Development of a computer-based quantification method for immunohistochemically-stained tissues and its application to study mast cells in equine wound healing (proof of concept)

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Abstract

Background: There is a growing interest in the scientific community to use computer-based software programs for the quantification of cells during physiological and pathophysiological processes.

Drawbacks of current computer-based methods used to quantify immunohistochemical staining are the complexity of use, expense of software and overly-simplified description of protocol thereby limiting reproducibility. The precise role of mast cells in equine cutaneous wound healing is unknown. Given the contribution of mast cells to the chronic inflammation observed in human keloid, a pathology similar to exuberant granulation tissue (EGT) in horses, mast cells might be present in high numbers in equine limb wounds predisposed to EGT. The main goal of this study was to develop a reliable and reproducible quantification method for immunostained tissues using a computer software that is widely available, at no cost, to the scientific community. At secondary goal was to conduct a proof of concept using the newly-established method to quantify mast cells during wound healing at different anatomical sites (body and limb) in horses to see if a different pattern is observed in limb wounds.

Results: A good intraclass correlation coefficient (ICC, 0.67 p < 0.05) was found between the computer-based ImageJ method and manual counting. An excellent intra-operator ICC of 0.90 (p < 0.01) was found for the ImageJ quantification method while a good interoperator ICC of 0.69 (p < 0.01) was measured. Mast cells were localized below the epidermis, around cutaneous appendages and blood vessels. Mast cell numbers did not differ significantly in relation to anatomical location or time of healing.

Conclusions: The computer-based quantification method developed is reliable, reproducible, available, cost-free and could be used to study different physiological and pathological processes using immunohistochemistry.

Background

Major drawbacks of current computer-based methods used to evaluate immunohistochemically-stained tissues are the complexity of use, expense of software and overly-simplified description of protocols limiting its reproducibility [1]. Another challenge is to ensure objectivity by validating the method with appropriate parameters including reliability, repeatability and reproducibility [2]. The development of new computer-based methods would be useful to facilitate quantification of cellular...
populations in immunostained tissues.

Wounds on the limbs of horses suffer a weak and prolonged inflammatory response compared to body wounds, that impairs healing and may lead to the development of exuberant granulation tissue (EGT) [3]. Mast cells are suspected to contribute to the chronic inflammation, as is the case in human keloid, a fibroproliferative pathology exhibiting features comparable to EGT [4]. Mast cells are immune cells that secrete vasodilating factors, and numerous pro- and anti-inflammatory cytokines during wound healing [5]. They also express a c-kit receptor (CD117) that enables cell communication with fibroblasts. Their precise role in cutaneous wound healing in horses is unknown.

The objectives of this study were to 1) develop a reliable and reproducible quantification method for immunostained tissues using a computer software that is widely available, at no cost, to the scientific community, and 2) conduct a proof of concept using the newly-established method to quantify mast cells during skin wound healing at different sites (body and limb) in horses in an effort to determine if a different mast cell pattern is observed in limb wounds that could explain their predisposition to EGT. It was hypothesized that 1) the computer-based method developed would have excellent repeatability, reproducibility and reliability parameters, and 2) mast cell numbers would be greater and would persist in limb wounds compared to body wounds throughout healing, since this cell is known to contribute to chronic inflammation in human keloids.

Results
One mare developed EGT on its limb wounds from 11 days post wounding until the end of the study and those specimens were excluded from the final analysis (Fig. 1, Additional file 4: Table S1). Specimens harvested from wounds that were bandaged for more than 7 days and/or received a treatment of anti-inflammatories, anti-microbials and/or negative pressure wound therapy (NPWT) were excluded from the study. Specimens harvested from wounds that showed healthy tissues during the healing process and had a granulation tissue elevation score of less than 2 were included in the study [6]. According to these criteria, 16 body and 10 limb specimens were included for a total of 26 healthy specimens (Fig. 1).
Validation of the ImageJ computer-based method

An intraclass correlation coefficient (ICC) value of 0.67 (p < 0.05) was found between the ImageJ method and the pathologist-based manual counting (Table 1). By looking at the raw data, a slight overestimation of mast cell counts was observed with the ImageJ method compared to the manual counting method and may relate to the exclusion of cells, by the pathologist, based on observable morphological parameters. An intraoperator ICC of 0.90 (p < 0.01) and an interoperator ICC of 0.69 (p < 0.01) were found for the computer-based method (Table 1).

Proof of concept: quantification of mast cells

Mast cells in wound margins were located in the subepidermal and deeper dermal layers and were mostly found around hair follicles, sweat and sebaceous glands, and blood vessels (Fig. 2A-b). Mast cells were also well distributed throughout the granulation tissue (Fig. 2A-c).

In the subepidermal and deep dermal layer, mast cell numbers were greater in body wounds and more variability was seen in limb wounds, but these changes were not statistically-significant (Fig. 2B). In the granulation tissue, mast cell kinetics between body and limb wounds were similar, except that the magnitude of the reaction seemed inferior in limb wounds (not statistically-significant) (Fig. 2B).

Discussion

The primary objective of this study was to develop a reliable and reproducible quantification method based on a widely-available cost-free computer software, for the objective evaluation of immunohistochemically-stained tissues. The ImageJ computer-based method developed in this study allows numerical analysis of cells with cell numbers (number of cells/mm² of tissue) as opposed to previously developed computer-based quantification methods allowing the quantification of the immunostaining intensity [7].

The intraoperator ICC value confirmed excellent repeatability of the ImageJ method while interoperator ICC value showed good reproducibility [8], suggesting that reliability is improved if the
method is used by a single operator. This is likely due to the subjective nature of the manual subtraction step required to delete structures considered to be stained unspecifically (Fig. 3D). Few histomorphology studies validated newly-developed computer-based quantification methods with ICCs to compare the reliability of the methods with the evaluation of a pathologist or a tissue specialist (e.g. surgeon), mostly considered as the gold standard method [9, 10]. To the author’s knowledge, this study is the first to develop and validate a computer-based quantification method, with ICCs, during equine wound healing. The ICC found between the ImageJ and the pathologist (0,67; p < 0.05) confirmed good reliability between the ImageJ and the gold standard methods [11]. It is difficult to compare such results with other studies aiming to develop computer-based methods applied to cutaneous wound healing since these studies are scarce and ICC agreement intervals may differ in the literature [8, 10-12]. In short, the hypothesis is partially confirmed since an excellent repeatability and good reproducibility and reliability were found.

As a proof of concept, the newly-developed ImageJ method was used to quantitate mast cells, for the first time, in specimens harvested from healing skin wounds of horses. The location of mast cells in the subepidermal and deep dermal layers agrees with other studies conducted on equine skin (Fig. 2A-b) [13, 14]. However, the scattered distribution within the granulation tissue of equine wounds has, to our knowledge, never been reported (Fig. 2A-c). The smaller numbers observed in limb compared to body wounds (Fig. 2B) may explain the weak inflammatory response seen in limb wounds of horses, refuting our hypothesis [3].

The principal limitation of the proof of concept portion of our study is the small number of horses, which may explain why no significant differences were found between limb and body wounds, since mast cell numbers are known to show great interindividual variation in horses [13]. However, the multiple sites and the longitudinal nature of the study added robustness for statistical analysis. Results obtained here should help to elucidate the role of mast cells in normal wound healing and future studies might include EGT-affected wounds to document the numbers of mast cells in this healing impairment common to horses.

Conclusions
In conclusion, a reliable, reproducible, available and cost-free computer-based method has been developed to objectively evaluate immunostained tissues. This method could be useful to study and quantify several types of cells implicated in physiological and pathophysiological processes.

Methods

Samples

Four, mixed breed mares bought from a private horse dealer with a median of 9 years of age (range 5-15) were included in a previous controlled and randomized study [15]. The experimental model is shown in Fig. 1. A total of 31 specimens were harvested and archived, of which 26 were included in the current study based on inclusion and exclusion criteria (n = 26) (Fig. 1, Additional file 4: Table S1) [6]. Specimens were fixed in 10% formalin, dehydrated, paraffin-embedded and then cut into 4 µm sections. Specimens included wound margins and granulation tissue [15].

Immunohistochemistry for mast cell staining

A rabbit polyclonal anti-CD117/c-kit antibody (1/87, #RB-9038, Thermo Fisher Scientific, Rockford, IL, USA) was incubated on the sections for one hour at room temperature [16]. An equine mast cell tumor was used as a positive control and rabbit serum replaced the primary antibody for the negative control (Fig. 4). The detailed protocol is presented in Additional file 1.

Image analysis and quantification

Immunostained cells were observed under light microscopy at 200x and scanned with the Panoptiq software program v.1. 4. 3 (ViewsIQ, Richmond, BC, CAN). Mast cell characterization and quantification was performed by 2 blinded observers in the wound samples harvested from a former study [15]. In the wound margin, mast cell quantification was performed in the subepidermal layer (0-296 µm under basal membrane) and in the deep dermal layer (296-1,184 µm) [14]. From each region, 5 high power fields (HPFs) were selected randomly. Mast cells were quantified using the newly-developed computer-based quantification based on the ImageJ software program (U. S. National Institutes of Health, Bethesda, MD, USA). Mast cells in six of these specimens were also manually
counted by an ACVP-certified pathologist and statistically compared to the ImageJ method. The quantification method is shown in Fig. 3 and the complete protocol is described in Additional file 2. Additional file 3 provides details about the chosen area range. Mast cell numbers were then compared between limb and body wounds.

**Statistical analysis**

Statistical analysis was done with SPSS software program v.25 (IBM, Armonk, NY, USA) to validate the quantification method by calculating ICCs, and with SAS software program v.9.3 (SAS Institute Inc, Cary, NC, USA) for mast cell quantification. A linear model for repeated measures was used to detect differences relating to time and to anatomical location of the wound (limb vs body). A p-value < 0.05 was considered statistically significant.

**List Of Abbreviations**

EGT, exuberant granulation tissue; ICC, intraclass correlation coefficient; NPWT, negative pressure wound therapy; HPF, high power field.

**Declarations**

**Ethics approval and consent to participate**

The protocol follows the guidelines for the care and use of laboratory animals as established by the Canadian Council on Animal Care and approved by the Institutional Animal Care and Use Committee of the Université de Montréal (# 15-Rech-1811).

**Consent for publication**

N/A

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

No competing interests have been declared.

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**Author’s contributions**

VD developed the quantification method, did the experimentation related to the project, analyzed the data, wrote the manuscript and participated in its revision. SL participated in data analysis and revision of the manuscript. HR provided technical support and helped to write the manuscript. MD helped for the validation of the quantification method. CT developed the study design, participated in data analysis and helped to revise the manuscript. All the authors have accepted the final version of the manuscript.

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**Author’s information**

N/A

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16. Masked for peer review.

Table

Table 1: Validation of the quantification method using statistical analysis (n = 6).

| Comparisons                        | ICC  | p    |
|------------------------------------|------|------|
| Same operator                      | 0.90 | 0.00 |
| Two different operators            | 0.69 | 0.01 |
| Mean of ImageJ vs pathologist      | 0.67 | 0.04 |

Pathologist: manual counting; ICC, Intraclass correlation coefficient; The ICC calculated between the ImageJ method and the pathologist-based manual counting method used the mean of the 2 counts done by each method p < 0.05 was considered statistically significant

**This table should be placed between lines 95 and 97.**

**Supplemental File Legends**

Additional file 1 (docx.): Detailed mast cell immunohistochemistry protocol. Full immunohistochemistry protocol for the mast cell identification in limb and body wounds of horses included in the study.

Additional file 2 (docx.): ImageJ quantification protocol used on mast cells. Full protocol of the quantification of mast cells in limb and body wounds of horses included in the study with the ImageJ
software program.

Additional file 3 (docx.): Establishment of protocol of mast cell area interval. This document illustrates how the range of mast cell area interval was established by using the ImageJ software program for the later quantification.

Additional file 4 (docx.): Table S1: Numbers and characteristics of the samples included in the study. Specimen characteristics are grouped in a table to show which specimens were included or excluded in the study and why.

Figures
Inclusion criteria:
- Wound had healthy tissues during the healing process (no signs of infection requiring antimicrobial therapy, no signs of necrosis)
- Wound had a granulation elevation score < 2 according to Bigbie et al. (1991)

Exclusion criteria:
- Wound received a treatment (NPWT, antimicrobials and/or inflammatories)
- Wound was bandaged more than 7 days post wounding (predisposition to EGT formation)
- Wound showed clinical signs of EGT (granulation elevation score ≥ 2 from Bigbie et al. (1991), swelling and inflammation)

Excluded specimens: 5 specimens (Limb n = 5)
Included specimens: 26 specimens (Limb n = 10, body n = 16)

Immunohistochemistry (Mast cells CD117+)

Figure 1
Study design. Full-thickness excisional wounds were created on the thorax (15 cm²) and the forelimb (6 cm²) (2 per anatomical site) on 4 mixed breed mares and left to heal by second intention. Samples were harvested alternatively between the two wounds/site on days 1, 3, 8 and 17. Based on inclusion and exclusion criteria, a total of 26 healthy wounds were included. Two specimens showed clinical signs of EGT and 3 others received NPWT treatment, all harvested from limb wounds, and were thus excluded. NPWT: negative pressure wound therapy, EGT: exuberant granulation tissue.

Figure 2
Photographs and quantification of CD117+ mast cells in skin wounds of horses. A) Photographs showing a) HPF of an equine CD117+ skin mast cell and b) mast cells distribution in the i-subepidermal and ii-deep dermal layer of the wound margins and in the c) granulation tissue of a 3-day old thoracic wound. Immunohistochemistry was performed with the avidin-biotin-alkaline phosphatase method and used Harris hematoxylin counter stain (x200). Arrow: epidermis, arrow head: blood vessel, asterisk: sweat gland. B) Graphs showing evolution of mast cells numbers (/mm²) over time and according to anatomical location in the subepidermal layer, deep dermal layer, and granulation tissue (left to right). Values represent mean ± SEM (n = 26).
Principal steps of mast cell quantification protocol using ImageJ software program. a) Original photo illustrating the subepidermal layer of a one-day old skin wound on the limb stained with the CD117 antibody. The blue line represents the border between the subepidermal and the deeper dermal layer. b) Staining of CD117+ mast cells after colour deconvolution and c) its binary transformation. d) Manual subtraction of stained cutaneous appendages; the deeper dermal region below the blue line was also deleted. e) Remaining cells stained by the antibody; blue cells are those included in the count by the software (cell area between 8-300 µm²). The original photo was taken at a 200X magnification. Arrow head points to a stained mast cell.
Positive and negative controls included in the immunohistochemistry technique. A) Positive control showing mast cells in an equine mast cell tumor. B) Negative control using rabbit serum for the replacement of the primary antibody on a 3-day old limb wound. No cellular staining was observed but unspecific staining of epidermis, sebaceous and sweat glands, and hair follicles, was noted. An additional blocking step of the endogenous biotin was added to the protocol and limited the unspecific staining. The technique used the avidin-biotin-alkaline phosphatase method with Harris hematoxylin counter stain (x200).

Supplementary Files
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Additional file 3.docx
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