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Effects of Chronic and Acute Oestrogen Replacement Therapy in Aged Animals after Experimental Stroke

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Abstract

The effect of oestrogen replacement therapy (ERT) on stroke incidence and severity has been extensively debated. Clinical trials of ERT have demonstrated an increased risk of stroke in treated women, although the study participants were well past menopause when therapy was initiated. It has been suggested that detrimental effects of ERT may be unmasked after prolonged periods of hypoestrogenicity. To date, very few studies have examined the effect of ERT in aged animals, although the timing of replacement may be critical to the neuroprotective effects of ERT. We hypothesised that chronic ERT initiated in late middle age would decrease infarct size in the brain after an induced stroke, whereas acute ERT would have no beneficial effects in aged females. To test this hypothesis, two paradigms of ERT were administered to aged mice of both sexes aiming to determine the effects on stroke outcome and to explore the possible mechanisms by which ERT interacts with age. Female mice that received chronic ERT from 17–20 months of age showed improved stroke outcomes after experimental stroke, whereas females that had acute ERT initiated at 20 months of age did not. Chronic ERT females exhibited diminished levels of nuclear factor kappa B (NF- κ B) translocation compared to acute ERT females after stroke. Acute ERT females demonstrated both an increase in nuclear NF- κ B and enhanced expression of pro-inflammatory cytokines. In addition, a sexual dimorphic effect of ERT was seen because males benefited from ERT, regardless of the timing of initiation. Aged males had significantly reduced expression of pro-inflammatory markers after stroke compared to age-matched females, suggesting a pro-inflammatory milieu emerges with age in females. These results are consistent with the emerging clinical literature suggesting that ERT should be initiated at the time of menopause to achieve beneficial effects. The present study demonstrates the importance of using appropriate animal models in preclinical studies.

Keywords

ageing; oestrogen; inflammation; sex differences; NF- κ B

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The authors declare that there are no conflicts of interest.

Supporting information

Fig. S1. Serum testosterone levels in each group. The mean level of testosterone in each group was below 1 ng / ml. There was no significant difference in testosterone level between any two groups.

Table S1. Serum monocyte chemotactic protein (MCP)-1 levels (pg / ml) in stroke and sham mice.

Table S2. Serum interleukin (IL)-6 levels (pg / ml) in stroke and sham mice.

Table S3. Serum tumour necrosis factor (TNF)- α levels (pg / ml) in stroke and sham mice.

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Stroke is a disease that mainly affects the elderly. Stroke rates more than double every 10 years after the age of 55 years in both men and women (1). However, functional outcomes after stroke are worse in women than in men, with higher associated disability and mortality (2). Most international databases consistently demonstrate that women enjoy a lower incidence of stroke relative to men until well after menopause, after which stroke incidence in women climbs dramatically (3, 4). Although the age-related risk of stroke is evident, very few studies have examined the neurochemical changes that occur in aged brains after stroke because most of studies have been exclusively performed in young animals.

Oestrogens, primarily 17β -oestradiol (E_2), are potent neuroprotective agents in various animal models, including experimental ischaemic stroke (5). Numerous observational and retrospective clinical studies have also suggested that oestrogen replacement therapy (ERT) protects postmenopausal women against age-related diseases, including cardiovascular disease and stroke (6). However, two important clinical ERT trials, the Women Estrogen Stroke Trial (WEST) and the Women Health Initiative (WHI) trial, reported an unexpected increase in stroke incidence in E_2 -treated women (7, 8). The explanation for these findings has been debated extensively in the literature (9). One major factor is the timing of E_2 replacement. Participants recruited in these trials were well beyond menopause when they received ERT; and it has been hypothesised that ERT should be initiated immediately at menopause to achieve its protective effects (10, 11), which is an effect recently modelled in preclinical studies. Prolonged periods of hypo-oestrogenicity led to an enhanced inflammatory response when ERT was administered, which exacerbated stroke damage; however, animals in that study were not aged. Intriguingly, the latest study (12) reporting on post-intervention outcomes in women involved in the WHI trial who were taken off ERT found that, after 10.7 years of follow-up, there was no significant difference in stroke risk between E_2 and placebo-treated groups.

Recent studies (13, 14) have revealed that brain ischaemia is a powerful stimulus that triggers a series of events leading to the activation of several transcription factors involved in the inflammatory responses, including nuclear factor kappa B (NF- κ B) (15). After ischaemia NF- κ B translocates from the cytoplasm to the nucleus and up-regulates the expression of many pro-inflammatory genes, such as tumour necrosis factor (TNF)- α , monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-6, etc. (15, 16). A growing body of evidence (17, 18) has shown that E_2 interacts with NF- κ B through oestrogen receptor (ER) α to down-regulate the expression of inflammatory cytokines. However, whether E_2 continues to have these beneficial effects in the aged brain is not yet known.

The present study aimed to evaluate the effects of either chronic or acute ERT on aged mice subjected to ischaemic stroke and to explore the possible mechanism underlying the effects of E_2 in the aged brain. The mice utilised in the present study were 17 months old, which is an age equivalent to a 50–55-year-old woman (19) (i.e. the approximate age of menopause). We hypothesised that only chronic ERT initiated early after reproductive senescence would exert neuroprotective effects on mice after stroke, and predicted that the effect is related to the down-regulation of NF- κ B activation as a result of continuous exposure to E_2 after menopause. We also performed studies in aged males, aiming to determine whether there were sex differences in the response to ERT and to control for other factors associated with ageing.

Materials and methods

Animals

C57BL / 6 mice (17 months old) of both sexes were purchased from the NIA aged mouse colony (Charles River Laboratories, San Diego, CA, USA) All experiments were performed

in accordance with NIH guidelines for the care and use of animals in research and under protocols approved by the UCHC Animal Care and Use Committee.

E₂ replacement

E₂ replacement was administered by implanting E₂ pellets s.c. as described previously (20). Briefly, E₂ was delivered s.c. by a silastic capsule (0.062 inch inner diameter; 0.125 inch outer diameter) filled with 0.035 ml of E₂ (180 mg / ml; Sigma, St Louis, MO, USA) in sesame oil or sesame oil alone (vehicle). Two protocols for E₂ pellet replacement were developed: for chronic ERT, pellets were implanted in mice at 17 months of age and replaced every month until 20 months of age; for acute ERT, E₂ pellets were only implanted once, at 20 months of age. Mice were exposed to anaesthesia at the same frequency as that of the chronic ERT mice to control for repeated anaesthetic effects. Mice from both groups were then subjected to ischaemic stroke at 20.5 months (Fig. 1). Control groups were treated with sesame oil pellets replacement therapy (ORT) correspondingly (chronic or acute ORT). Animals were randomised into treatment groups and stroke was performed by a blinded investigator.

Focal cerebral ischaemic model

Focal transient cerebral ischaemia was induced by reversible middle cerebral artery occlusion (MCAO) for 90 min followed by reperfusion as described previously (20). A 6–0 nylon suture, 0.23 mm in dimension, was utilised to occlude the MCA. During surgery and ischaemia, rectal muscle temperature was monitored with a Monotherm system (VWR LabShop, Batavia, IL, USA) and maintained at approximately 37 °C with an automated temperature control feedback system; after surgery, the mice were returned into a cage with a heating pad system that maintained temperature until reperfusion. Cerebral blood flow was measured by Laser Doppler flowmetry (LDF; Moor Instruments Ltd, Axminster, UK) for every single mouse. Occlusion was confirmed by a drop in LDF by 85% of baseline in all mice. Neurological deficit scores were recorded 4 h after stroke and at sacrifice. The scoring system was: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; 4, no spontaneous locomotor activity or barrel rolling. All mice were examined before MCAO for dermatitis or visible tumours and were subjected to necropsy after sacrifice to exclude any animals with gross tumours including those in the pituitary region or other visible comorbidities.

Histological assessment

For evaluation of stroke outcomes, the mice were allowed to survive for 24 h and then were sacrificed with pentobarbital overdose (i.p.). Animals were perfused transcardially with cold phosphate-buffered saline followed by 4% paraformaldehyde; the brain was removed from the skull and post-fixed for 18 h and subsequently placed in cyroprotectant (30% sucrose). The brain tissue was cut into 30- μ m free-floating coronal sections on a freezing microtome and every eighth slice was stained by cresyl violet (CV) staining for evaluation of ischaemic cell damage (21). Then images were digitalised by a charge-coupled device camera (Qiaming, Surrey, BC, Canada), and the infarct volumes [expressed as a percentage of (%) the contralateral hemispheric structure to correct for oedema] (Swanson's method) (22) and analysed using SIGMASCAN PRO5 (Systat Software Inc., Chicago, IL, USA) as described previously (20).

Subcellular fractionation

For analysis of protein expression, subcellular fractionation was performed as described previously (23). Briefly, brain samples were obtained by rapid removal of the brain, with the

subsequent removal of the cerebellum / occipital pole and olfactory / frontal pole to enrich for tissue supplied by the MCA. The ipsilateral hemispheres were homogenised in Dounce homogenisers with cold lysis solution [50 mM HEPES, pH 7.5, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 50 mM NaF, 0.25 mM sucrose, 1 mM dithiothreitol, Protease cocktail (dilution 1 : 50; Roche Diagnostics Corp., Basel, Switzerland)]. Homogenates were filtered and centrifuged at 800 *g* for 10 min at 4 °C. The pellet (P1) contained the nuclear fraction; the supernatant (S1) was further centrifuged at 15 000 *g* for 10 min to obtain the cytosolic (supernatant, S2) and the mitochondrial fraction (pellet, P2). The P2 pellet was washed in SEE buffer [10 mM HEPES, pH 7.4, 1 mg / ml bovine serum albumin (BSA), 0.5 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0], then centrifuged at 15 000 *g* for 10 min. The S2 supernatant (cytosolic fraction) was centrifuged at 20 200 *g* for 30 min. The resulting supernatant is the cytosolic fraction. The P1 pellet was resuspended in a Tris buffer and run through a sucrose gradient composed of 1.8 and 2.3 M sucrose and centrifuged in a SW41 rotor at 30 000 *g* for 45 min. The pellet (P3) was washed with a nuclei pure storage buffer (Sigma-Aldrich, St Louis, MO, USA), and centrifuged at 960 *g* for 10 min. The pellet (P4) was resolved with nuclear extraction buffer (20 mM HEPES, pH 7.9, with 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA and 25% glycerol) (Sigma-Aldrich), sonicated, and stored at -80 °C as the nuclear fraction.

Western blots

The fractionated protein concentration was determined by BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) and subjected to western blotting as described previously (23). Sample proteins were resolved on 4–15% sodium dodecylsulphate electrophoresis gels and transferred to a polyvinylidene fluoride membrane and then probed with NF- κ B (p65; dilution 1 : 1000; Abcam, Cambridge, MA, USA), ER α (dilution 1 : 500; Abcam), insulin-like growth factor (IGF)-1 receptor β (IGF-1R β) (dilution 1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Bcl-2 (dilution 1 : 500; Cell Signaling Technology, Beverly, MA, USA). Macrophage migration inhibitory factor (MIF; dilution 1 : 1000; Cell Sciences, Canton, MA, USA) and Histone H3 (dilution 1 : 4000; Abcam) were used as loading controls for cytosolic and nuclear fractions, respectively. All blots were incubated overnight in primary antibodies at 4 °C in Tris-buffered saline containing 4% BSA and 0.1% Tween 20. Secondary antibodies (goat anti-rabbit IgG, dilution 1 : 5000; goat anti-mouse IgG, dilution 1 : 5000; donkey anti-goat IgG, dilution 1 : 1000; Santa Cruz Biotechnology) were diluted and ECL detection kit (Amersham Pharmacia, Piscataway, NJ, USA) was used for signal detection. Densitometry of western blots was analysed with SCION IMAGE (Scion Corp., Frederick, MD, USA) as described previously (20).

Immunohistochemistry (IHC)

Floating brain sections were prepared as described previously (24). Briefly, free-floating sections were blocked in normal goat serum block solution (PBTGS buffer: phosphate buffer + 0.3% Triton X-100 + 10% goat serum) for 1 h at room temperature followed by incubation overnight at room temperature with primary antibodies diluted with PBTGS buffer: anti-NF- κ B p65 (dilution 1 : 200; Abcam); anti-neuronal nucleus (NeuN) (dilution 1 : 200; Chemicon International Inc., Temecula, CA, USA). The secondary antibodies (dilution 1 : 1000; Invitrogen, Carlsbad, CA, USA) were tagged with red or green fluorescent Fluor dye and diluted in PBTGS buffer. They were either goat anti-mouse or goat anti-rabbit depending on the primary antibody and were incubated for 2 h at room temperature. 4',6-diamidino-2-phenylindole (DAPI) (dilution 1 : 1000; Invitrogen) was added to the sample together with the secondary antibodies. The signal was visualised with a Zeiss Axiovert 200M microscope (Carl Zeiss, Oberkochen, Germany) using a X-Cite 120Q fluorescence illumination system (Lumen Dynamics Group Inc., Mississauga, ON, Canada)

using Zeiss image acquisition software (Zeiss LSM 510). All slices (from the same brain as for CV staining) were approximately 0.5 mm anterior to bregma and three \times 20 fields / animal ($n = 6$ animals per group) were analysed in the penumbral area of the infarct. Cells with NF- κ B and NeuN co-localised in the nuclei were counted using MACBIOPHOTONICS IMAGEJ software (<http://www.macbiophotonics.ca>) after a DAPI counterstain to confirm nuclear localisation. For each animal, the total numbers of cells in three \times 20 fields were used for the statistical analysis.

E₂, testosterone and cytokine analysis

Serum levels of E₂, testosterone, MCP-1, IL-6 and TNF- α were measured in each group by enzyme-linked immunosorbent assay (ELISA) (E₂; IBL Hamburg, Hamburg, Germany; testosterone; Calbiotech, Spring Valley, CA, USA; MCP-1, IL-6, TNF- α ; eBioscience, San Diego, CA, USA). The uterus was removed at sacrifice in all female mice and weighed at sacrifice to confirm the loss of end-organ oestrogen effects.

Statistical analysis

Data from individual experiments are presented as the mean \pm SEM and analysed with a t-test for two groups, and two-way ANOVA (with Tukey's post-hoc correction for multiple comparisons where appropriate) for the comparison of the means between the experimental groups. Neurological deficit scores were analysed with a Mann–Whitney U-test. $P < 0.05$ was considered statistically significant. Induction of ischaemia and all behavioural and histological assessments were performed by an investigator who was blinded to vehicle or E₂ replacement.

Results

Chronic and acute ERT exerted different effects on stroke outcomes in males and females

To evaluate the effects of E₂ replacement initiated at different times during the lifespan on ischaemic stroke, we designed two experimental paradigms: chronic and acute ERT (Fig. 1). We first measured stroke outcome 24 h after the induction of a 90-min MCAO. Brain slices were stained with CV and infarct volumes were digitally calculated (Fig. 2A–D). Consistent with our previous study performed in middle-aged mice (20), both chronic or acute ERT males had significantly smaller infarct volumes than their counterpart females. Both chronic and acute ERT decreased infarct volumes significantly in males compared to vehicle-treated groups (total infarct: chronic ERT versus chronic ORT $15.1 \pm 3.1\%$ versus $33.2 \pm 2.0\%$, acute ERT versus acute ORT $16.4 \pm 2.9\%$ versus $34.4 \pm 3.1\%$; $n = 12$ per group, $P < 0.05$). A different pattern was seen in females. Only chronic ERT reduced infarct volume compared to oil-treated mice (total infarct: chronic ERT versus chronic ORT $34.7 \pm 2.4\%$ versus $58.2 \pm 2.6\%$; $n = 9$ per group, $P < 0.05$) and there was no difference in infarct size between acute ERT and ORT groups (total infarct: acute ERT versus acute ORT $57.8 \pm 1.8\%$ versus $64.1 \pm 2.4\%$; $n = 10$ per group, $P > 0.05$). Neurological deficit scores reflected these changes in infarct size (Fig. 2E). There were no differences in preischaemic or intraschaemic arterial blood pressure, and blood gas measurements between any two groups; however, female mice in both the ORT and chronic ERT groups were significantly lighter than their male counterparts ($n=6$ per group, $P < 0.05$) (See Table 1).

NF- κ B translocation differed between the sexes after MCAO

With ischaemic injury, NF- κ B is activated and translocates from the cytosol to the nucleus to regulate gene expression of inflammatory cytokines (15). To examine the translocation of NF- κ B, we performed immunofluorescent staining to co-label brain slices with NF- κ B and NeuN. Because NeuN is also expressed in cytoplasm (25, 26), we further exposed the slices

with the nuclear stain, DAPI, to confirm the nuclear localisation of NF- κ B. As shown in Fig. 3(A), NF- κ B co-labelled with NeuN in the nucleus in stroke brains (NF- κ B translocation positive cells; indicated by arrows in the upper panel of Fig. 3A); however, in sham brains, NF- κ B was localised only in the cytoplasm (NF- κ B translocation negative cells; indicated by arrow heads in the lower panel of Fig. 3A). Quantification was performed in all the stroke groups (Fig. 3B–D). There were significantly less NF- κ B translocation positive cells in chronic ORT or acute ORT males compared to their counterpart females, and ERT did not decrease NF- κ B translocation significantly in males. Interestingly, chronic ERT females had significantly fewer positive cells than chronic ORT females; however, no difference in NF- κ B positive cell numbers was seen between acute ERT and ORT females.

Western blotting showed a reduction in NF- κ B expression in chronic ERT females

To confirm the IHC findings, and quantify nuclear NF- κ B expression levels, western blotting was performed to examine nuclear NF- κ B expression. Consistent with the IHC data, chronic ERT females had significantly less nuclear NF- κ B compared to either chronic ORT or acute ERT females (Fig. 4A–C). Acute ERT females exhibited higher NF- κ B expression in the nuclear fragment compared to acute ORT females. No significant difference in NF- κ B expression levels was seen between male cohorts.

Expression of pro-inflammatory cytokines differed after stroke in males and females

Because NF- κ B can regulate the expression of IL-6, MCP-1 and TNF- α , etc., we next examined the serum level of these cytokines by ELISA. We selected to assess cytokines 24 h after stroke. Proinflammatory signalling occurs in two peaks after stroke: the first peak is usually within 6 h and the second peak occurs 24–36 h after stroke (27). Stroke induced a significant increase in levels of these cytokines in every group compared to sham (see Supporting information, Tables S1–S3). Both MCP-1 and IL-6 levels in chronic ERT females were significantly lower than those in chronic ORT or acute ERT females; chronic ERT also reduced TNF- α levels compared to chronic ORT females (Fig. 5A–C; $n = 6$ per group, $P < 0.05$). In males, E₂ treatment did not decrease these cytokines compared to oil vehicle groups after stroke; however, chronic ORT or acute ORT males had significantly lower MCP-1 compared to their counterpart females. IL-6 and TNF- α levels were significantly lower in chronic ORT males compared to chronic ORT females.

Serum E₂, testosterone levels and uterine weights

Serum E₂ and testosterone levels were assessed in each group by ELISA. As expected, both chronic ERT or acute ERT paradigms led to significantly higher serum E₂ levels compared to those seen in the chronic ORT or acute ORT mice of both sexes (Fig. 6A), and were equivalent to basal circulating oestrous levels of cycling female mice (28–30). No difference in E₂ levels were seen between chronic and acute ERT mice. Uterine weights in all female mice showed the same pattern as serum E₂ levels (Fig. 6B). The mean testosterone level was below 1 ng / ml in all groups regardless of sexes, which is lower than the previously reported levels in young C57BL6 male mouse (> 10 ng / ml) (31, 32), and showed no significant differences between groups (see Supporting information, Fig. S1).

Effects of ERT on ER α , IGF-1R β , Bcl-2 expression

E₂ replacement led to a higher expression of cytosolic ER α in both chronic ERT males and females compared to their acute ERT counterparts after stroke. Intriguingly, acute ERT decreased ER α levels after stroke compared to sham groups in both males and females (Fig. 7A,C). ER α levels in both male groups were significantly lower than their female counterparts. E₂ can also regulate the expression of the anti-apoptotic protein Bcl-2, by interacting with IGF-1 (33). Therefore, we also examined IGF-1R β and Bcl-2 expression by

western blotting. We found that these two protein levels significantly increased after stroke in each group compared to sham mice, except for IGF-1R β in chronic ERT males (Fig. 7A,B) and Bcl-2 in acute ERT males (Fig. 7D,E); however, no significant differences were seen between sexes and treatments.

Discussion

The present study demonstrates several important new findings. First, the timing of administration of ERT is important, although only in the female brain. Both chronic ERT and acute ERT led to neuroprotection after induced stroke in males; however, only chronic ERT improved infarct outcome in females, whereas acute ERT did not. Oil-treated males had significantly smaller infarcts than oil-treated females, which is consistent with previous findings in middle-aged animals (20). Second, the expression of nuclear NF- κ B was different in males versus females after stroke. Male mice had less NF- κ B translocation after stroke compared to their counterpart females, except in the chronic ERT group. Chronic ERT robustly inhibited NF- κ B translocation in females after stroke compared to chronic ERT females, which reduced the level to that of chronic ERT males; however, acute ERT increased NF- κ B translocation beyond that seen with acute ERT, consistent with the proinflammatory effects of acute ERT. Third, the pro-inflammatory cytokines MCP-1, IL-6, and TNF- α induced by NF- κ B were inhibited by chronic ERT but not acute ERT in females. Fourth, the timing of E₂ therapy had an effect on expression of ER α after stroke (i.e. acute ERT decreased, whereas chronic ERT increased, cytosolic ER α levels in both males and females after stroke compared to shams). Two-way ANOVA also showed a sex effect in ER α expression (i.e. females had significantly higher level of ER α compared to males in each respective treatment group). Finally, although IGF-1 and Bcl-2 levels increased after stroke in both male and female mice, neither sex, nor the timing of E₂ therapy had an effect on levels of either protein.

Extensive debate continues regarding the potential neuroprotective effects of E₂ in both pre-clinical and clinical populations (9); the importance of the timing therapy of ERT has been increasingly recognised. The interval between menopause and the start of ERT plays a crucial role in the effectiveness of ERT in the vascular system (34). The present study found that only chronic ERT reduced infarct in aged females. We designed the chronic ERT treatment to initiate ERT in mice beginning at an age equivalent to a 50–55-year-old woman (19), the approximate age of menopause. Interestingly in both the WEST and WHI trial, both of which showed an increase in stroke incidence in treated women, the mean age of the participants at entry was 63 years (WHI) or 71 years (WEST), representing an age well past menopause. In a previous study (12) where young female mice were ovariectomised (OVX), it was found that E₂ treatment was neuroprotective only if treatment was initiated immediately after OVX, which is consistent with our results demonstrating that chronic ERT was neuroprotective. If E₂ treatment was delayed for 10 weeks after OVX, the infarct size was significantly larger (12); however, in the present study, acute ERT had no effect in aged females. The subtle difference between the previous and the present study may be a result of the different menopausal models used. In the present study, we used intact aged mice (17 months old), which have low circulating E₂, testosterone, and low uterine weights to model natural menopause and to determine the effects of ERT in the ageing brain. By contrast, the previous study was performed in young females after surgical menopause (11). Additionally, we utilised a reperfusion injury, where the suture was removed after 90 min, allowing for reperfusion, whereas a permanent occlusion model was used in the previous study. This model leads to larger infarcts (35, 36) that may be less responsive to neuroprotectants (37) compared to that of the reperfusion model. Previous studies of ERT in middle-aged rats subjected to MCAO demonstrated similar results to those reported in the present study. Immediate ERT after OVX decreased infarct volume in 9–12-month-old female rats (38),

whereas ERT initiated after an additional 9 weeks of E₂-deficiency significantly increased infarct size (39). Intriguingly, long-term ovarian hormone deprivation not only blocks the beneficial effects of ERT in ischaemic injury, but also attenuates the ability of E₂ to improve hippocampus-dependent memory in both young adult and middle-aged rats (40).

The mechanism by which E₂ loses its protective effects after a prolonged period of hypoestrogenicity is not yet known. E₂ is known to have profound effects on inflammation, which, in turn, can influence stroke outcome (41). In most cases, at least in models that have examined young animals, E₂ suppresses the inflammatory response. This occurs in central nervous system disorders (42), as well as in models of arthritis (43), wound repair (44) and trauma (45). However, the role E₂ plays in inflammation is complex. It is becoming increasingly clear that ERT administered at different time points during the lifespan can elicit very different effects (46). This is evident in many species, including primates. In surgically ovariectomised primates, a 70% reduction of atherosclerosis was seen when ERT was initiated simultaneously with an atherosclerotic diet but, when ERT was delayed 2 years after diet initiation, no protection against atherosclerosis was observed (47). The inflammatory responses induced by NF- κ B activation play crucial roles in the development of post-stroke injury (48). E₂ has long been known to inhibit NF- κ B activity in various diseases, including stroke (18, 49); the loss of E₂ with menopause promotes a pro-inflammatory state that is unmasked with acute injury. The present study revealed that chronic, but not acute, E₂ replacement suppresses this NF- κ B proinflammatory response, which likely contributes to the reduction of infarct volume after MCAO.

The timing effect of E₂ on ischaemic stroke in aged mice also showed a subtle sexual dimorphic effect in the present study. To date, no studies have examined the effects of ERT after MCAO in aged mice. Young mice have been increasingly used in focal ischaemia models as a result of the availability of transgenic and knockout strains (21). Only a few studies on ischaemic stroke have been performed in aged rats (50–52), and the results from these studies were quite controversial. This may be because of the variability of the sex of the animals examined. Sex differences have been well documented in ischaemic stroke studies using young animals (5, 23). The neuroprotective effects of E₂ are a major contributor to the smaller infarcts seen in young gonadally intact females. Numerous neurochemical and physiological changes occur with ageing (53), which likely lead to differences in ischaemic sexual dimorphism between young and aged animals. We have previously found that middle-aged male mice (15 months) had significantly smaller infarct volumes than females after MCAO (20), and the present study now extends these findings into the aged brain. These findings are consistent with clinical data demonstrating that aged women have higher disability and mortality than men after stroke (2). Despite the significant difference in infarct in oil-treated groups, no difference in neurological deficits were seen between males and females, suggesting that infarct size does not necessarily correlate with behavioural deficits, especially in aged animals.

Inflammatory responses have long been known to contribute to ischaemic injury (14), although how the inflammatory response changes with age and sex is not yet known. Serum levels of IL-6 differed in middle-aged male and female mice after stroke (20), which we investigated further in the present study. The expression of several pro-inflammatory markers, NF- κ B, MCP-1 and IL-6, were significantly up-regulated after stroke in females compared to males. The higher level of pro-inflammatory markers is consistent with clinical data (54) showing that women enjoy immunological and metabolic advantages until menopause, which then decrease as women age. The results of the present study suggest that differential inflammatory responses after stroke contribute, at least in part, to differences in stroke outcomes in aged male and female mice.

Chronic and acute ERT not only led to different stroke outcomes and inflammatory responses, but also induced age-related differences in ER α expression. E₂ receptor α (and not β) is a critical to E₂-mediated protection against brain injury (55) and was therefore the focus of these investigations. Oestrogens are known to autoregulate ER (56), and the expression of ER α increases greatly in the ischaemic cortex after stroke injury (41); therefore, it is not unexpected that chronic ERT induced high levels of ER α in both male and female mice after stroke. However, there was a different response of ER α to ERT in the acute ERT groups, with decreased ER α expression in both sexes after stroke. It has been reported that the expression of both ER α and ER β decreases with age in the cortex and hippocampus (57, 58), which could contribute to a decreased responsiveness to E₂ in the acute ERT groups. The results obtained in the present study suggest that the decreased expression of ER α in aged animals cannot be reversed by acute ERT, in contrast to chronic ERT. To counteract the decreased responsiveness caused by ageing, it may be necessary to start E₂ replacement early after menopause so that ER levels can be maintained. This is suggested by our results demonstrating that chronic ERT groups of both sexes had increased ER α expression compared to the corresponding acute ERT groups. The mechanism by which ER α expression decreases with age has not been elucidated; however, a recent study reported that methylation of 5' untranslated exons in DNA encoding ER α increases with age, leading to silencing of the ER α gene (59). Methylation of DNA is an epigenetic mechanism underlying gene silencing (31). Interestingly, increased methylation of DNA with age also exists in X-chromosome inactivation (XCI), a major mechanism for dosage compensation of X-linked genes in females. XCI becomes unstable with ageing, and XCI skewing may occur in ageing populations, as demonstrated by increasing levels of methylation of target genes (60). Gene silencing by methylation of DNA in ageing subjects is being increasingly recognised and requires further study.

Sex differences in ER α expression were also seen in the present study (i.e. males had significantly lower cytosolic ER α level than females in each group), consistent with previous studies (61, 62). Activation of ER α can have inhibitory effects on NF- κ B activity via enhancement of endogenous inhibitors of NF- κ B, or by direct inhibition of NF- κ B DNA binding activity (17). This is consistent with our finding that chronic ERT female mice had less nuclear NF- κ B than that of acute ERT females, which also had lower levels of ER α expression. However, acute ERT females, despite their higher ER α levels, had larger infarcts than acute ERT males. This suggests that acute ERT is unable to inhibit NF- κ B in females despite the higher level of ER α expression, and demonstrates that ER α levels do not correlate with infarct size. Both chronic and acute ERT males had low level of NF- κ B, although chronic ERT males had higher expression levels of ER α compared to acute ERT males. However, both acute and chronic ERT were neuroprotective in males. The complicated nature of the relationship between E₂ signalling and NF- κ B seen in aged male animals suggested that E₂ signalling may not be a major contributor to NF- κ B activity in males.

One major limitation of these results is that ER α levels were only measured in the cytosolic fractions. Therefore, the results must be interpreted with caution. It is possible that acute E₂ treatment led to receptor dimerisation with subsequent DNA binding and nuclear translocation. Indeed, decreased levels of cytosolic ER could be secondary to nuclear translocation and subsequent enhancement of ER signalling. However, it is clear that differences exist in the response of the ER to both E₂ treatment regimen and by sex. Further studies that directly evaluate nuclear ER levels or, even more importantly, directly assess the downstream consequences of ER activation in the aged stroke brain are needed. This will require the use of ER knockout mice.

Another important pathway through which E₂ may exert its neuroprotective effects is via interaction with IGF-1, leading to activation of the extracellular regulated kinase / mitogen-activated protein kinase signalling cascade and activation of transcription factor such as cAMP-response element binding protein, whose target genes include Bcl-2 (63, 64). In the present study, both IGF-1R β and Bcl-2 were increased after stroke compared to sham in each group, reflecting the activation of neuroprotective E₂-IGF-1 axis. However, no differences in the expression of these two proteins were seen between the acute and chronic ERT groups of both sexes, indicating that sex differences and timing effects of E₂ in ischaemic stroke in aged mice are not caused by activity of E₂-IGF-1 axis.

The present study has several limitations that must be kept in mind when interpreting the results. MCAO in mice is not a perfect model to compare to the WHI trial because we induced a stroke and infarct size was examined subsequently. This is very different from the WHI trial in which the outcome assessed was stroke incidence. In the present study, we administered ERT to mice before MCAO; however, in most clinical neuroprotection trials, agents are usually applied a few hours after stroke onset. It is possible that E₂ may be equally effective in both sexes if administered as an acute neuroprotectant. We also only evaluated acute stroke outcomes after 24 h of stroke; therefore, the long-term effects of ERT in aged mice of both sexes remain unknown. However, previous work in this model has shown that infarct is complete by 24 h (65) and infarct size at 24 h correlates well with that seen at 30 days, even in aged mice (66).

In conclusion, the effect of ERT on stroke in aged mice is modulated by several factors. Overall, sex differences are evident in that ERT led to neuroprotection in aged male mice regardless of the timing of ERT; whereas the effect is modulated in aged females based on the timing of initiation of therapy. Chronic ERT treated females showed improved stroke outcomes, whereas acute ERT had no beneficial effect on stroke induced brain injury. Inflammatory responses may mediate the differential effect of ERT in aged mice because NF- κ B translocation and serum levels of MCP-1, IL-6 and TNF- α paralleled the changes in infarct size. ER α expression exhibited sex differences in aged mice after stroke, and may contribute to the inflammatory response to ischaemic stroke in aged females.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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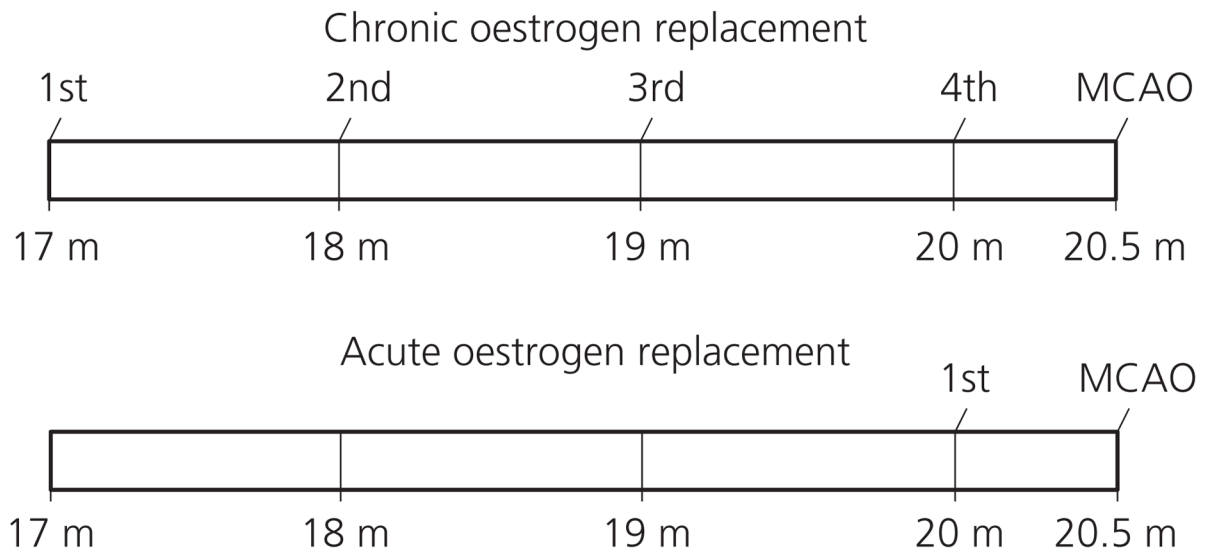


Fig. 1.

Schematic representation of the experimental design of 17β -oestradiol (E_2) replacement. In the chronic E_2 replacement group, E_2 pellets were first implanted to mice at 17 months old and replaced every month until the twentieth month; in the acute E_2 replacement group, the pellets were only implanted in the twentieth month. Mice were subjected to MCAO 15 days after the final implant of the pellets. Control groups used pellets that contain oil vehicle and were treated with the same experimental conditions as the E_2 -treated groups.

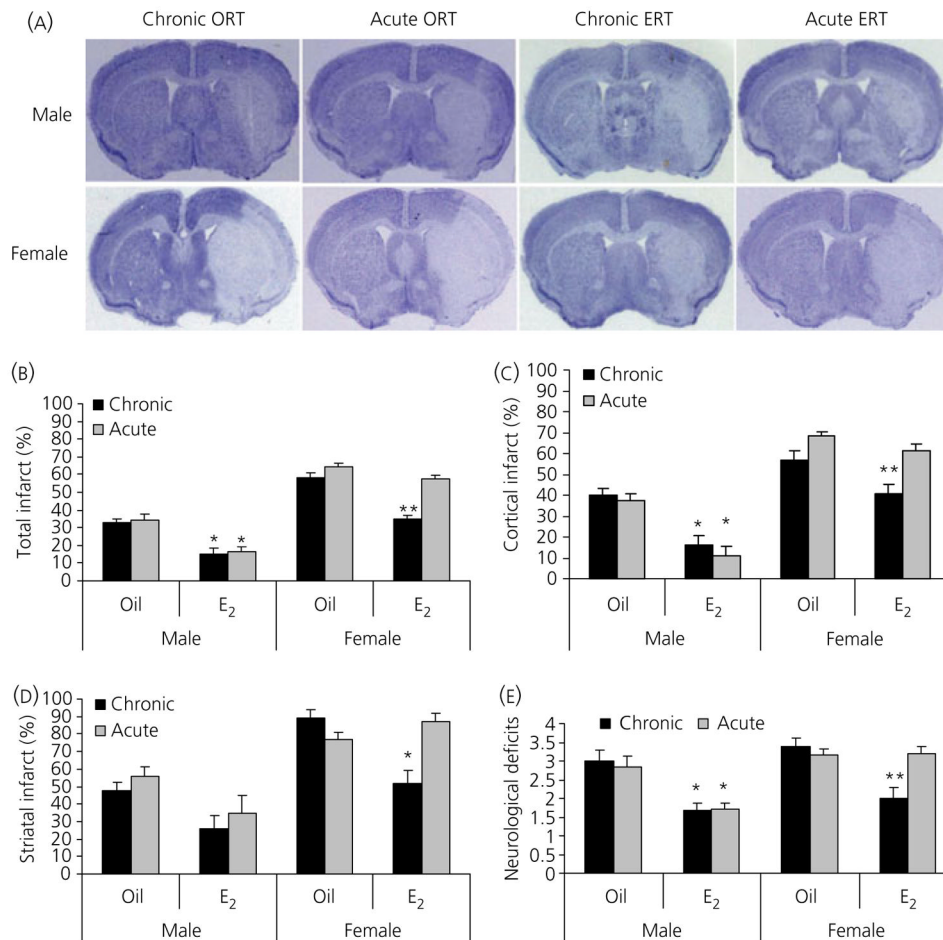


Fig. 2. Effect of 17β-oestradiol (E₂) replacement on infarct size in mice. (A) Representative image of a cresyl violet (CV)-stained brain for each group. The area lacking staining (white) reflects the infarct. (B–D) Quantification of infarct volumes based on CV staining in total hemisphere (B), cortex (C) and striatum (D). (E) Neurological deficit scores at 4 h of stroke. In all figures: *P < 0.05 versus oil-treated counterparts in males; **P < 0.05 versus chronic vehicle or acute E₂-treated female mice; n = 9–12 animals per group. ORT, oil pellets replacement therapy; ERT, oestrogen replacement therapy.

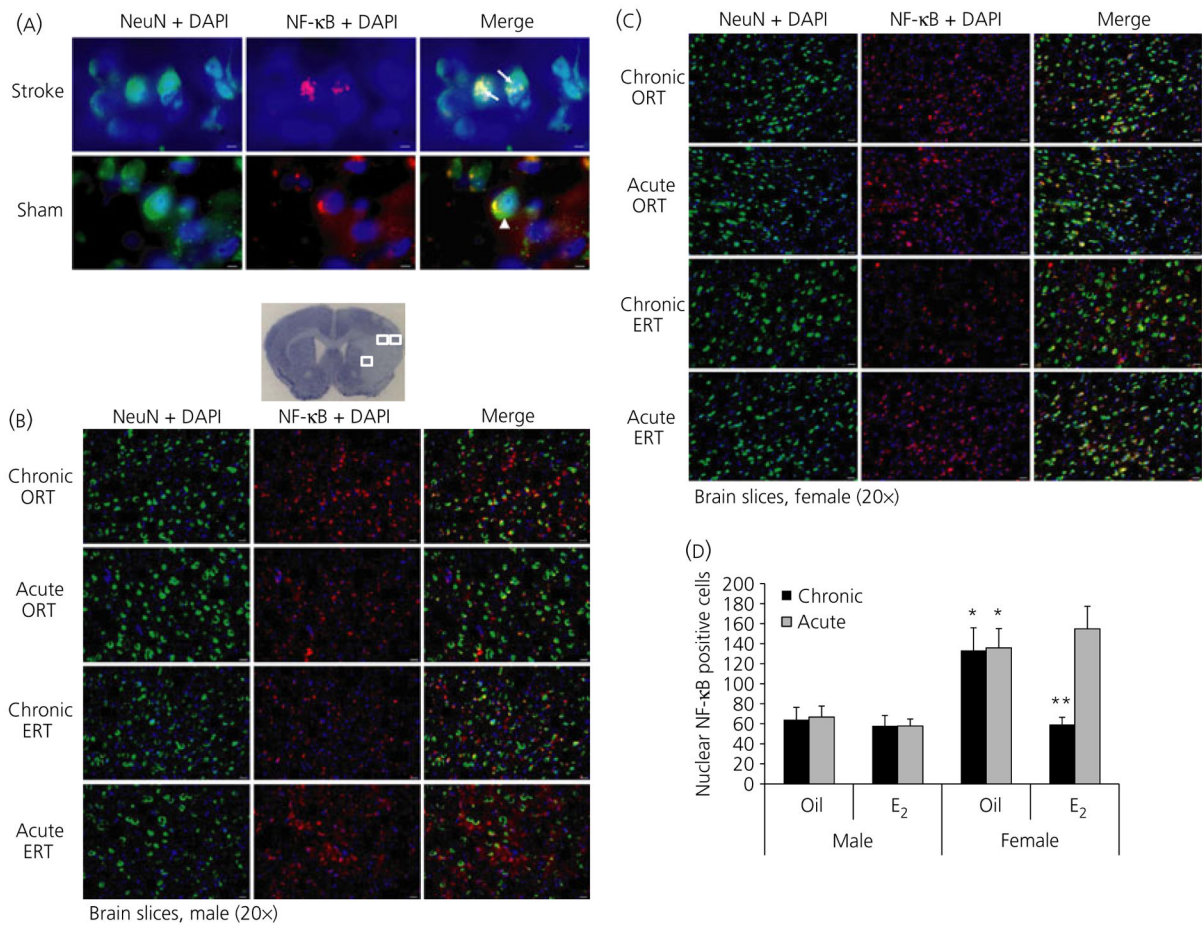


Fig. 3. Immunofluorescence of nuclear factor kappa B (NF- κ B), neuronal nucleus (NeuN) and 4',6-diamidino-2-phenylindole (DAPI). (A) Co-localisation of NF- κ B and NeuN (NF- κ B translocation positive cells indicated as arrows; negative cells as arrow heads) in the nucleus (upper panel; stroke groups) and the cytosol (lower panel; sham groups) ($\times 100$ magnification; scale bar = $20 \mu\text{m}$). (B,C) Co-labelling of NF- κ B and NeuN in aged male (B) and female (C) mice brain after stroke. Boxed areas in a cresyl violet stained slice illustrate the three examined regions in each slice ($\times 20$ magnification; scale bar = $50 \mu\text{m}$). (D) Semi-quantification of co-localisation of NF- κ B and NeuN in the nucleus. Each column stands for nine fields from six animals. *P < 0.05 versus oil or E₂ counterparts in males. **P < 0.05 versus chronic oil or acute E₂-treated group. ORT, oil pellets replacement therapy; ERT, oestrogen replacement therapy.

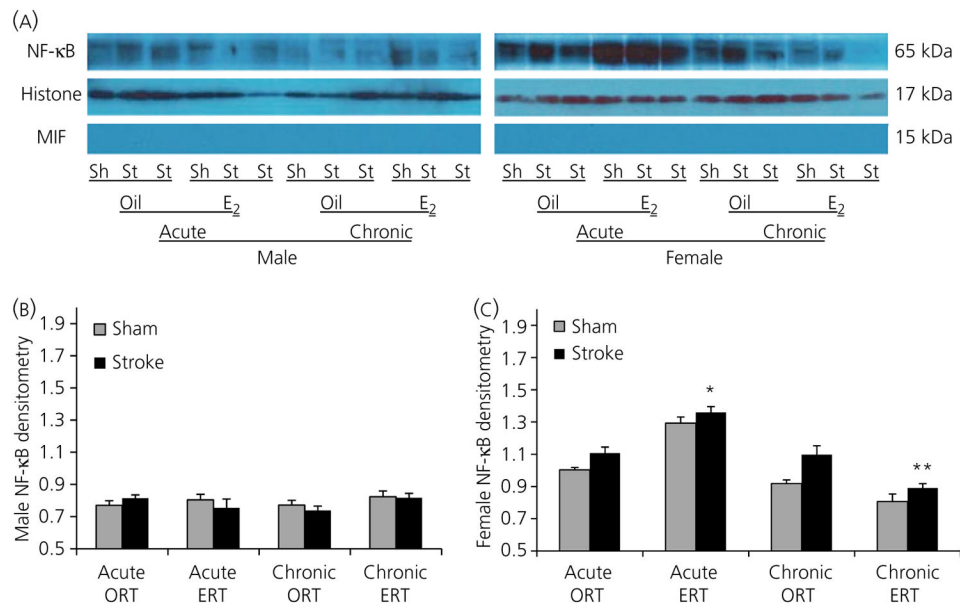


Fig. 4. Nuclear factor kappa B (NF- κ B) expression in the nucleus after stroke. (A) Representative western blots of NF- κ B protein levels in the nuclear fraction of brain homogenates from male (right) and female (right) mice 24 h after stroke onset. Histone H3 was used as a loading control and migration inhibitory factor (MIF) confirmed the lack of cytosolic contamination. (B,C) The optical density of samples in males (B) and females (C) was expressed as the ratio of NF- κ B bands to control bands (Histone). Six stroke and six sham brains were randomly aliquoted into three separate experiments for statistical analysis. * $P < 0.05$ versus stroke mice in acute oil pellets replacement therapy (ORT) group; ** $P < 0.05$ versus stroke mice in acute oestrogen replacement therapy (ERT) or chronic ORT group. Sh, sham; St, stroke.

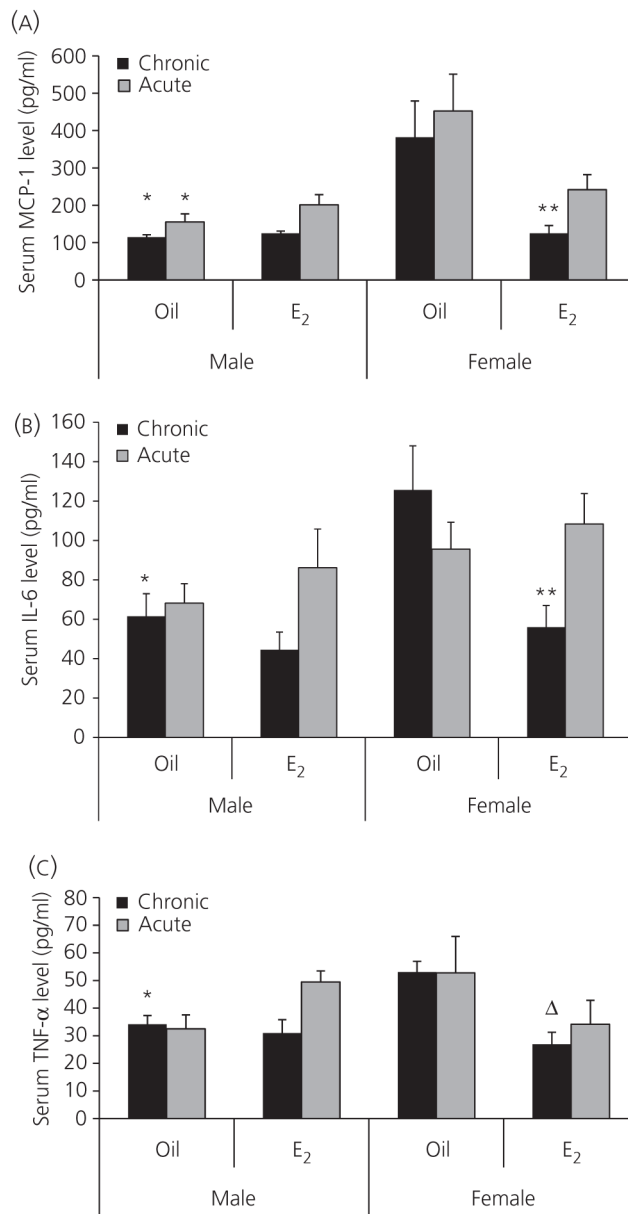


Fig. 5. Serum levels of monocyte chemoattractant protein (MCP)-1 (A), interleukin (IL)-6 (B) and tumour necrosis factor (TNF)- α (C) after stroke. * $P < 0.05$ versus female counterparts; ** $P < 0.05$ versus chronic oil or acute 17β -oestradiol (E_2)-treated group in females; $\Delta P < 0.05$ versus chronic oil group in females ($n = 6$ per group).

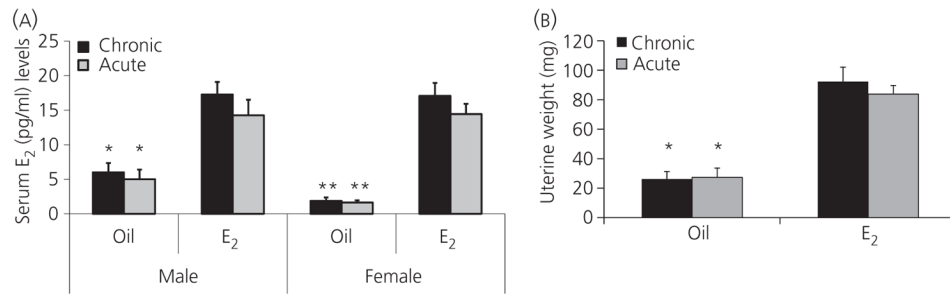


Fig. 6. Serum levels of 17β -oestradiol (E_2) and uterine weights after middle cerebral artery occlusion. (A) Serum E_2 levels in stroke mice. * $P < 0.05$ versus E_2 -treated counterparts in males. ** $P < 0.01$ versus E_2 -treated counterparts in females ($n = 6$ per group). (B) Uteruses of both oil and E_2 -treated female mice were separated, cut, and weighed after brains were removed. All the uteruses were handled and measured by the same investigator ($n = 6$ per group). * $P < 0.05$ versus E_2 -treated counterparts.

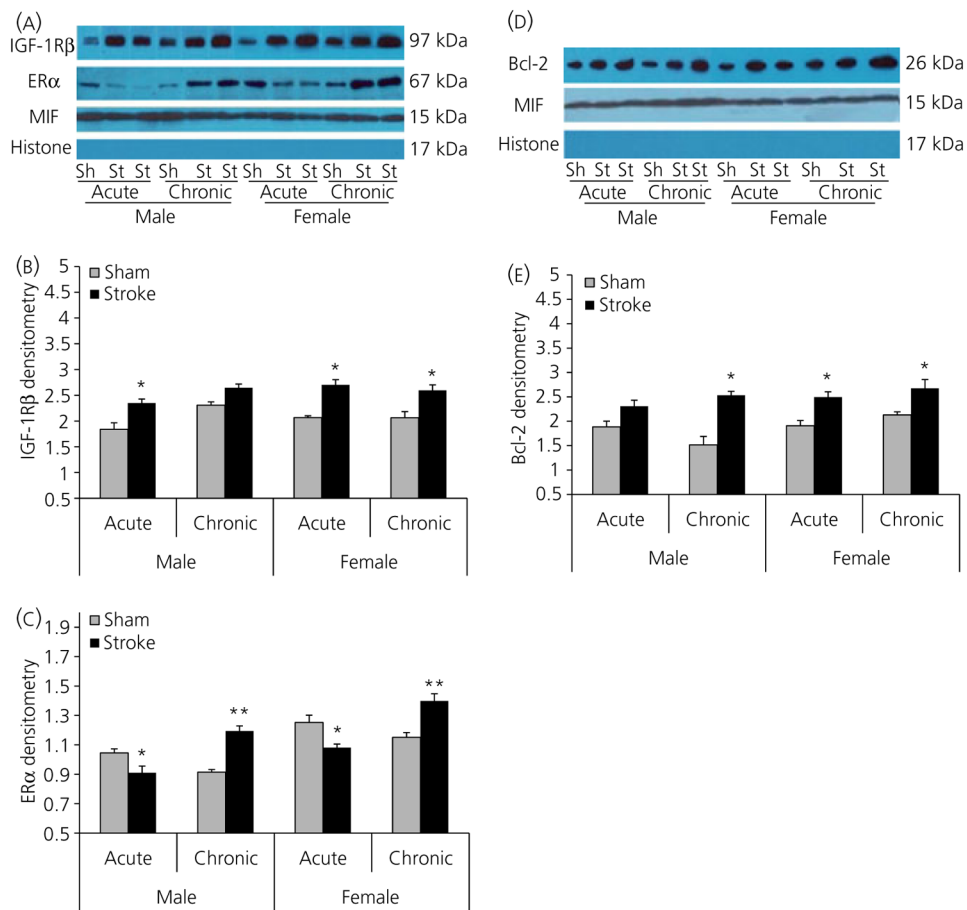


Fig. 7. Western blots of insulin-like growth factor (IGF)-1R β , oestrogen receptor (ER) α and Bcl-2 in each group. (A) Expression of IGF-1R β and ER α in the cytosolic fraction of brain homogenates from E₂-treated male and female mice 24 h after stroke onset. Migration inhibitory factor (MIF) was used as a loading control and Histone H3 analysis confirmed the lack of nuclear contamination. (B,C) The optical density of samples in (A) was expressed as the ratio of IGF-1R β (B) and ER α (C) bands to control bands (MIF). (B) *P < 0.05 versus sham groups. (C) *P < 0.05 versus acute sham groups; **P < 0.05 versus chronic sham groups. (D) Expression of Bcl-2 in the cytosolic fraction of brain homogenates from E₂-treated male and female mice 24 h after stroke onset. MIF was used as a loading control and Histone H3 confirmed the lack of nuclear contamination. (E) The optical density of samples in (D) was expressed as the ratio of Bcl-2 bands to control bands (MIF). *P < 0.05 versus sham groups. Six stroke and sham brains were randomly aliquoted into three separate experiments for statistical analysis. Sh, sham; St, stroke.

Table 1
Physiological Measurements in Each Group After Middle Cerebral Artery Occlusion MCAO).

Group	pH	PaCO ₂ (mm Hg)	PaO ₂ (mm Hg)	Glucose (mg / dl)	Mean arterial blood pressure (mmHg)	HCO ₃ (mM)	Body weight (g)
C ORT - M	7.38 ± 0.02	38.1 ± 2.9	105.3 ± 7.8	128 ± 12	67 ± 4	26.6 ± 2.3	41.8 ± 5.0
A ORT - M	7.35 ± 0.05	38.3 ± 4.3	89.3 ± 7.8	130 ± 21	76 ± 8	25.2 ± 1.4	45.7 ± 6.0
C ERT - M	7.41 ± 0.03	39.9 ± 4.4	90.7 ± 14.2	131 ± 14	67 ± 7	25.2 ± 2.8	42.6 ± 5.2
A ERT - M	7.38 ± 0.03	41.0 ± 4.6	97.3 ± 5.8	130 ± 5	65 ± 3	26.1 ± 1.5	37.3 ± 3.5
C ORT - F	7.40 ± 0.04	39.1 ± 4.3	91.3 ± 19.8	126 ± 8	79 ± 3	24.5 ± 1.1	31.8 ± 4.1
A ORT - F	7.38 ± 0.01	39.8 ± 3.8	81.7 ± 7.6	110 ± 20	69 ± 5	26.1 ± 3.2	32.7 ± 1.5
C ERT - F	7.38 ± 0.03	40.0 ± 3.7	92.7 ± 6.9	105 ± 16	71 ± 6	26.8 ± 2.5	33.6 ± 4.7
A ERT - F	7.38 ± 0.06	37.4 ± 2.8	92.7 ± 4.2	115 ± 17	65 ± 6	25.0 ± 2.3	33.3 ± 2.7

No differences were seen in physiological variables between oil- and E₂-treated mice of either sex prior to (not shown) and 60 min after MCAO, except body weight (*P < 0.05 versus male counterpart) (n = 4 per group, except body weight group, where n = 6 per group). C, chronic; A, acute; M, male; F, female; ORT, oil pellets replacement therapy; ERT, oestrogen replacement therapy.