Effects of hyperglycemia and ageing on the healing of colonic anastomoses in rats

Efeitos da hiperglicemia e do envelhecimento na cicatrização de anastomoses colônicas, em ratos

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ABSTRACT
Introduction: Despite the significant advances in the knowledge of the healing process, there is a limited number of studies demonstrating the relationships of this healing with ageing and elevated levels of glycemia. Purpose: To evaluate the effects of ageing and hyperglycemia on the healing of colonic anastomosis. Methods: 138 young and old male rats were utilized. Some of them were normoglycemic and others had hyperglycemia induced by streptozocin (50mg/kg). They were maintained under control for 90 days. They were then submitted to a termino-terminal anastomosis in the left colon. On the third, seventh and fourteenth days after surgery, their resistance was evaluated and a histopathological study of the anastomosis was carried out. Results: Gain in resistance was similar for both groups. The additive effect of age with hyperglycemia made a significant difference to the collagen I (p<0.001), III (p=0.022) and total (p<0.001). Among the old animals, the glycemia was a determining factor for the occurrence of a significant difference in total collagen (p=0.029) and collagen I (p=0.013). Among the normoglycemics, age determined a lower density of collagen I (p=0.002). Conclusion: There is delayed collagen synthesis and maturation of the scars of older animals, a situation that becomes more serious in older hyperglycemic animals, but insufficient to adversely affect the gain in resistance. Key words: Diabetes Mellitus, Experimental. Hyperglycemia. Collagen. Wound Healing. Colon. Anastomosis, Surgical.

RESUMO
Introdução: Apesar dos significantes avanços no conhecimento do processo cicatricial há um restrito número de estudos demonstrando as relações deste reparo com o envelhecimento e com níveis elevados de glicemia. Objetivo: Avaliar os efeitos do envelhecimento e da hiperglicemia na cicatrização de anastomoses colônicas. Métodos: Utilizaram-se 138 ratos machos, adultos jovens e velhos. Parte deles era normoglicêmico e parte teve hiperglicemia induzida pelo streptozocin (50mg/kg). Foram mantidos controlados por 90 dias. Após fez-se uma anastomose término-terminal no cólon esquerdo. No 3.º, 7.º e 14.º dia de pós-operatório avaliou-se a resistência e fez-se o estudo histo-patológico da anastomose. Resultados: O ganho de resistência foi semelhante entre os grupos. O efeito aditivo de idade com hiperglycemia determinou diferença significativa nos colágenos I (p<0,001), III (p=0,022) e total (p<0,001). Entre os velhos a glicemia foi determinante para ocorrer diferença significativa no colágeno total (p=0,029) e colágeno I (p=0,013). Entre os normoglicêmicos, idade determinou menor densidade de colágeno I (p=0,002). Conclusão: Existe atraso na síntese do colágeno e na maturação das cicatrizes nos animais velhos, situação que se agrava nos animais velhos e hiperglicêmicos, não suficiente para prejudicar o ganho de resistência. Descritores: Diabetes Mellitus Experimental. Hiperglycemia. Colágeno. Cicatrização de Feridas. Colo. Anastomose Cirúrgica.

Introduction

Healing can be affected by negative influences of many factors such as: disturbances of coagulation, metabolic disturbances such as diabetes and hypothyroidism, vascular alterations such as atherosclerosis, radiotherapy, the chronic use of medicines such as steroidal and non-hormonal anti-inflammatory drugs, advanced age, infection and others¹.

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With the advance of age, it is common for the coexistence of diseases to affect the healing process. It has been shown that although there are differences in the healing process for colonic anastomoses in young and old rats, like the higher number of inflammatory cells and the higher deposition of collagen I in the post-surgery phase of anastomoses in young rats, age by and of itself did not cause the failure to heal\(^2\). Nevertheless, age did delay re-epithelization\(^1\).

Several factors are known to contribute to deficient healing in individuals with diabetes. These include: a reduction in the production of growth factors, angiogenic response, macrophage function, collagen accumulation, epidermal barrier function, quantity of granulation tissue, proliferation and migration of keratinocytes and fibroblasts, basal beam healing and the balance between the accumulation of extra (MMPs)\(^3\)-12.

One of the greatest physio-pathological consequences of hyperglycemia is excessive chemical interaction of glucose with proteins, leading to their connection without the aid of enzymes, non-enzymatic glycosylation. It has been seen that diabetics glycosylated serum protein levels that are four times higher than non-enzymatic glycosylation. It has been seen that diabetics glycosylated proteins, leading to their connection without the aid of enzymes, non-enzymatic glycosylation. It has been seen that diabetics glycosylated serum protein levels that are four times higher than non-enzymatic glycosylation. It has been seen that diabetics glycosylated serum protein levels that are four times higher than non-enzymatic glycosylation. It has been seen that diabetics glycosylated serum protein levels that are four times higher than non-enzymatic glycosylation.

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Blood was collected from the end of the tail at the beginning of the study. The animals selected to belong to the hyperglycemic groups were weighed and received streptozocin (Zanosar®; Upjohn CO.), 50 mg/kg of weight by endovenous injection utilizing the dorsal vein of the penis and those selected for the normoglycemic groups received a corresponding volume. New glycemia checks were made after twenty-four hours and after ninety days, prior to surgery.

An anesthetic by an intramuscular injection of 0.2ml/100g of weight from a mixture of 1ml of ketamine (50mg) and 1ml of xylazine (20mg)31. The trichotomy of the ventral abdominal wall was done and anti-sepsis with polyvinylpyrrolidone-iodine.

The surgical procedure consisted of a median laparotomy with four centimeters. The transverse colostomy was done at 2.5cm from the peritoneal reflection, followed by termino-terminal anastomosis on a single sero-muscular layer with eight interrupted 5.0 monofilament nylon stitches. We then moved on to the laparorraphy at two levels: the first, peritoneal-muscular-aponeurotic; the second, of the skin, with continuous synthesis, utilizing 4.0 monofilament nylon.

After recuperating from the anesthetic, the rats were returned to their cages, where they had free access to standard commercial food and water. Immediately after surgery they were given an intramuscular dose of sodium diclofenac at a dosage of 10 mg/kg, as an analgesic and anti-inflammatory drug31.

The four groups were subdivided at random into three subgroups according to the date of euthanasia on the third, seventh and fourteenth days after surgery, for which a lethal dose of intra-peritoneal sodium thiopental was used at 120mg/kg32.

After euthanasia, another laparotomy was performed, enabling us to observe the abdominal cavity, the existence of peritonitis or abscess, adherences and the integrity of the anastomoses.

A four-centimeter segment of the colon was dries, containing anastomosis in the mid portion and a study of the resistance of anastomoses to insuflation, obtaining the pressure measurements at the time of rupture in millimeters of mercury.

After this evaluation, the surgical parts were opened lengthwise and extended on filter paper and set in 10% formalin and forwarded for histopathological study.

Seriated cuts were made with four micrometers of thickness which, placed on blades and stained by the Sirus-Red technique enabled us to evaluate the density, quality and ordering of the collagen33. The cuts were evaluated without prior knowledge of the meaning of the codes utilized to identify the samples.

In the Sirus-Red analysis of the cuts, the thickest and most birefringent collagen fibers had a reddish orange color (collagen I); and the thinnest and most dispersed fibers, weakly birefringent, had a greenish coloring (collagen III)33. The images were captured by a Sony CCD101 camera and transmitted to a Trinitron Sony color monitor, frozen and digitalized through the oculus TCX board. The images were analyzed using Image-Plus® 4.5 for Windows® from MediaCybernetics on a Pentium microcomputer. Five fields enlarged 200x and located on the line of the scar were analyzed in each cut. In each of the fields the percentage of area occupied by the red and yellow fibers (collagen I) and green fibers (collagen III) was calculated. Considering that the other types of collagen constitute small fraction, for practical purposes, the sum of collagens I and III was considered as being the total collagen of the scar.

For comparison of the four groups in this study, the variance analysis was used with a factor (ANOVA) and the LSD test for multiple comparisons, or the non-parametric Kruskal-Wallis test, when appropriate. The condition of normality of the data was evaluated by the Shapiro-Wilks test and the homogeneity of variances by the Levene test. Values of p<0.05 indicated statistical significance.

Results

Eight animals from the YH group and 8 from the OH group died during the ninety days prior to the surgery. One hundred and twenty-two were operated on. In the immediate post-operative period, 2 from the YH group and 2 from the OH group died. Therefore, 118 animals were left for monitoring.

All the animals induced with streptozocin developed hyperglycemia. The levels were higher after 24 hours, although they remained high until the end of the experiment (Figure 1).
No anastomotic dehiscence was registered and adherences were found, with different intensities, in the anastomoses of all groups. The anastomoses gained resistance as time passed in the four groups, without any significant difference between them (Figure 2).

The values of the total collagen density readings show that there was no difference between the groups in the evaluation on the third day ($p=0.987$), which was also the case on the seventh day ($p=0.0439$). However, there was a significant difference on the fourteenth day ($p<0.001$). The two by two comparison at this time, showed that there was more collagen in the anastomoses of the young normal animals than in the older hyperglycemic animals ($p<0.001$), demonstrating the interference of age and hyperglycemia. Colonic anastomoses in the older normal animals had more collagen than in the older hyperglycemic animals ($p=0.029$). In this case, as the two groups compared are of older animals, the factor of interference would be age. Comparing the collagen densities between young and old hyperglycemics, we observed that the young ones had more collagen ($p<0.001$) (Figure 3).
Analysis of the collagen fractions enabled us to verify that at the three times studied, the collagen III density was higher than that of collagen I and that the proportion between them was continually modified with the evolving healing process. There was no significant difference in the collagen III density between the groups on the third day (p=0.191), nor on the seventh day (p=0.773). On the fourteenth day, there was a difference between the groups (p=0.038). Compared two by two, we could see that in the colonic anastomoses of the young animals there was a higher density of collagen III than in the older hyperglycemic animals (p=0.022), showing the interference of age and the levels of glycemia. Comparison of the densities of this fraction of collagen between the old groups showed that the noromglycemics were higher (p=0.013). In this case the interference of glycemia is evident (Figure 4).

Analysis of the collagen I fraction was lower than that of collagen III at all times, although there was a gain with the progress of the healing process. At the first two times, the values were similar (third day – p=0.100 and seventh day – p=0.264). On the fourteenth day, the collagen I density in the anastomoses was different (p<0.001). For the normoglycemic groups, the colonic anastomoses of the young had more collagen I than the old (p=0.002), thus the interference of age. Colonic anastomoses in young normal animals had more collagen I than young hyperglycemic animals (p<0.001). In this case, this was evidence of the influence of glycemia. Comparing the colonic anastomoses of young normal animals with the old hyperglycemic, the former had more collagen I (p<0.001), strengthening the hypothesis that age and glycemia would be factors of interference (Figure 5).
Discussion

Intolerance of glucose is the most important endocrinial alteration in the elderly population, being around 45% more frequent than in the younger population\textsuperscript{14}.

One of the major consequences of hyperglycemia is non-enzymatic glycosylation of proteins\textsuperscript{13} such as hemoglobin, a albumin, crystalline proteins, collagen, elastin, myelinic proteins and other\textsuperscript{14}. There is an accumulation of end products of glycosylation until this deposition is irreversible, leading to structural and functional alterations\textsuperscript{14,35}. When these proteins are exposed to high levels of glycemia for some days, the reactions of glycosylation of formed products are still reversible, which is not the case after months of exposure\textsuperscript{14}. Hyperglycemia leads to end products of advanced glycosylation which are strengthened by aldolases by covalent reactions of amino-reactive groups. These end products of advanced glycosylation lead to increased oxidative stress and may activate the key to the transcription factor of the nuclear factor that is related to many cellular responses linked to cytokines. Thus these products appear to induce the production of PDGF, TNF-\alpha and IL-1-\alpha\textsuperscript{10}. These products may increase cross linking of collagen, inhibiting normal degradation.

A significant alteration has been shown in the degree of collagen glycosylation and its content in injuries after five days of hyperglycemia in rats\textsuperscript{37}. This finding implies that recently formed glycosylation products, although reversible, are harmful to the healing process. In this study, after three months of exposure to hyperglycemia, the animals probably had the end products of advanced glycosylation, a fundamental condition for evaluating the effects of hyperglycemia on collagen synthesis and the related effects of healing intestinal anastomoses.

In literature we find less activity of leucocytes with a consequent lower inflammatory response, bactericide capacity and the formulation of granulation tissue\textsuperscript{9,11,26}. Komesu \textit{et al.}\textsuperscript{26} described in rats with diabetes induced by alloxan an inflammatory response with a slower and more prolonged beginning. They also found lower density of neutrophils in the analysis of the third day. When they examined after a longer time, they noted that at the time when they were expecting higher density of macrophages, what they did find was a larger number of neutrophils, confirming a slow and drawn out beginning of the inflammatory phase. The glycoylated hemoglobin has reduced reactivity, with 2,3-dephosphoglycerate, which affects the transport of oxygen and results in tissue hypoxia\textsuperscript{11}. If we consider that proline and lysine hydroxylation depend on oxygen, its lack will result in lower collagen synthesis.

Insulin deficiency in diabetics leads to a reduction in the metabolism of carbohydrates, fats and proteins which are necessary for cellular activities and protein synthesis in the healing process. Insulin is necessary for the entry of glucose in the cells and to remove the energetic need for the synthesis of proteins and to inhibit lipolysis. Glucose is the main energy supply for the fibroblasts and for the polymorphonuclears. Thus, diabetics have reduced expression of shock proteins\textsuperscript{39}, lower antioxidant activities in these cells and therefore in healing\textsuperscript{38}. There is a significant fall of the levels of TGF-\beta and IGF-1 on the seventh day, the latter being an important stimulator of the proliferation of keratinocytes and fibroblasts.

Minimum disturbances in the synthesis balance, deposition, degradation and remodeling of the collagen may result in a deficiency in the healing of the anastomosis\textsuperscript{7}. The collagen fibers are responsible for the integrity of the intestinal wall\textsuperscript{6} and the organization of fibers is essential to understanding the anastomotic healing process, although the quality, in comparison with the quantity of collagen fibers, is what plays the fundamental role in maintaining the integrity of the intestinal wall during this process\textsuperscript{97}.

Several authors have reported in experimental studies that there is lower resistance and less collagen deposition in anastomoses done in the stomach, the ileum and the colon in animals with hyperglycemia\textsuperscript{18-23,50}.

Despite significant advances in the knowledge of the healing process for colonic anastomoses, there is a limited number of studies demonstrating the relationship between this healing mechanism and age and high levels of glycemia.

Stoop \textit{et al.}\textsuperscript{15} reported that colonic anastomoses had equal resistance in young and old animals and that the collagen content was higher in the old. Seyer-Hansen \textit{et al.}\textsuperscript{52}, studied colon anastomoses in old and diabetic rats and concluded that resistance was not reduced with age and with diabetes. Meanwhile, Onodera \textit{et al.}\textsuperscript{21} tested the integrity of colon anastomoses in diabetic rats and stated that resistance was significantly compromised with less accumulation of collagen I in the evaluation after seven days and that these parameters were the same after fourteen days.

In a previous study in our line of research we observed that the inflammatory process was extended over a longer period of time in the anastomoses of older rats, that resistance was similar and that the collagen content up to the seventh day was also resistant, becoming greater in colonic anastomoses in young rats on the fourteenth day\textsuperscript{7}. In another study, re-epithelization was seen to occur earlier in the anastomoses of young normoglycemic rats, as was the case with neovascularization\textsuperscript{1}. According to Spravchikov \textit{et al.}\textsuperscript{43} high concentrations of glucose, the effects of IGF-1 in the absorption of glucose by the keratinocytes and its proliferation and differentiation would be inhibited.

In this study the previous information was confirmed that resistance was not being affected, confirming the findings described by Seyer-Hansen \textit{et al.}\textsuperscript{52}. It was possible to see that colonic anastomoses in young and old normo or hyperglycemic rats had similar collagen density up to the seventh day. However, on the fourteenth day, the densities were different. There was less collagen in the anastomoses of the old and hyperglycemic animals (p<0.001). In this case, age and hyperglycemia influenced the result. It was also seen that the anastomoses in old normoglycemic animals had more collagen than the hyperglycemic animals (p=0.029) and in this case the factor responsible for this must have been the glycemia level.
By analyzing both young and old hyperglycemic rats, a lower density of collagen was found in the anastomoses of the latter and therefore age must be affecting the process. If we consider that the fraction of collagen I was higher in the anastomoses of the young normoglycemic rats than in the older normo and hyperglycemic rats, it is possible to suppose that not only was the collagen density affected but also the scar maturation.

Conclusions

1. Resistance of anastomosis is not affected by age or by hyperglycemia;
2. Collagen density is not affected in the early stages;
3. Collagen density is affected in later stages both by age and by high levels of glycemia;
4. Both fractions of collagen are affected, especially collagen I, which means that there is a delay in scar maturation.

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