Sex Differences in Cardiac and Cerebral Damage after Hypovolemic Cardiac Arrest

EGIDIJUS SEMENAS
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Abstract

Resuscitation from haemorrhagic shock and the subsequent circulatory arrest remains a major clinical challenge in the care of trauma patients. Numerous experimental studies in sexually mature animals have shown a gender dimorphism in response to trauma and haemorrhagic shock. The first study was designed to evaluate sex differences in outcome after resuscitation from hypovolemic circulatory arrest. We intended to examine innate sex differences, and chose to study sexually immature animals. The study showed that cerebral cortical blood flow was greater, blood-brain-barrier was better preserved and neuronal injury was smaller in female as compared to male piglets. The second study demonstrated that female sex was associated with enhanced haemodynamic response, cardioprotection, and better survival. This cardioprotective effect was observed despite comparable estradiol and testosterone levels in male and female animals, indicating an innate gender-related cardioprotection. In both studies (I and II) female sex was associated with a smaller increase in the cerebral expression of inducible and neuronal nitric oxide synthase (iNOS and nNOS). Thus in the study III we tested the hypothesis that exogenously administered 17β-estradiol (E2) could improve neurological outcome by NOS modulation. The results showed that compared with the control group, animals in the E2 group exhibited a significantly smaller increase in nNOS and iNOS expression, a smaller blood-brain-barrier disruption and a mitigated neuronal injury. There was also a significant correlation between nNOS and iNOS levels and neuronal injury. A hypothesis if female-specific cardioprotection may be attributed to a smaller NOS activity was tested in study IV. The animals received methylene blue (MB) during CPR, but were otherwise treated according to the same protocol as studies I-II. The female-specific cardioprotection could be attributed to a smaller NOS activity, but NOS inhibition with MB did not improve survival or myocardial injury, although it abated the difference between the sexes.

Keywords: Cardiopulmonary resuscitation, sex, haemorrhage, neuronal damage, estradiol, hypertonic saline, nitric oxide, methylene blue

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To the memory of my mother
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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<tr>
<td>ANOVA</td>
<td>Analyses of variance</td>
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<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
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<td>CA</td>
<td>Cardiac arrest</td>
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<tr>
<td>CCBF</td>
<td>Cerebral cortical blood flow</td>
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<td>CPR</td>
<td>Cardiopulmonary resuscitation</td>
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<tr>
<td>CO</td>
<td>Cardiac output</td>
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<td>CPP</td>
<td>Cerebral perfusion pressure</td>
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<td>E2</td>
<td>17β-estradiol</td>
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<td>HR</td>
<td>Heart rate</td>
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<td>HS</td>
<td>Hypertonic saline</td>
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<td>HSD</td>
<td>Hypertonic saline with dextran</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MB</td>
<td>Methylene blue</td>
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<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
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<tr>
<td>PARP1</td>
<td>Poly(ADP-ribose) polymerase 1</td>
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<tr>
<td>PEA</td>
<td>Pulseless electrical activity</td>
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<tr>
<td>PWCP</td>
<td>Pulmonary capillary wedge pressure</td>
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<tr>
<td>ROSC</td>
<td>Restoration of spontaneous circulation</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>SEM</td>
<td>Standard error of mean</td>
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<td>VF</td>
<td>Ventricular fibrillation</td>
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<td>Ventricular tachycardia</td>
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<td>8-iso-PGF2α,</td>
<td>8-isoprostaglandin F2α</td>
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<td>15-keto-dihydro-PGF2α</td>
<td>15-keto-13, 14-dihydro-prostaglandin F2α</td>
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Introduction

Trauma is a major public health problem. It is one of the leading causes of death world-wide. Globally, injury is the seventh leading cause of death, with 5.8 million deaths attributable to trauma in 2006 [1]. Trauma is a leading cause of years of life lost before the age of 75 as it affects primarily the young subjects. It is estimated that by year 2020 deaths from injury are predicted to increase up to 8.4 million world-wide [2] and uncontrolled haemorrhage will be responsible for 30% of these deaths [3].

Motor vehicle injuries lead the list of injury deaths during childhood and adolescence. Injuries cause 44% deaths among 1-4 year old children and 3 times more deaths than the next leading cause, congenital anomalies. For the rest of childhood and adolescence up to the age of 19 yr., 65% of deaths are due to injuries.

Despite improvements in resuscitation techniques and surgical management of trauma victims, survival rates remain extremely low in trauma patients who exsanguinate to cardiac arrest (CA) [4] [5] and have not improved significantly during the last decades [6] [7]. Thus, resuscitation from haemorrhagic shock and subsequent cardiac arrest is a major clinical challenge in the care of patients after motor vehicle accidents, gunshot or stab wounds, and combat. Nevertheless, even after successful restoration of spontaneous circulation (ROSC) following cardiac arrest, the morbidity and mortality depend mainly on the recovery of neurological function [8].
1. Background

1.1 Paediatric cardiopulmonary arrest

Cardiac arrest occurs in 2–6% of children admitted to a paediatric intensive care unit [9-11]. About 16 000 children per year (8-20 in 100 000 children per year) suffer out-of-hospital cardiac arrest in the United States [12]. Thus, the rate of in-hospital cardiac arrest is approximately 100-fold higher than the rate of out-of-hospital arrest [13]. The true incidence of paediatric cardiac arrest is difficult to estimate, because of inconsistent definitions and difficulty assessing pulselessness in children. According to Utstein guidelines [14] pulseless cardiac arrest is defined as the cessation of cardiac mechanical activity, determined by the absence of a palpable central pulse, unresponsiveness, and apnoea. Although outcomes from paediatric cardiac arrest were once considered dismal [15], recent data indicate that paediatric cardiopulmonary resuscitation (CPR) is improving [16-20]. Among children resuscitated for their cardiac arrest, about 8% survive to discharge after out-of-hospital cardiac arrest, and 27% following an in-hospital arrest [19, 20]. This is contrast previous report where performing CPR in children was considered futile [21].

1.1.1 Out-of-hospital cardiac arrest

Survival is much worse following paediatric out-of-hospital arrests than in hospital arrests [22, 23]. Recently Donoghue et al. [12] reviewed 41 articles on paediatric out-of-hospital cardiac arrest. The incidence of out-of-hospital arrest ranged from 2.6 to 19.7 per year per 100 000 paediatric population. In this series, 30% had ROSC, 24% survived to hospital admission, and 12% survived to discharge. This review also showed that almost 22% of cardiac arrests in children were associated with trauma, making this one of the most common causes of pre-hospital cardiac arrest and death [24, 25]. Traumatic injury is usually considered in subgroups of blunt trauma, penetrating trauma and “other”, including hypoxic events such as drowning, hanging and electrocution [26]. Survival following traumatic cardiac arrest is poor in adults and children [7, 27]. The high rate of bleeding associated with hypovolaemic cardiac arrest is unlikely to respond to conventional cardiopulmonary resuscitation [28].
1.2.1 In-hospital cardiac arrest

Respiratory failure and circulatory shock are the most common causes of in-hospital cardiac arrests [10, 19, 20]. Treatment of in-hospital paediatric cardiac arrest with CPR results in return of spontaneous circulation among approximately 43-64% of patients, and approximately 25-33% survive to hospital discharge [19, 20]. Almost 75% of survivors to hospital discharge have favourable neurological outcomes.

Data from the American Heart Association’s National Registry of Cardiopulmonary Resuscitation have established that children have better outcomes than adults following in-hospital cardiac arrest. The superior paediatric survival rate reflects a higher survival rate among children with asystole or pulseless electrical activity (PEA) compared to adults (24 versus 11%). Moreover, the superior survival rate among children is primarily due to much better survival among infants and pre-school children compared with older children or adults [20].

1.2 Etiologic and pathophysiologic categories of cardiac arrest

Cardiac arrests can result from many causes. The three most common aetiologies are respiratory arrests, ischemic arrests, and arrhythmogenic arrests. Asphyxial cardiac arrests are caused by acute hypoxia and/or hypercarbia and are the most common in children [19, 29, 30]. Ischemic arrests are precipitated by inadequate myocardial blood flow, in children most commonly due to shock from hypovolemia, sepsis, or myocardial dysfunction. Arrhythmogenic arrests are precipitated by ventricular tachycardia and fibrillation, or asystole. Asystole and pulseless electric activity are the most common rhythms observed after in-hospital paediatric cardiac arrest though ventricular fibrillation (VF) or pulseless ventricular tachycardia (VT) is not rare [19]. Approximately 25% of children suffering in-hospital cardiac arrest suffer from VF or VT [30]. Out-of-hospital VF cardiac arrest more often occurs in adolescents (15%) compared to infants and children (4-5%) [31].

1.3 Outcome prognostication after cardiac arrest

Prediction of neurologic outcome following paediatric cardiac arrest is a difficult task. Data suggest that burst-suppression pattern on post-arrest electroencephalogram is sensitive and specific for poor neurologic outcome [32]. Increased neuron-specific enolase concentrations in plasma are associated with poor neurologic outcomes when measured 48 h after ROSC.
in children suffering from cardiac arrest. Elevated serum S-100β concentrations are associated with increased mortality [33]. However, neither single test nor any combined scores has been validated to predict outcome with a high level of sensitivity and specificity.

1.4 Cardiac arrest during anaesthesia
Cardiac arrest attributable to anaesthesia occurs from 0.5 to 1 case per 10,000 interventions. Paediatric cases show a higher incidence (1.4 – 4.6 per 10,000) [34, 35], especially in new-borns and infants [36, 37]. Majority of cardiac arrests may be associated with a pre-existing patient condition, mainly cardiovascular disease or severe trauma. Excessive surgical bleeding comprised 70% of surgical procedure related deaths [38].

1.5 Phases of resuscitation: pre-arrest, no flow, cardiopulmonary resuscitation low flow and post-resuscitation
There are four phases of cardiac arrest: 1) pre-arrest, 2) no flow, 3) low flow and 4) post-resuscitation [39-42]. Interventions to improve outcome from paediatric cardiac arrest should be targeted to optimise therapies according to the cause, timing, duration, intensity and phase of resuscitation.

1.5.1 Pre-arrest phase
The pre-arrest phase is an ideal phase to decrease mortality and morbidity from cardiac arrest by decreasing the incidence of cardiac arrest events. The precipitating causes of most paediatric cardiac arrests are acute respiratory insufficiency and circulatory shock [19].

1.5.2 Cardiac arrest (no flow) and CPR (low flow) phase
During the no-flow phase of pulseless cardiac arrest focus is on early recognition of cardiac arrest and fast initiation of basic and advanced life support. The goal of effective CPR is to achieve optimal coronary and cerebral perfusion and blood flow to critical organs during the low-flow phase.

Over-ventilation and interruptions in compressions during adult and paediatric CPR are common and can substantially compromise venous return and cardiac output, contributing to worse survival [43, 44]. Medications are recommended to use during CPR for both paediatric and adult advanced life
support. Medications commonly used for paediatric cardiac arrests are summarised in guidelines [45, 46].

1.5.3 Post-resuscitation phase

The post-resuscitation phase is a high-risk period for brain injury, myocardial dysfunction and extension of ischemia/reperfusion injuries. Interventions during this phase are aimed at minimising reperfusion injury and supporting cellular recovery. Meticulous management of temperature, glucose, blood pressure, acid-base state, and oxygenation are important in order to improve outcome.

Temperature. Mild induced hypothermia (32–34°C) can improve outcome for comatose adults after resuscitation from VF cardiac arrest [47]. However, extrapolation of results from adult hypothermia studies to children is difficult. Hyperthermia following paediatric brain injury is common and is associated with poor neurological outcome [48].

Myocardial support. After successful ROSC post-arrest myocardial stunning is usually observed. The condition is similar to sepsis-related myocardial dysfunction. However the optimal treatment of the post-arrest myocardial dysfunction has not been established.

A goal-directed protocol improved outcomes following adult cardiac arrest [49]. Improvements in post-resuscitation care are a promising strategy to improve outcome from paediatric cardiac arrest, too.

1.6. Brain injury in children after cardiac arrest

Post-ischemic brain injury in children is similar to injury in adults. Nevertheless, there are two important differences: 1) the mechanism of cardiac arrest, as respiratory causes far outnumbering cardiac causes in children, 2) the developing brain has different vulnerability and potential for repair compared with the mature brain [50]. The histology of cerebral injury following asphyxia differs from that seen in VF. Safar et al. [51, 52] showed that brain damage from asphyxial cardiac arrest in dogs is more severe than the damage found after equivalent periods of cardiac arrest from VF. Morimoto et al. [53] described increased prevalence of brain oedema (diagnosed by head computer tomography) in adults remaining comatose following respiratory-induced cardiac arrest compared with cardiac arrhythmia–induced cardiac arrest. Selective vulnerability and delayed neuronal death is observed with both injury types. Thus, although an asphyxial injury may be more severe than a cardiac-mediated injury for an equivalent period of ischemia, asphyxial injuries may respond similarly to neuroprotective therapies.
1.7 Sex differences in trauma and cardiac arrest

Basic research studies suggest that sex influence survival following traumatic shock. In animal models of haemorrhagic shock, exposure to female sex hormones has been shown to improve humoral and cell-mediated immunity, leading to improved survival [54-56]. A review by Wohltmann et al. [57] suggests that women have better survival after trauma. However, two large clinical database reviews by Napolitano et al. [58] and Gannon et al. [59] failed to show any difference in outcomes between sexes. The independent effect of sex on paediatric trauma is largely unknown. Haider et al. [60] showed that adolescent girls had lower mortality than boys following traumatic shock. However, this effect was not seen in preadolescent children.

Clinical studies have found gender differences in the incidence of cardiac arrest, survival, and outcome [61, 62]. Compared with men, women have a lower incidence of sudden cardiac arrest, are more likely to be successfully resuscitated, and reach the hospital alive more frequently than men [61]. However, sex-specific long-term survival and functional recovery have not been intensively studied.

Sex difference in survival after cardiac arrest has been observed in several human studies [61, 63]. Higher estradiol serum levels were associated with higher mortality. Besides, the risk of death increased with serum levels in critically ill adult population [64]. However, in two clinical studies a higher survival rate after trauma was observed in adolescent girls [60, 65]. No difference in survival was found in prepubescent children, suggesting the potential involvement of female sex hormones in survival after traumatic injury.

1.8 Estradiol in cardiac arrest

The potential neuroprotective role of sex hormones in acute brain ischemia following cardiac arrest is of a great therapeutic interest in order to improve neurological outcome after CA. Numerous experimental studies have confirmed that ischemic cell death pathways are sexually dimorphic [66, 67]. This dimorphism can be due to gender specific differences in gene expression or by the effects of sex hormones. Indeed, numerous studies have shown that estradiol is neuroprotective in brain injury [68]. Long term pre-treatment with estradiol and other estrogens affords a robust neuroprotection in male and female rodents subjected to global ischemia [69, 70]. The mechanisms through which it protects the neurones are not fully known, but likely involve antioxidant actions, promotion of blood flow, modification of nitric oxide synthase expression and nitric oxide production [68, 71]. Techniques aimed at reducing neuronal nitric oxide expression appear to
selectively protect the brain in males [72], but enhance ischemic injury in females [73].

1.9 Methylene blue and cardiac arrest

The efficacy of methylene blue (MB) added to hypertonic-hyperoncotic solution was shown on short time survival in extended normovolemic circulatory arrest [74]. Reactive oxygen species and other free radicals, such as nitric oxide (NO), have long been suspected of being involved in cerebral injury occurring during cardiac arrest and especially reperfusion [75]. The protective effect of methylene blue after global ischemia is considered to be due to inhibition of nitric oxide synthesis [76, 77] and inhibition of formation of free oxygen radicals and superoxide generation [78, 79]. The pathophysiological effects of MB are nevertheless not limited to NOS inhibition. MB can inhibit monoamine oxidase [80] and decrease platelet activation and thromboxane A2 levels, as well as endothelial prostacyclin I2 production [81]. It has reductive properties, but in higher doses it can oxidise haemoglobin to methemoglobin.

2.0 Nitric oxide synthases and brain

Cerebral ischemia/reperfusion injury triggers multiple and distinct cell signalling pathways, which may lead to cell survival or damage [82]. NO is a potent vasodilator and is released during normal synaptic activity, as well as during ischemia. It is also considered to be an important factor in the development of functional and reactive hyperaemia and subsequent blood-brain barrier disruption [83]. Furthermore, there is strong evidence showing that NO is involved in biological cascades that lead to an increased neurotoxicity after cerebral ischemia [84]. NO is synthesised by three distinct NO synthase (NOS) isoforms (neuronal, inducible, and endothelial NOS). nNOS is the most abundant isoform in central nervous system. Neurons produce NO by activation of nNOS [85] and glial cells – by iNOS activation [86]. Cerebral ischemia up-regulates all three isoforms [87] Previous studies suggest that NO resulted from activation of iNOS and nNOS is detrimental, and that NO derived from endothelial NOS is beneficial [86, 87]. Whereas post-ischemic iNOS induction may exacerbate neuronal damage by enhancing the NO dependant release of excitatory neurotransmitters, eNOS activation is suggested to improve post-ischemic blood flow by enhancing collateral circulation and preventing microvascular plugging by platelets and leukocytes [88].
2. Aims of the study

- To evaluate the innate effect of gender in cerebral injury parameters after haemorrhage and ventricular fibrillation, irrespective of the choice of fluid resuscitation regimen (Paper I).
- To evaluate the innate effect of gender in hemodynamic parameters and tissue injury after haemorrhage and ventricular fibrillation, while using hypertonic-hyperoncotic fluid for resuscitation (Paper II).
- To evaluate if 17β-estradiol (E2) improves the neurological outcome by NOS modulation in experimental haemorrhagic cardiac arrest (Paper III).
- To evaluate if intervention with methylene blue at the onset of reperfusion to inhibit nitric oxide synthase and nitric oxide action improves recovery of left ventricle dysfunction and diminishes cardiac damage due to differences in NOS expression in pre-pubertal piglets (Paper IV).
3. Materials and Methods

The Uppsala Institutional Review Board for Animal Experimentation approved all studies. They were the prospective, randomised, laboratory animal studies. The piglets were handled according to the guidelines of the Swedish National Board for Laboratory Animals and the European Convention of Animal Care. Qualified and experienced personnel in the experimental laboratory at the University Hospital took the care of animals.

3.1 Animals

The animals in all studies were 11-16 weeks old and without any evidence of illness (Table 1). They were Swedish country triple breed piglets of both genders. All piglets were obtained from the same provider and were delivered to the laboratory on the morning of the experiment. All animals were kept fasting with unrestricted access to water during the night before the experiment.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of piglets</th>
<th>Weight (mean±SD)</th>
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<tr>
<td>Paper I</td>
<td>32 (14 males and 18 females)</td>
<td>26.7 ± 2.6 kg</td>
</tr>
<tr>
<td>Paper II</td>
<td>21 (12 male and 9 female piglets)</td>
<td>26.3 ± 2.2 kg</td>
</tr>
<tr>
<td>Paper III</td>
<td>39 (20 male and 19 female piglets)</td>
<td>25.9 ± 1.9 kg</td>
</tr>
<tr>
<td>Paper IV</td>
<td>20 (10 male and 10 female piglets)</td>
<td>25.5 ± 2.9 kg</td>
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3.2 Anaesthesia

General anaesthesia was induced with an intramuscular injection of 6 mg·kg⁻¹ tiletamine-zolazepam mixed with 2.2 mg·kg⁻¹ xylazine and 0.04 mg·kg⁻¹ atropine, and an intravenous injection of 20 mg morphine and 100 mg ketamine. Anaesthesia was maintained with intravenous infusions of pentobarbital at 8 mg·kg⁻¹·h⁻¹ and morphine at 0.5 mg·kg⁻¹·h⁻¹. Baseline hemodynamic parameters and sympathetic response were used to assess and adjust the anaesthetic depth before haemorrhagic shock and cardiac arrest. Absences of motor (involuntarily movements of extremities) and
sympathetic response to painful stimuli were the signs of adequate anaesthesia. The piglets were the paralysed with pancuronium bromide at 0.25 mg·kg\(^{-1}\)·h\(^{-1}\), tracheotomized and mechanically ventilated with 30\% oxygen at 25 breaths min\(^{-1}\) (Servo 900C, Siemens-Elema, Solna, Sweden). Tidal volume was adjusted to yield arterial PaCO\(_2\) between 5.0 and 5.5 kPa. The capnogram and peripheral oxygen saturation were displayed continuously (CO\(_2\)SMO Plus-8100, Novametrix, Wallingford, CT, USA), as were leads II and V5 of electrocardiogram.

### 3.3 Fluid administration
Fluid losses were compensated by infusion of 30 ml·kg\(^{-1}\) of acetated Ringer’s solution during the first hour of preparation, followed by a continuous infusion of 2.5\% glucose-electrolytes solution 8 ml·kg\(^{-1}\)·h\(^{-1}\), and acetated Ringer’s solution 10 ml·kg\(^{-1}\)·h\(^{-1}\).

### 3.4 Preparation and procedures
A laser-Doppler flowmetry probe (MT B500 – 43, Periflux PF 2B Laser-Doppler Flowmeter, Perimed, Stockholm, Sweden) was inserted through a 10-mm burr hole between the occipito-parietal and coronal sutures for continuous monitoring of the cerebral blood flow. A 18-gauge arterial catheter was advanced into the aortic arch via a branch of the right external carotid artery. A 14-gauge central venous catheter was inserted through the right external jugular vein. A 7 Fr Swan-Ganz catheter was inserted into the pulmonary artery. The left internal jugular vein was cannulated and a 18-gauge catheter was advanced in the cephalad direction for blood sampling. A 14-gauge catheter (Radifocus Introducer II, Terumo Corporation, Tokyo, Japan) was inserted to the right femoral artery for blood withdrawal. A median sternotomy was performed for open-chest cardiopulmonary resuscitation. A minor vesicotomy was performed and a urinary catheter was introduced into the bladder for urine collection. In Paper III Camino™ intraparenchymatous pressure transducer (Camino M420, Camino Laboratories, San Diego, CA, USA) was inserted through 2-mm burr hole over the right hemisphere for measurement of intracranial pressure.
3.5 Measurements

3.5.1 Hemodynamic variables

Hemodynamic parameters, including lead II and V5 electrocardiogram recordings, heart rate (HR), systemic arterial blood pressure, right atrial pressure, and pulmonary artery pressure were continuously monitored (Solar 8000 monitor, Marquette Medical System, Milwaukee, WI, USA) and recorded (BioPac MP100, Acknowledge, version 3.8.1 software, BioPac Systems, Santa Barbara, CA). Cardiac output (CO) was measured as a mean of three recordings using thermodilution technique. Pulmonary capillary wedge pressure (PWCP) was recorded at the same time points as cardiac output. Coronary perfusion pressure was calculated as the difference between diastolic aortic pressure and the simultaneously measured right atrial pressure. Stroke volume was derived from a conventional formula (CO/HR).

3.5.2 Cerebral variables

Cerebral cortical blood flow was recorded every 5 seconds by a computer and presented as a fraction of the steady-state baseline flow before induction of ventricular fibrillation. Cerebral oxygen extraction ratio was calculated as the ratio of arterial-jugular venous oxygen content difference with the arterial oxygen content. In Paper III Intracranial pressure was measured and recorded at baseline, after exsanguination, once every minute during CPR, and 5, 15, 30, 60, 120 and 180 minutes after restoration of spontaneous circulation (ROSC). Cerebral perfusion pressure (CPP) was calculated as the difference between mean arterial pressure and intracranial pressure at the same time points.

3.6 Samples

Hemodynamic variables, temperature and laser Doppler flow measurements were recorded at baseline, after haemorrhage, once every minute during CPR, 5, 15, 30, 60, 120, and 180 min after ROSC. Samples of arterial and internal jugular venous blood were taken for blood gas analysis and acid-base balance (ABL 300 Radiometer, Copenhagen, Denmark) at baseline, after haemorrhage, 5, 15, 30, 60, 120, and 180 min after ROSC. Oxygen saturation and haemoglobin were determined simultaneously on Hemoximeter OSM-3 (Radiometer, Copenhagen, Denmark). Jugular venous blood samples were collected at baseline, 5, 15, 30, 60, 120, and 180 min after ROSC, the plasma was separated and stored at -70°C until analysed for 8-iso-PGF2α, 15-keto-dihydro-PGF2α and for protein S-100β (indicator of neurological injury). Plasma glucose, lactate and electrolyte concentrations
were determined (ABL 700, Radiometer, Copenhagen, Denmark) at baseline, 120, and 180 min after ROSC. The plasma obtained after centrifugation was stored at -20°C until analysed for estradiol and testosterone. In Paper III estradiol concentration in plasma was determined at baseline, 60 and 180 mins after ROSC.

3.7 Analytical methods

Plasma concentrations of isoprostane 8-iso-PGF$_{2\alpha}$ (indicator of peroxidative injury) and 15-keto-dihydro-PGF$_{2\alpha}$ (indicator of inflammation) were measured according to previously described methods. The detection limit for 8-iso-PGF$_{2\alpha}$ was 23 pmol/L. Prostaglandin F$_{2\alpha}$ is one of the major prostaglandins formed at the site of inflammation and is quantified by measurement of 15-keto-dihydro-PGF$_{2\alpha}$ with a detection limit of 45 pmol/L. Myocardial and cerebral damage were assessed by serum concentrations of cardiac troponin I and astroglial protein S100. Serum S100 levels were measured by an immunoluminometric assay (LIA-mat, Sangtec® 100) with a detection limit of 0.02 ng/mL and a cutoff level of <0.12 ng/mL. Troponin I were measured with monoclonal/polyclonal mass immunoassays (ADVIA Centaur, Bayer) with normal values below 0.12 µg/L for troponin I. Estradiol and testosterone measurements were performed on an automated immunoassay system (Modular E170, Roche Diagnostics GmbH, Mannheim, Germany).

3.8 Experimental protocol

After preparation piglets were allowed to stabilise for 1 hr, after which baseline measurements were made. Heparin (60 IU/kg) was injected intravenously and haemorrhage was started from the catheter in the femoral artery. Blood was collected in citrate-phosphate-dextrose anticoagulant solution containing 450 ml bags (Baxter Healthcare Corporation, Deerfield, Illinois, USA) for later re-infusion. A controlled, fixed pressure haemorrhage model was used. The speed of haemorrhage was controlled by adjusting the blood flow through the femoral catheter. Bleeding was stopped when a mean arterial blood pressure of 35 mm Hg was reached. Total blood volume in a piglet was calculated as 67 ml/kg. Ventricular fibrillation was then induced in all animals with a 50-Hz, 20- to 40-V transthoracic alternating current application via two subcutaneous needles placed on both sides of thorax. FiO$_2$ of 1.0 was delivered during cardiac arrest and CPR.

After 2 min of circulatory arrest, open-chest CPR was started with a compression rate of 60-80 per min.
See specific protocol description (section 3.8.1, 3.8.2, 3.8.3 and 3.8.4).
ROSC was defined as return of co-ordinated electrical activity resulting in a pulsatile cardiac rhythm with a systolic blood pressure of >60 mm Hg for at least 10 consecutive minutes. After 5 min of ROSC, FiO₂ was reset at 0.3. If arterial pH was less than 7.20 or the base deficit more than 10 mmol/l at 5 min and 15 min after ROSC, acidosis was corrected with Tris buffer mixture 1mmol/kg and 0.5 mmol/kg respectively, and by increasing the minute ventilation. Minute ventilation was adjusted to maintain an arterial PaCO₂ within the range of 5.0 and 5.5 kPa. Dobutamine was administered, as required, to maintain systolic blood pressure >70 mmHg. Three hours after ROSC the last measurements were made and piglets received a lethal intravenous injection of potassium. In all the animals within 5 min after death the skull was opened in prone position and the brain was taken out for histopathological analysis. Samples of myocardium were taken immediately and stored in formaldehyde for later histological analysis (Paper IV).

3.8.1 Protocol I

After 1 min of CPR, all the piglets received 0.4 units/kg of vasopressin (Arg⁸-vasopressin, PolyPeptide Laboratories, Wolfenbuttel, Germany) via the right atrial catheter. Piglets of both sexes then randomly received one of the following three resuscitation fluids: whole blood 12 ml·kg⁻¹ (5 female and 5 male piglets), hypertonic saline (7.5%) with dextran (6%) solution (RescueFlow®, Biophausia, Uppsala, Sweden) (HSD) 3 ml·kg⁻¹ (5 female and 5 male piglets) or, whole blood 10 ml·kg⁻¹, acetated Ringer’s 10 ml·kg⁻¹ and methylene blue (Metyltioninklorid™ 10 mg/ml, Apoteket, Umeå, Sweden) 2.5 mg·kg⁻¹ (4 male and 6 females). The infusions were given through the right atrial catheter and continued for 20 minutes.

After 2 min of open-chest CPR, an internal monophasic counter-shock was delivered at the energy level of 20 J (Medtronic Physio-Control, Seattle, WA). Subsequent defibrillatory shocks were increased to 40 J and continued up to 8 minutes. CPR was discontinued if ROSC was not achieved during this period. If ROSC was not achieved after 6 shocks, a 20 µg/kg bolus of epinephrine was administered through the right atrial catheter. Twenty three minutes after cardiac arrest the resuscitation fluid infusion rates were reduced as follows: animals receiving whole blood were given the remaining blood (collected during exsanguination) over 1 hr; HSD was stopped and the remaining blood was given over 1 hr; acetated Ringer’s was stopped and the remaining blood was given over 1 hr, and 1.5 mg·kg⁻¹ methylene blue was administered IV over 40 min. The dose of MB was chosen after pilot experiments indicating the necessity of continuous infusions and a total dose of about 4 mg/kg.
3.8.2 Protocol II

After 1 min of CPR, all the piglets received 0.4 units/kg of vasopressin (Arg8-vasopresin, PolyPeptide Laboratories, Wolfenbuttel, Germany) via the right atrial catheter. At the same time all piglets received 3 ml·kg⁻¹ hypertonic saline (7.5%) with dextran (6%) solution (RescueFlow®, Biophausia, Uppsala, Sweden), infused through the right atrial catheter over 20 minutes. After 2 min of open-chest CPR, an internal monophasic counter-shock was delivered at the energy level of 20 J (Medtronic Physio-Control, Seattle, WA). Subsequent defibrillatory shocks were increased to 40 J and continued up to 8 minutes. CPR was discontinued if ROSC was not achieved during this period. If ROSC was not achieved after 6 shocks, a 20 µg/kg bolus of epinephrine was administered through the right atrial catheter. Twenty three minutes after cardiac arrest the resuscitation fluid infusion rates were reduced as follows: HSD was stopped and the remaining blood was given over 1 hr.

3.8.3 Protocol III

After the haemorrhage E2 (water soluble, cyclodextrin encapsulated 17β-estradiol; E4389, Sigma, St.Louis) 50 µg/kg was given intravenously to estradiol group (n=10 males, and 9 females), while control group received equal amount of isotonic saline (n=10 males and 10 females). Ventricular fibrillation was induced in all animals after 5 min with a 50-Hz, 20- to 40V transthoracic alternating current application via two subcutaneous needles placed on both sides of thorax. FiO2 of 1.0 was delivered during cardiac arrest and CPR. After 4 min of circulatory arrest, open-chest CPR was started with a compression rate of 60-80 per min. At 5 min of CA all piglets received vasopressin 0.4 U/kg (Arg8-vasopresin, PolyPeptide Laboratories, Wolfenbuttel, Germany), amiodarone 0.5 mg/kg and hypertonic saline (7.5%) with dextran (6%) solution (RescueFlow®, Biophausia, Uppsala, Sweden) 3 ml×kg⁻¹ infusion through the right atrial catheter. The infusion was continued for 20 minutes. After 2 min of open-chest CPR, an internal monophasic counter-shock was delivered at the energy level of 20 J (Medtronic Physio-Control, Seattle, WA). Subsequent defibrillatory shocks were increased to 40 J and continued up to 15 minutes. CPR was discontinued if ROSC was not achieved during this period. If ROSC was not achieved after 6 shocks, vasopressin 0.4 U/kg bolus was administered through the right atrial catheter. Twenty three minutes after cardiac arrest the resuscitation fluid infusion rates were reduced as follows: HSD was stopped and the remaining blood was given over 1 hr.
3.8.4 Protocol IV

After 2 min of circulatory arrest, open-chest CPR was started with a compression rate of 60-80 per min. After 1 min of CPR, all the piglets received 0.4 units/kg of vasopressin (Arg8-vasopresin, PolyPeptide Laboratories, Wolfenbuttel, Germany) via the right atrial catheter. At the same time 3 ml·kg⁻¹ hypertonic saline (7.5%) with dextran (6%) solution was administered, with 2.5 mg·kg⁻¹ methylene blue (Metyltioninklorid™ 10 mg/ml, Apoteket, Umeå, Sweden) infusion over 20 min. The infusions were given through the right atrial catheter. After 2 min of open-chest CPR, an internal monophasic counter-shock was delivered at the energy level of 20 J (Medtronic Physio-Control, Seattle, WA). Subsequent defibrillatory shocks were increased to 40 J and continued up to 8 minutes. CPR was discontinued if ROSC was not achieved during this period. If ROSC was not achieved after 6 shocks, a 20 µg/kg bolus of epinephrine was administered through the right atrial catheter. Twenty three minutes after cardiac arrest the resuscitation fluid infusion rates were reduced as follows: HSD was stopped and the remaining blood was given over 1 hr, and 1.5 mg·kg⁻¹-methylene blue was administered IV over 40 min. The dose of methylene blue was chosen after pilot experiments indicating the necessity of continuous infusions and a total dose of about 4 mg/kg [89].

3.9 Neurological injury and immunochemistry

3.9.1 Fixation

One of the brain hemispheres was immersed in 4% buffered formalin and stored at 4° C for 1 week. Small tissue pieces (<3x5 mm) from cerebral cortex were cut and processed for histology or immunohistochemistry. The tissue pieces were dehydrated in a graded series of alcohol, rinsed in xylene, and embedded in low-temperature paraffin (56-58° C) according to a standard protocol. About 3 to 5 µm thick multiple sections (6 to 8) were cut from each tissue block and collected on glass slides. After deparaffinization, duplicate sections were stained with either Nissl (Cresyl violet) or hematoxylin and eosin using a commercial protocol.

3.9.2 Cerebral tissue injury (histological evidence)

Three-micrometer paraffin sections from identical tissue blocks from the cerebral cortex were cut and stained with Hematoxylin and eosin or Nissl for light to analyse cellular changes. The number of distorted neurons in one whole section was counted in each animal at least three times in a blinded fashion and the median values were recorded for data analysis.
3.9.3 Albumin immunostaining

Immunohistochemistry for albumin was performed on paraffin embedded (3-µm thick) sections using a sheep polyclonal anti-rat albumin antibody (Sigma, USA) and the streptavidin-HRP-biotin technique as described previously. The numbers of albumin-positive cells were counted in one identical area of the cortex from each animal, in a blinded fashion three times. The median value was used for the final calculation.

3.9.4 Immunohistochemistry of nitric oxide synthase

Immunostaining was performed on 3 µm thick paraffin sections using a monoclonal NOS antiserum as described previously [90]. In brief, the antibodies of inducible and neuronal nitric oxide synthases (iNOS and nNOS, respectively) and were diluted 1:5000 and applied for 48 h with continuous shaking at room temperature. The immune reaction was developed using a peroxidase-antiperoxidase technique and visualised at light microscopes. No immunoreactivity was detected in controls where the primary antibody step was omitted. The number of nNOS and iNOS positive cells in each group was counted in a blind fashion. In Paper III eNOS positive cells were analysed using the same technique as described for iNOS and nNOS.

3.9.5 Quantification of histological and immunohistochemical results (Papers I-III)

Counting of distorted or damaged neurones or immunopositive nerve cells have been used in numerous neuropathological articles earlier [91]. It is presumed that in a well-defined area, number of distorted neurones is proportional to injury severity and thus less number of damaged or distorted neurones represents neuroprotection. In histological sections, neurones could be easily identified because of their triangular shape. Glial cells are often round in shape and could be distinguished from neurones easily. Only NOS immunopositive neurones were counted. The non-neuronal cells even if they are positive were not included into the semiquantitative study. A leakage of albumin within the microenvironment of the brain denotes breakdown of the BBB because under normal circumstances, albumin is not present in the brain or cerebrospinal fluid compartments [91]. When albumin labelled neurones are seen it means that neurones are probably severely damaged and thus show uptake of albumin in their cell cytoplasm.
3.9.6 Histopathologic heart examination (Paper IV)

Immediately after sacrificing the piglets the hearts were fixed in 4 % neutral formaldehyde. Subsequently two samples were taken from each heart; one from the ventral part and one from the dorsal part of the left ventricle. Both samples were cut transversely to the longitudinal axis of the heart in order to give a view encompassing the entire wall including the epicardium and the endocardium. The specimens were embedded in paraffin and sections 3 – 5 µm thick and were cut with a regular microtome knife. Picro-Mallory [92] and polymer-based immunohistochemistry C9 complement (Leica Microsystems NcL-CCC9) [93] staining methods were used. These methods were chosen because they are able to detect early myocyte damage. In Picro-Mallory stain early myocyte damage is seen as contraction bands disrupting the ordinary cross striation of the myocyte. In C9 staining damage is seen as diffuse or granulated cytoplasmatic uptake of the reagent. All samples were examined without the microscopist knowing to which group the specimens belonged. Before examination a protocol was prepared. The protocol variables were 1) Cell damage Picro-Mallory stain 2) Cell damage C9, 3) Acute inflammatory cells, 4) Lymphocytic infiltration. Each variable was ranked: 0 = no finding, 1 = moderate finding and 2 = pronounced finding. Each rank value was added to each other in order to get a rank sum for each case.
4. Statistical analysis

In all studies p-values of <0.05 were considered statistically significant. All data were presented as mean ± standard error of mean (SEM). The following statistical software was used: GraphPad PRISM® version 4 and 5, GraphPad Software Inc., San Diego, CA, USA and StatView for Windows, SAS Institute Inc., version 5, Cary, NC, USA.

4.1 Paper I

Normally distributed parameters (except cortical cerebral blood flow, histological and immunohistochemical data) were analysed with 2-way repeated measures analyses of variance [94]. No interaction was observed between sex and fluid regimen groups. Data not accepted as normally distributed were analysed using Mann-Whitney test. In order to secure normally distributed data, statistical analysis of 8-iso-PGF2α and 15-keto-dihydro-PGF2α results were done after the logarithmic transformation. If significant sex groups’ effects were observed, the specific differences between groups were determined with one way ANOVA and a Bonferroni-Dunn post hoc test. Differences in the resuscitation rate among groups were assessed by Fisher’s exact test for independent samples. Chi-square test was used to evaluate survival.

4.2 Paper II

After the data had been shown to be normally distributed, all parameters (except cerebral cortical blood flow was analysed with 2-way repeated measures ANOVA. Data not accepted as normally distributed were analysed using Mann-Whitney test. If significant sex groups’ effects were observed, the specific differences between groups were determined with one way ANOVA and a Bonferroni-Dunn post hoc test. Differences in the resuscitation rate among groups were assessed by Fischer’s exact test. Survival analysis was performed by Kaplan and Meier method.
4.3 Paper III

Normally distributed parameters (all except histological and immunohistochemical data) were analysed with 2-way repeated measures ANOVA. Data not accepted as normally distributed were analysed using Mann-Whitney test. In order to secure normally distributed data, statistical analysis of 8-iso-PGF2α and 15-keto-dihydro-PGF2α results were done after logarithmic transformation. For data included in ANOVAs that were statistically significant, a Bonferroni-Dunn multiple comparison procedure was performed. When a statistically significant interaction between a group and the time factor occurred, one-way ANOVAs were performed separately at each time point in order to compare the response between the groups. Differences in the resuscitation rate among groups were assessed by Fischer’s exact test. Survival analysis was carried out using the method of Kaplan and Meier. Maximum eicosanoid values and the total number of distorted neurones were correlated to NOS levels using Pearson correlation.

4.4 Paper IV

Normally distributed parameters (except cerebral cortical blood flow and histopathologic data) were analysed with 2-way repeated measures ANOVA. Data not accepted as normally distributed were analysed using Mann-Whitney test. If significant sex groups’ effects were observed, the specific differences between groups were determined with one way ANOVA and a Bonferroni-Dunn post hoc test. Differences in the resuscitation rate among groups were assessed by Fischer’s exact test. Survival analysis was carried out using the method of Kaplan and Meier.
5. Results

5.1 Bleeding volume and time

There were no differences between female and male animals in baseline variables, or the duration or volume of haemorrhage in all studies.

Table 2. Haemorrhage volume and time in Papers I-IV.

<table>
<thead>
<tr>
<th>Gender/group</th>
<th>Duration of haemorrhage (mins) until a mean arterial blood pressure of 35 mm Hg was reached</th>
<th>Bleeding volume (% of calculated total blood volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paper I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=14)</td>
<td>16.4 ± 3.6</td>
<td>28.6 ± 3.5</td>
</tr>
<tr>
<td>Female (n=16)</td>
<td>19.3 ± 4.7</td>
<td>31.5 ± 5.5</td>
</tr>
<tr>
<td><strong>Paper II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=11)</td>
<td>15.9 ± 2.3</td>
<td>26.8 ± 4.3</td>
</tr>
<tr>
<td>Female (n=9)</td>
<td>17.3 ± 4.6</td>
<td>28.4 ± 5.7</td>
</tr>
<tr>
<td><strong>Paper III</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol (n=20)</td>
<td>14.1 ± 1.0</td>
<td>29.24 ± 4.76</td>
</tr>
<tr>
<td>Control (n=19)</td>
<td>14.0 ± 1.1</td>
<td>29.99 ± 4.36</td>
</tr>
<tr>
<td><strong>Paper IV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=10)</td>
<td>14.7 ± 1.1</td>
<td>28.6 ± 4.2</td>
</tr>
<tr>
<td>Female (n=10)</td>
<td>14.4 ± 0.9</td>
<td>27.5 ± 5.6</td>
</tr>
</tbody>
</table>

The values are presented as means ± SD.

5.2 Survival

5.2.1 Paper I

Two female piglets were excluded due to surgical bleeding from damaged intercostal veins and an accidental hole of right atrium during open-chest CPR, leaving 14 male and 16 female animals for the final analysis. ROSC was achieved in 11 out of 14 male piglets and in 10 out of 16 female piglets.
Survival was not different between sex groups (p=0.27). Males piglets that achieved ROSC needed 5 defibrillatory shocks (mean, range: 1–11) and female piglets needed 4 defibrillatory shocks (mean, range: 1–11).

5.2.2 Paper II

One male piglet was excluded from the study due to macroscopic signs of chronic pericarditis upon post-mortem examination, leaving 11 male and 9 female animals for the final analysis. ROSC was achieved in 7 of 11 male and 9 of 9 female piglets (p=0.09). No significant group differences were observed in the CPR duration or the number of defibrillatory shocks needed to achieve ROSC (Table 3). Kaplan-Meier survival curve and the log-rank test demonstrated an improved 3-hr survival in female compared with male animals (p=0.048).

Table 3. Resuscitation variables in Paper II.

<table>
<thead>
<tr>
<th>Resuscitation Variables</th>
<th>Male (n=11)</th>
<th>Female (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of deaths after ROSC</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Survival (number of survivors/total)</td>
<td>7/11</td>
<td>9/9</td>
</tr>
<tr>
<td>Number of defibrillatory shocks (mean, minimum – maximum)</td>
<td>10 (2-21)</td>
<td>5 (1-11)</td>
</tr>
<tr>
<td>Duration of cardiopulmonary resuscitation, mins (mean ±SD)</td>
<td>7 (5-10)</td>
<td>6 (4-8)</td>
</tr>
</tbody>
</table>

5.2.3 Paper III

ROSC was achieved in 19 out of 19 piglets in control group and in 18 out of 20 in estradiol group (p=1.0). No significant differences were observed in either CPR duration or the number of defibrillatory shocks needed to achieve ROSC between the groups (data not shown. In control group two male and two female piglets experienced VF and died at 16 and 80 mins, and at 12 and 78 mins, respectively, after ROSC. In estradiol group one male and two female piglets had an episode of VF and died at 137 mins, 10 and 168 mins, respectively, after ROSC.

5.2.4 Paper IV

One male piglet was excluded from the study due to macroscopic signs of chronic pericarditis upon postmortem examination, leaving 10 male and 9 female animals for the final analysis. ROSC was achieved in 8 of 10 piglets in each group. All resuscitated piglets survived until the end of the experiment.
5.3 Hemodynamic parameters

All successfully resuscitated piglets needed a constant dobutamine infusion until the end of experiment (all papers). No significant differences were observed in dobutamine requirement/dose between sexes in all papers.

5.3.1 Paper I

There were significant differences between male and female piglets in their heart rate (p<0.0001), systolic (p=0.04) and mean arterial blood pressures (p=0.02) after resuscitation from cardiac arrest (Figure 1).
Figure 1. Haemodynamic variables: heart rate (HR), systolic arterial blood pressure (SABP) and mean arterial blood pressure (MABP) at baseline, during cardio-pulmonary resuscitation (CPR) and after restoration of spontaneous circulation (ROSC). VF, ventricular fibrillation. Data provided as a mean ± SEM. Male piglets, n = 11; female piglets, n = 10. * denotes significant difference between male and female piglets (2-way repeated measures ANOVA).
5.3.2 Paper II

Heart rate was significantly higher (p<0.001) and mean arterial blood pressure was lower (p=0.03) in male piglets compared with female animals after ROSC (Figure 2). In female piglets systolic blood pressure was greater at 15, 120 and 180 mins (p=0.02 p=0.05 and p=0.008, respectively), and stroke volume was greater at 120 and 180 mins (p=0.02 and p=0.05, respectively) after ROSC, when compared with male animals. Male piglets also had a greater rate pressure product (heart rate x systolic blood pressure) at 60 and 120 mins (p=0.02, and p=0.008, respectively) after ROSC. Female piglets had greater coronary perfusion pressure at 180 mins (p=0.046) compared with male piglets after ROSC (Table 4).
Figure 2. Haemodynamic variables: heart rate and mean arterial blood pressure (MABP) at baseline, during cardio-pulmonary resuscitation (CPR) and after restoration of spontaneous circulation (ROSC). VF, ventricular fibrillation. Data provided as a mean ± SEM. Male piglets, n = 7; Female piglets, n = 9. # denotes significant difference between male and female piglets (2-way repeated measures ANOVA, p<0.05).
Table 4. Stroke volume, coronary perfusion pressure, rate pressure product, systolic arterial blood pressure at baseline and after restoration of spontaneous circulation (Paper II).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Stroke volume</th>
<th>Coronary perfusion pressure, mmHg</th>
<th>Rate pressure product</th>
<th>Systolic arterial blood pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.032±0.002</td>
<td>0.031±0.002</td>
<td>67.7±5.1</td>
<td>81.4±3.8</td>
</tr>
<tr>
<td>After haemorrhage</td>
<td>0.014±0.001</td>
<td>0.015±0.001</td>
<td>25.6±0.69</td>
<td>24.8±0.9</td>
</tr>
<tr>
<td>Circulatory arrest and CPR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Restoration of spontaneous circulation</td>
<td>5</td>
<td>0.018±0.002</td>
<td>68.1±12.8</td>
<td>63.9±11.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.022±0.003</td>
<td>50±4.1</td>
<td>58.8±4.0</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.021±0.002</td>
<td>52.4±3.5</td>
<td>58.4±3.0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.021±0.003</td>
<td>52.3±4.4</td>
<td>53.2±2.1</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.021±0.002*</td>
<td>53±5.8</td>
<td>61.8±3.4</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>0.02±0.002*</td>
<td>51.9±5.9*</td>
<td>65.6±3.2</td>
</tr>
</tbody>
</table>

CPR, cardio-pulmonary resuscitation. Data provided as a mean ± SEM.
Male piglets, n = 7; Female piglets, n = 9. * denotes significant difference between male and female piglets.
5.3.3 Paper III
No significant differences were observed in any hemodynamic parameters or troponin I levels between groups.

5.3.4 Paper IV
Female piglets had higher systolic blood pressure ($p=0.02$) and mean arterial blood pressure ($p=0.03$) compared with male piglets.

5.4 Cardiac enzymes (troponin I)

5.4.1 Paper II
Male piglets had significantly higher levels of troponin I in comparison with female piglets after ROSC ($p=0.02$) (Figure 3).

Figure 3. Plasma levels of troponin I at baseline and after restoration of spontaneous circulation. Data provided as a mean ± SEM. Male piglets, $n=7$; Female piglets, $n=9$. # denotes significant difference between male and female piglets (2-way repeated measures ANOVA, $p<0.05$).

5.4.2 Paper IV
Male piglets had higher levels of troponin I in comparison with female piglets at all time points after ROSC ($p=0.2$). (Table 5).
Table 5. Coronary perfusion pressure, stroke volume and troponin I in plasma at baseline and after restoration of spontaneous circulation (Paper IV).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Coronary perfusion pressure (mmHg)</th>
<th>Stroke volume</th>
<th>Troponin I (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Baseline</td>
<td>65 ± 4</td>
<td>76 ± 4</td>
<td>0.032 ± 0.001</td>
</tr>
<tr>
<td>After haemorrhage</td>
<td>26 ± 0.8</td>
<td>26 ± 0.6</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>Circulatory arrest and CPR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Restoration of spontaneous circulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>75 ± 8.7</td>
<td>74 ± 6</td>
<td>0.016 ± 0.002</td>
</tr>
<tr>
<td>15</td>
<td>50 ± 4.2</td>
<td>52 ± 3.7</td>
<td>0.022 ± 0.002</td>
</tr>
<tr>
<td>30</td>
<td>49 ± 2.3</td>
<td>58 ± 2.8*</td>
<td>0.022 ± 0.002</td>
</tr>
<tr>
<td>60</td>
<td>50 ± 4.2</td>
<td>61 ± 4.2</td>
<td>0.023 ± 0.003</td>
</tr>
<tr>
<td>120</td>
<td>56 ± 3.7</td>
<td>60 ± 4.1</td>
<td>0.023 ± 0.002</td>
</tr>
<tr>
<td>180</td>
<td>52 ± 3.8</td>
<td>65 ± 4.1*</td>
<td>0.021 ± 0.002</td>
</tr>
</tbody>
</table>

CPR, cardio-pulmonary resuscitation. Data provided as a mean ± SEM.

Male piglets, n = 8; Female piglets, n = 8. Denotes significant difference between male and female piglets (one-way ANOVA, p<0.05).
5.5 Cerebral cortical blood flow, oxygen extraction ratio, cerebral perfusion pressure and intracranial pressure

5.5.1 Paper I
Cerebral cortical blood flow (CCBF) was higher in female piglets compared to male piglets 5 min after ROSC (p=0.01). Female piglets had a lower cerebral oxygen extraction ratio in comparison to male piglets at all time points after ROSC (non-significant) (Table 6).
Table 6. Cerebral oxygen extraction ratio, arterial and jugular venous pH and base excess (mmol/l) at baseline and after restoration of spontaneous circulation (Paper I).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Cerebral oxygen extraction ratio</th>
<th>Arterial pH</th>
<th>Jugular venous pH</th>
<th>Arterial base excess</th>
<th>Jugular venous base excess</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male Female</td>
<td>Male Female</td>
<td>Male Female</td>
<td>Male Female</td>
<td>Male Female</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.357±0.046</td>
<td>0.340±0.045</td>
<td>7.49±0.01</td>
<td>7.48±0.01</td>
<td>7.40±0.01</td>
</tr>
<tr>
<td>After haemorrhage</td>
<td>0.586±0.049</td>
<td>0.579±0.050</td>
<td>7.50±0.01</td>
<td>7.50±0.01</td>
<td>7.35±0.01</td>
</tr>
<tr>
<td>Circulatory arrest, CPR, and restoration of spontaneous circulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.421±0.066</td>
<td>0.352±0.046</td>
<td>7.30±0.03</td>
<td>7.35±0.02*</td>
<td>7.19±0.01</td>
</tr>
<tr>
<td>15</td>
<td>0.419±0.073</td>
<td>0.401±0.049</td>
<td>7.29±0.01</td>
<td>7.30±0.01</td>
<td>7.17±0.02</td>
</tr>
<tr>
<td>30</td>
<td>0.468±0.063</td>
<td>0.371±0.060</td>
<td>7.38±0.01</td>
<td>7.37±0.01</td>
<td>7.26±0.02</td>
</tr>
<tr>
<td>60</td>
<td>0.396±0.051</td>
<td>0.327±0.042</td>
<td>7.44±0.01</td>
<td>7.44±0.01</td>
<td>7.34±0.01</td>
</tr>
<tr>
<td>120</td>
<td>0.384±0.066</td>
<td>0.298±0.031</td>
<td>7.46±0.01</td>
<td>7.48±0.01</td>
<td>7.38±0.01</td>
</tr>
<tr>
<td>180</td>
<td>0.333±0.057</td>
<td>0.290±0.034</td>
<td>7.46±0.02</td>
<td>7.47±0.01</td>
<td>7.40±0.02</td>
</tr>
</tbody>
</table>

CPR, cardio-pulmonary resuscitation. Data provided as a mean ± SEM. Male piglets, n = 11; female piglets, n = 10. * denotes significant difference between male and female piglets.
5.5.2 Paper II
CCBF was significantly higher in female animals in comparison with male piglets at 5, 15, 30, 60, and 120 min after ROSC (p=0.02, p=0.04, p=0.05, p=0.04 and p=0.01, respectively) (Figure 4).

Figure 4. Cortical cerebral blood flow (CCBF) at baseline and after restoration of spontaneous circulation (ROSC). VF, ventricular fibrillation; CPR, cardiopulmonary resuscitation. Data provided as mean ± SEM. Male piglets, n = 11; female piglets, n = 9. * denotes significant difference between male and female piglets (p<0.05, Mann-Whitney test).

5.5.3 Paper III
Cerebral cortical blood flow, jugular venous oxygen extraction ratio, cerebral perfusion pressure and intracranial pressure were not significantly different between groups at any time point (Figure 5).
5.5.4 Paper IV
Female piglets had a lower cerebral oxygen extraction ratio in comparison to male piglets at 15 and 180 mins after ROSC (p=0.0006 and p=0.05, respectively) and had lower CCBF at all time points.

5.6 Cerebral neurological injury (protein S-100β)
5.6.1 Paper I
Protein S-100β levels were numerically higher in male piglets compared with female piglets after ROSC, though it did not reach statistical significance.

5.7 Systemic and jugular venous acid-base status
5.7.1 Paper I
Compared to female animals, arterial and jugular venous pH (p=0.02 and p=0.04, respectively) and base excess values (p=0.003 and p=0.02, respectively) were lower in male piglets at 5 min after ROSC.
5.7.2 Paper II-IV
No significant differences were observed between genders (Papers II, IV) or groups (control vs estradiol in Paper III) regarding systemic and jugular bulb acid-base parameters (data not shown).

5.8 Cerebral oxidative and inflammatory injury
Jugular venous 8-iso-PGF$_{2\alpha}$ and 15-keto-dihydro-PGF$_{2\alpha}$ levels were determined in all animals as the indicators of oxidative and inflammatory injury, respectively. In Paper I and II 8-iso-PGF$_{2\alpha}$ and 15-keto-dihydro-PGF$_{2\alpha}$ values were not significantly different between sexes and groups (control vs estradiol in Paper III).

Table 7. Paper I. Indicators of brain peroxidation (8-iso-PGF$_{2\alpha}$) and inflammation (15-keto-dihydro-PGF$_{2\alpha}$) in jugular plasma at baseline and after restoration of spontaneous circulation.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>8-iso-PGF$_{2\alpha}$ (pmol/l)</th>
<th>15-keto-dihydro-PGF$_{2\alpha}$ (pmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Baseline</td>
<td>67 ± 8.0</td>
<td>71 ± 13.7</td>
</tr>
<tr>
<td>Restoration of spontaneous circulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>167 ± 23.4</td>
<td>154 ± 26.4</td>
</tr>
<tr>
<td>15</td>
<td>156 ± 12.8</td>
<td>175 ± 35.8</td>
</tr>
<tr>
<td>30</td>
<td>152 ± 7.5</td>
<td>174 ± 33.6</td>
</tr>
<tr>
<td>60</td>
<td>118 ± 11.2</td>
<td>136 ± 25.9</td>
</tr>
<tr>
<td>120</td>
<td>84 ± 6.8</td>
<td>93 ± 19.3</td>
</tr>
<tr>
<td>180</td>
<td>90 ± 9.5</td>
<td>116 ± 24.1</td>
</tr>
</tbody>
</table>

CPR, cardio-pulmonary resuscitation. Data provided as a mean ± SEM. Male piglets, n = 11; female piglets, n = 10.

5.9 Electrolytes, lactate, glucose, temperature
In all papers no significant differences were observed between genders (Papers I and II) and groups (control vs estradiol in Paper III) regarding plasma lactate, potassium, sodium, glucose concentrations or body temperature (data not shown).
5.10 Immunochemistry (NOS, albumin) and neurological injury

5.10.1 Paper I
Inducible and neuronal nitric oxide synthases (iNOS and nNOS) activity in cerebral cortex was higher in males compared with females at 180 mins after ROSC (p=0.01 and p=0.0006, respectively). As a measure of neuronal injury the number of distorted neurones was increased in male piglets compared with female group at 180 min after ROSC (p=0.0002). Albumin immunoreactivity was higher in male piglets compared with female animals at 180 min after ROSC (p=0.011) (Figure 6).

5.10.2 Paper III
The number of distorted neurones was greater in control group animals as compared with estradiol group at 180 min after ROSC (p<0.0001) (Figure 7). Albumin immunoreactivity was higher in control group compared with estradiol group animals at 180 min after ROSC (p<0.0001) (Figure 7). There was greater activation of nNOS and iNOS in control group compared with estradiol group at 180 mins after ROSC (p<0.0001 and p<0.0001,
respectively) (Figures 8-9). No differences were observed in eNOS activation between groups (p=0.21).

Figure 7. Neuronal injury and albumin reactivity in cerebral cortex at 180 min after ROSC. Data are expressed as mean±SEM. The grey bar represents estradiol group. The white bar represents control group. Estradiol group, n = 15, female animals n=6, male animals n=9. Control group, n = 15, female animals n=8, male animals n=7. * denotes significant difference between control and estradiol groups (Mann-Whitney test, p<0.05). # denotes significant difference between male and female animals in control and estradiol groups, respectively (Mann-Whitney test, p<0.05).
Figure 8. Neuronal nitric oxide synthase (nNOS) activity in cerebral cortex at 180 min after ROSC. Data are expressed as mean±SEM. The grey bar represents estradiol group. The white bar represents control group. Estradiol group, n = 15, female animals n=6, male animals n=9. Control group, n = 15, female animals n=8, male animals n=7. * denotes significant difference between control and estradiol groups (Mann-Whitney test, p<0.05). # denotes significant difference between male and female animals in control and estradiol groups, respectively (Mann-Whitney test, p<0.05). nNOS activity was correlated to number of distorted neurons (Y=16.84+0.691X; R²=0.62, p<0.0001).

Figure 9. Inducible nitric oxide synthase (iNOS) activity in cerebral cortex at 180 min after ROSC. Data are expressed as mean±SEM. The grey bar represents estradiol group. The white bar represents control group. Estradiol group, n = 15, female animals n=6, male animals n=9. Control group, n = 15, female animals n=8, male animals n=7. * denotes significant difference between control and estradiol groups (Mann-Whitney test, p<0.05). # denotes significant difference between male and female animals in control and estradiol groups, respectively (Mann-Whitney test, p<0.05). iNOS activity was correlated to number of distorted neurons (Y=13.01+0.904X; R²=0.57, p<0.0001).
**Figure 10. Paper III.** Representative examples of albumin immunostaining in the parietal cortex of pig brain from control group (upper left panel, CA a) and estradiol group (upper right panel, CA+ESTRO b). Nerve cells showed dark albumin immunostaining of the nerve cell cytoplasm and/or the karyoplasm (arrows). The neuropil is swollen and sponginess is evident around the damaged neurones. **Lower panel.** Representative examples of neuronal damages in the parietal cortex in pig brain after 180 min after cardiac arrest. Neuronal changes were examined using Nissl (Cresyl violet) staining on 3 μm thick paraffin sections. Presence of several eosinophilic shrunken and dark neurons (arrows) is shown in control group (lower left panel, CA c) as compared to estradiol group (lower right panel, CA+ESTRO d).
Figure 11. Paper III. Representative examples of neuronal (upper panel), inducible (middle panel) and endothelial (lower panel) nitric oxide synthase (NOS) expression in the parietal cortex of pig brain from control group (CA a, c, e) and estradiol group (CA+Estro b, d, f). The nNOS expression is seen in deformed neurones and axons distributed in the neuropil (arrows, upper panel, a, b). Intense nNOS expression is seen in deformed and damaged nerve cells showing marked perineuronal edema. Several neurones show iNOS expression (arrows, middle panel, c, d). The eNOS-containing cells are deformed and show intense immunoreactivity, largely in the neuronal cytoplasm (arrows, lower panel, e, f).
5.11 Sex hormones

Table 8. Sex hormones concentration at baseline in Papers I, II and IV (mean ±SD)

<table>
<thead>
<tr>
<th></th>
<th>Estradiol concentration, pmol/l</th>
<th>Testosterone concentration, nmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paper I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>92.80 ± 13.19</td>
<td>0.77 ± 0.27</td>
</tr>
<tr>
<td>Male</td>
<td>94.14 ± 7.96</td>
<td>0.64 ± 0.14</td>
</tr>
<tr>
<td><strong>Paper II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>71.8 ± 4.7</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>Male</td>
<td>94.0 ± 8.1</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td><strong>Paper IV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>84.0 ± 5.8</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>Male</td>
<td>81.7 ± 4.09</td>
<td>0.5 ± 0.0</td>
</tr>
</tbody>
</table>

5.11.1 Paper III

Male animals: The mean testosterone concentration was 0.54 ± 0.04 nmol/l in estradiol group and 0.54 ± 0.0 nmol/l in control group. The mean estradiol concentration was 70 ± 5 pmol/l in control group at baseline. The mean estradiol concentration was 108 ± 25 pmol/l at baseline, 4351 ± 683 pmol/l at 60 mins and 698 ± 131 pmol/l at 180 mins after ROSC in estradiol group.

Female animals: The mean testosterone concentration was 0.3 ± 0.07 nmol/l in estradiol group and 0.56 ± 0.15 nmol/l in control group. The mean estradiol concentration was 78 ± 8 pmol/l in control group at baseline. The mean estradiol concentration was 98 ± 13 pmol/l at baseline, 3818 ± 609 pmol/l at 60 mins and 694 ± 156 pmol/l at 180 mins after ROSC in estradiol group.
6. Discussion

Data about sex differences after hypovolemic normothermic circulatory arrest is lacking. We intended to examine innate sex differences, and hence chose to study sexually immature animals to control for the effects of the sexual hormones.

The first study showed that cerebral cortical blood flow was greater, blood-brain-barrier was better preserved and neuronal injury was smaller in female as compared to male piglets. The second study demonstrated that female sex was associated with enhanced hemodynamic response, cardioprotection, and better survival. This cardio- and cerebro-protective effects were observed despite comparable estradiol and testosterone levels in male and female animals, indicating an innate gender-related cardio- and cerebro-protection, independent of sex hormone effects. In the third study we tested a hypothesis if 17β-estradiol administration could improve neurological outcome by NOS modulation. The results showed that compared with the control group, animals in the E2 group exhibited a significantly smaller increase in nNOS and iNOS expression, a smaller blood-brain-barrier disruption and a mitigated neuronal injury. There was also a significant correlation between nNOS and iNOS levels and neuronal injury. The last study was designed to test a hypothesis if female-specific cardioprotection might be attributed to a smaller NOS activity by administration of methylene blue. The female-specific cardioprotection could be partly attributed to a smaller NOS activity, however NOS inhibition with MB did not improve survival or myocardial injury.

6.1 The experimental model of haemorrhage and CPR

In all studies we used a controlled, fixed pressure haemorrhage model. Haemorrhage was started from the catheter in the femoral artery and stopped when a mean arterial blood pressure of 35 mmHg was reached. This blood pressure was maintained for 5 minutes by withdrawing more blood or administering small volumes of shed blood. The bleeding times are presented in Table 2. The decision to bleed to 35 mmHg was based on pilot studies performed at our laboratory, as it was almost impossible to achieve ROSC if piglets were bled to lower blood pressure. This level of blood
pressure is arbitrary and different results could have been obtained if lower blood pressure or longer bleeding time was used. Besides, in clinical reality cardiac arrest usually occurs when blood pressure is lower. However, this was not feasible at the experimental setting. In our experimental model the time selected for CA was 2 min and duration of CPR was 8 minutes (Papers I-II, and Paper IV). In Paper III we extended CA time to 4 min and duration of CPR to 15 minutes. We tried to mimic a clinical situation when VF occurs as a consequence of rapid and uncontrolled haemorrhage. In order to increase chances of successful ROSC, open-chest CPR was used. Clinical experience shows that surgical thoracotomy is possible in a few minutes if an experienced surgeon is present [95, 96]. The protocol of all studies included performance of CPR before defibrillation according to 2005 guidelines for CPR. Vasopressin was used as a primary vasoactive drug and epinephrine as a rescue medication. Although animal studies indicate that epinephrine can improve initial ROSC resuscitation success after both asphyxial and ventricular fibrillation cardiac arrests, no single medication has been shown to improve survival outcome from paediatric cardiac arrest. Vasopressin during prolonged paediatric cardiac arrest may result in ROSC when standard medications have failed [97]. There is currently no conclusive evidence to support or reject the use of vasopressin as an alternative to, or in combination with, adrenaline in any cardiac arrest rhythm in adults [98] or children [99, 100]. In order to increase defibrillation success we gave amiodarone 0.5 mg/kg dose together with vasopressin in Paper III.

6.2 Open-chest CPR

Our experience from pilot studies has shown that open cardiac access is mandatory for success [101]. Good quality closed chest CPR generates approximately 10-15% of baseline myocardial blood flow and approximately 50% of cerebral blood flow. By contrast, open-chest CPR can generate a near normal cerebral blood flow. Besides, a relatively simple technique for resuscitative thoracotomy has been described recently [95, 96], and open-chest CPR is often provided to children after open-heart surgery and sternotomy [102]. In our experiments a median sternotomy was done in all piglets before haemorrhage.

6.3 Resuscitative fluids

The project was designed to mimic a clinical situation when ventricular fibrillation occurs as a consequence of rapid haemorrhage, a common cause of death in rapidly exsanguinating patients in many operating facilities or emergency rooms, or in out-of-hospital cases of hypovolemic cardiac arrest.
The first study was planned to test the hypothesis that sex differences are present despite different resuscitation regimens. The rationale behind selection of fluids was based on the current practice of using different fluids in the management of hypovolemic cardiac arrest. Crystalloids and colloids are used in the pre-hospital phase, plus blood products in the hospital [103]. Thus one group of animals received standard resuscitation with whole blood. In another group, we used hypertonic saline (HS) as in experimental studies it has been reported to increase resuscitation success rate and improved myocardial and cerebral reperfusion during CPR [104, 105]. We chose to modify the resuscitation fluid approach used by Fisher et al. [106], in which successful resuscitation was reported with 4 ml/kg of HS during 20 min in an animal model of cardiac arrest, by adding dextran to HS to enhance its volume expansion capacity and intravascular persistence [107]. Hypertonic-hyperoncotic saline, including 7.5% saline and 6% dextran (hypertonic saline dextrane, HSD), have been shown to be effective in resuscitation from severe haemorrhage [108]. Analogously, HSD has been used during CPR not only as a resuscitative fluid, but also as anti-inflammatory agent. Finally, we chose to treat a group of animals with a combination of a crystalloid (acetated Ringers’ solution) and whole blood. Methylene blue was also added to the latter fluid regimen as it has been recently shown to improve short-time survival after extended normovolemic circulatory arrest [74]. Besides, when added to electrolyte solutions, MB has been shown to increase the mean arterial pressure and to reduce the volume of blood and fluid requirement during resuscitation after haemorrhagic shock [109]. During human experimental haemorrhage studies performed in our laboratory, it was demonstrated that relative volume expansion effectiveness for HSD is 4:1 versus whole blood. When compared to lactated Ringer’s solution, its relative volume expansion is reported to be 10:1, approximately [107, 110]. As rapid administration of large volumes of blood or isotonic solutions during CPR is shown to decrease myocardial perfusion pressure and blood flow [111], we chose a more gradual volume expansion and administered the resuscitation fluids over a 20 min period of time. A major limitation of these volumes, relevant to its clinical applicability, is that the level of haemorrhage may be different and may require larger or smaller volumes of crystalloids or colloids. The fluid selection and the used doses are arbitrary and may differ among some colleagues in clinical practice. In the second and third paper we decided to use only HSD solution as it was more practical and easier to administer in experimental setting. In the fourth paper HSD solution was combined with methylene blue infusion. In all papers resuscitated animals received remainder of the previously exsanguinated blood at 23 min after CA, simulating the time needed to receive and administer blood in operating theatre.
6.4 Sexual immaturity of animals

In order to investigate sex differences, sexually mature animals, in particular proestrus females vs males are often used. Studies done to date have concentrated on non-genomic effects by investigating proestrus females, ovariectomised and aged females, by regulating key sex hormones using selective enzymatic blockade, by selective blockade of hormone receptors or by administration of sex steroids. Gender differences in outcome are well documented in animal models of trauma and haemorrhage [112]. These differences are often attributed to physiological effects of sex hormones, as sexually mature animals are routinely used for these experiments. Although the contribution of sex hormones in the pathophysiology of post-resuscitation organ injury is unambiguous, the primary goal of our studies was to investigate innate sex-related differences by using sexually immature animals, a model that is of particular relevance to the paediatric and young adolescent victims of haemorrhagic cardiac arrest. The findings reported herein are, hence, important as sexual hormone levels were equal in male and female animals, confirming that the observed hemodynamic, cerebral and outcome differences are independent of the hormonal effects, and can therefore be attributed to innate gender differences. This approach, therefore, enabled us to obtain this important data without the need to sacrifice additional sexually mature animals. The traditional approach of a male-female comparison would, in other words, not allow us to differentiate the effect of hormones versus those of the genes. Besides, as puberty seldom occurs prior to 150 days in female piglets, we also suggest that hormonal factors have minimal effect, if any, in the female piglets used in our study [113]. As males lack the homologous pair of their X chromosome, and many genes that encode key metabolic and regulatory proteins (e.g. apoptosis and inflammation) reside on the X chromosome, it is possible that the observed sex differences are related to an unopposed expression of these genes in males.

6.5 Cerebral immunochemistry

In normal conditions, neurones in the brain represent typical triangular shape with a centrally placed clear nucleus containing small round nucleolus. Under pathological conditions, these standard shapes of neurones together with nucleus and nucleolus relations are severely altered. Thus, the triangular shape of neurones is altered that could be followed by dark nucleus with sometime eccentric nucleolus. The cytoplasm of nerve cells, that is normally very clear and translucent show dark staining and granular in nature. Presence of any such characteristics, i.e. altered shape of neurones, displacement of nucleus or nucleolus and dark cytoplasm is referred to as
“distorted neurones”. In our studies, we counted distorted neurones in the specifically defined area of cerebral cortex as described in details elsewhere [91]. In the results section of our manuscript Nissl stained neuronal damage, showing distorted neurones are already included and clearly marked with arrows (see Fig.10).

We did not express our results as percentage of neurones that were distorted, but we counted only distorted neurones present in a well-defined region of cerebral cortex as described earlier [114, 115]. Our observations are thus semiquantitative. It is presumed that in a well-defined area, number of distorted neurones is proportional to injury severity and thus less number of damaged or distorted neurones represents neuroprotection. In histological sections, neurones could be easily identified because of their triangular shape. Glial cells are often round in shape and could be distinguished from neurones easily. We counted NOS immunopositive neurones only. The non-neuronal cells even if they are positive were not included into our semiquantitative study.

Albumin is often present in nerve cells if the cell membranes of neurones are damaged extensively. What we see is the albumin labelled neurones that means they are probably severely damaged and thus show uptake of albumin in their cell cytoplasm. This is a well-known phenomenon and several authors have reported this in a wide variety of neurological diseases especially where oedema and neuronal damage are most prominent after the breakdown the BBB function [91, 116].

6.6 Validity of eicosanoids and protein S-100β measurements

Jugular bulb isoprostanes and PGF$_{2\alpha}$ metabolites have previously been demonstrated to serve as biomarkers of oxidative free-radical damage and inflammatory response in the brain after reperfusion injury [117, 118]. The jugular bulb plasma concentrations of 8-isoPGF$_{2\alpha}$ and 15-keto-dihydro-PGF$_{2\alpha}$ are proportional to both the duration of ischemia and the resulting neurologic deficit after experimental CA and CPR [75].

Protein S-100β is predominantly and normally located in astroglial cells and is a sensitive marker of hypoxic brain injury and disruption of the blood-brain barrier when found in blood [119]. The level of marker in jugular venous blood closely reflects the level of blood-brain-barrier disruption.
6.7 Survival

While it is no problem to resuscitate a normovolemic, normothermic CA piglet after 12 min [75], it is very difficult to achieve resuscitation in a hypovolemic CA piglet after even a couple of minutes. It is in agreement with the experience of many clinical colleagues that hypovolemic normothermic CA is very difficult to resuscitate. The reason for this great difference on the physiological, cellular and molecular levels is largely unknown. The project has been designed to mimic a clinical situation when CA occurs unexpected and thus hypothermia or suspended animation is not an alternative within a few minutes. Currently this is the most frequent situation in most operating facilities. Consistent with previous reports on resuscitability after exsanguinations cardiac arrest, ROSC was not easily achieved in the first paper despite maximisation of the blood flow with open-chest CPR, fluid administration and vasopressin and epinephrine boluses (no gender differences). In the second paper female animals had a significantly better short-term survival compared to male animals. HSD was used as a resuscitation fluid in the second paper compared with three different resuscitation fluids in the first paper (blood, HSD, crystalloid plus methylene blue plus blood). However, neither estradiol (Paper III), no methylene blue (Paper IV) had any effect on survival.

6.8 Hemodynamic differences between genders

Sex differences in myocardial recovery have been reported after acute ischemia and reperfusion injury [120]. In some animal studies intact premenopausal females compared to males exhibit reduced ischemia-reperfusion injury [121, 122]. In the current experiment (Papers I and II) the post-arrest hemodynamic profile in female piglets appeared to correspond with a mitigation of the myocardial injury (lower troponin I levels) and dysfunction. This may be explained by an enhanced or better-preserved oxygen supply-demand ratio after successful ROSC in female piglets (higher mean blood pressure and lower heart rate/rate pressure product). Although female and male animals received similar doses of dobutamine, different hemodynamic response in females may also be attributed to a sex-difference in receptor sensitivity and response to catecholamines as was previously suggested in experimental studies of trauma and haemorrhagic shock [55, 112, 123].

Programmed cell death (apoptosis) also plays a key role in the pathophysiology of ischaemia-reperfusion injury, and is likely precipitated by the oxidative stress [124] and inflammation that is mediated through different signalling cascades, such as the p38 mitogen-activated protein kinase (MAPK), nuclear factor κB (NFκB), and expression of
proinflammatory cytokines such as TNFα and IL-1β [122, 125]. Importantly, several genes that encode these key metabolic and regulatory proteins, e.g. apoptotic cascade and nuclear factor κB, reside on the X chromosome [126, 127]. It appears that the susceptibility of the cardiomyocytes to ischemia-reperfusion injury may be affected by an X-linked genetic polymorphism and cellular mosaicism in females. Females have a homologous pair of their X-linked genes, and are therefore likely to manifest phenotypic differences, as compared to male animals, in response to ischemic injury. Based on the above findings, we posit that phenotypic expression of unopposed X-linked alleles in male animals provides a less adaptive and balanced response to ischemia-reperfusion in our model of exsanguination circulatory arrest. However, we did not collect or analyse genomic data, as we considered these studies beyond the scope of the current project.

As female piglets had smaller myocardial injury in Paper I and II, we tested a hypothesis if an exogenous administration of E2 may improve cardiovascular outcome (Paper III). In this study we failed to observe any differences in hemodynamic parameters between groups or genders. Furthermore, the level of troponin I (marker of cardiac injury) was not different between groups/genders. This is in contrast to other studies, which have shown that administration of E2 in males and ovariectomised females protected cardiac function following trauma-haemorrhage [123, 128]. Different results can be obtained in different experimental models. The duration of CA was 2 min in Papers I and II, and 4 minutes in Paper III. We may speculate that after 4 minutes cardiac arrest the insult is substantial and the difference between genders disappears.

6.9 Methylene blue and hemodynamic effects

Some studies have reported that females have reduced ischemia-reperfusion injury [121, 129]. Other studies have failed to observe a male-female difference in ischemia-reperfusion injury [130]. Nitric oxide is suggested to mediate expression of cyclooxygenase 2 which has also been involved in cardioprotection [131]. An increase in NO is involved in cardioprotection via activation of protein kinase G which leads to activation of mitochondrial pathways including activation of mitochondrial potassium ATP channel [132]. At the onset of reperfusion the dynamic transition that occurs within the ischemic myocardium is a critical time for successful resuscitation and is characterised by rapid changes in oxidant stress, Ca\(^{2+}\) flux and contractile function [133, 134]. Studies have shown that nitric oxide has both cardio-protective and cardio-depressive effects depending on the oxidant stress level in the system [135].

MB has a mixture of effects, nitric oxide synthase inhibition being one of them, and different results can be achieved in various experimental models.
Haemorrhagic shock is implicated in the induction of inducible NOS and the down-regulation of the eNOS isoforms [136, 137]. Survival and hemodynamic benefits in blocking NOS were achieved in protracted (3 hours) haemorrhagic shock [138]. Besides, Wu et al. [139] noted that eNOS and nNOS derived NO showed predominantly protective effects while iNOS derived NO played a detrimental role in CPR. However, only several studies evaluated NOS inhibition started during or after CPR. Krismer et al. performed a study (a swine VF cardiac arrest model) where L-NAME was given after 3 and 13 min of CPR and showed improved coronary perfusion pressure and better initial resuscitation compared with placebo control [140]. Falk et al. [141] used isolated rat heart model and showed that infusion of L-NAME during reperfusion significantly improved myocardial recovery.

Previous research from our group suggested that MB co-administered with HSD might be an effective mechanism for attenuation of oxidative, inflammatory and neurologic injury [74, 142]. The contribution of MB to those effects may be explained by inhibition of nitric oxide production and reduced effects of excess NO as well as by scavenging of oxygen-derived free radicals [74, 142]. In Paper IV addition of MB had no effect on survival in contrast to Paper II where resuscitation with HSD resulted in improved survival in female piglets [143]. Compared with our previous studies (Paper II), addition of MB showed improvement in hemodynamics (increased not only mean arterial pressure but also systolic blood pressure) in female piglets and a tendency to reduce myocardial damage in male piglets. When MB was added, however, the protective effect of female sex was abated, implying an association between NOS expression and gender-related cardioprotection. NOS inhibition with MB in Paper IV abated the disadvantageous hemodynamic profile (higher heart rate and rate-pressure-product with lower stroke volume) in males observed in previous experiment [143]. However, troponin I levels increased in both genders (albeit more so in females) when MB was added to the resuscitation regimen. This observation indicates a potential untoward effect by MB administration in this setting, which leads to an increased myocardial injury. A dose related toxicity has been described when MB doses exceeding 2 mg/kg are administered, although considerably higher doses have also been used without ill effects [144]. Moreover, studies using high doses of NOS inhibitors (i.e. >IC50) provide evidence that overzealous NOS inhibition can increase myocardial injury and infarct size, possibly because of an increase in myocardial oxygen requirement associated with higher arterial blood pressure [145]. In the post-resuscitation phase we observed a tendency to increase in total peripheral and pulmonary resistance in the female group compared with the male animals. However, the increase in the afterload did not worsen myocardial injury as troponin I was lower in the female group compared with the male group during the whole experiment. Myocardial contraction is affected by NO in a dose-dependent manner, with low production of NO resulting in positive inotropic
effects and higher concentrations exerting negative inotropic effects [146]. These result differ from Paper II where male piglets had significantly higher troponin I values compared with female animals after resuscitation with HSD [143].

Although a causative relationship between NOS inhibition and cardioprotection is difficult to prove, a coherent NOS etiology is unlikely to account for the outcome difference between male and female animals after hypovolemic CA arrest. However, our results do implicate NOS in the pathophysiology of post-arrest cardiac injury, as NOS inhibition diminished the protective effect of female sex (survival and troponin levels). This MB-induced effect may be dose-related and can potentially be avoided if smaller MB doses are administered. Overall, our data is in agreement with previously published reports and implicate NOS in the male-female difference in cardioprotection [120]. We can postulate that NOS expression is different in pre-pubertal piglets and that inhibition of NOS by MB favourably affects male piglets despite significantly lower systolic and mean arterial blood pressures. As histopathological evaluation of heart did not show any difference between genders, our interpretation is that experiment time (i.e. 3 hrs) was too short to observe significant cardiac damages.

6.10 Protection from cerebral injury in female piglets

Previous animal studies suggest that the post-resuscitation hyper-perfusion plays a pivotal role in the ischemia-reperfusion injury and blood-brain-barrier disruption, but may also be important in providing the blood supply and oxygen required for the restoration of cellular homeostasis and to mitigate the post-ischemic no-reflow phenomenon [147]. In areas with impaired reperfusion, a mismatch between cerebral oxygen delivery and metabolism (and oxygen consumption) augments the neuronal injury, which can worsen the functional outcome [83]. This supply-demand relation is clinically mirrored by a change in cerebral oxygen extraction ratio, with higher numbers indicating a decreased tissue oxygen delivery and compromised blood flow [148]. Conversely, lower cerebral oxygen extraction ratios that were documented in female animals in Paper I implicate an improved global cerebral perfusion. This observation together with the finding of greater cerebral cortical blood flows in female piglets also implies better preservation of cerebral autoregulation, a conclusion that is supported by the evidence for decreased blood-brain-barrier (BBB) disruption in these animals.

Despite the aforementioned benefits of post-resuscitation hyperaemia, it has in some studies been associated with an increased tissue injury, possibly
by aggravating the post-ischemic lipid peroxidation [149]. This hypothesis is supported by our finding of a slight amelioration of cerebral inflammation and lipid peroxidation in male piglets, exhibited by numerically lower jugular venous blood levels of 15-keto-dihydro-PGF$_{2\alpha}$ and 8-iso-PGF$_{2\alpha}$, respectively (Paper I). These findings, nevertheless, do not exclude focal cerebral ischemia, with possible redistribution of the cerebral blood flow towards the cortex. The observation that histological outcome is improved in females is, therefore, of particular importance supporting the hypothesis that female animals have a more favourable distribution of cerebral blood flow.

Cerebral ischemia/reperfusion injury triggers multiple and distinct cell signalling pathways, which may lead to cell survival or cell damage [82]. It has been suggested that females are exquisitely sensitive to caspase-mediated cell death, whereas cell death in males is triggered by caspase-independent pathways involving apoptosis-inducing factor and Poly(ADP-ribose) polymerase (PARP1) activation [73, 150, 151] Emerging data also confirms that ischemic cell death pathways are sexually dimorphic [66, 67] Reducing neuronal nitric oxide or PARP1 activation protects only the male brain [72] and enhances ischemic injury in females [73]. As nNOS and iNOS upregulation was mitigated in female animals in Paper I, we suggest that the aforementioned sexual dimorphism may be attributed to a difference in NOS expression.

### 6.11 Neuroprotective effects of estradiol

As female sex was associated with a smaller increase in the cerebral expression of iNOS and nNOS (Papers I and II), in Paper III we tested the hypothesis that exogenously administered 17β-estradiol (E2) can improve neurological outcome by NOS modulation. This issue is clinically relevant as the estradiol could be used in circumstances associated with known brain hypoperfusion or cerebral ischemia (for example, cardiac surgery). Besides, if a therapy is not effective given before insult (i.e. prophylactic use), the chances of its success after insult are even smaller. A single intravenous injection of 17β-estradiol (50 μg/kg) was given immediately before CA and attenuated brain injury in immature piglets.

The biological effects of estrogens are divided into rapid non-genomic actions [152] and slower genomic effects [153]. It appears that physiological levels of estradiol usually protect via a mechanism that require pre-treatment and involve estrogen receptors and changes in gene expression. In contrast, pharmacological levels of estradiol appear to by-pass action of estrogen receptors and involve mechanisms that involve antioxidant actions, promotion of blood flow and / or nitric oxide production [68, 71]. It is unlikely that E2 acted via traditional transcriptional mechanisms in our study since a single injection of E2 provided more or less immediate protection
and brain damage was evaluated after a relatively short period (i.e. only 3 hours after ROSC). Similarly, antioxidant mechanisms were unlikely as such mechanisms require micromolar concentrations of E2 [154] and in Paper III E2 concentration was in picomolar range. Several investigators reported that E2 reduces lipid peroxidation in several different neuronal cell systems and that decrease in lipid peroxidation correlated with reduced cell death [155]. However, doses of E2 required for neuroprotection in these systems parallel doses required for antioxidant activity. In addition, we failed to observe any differences in jugular venous 8-iso-PGF$_{2\alpha}$ and 15-keto-dihydro-PGF$_{2\alpha}$ levels (indicators of peroxidative and inflammatory injury, respectively) between the groups. Importantly, we failed to show a correlation between 8-iso-PGF$_{2\alpha}$ and 15-keto-dihydro-PGF$_{2\alpha}$ levels and neuronal damage was observed.

In Paper III a significant correlation between nNOS and iNOS activation levels and histological brain injury was observed. Based on the reported association between NOS activation and cerebral protection, we suggest that suppression of iNOS and nNOS may provide a mechanistic link between E2 administration and neuroprotection in this model of hypovolemic CA.

The protective effect resulting from suppression of iNOS by estrogens is well documented in cell culture and in models of brain inflammation [156], however the role of iNOS in the protection afforded by estrogens in ischemic brain injury is less well defined. In Paper III E2 lowered iNOS levels in both sexes. Other studies have also shown that iNOS expression exacerbates injury during stroke in male mice [157] and down-regulation of iNOS is neuroprotective in wild-type female mice [158]. Quite interestingly nNOS activation was also lowered by estradiol. This contrasts to most reports as estrogen is reported to increase nNOS expression and level of activation [159, 160].

Thus, Paper III results showed that E2 could decrease brain damage in immature piglets of both sexes even though experimental studies have confirmed that ischemic cell death pathways were sexually dimorphic [66, 67]. Thus despite gender differences in cerebral injury observed in Paper I, E2 diminished cerebral damage in both sexes by modulating NOS system. Other neuroprotection mechanisms may have also played role.
7. Conclusions

- After resuscitation from exsanguination and circulatory arrest, cerebral cortical blood flow is greater, blood-brain-barrier is better preserved and neuronal injury is smaller in female as compared to male piglets. This important difference in outcome is independent of the physiological effects of sexual hormones (Paper I).
- After resuscitation from haemorrhage and circulatory arrest, hemodynamic parameters are better preserved and myocardial injury is smaller in female piglets. This difference in outcome is independent of sexual hormones (Paper II).
- After resuscitation from hypovolemic CA, E2 is neuroprotective in immature male and female piglets. E2 down-regulates iNOS and nNOS expression and results in decreased BBB permeability and smaller neuronal injury both in male and female piglets (Paper III).
- The female-specific cardiac protection may be attributed to a smaller NOS activity, but NOS inhibition with MB does not improve survival or myocardial injury (Paper IV).
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9. References


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