Complement Inhibitor APT070 Dramatically Reduces the Need for Resuscitation and Improves Survival in Controlled Isobaric Rat Hemorrhage Model

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ABSTRACT

The complement system (C) becomes activated during hemorrhagic shock in a rat isobaric hemorrhage model, a process significantly aggravating the outcome of shock. Consequently, inhibition of C activation may have beneficial effects on both survival and metabolic status of animals subjected to shock.

1.0   INTRODUCTION

Earlier data showed that complement activation takes place during hemorrhagic shock in pigs[6]. The exact cause of this activation is not known, but it is likely that the C5a and C3a anaphylatoxins released during the activation of the complement cascade may have a profound role in the pathophysiological changes that characterize trauma and shock[7]. It has been hypothesized that by inhibiting the complement system, it may be possible to minimize the harmful effects of hemorrhagic shock. To address this question, we have used an isobaric hemorrhage model in rats for the testing of complement inhibitors.

The complement system is complex with three distinct pathways known to initiate the serine protease cascade and produce anaphylatoxins (Fig. 1). The classical pathway is triggered by antibody–antigen complexes while the alternative pathway includes the generation of C3 convertase by foreign surfaces

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* Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

(lacking membrane associated or soluble complement inhibitors) and the stabilization of activated C3 by factor D or B. The third pathway involves lectins that bind to bacterial surfaces and initialize C3 activation. All three pathways lead to the cleavage of C3 and release of C3a.

The activation pathways merge into the *terminal pathway* with formation of the membrane attack complex (MAC, C5b-9) that lyses foreign particles as well as activates inflammatory cells for particle elimination. Complement activation is strictly regulated by natural inhibitory proteins, and there are numerous synthetic molecules that block the activation chain at different sites (Fig. 2). Thus, various reagents with potential therapeutic applications have been developed to target C activation and function (indicated by red asterisks in Fig. 1) but the work reported here has used a modified version of a CR1 (complement receptor type 1) fragment that has been shown to interfere with the early amplification phase in which C3 convertase generates more C3b. The molecule has been modified to be made more effective by tagging it with a positively charged peptide and hydrophobic anchor that allows it to be concentrated on the cellular surface and protect the cell surface vigorously from complement activation. It is know as APT070 (Adprotec, Inc.) (Linton et al. 2590-97, Smith 1037-41). APT070 effectively blocks and reduces the generation of the activated form of C3, and thwarts the release of both C3a and C5a. APT070 is a chemical conjugation of APT154, a region taken from the Complement Receptor type 1 molecule, APT542, and myrystoic acid.
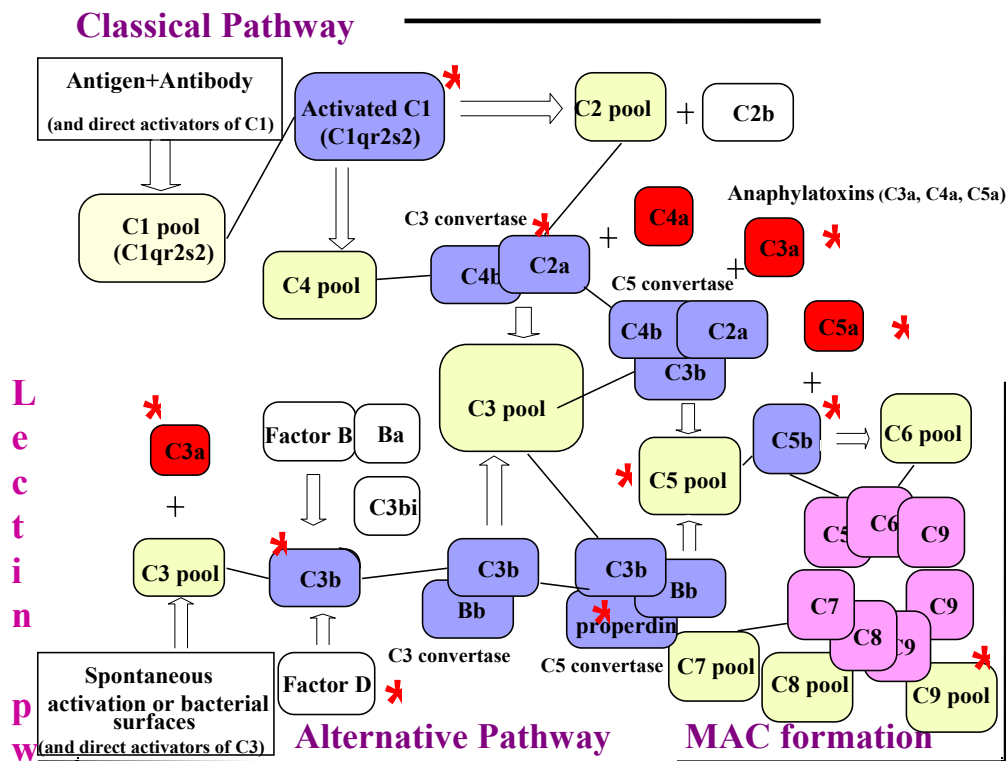
Figure 1. The key elements in complement system are the serine protease pro-enzymes, which themselves serve as substrate for the next step in the cascade. It represents one of the most powerful signal amplification system ever known. The products of the cascade are **a)** the membrane attack complex (MAC), that can bore holes in membranes, and has cytolytic properties, **b)** the opsonins mainly C3b,C3bi, and **c)** anaphylatoxins (C3a and C5a) and other signalling molecules such as C3d, C4d and C4dg etc). The man-made inhibitors act in the steps marked by red * .

2.0 METHODS

2.1 Animals

Sprague-Dawley rats (300-600 g) were from Charles River Laboratories, Wilmington, MD. Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations related to animals and experiments involving animals adheres to principles stated in the Guide to the Care and Use of Laboratory Animals, National Research Council.

2.2 Chemicals

Isoflurane (Viking Medical Products, Medford Lakes, NJ); pentobarbital (Sigma, St. Louis, MO); 6 IU / mL heparin (Elkins-Sinn, Cherry Hill, NJ); IvIg F(ab)$_2$ (Centeon Pharm. GmbH, Wien, Austria); APT070 (ADPROTECH Co., Little Chesterfort, UK), Physiological saline solution (Gibco, Grand Island, NY).

2.3 Equipment for Data Collection

Digital balance (PM300, Metler-Toledo, Columbus, OH); Peristaltic pump (P730, Instech Inc., Plymouth Meeting, PA); Radiometer, Automated Blood Analyzer (ABL 7000, Radiometer America Inc., Westlake, OH); Amplifiers (BPA-400A, Digi-Med, Louisville, KY); MIO6325 DAQ board (National Instruments, Austin, TX); Pentium class PC; DataLyser and REDIREC proprietary data acquisition and analysis software, proprietary isobaric hemorrhage computerized data acquisition and process control software developed at WRAIR using LabView (National Instruments, Austin, TX).

2.4 Induction of isobaric hemorrhagic shock in anesthetized, unconscious rats

Sprague-Dawley rats were temporarily anesthetized with 5%v/v isoflurane gas. The rats were weighed (300-600 g), and single dose of 50mg/kg pentobarbital bolus was injected intra-peritoneally to anesthetize the rat.
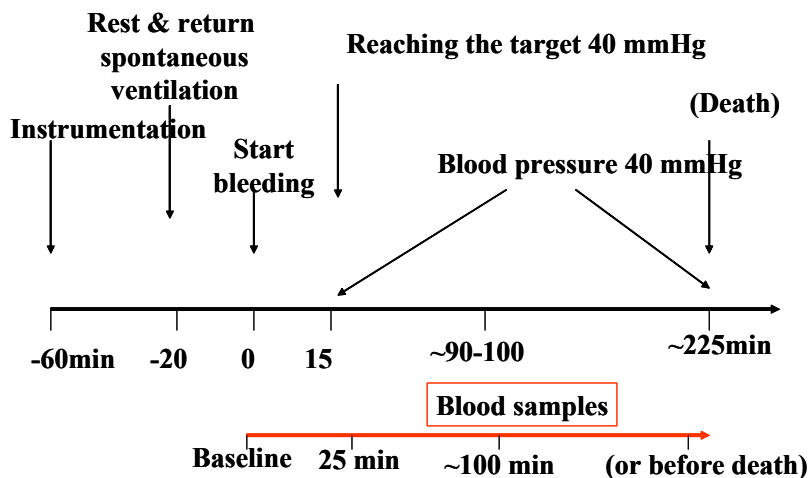

Figure 2a. Isobaric hemorrhage: the timeline in the anesthetized, unconscious rat model.

Once the rat was unconscious, tracheotomy and bilateral femoral artery cannulations were performed. Once the rat was unconscious, tracheotomy and bilateral femoral artery cannulations were performed. The femoral artery cannula was linked to a computer-controlled peristaltic pump (P730) system and a reservoir to maintain desired mean arterial blood pressure (MABP) through the withdrawal of blood under the control of the hemorrhage software. All measurement signals were recorded every five seconds for subsequent analysis. The pentobarbital-anesthetized animals were allowed to stabilize during a control period of 20 minutes, (Fig 2a). Bleeding followed this period, and was conducted with computer-controlled peristaltic pump. The

targeted mean arterial pressure (MBP) of 40 mmHg was reached in 15 minutes with the withdrawal of 3-5 ml of blood. Blood samples were obtained at 0 (baseline), 60, 80, 120, 150, and 180 minutes after bleeding began, and the experiment was terminated after 225 minutes.

2.5   Induction of isobaric hemorrhagic shock in resuscitated, conscious rats

Male Sprague-Dawley rats were anesthetized with isoflurane (5% induction, 1.5-2% maintenance) and cannulas were inserted surgically into femoral artery and vein. The cannulas were then lead subcutaneously to the neck area, exteriorized, passed through a metal spring cannula guard and connected to a two channel fluid swivel. The swivel connected the venous cannula to the peristaltic pump and the arterial cannula to the blood pressure sensor. The animals recovered from the anaesthesia for two hours and then underwent the hemorrhage and resuscitation while conscious and freely moving. After a 20 min control period, blood was withdrawn under computer control lowering MABP over 15 min to 40 mmHg where it was held for 30 min. The animals received lactated Ringers to during a prolonged hypotensive resuscitation (MABP supported at 60 mmHg for 4 hrs) followed by full resuscitation to 80 mmHg and 24 hr survival (Fig. 2b). Treated animals received the APT070 complement inhibitor (10 mg/kg bolus in 0.7 mL**)** one minute before the start of the hypotensive resuscitation (at 64 min). Control animals received an equivalent volume of saline at the same time point. Blood samples were taken prior to hemorrhage (baseline), 65 min (end of hemorrhage period), 335 min (end of resuscitation) and the last sample was collected when the animal was nearly moribund or at 24 h, when the observation period ended. The blood samples were analyzed using a blood gas analyzer (ABL 735, Radiometer America Inc., Westlake, OH) to determine blood chemistry.
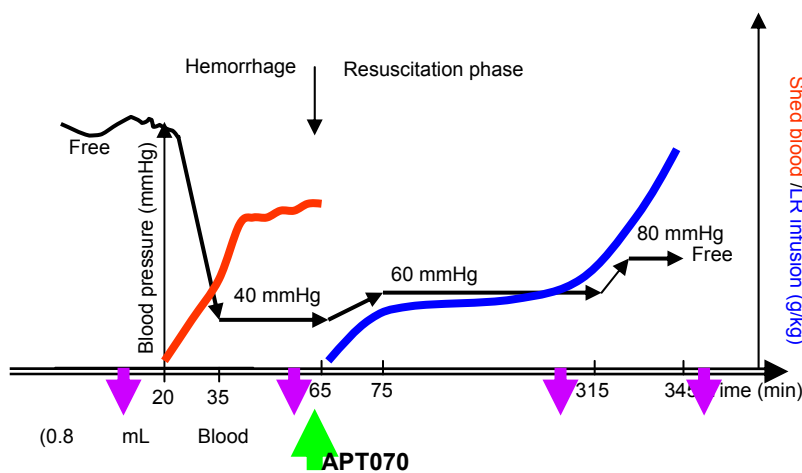
Figure 2b
Unanaesthetized, survival rat model of isobaric hemorrhagic shock, with resuscitation. Blood pressure data, shed blood volume, the volume of resuscitation fluid and survival time is recorded. Rats recovered 2 h after anaesthesia before start of experiment.

2.6   Statistical analysis

Students' unpaired t-test was used to compare the mean values in treated and untreated groups, as implemented in GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

3.0 RESULTS

Complement activation is found early in hemorrhaged rats, as measured by a decrease in residual hemolytic activity of the serum using the Ch50 analysis. The shed blood volume reached it maximum at about 90 min and since blood was only removed and no resuscitation fluid was given up to that time, there was no significant plasma dilution to account for the decrease in the hemolytic activity. Therefore, it is concluded that strong complement activation precedes the development of decompensated hemorrhagic shock (Fig. 3).

Figure 3. Complement activation in hemorrhaging rats, representative data of n>10)
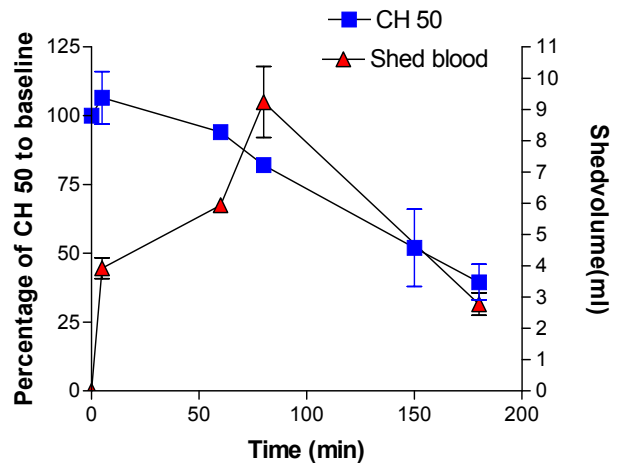
Figure 4.   Shed blood profile in hemorrhage experiment. Shed blood reached a maximum after 90 min and infusion of resuscitation fluid was necessary to maintain the targeted blood pressure of 40 mmHg.
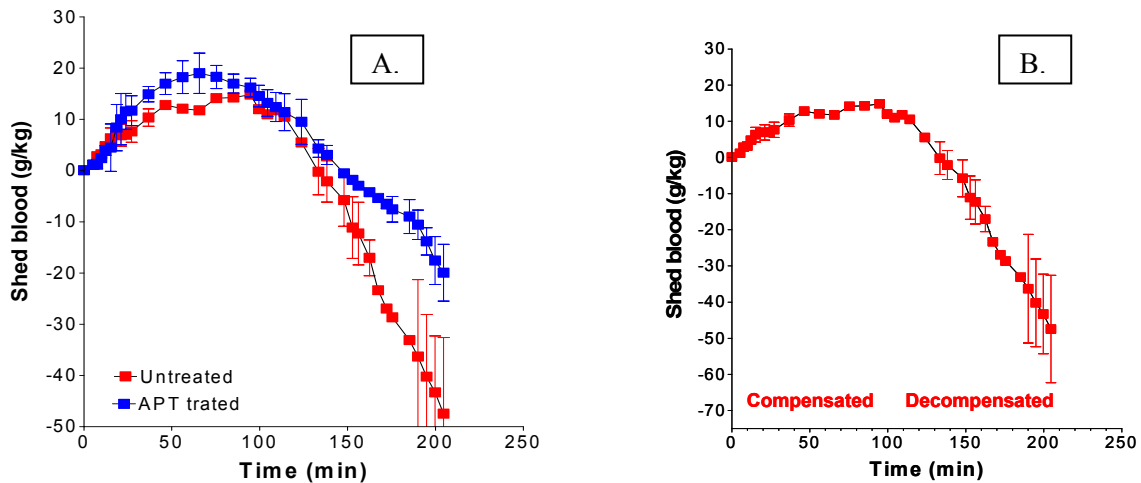
Shed blood is the amount of blood removed from a rat to lower its blood pressure to 40 mmHg. More blood is removed, if the animal can keep its blood pressure at a higher level. The apparent increase in the amount of shed blood in rats pre-treated with APT070 while all of the pre-treated rats survived confirms our hypothesis, that blood loss in itself is not the most important factor for the early deaths (difference in death rate by 225 minutes is statistically significant, $P<0.001$). As Figure 4b indicates, APT070 treatment reduced the need for infusion of lactated ringer solution as well, blood loss induced severe hypoxia and physiological changes characteristic to the late phase.

The blood chemistry of rats undergoing hemorrhage show several large and potentially important changes. Blood lactate concentration increases are due to the development of tissue hypoxia and the consequent switchover from aerobic to anaerobic respiration. Reduced hepatic flow may also contribute to lactate elevation since the liver under aerobic conditions normally takes up lactate produced by other tissues, Fig. 5a). It is worth noting that the APT treated rats lost slightly more blood, and if fact are expected to have more severe hemorrhage, however the survival times and blood chemistry illustrated that the opposite is the case, the are better of than the controls, and complement inhibiton is highly beneficial.
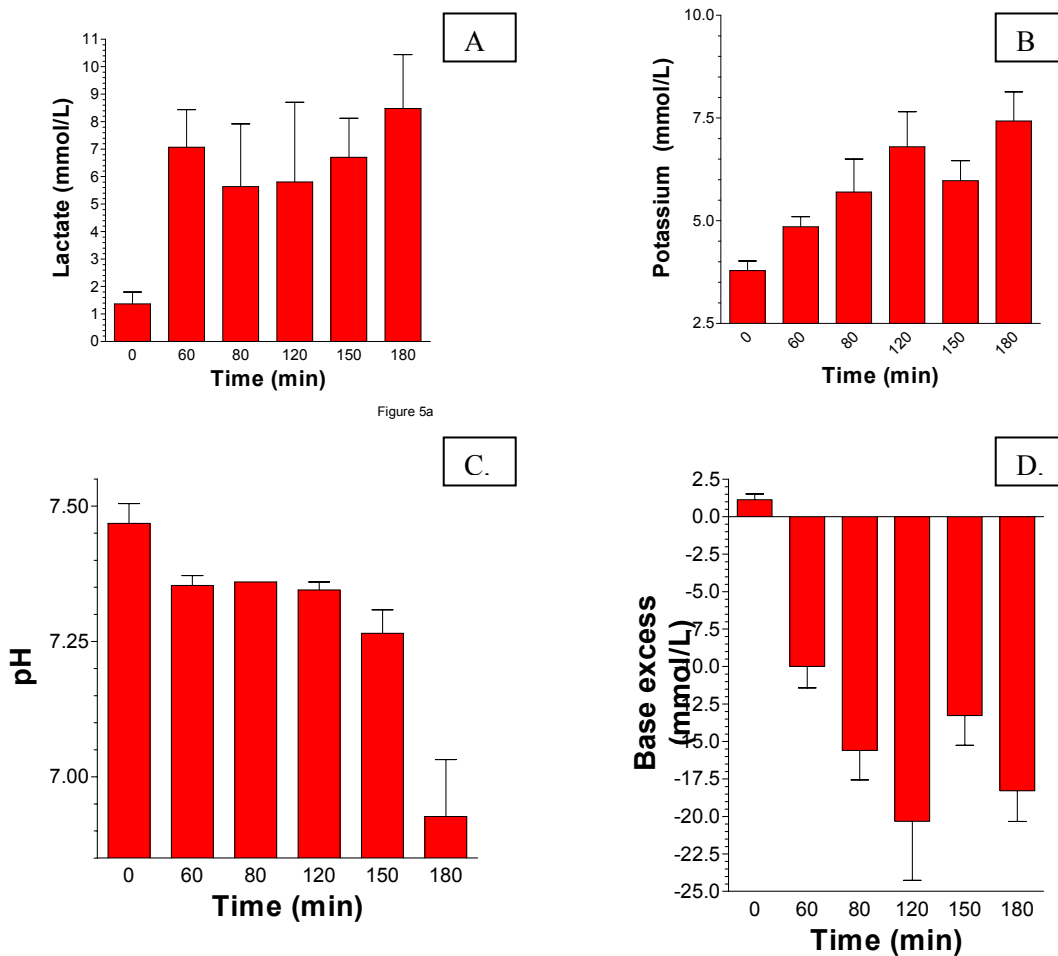

Figure 5. Changes in blood chemistry in rats undergoing hemorrhage.

Under normal conditions, potassium is actively stored in cell cytoplasm, requiring energy to maintain this state. Rats in the untreated and control groups have increased potassium levels, as hypoxia sets in and energy supply is insufficient to maintain the active storage of the potassium ions (Fig. 5b). Due to a higher shed blood volume (and therefore, more severe hypoxia and impaired energy production), the blood potassium level is expected to be more severe. However as we show in the presentation, it is not the case. Blood pH is normally slightly alkaline (7.4). During hemorrhage and other hypoxic conditions, lactate is produced through anaerobic respiration and contributes to shifting the plasma to acidic pH (Fig. 5c) the acidosis is also marked by negative base excess values, indicating that the bicarbonate/CO_2 buffer system cannot compensate for the observed shift in pH (Fig. 5d). At the baseline measurement, all groups are within a normal range. This also indicates changes in gas exchange and breathing patterns. During hemorrhage the APT070 pre-treated animals were partially protected from these severe changes as we show in the presentation. Plasma in hemorrhagic rats shifts to acidic pH (Fig. 5c) and metabolic acidosis is also marked by negative base excess values, indicating that the bicarbonate/CO_2 buffer system can not compensate for the observed shift in pH (Fig. 5d).

4.0 DISCUSSION & CONCLUSIONS

In the isobaric hemorrhage model, hemorrhage occurs in three distinct phases. The first, or compensated stage, occurs when bleeding has begun, but the rat's cardiovascular system is still capable of maintaining blood pressure above the 40 mmHg preset threshold. Approximately 45 minutes into the compensated stage, blood loss reaches a plateau, at which point the cardiovascular system collapses; in a sense, it is no longer able to maintain blood pressure above 40 mmHg and requires blood or resuscitative fluid to be continually infused for the animal to survive.

Earlier measurements indicated that the complement system is activated during hemorrhage. Since C5a and C3a are anaphylatoxins that are implicated in inducing hemorrhagic shock, a theory was developed that inhibiting the anaphylatoxins would prevent the adverse affects of hemorrhagic shock. APT070 blocks complement activation on cell surface, and strongly reduce the release of the anaphylatoxins C3a and C5a. Its demonstrated benefits in our model hold promise for using this C inhibitor as adjunct therapy for hemorrhagic shock. However, further studies are necessary to bear out this proposition.

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