Estrogen and experimental ischemic stroke; a systematic review.

Claire L Gibson, PhD[1,2], Laura Gray, MSc[2], Sean P Murphy, PhD[1,2], and Philip MW Bath, MD, FRCP[2]

[1]Institute of Cell Signalling, [2]Institute of Neuroscience, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK.

Correspondence addressed to Dr Claire Gibson, Division of Stroke Medicine, D Floor, South Block, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK.

Phone: +44 (0) 115 875 4513

Fax: +44 (0) 115 875 4506

claire.gibson@nottingham.ac.uk

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Short Title: Estrogen and experimental ischemic stroke

Abstract

Estrogen is believed to provide females with endogenous protection against cerebrovascular events although clinical trials studying long-term hormone replacement have yielded disappointing results. In contrast, estrogen may be neuroprotective following experimental ischemia. We performed a systematic review of controlled experimental studies that administered estrogen prior to, or following, cerebral ischemia and measured lesion volume. Relevant studies were found from searching PubMed, Embase and Web of Science. From 161 identified publications, 26 studies using 1,247 experimental subjects were analysed using the Cochrane Review Manager software. Estrogen reduced lesion volume in a dose-dependent manner, following either transient ($P < 0.001$) or permanent ($P < 0.001$) ischemia and whether it was given before or up to 4 hours after ischemia onset; no studies assessed efficacy for later time periods. The effect size for estrogen decreased with increasing quality scores for studies of transient ischemia. Estrogen reduced lesion volume when administered to ovariectomised females and young adult males, but had no effect in intact females. Limited data were present for aged animals and the full dose-response relationship was not available in all experimental groups. On the basis of these data, estrogen is a candidate treatment for ischemic stroke, although further pre-clinical studies are also warranted.

Keywords: estrogen, estradiol, ischemia, neuroprotection, stroke, systematic review.

Introduction

Prior to the menopause, women have a lower risk of stroke relative to men of the same age (Kannel and Thom, 1994; Sacco et al., 1997). Following the menopause, the incidence of stroke in women rapidly increases (Wenger et al., 1993), coincident with diminished circulating levels of estrogen and progesterone. Thus, steroid hormones appear to provide females with a certain degree of endogenous protection against stroke occurrence and stroke damage.

Experimental studies also support the concept that steroid hormones provide females with endogenous protection against stroke. These animal studies demonstrate that; females suffer less ischemic damage than males, the protection in females is absent following ovariectomy, and exogenously applied estrogen reduces ischemic damage in males and females (for reviews, see Murphy et al., 2004; Simpkins et al., 2005). Such observations have been replicated across a variety of species, strains, and research laboratories, and the outcomes from experimental animal studies have been fundamental in supporting the need for clinical trials of estrogen.

Hormone replacement therapy (HRT) has been widely tested in clinical trials aimed at reducing the occurrence of vascular events. Whilst some studies reported a significant reduction in stroke occurrence after HRT (Finucane et al., 1993; Henderson et al., 1991), others found no significant benefit (Bushnell et al., 2001; Pedersen et al., 1997; Petitti et al., 1998; Viscoli et al., 2001). More recently, the Women's Health Initiative (WHI) trial assessing the effect of HRT on incidence and outcome after stroke and other events, was halted prematurely because of increased hazard (Wassertheil-Smoller et al., 2003). A systematic review of completed clinical trials found that HRT was associated with an elevated risk of stroke, which was ischemic in type and of increased severity (Bath and Gray, 2005).

The discrepancy in findings between long-term clinical use (HRT increases stroke) and short-term use in pre-clinical models of ischemia (estrogen may be neuroprotective) findings remains unexplained. Key factors include the relationship between the design of

clinical and experimental studies and interpretation of the latter in terms of dose, timing of administration, sex, and age.

In order to evaluate the neuroprotective potential of estrogen we have performed a systematic review to investigate the neuroprotective properties of estrogen on lesion volume following experimental stroke, including with regards to timing of treatment, therapeutic dose, and effectiveness according to sex and age.

Materials and methods

Study Identification

Experimental controlled studies of the effects of exogenous estrogen on infarct size in animal models of stroke were identified from Pubmed, Embase, and Web of Science by searching for all articles published by the end of 2004. Additional publications were identified from reference lists of all identified publications and non-systematic review articles (Garcia-Segura et al., 2001; Green and Simpkins, 2000; Hurn and Brass, 2003; Hurn and Macrae, 2000; McCullough and Hurn, 2003; Wise, 2002; Wise et al., 2001; Yang et al., 2001). The search strategy employed the following keywords: estrogen or estradiol, ischaemia, and cerebral.

Data Extraction

Two authors (CG, LG) independently extracted data from relevant publications on animal species, number, gender and estrogen status, model if ischemia (permanent, transient), intervention (estrogen dose, timing relevant to induction of ischemia), and infarct volume (mm^3, % of normal brain, mean, standard deviation). Where the number of animals per group was reported as a range, the lowest numerical value given was used. If studies used multiple groups, e.g. to assess dose-response relationships, then the data from each group were individually extracted for analysis. Infarct volumes were classified as total, and, if available, subcortical and cortical. Occasionally, numerical data were not reported in text and these were extracted from enlarged, photocopied figures.

The methodological quality of each study was assessed using an 8-point 'STAIR' (1999) rating, as previously described (Horn et al., 2001; Willmot et al., 2005). One point was given for written evidence of each of the following criteria; presence of randomisation; monitoring of physiological parameters; assessment of dose response relationship; assessment of optimal time window; blinded outcome measurement; assessment of outcome at days 1-3; assessment of outcome at days 7-30; combined measurement of lesion volume and functional outcome.

Data Analysis

The data were analysed as forest plots using Cochrane Collaboration Review Manager (RevMan Version 4.2) software; an example is given in figure 1. Results are given as standardised mean difference (SMD) which allows the merging of data measured on different scales, with 95% confidence intervals (CI). A random effects model was used because statistical heterogeneity was likely to be present due to the use of different protocols. Statistical heterogeneity was assessed with a χ^2 test. Study data were grouped by pre-specified criteria: experimental model (permanent or transient) and location of lesion (total, cortical, sub-cortical).

In order to examine the effects of study characteristics and potential sources of heterogeneity on outcome, stratified meta-analyses were performed with experiments grouped according to: (i) trial quality score; (ii) population grouping - all animals, adult males, ovariectomised females, intact females, aged females, aged males; (iii) lesion location; (iv) estrogen dose; and (v) timing of estrogen administration in relation to onset of ischemia.

A random effects model was used because statistical heterogeneity was likely to be present due to the use of different protocols. Sensitivity analyses were performed to identify possible sources of heterogeneity including: study quality, lesion location, population grouping, dose range, and timing of estrogen administration in relation to onset of ischemia. Publication bias was assessed using Egger's asymmetry test (STATA function "metabias" Egger et al., 1997). Significance was set at $P < 0.05$.

Results

Design of Studies

The literature search identified 161 potential articles, although a large number of these were excluded for the reasons given in Figure 2. The characteristics of the remaining 26 studies are reported in Table 1. All of the included studies reported the effect of exogenously applied estrogen on infarct volume following cerebral ischemia. The 26 studies represented the outcome from 9 independent research groups. Within the 26 studies, data from a total of 1,247 experimental subjects were included for analysis.

The majority of studies employed a model of transient focal ischemia (17 studies) with 9 studies reporting the effect of estrogen following permanent focal ischemia. A study of estrogen following global ischemia (Horsburgh et al., 2002) was excluded since data on lesion volume were not reported.

Various rat strains (Wistar, Sprague-Dawley, Spontaneously Hypertensive, and Reproductively Senescent) were used in 24 out of the 26 included studies; 2 studies used mice. Methodological design was variable as far as drug administration was concerned. Several routes of administration (subcutaneous, intraperitoneal, intravenous, intracerebroventricular) were used with first dose timings in relation to onset of ischemia varying from 10 days prior, to 4 hours following.

In terms of outcome measure, lesion volume was assessed by histological staining and reported as: lesion volume (mm^3), % of total cross-sectional area, or % of ipsilateral non-ischemic total/region. An exception to this was the study by Shi et al (2001), which used structural MRI to determine lesion size.

Publication bias was present for studies reporting the effect of estrogen administration on lesion volume in permanent (Egger's test $P = 0.047$) and transient (Egger's test $P = 0.011$) models.

Reported Study Quality

The median STAIR rating for included articles was 3 (range 1-4 out of 8). Animals allocated treatment by randomisation was reported in only two studies (Toung et al., 1998, 2004). All but two studies (Wise, 2000; Yang et al., 2003) reported the monitoring of physiological parameters, with most only monitoring body temperature. Eight studies assessed dose-response relationships (Choi et al., 2004; Culmsee et al., 1999; Dubal et al., 1998, 2001; Rusa et al., 1999; Toung et al., 1998; Vergouwen et al., 2000; Yang et al., 2000) and eight studies investigated the optimal time window of estrogen administration (Dubal et al., 1998; Fukada et al., 2000; McCullough et al., 2001; Rau et al., 2003; Saleh et al., 2001; Simpkins et al., 1997; Vergouwen et al., 2000; Yang et al., 2000). All but two studies (Saleh et al., 2001; Shi et al., 2001) assessed outcome at days 0-3; one study (Simpkins et al., 1997) assessed outcome at days 7-30, and another (Santizo et al., 2002) reported combined measurement of lesion volume and functional outcome. The latter was the only study to report outcome measures being blinded to treatment.

Following permanent ischemia a beneficial effect of estrogen treatment was observed regardless of reported quality score ($P < 0.001$, Figure 3A). The majority of studies utilised the model of transient focal ischemia and the beneficial effect of estrogen administration decreased with increasing reported quality score (Figure 3B). In fact, there was no beneficial effect of estrogen administration in studies that obtained a quality score of 4 which was the highest score awarded ($P = 0.39$).

Infarct Volume According to Hormonal Status/Age

The effects of estrogen on total, cortical and subcortical lesion volume were analysed (Figure 4) according to population groupings based on hormonal status/age (i.e. males, ovariectomised females, intact females, aged females, aged males).

Estrogen administration significantly reduced total lesion volume following permanent ($P < 0.001$) and transient ischemia ($P = 0.002$, Figure 4A). Following permanent ischemia estrogen had a slightly greater beneficial effect in ovariectomised females compared

to males. Following transient ischemia, the largest effect of estrogen on total lesion volume was also seen in ovariectomised females. However, estrogen treatment appeared to have a detrimental effect when administered to intact females (Figure 4A) although this was not significant ($P = 0.06$). For total lesion volume, hormonal status accounted for a significant amount of between-group heterogeneity following both permanent ($\chi^2 = 61.3$, df = 35, $P = 0.004$) and transient ischemia ($\chi^2 = 107.7$, df = 40, $P < 0.00001$).

Estrogen treatment significantly reduced cortical lesion volume when measured following permanent ($P < 0.001$) and transient ischemia ($P < 0.001$, Figure 4B). The effects of estrogen on cortical lesion volume following permanent ischemia have only been reported in ovariectomised females and between-group heterogeneity analysis was not possible. Following transient ischemia, estrogen treatment was most effective at reducing cortical lesion volume in aged females, although this was based on data extracted from one published study (Figure 4B). For cortical lesion volume following transient ischemia, hormonal status accounted for a significant portion of between-group heterogeneity ($\chi^2 = 21.9$, df = 11, $P = 0.02$).

In addition, estrogen treatment significantly reduced subcortical lesion volume when measured following permanent ($P < 0.001$) and transient ischemia ($P < 0.001$, Figure 4C). Again, the effects of estrogen on subcortical lesion volume following permanent ischemia were only examined in ovariectomised females. Following transient ischemia (one study), estrogen treatment was most effective at reducing cortical lesion volume in aged females (Figure 4C). For subcortical lesion volume following transient ischemia, hormonal status accounted for a significant portion of between-group heterogeneity ($\chi^2 = 23.6$, df = 13, $P = 0.04$).

Estrogen dose

Taking all animals into account, i.e. regardless of age/hormonal status, estrogen significantly reduced lesion volume following permanent ($P < 0.001$) and transient ischemia ($P < 0.004$,

Figure 5A). The effect of estrogen on reducing lesion volume appeared to increase with increasing dose. Estrogen dose accounted for a significant amount of between-group heterogeneity following both permanent (χ^2 = 61.21, df = 34, P = 0.003) and transient ischemia (χ^2 = 113.56, df = 41, P < 0.001).

In an attempt to identify any gender differences in response to different estrogen doses, the data were analysed for males (Figure 5B) and ovariectomised females (Figure 5C). In males, the effect of estrogen on reducing lesion volume was increased with increasing dose (Figure 5B). However, in ovariectomised females no studies have reported the effect of administering estrogen within the dose range (3.1 - 30.0 mg/kg) that gave the best result in males.

Timing of estrogen administration in relation to onset of ischemia

Both pre- and post-ischemic estrogen administration were effective at reducing lesion volume in all animals following permanent (P < 0.001) and transient ischemia (P = 0.006, Figure 6A). However, no studies examined the effects of administering estrogen later than 6 hours following onset of ischemia. Timing of estrogen administration accounted for a significant portion of between-group heterogeneity following both permanent (χ^2 = 61.2, df = 34, P = 0.003) and transient ischemia (χ^2 = 112.2, df = 40, P = <0.001).

For permanent ischemia, estrogen only significantly reduced lesion volume in males when administered -21 days to -2 hours prior to surgery (Figure 6B). Due to the small number of studies investigating the neuroprotective effects of estrogen in males following transient ischemia it is not possible to identify an ideal time of administration.

In ovariectomised females, estrogen treatment had the greatest effect when administered immediately following (i.e. 0h to +2h) permanent ischemia (P < 0.001) or immediately before (i.e. -2h to 0h) transient ischemia (P = 0.02, Figure 6C). However, the majority of studies administered estrogen between 21 days and 2 hours prior to ischemia where it was effective at reducing lesion volume following permanent and transient ischemia.

Discussion

This systematic review has found that estrogen reduces lesion volume following either transient or permanent ischemia. However, following transient ischemia, the effect of estrogen treatment decreased with increased reported quality score. When studies were grouped according to hormonal status and age, estrogen treatment was only effective in ovariectomised females and young adult males. Importantly, studies of estrogen in aged animals and its administration later than 4 hours following ischemia were lacking. Additionally, adequate dose response relationships of estrogen has not been fully investigated in all experimental groups.

This review focuses only on the effect of estrogen on lesion volume following stroke, due to insufficient data regarding other outcomes such as behaviour; only one study examined the functional benefits of estrogen administration in both males and females up to 14 days following experimental ischemia (Li et al., 2004). However, lesion volume is of limited value when interpreting whether a treatment is beneficial. Functional outcome, in combination with histopathological outcome, is as important in terms of assessing benefit (STAIR, 1999). Infarct size may (Rogers et al., 1997) or may not (Hattori et al., 2000; Reglodi et al., 2003; Wahl et al., 1992) correlate with neurological impairment. The STAIR criteria (STAIR, 1999) emphasise the need to determine functional effects of interventions.

Whilst estrogen appeared to be effective in young adult males and ovariectomised females, it may have been hazardous in intact females. Assessments in ovariectomised females are not an ideal model of post-menopausal women. In experimental studies young female animals are subjected to a sudden surgical removal of estrogen supply, whereas humans who enter the menopause, at a mean age of 51 years in the USA (Creasman et al., 2003), experience a slow decline of circulating estrogen levels over months or even years. Importantly, there is a substantial lack of data in the literature reporting the effects of estrogen administration in aged animals, yet the incidence of stroke in humans is strongly age-

dependent (Wolf et al., 1992). Although studies on aged animals are not usually undertaken because of expense, these represent but a fraction of the costs of failed clinical trials.

The neuroprotective effects of estrogen are dose-dependent, with studies administering estrogen at doses considered to be either physiological (Dubal et al., 1998) or pharmacological (Simpkins et al., 1997). In males and ovariectomised females the effect of estrogen on reducing lesion volume increased with increasing dose. However, the higher doses administered in males have not been applied to ovariectomised females. Thus, there is a need to further explore the dose-response relationship of estrogen and neuroprotection. Additionally, the long term benefits of estrogen treatment are not fully understood. In fact, where histological outcome was assessed at 7 days following ischemia, physiological levels of estrogen had a detrimental effect on cell death in the vulnerable CA1 region of the hippocampus (Harukuni et al., 2001). Importantly, no studies reported administering estrogen at later than 4 hours following ischemia. As the majority of those at greatest risk of suffering a stroke are not receiving HRT (i.e. men and post-menopausal women), such a time window of application is not clinically relevant. Thus, this review emphasises the need for bi-directional translational research between experimental and clinical studies.

Although it is well recognised that premenopausal women have an endogenous protection against vascular events, the importance of other steroid hormones must also be considered. For example, progesterone has been shown to reduce lesion volume following both permanent (Gibson et al., 2005) and transient experimental ischemia (Gibson and Murphy, 2004; Murphy et al., 2002), and also to improve functional outcome (Gibson and Murphy, 2004). Although experimental studies have shown that progesterone is neuroprotective when administered prior to or following cerebral ischemia in males and ovariectomised females, no clinical studies to date have investigated the effects of progesterone on outcome following stroke.

Systematic review and meta-analysis are fundamental tools in the interpretation of the effectiveness of a particular treatment across a large number of studies. However, they do have various limitations. Firstly, analyses can only include available data, usually only

available in published studies. Negative or neutral studies are less likely to be published so the results meta-analysis may overstate effect size. In fact, Egger's asymmetry test did suggest publication bias was likely which could have resulted from the lack of reporting neutral or negative studies. Consequently, the benefits of estrogen on infarct volume might have been either over or underestimated. Additionally, non-publication will limit available information on the effect of treatment within certain protocol aspects such as dose or time of administration.

In respect of study quality, we could only judge the studies as reported; a low quality score could reflect either that the authors did not undertake that procedure, e.g. randomise animals to treatment, or that they did not report it. Authors, journal reviewers and editors need to be more stringent in reporting key methodological details. A particular area of concern is that the majority of studies did not report randomisation and/or blinded assessment of outcome. Lack of randomisation and blinded assessment of outcome are key sources of bias and will over-emphasise treatment efficacy. Additionally, whilst most studies reported physiological parameters, the majority only assessed body temperature. Although body temperature is a useful indicator, additional physiological parameters also give invaluable information about the physiological effects of a particular treatment which could be crucial when considering the design of clinical trials.

There is a need to determine precisely the cellular targets and molecular events triggered by estrogen administration. Animal studies can be fundamental in determining the optimal estrogen treatment in terms of timing and dosing amongst different populations. However, this review has highlighted fundamental areas where experimental evidence demonstrating a protective effect of estrogen is lacking. It is these areas which should focus research in order to determine the most relevant design of further clinical studies.

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Figure Legends

FIG. 1 A forest plot showing total lesion volume following permanent ischemia. N, number of animals; SMD, standardized mean difference; 95% CI, 95% confidence interval; SD, standard deviation.

FIG. 2 Search process showing reasons for exclusions of studies. N = number of studies.

FIG. 3 SMD and 95% CI by reported STAIR score following permanent (**A**) and transient (**B**) ischemia. N = number of animals; S = number of studies.

FIG. 4 SMD and 95% CI for total (**A**), cortical (**B**), and subcortical (**C**) lesion volume. Data shown following permanent and transient ischemia is grouped according to age and hormonal status of animals. OV, ovariectomised; N, number of animals; S, number of studies.

FIG. 5 SMD and 95% CI for total lesion volume following permanent and transient ischemia in all animals (A), males (B), and ovariectomised females (C). Data is grouped according to dose of estrogen given (mg/kg, where reported). N, number of animals; S, number of studies.

FIG. 6 SMD and 95% CI for total lesion volume following permanent and transient ischemia. Data is grouped according to time of first administration of estrogen. N, number of animals; S, number of studies.

Table 1 Characteristics of Included Studies.

Study	Year	Species	Sex/hormonal status	Age	Model of ischemia	Dose range (mg/kg)	1st dose timing (hr/day)	Route	Measure of infarct	Other outcome(s)
Alkayed	2000	WR	M, RSF	A, N	F, T	0.025	-7d	s.c.	%	CBF
Choi	2004	SDR	M	N	F, T	1.0 - 10.0	-24h	i.p.	%	
Culmsee	1999	NM	M	N	F, P	0.0003 - 30.0	-24h	s.c., i.p.	mm^3	
Dubal	1998	SDR	OF	N	F, P	NR	-7f	s.c.	mm^3	
Dubal	2001	SDR	OF	A, Y	F, P	NR	-7d	s.c.	mm^3	
Dubal	2001	CM	OF	N	F, P	NR	-7d	s.c.	mm^3	
Fan	2003	SDR	OF	N	F, T	0.1	-2h	s.c.	%	
Fukada	2000	SHR	OF	N	F, T	0.2	-21d	s.c.	mm^3	CBF
Green	2001	SDR	OF	N	F, T	0.1	-2h	s.c.	%	
Hawk	1998	SDR	M	N	F, T	NR	-7d	s.c.	%	
Liu	2002	SDR	OF	N	F, T	0.1	-2h	s.c.	mm^3	CBF
McCullough	2001	WR	M	N	F, T	1.0	0h	i.v.	%	CBF
Rau	2003	SDR	OF	N	F, P	NR	-7d	s.c.	mm^3	

Rusa	1999	WR	OF	N	F, T	0.25 - 1.0	-7d	s.c.	%	CBF
Saleh	2001	SDR	M	N	F, P	NR	-0.5h	i.c.v.	%	
Santizo	2002	SDR	OF	N	F, T	0.1	-7d	i.p.	%	NS
Shi	2001	SDR	OF	N	F, T	0.1	-2h	s.c.	MRI	CBF
Simpkins	1997	SDR	OF	N	F,T	1.0	-24h	s.c.	%	
Toung	1998	WR	M	N	F, T	0.025 - 1.0	-7d	s.c.	%	
Toung	2000	WR	M	N	F, T	0.025	-10d	s.c.	%	
Toung	2004	RSFR	RSF	A	F, T	0.025	-7d	s.c.	%	
Vergouwen	2000	WR	M, F, OF	N	F, T	0.1 - 1.0	-10d	i.v.	mm^3	
Wise	2000	SDR	OF	N	F, P	0.18	-7d	s.c.	mm^3	
Yang	2000	SDR	F, OF	N	F, P	0.1	+4h	s.c.	%	CBF
Yang	2003	SDR	OF	N	F, P	0.1 - 5.0	+0.5h	s.c.	mm^3	
Zhang	1998	SDR	OF	N	F, T	1.0	+0.6h	i.v.	%	

Abbreviations: WR, Wistar rats; SDR, Sprague-Dawley rats; NM, NMRI mice; CM, C57 mice; SHR, spontaneously hypertensive rats; RSFR, reproductively senescent female rats; M, males; OF, ovariectomised females; RSF, reproductively senescent females; F, intact females; A, aged; N, normal adult; Y, young;

F, focal; T, transient; P, permanent; NR, not reported; s.c., subcutaneous; i.p., intraperitoneal; i.c.v, intracerebroventricular; i.v., intravenous; CBF, cerebral blood flow; NS, neurological score.