Significance of oxygen free radicals in the pathophysiology of hemorrhagic shock – A protocol

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ABSTRACT

Oxygen free radicals have been implicated as the deleterious agent in a variety of organ systems undergoing ischemia and subsequent reperfusion. Hemorrhagic shock represents a clinical situation that carries a high rate of morbidity and mortality despite adequate fluid resuscitation. Since this entity represents, in its most simplified sense, total body ischemia followed by reperfusion, it is likely that the generation of oxygen free radicals has some significance in the pathophysiology of this delayed morbidity. This is a research protocol, where rabbits will be subjected to severe hemorrhagic shock followed by adequate fluid resuscitation. In the first part of the experiment, free radical generation will be measured directly by Electron Paramagnetic Resonance (EPR) spectroscopy in various organ systems in rabbits before and during shock, and following resuscitation. In the second part, free radical scavengers will be introduced as an adjunct to fluid resuscitation in a group of rabbits subjected to hemorrhagic shock to see if mortality rates are affected. By acquiring a better understanding of the molecular mechanisms that may be responsible for the delayed morbidity in reperfusion injury in general, and hemorrhagic shock in particular, we will be able to better address the long-standing problem of multi system organ failure (MSOF) that often follows a successful resuscitation.

1. Introduction

1.1. Specific aims

Despite adequate and presumably successful hemodynamic resuscitation, morbidity and mortality from hemorrhagic shock remains high due to the high incidence of multi system organ failure (MSOF) [1–4]. Common complications in such patients include Acute Respiratory Distress Syndrome (ARDS), acute renal failure (ARF), myocardial depression, and liver dysfunction. The delayed onset of these complications points to an additional mechanism of injury besides ischemia alone. We hypothesize that the production of toxic free radicals in the post-ischemic reperfusion phase may contribute to this organ damage.

We will attempt to demonstrate this in two steps. First, we will directly measure and quantitate the production of free radicals during ischemia and reperfusion in the lungs, kidneys, heart, and liver in animals being resuscitated following hemorrhagic shock. Second, we will attempt to determine if the administration of free radical scavengers during the period of resuscitation can reduce mortality in animals recovering from profound shock [5,6]. Positive data should provide both direct and indirect evidence that the production of free radicals plays a significant role in the delayed morbidity and mortality seen in hemorrhagic shock.

2. Significance

Free radicals, especially those derived from molecular oxygen, have been implicated in a variety of disease processes. In 1983, Schoenberg and colleagues demonstrated that the administration of free radical scavengers could reduce the amount of damage to the small bowel undergoing ischemia and reperfusion [7]. Jolly and co-workers have demonstrated a comparable effect in the myocardium [8]. Other studies carried out on the effect of antioxidant therapy following hemorrhagic shock on the liver [9,10] and overall survival [11] have shown similar results. There is also a growing body of experimental evidence that implicates free radical

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dependent lipid peroxidation in injury to the central nervous system (CNS), as well as the myocardium [12,13]. Overall, there have been many investigators who have indirectly demonstrated that free radicals play an important role in the pathogenesis of reperfusion injury in multiple organ systems. Some investigators (including ourselves) have been able to quantitate free radical production by electron paramagnetic spectroscopy (EPR) [14–16]. Further, a novel fluorescence probe, 2-[6-(4-hydroxyl)phenoxy-3H-Xanthen-3-on-9-yl]benzoic acid (HPF) has been used by some workers to investigate the generation of highly reactive oxygen species (hROS) under ischemia, both *in vivo* and *in vitro* [17]. These techniques have directly demonstrated the production of free radicals during reperfusion after a given period of ischemia to the heart. It has also been demonstrated that there is a significant rise in free radicals in ischemic lung tissue, and are probably involved in the pathogenesis of ischemia reperfusion injury [18].

Due to the ubiquitous nature of this molecular species and the growing evidence of its production during ischemia-reperfusion sequences, we suspect that free radicals may play some role in the pathogenesis of delayed morbidity following shock. It is possible that reperfusion of ischemic organ systems during the period of resuscitation results in the production of free radicals that create additional tissue damage through membrane destabilization. Furthermore, free radicals catalyze lipid peroxidation, a chain reaction mediated by polymorphonuclear leukocytes (PMN), and a phenomenon extremely toxic to cellular membranes.

Our experiments will directly measure the production of free radicals during shock and resuscitation in organ systems that have been clinically shown to be the most vulnerable to delayed morbidity. Confirmation of the generation of significant quantities of free radicals may be of great clinical importance, since it represents a potentially treatable regional molecular event that could otherwise be responsible for a serious clinical problem. Further information regarding increased survival following the administration of free radical scavengers or iron chelators that could interrupt lipid peroxidation would have an obvious clinical significance. The second part of the experiment would potentially provide such data, thus opening the possibility for future clinical trials using scavengers.

3. Background

Literature is abound with evidence that the administration of Superoxide Dismutase (SOD – a superoxide scavenger) or Mannitol (a hydroxyl free radical scavenger) can reduce reperfusion injury. This phenomenon has been documented in the intestines [19,20], liver, kidneys, pancreas, and in the heart both *in vivo* [8,20] and in the global ischemic model [21]. Our laboratory has reported that the administration of either Superoxide Dismutase/ Catalase or Mannitol will significantly reduce the mortality of *Escherichia coli*/Hemoglobin-induced peritonitis in the rat [22]. In this study, the suspected mechanism of action is that free radical scavengers prevent overwhelming production of hydroxyl free radicals generated by the Fenton reaction (driven by PMN in the setting of bacteria and hemoglobin within the peritoneal cavity).

Other experiments with iron chelators have suggested that free radical production continues long after the initial burst due to the lipid peroxidation chain reaction that is mediated by PMN. Deferoxamine was found to attenuate shock-induced oxidative stress in the hepatocytes [23]. Some investigators have found a decrease in CNS damage following ischemia and reperfusion in animals treated with Deferoxamine, an iron chelator [24]. A study done by Liachenko et al. showed a significant increase in cerebral perfusion following the administration of deferoxamine [25]. This is thought to be secondary to the interruption of lipid peroxidation, a process that generates intracellular free radicals and requires iron as a cat-

alyst. A similar study has been carried out in cardiocytes with comparable results [26].

Free radicals in the ischemia-reperfusion setting may be derived from a neutrophil oxygen burst or from the metabolism of Hypoxanthine to Urea with the consequent generation of oxygen. Our group [14,15] and a group at Johns Hopkins University [16] have been successful in measuring free radical production in peri-ischemic conditions. Our work revealed that in the isolated rabbit heart model, global ischemia resulted in a decrease of free radicals, but that free radical production more than doubled upon reperfusion of the heart [143]. The free radicals were measured by freezing the tissue in liquid nitrogen (77 K) and measuring signal size on the electron paramagnetic resonance spectrometer. This work provided direct evidence that free radicals are indeed produced in abundance during reperfusion, and may mediate reperfusion injury. Similarly, in a study carried out on a rat model of focal cerebral ischemia, transient occlusion of the middle cerebral artery generated more reactive oxygen species in comparison to permanent occlusion [17].

Carrying out the experiment we propose requires experience in the use of free radical scavengers, the measuring of free radicals, and the development of a suitable hemorrhagic shock model; all of which are well established procedures in our laboratory. We have also used Superoxide Dismutase/ Catalase [27], Mannitol [28], Deferoxamine [29], Hyaluronidase [30], and perfluorocarbons [31] in an attempt to reduce myocardial reperfusion injury in the baboon.

Our work with the isolated rabbit heart represented one of the first successful attempts to demonstrate a decrease in free radical production during ischemia, followed by a dramatic increase after reperfusion. We have extensive hands-on experience with the use of spin traps for measurement of free radicals using EPR spectroscopy. Our laboratory has also developed a suitable hemorrhagic shock model in rabbits that affords an 80% mortality rate if treated only with resuscitation [32]. This model provides an excellent vehicle through which to test the hypothesis.

To our knowledge, there is no study that has attempted to measure the production of free radicals in organ systems vulnerable to reperfusion injury following massive hemorrhage. There have been few attempts to study survival curves in animals resuscitated from shock, additionally treated with various free radical scavengers (including iron chelators). When one takes into account the recent experimental observations in the field of free radicals and reperfusion injury (summarized below), these tasks are the next logical steps.

Our appraisal of the current state of knowledge is as follows:

1. In isolated organ systems, free radical scavengers have been shown to reduce reperfusion injury in a variety of tissues and organ systems, but little work has been done on systemic ischemia and reperfusion.
2. In shock induced by peritonitis, free radical scavengers have been shown to improve survival.
3. Efforts to quantitate the production of free radicals in the heart have consistently shown a dramatic rise in free radical groups following reperfusion.

These points logically lead to our experimental premise for the following reasons:

1. Delayed morbidity continues to be a problem in hemorrhagic shock despite adequate hemodynamic resuscitation.
2. Many of the organs that are affected have been shown to be damaged by free radical formation and to have less injury when treated concomitantly with free radical scavengers (in isolated reperfusion studies).

3. Other types of shock (peritonitis) have been shown to be less lethal in the presence of free radical scavengers.
4. Hemorrhagic shock with resuscitation can be viewed as total body ischemia and reperfusion.
5. A better understanding of multi system organ failure (MSOF) following shock is a critical next step toward improving survival in patients involved in major trauma.

4. Experimental design

The experimental protocol is described in two parts. The first (A) is designed to measure free radical production in various organs in rabbits subjected to profound hemorrhagic shock, followed by resuscitation (reperfusion). The second part (B) will attempt to study the effects that free radical scavengers and iron chelators have on survival when used as an adjunct to resuscitation.

A. Quantitative assessment of free radical production during shock and subsequent resuscitation in multiple organ systems in the rabbit

In this part of the experiment, four groups of rabbits will be used. All groups will have their lungs, heart, liver, intestines, and kidneys sampled at different stages of shock. These tissues will be frozen with liquid nitrogen (77 K) and powdered. This powder will then be analyzed by EPR spectroscopy for the presence of free radicals. The first group will consist of animals that are anesthetized, then sacrificed before the initiation of shock. The second will be sampled during the period of shock itself. The third group will be sampled immediately after reperfusion (resuscitation). The fourth will be sacrificed and sampled, 12 h post-shock or upon expiration, whichever comes first. In this manner, a comparison can be made as to the quantity of free radicals produced during each phase by comparing the size of the EPR signals.

5. Specific protocol

Initially, forty New Zealand white rabbits will be used (ten per group in four groups). They will be anesthetized with subcutaneous injections of Ketamine Hydrochloride (10 mg/kg). Sedation will be maintained with Ketamine and Diazepam (Valium, 5 mg/ml) in combined doses of 0.2 ml through an ear vein catheter. The rabbits will be secured in a supine position on a heating pad to maintain normothermia. Femoral cutdowns will be performed and all four vessels cannulated to monitor femoral arterial pressure, central venous pressure (CVP), body temperature, and cardiac output (CO). Routine arterial samples will be drawn to monitor pH and blood gases. All lines will be periodically flushed with heparinized saline (4 NIH units of Heparin/ml).

Baseline measurements will be taken, including arterial blood gases (ABG), mean arterial pressure (MAP), CVP, heart rate (HR), and CO. A control group of ten animals will be sacrificed at this point of the experiment. Biopsies of the lungs, heart, liver, intestines, and kidneys will be taken with a Van Doren clamp. The tissues will be immediately frozen in liquid nitrogen (77 K) and maintained in that state. The remaining animals will be slowly bled over 15 min to a MAP of 44 mm Hg. This blood will then be stored with 2 ml Heparin/50 ml blood. Aliquots of blood will either be withdrawn or infused to maintain the MAP at 44 mg Hg for 2½ h. At the end of this shock period, the withdrawn blood will be re-infused along with lactated Ringer's solution until a MAP is obtained that is within 20% of the baseline or until a maximum of 100 ml of fluid is delivered. The animals will then be given maintenance IV fluid for one hour and then a bolus of three times the

normal rate. The catheters will then be removed from the femoral vessels, and the vessels will be ligated. The wounds will be closed in layers and the animals returned to the cages for observation.

Of the three groups of animals undergoing shock, one group will be biopsied during the period of shock itself (Group 2). Group 3 will be biopsied immediately after reperfusion (5 min after resuscitation). The final group will be biopsied 12 h after the shock period, or the instant the animal dies, whichever occurs first. The tissue from these biopsies will be treated in an identical fashion to the control group.

The frozen, powdered tissue will then be placed in EPR tubes with a diameter of 3 mm and filled to a height of 7 mm. The EPR spectra of the tissues will be recorded on a Varian Model E-9 spectrometer (frequency = 9.19 GHz, power = 1.0 mW, modulation amplitude = 2.5 G). The amplitude of all signals generated will be measured and averaged for all rabbits in each group. The mean signal size for each of the groups will then be compared. Using calculated g values, the types of free radicals generated can be identified.

B. The effect of free radical scavengers on survival in hemorrhagic shock and subsequent resuscitation in the rabbit

This part of the experiment will also consist of 4 groups of rabbits. All groups will undergo hemorrhagic shock to a MAP of 44 mm Hg for 2½ h. This degree of shock has been shown in our laboratory to be associated with a 20–30% survival rate, with the mean time of death for rabbits being approximately 20 h post-shock. The control group will be resuscitated with blood and lactated Ringer's solution alone. The first treated group will receive superoxide Dismutase/Catalase along with the infusion. The second will receive the iron chelator, Deferoxamine. The third group will receive both drug regimens along with the standard reperfusion. Survival curves will be compared.

6. Specific protocol

Sixty New Zealand white rabbits (15 per group) will be surgically prepared in an identical fashion as was proposed in Part A of the experiment. Shock will be induced by slowly bleeding the rabbits over a 15-min period to a MAP of 44 mm Hg. This blood will be stored with 2 ml Heparin/50 ml blood. Aliquots of blood will either be withdrawn or infused to maintain the MAP at 44 mm Hg for 2½ h.

The control group will be resuscitated at the end of the 2½ h period with the shed blood and lactated Ringer's solution until the MAP achieved is within 20% of baseline or until a maximum of 100 ml of fluid is given. Group 2 will be resuscitated in the same manner, but will receive in addition a 50 mg/kg bolus of Superoxide Dismutase and a 5 mg/kg bolus of Catalase at the start of resuscitation, followed by a 1 mg/kg/min drip of each of Superoxide Dismutase and Catalase during the one hour maintenance period. The second treated group (Group 3) will receive Deferoxamine with the fluid resuscitation, at a dose of 50 mg/kg given as a continuous infusion, at the start of resuscitation and ending after the one hour maintenance period. The final group will receive all treatment modalities (Fluids, Superoxide Dismutase/Catalase, and Deferoxamine).

Once the resuscitation phase is complete, all animals will undergo a one hour period of maintenance IV fluid administration followed by a fluid bolus of three times the maintenance prior to closing. They will then be returned to their cages and allowed food and water. Survival will be assessed for each group up to a period of five days. At five days, all surviving animals will be sacrificed and autopsied. The lungs, heart, liver, intestines, and kidneys will be

biopsied and examined grossly and microscopically for evidence of necrosis. Survival curves will be constructed for all four groups and statistical significance will be determined by use of the Kaplan–Meier methods.

7. Vertebrate use

New Zealand white rabbits, all young adults, will be used for this experiment. A total of 100 (40 for Part A and 60 for Part B) will be necessary for the two parts of the experiment. These animals will weigh approximately 2–3 kg. They are relatively inexpensive and freely available from local sources.

1. *Rationale for the use of rabbits*New Zealand white rabbits are not expensive, easily obtained, and easy to work with. Through extensive prior experience, we have accumulated satisfactory working knowledge in this species and successfully developed a shock model that meets the requirements of our current study. The existence of a proven shock model will help save additional time, money and resources required in developing a model from ground up. The number of animals that we will require to complete both parts of our study
($n = 100$) are such that a relatively inexpensive and easily obtainable animal species is necessary. The number of groups that we have established is necessary to prove our hypothesis, and the number of animals per group is necessary to ensure statistical significance.
2. *Procedure for maintenance and care*
We have a well-established animal care facility that has been in existence for 28 years. We have relocated to a newly renovated facility within the past two years complete with new cages and animal maintenance facilities that meet the requirements of the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use of Laboratory Animals, National Institutes of Health, DHEW Publication No. (NIH) 78-23. A veterinarian employed by the hospital consults on the facilities, and animal care. There is an animal caretaker who feeds and cleans the animals daily. All animals are adequately sedated or anesthetized before any procedure, and the sedation is continued until the conclusion of the experiment. All surgically prepared animals are recovered under observation for a minimum of four hours.

Consultants

A computer and statistical consultant is required to be on staff or available for data analysis.

Consortium agreements

Such agreements may be necessary, especially in relation to equipment which may not be available in the principal investigator's laboratory, where the work will be conducted.

Author statement

There are no author disclosures. This is a protocol and does not have any financial issues.

Conflicts of interest

None.

Funding

This was a protocol and sources of funding are not applicable.

Ethical approval

None.

Consent

None.

Author contribution

Shyamal Premaratne: planning of experiment, protocol development, literature search, manuscript preparation.
Dhanushya Amaratunga: protocol development, literature search.
Francis Mensa: protocol development, statistical analysis.
Late J. Judson McNamara: principal investigator on the project, chairman of the department.

Guarantor

Dr. Shyamal Premaratne and Dr. J. Judson McNamara were the initial guarantors. However, with the passing of Dr. McNamara, Dr. Premaratne will assume that role.

Registration research studies

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.isjp.2018.04.002.

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