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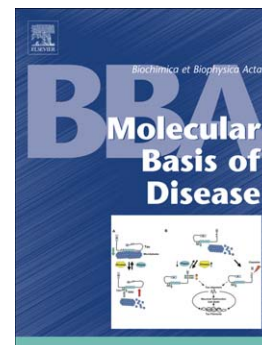
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**Conserved aquaporin 4 levels associated with reduction of brain edema is mediated by estrogen in the ischemic brain after experimental stroke**

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**Abstract**

Aquaporin 4 (AQP4), the most abundant water channel protein in the brain, is involved in brain edema induced by ischemic insults. To evaluate whether the neuroprotective effects of estrogen are associated with AQP4 expression and edema formation, changes in AQP levels and ischemic edema were examined in the brains of male and female mice subjected to transient middle cerebral artery occlusion. Infarct volume and edema formation were markedly less in females than in males. AQP4 expression in the ischemic cortex of females was relatively well preserved, whereas it was significantly decreased in males. These effects disappeared in ovariectomized females but were reversed by estrogen replacement. Furthermore, AQP4 expression was decreased with increased brain edema in females treated with ICI182,780, an estrogen receptor antagonist. These findings suggest that the estrogen effect on the reduction of ischemic brain edema is associated with the preserved level of AQP4 that is partly mediated by estrogen receptors.

**Research highlights**

- (1) Vasogenic edema of the ischemic brain was reduced in female mice compared males.
- (2) AQP4 levels in the ischemic cortex were maintained in female mice.
- (3) Down-regulated AQP4 levels by ovariectomy were reversed by estrogen replacement.
- (4) An ERs antagonist reduced AQP4 levels with an increase of vasogenic edema.
- (5) Estrogen receptors may regulate AQP4 levels to prevent the ischemic brain edema.

**Keywords**

Aquaporin 4, brain edema, estrogen, estrogen receptors, ischemic stroke, sex difference

## 1. Introduction

Development of brain edema is the major cause of death in patients with sizable ischemic stroke because it produces elevated intracranial pressure that leads to secondary brain damage, such as reduction in perfusion pressure and compression of vital areas of the brain [1, 2]. Despite its clinical importance, medical management has been ineffective at controlling brain edema in patients with stroke [1], indicating that a better understanding of the pathophysiology of edema formation in the ischemic brain is required for the management of edema.

Aquaporin 4 (AQP4) is a water channel protein and is the most abundant aquaporin in the brain. It is located in astroglial endfeet around the microvessels associated with the blood-brain barrier (BBB), glia limitans, and ependyma, suggesting that AQP4 has an important role in the movement of water into and out of the brain [3, 4]. Animal studies of brain injuries indicate that AQP4 is implicated in the pathogenesis of cerebral edema, as shown by an association of altered expression with the degree of edema formation in injured brain. Direct studies using AQP4 null mice have shown different roles for AQP4 according to the type of edema, that is, cytotoxic (fluid accumulation within cells) and vasogenic edema (extracellular swelling caused by increased permeability of the BBB) [5]. Brain swelling and mortality of mice with cytotoxic edema were reduced, whereas vasogenic edema was increased in AQP4 null mice compared to wild-type mice [6, 7]. In an ischemic stroke model, AQP4 null and alpha syntrophin null (functionally disrupted AQP4) mice showed a reduction in hemispheric swelling [6, 8], and the authors suggested that AQP4 contributes to edema formation. However, other studies showed the increased expression of AQP4 in the borderzone or periinfarct area, but not in the central part of ischemic brain [9, 10], suggesting positive roles of AQP4 in the salvageable brain. Therefore, the functional significance of up- or down-regulated AQP4 in ischemia-induced edema formation remains unclear.

It has been well documented that the female brain is resistant to ischemic injury during the

reproductive period compared to that of males and that estrogen plays an important role in this neuroprotection [11]. Multiple studies have shown a smaller infarct volume in females, but few studies have evaluated the effects of sex and sex hormones on ischemic edema, and furthermore, the findings are inconsistent [12-15]. Recent studies demonstrated no sex difference in ischemic brain edema [12, 13], whereas others reported that estrogen reduces brain edema induced by focal ischemic stroke [14, 15]. In traumatic brain injury (TBI) models that induce vasogenic edema, the lesion volume is reduced in females compared to males, and estrogen and progesterone administration reduces brain edema in males and ovariectomized rodents [16-18]. These results and the findings showing that sex hormones modulate AQP4 expression in the brain [18-20] led us to hypothesize that the vasogenic component of ischemic edema can be influenced by sex hormones through regulation of AQP4 expression.

Using ovariectomized mice given estrogen replacement, we previously demonstrated sex differences in the infarct volume following experimental ischemic stroke, and confirmed that estrogen was responsible for the neuroprotective effects in females [21]. Our current study was conducted to define sex differences in brain edema that is induced by ischemic stroke, and the effects of estrogen on AQP4 expression and edema formation. In addition, the role of estrogen receptors (ERs) in AQP4 expression was evaluated in the ischemic brain by treating female mice with an ER antagonist, ICI182,780.

## **2. Materials and methods**

### *2.1. Animals*

Experiments were performed in male and female C57BL/6 mice (age 10 to 11 weeks, Orient Bio Inc., Seongnam, Republic of Korea). All procedures were approved by the Institutional Animal Care and Use Committee at the Medical School of Ewha Womans University and conformed to international guidelines on the ethical use of animals. Before the

experiment, mice were acclimatized for 1 week in an animal room under a 12-h light/dark cycle at  $22 \pm 2^\circ\text{C}$ . The number of animals used for the study was minimized to reduce animal suffering.

## 2.2. *Transient middle cerebral artery occlusion (MCAO)*

Procedures for transient MCAO were described previously [22]. Briefly, mice were anesthetized with isoflurane, and a fiber optic probe was attached to the right parietal bone (2 mm posterior and 5 mm lateral to bregma) and connected to a laser-Doppler flowmeter (Periflux System 5010, Perimed, Sweden). Cerebral blood flow (CBF) was continuously recorded during MCAO and reperfusion periods with a computer-based data acquisition system (Perisoft, Perimed, Sweden). A 6-0 silicon-coated black monofilament surgical suture (Doccol Cooperation, Redlands, CA, USA) was inserted into the exposed right external carotid artery, advanced into the internal carotid artery, and wedged into the circle of Willis to obstruct the origin of the MCA. The filament was left in place for 30 min and then withdrawn to re-establish CBF. Only animals that exhibited a reduction in CBF  $> 85\%$  during MCA occlusion and in which CBF recovered by  $> 80\%$  after 10 min of reperfusion were included in the study. Rectal temperature was maintained at  $37.0 \pm 0.5^\circ\text{C}$  with a thermostatically controlled heating pad during surgery and recovery until the animal regained consciousness.

## 2.3. *Ovariectomy, drug administration, and measurement of estrogen activity*

Two weeks before MCAO, female mice underwent aseptic bilateral surgical ovariectomy via a dorsal incision under isoflurane anesthesia. Ovariectomized (OVX) female mice were divided randomly into vehicle and estrogen replacement groups, and vehicle (0.1 ml of sesame oil) or estrogen benzoate (1  $\mu\text{g}$  in 0.1 ml of sesame oil; Sigma-Aldrich, St. Louis, MO, USA) was administered subcutaneously for 6 days starting 3 days before MCAO. The ER antagonist ICI182,780 (100  $\mu\text{g}$  in 0.1 ml of sesame oil, subcutaneously; Tocris Bioscience, Bristol, UK) was given to gonadally intact female mice for 10 days starting 7 days before

MCAO. Naïve control mice for vehicle, estrogen benzoate, or ICI182,780 were treated with each drug for 10 days without MCAO. The doses of estrogen benzoate and ICI182,780 were adopted from previous studies that showed their effects in the brains of female mice [21, 23]. To measure estrogen activity, blood was collected at the time of euthanasia, and plasma estradiol levels were measured with an ELISA assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Uterine weight was also measured at the end of experiment to confirm estrogen activity.

#### *2.4. Measurement of infarct volume and brain edema*

Infarct volume was measured according to procedures described previously [24]. Mice were euthanized 72 h after MCAO, and brains were removed, frozen, and sectioned (30  $\mu\text{m}$  thick) with a cryostat. Brain sections were collected serially at 600- $\mu\text{m}$  intervals, and stained with cresyl violet. Infarct volume was determined with an image analyzer (Axiovision LE 4.1, Carl Zeiss, Jena, Germany). Values were reported after correcting for post-ischemic swelling. To measure brain edema, the whole brain was quickly removed and dissected into right and left hemispheres, which were weighed (wet wt). Dry weight (dry wt) of each hemisphere was determined after drying the tissue for 3 days at 100°C in an oven. The hemispheric water content was determined as % water content =  $(1 - \text{dry wt}/\text{wet wt}) \times 100$  [24], and then edema index was calculated by putting water content in ipsilateral over that in the contralateral hemisphere [13].

#### *2.5. Measurement of Evans blue extravasation*

Ten minutes after reperfusion, 5 ml/kg of 2% Evans blue (Sigma-Aldrich) in normal saline was injected into the tail vein. Six or 24 hours after reperfusion, mice were anesthetized with sodium pentobarbital (120 mg/kg, i.p.) and perfused transcardially with normal saline. Brains were removed, and each hemisphere was homogenized in 1 ml 50% trichloroacetic acid and centrifuged at  $15,700 \times g$  for 20 min at 4°C. The supernatants were diluted 4-fold with

ethanol, and analyzed at 650 nm by spectrophotometry [25]. Evans blue extravasation was quantified as  $\mu\text{g}$  per hemisphere in comparison with a standard curve, and Evans blue index was calculated by putting Evans blue concentration in ipsilateral over that in the contralateral hemisphere [13].

## 2.6. RNA isolation and real-time PCR analysis

RNA was isolated from the ipsilateral hemisphere cortex and subjected to quantitative real-time PCR. Total RNA was prepared with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Tissues were homogenized by sonicating in 0.5 ml Trizol, and RNA was purified using chloroform/isopropanol precipitation and dissolved in diethylpyrocarbonate-treated water (iNtRON Biotechnology, Seongnam, Republic of Korea). First strand synthesis was performed with 1  $\mu\text{g}$  of total RNA from each sample and 1  $\mu\text{l}$  (6.7  $\mu\text{M}$ ) of oligo (dT)<sub>15</sub>, with a Power cDNA Synthesis kit (iNtRON Biotechnology). A 2- $\mu\text{l}$  aliquot of diluted cDNA (1:10) was amplified with SYBR<sup>®</sup> Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a final volume of 20  $\mu\text{l}$ . PCR was performed in an ABI Prism 7000 sequence detector (Applied Biosystems). PCR cycles consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s. The primers for cyclophilin (forward: 5'-TAGAGCTGTCCACAGTCGGA-3'; reverse: 5'-TTCATCTGCACTGCCAAGAC-3') and AQP4 (forward: 5'-GGAAGGCTAGGTTGGTGA CTTC-3'; reverse: 5'-TGGTGACTCCCAATCCTCCAAC-3') were purchased from Bioneer (Daejeon, Republic of Korea). Cycling threshold (Ct) values of AQP4 were normalized to the Ct values of cyclophilin, and the relative expression level was calculated with the  $2^{-\Delta\Delta\text{Ct}}$  method [26]. All samples were run in triplicate, and naïve animals (n = 3) served as controls.

## 2.7. Western blot analysis

Cortical tissues from the ipsilateral hemisphere of each animal were subjected to



simultaneous western blotting. Tissues were lysed in 10 ml sodium dodecyl sulfate (SDS) buffer (62 mM Tris-HCl, 1 mM ethylenediamine tetraacetic acid, 2% SDS, pH 6.8–7.0) containing a protease inhibitor cocktail (Complete Mini, Boehringer Mannheim, Germany), incubated for 20 min on ice, and centrifuged at  $15,700 \times g$  for 10 min at 4°C. Protein concentration of the supernatant was determined (Bio-Rad Laboratories, Hercules, CA, USA), and 80 µg of protein was loaded for SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P transfer (Millipore Corporate, Billerica, MA, USA) with an electroblotting apparatus. Membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% dry milk for 1 h, incubated overnight with antibodies against AQP4 (1:1000; Abcam, Cambridge, UK), GFAP (1:1000; Sigma, USA), or Kir4.1 (the inward rectifying K<sup>+</sup> channel of astrocyte, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and washed three times (20 min each) with TBS containing 0.1% Tween-20. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h each and washed three times (20 min each) with TBS containing 0.1% Tween-20. Protein bands were visualized with the Western Blotting Luminal Reagent (Santa Cruz Biotechnology). Each membrane was re-blotted with anti-actin (1:1000; Santa Cruz Biotechnology) after stripping with an antibody stripping solution (Chemicon international, Temecula, CA, USA) according to the manufacturer's instructions. For quantification, densities of AQP4, GFAP, and Kir4.1 bands were normalized to the density of actin bands measured with Image J 1.37v program (NIH, USA).

### 2.8. Immunofluorescence staining

Animals were anesthetized with sodium pentobarbital (120 mg/kg, i.p.) and perfused transcardially with saline followed by 4% cold formaldehyde in 0.1 M sodium phosphate buffer (pH 7.2). The brains were removed, incubated overnight in fixative, and stored in a 30% sucrose solution. Serial coronal brain sections (20-µm thick, 600-µm interval) were

collected in a cryostat in regions between +1.4 and -1.0 mm from bregma. Brain sections were incubated in TBS containing 0.1% Triton X-100, 5% normal serum, and 1% bovine serum albumin for 1 h and then incubated with AQP4 antibody (1:500; Santa Cruz Biotechnology) and GFAP antibody (1:500; Sigma). On the following day, the secondary antibodies Alexa fluor<sup>®</sup>555 (1:1000; Molecular Probes, Eugene, OR, USA) and FITC (1:1000; Vector Laboratories, INC., Burlingame, CA, USA) were applied to sections for 1 h. Sections were washed with TBS between all steps. The sections were mounted with Vectashield mounting medium (Vector Laboratories, INC.). Fluorescence images of lateral cortex were obtained at a 4- $\mu$ m thickness through the z axis of the section covering a total of 20  $\mu$ m in depth with a confocal microscope (LSM5 PASCAL; Carl Zeiss) equipped with a filter set with excitation at 488 nm and 543 nm.

### 2.9. Statistical analysis

The data are expressed as the mean  $\pm$  S.E.M. Comparisons between two groups were analyzed with an unpaired Student's t-test. Multiple comparisons were evaluated with one-way analysis of variance (ANOVA), followed by a *post-hoc* Fisher's protected least significant difference (PLSD) test with Statview (Statview version 5, SAS Institute Inc., Cary, NC, USA). Differences were considered significant at  $p < 0.05$ .

## 3. Results

### 3.1. Reduced ischemic brain edema and infarct volume in female mice

First, we examined the infarct volumes between the sexes to confirm the sex difference in our stroke model. The total infarct volume was markedly reduced in females compared to males ( $p < 0.001$ ), and the reduction was only significant in the cortex ( $p < 0.001$ ) and not in the striatum, which is the ischemic core (Fig. 1B and C). CBF changes during MCAO and reperfusion were not different between males and females (Fig. 1A). These findings are

consistent with previous results showing that the female brain is resistant to focal ischemic insult and that the spared brain was the cortex, the area of ischemic penumbra [11, 21, 27]. Next, the time course of brain edema was examined in male brains to determine the time for comparison between sexes. Ischemic brain edema was not significant at 6 h, but was significantly increased at 24 h ( $p < 0.001$ ) and further enhanced at 72 h after reperfusion compared to naïve control mice (Fig. 2A). To determine whether brain edema formation was related to BBB breakdown, Evans blue extravasation was measured at 6 and 24 h after reperfusion. Evans blue was detected at 6 h ( $p = 0.364$ ), but it was significantly increased at 24 h ( $p = 0.022$ ) compared to control (Fig. 2B). These findings suggest that vasogenic edema caused by BBB breakdown contributes to edema formation at 24 h after reperfusion. Based on these results, we compared brain edema between sexes at 24 h and 72 h after reperfusion and observed less edema formation in female brains compared to male up to 72 h (Fig. 2C). These results showed that not only infarct volume but also edema formation was reduced in the brain of females, supporting the view that the extent of edema formation is associated with the degree of ischemic brain damage [25, 28].

### *3.2. The expression of AQP4 in the ischemic cortex is different between sexes*

To evaluate whether the difference in brain edema between sexes was related to the expression of AQP4, mRNA and protein levels of AQP4 were examined. Because the infarct volume of the cortex was different between males and females (Fig. 1C), the remaining experiments were done with ipsilateral cortical tissues. The basal levels of AQP4 mRNA in controls were not statistically different between sexes ( $p = 0.43$ ), although a slight increase was noted in females. After ischemic reperfusion, AQP4 mRNA expression decreased in males at 24 h and 72 h, whereas levels were maintained in females up to 72 h (Fig. 3A). Therefore, sex differences in AQP4 mRNA expression were observed at 24 and 72 h ( $p = 0.005$  and  $p = 0.012$ , respectively). AQP4 protein levels were decreased in both males and

females from 24 h to 72 h, but the reduction was significantly smaller in females than in males ( $p = 0.001$  at 24 h and  $p < 0.001$  at 72 h, Fig. 3B). To determine whether the reduction in AQP4 expression was due to loss of cells following ischemic insult, the levels of the astrocyte marker GFAP were examined. GFAP protein levels in the ischemic cortex of males were not statistically different compared to controls, although there was a trend towards decreased expression with time after reperfusion (Fig. 3B). In female brains, a reduction in GFAP was observed only at 72 h compared to naïve controls ( $p = 0.025$ ). However, there was no sex difference in GFAP levels, indicating that AQP4 reduction in males was not caused by loss of astrocytes. We also examined protein levels of inwardly rectifying  $K^+$  channel subunit 4.1 (Kir4.1) that colocalizes with AQP4 in astrocytes [29] and may be associated with AQP4-mediated water flux [30]. As GFAP expressions, its level was not different between sexes at any reperfusion periods (Fig. 3C), suggesting that AQP4 expressions of astrocytes were specifically decreased in male brains by ischemic injury. In addition, immunofluorescence staining of AQP4 in the ischemic cortex 24 h after reperfusion supported the result of AQP4 protein level that its expression in female brains was maintained (Fig. 4J). In male brains, the intensity of AQP4 was weak, and its outline around vessels was less clearly defined (Fig. 4F). Vessel-shaped AQP4-positive cells were colocalized with endfeet of astrocytes stained with GFAP in females (yellow, Fig. 4K & L), but this colocalization was not clearly observed in males (Fig. 4G & H).

Considering the correlation between the extent of brain edema and the levels of AQP4 at 24 h and 72 h after reperfusion (Fig. 2C), conserved AQP4 expression at both the mRNA and protein levels may contribute to reduced edema formation in female ischemic cortex.

### 3.3. *Effect of estrogen on AQP4 expression and ischemic brain edema*

To evaluate whether AQP4 expression was affected by the female sex hormone estrogen, estradiol was administrated to OVX mice, and AQP4 levels were examined 24 h after reperfusion, the time at which sex differences in AQP4 expression were apparent. OVX mice

showed decreased levels of AQP4 compared to naïve controls, whereas estrogen replacement reversed the reduction of both mRNA ( $p = 0.033$ ) and protein ( $p = 0.036$ ) levels 24 h after reperfusion (Fig. 5A and B). To determine whether Kir4.1 expression was also affected by estrogen, Kir4.1 protein levels were examined. Kir4.1 levels were not changed in the ischemic brain, and levels were not different between OVX mice with and without estrogen replacement (Fig. 5B). GFAP protein was also not affected by estrogen. The findings indicate that AQP4 expression in astrocytes is specifically affected by estrogen in the ischemic female brain. Next, estrogen effects on infarct volume and edema formation were examined. We confirmed the protective effect of estrogen by showing that estrogen replacement decreased the total and cortical infarct volumes ( $p < 0.001$  compared to OVX), which were increased by OVX (Fig. 5C). In addition, increased ischemic edema in OVX mice was reduced by estrogen 24 h after reperfusion ( $p = 0.01$ , Fig. 5D). Serum estradiol levels and uterine weights were decreased by OVX and were reversed by estrogen replacement to the level of gonadally intact females (Table 1). These results suggest that preserved AQP4 expression and reduced edema formation in the female ischemic brain is regulated by estrogen.

#### 3.4. *AQP4 expression in the ischemic cortex is mediated by ERs*

Next, to examine whether estrogen maintained AQP4 levels in ischemic brain via ERs, AQP4 expression was examined in females treated with ICI182,780, an ER antagonist. Compared to gonadally intact females, ICI182,780 reduced the levels of AQP4 mRNA and protein ( $p = 0.038$  and  $p = 0.039$ , respectively, Fig. 6A and B), whereas GFAP and Kir4.1 were not affected by ICI182,780 (Fig. 6B). These data indicate that AQP4 expression is maintained by ERs. To confirm the role of ERs in neuroprotection afforded by estrogen, the infarct volume was measured in females treated with ICI182,780. The total and cortical infarct volumes were significantly increased by ICI182,780 ( $p < 0.001$  compared to untreated females, Fig. 6C). ICI182,780 did not affect serum estradiol levels but decreased uterine

weight compared to untreated females (Table 1). These findings are in agreement with a previous study showing receptor-mediated estrogen effects in the ischemic brain [23]. To determine the effects of ERs on ischemic edema formation, brain edema in females treated with ICI182,780 was compared to untreated females. Ischemic edema was significantly increased by ICI182,780 ( $p = 0.029$  between treated and non-treated females, Fig. 6D). These results suggest that ER-mediated regulation of AQP4 levels contributes to reduction of ischemic edema formation.

#### 4. Discussion

Our current study showed that ischemic brain edema was reduced in females and that the extent of edema was correlated with preserved expression of AQP4. Furthermore, we provided evidence that the expression of AQP4 mRNA and protein is affected by estrogen and that ER-mediated AQP4 expression contributes to prevention of edema formation.

During the last decade, the role of AQP4 in the ischemic brain has been actively investigated. Since the study that showed a reduction of hemispheric enlargement after permanent MCAO in AQP4 knockout mice was reported [6], the notion that increased AQP4 expression contributes to edema formation [31, 32] and that suppression of AQP4 levels by therapeutics [33-36] reduces edema and infarct volume has been supported by other studies. These studies have demonstrated that the increase in AQP4 levels is correlated with the time of brain edema formation after ischemic insult. However, other studies have shown that changes in AQP4 expression are different according to ischemic regions in MCAO models. Perivascular AQP4 expression is decreased or nearly abolished in the ischemic core and central part of the ischemic cortex, whereas it is partially decreased or increased in the penumbra and border zone areas at the time of vasogenic brain swelling [8-10, 31]. In particular, abolished AQP4 expression in the ischemic core did not return to control levels until 72 h or 7 days after reperfusion [10, 31]. Consistent with these studies, our results

showed that AQP4 was gradually decreased in male cortex (central part of the ischemic cortex) and relatively well preserved in female cortex that was rescued from ischemic insult, corresponding to the penumbra, from 24 h to 72 h after reperfusion (Fig. 3). This finding may explain why our data are different from previous studies that reported no sex difference in AQP4 levels [12, 13]. In previous studies, there was no statistical sex difference in water content in the ischemic brain [13], although edema was slightly decreased in females [12]. Furthermore, infarct volume in female brains was reduced in caudoputamen, not in the cortex, compared to males, but AQP4 levels were examined in the cortex [12]. The absence of statistical differences in AQP4 expression shown in previous studies may result from using ischemic cortical tissues where infarct volumes were similar between sexes. We chose the time point that showed sex differences in edema formation (24 h) and examined ipsilateral cortical tissue that was rescued from ischemic insult in female brain. We also demonstrated that preserved AQP4 levels in females did not result from survival of astrocytes compared to males, as shown by similar GFAP and Kir4.1 expression in the sexes. Moreover, sex differences in the AQP4 levels in the ischemic brain were supported not only by quantitative measurement of mRNA and protein levels (Fig. 3), but also by immunofluorescence staining (Fig. 4). Results of the time course changes of AQP4 expression after reperfusion showed that decreased expression was inversely correlated with edema formation in male brains (Fig. 2C, 3A and B). Considering the bidirectionality of water flow through AQP4 [7, 8], our results suggest that preserved AQP4 levels in the ischemic cortex of females contributes to water clearance during ischemia-induced vasogenic edema.

It is well documented that estrogen provides neuroprotection from ischemic stroke in rodent models. However, few studies have evaluated estrogen effects on edema and AQP4 levels in the injured brain. Single physiological or pharmacological doses of estradiol given to OVX females were shown to be effective in reducing brain edema following TBI, which causes BBB breakdown and vasogenic edema [16, 17]. In focal ischemic stroke models, both

types of brain edema evaluated by MRI (diffusion-weighted imaging for cytotoxic edema, and T2-weighted imaging for vasogenic edema) were reduced in OVX mice by estrogen replacement [14, 15]. Our results are consistent with these findings showing that estrogen replacement reversed the increase in brain edema in OVX mice (Fig. 5D). We also showed that AQP4 levels were preserved in estrogen-treated OVX mice, suggesting that estrogen reduces brain edema by regulating AQP4 expression. These findings are in contrast to previous studies reporting that reduction in brain edema by estrogen and progesterone was associated with a decrease in AQP4 levels in brains injured by direct LPS injection or trauma [18, 20]. The difference may be due to different experimental models that were used. The previous studies demonstrated induction of AQP4 levels following insults, but in our current study, AQP4 expression was not increased but reduced in the ischemic cortex within the time frame we examined. AQP4 expression was correlated with edema formation rather than edema resolution. Therefore, levels of AQP4 maintained by estrogen may provide resistance to the development of brain swelling. Similar to our findings, recent reports using transient MCAO models suggested a positive role for AQP4 in edema reduction with neuroprotective therapeutics. Thrombin preconditioning reduces edema and increases AQP4 levels in the striatum at 1 h, an early time point after reperfusion [28]. In addition, administration of an arginine-vasopressin antagonist during ischemic insult reduces water content and increases AQP4 expression in the cortex [37]. Thus, up-regulation of AQP4 may be involved in neuroprotection that is afforded by some therapeutic strategies including estrogen.

Estrogen effects on AQP4 expression were further supported by experiments with females treated with the ER antagonist, ICI182,780. This drug blocks the action of estrogen on AQP4 expression selectively, indicating that AQP4 levels are modulated by genomic pathways via ERs at physiological levels of estrogen. Because AQP4 mRNA levels were maintained in females until later reperfusion periods (Fig. 3A), estrogen may maintain the transcription level of AQP4 in the ischemic brain. However, there is currently no direct evidence that estrogen



response elements exist in the promoter of the AQP4 gene, and that ER-binding estrogen response elements results in induction of AQP4 transcription. In addition, the increases of infarct volume and brain edema by ICI182,780 treatment were less than those of OVX (Fig. 5 and 6), indicating that non-genomic effects of estrogen such as activation of signaling pathways are involved in the reduction of brain edema. In fact, activity of AQP4 water permeability can be regulated by phosphorylation via activation of protein kinase A, calcium/calmodulin-dependent protein kinase, and protein kinase C [38], which are also affected by estrogen [39]. Therefore, further detailed molecular work to define the mechanism by which estrogen regulates both AQP4 levels and AQP4 activity is required. Moreover, because estrogen has various neuroprotective effects such as antioxidant, anti-inflammatory and vasodilatory activities that may prevent BBB breakdown and edema formation [11], it is possible that not only regulation of AQP4 expression but also other protective actions may contribute to reducing ischemic brain edema in a synergistic or additive manner. Future studies of genetic or pharmacological suppression of AQP4 expression in the ischemic brain during reperfusion periods will help define the estrogen effects on brain edema.

In conclusion, sex differences in ischemic brain edema were associated with difference in AQP4 expression, and estrogen contributed to reduction of brain edema by maintaining the levels of AQP4 in part via ERs. These findings suggest a role for estrogen in regulating AQP4 levels to prevent development of ischemic brain edema.

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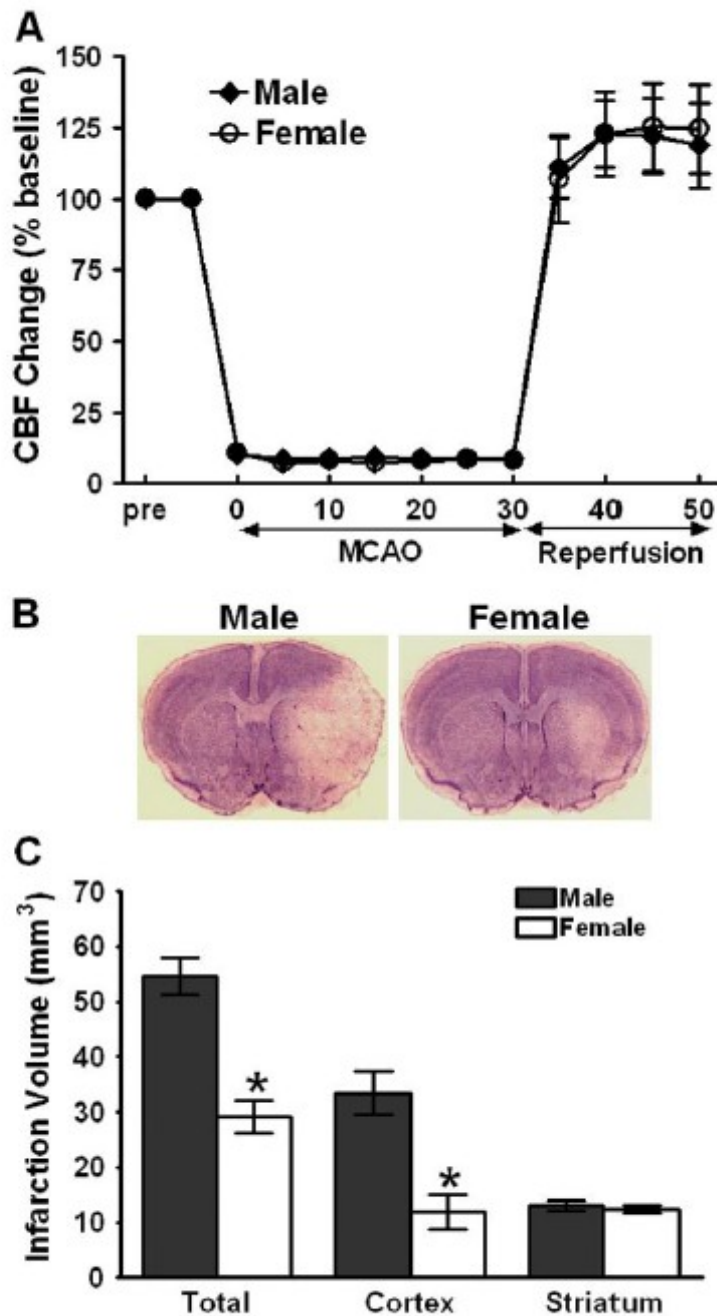
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Figure 1



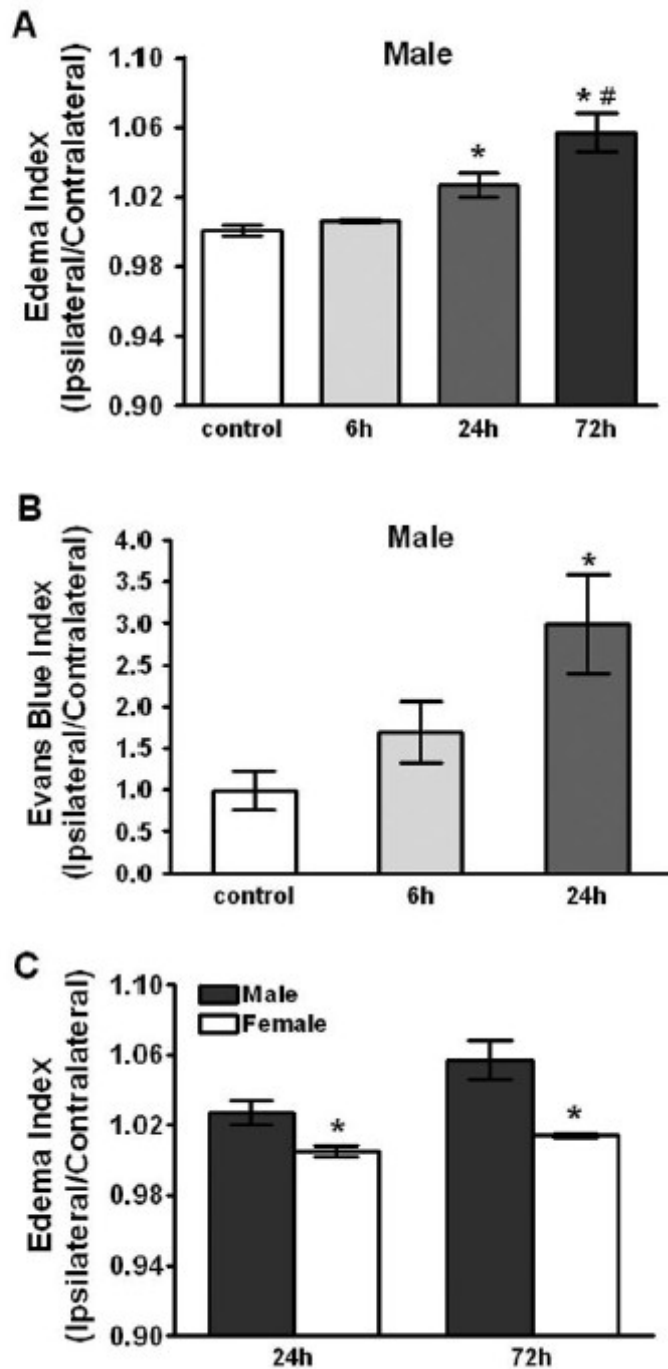
**Fig. 1.** Reduced infarct volume in female mice. **(A)** Cerebral blood flow changes during ischemia and reperfusion in males ( $n = 9$ ) and females ( $n = 10$ ). **(B)** Representative brain sections of cresyl violet staining 3 days after MCAO in male and female mice. **(C)** Infarct volumes in total hemisphere, cortex and striatum in male ( $n = 9$ ) and female ( $n = 10$ ) mice 3 days after MCAO. \* $P < 0.05$  compared to the corresponding infarct volume of males

(Student's t-test). The data are expressed as the mean  $\pm$  S.E.M.

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Figure 2

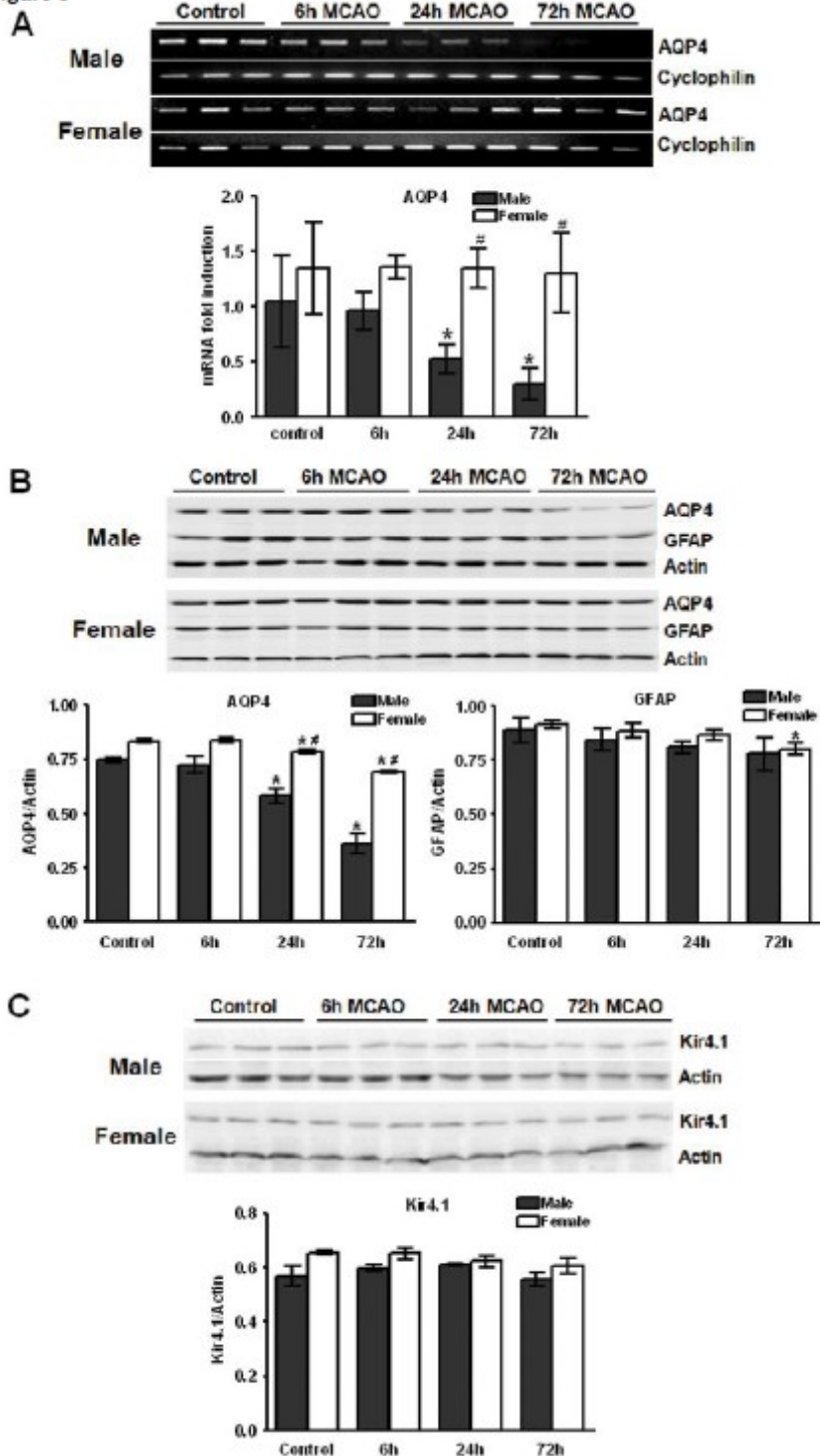


**Fig. 2.** Reduced ischemic brain edema in female compared to male mice. **(A)** Time course of hemispheric brain edema after MCAO in males ( $n = 4-5$ ). \* $P < 0.05$  compared to control and 6 h after reperfusion. # $p < 0.05$  compared to 24 h after reperfusion (ANOVA with *post-hoc* Fisher's PLSD test). **(B)** Evans blue extravasation at 6 and 24 h after reperfusion (control;  $n = 3$ , 6 and 24 h;  $n = 5$ ). \* $P < 0.05$  compared to control and 6 h after reperfusion (ANOVA with

*post-hoc* Fisher's PLSD test). (C) Brain edema in male and female mice at 24 h and 72 h after MCAO (n = 4-5). Male data are the same as in Fig. 2A, and are reproduced here for comparison. \*P < 0.05 compared to males (Student's t-test). The data are expressed as the mean  $\pm$  S.E.M.

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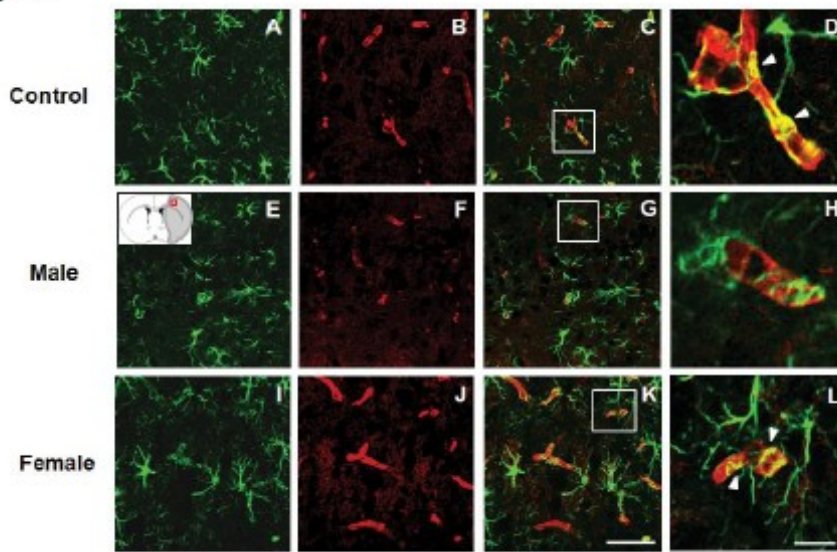
Figure 3



**Fig. 3.** Sex difference in AQP4 expression in the ischemic cortex. (A) Time course of AQP4 mRNA expression by PCR and quantitative real-time PCR in the ischemic cortex after MCAO in males and females ( $n = 4$  per time point for males and females except control;  $n = 3$ ). Values are expressed as the ratio versus male controls (given a nominal value of 1). \* $P < 0.05$ , # $P < 0.05$ .

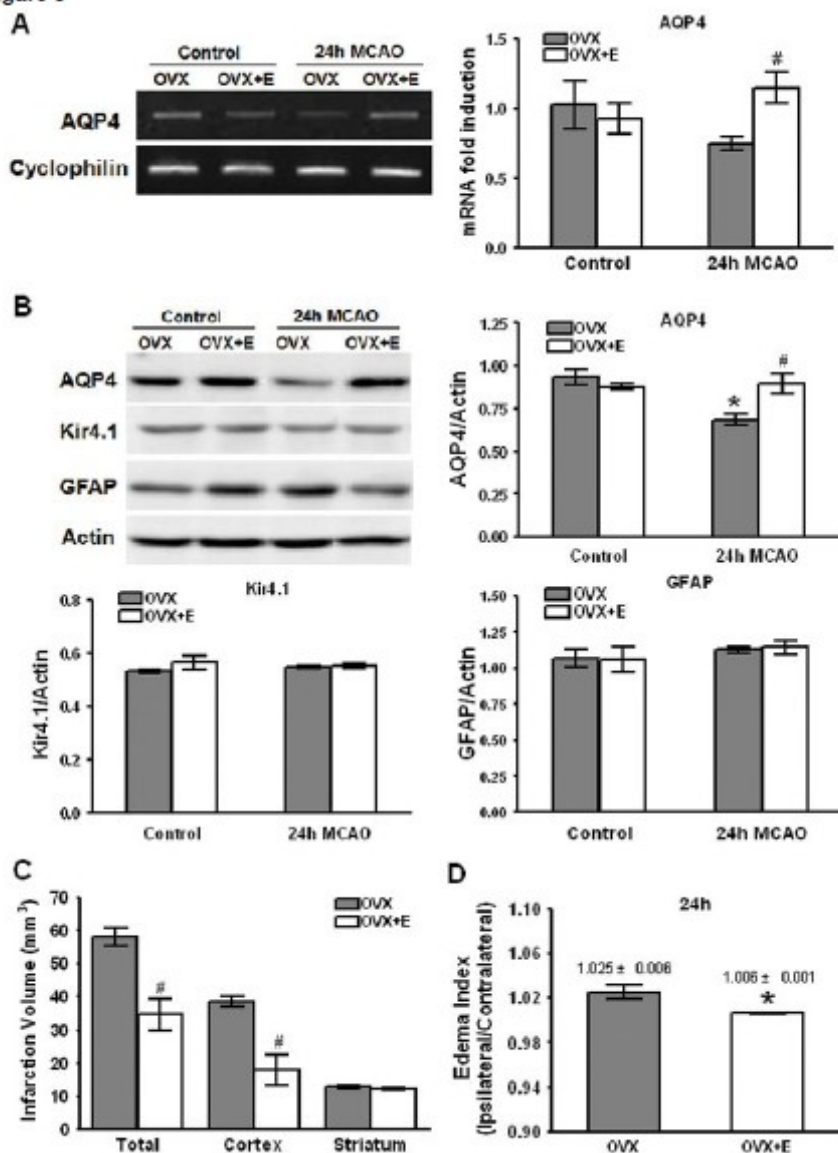
0.05 compared to male control, #p < 0.05 compared to the corresponding time point in males (Student's t-test). **(B)** Time course expression of AQP4 and GFAP proteins with western blotting and their quantitative levels normalized to actin in the ischemic cortex after MCAO in males and females ( n = 4 per time point for males and females except control; n = 3). \*P < 0.05 compared to each control, #p < 0.05 compared to the corresponding time point in males (Student's t-test). The data are expressed as the mean  $\pm$  S.E.M. **(C)** Time course expression of Kir4.1 proteins with western blotting and their quantitative levels normalized to actin in the ischemic cortex after MCAO in males and females (n = 3). The data are expressed as the mean  $\pm$  S.E.M.

Figure 4



**Fig. 4.** Vessel-shaped AQP4 expression is preserved in the ischemic cortex of females. Immunofluorescence staining for GFAP (green, **A, E, I**), AQP4 (red, **B, F, J**), the merged images (yellow, **C, G, K**), and enlarged images of white boxed areas (**D, H, L**) in male control (**A - D**) and ischemic cortices of male (**E - H**) and female (**I - L**) 24 h after MCAO. An area taken in the ischemic cortex (red box) for image analysis is depicted in **E**. Arrow-heads indicate colocalization of GFAP and AQP4. Representative images were obtained from one set of experiments, and three experiments were done independently. Scale bar = 50  $\mu\text{m}$  in **A-C, E-G, I-K**; 10  $\mu\text{m}$  in **D, H, L**.

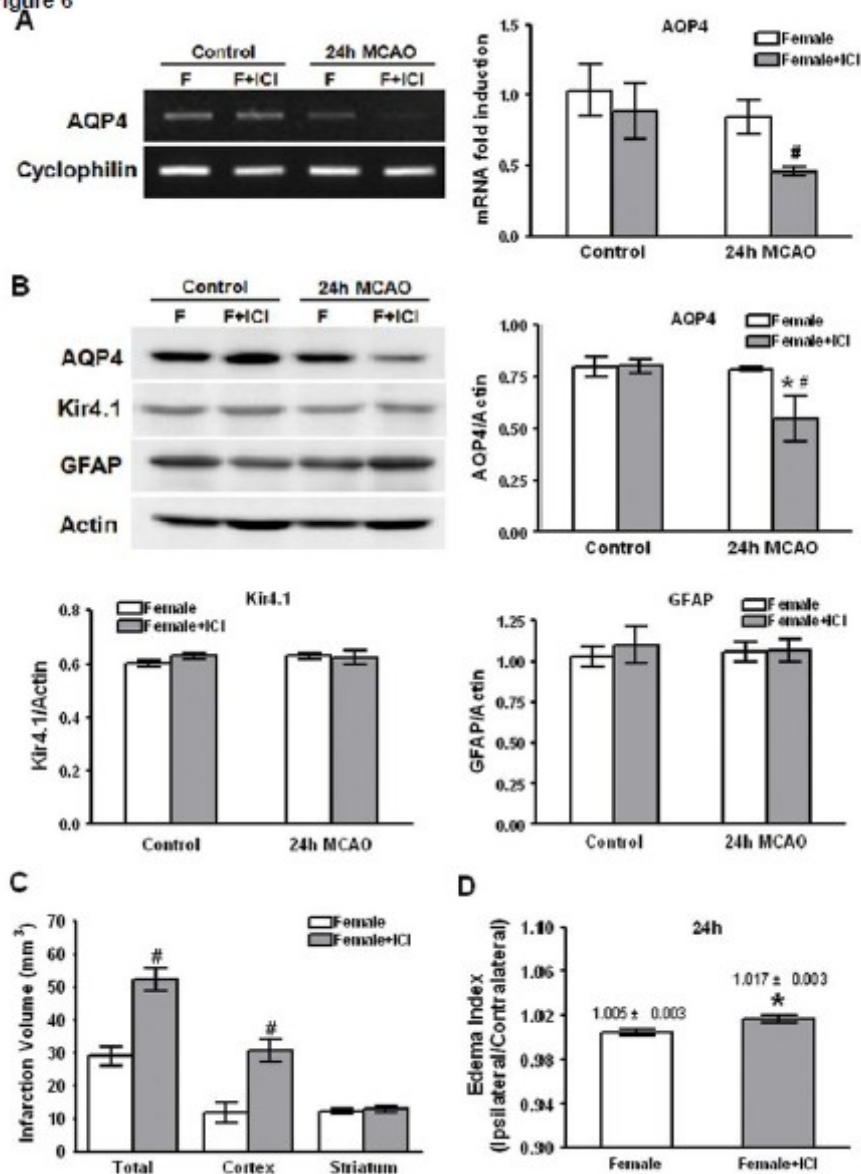
Figure 5



**Fig. 5.** Estrogen maintains AQP4 expression in the ischemic cortex and reduces ischemic edema. **(A)** Representative blots and quantitative levels of AQP4 mRNA in control and ischemic cortices of OVX mice and OVX mice given estrogen replacement (OVX + E) 24 h after MCAO ( $n = 3$  per group). Values are expressed as the ratio versus OVX controls (given a nominal value of 1).  $\#P < 0.05$  between OVX mice and OVX + E mice 24 h after MCAO (Student's t-test). **(B)** Representative blots and quantitative levels of AQP4, Kir4.1, and GFAP proteins in control and ischemic cortices of OVX and OVX + E mice 24 h after MCAO ( $n = 3$  per group).  $*P < 0.05$  compared to OVX control mice,  $\#p < 0.05$  between OVX mice and

OVX + E mice 24 h after MCAO (Student's t-test). (C) Infarct volumes in total hemisphere, cortex and striatum from OVX (n = 9) and OVX + E (n = 10) mice 3 days after MCAO. #P < 0.05 compared to the corresponding infarct volume of OVX mice (Student's t-test). (D) Brain edema in OVX (n = 4) and OVX + E (n = 4) mice 24 h after MCAO. \*P < 0.05 compared to OVX mice (Student's t-test). Estrogen (1 µg/mouse, s.c.) was given to OVX mice for 6 days starting 3 days before MCAO. The data are expressed as the mean ± S.E.M.

Figure 6



**Fig. 6.** Estrogen receptor-mediated AQP4 expression in the ischemic brain partly contributes to reduction of edema. **(A)** Representative blots and quantitative levels of AQP4 mRNA in control and ischemic cortices of gonadally intact females and females treated with ICI182,780 (female + ICI) 24 h after MCAO ( $n = 3$  per group). Values are expressed as the ratio versus female controls (given a nominal value of 1). # $P < 0.05$  between female and female + ICI 24 h after MCAO (Student's t-test). **(B)** Representative blots and quantitative levels of AQP4, Kir4.1, and GFAP proteins in control and ischemic cortices of females and female + ICI 24 h after MCAO ( $n = 3$  per group). \* $P < 0.05$  compared to control of female + ICI, # $p < 0.05$



between females and female + ICI 24 h after MCAO (Student's t-test). (C) Infarct volumes in total hemisphere, cortex, and striatum from female (n = 9) and female + ICI (n = 7) mice 3 days after MCAO. The data of the infarct volumes in females are the same as in Fig. 1 and are reproduced here for comparison. #P < 0.05 compared to the corresponding infarct volume of females (Student's t-test). (D) Brain edema in female (n = 4) and female + ICI (n = 4) mice 24 h after MCAO. The data of brain edema in females are the same as in Fig. 2C and are reproduced here for comparison. \*P < 0.05 between female and female + ICI (Student's t-test). ICI182,789 (100 µg/mouse, s.c.) was administered to gonadally intact females for 10 days starting 7 days before MCAO. The data are expressed as the mean ± S.E.M.

**Table 1.** Serum estradiol levels and uterine weights in females according to treatments.

Treatment	Female (n = 5)	Female + ICI182,780 (n = 5)	OVX (n = 5)	OVX + E (n = 5)
Serum estradiol (pg/ml)	3.98 ± 0.24	3.32 ± 0.57	1.88 ± 0.43*	4.30 ± 1.0
Uterine weight (mg)	90.4 ± 25.17	27.8 ± 0.92*	13 ± 1.27*	100.52 ± 6.04

OVX; ovariectomy, OVX + E; OVX with estrogen replacement.

\*P < 0.05 compared to gonadally intact female and OVX + E.