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Original communication

The impact of force on the timing of bruises evaluated in a porcine model

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ABSTRACT

In animal models developed in order to estimate the age of bruises, focus has been on the changes over time and not considering the force used to inflict the trauma. In the present study, gross and histological changes in 2, 4, 6 and 8 h old bruises which were inflicted with a low, moderate and high force were compared.

Twelve experimental pigs were randomly assigned to three groups of force (low, moderate and high force). All pigs were anesthetized, and on each animal four blunt traumas were inflicted on the back with the low, moderate or high force according to the groups. The pigs were kept in anesthesia for 2, 4, 6 or 8 h, after which they were euthanized, and skin and muscle tissues were sampled for histology. As control, two pigs were included.

The gross appearance of bruises developed similarly until 0.5 h after infliction at which time the visibility of the bruises depended on the force. The infiltration of subcutaneous neutrophils depended on the time and force used which was confirmed by both manual evaluation and image analysis of immunostained skin sections. In the muscle tissue, the number of macrophages was found useful for age determination in bruises inflicted with the highest force. Therefore, when evaluating forensic cases of bruises in both human and veterinary pathology the impact of force and not only the timing should be taken into consideration.

1. Introduction

Attempts to estimate the age of bruises in animal models are multiple and include color assessment, histology, various histochemical techniques and biochemical methods, real-time quantitative PCR and electric impedance spectroscopy.[1–12] However, in all of these, focus has been on the changes in bruises over time and not considering the impact of the force used to inflict the trauma causing the lesions. In a single model, bruises were inflicted with high and low force, but the development in the histological changes over time was not presented.[13]

In a recent study, a highly reproducible method for inflicting experimental bruises in pigs was developed and validated.[14] In that model, the number of subcutaneous neutrophils and macrophages in the underlying muscle tissue was found suitable for estimating the age of bruises.

In the present study, gross and histological changes in bruises inflicted with a low, moderate and high force during time were compared. Histologically, the amount of hemorrhage, necrotic muscle fibers, infiltrations of neutrophils and macrophages were evaluated. The counting of neutrophils in the subcutis was evaluated both manually and by image analysis.

2. Materials and methods

2.1. Animals

In total, 14 (12 experimental and 2 controls) specific pathogen free pigs, female, Yorkshire-Landrace crossbred pigs with a mean body weight of 32.1 kg (23–38 kg) were used. All pigs were acclimatized for one week and housed in pens of two animals. The pigs were fed a commercial pig diet (NAG, Helsinge, Denmark) twice a day and had free access to tap water. All animals remained healthy during the period of acclimatization. The pigs were randomly

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divided into three groups with four animals in each (groups 1, 2 and 3) and a control group of two pigs (Table 1).

2.2. Experimental procedure

The experimental procedure was approved by the Danish Animal Inspectorate (2013–15–2934–00849). All pigs were anesthetized using the same protocol as recently described by the authors.[14] Four blunt traumas (area of impact Nos. 1, 2, 3 and 4) were inflicted on the back along the M. longissimus dorsi of each pig during a period of 3–4 min using a mechanical device and procedure described recently by the authors.[14] In groups 1, 2 and 3, blunt traumas were inflicted with an impact force of 2.14 N ± 0.22 N (low force), 4.24 N ± 0.16 N (moderate force) and 6.52 N ± 0.17 N (high force), respectively. Force of impact was measured at the Danish Technological Institute (Taastrup, Denmark).[14] After infliction of the traumas, pigs were left in anesthesia and euthanized following 2, 4, 6 or 8 h with an overdose of pentobarbital given intravenously (Glostrup Apotek, Glostrup, Denmark) (Table 1). Two control pigs were anesthetized for 6 h and then euthanized without having blunt trauma inflicted. Data from the pigs inflicted with the highest force (group 3) and the control pigs, which were the basis for developing the model and documentation of the reproducibility of the mechanical device used for infliction of bruises, were recently published.[14]

2.3. Gross pathology

During the time of anesthesia and post-mortem, each pig was subjected to gross evaluation of each of the four areas of impact, and the dimension of the bruises was measured using a ruler. Post-mortem, skin areas were cut out *in toto* including the underlying part of M. longissimus dorsi and cross-sectioned in slices of 0.5–1 cm. The presence of hemorrhages in the subcutis and muscle tissues was recorded before skin and muscle tissues were sampled for histology. Finally, all pigs were subjected to a complete necropsy following the instructions by Madsen and Jensen.[15] From the according areas on the back of the control pigs, skin and muscle tissues were sampled, too.

2.4. Histology

From the areas of impact, 4 to 5 slices of skin and muscle tissue were immersion fixed in 10% neutral buffered formalin for up to 5 days. Then processed through graded concentrations of ethanol and xylene and finally embedded in paraffin wax. Tissue sections were cut at 4–5 μm and stained with hematoxylin and eosin (HE).[16] From each of the areas of impact, multiple sections (3–5) were made and evaluated. From each area of impact, the tissue section with the most extensive hemorrhage in the subcutaneous tissue was selected for further examination.

Damage to the epidermis was registered as present/absent. In the dermis and muscle tissue, hemorrhage was registered as present/absent. In the subcutis, the density of hemorrhage was registered as the percentile area of extravasated erythrocytes using a 10× objective and scored as: 0: absent, 1: <12.5%, 2: 12.5–25% or 3: >25%. Neutrophils and macrophages were counted on a semi-quantitative scale: 0: Absence of neutrophils/macrophages; 1: 1–10 neutrophils/macrophages; 2: 11–30 neutrophils/macrophages; and 3: >30 neutrophils/macrophages. The scoring was carried out in the dermis, subcutis and muscle tissue using a 40×/0.65 objective in the area with the highest density of neutrophils/macrophages, and an average score of the four bruises in each pig was calculated. Neutrophils were identified by their multilobulated nucleus, homogenous, eosinophilic cytoplasm and an approximate size of 10–12 μm. Macrophages were identified by their circular nucleus, abundant cytoplasm and an approximate size of 15–80 μm.[17]

In the muscle tissue, the percentile area of necrotic muscle fibers was scored: 0: absence of necrotic muscle fibers; 1: <12.5%; 2: 12.5–50%; and 3: >50% using a 10×/0.25 objective in the area with the most necrotic muscle fibers.

Table 1
Force of impact, number of pigs and age of bruises in the three experimental groups and the control group.

Group	Force of impact	No. of pigs	Age of bruises
1	Low (2.14 N ± 0.22 N)	4	2, 4, 6 and 8 h
2	Moderate (4.24 N ± 0.16 N)	4	2, 4, 6 and 8 h
3	High (6.52 N ± 0.17 N)	4	2, 4, 6 and 8 h
Control	No trauma	2	Not relevant

2.5. Immunohistochemistry

For immunohistochemistry, 4–5 μm tissue sections of area of impact No. 1 from each pig (skin only) were mounted on adhesive glass slides (Thermo Fisher Scientific, Menzel GmbH & CoKG, Baunschweig, Germany). The tissue was deparaffinised and subjected to heat-induced antigen retrieval in tris-EDTA-buffer pH 9 for 1 h at 95 °C. Blocking of endogenous peroxidase activity by 0.6% H_2O_2 for 20 min and blocking of unspecific protein binding by Ultra V Block (Thermo Fisher Scientific) were carried out. Then the tissue was incubated overnight with primary murine monoclonal antibodies towards MCA874G/MAC387 (MAC) (AbdSerotec, Oxford, UK). Primary antibody enhancer and horseradish peroxidase (HRP) polymer were applied according to the manufacturer's instructions (Thermo Fisher Scientific). The immunostaining was performed by application of the Ultravision LP Detection System HRP (Thermo Fisher Scientific, Fremont, CA, USA). The reaction was developed in 3,3 diaminobenzedine tetrahydrochloride pH 7.0 (DAB) (KemEnTec Diagnostics, Taastrup, Denmark). Throughout the protocol, tissue slides were washed in tris-buffered saline, pH 7.6.[18] All sections were counterstained by Mayer's hematoxylin. All tissue slides were made by a single skilled technician to minimize variation in the thickness of the sections and the staining procedure.

2.6. Image analysis and evaluation

All immune-stained tissue sections were scanned using a 10× objective (Zeiss Axio Scan.Z1) and imported into Visiopharm Integrator System (VIS) (Visiopharm Integrator System, Version 4.6.857) for image analysis. The VIS software was trained to recognize MAC-positive cells and staining them green (Fig. 1). To improve correct identification, the classifier K-means clustering and the post-processing steps outline cells (15 μm), change small (20 μm^2), change non-circular (4), separate objects (10 μm, "assume object are elliptical") and change by intensity (Threshold 80%, 75%) were applied (Fig. 1). The adipocytes and fibrous tissues were stained red and blue, respectively, for the purpose of maintaining the appearance of the subcutis (Fig. 1).

Using the according HE tissue section, the borders of the two areas of hemorrhage in the subcutis were identified on the immunostained tissue sections and 24 fields of 1,000,000 μm^2 were marked. The VIS software was used to identify and calculate the number of MAC-positive cells in all 24 fields in each of the tissue sections. In the controls, 24 fields were marked in the center of the sections.

In the two control samples, a total of 645 and 805 MAC-positive signals was counted, respectively. However, when manually