The effect of abdominal sepsis in the healing of skin on diabetic rats

Efeito da sepse abdominal na cicatrização da pele de ratos diabéticos

Nathália Eufrásio de Lima, Isaac Felipe Leite Braz, Layssa Carolinne de Sousa Carvalho, Débora Lopes Emerenciano, Ítalo Medeiros Azevedo, Vítor Brasil Medeiros, Rafael Pereira do Nascimento, Aldo Cunha Medeiros

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Correspondence address: Aldo Cunha Medeiros, Department of Surgery, Federal University of Rio Grande do Norte,
at Av. Nilo Peçanha 620, Natal, RN, Brazil, Email: cirurgex.ufrn@gmail.com
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ABSTRACT

Background/purpose: There is an increasing discussion concerning the deleterious effect of hyperglycemia on the wound healing. Patients with sepsis are subjected not only to high risk of death, but are also vulnerable to developing problems related to deficient healing. The aim of this study was to examine the interference of abdominal sepsis and diabetes on the healing of skin in a rat experimental model. **Methods**: Wistar rats (*Rattus norvegicus*) weighing 282±34g randomly distributed into 4 groups of 6 animals each. Twelve animals were subjected to abdominal sepsis with cecal ligation and puncture (CLP) and 12 without sepsis. Six animals from each subgroup had induction of diabetes induced with streptozotocin 50 mg/kg, i.p. and six were non-diabetic. Six days after sepsis induction, a tensile strength test of skin scar from abdominal wall and histopatology of skin wound were carried out. **Results:** The tensile strength showed a significant difference between groups. The control group rats had a tensile strength of 219.0±12.4 gf/cm2, significantly higher than in the diabetes group (185.2±5,9gf/cm2) and in the sepsis group (107.0±9,8mg/cm2) respectively. The group of animals subjected to sepsis + diabetes had the lowest mean tensile strength (86.3±6,6gf/cm2), significantly lower than the results from other groups ($p<0.0001$). The isolated sepsis was more detrimental to wound healing than the isolated diabetes. The existence of the two comorbidities resulted in the lower tensile strength of the scar tissue. The control group rats exhibited an inflammatory reaction score (4.67±0.16) significantly lower ($p=0.002$) than the scores exhibited by the sepsis + diabetes group (5.98 ± 0.27), the sepsis group (5.04 ± 0.23) and the diabetes group (4.95 ± 0.32). **Conclusion:** Sepsis and diabetes, alone or in combination, negatively influenced the healing of skin wounds in rats. Therefore, the data indicates that sepsis and diabetes may operate as deleterious to the wound healing.

Key words: Sepsis. Diabetes. Healing. Skin wound.

RESUMO

Introdução/objetivo: Há uma discussão crescente a respeito do efeito da hiperglicemia na cicatrização de feridas. Os pacientes com sepse estão sujeitos não só a alta mortalidade, mas também são vulneráveis a problemas com a cicatrização de feridas cirúrgicas. O objectivo deste estudo foi examinar a infuência da sepse abdominal e diabetes na cicatrização da pele em modelo experimental. **Métodos:** Ratos Wistar (Rattus norvegicus) com peso 282±34g foram distribuídos aleatoriamente em 4 grupos de 6 animais cada. Doze animais foram submetidos a sepse abdominal com ligadura e punção cecal e 12 sem sepse. Seis animais de cada subgrupo tiveram indução de diabetes com estreptozotocina 50 mg / kg, i.p. e seis eram não-diabéticos. Seis dias após a indução da sepse, um teste de resistência à tração da cicatriz de pele da parede abdominal, e exame histopatológico da ferida de pele foram realizados. **Resultados:** A resistência à tração da ferida de pele mostrou diferença significativa entre os grupos. Os ratos do grupo de controle tiveram uma resistência à tração de 219,0±12,4gf/cm^2, significativamente maior do que no grupo diabetes (185,2±5,9gf/cm^2) e do que no grupo sepse (107,0±9,8mg/cm^2), respectivamente. O grupo de animais submetidos à sepse + diabetes teve a menor resistência média à tração (86,3±6,6gf/cm^2), significativamente menor do que os resultados de outros grupos (p<0,0001). A sepse isolada foi mais prejudicial para a cicatrização de feridas que o diabetes isolado. A existência das duas co-morbidades resultou na menor resistência à tração do tecido cicatricial. Os ratos do grupo controle apresentaram escores de reação inflamatória (4,67±0,16) significativamente menor (p=0,002) do que os escores apresentadas pela grupo sepse + diabetes (5,98±0,27), o grupo de sepse (5,04±0,23) e do grupo de diabetes (4,95±0,32). **Conclusão:** A sepse e diabetes, sozinhos ou em combinação, influenciaram negativamente a cicatrização de feridas da pele em ratos. Entretanto, a sepse associada a diabetes atuaram como os fatores mais deletérios à cicatrização no modelo em estudo.

Descritores: Sepse. Diabetes. Cicatrização. Ferida de pele.

INTRODUCTION

The wound healing is dynamic, interactive, regulated by multiple factors and cytokines, and it involves four phases: haemostasis, inflammation, proliferation and maturation (or remodelling)[1,2]. First, the events of vasoconstriction and coagulation, characteristic of the haemostasis phase, occur. Subsequently, there is the processing of an action mediated by neutrophils, which intends to prevent the bacterial contamination and removal of any unviable tissue. There is also the formation of granulation tissue. In this process, chemotactic factors attract monocytes to the wound site. Those monocytes differentiate into macrophages, whose main functions are phagocytosis and the expression of important growth factors for fibroplasia and angiogenesis[3].

The formation of this tissue and of new blood vessels, on the other hand, is contained within the proliferation phase. Simultaneously, the process of epithelialization

initiates, with the purpose of encasing the granulation tissue in a protective barrier. Finally, the maturation phase depends on a delicate balance between the synthesis and degradation of collagen, prompting the substitution of the thinner collagen (type III), which is produced initially, with a thicker collagen (type I), resulting in a more resistant scar tissue[3].

However, previous studies[4-6] suggest that this natural sequence is negatively influenced by pathologic processes that cause prolonged or exaggerated inflammatory response, such as diabetes and sepsis. In the case of diabetes, the formation of foot ulcers is a very common and severe complication[2]. Considering this, there is an increasing discussion concerning the deleterious effect of hyperglycemia on the healing process of wounds involving the formation of glycation products which induce the production of inflammatory molecules (TNF-α, IL-1) that interfere with collagen synthesis[2]. From a molecular point of view, the IL-1β, important for the epithelialization, angiogenesis, and lymphocyte chemotaxis, is incorrectly expressed, during the initial stages of healing, by the inflammatory cells present in the diabetic wound location[4].

The exposure to high levels of glycose is associated to abnormalities in cell morphology, proliferation, and keratinocyte differentiation[2]. Because of these changes in the wound healing process, the ulcerated wounds in diabetic feet become persistent[2]. Therefore, with the high prevalence of diabetes worldwide in mind, and the statistic data that reports that up to 15% of the population from The United States and United Kingdom, either diagnosed with Diabetes Mellitus or not, develop feet ulcers at some point in their lives, it becomes clear how important it is to identify the factors associated with the poor wound healing of patients with diabetes[4].

Concerning to the patients with sepsis, generally admitted to intensive care units, these are subjected not only to a higher risk of death, but are also vulnerable to developing problems related to deficient healing. This is a result of the interruption of repairing and remodelling events, once, during sepsis, there ishighoxidative stress, insufficient collagen production, and exaggerated immune response, with high levels of proinflammatory cytokines, especially TNF-α[5-8].

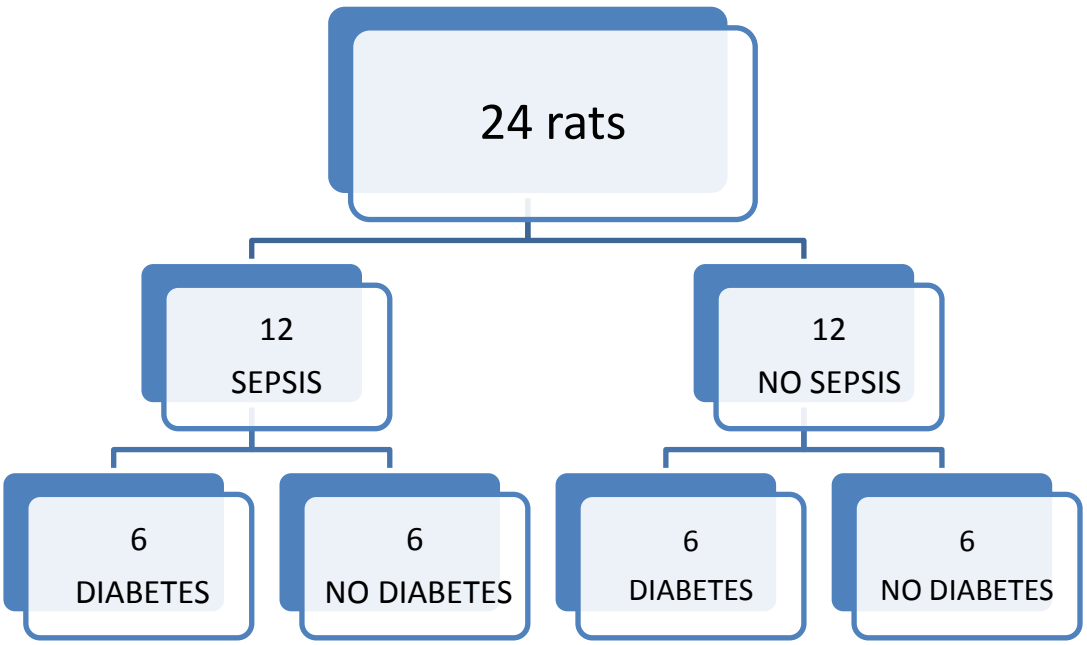
Nevertheless, these patients frequently require surgical procedures, and the inadequate healing of their wounds can cause a number of complications, such as the dehiscence of sutures of the abdominal wall, which is an incident associated with high rates of morbidity and death. A decrease of tensile strength in intestinal anastomoses and sutures of the abdominal wall, has been shown in studies with rats with peritoneal sepsis[8-10]. Thus, the detection of factors relating to deficient healing in patients with sepsis will be able to improve therapeutic procedures[6].

As for the weight of these two diseases on the health care system, in 2005, DATASUS registered 54.365 hospitalizations due to sepsis (approximately 0.5% of the total), with a high cost[11,12]. So, the effects of sepsis in the wound healing process of the integument in diabetic rats can show how these two factors might together influence it.

The aim of this study was to examine the interference of abdominal sepsis and diabetes on the healing of skin in a rat experimental model.

METHODS

We used 24 Wistar rats (*Rattus norvegicus*) weighing 282±34g randomly distributed into 4 groups. This study was submitted to and approved by the Institutional Ethic Comission for the use of Research Animals (Protocol n° 024/2014). The handling and care of these animals followed the current regulation for the scientific use of animals in Brazilian legislation (Law nº11.794/2008). The study was performed at the Nucleus of Experimental Surgery, Federal University of Rio Grande do Norte, Brazil. Rats were observed in individual cages throughout the entire experiment, receiving water and rodents chow *ad libitum*. The distribution of groups and experimental design was determined as such: 24 Wistar rats were randomly divided into 4 groups with 6 animals each. Twelve animals were subjected to abdominal sepsis model and 12 without sepsis. Six animals from each subgroup were subjected to induction of diabetes and another six non-diabetic, as shown in the following schem.



Diabetes was induced with streptozotocin (SIGMA origin) 50 mg/kg, intraperitoneally (i.p.). On day 2, the rats glycemia was determined using the Accu-Chek (Roche, USA). Animals were included in the study when blood glucose was above 250 mg/dL.

Abdominal sepsis induction

After an overnight fast of 12 hours, the rats in the sepsis group were anesthetized using a dose of xylazine of 10mg/kg and 70mg/kg of ketamine, i. p. Followed by the abdominal skin hair removal, the rats were immobilised on supine position on the operating table with adhesive tapes, and skin antisepsis with 70% alcohol. Using aseptic technique, the rats underwent a 4cm laparotomy. The sepsis group rats were submitted to cecum ligation and puncture (CLP). For ligation we used cotton thread 2-0. Four punctures were made in the cecum with a number 25F needle and gentle pressure applied on the cecum, allowing the outflow of a small amount of feces. Thereafter, the cecum was returned to the abdominal cavity, the abdominal incision was sutured with 4-0 nylon and the animals were returned to their cages. Postoperative pain was controlled with meperidine 10 mg/kg, subcutaneously, one dose every 12 hours. The animals in the control group (without sepsis) underwent only laparotomy and suture using the same technique.

Seven days later, animals were anesthetized again, and a rectangular segment of abdominal skin with 3 cm in width and 6 in length, containing the scar inside, was resected. Half of this segment was subjected to a tensile strength test and the other half was subjected to histopathological examination of scar tissue. After the biopsy procedure, euthanasia procedure was conducted with an overdose of anaesthetic (thiopental 100 mg/kg, i.p.).

Scar tissue tensile strength test

The tensile strength test was carried with the aid of a Universal Testing Machine (EMIC, Paraná, Brazil). The rectangular fragment of skin tissue, containing the scar had the standard width of 1.5 cm. The skin was secured by its extremities through special claws and extended at a speed of 5 cm/min. The tensile strength was measured at the time of rupture of the scar, and expressed in gf/cm².

Histopathology

Skin samples were fixed in 10% formalin for 2 days and then embedded in paraffin blocks. Sections (5 µm thick) were stained with hematoxylin-eosin.The histopathological changes were examined through optical microscopy by an experienced pathologist without prior knowledge of the respective groups. The quantitative analysis (histomorphometry) was performed concerning the amount of leukocytes, fibroblasts and other inflammatory cells, using a scanner and an image analyser system. The total area of the microscopic fields was observed through an optical microscope (Olympus BX50), and the image was captured by the Olympus SC30 high resolution camera and scanned through the Software ImagePro-plus (Media Cybernetics, LP, USA). Each digitized field was quantified in pixel units with defined coordinates, six random microscopic fields being evaluated per slide. After selecting the desired resolution, the images were stored to quantify the density of the inflammatory reaction in the regions of interest (ROI). To render more appropriate the quality and representation of the data, alogarithm was used, so that the variable density of the histological findings was expressed in logROI.

Statistics

The statistical analysis was performed using the SPSS 17.0 software, using the analysis of variance test (ANOVA), followed by the Tukey's test, considering significant differences at $p<0.05$.

RESULTS

Table 1 – Descriptive statistical results of the variables: weight, tensile strength, glycemia and their inferential statistical tests.

Variables	Groups				p-value
	Sepsis+diabetes	Sepsis	Diabetes	Control	
Weight (g)	289,2±8,4	286,3±7,7	282,8±12,7	288,7±11,5	0,704[1]
Tensile strength of the skin (gf/cm^2)[2]	86,3±6,6[§]	107,0±9,8[§]	185,2±5,9[§]	219,0±12,4[§]	< 0,001[1]
Glycemia (mg/dL)[2]	267,2±10,0[♦§]	86,8±4,7[§¥]	273,2±13,8[¥]	84,5±5,4[♦¥]	0,001[1]

Average ± Standard deviation
1 – p-value of the analysis of variance (ANOVA).
2 – Values followed by similar symbols showed statistically significant differences between groups, Tukey's test.

Table 2 – Histopathological examination of skin, 200X magnification, HE stain.

Variables	Groups				p-value
	Sepsis+diabetes	Sepsis	Diabetes	Control	
Area–region of interest (log ROI)	5,98±0,27[§¥]	5,04±0,23[♦]	4,95±0,32[¥]	4,67±0,16[♦§¥]	0,002[1]

Average ± Standard deviation
1 – p-value of the analysis of variance (ANOVA).
2 – Values – on same row - followed by similar symbols showed statistically significant differences between groups, through Tukey'stest,at a significance level of 5%.

In Table 1, we observe that the tensile strength showed a significant difference between groups. Compared with the control group, all groups that underwent other interventions along with the skin incision experienced a significant reduction in the tensile strength of the cicatricial tissue. The control group rats had a tensile strength of 219.0 ± 12.4 gf/cm2, significantly higher than that observed in the diabetes group (185.2 ± 5,9gf/cm2) and in the sepsis group(107.0 ± 9,8mg/cm2) respectively. The group of animals subjected to sepsis + diabetes had the lowest mean tensile strength (86.3 ± 6,6gf/cm2), significantly lower than the results from other groups ($p<0.0001$). Through this data, we observed that the isolated sepsis was more detrimental to wound healing process than the isolated diabetes. The existence of the two comorbidities resulted in the lower tensile strength of the scar tissue.

As for glycemia, we found that diabetes was sufficiently induced in the animals of groups sepsis + diabetes (267.2 ± 10.0 mg/dL) and diabetes (273.2 ± 13.8 mg/dL), while in the sepsis group and the control group, which were not treated with streptozotocin, the rats glycaemia levels reached 86.8 ± 4.7 mg/dL and 84.5 ± 5.4 mg/dL respectively.

Regarding the weight, there was no statistically significant difference between the groups ($p>0.05$), suggesting that the environmental variables were properly controlled and that the interventions were not sufficient to significantly alter the weight of the animals during the experiment.

In table 2, we see that the histopathological inflammatory changes observed in the healing of skin were significantly higher in the group of rats subjected to sepsis + diabetes than in the animals from the diabetes group and the control group ($p<0.05$). As for the animals in the sepsis group, when compared with the animals from the sepsis + diabetes group and the ones from the diabetes group, there was no significant difference regarding the histopathological reaction parameter in the skin tissue during the wound healing process. However, the control group animals exhibited an inflammatory reaction score (4.67 ± 0.16) significantly lower ($p = 0.002$) than the scores exhibited by the sepsis + diabetes group (5.98 ± 0.27), the sepsis group (5.04 ± 0.23) and the diabetes group (4.95 ± 0.32).

Hematoxylin and eosin staining was used to examine the morphological alterations in the healing of skin tissue. Images 1, 2 and 3 show that the animals from the sepsis + diabetes group displayed a more intense inflammatory reaction (Image 1A, B, C, D) than the ones from the sepsis group (Image-2 A, B, C, D) and from the diabetes group (Image-3A, B, C, D). The histomorphometric results confirm the descriptive observations.

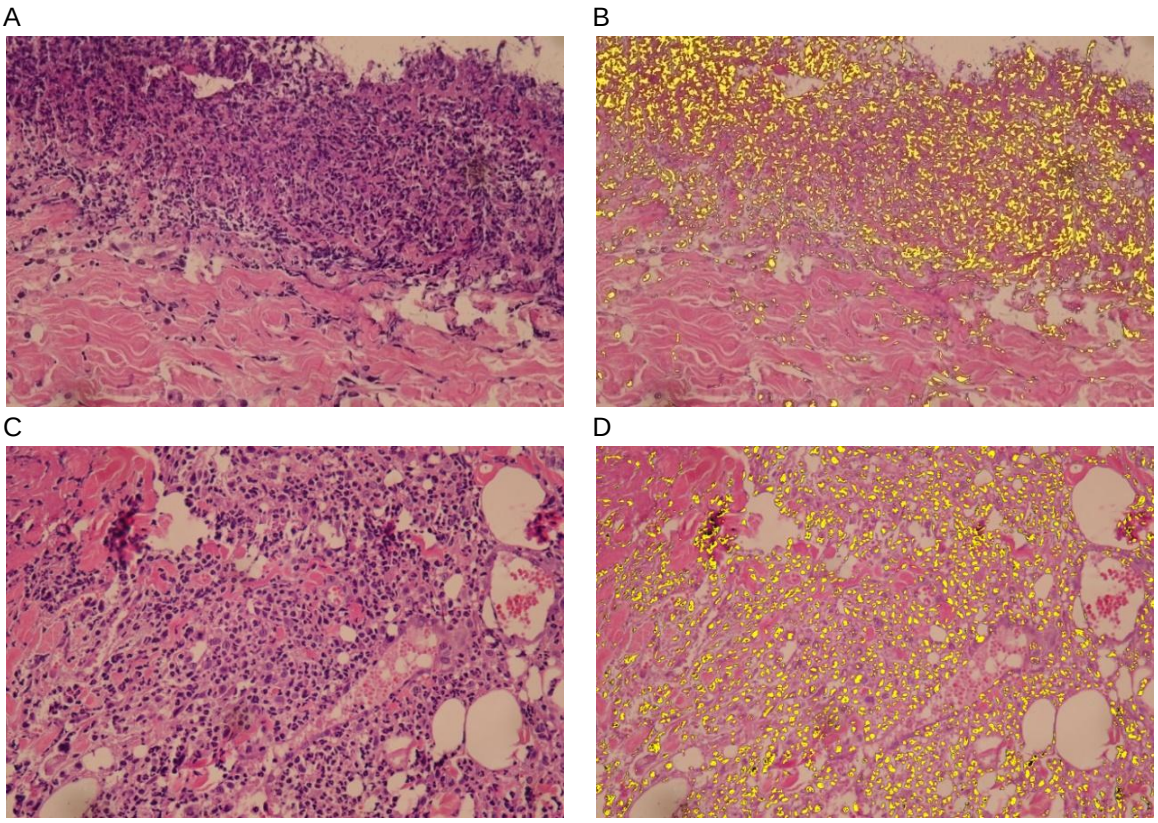


Figure 1 – HE staining of skin from the sepsis + diabetes group during healing. (A) A large number of leukocytes was identified and highlighted in yellow for quantification (B). (C) Quantitative histomorphometry showed inflammatory cells, which in image D are highlighted in yellow by the software ImageProPlus, for the purpose of counting. (200x magnification).

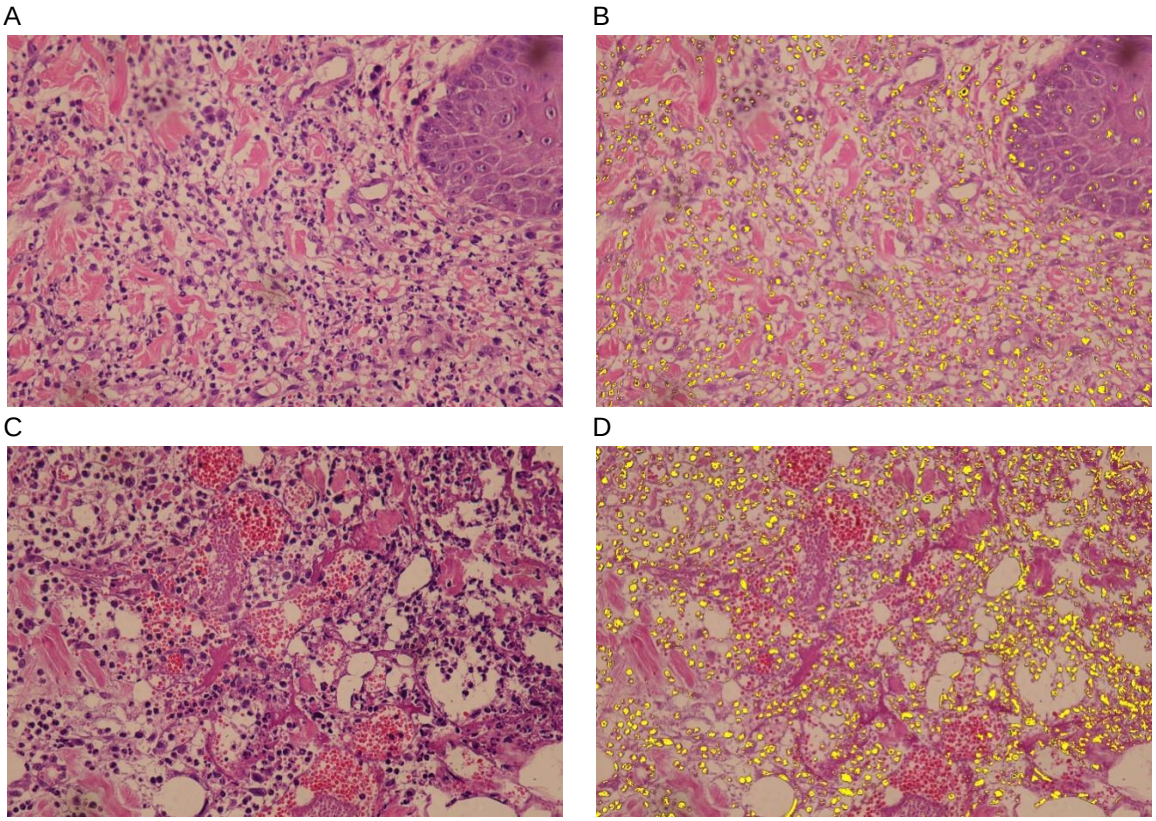


Figure 2 – HE staining of skin healing from the sepsis group. (A) A moderate number of leukocytes was identified and highlighted in yellow for quantification (B). (C) Quantitative histomorphometry showed inflammatory cells and an expressive number of erythrocytes; in image D the inflammatory cells are highlighted in yellow by the software ImageProPlus, for the purpose of counting. (200 x magnification).

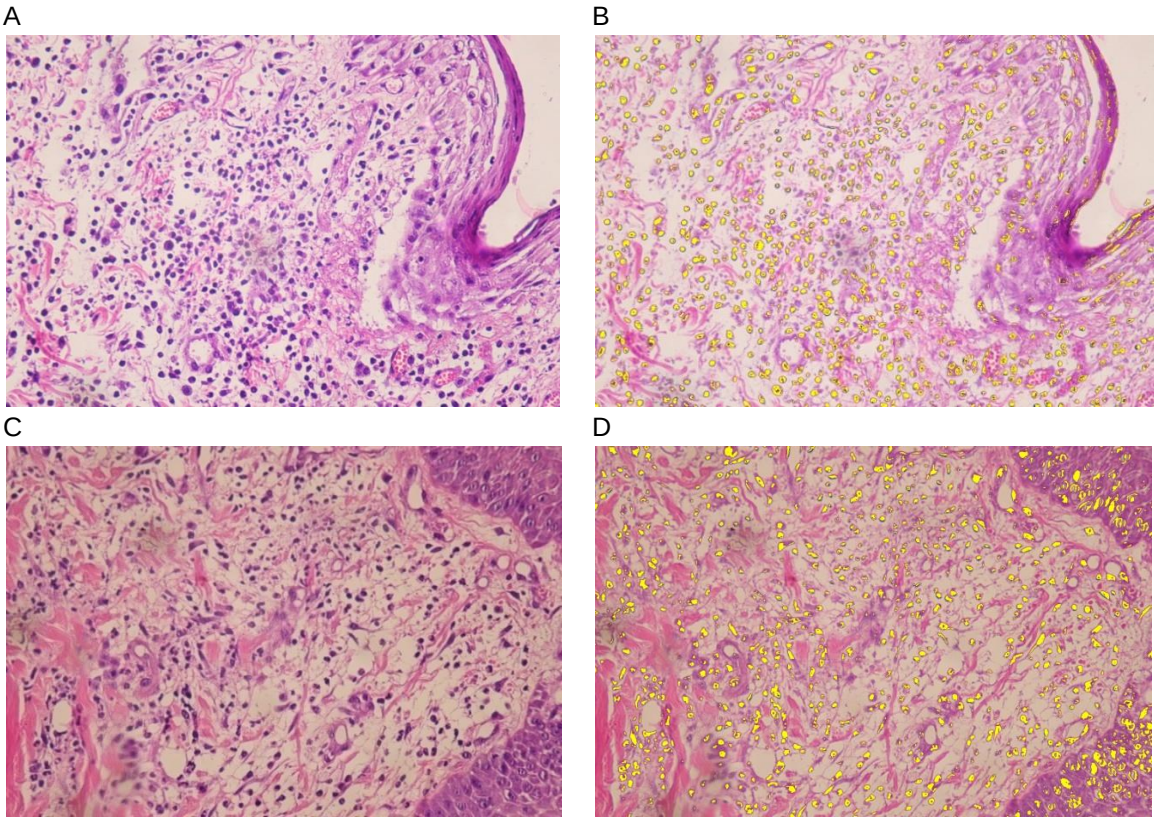


Figure 3 – HE staining of skin healing from the diabetes group. (A) A large number of leukocytes was identified and highlighted in yellow for quantification (B). (C) Quantitative histomorphometry showed inflammatory cells, which in Figure D are highlighted in yellow by the software ImageProPlus, for the purpose of counting. (200 x magnification).

DISCUSSION

Considering the group with diabetes induced with streptozotocin, responsible for the destruction of pancreatic beta cells, we recognised that the tensile strength of the skin scar of diabetic rats was significantly lower than in the control group, as demonstrated in other studies[2,4,13]. Currently, more than 100 physiological alterations are recognized as deleterious factors to wound healing in diabetic patients, involving issues from the formation of advanced glycation end products to the inability of macrophages, endothelial cells, fibroblasts, keratinocytes and platelets to properly coordinate the healing process, due to changes in the expression and secretion of growth factors and cytokines[13].

In previous studies about rats with Streptozotocin-induced diabetes, a reduction in the expression of Stromal Cell-derived Factor 1-Alpha (SDF-1 α) by epithelial cells and myofibroblasts was confirmed, and this reduction led to a deficit on the neovascularization of animals' wounds, as this recruiting molecule is responsible for guiding endothelial progenitor cells[13]. Thus, the microenvironment of the diabetic wound remains hypoxic, causing it to be predisposed to infections[14]. In clinical practice, the absence of such

chemotactic factor has been suggested as one of the causes of the deficient responses of diabetic patients to therapeutic procedures for the healing of wounds[13].

In the present study, inflammatory histopathological changes were observed to be significantly more intense in diabetic animals than it was in the control group. Once the abdominal sepsis was combined with the diabetes, the inflammation was shown to be even more intense. Diabetes in our rats was induced sharply, for this reason we did not expect any chronic complications that could potentially interfere with the healing process, such as neuropathy and vasculopathy. However, although they were exposed to the diabetic condition for a relatively short period of time, the rats showed a significant impairment in the healing process, possibly caused by the absence of insulin and the hyperglycemic environment.

It is known that insulin has anabolic effects: it has been demonstrated that diabetic patients show improvement of fibroblast proliferation and of collagen synthesis with the use of insulinotherapy, demonstrating that insulin contributes to the healing of wounds[17]. As for local hyperglycemia, a study that evaluated the effects of high glucose concentration in cultures of human keratinocytes, revealed a reduction in beta-defensin-2 expression, a molecule related to inflammation, re-epithelialization and angiogenesis in the wound healing context[4]. In contrast, the study mentioned above, with diabetic pigs[16], found that there is no statistical difference between the healing speed of a wound in a normoglycemic environment and a wound in a hyperglycemic environment. Therefore, this issue remains controversial.

In CLP rats, the sepsis conferred a deterioration of the skin healing, as the group exhibited significantly lower tensile strength necessary to rupture the surgical wound than in the control group. From a clinical point of view, this means that a patient with sepsis, relative to a healthy patient, carries a high risk of wound dehiscence.

The pathophysiology of sepsis can justify such damage to healing, as it includes an altered cytokine production that can destabilize the hemodynamics, the metabolism and the inflammatory phase present during a normal healing process. When prolonged, sepsis increases the production of TNF-α, IL-1β, IL-6, IL-8 and other cytokines[14], and so, the excess of TNF-α can damage the wound healing, inhibiting collagen and fibronectin production, and it also induces collagenase synthesis. Furthermore, the excess of IL-1β and IL-6 also can be extremely detrimental to the wound healing, since the imbalance between IL-1β production, and its inhibition may lead to tissue damage and organ dysfunction. IL-6 has been shown to be responsible for the suppression of fibroblast proliferation[14.]

Another important aspect of sepsis relates to the fact that abnormal activation of the complement, kinin, and the coagulation cascade, result in reduced microcirculatory flow and increasing peripheral shunts, with consequent damage to the perfusion of peripheral organs, such as the skin[14]. Thus, hypoxia leads to an increased production of cytokines (TNF-α and IL-1β) for a long period of time, resulting in the increased production of matrix metalloproteinase (MMPs) that interferes hindering the healing process[14, 15]. This tissue hypoxia also prevents the formation of reactive oxygen species that are essential for healing.

In general, there is an escalation of the inflammatory phase of the healing process through maintaining an elevated levels of pro-inflammatory cytokines and bacterial endotoxins, which impair the integrity of the wound in process of healing. In our laboratory, factors influencing wound healing have been studied[17-20].

When comparing the data from the isolated sepsis group and the isolated diabetes group, we ascertained that the tensile strength of sutures of septic rats was considerably lower than that of diabetic rats, with a statistically significant result. However, we could not find studies that compared the effect of these two diseases on wound healing process, so we did not have enough knowledge to explain it.

Finally, observing the outcome of the sepsis + diabetes group, we identified that it had obtained the worst result in the tensile strength of skin and in the intensity of the inflammatory reaction. As mentioned, both the sepsis and the diabetes, alone, have deleterious effects on the healing process, and with the results of our study, we can ascertain that these two factors, when combined, act synergistically and deteriorate the skin healing. Based on studies evaluating the effect of insulin in skin healing in catabolic states[17], we can suggest a hypothesis for the effects of an association between sepsis and diabetes in tissue repair: in sepsis, there is an increased release of counter-insulin hormones (glucagon, corticosteroids, growth hormone, and catecholamines), resulting in hypermetabolism and increased protein catabolism of connective tissue. Added to the absence of the anabolic effects of insulin, which is present in diabetes, this should cause tissue repair to be further impaired.

CONCLUSION

In conclusion, sepsis and diabetes, alone or in combination, negatively influenced the healing of skin incisions in rats. Therefore, the data indicates that sepsis and diabetes may operate together in a way that is deleterious to the wound healing.

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