in rats.

### Materials and Methods

Forty-nine male albino Wistar rats, each weighing between 240 and 280 g, were used in this study. The experiment was performed in accordance with institutional guidelines for the use of experimental animals and after approval was granted by the Ethics Committee at Hacettepe University Medical Faculty (protocol number 2004/4–8). The animals were kept at the same environmental temperature and humidity in normal daylight conditions. Throughout the experiment, the rats had free access to food and water. Intramuscular injections of ketamine hydrochloride 50 mg/kg (Ketalar®; Pfizer Inc., New York, N.Y., U.S.A.) and xylazine 5 mg/kg (Rompun®; Bayer AG, Leverkusen, Germany) were administered before all procedures. The solution of raloxifene (Evista®; Eli-Lilly and Co., Indianapolis, Ind., U.S.A.) was prepared at the Hacettepe University Medical Faculty Pharmacology Department. Normal raloxifene tablets were pulverized manually and dispersed in physiological saline solution containing gelatine (0.1%) by ultrasonic and mechanical mixing. No precipitation was observed in the prepared solution.

I. Induction of SAH

The rat was immobilized in the prone position using a stereotactic frame. A midline occipitocervical incision was performed and the suboccipital muscles were dissected to reveal the atlantooccipital membrane. The membrane was punctured with a 27 gauge needle and 0.4 ml/kg of cerebrospinal fluid (CSF) was removed, then the same amount of fresh autologous blood obtained from the tail artery was slowly injected into the cisterna magna. All animals were kept in the 45° head-down position for 30 minutes to ensure the blood disseminated to all basal cisterns while the wound was closed. The rat was then returned to the cage.

II. Experimental design and morphometric analysis

Seven experimental groups of seven rats each were designated in this study, as shown in Table 1. Raloxifene was administered as a bolus at 24-hour intervals starting 4 hours after induction of SAH.

At the end of the experimental procedure the rats were sacrificed with a high dose of intraperitoneal pentobarbital and were transcardially perfused with 50 ml of 30 mM phosphate-buffered saline and then 100 ml of 4% paraformaldehyde. Groups 2, 3, 4, and 6 were sacrificed at 72 hours following SAH and Groups 5 and 7 at 96 hours. After perfusion and fixation, the brain was removed, placed in 4% paraformaldehyde solution and stored overnight. The brainstem was separated from the hemispheres of the brain, conserving the pons and basilar artery. The tissues were embedded in paraffin and cut into 6-μm slices using a Motorized Microtome HM360 (Mikron Instruments, Inc., San Marcos, Calif., U.S.A.). Three sections from the top, middle, and bottom of the basilar artery were used for measurements. Each section was digitally photographed at 20× magnification using a Leica DMR Research Microscope System and DC500 digital camera at 1300 × 1030 resolution (McBain Instruments, Chatsworth, Calif., U.S.A.). Measurements were made with Leica QWin image analysis software (Leica Microsystems GmbH, Wetzlar, Germany) after calibration for 20× magnification. The luminal area of the artery was measured on each section. The mean value was then calculated for the basilar artery.

III. Statistical analysis

The groups were compared with the analysis of variance (ANOVA) test using SPSS for Windows (version 14.0). Values of p < 0.05 were considered to be significant. Following the one-way ANOVA test, a post-hoc Bonferroni test was performed (ANOVA test, F = 14.071, DF = 6 between groups, DF = 42 within groups, DF = 48 total p < 0.0001).

### Results

There were no statistically significant differences between the groups for the physiological parameters recorded (body weight and mean arterial blood pressure). The experimental procedure was well tolerat-
Fig. 1  Graph of cross-sectional areas of the basilar arteries of the groups. *Significantly different (p < 0.05) from Group 2 (subarachnoid hemorrhage [SAH] only). **Significantly different (p < 0.05) from Group 3 (SAH plus vehicle). Groups are defined in Table 1.

Fig. 2  Photomicrographs of representative slices showing the basilar arteries from different groups. Groups 2–4 show some characteristic features of cerebral vasospasm such as corrugation of the internal elastic lamina, thickening of the arterial wall, vacuolization of the arterial wall, and narrowing of the cross-sectional area of the basilar artery. Hematoxylin and eosin stain, original magnification × 200.

**Discussion**

Overproduction of ROS and increased lipid peroxidation, possibly involving oxyhemoglobin, appear to be the major contributing factors in the pathogenesis of cerebral vasospasm and secondary brain damage after SAH. The expression of iNOS and eNOS in spastic cerebral arteries after SAH is upregulated and downregulated, respectively, whereas the effect of E2 is opposite. Massive production of NO by iNOS, but not eNOS, combined with free radicals produces peroxynitrite, which inflicts cellular damage through lipid peroxidation, protein degradation, deoxyribonucleic acid (DNA) damage, mitochondrial dysfunction, or degradation of structural proteins, resulting in deterioration of the vascular structure and function. Excessive production of reactive oxygen, especially superoxide, occurs in the cerebral vasculature after SAH. The reactions between superoxide and NO may contribute to vasospasm after SAH by reducing the bioactivity of NO. Subsequently, endothelium-dependent relaxation is impaired and vasospasm occurs. Exogenous E2 administration can reduce SAH-induced vasospasm through interference in p65/iNOS DNA binding, which suppresses iNOS expression in male rats, and prevents oxidative stress through upregulation of manganese superoxide dismutase and extracellular superoxide dismutase in ovariectomized rats. The beneficial effects of estrogen on delayed vasospasm may arise from preservation of eNOS expression and prevention of iNOS induction by SAH. Oxyhemoglobin participates in the excessive production of ROS and reactive nitrogen species, especially $\text{O}_2^-$ and NO. $\text{O}_2^-$ strongly binds NO, so can lead to reduction of NO bioavailability, and production of highly cytotoxic peroxynitrite and hydroxyl, together with other active radicals. High levels of NO have a toxic effect on smooth muscle cells directly or as part of an inflammatory response to SAH, resulting in increased nitrite/nitrate levels in CSF and all are proposed mechanisms leading to delayed cerebral vasospasm.

Raloxifene has the potential to block or reverse the effects of cerebral vasospasm after SAH. Theoretically, raloxifene is involved in the blockage of many pathways involved in cerebral vasospasm such as inhibition of Ca$^{2+}$ influx through L-type Ca$^{2+}$ chan-
nels, inhibition of iNOS, estrogen receptor-dependent reduction in the release of reactive oxygen species from vascular cells, antioxidative activity on low density lipoproteins, antagonistic effect on activation of nuclear factor κB, prevention of nuclear factor κB transactivation, inhibition of the vascular cellular adhesion molecule-1, inhibition of the proliferation of vascular smooth muscles by binding estrogen receptors, downregulation of the iNOS, and upregulation of the superoxide dismutase.

The present study found that raloxifene decreased SAH-induced cerebral vasospasm in all treatment groups, and suggested no difference between intraperitoneal and intrathecal application, or between 3 days and 4 days of raloxifene treatment. Cerebral vasospasm reached its highest level 48 hours after SAH in rats in one hemorrhage model. We preferred to sacrifice our rats at 72 hours and 96 hours after SAH to observe the late effects of the raloxifene administration. The drug dose used in this study was chosen based on the doses used in the previous animal studies with raloxifene. However, we could not find any data on the intrathecal use of raloxifene and so decided to use a smaller dose than the intraperitoneal dose. Oral, intraperitoneal, and subcutaneous application of raloxifene has been reported at doses between 1 mg/kg and 10 mg/kg, and the duration of the application has been 5 days to 60 days.

The limitations of the present study were the low number of rats in each group and the use of a commercially available form of raloxifene, which also contains other additives. The diffusion of raloxifene through the blood-brain barrier following intraperitoneal administration after SAH in rats remains unclear. The effective treatment dose of raloxifene and its molecular effect on cerebral vasospasm following SAH need further investigation. However, the present study indicates that raloxifene is a potential therapeutic agent for the treatment of cerebral vasospasm after SAH.

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Commentary

As a prominent member of the selective estrogen receptor modulators family, raloxifene acts as estrogen receptor antagonist in the breast and uterus, omitting the harmful effects of estrogen but presumably preserving the beneficial effects of estrogen on bone, lipids and cardiovascular system.23 It improves endothelial function and induces vasodilation by NO release and calcium channel blockade in rabbit coronary arteries.23 In this article, the authors studied the effect of raloxifene on cerebral vasospasm following subarachnoid hemorrhage (SAH). The authors measured the luminal area of the basilar arteries in a single-injected SAH model of rats subsequently managed with vehicle, 3 days intraperitoneal raloxifene, 4 days intraperitoneal raloxifene, 3 days intrathecal raloxifene, or 4 days intrathecal raloxifene. The authors found that intraperitoneal or intrathecal raloxifene decreased SAH-induced narrowing of the basilar arteries. Despite some research limitations, the issue raised by this is intriguing, and warrants further investigation.

Care is required to use animal models of SAH and vasospasm. Because primates are phylogenetically closest to humans, these models have provided results that have most applicability to vasospasm in humans. However, the cost and ethical issues have become considerable. Rat and rabbit models are inexpensive, SAH is technically easy to induce, but the applicability of results is less certain. The most widely used animal model is double-injection of autologous blood in the dog cisterna magna for drug screening and may be useful before advancing to primate experiments. The double-injected rat SAH model would be more reliable than the single-injected one, and besides the measurement of the basilar artery, the following parameters, such as neurological score, cerebral blood flow in relation to muscle blood flow by laser Doppler monitoring, or perfusion weighted imaging of magnetic resonance should be included.23 It is pity that the authors did not explain why there was no significant effect of intraperitoneal raloxifene application at 3 days compared with that of vehicle application. Does intraperitoneal application of raloxifene have little effect at the early stage after SAH due to poor diffusion through the blood-brain barrier, or does it decrease cerebral vasospasm in a dose-dependent manner?