Comparing open wound measuring methods popularly used in experimental studies

Estudo comparativo de métodos de avaliação de contração de feridas cutâneas abertas

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Abstract

Tissue repair is a response reaction to lesions and aggressions that constitutes a dynamic process to maintain the integrity of the organism. Wound healing experiments have used several approaches in order to assess and compare treatment methods, and these discrepancies hamper comparisons among assays. This study assessed three different methods of wound measurement commonly used in healing assays: clock method, graph paper method, and computer-assisted image analysis. We used 30 Wistar rats, kept in appropriate conditions for animal well-being. After anesthesia, and using an eight-millimeter punch, two lesions were made in the back region of each rat. The wounds were assessed on days four, seven, and 14 after infliction. At four days, all methods generated similar results. By day seven, the clock method had lost precision, likely due to wound shrinkage, and yielded greater means compared to the other two methods. On the last assessment, the computer-assisted method appeared to have more precise results, with the other two generating statistically higher means. Computer-assisted image analysis seems to have maintained wound measuring precision throughout this experiment, even when faced with small lesions. Considering these results, the authors recommend the use of computer-assisted measurements in future experiments.

Keywords: Healing. Wound contraction. Granulation tissue. Animal experimentation.

Resumo

O reparo tecidual surge em resposta às lesões e constitui-se de um processo dinâmico para manutenção da integridade do organismo. Nos estudos sobre cicatrização de feridas, tem se utilizado várias técnicas buscando-se uma avaliação da eficácia de meios de tratamento local. Sendo assim, o presente trabalho objetivou comparar três métodos de avaliação experimental de áreas de feridas, sendo eles: o método do relógio, papel milimetrado e avaliação computadorizada. Para realização do experimento, foram utilizados 30 ratos Wistar em condições de bem-estar. Após realizada a anestesia, foram confeccionadas duas lesões no dorso de cada animal com punch de 8 mm, sendo as feridas avaliadas após quatro, sete e 14 dias de tratamento. Observou-se que aos quatro dias de tratamento, todos os métodos apresentaram o mesmo desempenho, enquanto aos sete e 14 dias, o método do relógio, apresentou maior média de área, perdendo precisão, e aos 14 dias, a avaliação computadorizada apresentou resultados mais precisos em relação a ambos os outros métodos. Concluiu-se que a avaliação computadorizada através de *software* de áreas de feridas cutâneas apresenta resultados mais precisos em relação aos métodos do relógio e papel milimetrado, principalmente em lesões menores.

Palavras-chave: Cicatrização. Contração da ferida. Tecido de granulação. Experimentação animal.

Introduction

The healing process constitutes a dynamic series of events, aimed at maintaining the integrity of an organism. It involves a series of steps, mainly: inflammation, chemotaxis, cellular proliferation, differentiation, and maturation (MANDELBAUM; DI SANTIS; MANDELBAUM, 2003). Tissue repair is a response to lesions, either caused by trauma or surgically induced (GARROS et al., 2006). Humans have long developed ways to interfere in this process, seeking to reduce the healing period and promote

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better aesthetic and functional results (BRANCO NETO et al., 2006).

In veterinary practice, wound healing studies are essential, considering the important role skin plays in the protection against microorganisms, sun, heat, etc. (MANDELBAUM; DI SANTIS; MANDELBAUM, 2003). Furthermore, knowledge of species peculiarities will allow the appropriate measures to be taken. For instance, horses will produce exuberant granulation tissue when healing wounds; while dogs have a highly vascularized skin, when compared to cats, among other differences (BOHLING; HENDERSON, 2006).

Regardless of the animal species, during the healing process, the wound contracts in order to approximate the borders. This process is favored where the skin is looser, such as the abdomen and trunk (COELHO; REZENDE, TENÓRIO, 1999). Wound contraction is one of the most important aspects when assessing the healing process of open skin wounds (MEDRANO, 2010), and several methods and tools are used to measure the evolution of this phenomenon in wound healing assays (FALCÃO et al., 2001). A widespread method used in research is measurement with the help of a caliper rule, having a clock as a measuring guide (MEDRANO, 2010), which may be enhanced to yield better results. Graph paper is also frequently used, and is considered more trustworthy than the aforementioned method (FALCÃO et al., 2001).

The use of computer software to assess wound size in healing tests may allow experiments with greater precision (ABDALLA et al., 2009). Regarding these arguments, the objective of this study was to compare the results generated by these three popular methods of wound measurement: caliper rule/clock method, graph paper method, and computer software method.

Material and Methods

Animals

The ethics committee for animal experimentation and well-being of the Universidade Federal de Pelotas (UFPel) (CEEA 7605) approved this study. Thirty adult Wistar rats (*Rattus norvegicus*) were used in this experiment. These were, on average, 60 days old, 200g animals, kept in appropriate housing conditions with water and food *ad libitum*. To produce the lesions, each animal was anesthetized with subcutaneous atropine (5 mg/Kg), and intramuscular xylazine (10 mg/Kg) and ketamine (100 mg/Kg). After shaving with an electric clipper, two lesions were made in the back of each animal, and an eight millimeter punch blade was used to produce the circular wounds. The animals were divided into three groups regarding their assessment dates (four, seven and 14 days). Half of the wounds in each group were treated with saline solution and the other half with petroleum jelly (Vaseline), typically used as mock treatments to control wound healing assays (LOPES et al., 2005; OLIVEIRA et al., 2010; NAYAK et al., 2011). After the experimental period, the animals were euthanized according to resolution nº 1000 may 12th 2012 (CFMV).

After the experimental period, the animals were euthanized, first receiving pre-anesthetic acepromazine, and then receiving a lethal dose of injectable anesthetic, according to resolution nº 1000 may 12th 2012 (CFMV).

Clock method

For this assessment, a clock face was used as a measuring guide, where the lesions were measured using a digital caliper rule. Measurements were taken in two directions, corresponding to the 12 and 6 o'clock positions for height and the 9 and 3 o'clock for width. The area was calculated by multiplying height x width, and the results were expressed in mm^2 (MEDRANO, 2010).

Graph paper method

For this assessment, wound borders were traced on transparent paper using a 4mm thin marker pen. After this, the stencil was transferred to a sheet of graph paper and the number of squares inside the traced area was used to determine the area of the wound, as described (FIKRU et al., 2012).

Computer software method

For this assessment, the animals were placed in a ventro-dorsal position, and a digital camera (Sony DC20®), fixed at 15 cm height by a tripod, was used to obtain the photographic records, and a ruler was placed beside the wound for scale. The image was then transferred to a computer where it was processed in the GIMP2 and ImageJ® software. GIMP2 was used to treat the images in order to fill the wound area with a single color. To accomplish this the following tutorial was used: (1) menu: file/open: select desired photograph; (1.1) menu: file/save as: save file in the "xcf" format; (2) menu: image/resize image: width 800, height 600, and select the cubic interpolation option; (3) menu: view/zoom/200%; (4) menu: tools/selection tools/contiguous selection; (5) identify the wound area in order to continue with the selection; (5.1) while holding down the SHIFT key, click the desired area; (5.2) menu: selection/ turn quick mask on/off – observe the appearance of a red mask in the entire image, except in the selected area; after this evaluation, unselect the quick mask option; (5.3) menu: layer/new layer: the transparency option will be marked and a new image layer will be formed, this will now be the active work layer; (6) menu/dialogs/colors – determine a distinct color for analysis; (6.1) use the brush tool on the new layer, completely paint the prepared area which represents the wound; (7) menu/file/save a copy; click "export".

To obtain the area measurements for the wounds in mm^2, ImageJ® software was used, according to the following tutorial: (1) menu: file/ open: select the ".gif" file that was generated using the GIMP2 software; (2) determine scale on the image ruler using the "straight line selections" option, mark one centimeter; (2) menu: analyze set scale: "known distance": 10, "unit of length": mm, click OK; (3) click the painted wound area with the "wand (tracing) tool", the outline of this area should now be in yellow; (4) menu: analyze/ measure: a new window will pop up with a column entitled "area", where the area of the wound will be shown in mm^2 (Figure 1).

Statistical analysis

The data were expressed in mean and standard deviation. In order to compare the different measuring methods, an ANOVA and Tukey's test were used to compare means (Statistix 9.0).
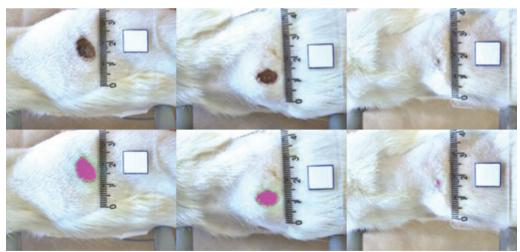

Figure 1 – Computer-assisted image analysis of the wound area through the GIMP2 software. The wounds were assessed at four (A), seven (B), and 14 (C) days post-infliction. (A1), (B1), and (C1) represent the same images as above, highlighting the wound area considered for analysis, after color treatment in the GIMP2 software (shown here in white for contrast)
Source: (FERNANDES et al., 2014)

Results

All the animal procedures were performed according to the description, and all the lesions were successfully produced. No difference was observed among treatments, as was expected, since both are humectants, with little or no further therapeutic properties.

Concerning the wound measurement assays, at four days after the wounds were produced, no difference could be observed among the different measuring methods (p = 0.644). The mean value and the standard deviation can be seen in table 1. After seven days, the clock method presented a larger mean area result (28.95 mm^2), when compared to the other methods (19.30 mm^2 and 17.87 mm^2 for the graph paper and the computer software, respectively), representing a statistically significant difference (p < 0.01). At 14 days, both the clock and graph paper methods had higher mean area results (3.63 mm^2 and 2.75 mm^2, respectively), when compared to the computer software method (0.85 mm^2), representing a difference of statistical significance (p < 0.01). The full results can be seen in table 1.

Discussion

Healing assays using experimental animals, particularly those assessing wound healing promoter substances, are conducted worldwide (LOPES et al., 2005; OLIVEIRA et al., 2010; NAYAK et al., 2011). Wound contraction is frequently assessed in these experiments because it allows for a better understanding of the healing process evolution and the maturation

Table 1 – Mean (mm²) and standard deviation of the wound area according to the different measuring methods assessed – Capão do Leão – 2014

Method	Day 4	Day 7	Day 14
Graph paper	40.40 ± 10.05 **a**	19.30 ± 7.89 **b**	2.75 ± 1.94 **a**
Clock	39.20 ± 9.34 **a**	28.95 ± 10.60 **a**	3.16 ± 3.38 **a**
Computer software	32.25 ± 14.91**a**	17.87 ± 12.00 **b**	0.85 ± 0.11 **b**

Different letters indicate statistically different results in the column (Tukey's test, p < 0.05)

of the scar, which represent myofibroblast activity, and the buildup and maturation of collagen fibers (COELHO et al., 1999). Morphometric evaluation assays are employed to measure the diameter of the lesion, in order to establish a comparison of the initial measurement and subsequent results throughout the healing process (LIMA et al., 2012). The use of an appropriate method to assess wound contraction will allow a standardization of this stage, with precise data and an estimate of the actual therapeutic value of the pharmaceuticals used in the treatments (SANTANA et al., 2008). Studies comparing graph paper and computer image methods have been conducted in humans (GRIFFIN et al., 1993). However, to the authors' knowledge, no assay has compared the methods, described herein, to measure contraction in induced wounds in experimental animals, since the different researchers in this field use the methodology they consider appropriate.

Our results show that, at four days after the onset of the wounds, all the considered measuring methods generated similar area measurements, lacking statistical difference. On the other hand, at seven and 14 days the clock method, using the digital caliper rule, yielded larger areas (Table 1). This may have occurred because at four days the wound area is still of considerable size, which may facilitate manual assessment, through the caliper rule, and minimize error. With the daily reduction of the wound through myofibroblast activity, manual measurement seems to have lost its precision by day seven. Furthermore, height multiplied by width is not an appropriate manner to measure a circular area, and results higher than reality would be expected when applying it in this manner. Regarding the graph paper method, it seems to have maintained precision in days four and seven, yielding results similar to those produced by the image analyzing software; however, by day 14 it had lost this precision. While there is no universal method to determine wound size, it is safe to consider that the computer software method had greater precision,

mainly because it considers the uneven outline of the wound in the advanced stages of healing, especially in small wounds, with areas as little as 1 mm^2.

In a study assessing skin ulcers in diabetes patients (humans), the computer software method showed greater precision compared to the manual measurement in transparent paper (SANTANA et al., 2008). Graph paper is used in several wound healing assays (NAYAK et al., 2011; FIKRU et al., 2012), possibly because it is a low-cost technique; however it requires utmost care so as not to disturb the wound when tracing it, while trying to obtain the most similar trace possible. Digital planimetry can be done using free software available for download on the Internet. To perform this technique, only a digital camera and a tripod are required, with no further costs. The use of these computer methods seem to generate the best results, not only because they are the most precise (FALCÃO et al., 2001; ABDALLA et al., 2009), but also because they do not compromise the wound area through manipulation or contamination.

Conclusion

Assessing skin wound size through computer software will generate more trustworthy results, when compared to the clock and graph paper methods, especially in smaller wounds. The authors therefore suggest that all future studies regarding the subject use the software-mediated method when the appropriate means are available.

Acknowledgment

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