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Pig as an experimental model for the study of snake venom induced local tissue necrosis

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ABSTRACT

Local tissue necrosis due to snake envenoming has resulted in considerable chronic disability. Specific antivenom, though highly useful in systemic poisoning, is not very effective in preventing the local tissue damages which occur within minutes of envenomation. Most of the studies on local tissue necrosis have been done in rodents whose skin structures are significantly different from human skin structure. In this study, pig, which is similar to human in this respect, was used as an experimental model for the study of local tissue damage caused by snake venom.

An intradermal (i.d.) injection of *Calloselasma rhodostoma* (CR) venom induced induration and hemorrhage at the same injection site, the areas of which could be estimated at 30–45 min and at 4 h after injection, respectively. Both the hemorrhage and induration were dose-dependent and a total of 24 data points of each assay per pig could be obtained. Catheterization of the jugular vein for gentle serial blood collection could be performed without any hematologic sequelae. Venom injected subcutaneously induced myonecrosis as demonstrated by the increment of serum creatine kinase (CK) level which peaked at 23 h. Furthermore, biopsies at varying distances and depths around the venom injection sites could be made within seconds of injection to study the pathological changes caused by snake venom. These results demonstrated that pig should be a useful animal model for the quantitation, pathogenesis and wound healing studies of snake venom induced local tissue necrosis, and for the search for effective treatment modality.

1. Introduction

Snake envenomation is an important medical problem in many regions of the world. The sequelae that follow may be systemic toxicity and/or local tissue damage (Warrell et al., 1977; Pugh and Theakston, 1987). While systemic poisoning can be effectively treated with specific antivenom, such treatment is relatively inefficient in preventing local tissue damage. The local effects of snake venoms have been classically defined to include edema, hemorrhage and myonecrosis. Many toxins and enzymes including metalloproteinases, phospholipases and other proteases, are thought to be involved in local tissue damage (Gutierrez and Rucavado, 2000; Perez et al., 2007). These toxins and enzymes act in some cases synergistically, to cause tissue damage within a few minutes of envenomation (Gutierrez and Lomonte, 1989), the manifestations of which appear hours or days later. The pathogenesis processes, especially those caused by hematotoxic venoms, although not well understood, cannot be studied by biopsy in man.

Because the victims usually arrive at the health centers hours, if not days after the bites, local tissue damage has been perceived as impossible to prevent or reduce.

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Recently, studies carried out by many investigators have shown that injections of various enzyme inhibitors immediately after venom injection could reduce or prevent local tissue damage (Rucavado et al., 2000; Yingprasertchai et al., 2003; Howes et al., 2007; Azofeifa et al., 2008). However, those experiments were carried out in rodents whose skin structures are very different from those of human, and a better experimental animal model is required to study this important problem.

The usefulness of pig in biomedical research is widely acknowledged. Several characteristics common to pig and man indicate that the pig can serve as a good experimental model for man. Pig has been used as a model for the human musculoskeletal system, in wound healing and in cardiovascular research (Ekman, 1996; Sullivan et al., 2001; Hughes, 1986). Since the skins of pig and man have many similar characteristics, e.g. sparsely covered with hair, thick epidermis and similar cutaneous vascular anatomy, it has been recently used in snake venom research as a model to evaluate the efficacy of pressure-immobilization, fasciotomy and venom extraction device as well as in the study of compartment syndrome (German et al., 2005; Tanen et al., 2004; Bush et al., 2004; Bania et al., 1998).

The aim of the present study was to assess the feasibility of quantifying edema, hemorrhage, and myonecrosis in pig envenomation model. The experiments were designed to be short so as to minimize suffering while maximizing the number of data points per animal. The results reported here indicate that pig should be a good experimental model for the study of local tissue damage caused by snake venoms and in the search for its treatment modalities.

2. Materials and methods

2.1. Snake venom

Pooled *Calloselasma rhodostoma* venom obtained from adult specimens was purchased from Queen Saovabha Memorial Institute, Bangkok. It was assayed for the protein content (Lowry et al., 1951) and kept frozen at −20 °C.

2.2. Animals

Immature female commercial pigs weighing 18–25 kg were housed individually for 5 days before starting the experiment. The animals were fed twice daily with 0.7 kg of commercial diet and water *ad libitum*. The care and handling of animals were in accordance with the National Research Council of Thailand guidelines for ethical animal research. The experimental protocol was approved by the Laboratory Animal Committee, Faculty of Veterinary Science, Mahidol University.

2.3. Edema-inducing activity

Pigs were premedicated by intramuscular injections of Azaperone (4 mg/kg) and Atropine sulphate (0.04 mg/kg). After 15 min, anesthesia was induced by intravenous administration of pentobarbital sodium (15 mg/kg). The pig was then transferred to a restraining device which held it in normal standing position, but its feet were approximately 10 cm above the floor level (Fig. 1). The pig skin was shaved, cleaned and then marked on each lateral side of the body with permanent markers to provide as many as 12 injection sites at least 5 cm apart. Thus, a total of 24 different treatments per pig could be made.

Venom was diluted in normal saline solution to give concentrations of 15.6, 31.2, 62.5, 125, 250 and 500 μg in 200 μl per injection. Each venom concentration was intradermally administered in duplicate sites. The induration which was hard and tense as described in CR venom poisoning in human victims, developed at these sites. The circumferences of the induration were marked using color markers on the pig skin by determining the edge of the elevated area around the injection site. This procedure was done by the veterinarian at 15, 30, 45, 60, 75, 90, 105 and 120 min after venom injection. The marked area of induration was subsequently copied to a transparent sheet which was later scanned together with a standard length marker for further analysis of the induration area by ImageJ® software (National Institutes of Health, USA).

2.4. Hemorrhagic activity

Hemorrhagic activity was studied as described in the edema-inducing activity. Briefly, 4 h after intradermal injection of various concentrations of CR venom, at which time the induration study has been completed, pigs were euthanatized by the intravascular administration of pentobarbital sodium (30 mg/kg). The skin was dissected to expose the subcutaneous hemorrhage. Photomicrographs of the lesions were analysed by ImageJ® software for the gray scale representing color intensity and area.

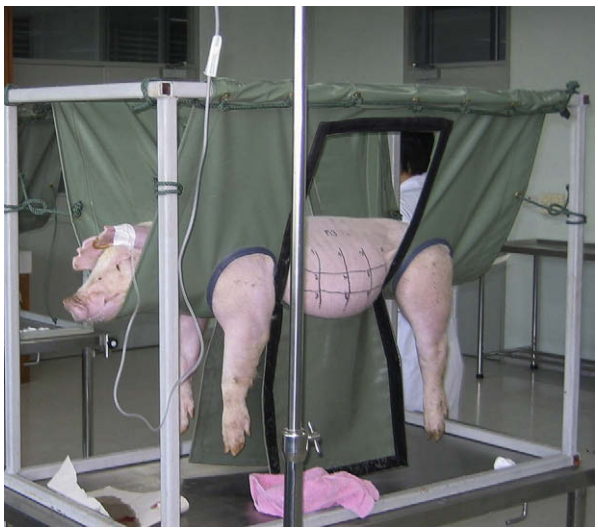

Fig. 1. A restraint used for holding a pig in the experiments on venom induced edema and hemorrhage. The openings at the lateral sides of the pig were designed for administration of the venom and quantitation of the venom effects.

2.5. Catheterization procedure and serial blood collection

In experiments where sequential blood sampling was required, catheters were implanted in order to minimize stress and trauma during blood collection. Pigs were fasted for 12 h prior to surgical catheterization. Following pre-medication with i.m. azaperone (4 mg/kg) and atropine sulphate (0.04 mg/kg), anesthetic induction was achieved with i.v. zolazepam (5 mg/kg), and analgesia was induced by butorphanol (0.3 mg/kg). Venous catheterization was carried out essentially as described by Rodriguez and Kunawongkrit (1983). Penicillin G (40,000 IU/kg, i.m.) was administered once post-surgically. The animals were allowed 5 days of recovery before beginning the experiment.

During the 5-day recovery period, blood samples were collected twice daily. Prior to collecting the samples, the heparinized blood which previously filled the catheter, was removed. After each blood sample was collected, the cannulation tubing was flushed with 1.5 ml of heparinized saline to maintain patency of the catheter. Blood samples were analysed for hematological parameters by the Analyzer ABC VET (ABX Diagnostics, France). The body temperature and the animal behavior were recorded daily.

2.6. Determination of creatine kinase (CK) and interleukin-6 (IL-6) levels

Pigs were catheterized in the jugular vein and housed for 5 days as described above. A group of 4 pigs were injected subcutaneously on the back with 16 mg of CR venom in a total volume of 250 μl. Each pig was injected at 2 injection sites, thus resulting in a total of 32 mg of CR venom per pig. In the untreated control group, pigs were injected with NSS only. At 0, 2, 4, 6, 12, 23, 47 and 72 h after venom injection, blood samples were collected from the catheterized vein. The serum as well as heparinized plasma were prepared, aliquoted and stored at −70 °C for further analysis. Creatine kinase was measured using a commercial kit (Randox Laboratories Ltd., UK). Porcine IL-6 immunoassay kit was used for the assay of plasma IL-6 according to the manufacturer's instruction (R&D Systems Inc., USA).

2.7. Histopathology study

In this experiment, an anesthetized pig which was under study on edema and hemorrhage was used, and CR venom (2.5 mg/200 μl) was injected s.c. at the back of the pig by keeping the needle perpendicular to, and 5 mm deep into the skin. Punch biopsies were taken 1, 3 and 18 min after venom injection approximately 4 mm from the injection mark using a 2 mm diameter Biopsy Punch (Stiefel Lab., UK). The tissues were fixed in 10% buffered formalin for 24 h. Sections of 3 micron were stained with hematoxylin and eosin and observed under light microscope.

3. Results

3.1. Edema-inducing activity

The time course of induration induced by different concentrations of the venom was followed over a 120-min period. All doses of the venom induced hard and tense induration within 15 min and peaked at about 50 min after injection. The induration areas measured at 30 and 45 min after injections when plotted against the CR concentrations showed a dose–response relationship (Fig. 2). BSA, which was used as control, induced soft and weak response of induration in a non-dose-dependent manner (data not shown).

3.2. Hemorrhage

Intradermal injection of CR venom induced hemorrhagic spots on the internal side of the pig skin. At 1 h after injection, CR venom as low as 15 μg induced faint reddish spots with the average diameter of about 10 mm. The spots were larger and appeared in dark-red when higher concentrations of the venom were used. The hemorrhages, both in area and in color intensity, were pronounced when the time was extended to 4 h (data not shown). Fig. 3 shows the dose-dependent response of hemorrhage; BSA, which was used as a control protein, produced no observable hemorrhagic spots at all concentrations tested (Fig. 3).

3.3. Effect of catheterization on the pig

Table 1 shows the results on hematologic parameters before the cannulation and during the 4 days of recovery. The only significant change was an increase in hemoglobin concentration during the first 2 days of catheterization. All pigs showed normal feeding behavior and body temperature (data not shown). Blood withdrawal could be done gently during feeding without any signs of pain and discomfort. When the cannula were removed at the end of the experiments, there were no signs of inflammation or infection and the cannula were secured in their primary positions.

3.4. Myonecrosis

Sera serially obtained from each pig were assayed for creatine kinase (CK). The level of CK continuously increased

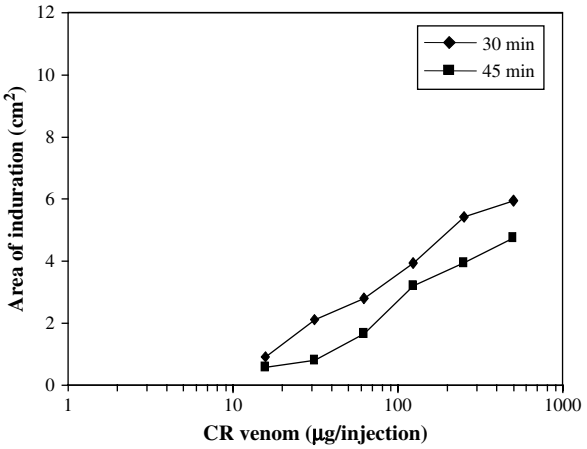

Fig. 2. Edema-inducing activity of CR venom in pig. Various concentrations of CR venom were injected intradermally and the induration areas were measured. Values are means area of 2 injection sites.

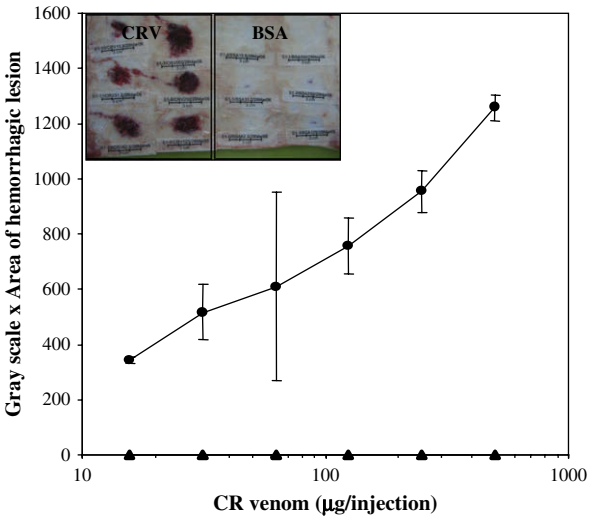

Fig. 3. A dose–response relation between hemorrhagic effect and venom concentration. CR venom (●) or BSA (▲) at varying concentrations were injected intradermally. Skin dissection at 4 h after injection was analysed for the areas and color intensity of the hemorrhagic lesions. Values are means ± SD from 4 independent injection sites. Inset shows the photograph of one representative dissected skin.

after subcutaneous injection of CR venom. The maximum response was observed at 23 h and then gradually subsided (Fig. 4). At 72 h, when the experiment was terminated, the CK level was still higher than that in the untreated control group. There was no obvious physical or behavioral changes in the pigs throughout the study period.

3.5. Inflammatory responses

The inflammation induced by subcutaneous injection of CR venom was assessed by determining the pro-inflammatory cytokine IL-6 in the pig plasma. No detectable level of IL-6 was observed during the 72-h period of the experimental study (data not shown). The detection limit of the assay employed was 10 pg/ml.

A thermal infrared camera with a temperature resolution of ±1 °C was used to detect changes of the surface temperature which is one of the classic signs of inflammation reaction. When CR venom was injected i.d. in the edema and hemorrhage experiments (#2.3 and #2.4) there was no observable change in temperature around the injection sites.

3.6. Histopathology study

This experiment was carried out simply to study the feasibility of taking serial biopsy specimens after s.c. injection of venom, and not to investigate the pathological change caused by the CR venom. It was found that specimens could be readily taken less than 1 min after venom injection, and at various distances and depths from the injection site. An example of serial biopsies is shown in Fig. 5 where no s.c. hemorrhage was observed at 1 min after venom injection (A). At 3 min (B) and 18 min (C) increasing s.c. hemorrhage was evident.

4. Discussion

Pigs are well accepted as being one of the best models of human because pigs and humans are remarkably similar anatomically and physiologically (Sullivan et al., 2001). In addition, the relatively large size of the pig makes it feasible to perform various different experimental studies of local tissue necrosis on the same animal. Such study would not be possible with the rodent model, thus resulting in the use of large number of animals and the accompanying biological variations. Moreover, juvenile domestic pigs are relatively easy to handle and house.

In the present study, CR venom which causes extensive tissue necrosis in human victims was used. Venom injections were made intradermally for edema and hemorrhage studies since this route gave easily quantifiable results and also used less venom. Hemorrhage has been studied using i.d. injection in rodent models. However, in experiments on CK and cytokine levels, s.c. injection was used since it represents the most prevalent route of venom injection in the human, and it is expected to result in measurable CK responses.

CR venom induces two types of edema responses in mouse paws: (i) a rapid onset edema which peaks within 1 h and declines thereafter; and (ii) the delayed type which reaches the peak at 4–6 h after injection (Tan and Saifuddin, 1990). In the pig, CR venom induced rapid and transient edema which was evaluated by measuring the area of induration developed at the site of injection. The response was dose-dependent.

Hemorrhage is one of the most important clinical features associated with *C. rhodostoma* envenoming. In the pig, the hemorrhagic spots were clearly observed at 1 h and persisted through 4 h. Both the changes in the area and in the color intensity of the hemorrhagic lesions could be evaluated using image analysis software. The gray scale

Table 1
Hematologic parameters of pigs before and after catheterization.

Parameters*	Day of catheterization				
	0	1	2	3	4
WBC (cells/μl)	11,720 ± 2130	11,140 ± 1289	10,720 ± 1379	11,750 ± 1705	11,740 ± 2244
RBC ($\times 10^6$ cells/μl)	4.77 ± 0.15	5.28 ± 0.12	5.19 ± 0.10	5.32 ± 0.26	5.04 ± 0.16
Hgb (g/dl)	6.88 ± 0.18	7.79 ± 0.18$^+$	7.74 ± 0.18$^+$	7.26 ± 0.35	7.14 ± 0.19
Hct (%)	25.6 ± 0.58	28.5 ± 0.78	27.3 ± 0.62	27.8 ± 1.11	27.2 ± 0.66
Platelet ($\times 10^3$/μl)	528 ± 54.2	550 ± 60.5	713 ± 92.0	661 ± 120.1	588 ± 137.5

*$n = 5$–10 pigs; $^+p < 0.05$.

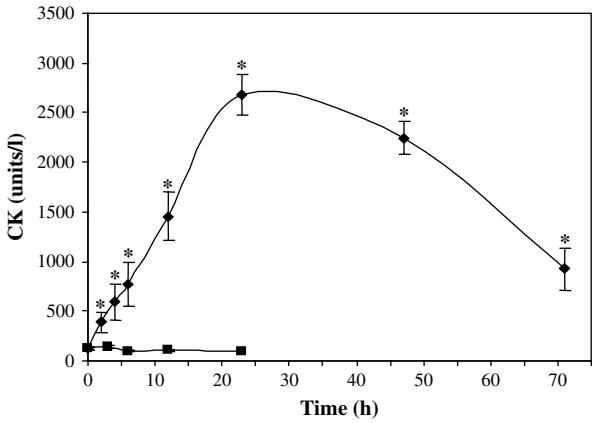

Fig. 4. Changes of serum creatine kinase (CK) in pig envenomation. Pigs were injected subcutaneously with CR venom (◆, $n = 4$). At different time points, blood samples were collected from implanted catheterized jugular vein, and the sera were assayed for CK. In the untreated group (■, $n = 6$–10), pigs were injected with normal saline only. Results are expressed as means ± SEM. $^{*}p < 0.05$ compared to the CK level of the untreated group.

corresponding to the color intensity times the area of hemorrhagic spots gave the hemorrhagic response which was dose-dependent.

When a pig is used, both the edema and hemorrhagic responses can be conveniently studied from the same venom injection sites and quantitated at 30 min and 4 h, respectively, after venom injection. Thus, a pig can provide at least 24 data points each for edema and hemorrhage. Moreover, experiments can be designed such that the controls and the experiments are performed in same animal, thereby further minimizing biological variations. This approach is applicable to the comparative studies on various therapeutic modalities, on wound healing and on the pathogenesis by serial biopsy of the venom injected areas.

Excitement and trauma associated with restraint and needle pricks have been reported to increase stress (Takahashi, 1986) which may confound the interpretation of the research results e.g., CK and IL-6 levels. The catheterization procedure described in this study was used successfully and satisfactorily in the kinetic study. Additionally, the collection of serial blood samples via indwelling catheters was rather gentle and simple and took a few minutes while the pigs were feeding.

Myonecrosis is usually quantitated by the level of serum creatine kinase. In this experiment, the pig was injected s.c. with CR venom at two sites in the back. The reasons for this were, first to mimic the two fangs of snakebite and secondly, in experiment on testing treatment modalities, one site can serve as experimental while the other site can serve as control. CK in the pig serum peaked at 23 h after s.c. injection of the venom, reaching approximately 250-fold higher than the untreated control group. In the previous study using purified hemorrhagic protease from CR venom, a 4-fold increase in mouse CK level at 1 h post injection was observed (Bando et al., 1991). The muscle damage as indicated by high and prolonged CK level in the present study correlated with a previous observation of damaged muscle cells in a 'late phase', i.e., 48–96 h after venom injection followed by a 'final phase' (1–4 wks) with regenerating muscle cells (Ownby and Colberg, 1988).

Although a number of studies have demonstrated the increment of inflammatory cytokines in response to snake venoms (Petricevich et al., 2000; Lomonte et al., 1993), the present study failed to demonstrate the increase in plasma IL-6 in pig model injected with CR venom. Severe envenomation induced in mice by intraperitoneal injection of *Bothrops asper* and *B. jararaca* venom has been shown to induce prominent elevations of several cytokines including IL-6 (Petricevich et al., 2000). In another study, the s.c. administration of sublethal dose of *Bothrops* venom in the mouse footpad resulted in the increment of IL-6, but not IL-1 nor TNF-α (Lomonte et al., 1993). The doses used in our study (32 mg of CR venom/20 kg pig) might be low and might not be able to induce systemic cytokine response in the present study. A local increase in IL-6 level might not be excluded.

The pathogenesis of local tissue necrosis induced by various snake venoms is most likely to be different due to qualitative and quantitative diversity in toxin and enzyme composition of the venoms. Thus, effective therapeutic measures may not be the same for different groups of venoms. In the pig, punch biopsies could be readily performed within a few seconds after venom injection and at varying distances and depths from the venom injection sites. This type of study, together with biochemical

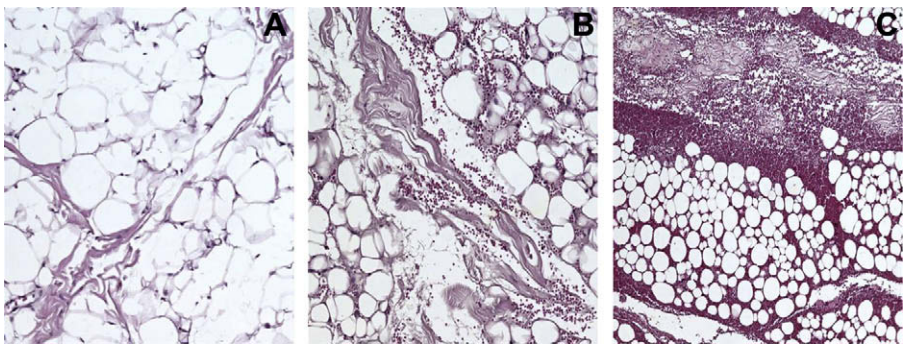

Fig. 5. Serial biopsies of the subcutaneous tissue of the pig taken at 1 min (A), 3 min (B) and 18 min (C) after subcutaneous injection of CR venom. Magnification: A and B, 200×; C, 100×.

information, should shed light on the early events of pathogenesis and could lead to a more rational and effective therapeutic approach for local injury caused by different groups of venoms. Moreover, by carrying out experiments in the same animal, objective and accurate comparison of various treatment modalities can be made.

In summary, the pig has several advantages over rodents in the study of local tissue necrosis induced by snake venoms. Pigs can be used in much smaller numbers than when rodents are used, with a reduction in the number of live animals used and less biological variation. Given the number of data points that can be obtained from a pig, the cost of a 20 kg pig (US $ 50) is only slightly more expensive than that of 2 dozen mice (US $ 36) in Thailand. More importantly, the anatomical and physiological similarities between pig and man make it more accurate for the extrapolation of experimental results to human.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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