Protective Effects of Progesterone on Neurological outcomes in a Rat Model of Cardiac Arrest

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Abstract

Objective: A significant percent of survivors from cardiac arrest and resuscitation suffer permanent brain damage. Previous studies have demonstrated the Neuroprotective effects of progesterone on regional brain ischemia and injury. In the present study, we investigate the Neuroprotective effects of progesterone on global brain ischemia as a result of cardiac arrest and resuscitation.

Subjects: Twenty-six male Sprague-Dawley rats

Interventions: Two groups of animals were randomized: 1) progesterone group (PG, n=13) and 2) saline placebo group (SG, n=13). After 8 mins of untreated ventricular fibrillation, progesterone (8 mg/kg) or saline was injected at the onset of cardiopulmonary resuscitation (CPR). Precordial compression and mechanical ventilations were initiated for 8 mins. Hemodynamics was measured at baseline, 2 and 4 hrs after the return of spontaneous circulation (ROSC).

Measurements and Main Results: Electroencephalograph (EEG) was continuously recorded from baseline to the end of the 4 hr observation. Evans blue measurement was performed in 5 animals of each group to investigate blood-brain barrier (BBB) permeability. The duration of survival was evaluated during the 72 hrs after resuscitation. All rats except for one in the placebo group were successfully resuscitated. The BBB permeability was less impaired in the PG group and the recovery of EEG was earlier in the PG group.

Conclusion: Administration of progesterone during CPR reduces cerebral BBB permeability and accelerates the recovery of neurological function in a rat model of prolonged VF.

ABBREVIATIONS

PG: Progesterone Group; SG: Saline Group; CPR: Cardiopulmonary Resuscitation; ROSC: Return of Spontaneous Circulation; ETCO₂: End-Tidal Carbon Dioxide; EEG: Electroencephalograph; BBB: Blood-Brain Barrier; CA: Cardiac Arrest; VF: Ventricular Fibrillation; CPP: Coronary Perfusion Pressure; EB: Evans Blue; MPI: Myocardial Performance Index, EF: Ejection Fraction; NMDA: N-Methyl-D-Aspartate, GABA: γ-Aminobutyric Acid Type A; PR: Progesterone Receptor

INTRODUCTION

Despite advances of the sciences and technologies in cardiopulmonary resuscitation (CPR), only 9% of out-of-hospital cardiac arrest patients survive to hospital discharge and up to 60% of those survivors have moderate to severe neurological disabilities up to three months after resuscitation [1]. Although therapeutic mild hypothermia is recommended by the American Heart Association and the European Resuscitation Council for all unconscious patients after cardiac arrest (CA) to mitigate the impairment of cerebral function, the outcomes of CPR, remain disappointing [2-5].

Studies have demonstrated that females have better neurological outcomes from focal/global ischemic and traumatic brain injury; the hormone differences are considered as the potential mechanism [6,7]. In the past decades, a number of focal/global ischemic brain injury studies have consistently demonstrated that female animals sustain less tissue damage than males after similar insults [8,9].

Progesterone, a female hormone, is an effective neuroprotectant in animal models of traumatic brain injury or cerebral ischemic injury. Research has demonstrated that progesterone reduces cerebral edema, necrosis, apoptosis, blood-brain barrier (BBB) impairment and pro-inflammatory mediators in a rat model of traumatic brain injury [10-14]. Chronic administration of progesterone has also been shown to
reduce the amount of nerve cell death following an acute episode of global ischemia in cats [15].

After CA and resuscitation, the brain is subjected to a transient period of complete ischemia followed by reperfusion and most of cerebral injury occurs during the early reperfusion period, during which there are two stages of BBB permeability impairment [16]. The first stage starts after the second minute of ischemia and involves the first post-resuscitation hour. BBB opens again 6hrs after ischemia and remains open 24 hrs after cardiac arrest and resuscitation [17]. Progesterone and its metabolites in the brain can induce neuroprotective effects in rats and the half-life of drugs in the brain of rats is approximately 1 hr [13,18]. A previous study indicates that IV administration of progesterone at a dose of 8 mg/kg significantly improves neurological outcomes after focal cerebral ischemia in rats. Treatment with 4 mg/kg or 32 mg/kg of progesterone fails to provide any therapeutic benefit [19]. Despite all the beneficial effects of progesterone on neurons, its protection against acute global cerebral ischemic injury during and following cardiac arrest and resuscitation is unknown. In this study we used a single-dose of 8 mg/kg of progesterone administered during CPR to investigate its acute effects on the neurological outcomes in a rat model of CA. We hypothesized that progesterone administered during CPR improves neurological outcomes after resuscitation.

MATERIALS AND METHODS

All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health (8th edition, Washington, DC, National Academy Press, 2011). The protocol was approved by the Institutional Animal Care and Use Committee of the Weil Institute of Emergency and Critical Care Research at VCU.

Animals were supplied by Harlan Sprague-Dawley Laboratories.

Animal Preparation

Male Sprague-Dawley rats weighing 450-550 g were fasted overnight except for free access to water. The detailed animal preparation was previously published [20]. The animals were anesthetized by intraperitoneal injection of pentobarbital (45 mg/kg) and additional doses (10 mg/kg) were administered at intervals of approximately 1 hr or when required to maintain anesthesia. No anesthetic agents were administered 30 mins before the induction of CA. The trachea was orally intubated with a 14G cannula. End-tidal carbon dioxide (ETCO2) was continuously monitored with a side-stream infrared CO2 analyzer (Model 200; Instrument Laboratory, Lexington, MA). A conventional lead II ECG was continuously monitored. The animals breathed room air spontaneously. For the measurement of electroencephalogram (EEG), an incision on the scalp was made to expose the skull. The electrodes were implanted 2 mm lateral and 2 mm anterior or posterior to the bregma. A ground electrode was implanted 2 mm posterior to the lambda in the midline. The dura was kept intact. All electrodes were fixed by dental cement and the incision was then closed. The electrodes were connected to a data acquisition system (Data Q, Akron, OH) via EEG amplifier (PRE-ISO.EEG100, Xiangyun Computing Technology, Beijing, China) [21]. A polyethylene catheter (PE-50; Becton-Dickinson, Franklin Lakes, NJ) was advanced into the descending aorta from the left femoral artery for the measurement of arterial pressure, blood gas analysis and infusion of drugs. An additional PE-50 catheter was advanced from the left external jugular vein into the right atrium for the measurement of right atrial pressure. Aortic and right atrial pressures were measured with high-sensitivity transducers (Model 42582-08; Abbott Critical Care Systems, North Chicago, IL, USA). A thermocouple microprobe, 10 cm in length and 0.5 mm in diameter (9030-12-D-30, Columbus Instruments, Columbus, OH), was inserted from the left femoral vein into the inferior vena cava for the measurement of blood temperature. A 3-Fr PE catheter (model C-PM3-301, Cook Critical Care, Bloomington, IN) was advanced from the right external jugular vein into the right atrium. A pre-curved guide wire was then advanced through the catheter into the right ventricle to induce ventricular fibrillation (VF), as confirmed by an endocardial electrocardiograph. The blood temperature for all animals was maintained at 37±0.5 °C with a heat lamp. All the catheters were flushed intermittently with saline containing 2.5 IU/ml of crystalline bovine heparin.

Experimental Procedures

All animals were randomized into two groups: 1) progesterone group (PG, n=13) that included progesterone (8 mg/kg, P-7556, Sigma-Aldrich Co., St. Louis, MO, USA) alone (n=8) and progesterone plus Evans blue (160 mg/kg, E2129, Sigma, St. Louis, MO, USA) (n=5); 2) saline placebo group (SG, n=13) that included saline alone (n=8) and saline plus Evans blue (n=5) [19,22].

VF was electrically induced with progressive increases in 60-Hz current to a maximum of 3.5 mA delivered to the right ventricular endocardium. The current flow was continued for 3 mins to prevent spontaneous defibrillation. Mechanical ventilation was discontinued after the onset of VF. After 8 mins of untreated VF, progesterone or saline was injected. 8 mins of untreated VF provides reasonable resuscitation and post resuscitation myocardial and cerebral dysfunction. Precordial compression at a rate of 200 min⁻¹ and mechanical ventilation at FiO₂ of 1.0 were simultaneously initiated after 8 mins of untreated VF and synchronized to provide a compression-ventilation ratio of 2:1 with equal compression-relaxation duration as previously described [20]. The depth of compression was adjusted to maintain the coronary perfusion pressure (CPP) at 22±2 mmHg [23]. Resuscitation was attempted with up to three 4-J biphasic waveform counter shocks (Code Master XL, Heart stream Operation, Philips; Seattle, WA) 8 mins after the start of Precordial compression. Resuscitation was defined as the return of supraventricular rhythm with a mean aortic pressure of 50 mm Hg or greater for a minimum of 5 mins.

Following resuscitation, mechanical ventilation was continued with 100% oxygen for 1 hr, 50% for the second hr and 21% thereafter. Eight animals of each group were randomized and observed by the investigators for 4 hrs after the return of spontaneous circulation (ROSC). After resuscitation, Ketorolac (5 mg/kg IM) was used after the rats were awake or as needed to relieve pain to avoid the effect of pentobarbital on the EEG. All catheters including the endotracheal tube were then removed. The animals were observed for an additional 68 hrs. Five animals

of each group were randomized and administered Evans blue (EB) 6 hrs after ROSC with subsequent circulation of EB for 2 hrs. The animals were then euthanized to harvest brain tissue.

**Measurements**

Aortic and right atrial pressures, electrocardiogram, body temperature and ETCO₂ values were continuously recorded on a personal computer-based data-acquisition system supported by CODAS hardware and software (DataQ, Akron, OH).

At baseline, 2 and 4 hrs after ROSC, ejection fraction (EF) and myocardial performance index (MPI) were measured by echocardiography (Model HD11XE, Philips Medical Systems, Eindhoven, Netherlands) with a 12.5 Hz transducer, which were adopted to estimate the myocardial function. The MPI and EF were reviewed and confirmed by two blinded investigators. MPI was obtained as both systolic and diastolic functions; it is the ratio of total time spent in isovolumic activity (isovolumic contraction and relaxation times) to the ejection time and measured from the mitral inflow and left ventricular outflow time intervals [24,25]. EF served as an indicator of myocardial contractility.

EB bound to serum albumin after injection was used to evaluate permeability of BBB [22]. EB solution was injected from the left femoral artery at 6 hrs after ROSC with subsequent circulation of EB for 2 hrs. After being euthanatized, the rats were transcardially punctured and then perfused with saline (50 ml) until colorless fluid was obtained from the right atrium. The brain was quickly removed and each hemisphere was weighed and frozen in liquid nitrogen and stored at −80 °C. To determine the content of EB in the brain tissue, samples were homogenized in 3.5 ml of phosphate buffered saline (0.1 mmol/L, PH 7.4) and mixed by vortexing for 2 mins after the addition of 2 ml of form amid (60%, F9037, Sigma-Aldrich Co., St. Louis, MO, USA) to precipitate the protein. The mixture was then incubated at 50°C for 72 hrs. The sample was centrifuged for 40 mins at 4000 rpm to pellet the brain tissue. Absorption of the supernatant was measured at a wavelength of 610 nm using a spectrophotometer (iMark, Bio-RAD). EB was calculated as µg/g of brain tissue via a standard curve [26].

EEG signals were recorded from both hemispheres at a sampling rate of 250 Hz [21]. All EEG patterns were analyzed and confirmed by two blinded investigators. During the 4 hr observation period, the EEG patterns were classified as one of the three following categories: suppression/isolectric, burst suppression and recovery of continuous background and EEG activity [27]. Suppression/isolectric was defined as the total suppression and recovery of continuous background and EEG activity [27]. Burst suppression was defined by the presence of EB bound to serum albumin after injection was used to evaluate permeability of BBB [22]. EB solution was injected from the left femoral artery at 6 hrs after ROSC with subsequent circulation of EB for 2 hrs. After being euthanatized, the rats were transcardially punctured and then perfused with saline (50 ml) until colorless fluid was obtained from the right atrium. The brain was quickly removed and each hemisphere was weighed and frozen in liquid nitrogen and stored at −80 °C. To determine the content of EB in the brain tissue, samples were homogenized in 3.5 ml of phosphate buffered saline (0.1 mmol/L, PH 7.4) and mixed by vortexing for 2 mins after the addition of 2 ml of form amid (60%, F9037, Sigma-Aldrich Co., St. Louis, MO, USA) to precipitate the protein. The mixture was then incubated at 50°C for 72 hrs. The sample was centrifuged for 40 mins at 4000 rpm to pellet the brain tissue. Absorption of the supernatant was measured at a wavelength of 610 nm using a spectrophotometer (iMark, Bio-RAD). EB was calculated as µg/g of brain tissue via a standard curve [26].

**Statistical Analyses**

Statistical analysis was performed with SPSS 18.0 software (SPSS Inc., Chicago, IL). All data were presented as mean ± SD. After confirmation of normal distribution with the Kolmogorov-Smirnov-Test, all variables were compared either using the parametric tests (independent-samples T test) or non-parametric tests (Mann-Whitney-U-test). The survival rate was compared using Fisher’s exact test. The survival time was analyzed using the Kaplan-Meier methods and compared using log rank test. A value of *p<.05* was considered significant.

**RESULTS AND DISCUSSION**

Thirty three rats were used for this study, seven of which were excluded due to instrumentation or technical failure. All animals except for one in the SG group were resuscitated. Baseline hemodynamics, blood temperature, blood gas and blood lactate levels did not differ between the PG and SG groups (Table 1). There was no difference in the quality of CPR between the two groups (Table 2). There was also no difference in myocardial function between the PG and SG group’s at 4 hrs after resuscitation (Figure 1).

As shown in Figure (2), the EB dye that passed through the BBB in the PG group was less than that in the SG group (3.2 ± 0.9 µg/g vs. 9.1 ± 0.8 µg/g, *p<.01*).

During 4 hrs of observation after resuscitation, all animals demonstrated the same EEG recovery pattern in the proper sequence of suppression/isolectric, burst suppression and continuous background EEG activity (Figure 3). However, the initial time of identifiable burst was shorter (17.1 ± 4.1 mins vs. 27.0 ± 4.2 mins, *p<.01*) in the PG group when compared with the SG group (Figures 3, 4). For all resuscitated rats, the frequency of burst was increased during the first 90 mins after resuscitation. It was higher in the PG group when compared with the SG group (29.2 ± 5.0 /min vs. 16.3 ± 8.4 /min at PR30, 36.0 ± 4.0 /min vs. 27.3 ± 6.0 /min at PR60, 38.6 ± 3.1 /min vs. 31.1 ± 3.3 /min at PR90, respectively, all *p<.01*) (Figure 4). At 2 hrs post-resuscitation, continuous background EEG activity occurred in the PG and SG groups (Figure 3). There was no difference in the frequency of burst between the two groups (42.8 ± 4.9 n/min vs. 39.1 ± 4.5 n/min at PR120, *p=.10*) at 2hrs post-resuscitation (Figure 4).

### Table 1: Baseline characteristics.

<table>
<thead>
<tr>
<th>Group</th>
<th>PG (n=13)</th>
<th>SG (n=13)</th>
<th><em>p</em></th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>515 ± 10</td>
<td>509 ± 14</td>
<td>NS</td>
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<tr>
<td>Temperature, °C</td>
<td>36.8 ± 0.15</td>
<td>36.9 ± 0.12</td>
<td>NS</td>
</tr>
<tr>
<td>Heart rate, beat/min</td>
<td>358 ± 9</td>
<td>359 ± 13</td>
<td>NS</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>143 ± 9</td>
<td>146 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>RAP, mmHg</td>
<td>1.5 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>End-tidal CO₂, mmHg</td>
<td>40 ± 2</td>
<td>39 ± 3</td>
<td>NS</td>
</tr>
<tr>
<td>EF, %</td>
<td>71 ± 4</td>
<td>72 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td>102 ± 10</td>
<td>105 ± 8</td>
<td>NS</td>
</tr>
<tr>
<td>MPI</td>
<td>0.66 ± 0.08</td>
<td>0.68 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>pH</td>
<td>7.49 ± 0.02</td>
<td>7.5 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Lactate, mmol/L</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>NS</td>
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</table>

Values are presented as mean ± SD. PG: Progesterone Group; SG: Saline Group; EF: Ejection Fraction; MPI: Myocardial Performance Index; MAP: Mean Arterial Pressure; RAP: Right Atrial Pressure; NS: No Significant
Although no significant differences in survival rate were observed between the PG and SG groups (5/8 vs. 2/7 at 24 hrs, 4/8 vs. 1/7 at 48 hrs, 4/8 vs. 0/7 at 72 hrs, p=.214, p=.182, p=.051, respectively), there was a trend toward increased survival rate in the PG group when compared with the SG group (Table 3). Mean survival time was 47.5 hrs (95% CI: 30.5-64.5 hrs) in the PG group and 25.0 hrs (95% CI: 13.1-36.9 hrs) in the SG group (p<.05) (Table 3).

Discussion

The present study demonstrated administration of progesterone during CPR significantly reduced BBB permeability and improved recovery of early post-resuscitation EEG by shortening the isoelectric period and increasing the burst frequency during the first 90 mins after resuscitation. There was no difference in the survival rate between the PG and SG groups.

<table>
<thead>
<tr>
<th>Table 2: Quality of CPR.</th>
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<tr>
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<td><strong>CPP, mm Hg</strong></td>
</tr>
<tr>
<td>PG (n=13) SG (n=13) p</td>
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<tr>
<td>PC1 22.7 ± 0.4 22.8 ± 0.3 NS</td>
</tr>
<tr>
<td>PC8 23.1 ± 0.5 23 ± 0.7 NS</td>
</tr>
<tr>
<td>Duration of CPR, s 483 ± 3 484 ± 5 NS</td>
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<tr>
<td>Defibrillations, n 1.77 ± 0.7 1.67 ± 0.8 NS</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD. PG: Progesterone Group; SG: Saline Group; CPP: Coronary Perfusion Pressure; PC 1, At 1 Minute of Precardial Pressure; PC 8, At 8 Minutes of Precardial Pressure; CPR: Cardiopulmonary Resuscitation; NS: No Significant

<table>
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<th>Table 3: Survival rate and survival time after resuscitation.</th>
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<tr>
<td><strong>Survival rate, n/N</strong></td>
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<tr>
<td>24h 48h 72h</td>
</tr>
<tr>
<td>PG 5/8 4/8 4/8</td>
</tr>
<tr>
<td>SG 2/7 1/7 0/7</td>
</tr>
<tr>
<td><strong>Survival time, hrs</strong></td>
</tr>
<tr>
<td>PG 47.5 (95% CI: 30.5, 64.5)*</td>
</tr>
<tr>
<td>SG 25.0 (95% CI: 13.1, 36.9)</td>
</tr>
</tbody>
</table>

PG: Progesterone Group; SG: Saline Group *p<.05 vs. SG.
In this study, the leakage of BBB was observed in all rats after resuscitation. Following the onset of induced VF, the cerebral “no-flow” phenomenon existed minutes after CA, while the energy requirements of the brain were dependent on complete oxidation of glucose, which was depleted within 2 to 4 mins [29,30]. The shortage of energy resulted in variation of the ionic environment by abnormal cellular influx of potassium and cellular influx of sodium, which causes extracellular accumulation of excitatory amino acids, swelling of brain cells and an increase of BBB permeability [31]. Under the condition of global cerebral ischemia, excessive activation of N-Methyl-D-aspartate (NMDA) receptors results in calcium overload and glutamate excitotoxicity [32]. This pathology has been demonstrated in patients recovering from CA that suffer global cerebral ischemia [33]. Strategies that interrupt the propagation of these cascades would theoretically favor neuronal survival. Interestingly, the systemic administration of progesterone at a single dose of 8 mg/kg significantly reduced the post-resuscitation impairment of BBB when the treatment was initiated at the onset of CPR. There are two different molecular mechanisms underlying its neuroprotection against brain damage: an acute protection by antagonizing α1 receptor to inhibit N-Methyl-D-aspartate (NMDA)-induced Ca2+ influx and a delayed protection by an activation of progesterone receptor (PR)-mediated Src-ERK1/2 signaling pathway [34,35]. NMDA activation causes increased BBB permeability. In addition, progesterone and its metabolites, allopregnanolone and tetrahydrodoc, rapidly alter neuronal excitability through γ-aminobutyric acid type A (GABA) receptor binding through sites on neurotransmitter-gated ion channels [36,37]. The acute administration of progesterone not only attenuates the NMDA-induced Ca2+ influx increase but also rebalances between excitatory and inhibitory neurotransmission by enhancing GABAergic activity in the CNS after resuscitation, which in turn preserves the BBB permeability and reduces cerebral edema [15, 38-40].

Ischemic cerebral injury affects synaptic transmission and the action potential of neuronal cells in a sequential manner and plays a critical role in determining characteristics of EEG [41]. In this study we first used EEG measurement in a rat model of prolonged VF and the recovery of EEG activity was consistent with earlier animal studies [21,27,28]. The studies reported that shorter isoelectric period and higher bursting frequency on EEG analysis of earlier post-resuscitation were correlated with better neurological recovery in a rat model of pulse less electrical activity (PEA) and asphyxia [21]. In this rat model of prolonged VF, we demonstrated decreased BBB permeability and earlier burst firing and higher EEG burst frequency in PG rats. The EEG measurement could be an early indicator of injury and neurological recovery after CA.

Our study reveals significant protective effects of acute progesterone administration on the neurological outcome of CA/Resuscitation in rats. The injection of progesterone during CPR significantly improves neurological function after resuscitation. This may relate to the reduction of cerebral edema. Compared with chronic treatment of progesterone by which neurons recover from ischemia-induced dysfunction through the P4R-activated Src/ERK signaling pathway, the ability of acute administration of progesterone to prevent the post-CA cerebral injury and promote survival in rats is clinically relevant. This may serve as the experimental basis for future clinical trials in which neuroprotective effects of progesterone on the outcome after CA/CPR are examined [40].

There were some limitations in this study. First, it is possible that in implanting the electrodes in the parietal bone during craniotomy, intracranial pressure may be affected. Yet, the dura mater remained intact and the incision was sutured thereafter, so there was no overt effect of cerebral function. Second, we investigated the effect of exogenously administered progesterone only on male rats; we did not use female rats because we wanted to exclude complicating effects on ischemic cell damage of hormonal fluctuations during the estrus cycle. Third, because of the different topographic anatomic characteristics between the rodent brain and human brain, the outcome of this study remains to be proven in larger animals and clinical studies. Finally, our study was performed on healthy rats which were free of underlying disease and direct applicability to human victims is not assumed.

CONCLUSION

The acute administration of progesterone during CPR improves BBB integrity, accelerates the recovery of neurological cells function and thus improves neurological outcomes in a rat model of prolonged VF. This may provide an additional means of cerebral protection.

ACKNOWLEDGEMENTS

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REFERENCES


