Tissue microarray technology and collagen evaluation to analyze surgical trauma performed with usual blade or ultrasonic harmonic scalpels in rats[1]

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Abstract

Purpose: To compare wound healing performed with cold blade (CSB) and ultrasonic harmonic scalpel (UHS) in the abdominal aponeurosis of rats.
Methods: Eighty Wistar rats divided into two groups and underwent midline incision in the linea alba with cold blade and harmonic ultrasonic scalpel. Analysis were performed in subgroups of 10 animals after 3, 7, 14 and 21 days. Macroscopically was observed the presence of hematoma, infection, wound dehiscence, fistula and adherences. Microscopically were used collagen and immunohistochemical staining methods.
Results: Macroscopic, complications showed no statistical difference. Immunohistochemical analysis for MMP-9 was more intense in UHS group ($p<0.05$). TGF β presented its lower expression in UHS group at 14 and 21 days, with no statistical difference at 3 and 7 days ($p<0.05$). α-AML expression appeared higher in UHS group after 14 days and remained similar in others ($p<0.05$). Collagen deposition had no change in type I, and increased in type III in UHS; at 7th day the deposition was higher in CSB group; at 14th was similar in both groups ($p<0.001$).
Conclusion: UHS compared to the CSB has higher lesion area at the time of the incision; as well as it led to the delay of regeneration and scar maturation process.
Key words: Wound Healing. Aponeurosis. Ultrasonics. Rats.

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■ Introdution

Incision and hemostasis procedures are performed with different devices in order to provide effective hemostasis and surgical time reduction, without prejudice to tissue healing.

The cold blade scalpel (CBS) is the most widely used instrument for dieresis; it is easy to handle and has predictable tissue damage, but incision bleeding interferes with operative field visibility requiring hemostasis.

Since the introduction of electrocautery by Bovie[1] several models were developed. The ultrasonic harmonic scalpel (UHS) was initially used in laparoscopic surgery in 1995. With it were introduced new features, like no smoke, good cut, coagulation as monopolar scalpel, dissection with minimal tissue injury, almost no waste, without tissue carbonization and with no electric current transmition to the patient's body. It is being used in both open and endoscopic operations[2].

The UHS perform hemostasis by combining dissection with blood clotting and consequent obstruction of the vessel. The ultrasound acts as a source of energy through the instrument tip (blade) causing unidirectional vibrations of ultra-high frequencies (300-3000 MHz), which leads to tissue dissection and, at the same time, to blood coagulation. When the blade vibrates at 55.500 Hz frequency, the sound is converted into mechanical vibratory energy, and acts to promote cutting and coagulation. It provides better coagulation because its blade is blunt and therefore generates slow cutting. The ultrasonic energy allows to cut and coagulate at the precise point of impact with minimal surrounding tissue thermal damage. It also offers great precision in small spaces and good visibility in the surgical field due to minimal smoke production.

Tissue heating caused by UHS has minimum depth compared to laser, radiofrequency and electrocautery, with reduced tissue damage**.**

This study aims to compare the healing process of incisions made with cold blade and ultrasonic harmonic scalpels in the abdominal wall of rats.

■ Methods

This study was conducted at the Institute of Medical Research, Postgraduate Program in Principles of Surgery, Faculdade Evangélica do Paraná, and was approved by the Research Ethics Committee of the Beneficent Evangelical Society of Curitiba.

Were used 80 rats (*Rattus norvegicus albinus*, Rodentia mammalia) Wistar male adult with three months old and weighing 250-300 g. They were divided into two groups of 40. The first had aponeurotic incision in the midline (linea alba) with cold blade scalpel, and the second with ultrasonic harmonic scalpel. The surgical skin incision in all animals was done using cold blade. Each group was subdivided into four subgroups of 10, with programmed euthanasia in 3, 7, 14 and 21 days.

The cold scalpel consisted of mobile cable nº 3 and blade 15. The ultrasonic generator model was 300 GEN04 and Ultracision model CS-14C (Ethicon EndoSurgery®, Inc).

The animals were kept in a vivarium in circadian light conditions (12/12 h) with stable temperature without noise and housed in standard cages, covered with sawdust and fed by ration for the species, free water and subjected to preoperative fasting for 12 h. Anesthesia was performed with 10% ketamine hydrochloride and xylazine 2% in the usual standard for the species.

After anesthesia the animals were positioned supine being fixed by the ends on the operating table; were submitted to ventral wall tricotomy, antisepsis with iodopolipovidone 10% and covered by surgical

dressing delimiting the operative area.

The surgical procedure was initiated by incision with cold scalpel with digital hemostasis in all 80 animals, and exposure of the linea alba with 7 cm long. Next, the first 40 animals group had aponeurosis section made until peritoneum with cold scalpel blade, 6 cm in length, and the same procedure was done using ultrasonic harmonic scalpel on level 3 (Figure 1A) in the second 40 animals group.

The aponeurosis synthesis was performed with polypropylene continuous sutures in both groups (Figure 1B) and skin´s with 5-0 monofilament nylon.

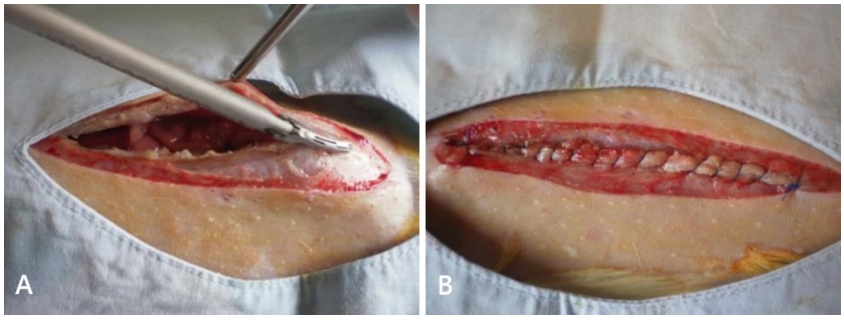

Figure 1 – Incision (**A**) with ultrasonic harmonic scalpel in the linea alba and continuous suture (**B**) for closing.

The animals returned to their pre-operative housing and diet, having the procedure date and scalpel type used in the operation marked, and also marked the day proposed for euthanasia. They underwent postoperative analgesia with paracetamol 200 mg/ml oral dose containing 40 drops for each 50 ml of water in the first two days.

The macroscopic evaluation was daily, with observation of the surgical wound. According to the schedule the rats were submitted to euthanasia by intraperitoneal injection of anesthetic using lethal dose - twice the conventional – at the 3rd, 7th, 14th and 21st days after surgery. After death, the abdominal wall was analyzed for the appearance of the scar, presence of hematoma, infection, wound dehiscence (incisional hernia). Soon after, was performed incision in the skin and subcutaneous tissue in craniocaudal direction, section of the entire ventral wall (8 cm long and 6 cm wide) encompassing the entire incision and analyzing presence or absence of hematoma, abscesses, fistulas, dehiscences and adhesions.

Macroscopic healing evaluation

Was conducted macroscopic evaluation of the wounds considering presence or absence of the following parameters: hematoma, infection, wound dehiscence, fistula and adhesions. Regarding the adhesions, was used the parameters of Goncalves *et al*.[3], which classifies them in relation to the intensity (Chart 1).

Chart 1 - Classification of abdominal adhesions[12].

Intensity	Parameters
0	Complete absence of adhesions
1	Adhesion of the greater omentum to the surgical wound
2	Adhesion of the greater omentum and small intestine to wound
3	Multiple intracavitary adhesions

Microscopic evaluation of the healing

Immunohistochemistry method

Tissue microarray technique was used (Tissue microarray-TMA) in the center of the scar in standard marked point to paraffin blocks (donor block), and using punch was withdrawal cylindrical tissue of 4 mm diameter throughout its depth, pressing on the marked place (Figure 2).

This cylinder was then inserted into a new block (receiving block), previously prepared with empty bores (Figure 3). The cylinders of several cases were successively added to the receiving block and the position of each sample was identified in spreadsheet with column and row references (X and Y axes). At the end, it was achieved a receiver block with 10 different samples. This block had sequential histological sections numbered slides treated with adhesive that allowed carrying out multiple reactions[4].

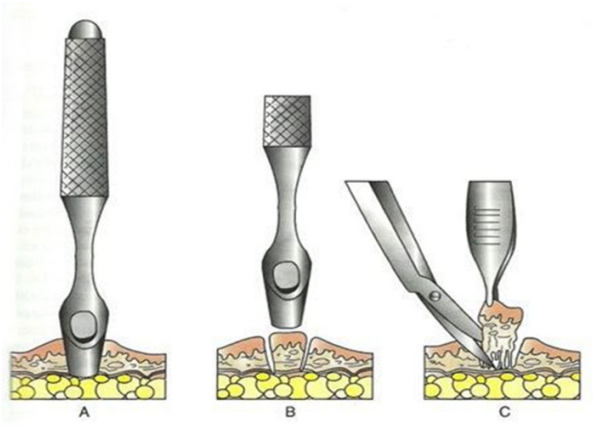

Figure 2 – Removal of cylindrical tissue after session with punch.

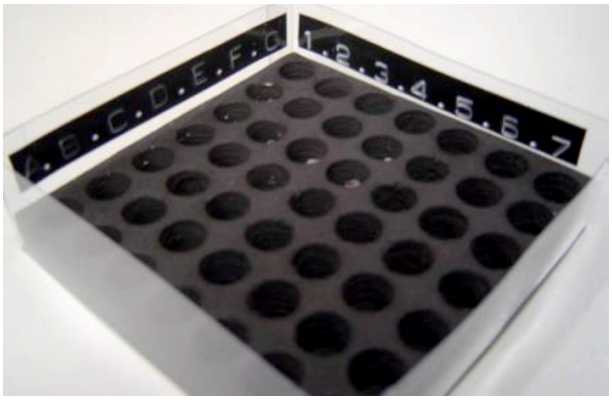

Figure 3 – Block receiver mounted with their identifications as map.

In this study, each block corresponding to a sub-group with 10 samples each were identified as: CBS 3, CBS 7, CBS 14 and CBS 21, or UHS 3, UHS 7, UHS 14 and UHS 21 according to programmed death after 3, 7, 14 and 21 days.

The staining was performed by standard immunohistochemical method used for matrix metalloproteinase 9 (MMP-9), conversion factor beta (TGF-β) and alpha smooth muscle actin (α-MLA). The pathologist was unaware of the animal study group.

The immunostained slides were subjected to reading through optical microscope connected to the camcorder Dinoeye and computer image analysis software Image Pro Plus™ (Maryland, USA). Four images in high-power field were captured (CGA=400x), with total area of 115226,1 μm^2 and resolution of 1024 x 768 pixels. The positive control reaction was scanned and an CGA image was chosen as a mask, containing the appropriate positive for the selected biomarker (Figure 4).

The area μm^2 generated by this method was then divided by the constant 115226.1 μm^2 which is the total area of the evaluated field, generating percentage of immunopositive area per CGA. Mean 4 CGA percentages was calculated in each case (Table 1).

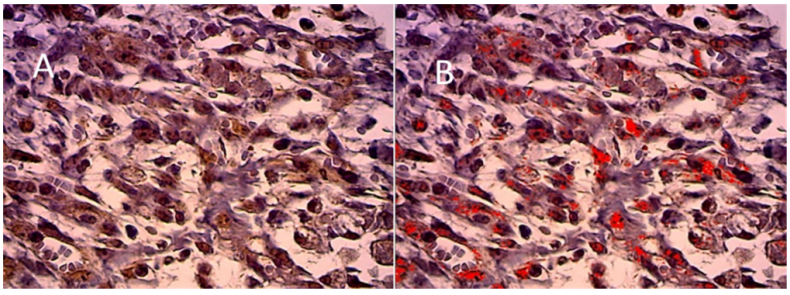

Figure 4 - Immunohistochemistry TGF-β CBS 3 subgroup: **A**) Submitted to immunohistochemical method; **B**) Immunopositive area in red.

Table 1 - Example of 4 immunopositive areas of each sample, with CBS 3 subgroup medium area of each animal.

	MMP 9						
	TMA	**Photo 1**	**Photo 2**	**Photo 3**	**Photo 4**	**Animal average**	**% Positive area**
CBS 3	1A	2398.8635	1634.8127	5156.5708	3829.6743	3254.980325	3.32%
	1B	7868.6494	6753.7031	4863.9614	4415.334	5975.411975	5.18%
	1C	8057.3438	7038.5112	2989.8218	5314.2085	5849.971325	5.07%
	1D	4967.1401	1939.3439	1648.2068	897.99158	2363.170595	2.05%
	2A	2074.0205	1570.3446	4280.2153	1444.4991	2342.269875	2.01%
	2B	6438.1328	8201.7354	4433.7324	7151.4043	6556.251225	5.68%
	2C	5418.123	6656.7065	5387.8022	6749.729	6053.090175	5.25%
	2D	9160.8096	3207.6594	7015.8442	6408.4014	6448.17865	5.59%
	3A	3446.9866	4144.0669	4818.0391	2552.969	3740.51540	3.24%
	3B	4081.9539	2637.6018	6509.9604	6032.9258	4815.610475	4.17%
	General average					4739.945002	4.11%

Picrosirus red method

Collagen analysis was made by Sirius Red technique and examining them under polarized light. The collagen type III fibers, thinner and dispersed under polarized light, are weakly birefringent and acquire shades ranging from yellow to green, while the type I, thicker and highly birefringent, shades ranging from orange to red.

The slides were examined without established identification, with the same equipment and facilities cited for immunostaining. Initially, the slide image was taken by computer, and with the help of the Image Pro-Plus was selected a photomicrograph that serialized, as mask, the measuring the interest areas. With the eyedropper tool in photomicrograph identified as a mask, the colors of interest were selected (shades ranging from yellow to green to collagen type III and ranging from orange to red to collagen type I) and the program automatically identified collagens I and III for its color under polarized light by highlighting them in photomicrograph. This mask containing the color selection was superimposed on all other photomicrographs, automatically identifying the areas of collagen I and III. Since the total area of examination

was constant, the program supplied the area and the percentage of it in the study object, namely type I and III collagen. Were measured three photomicrographs (x20 objective lens) per histologic section on each slide, yielding, so, the average of the reading section.

Statistical analysis

Quantitative variables were described by the mean and standard deviation statistics. For comparison of the types of scalpel in each time, was used Student t test for independent samples; for comparison two moments together, it was considered the Student t test for independent samples, considering the model analysis of variance as source of variation to estimate the variance within moments of evaluation. To maintain the overall level of significance was considered the Bonferroni procedure. To assess the normalcy of variables, within each type of scalpel and each time was used Jarque-Bera test. In case of rejection of normality hypothesis was investigated data transformation to meet that condition. To compare the times within each group, in relation to the classification hematoma, dehiscence and compliance, it was considered the chi-square test. To compare the groups at each time, with respect to adherence classification was used Fisher's exact test. For the analysis of variance on the type of collagen and its concentration was used the nonparametric Mann-Whitney. p values less than 0.05 were considered statistically significant. Data were analyzed with the computer program IBM SPSS v.20

■ Results

Macroscopic evaluation

No deaths were recorded. One animal CBS3 subgroup had hematoma without statistical significance (p=1). Two others UHS 3 subgroup had infection and dehiscence in suture line observed after their death, without statistical significance (p=1). In relation to adhesions, they were observed only in intensity 1 (Table 2). No fistulae were observed.

Statistical analysis on the CBS (p=1) and UHS (p=0.595) groups showed no significant difference between them.

Table 2 - Adhesions classified by grade and observation periods.

Adhesion	CBS 3	UHS 3	CBS 7	UHS 7	CBS14	UHS 14	CBS21	UHS 21
Grade 0	9	9	9	7	9	8	10	9
Grade 1	1	1	1	3	1	2	0	1
Grade2								
Grade 3								
Total	10	10	10	10	10	10	10	10

Microscopic analysis

Immunohistochemistry

Matrix metalloproteinase 9 (MMP-9) expression of MMP-9 in CBS follow-up, a progressive increase in its concentration, more evident from 7 to 14 days, with less progression to 21. In the series where were used UHS, 3rd day already had high levels, up to the 7th day and decreased to 14th and the level was maintained until day 21. Statistical analysis of MMP-9 expression in the comparison of the two scalpel types were significant at the 3rd, 7th and 21th days (Table 3 and Figure 5).

The expression of MMP-9 was observed in all cases (Figure 6) varying the tissue immunostaining between 2.65% and 11.50%.

Table 3 - MMP-9, TGF-β and α-AML (expressed as mean+SD) for CGA and statistical analysis in cold and harmonic scalpels.

Scalpel		Subgroups			
		3 days	7 days	14 days	21 days
Cold blade	MMP-9	4.739.95±1.676.9	6.633.82±1.867.31	10.560.1±1.653.4	11.533.89±1.161.7
	TGF-β	1.878.74±1.496.4	636.02±879.69	1.172.74 ±633.35	1.078.6 ± 556.78
	α-AML	1.836.81±724.47	2.011.21±516.91	1.879.22 ±721.32	2.022.17±1.007.34
Harmonic ultrassonic	MMP-9	9.185.9±1.502.61	12.036.44±1.216.7	9.104.05±2.201.6	9.171.86±1.785.63
	TGF-β	946.69 ± 638.38	744.66 ± 1.099.5	547.56 ±1.006.31	195.11±157.31
	α-AML	1.975.41 ±653.38	2.450.35 ± 773.47	2.832.99 ±893.45	2.741.54 ± 699.27
p value*	MMP-9	**< 0.001**	**< 0.001**	0.099	**0.001**
	TGF-β	0.081	0.826	**0.010**	**< 0.001**
	α-AML	0.665	0.140	**0.011**	0.091

*Student t test for independent samples;(p<0.05).

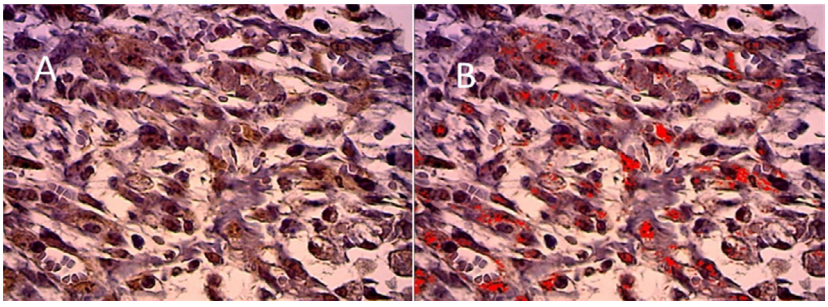

Figure 5 - Average of immune tissue expression (in square micrometers per CGA) of MMP-9 in relation to the CBS and UHS groups at 3rd, 7th,14th and 21st days.

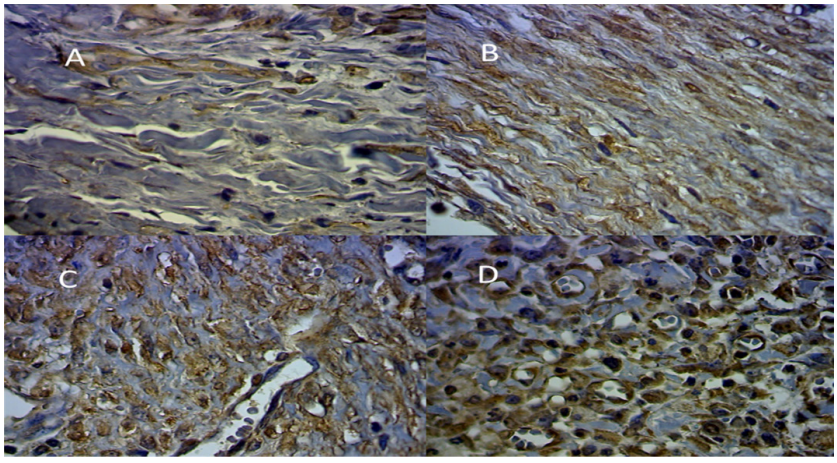

Figure 6- Average area of immunostaining histological samples with MMP-9 (x200): **A**) 2.65% CBS 3; **B**) 4.20% UHS14; **C**) 8.84% UHS 21; **D**) 11.50% UHS 7.

Transformation growth factor-beta (TGF-β)

TGF-β in incision with cold blade got highest level at the 3rd day, falling down on the 7th, going up on 14th, with a slight drop to 21st day. In the series where the incision with UHS was held, it was higher after 3 days, with progressive decline in 7, 14 and 21 days.

Statistical analysis comparing the two scalpel types with TGF-β expression found statistic significance at 14 and 21 days (Table 3 and Figure 7).

The expression of TNF-β was observed in all cases varying the tissue immunostaining between 0.03% and 2.92% (Figure 9).

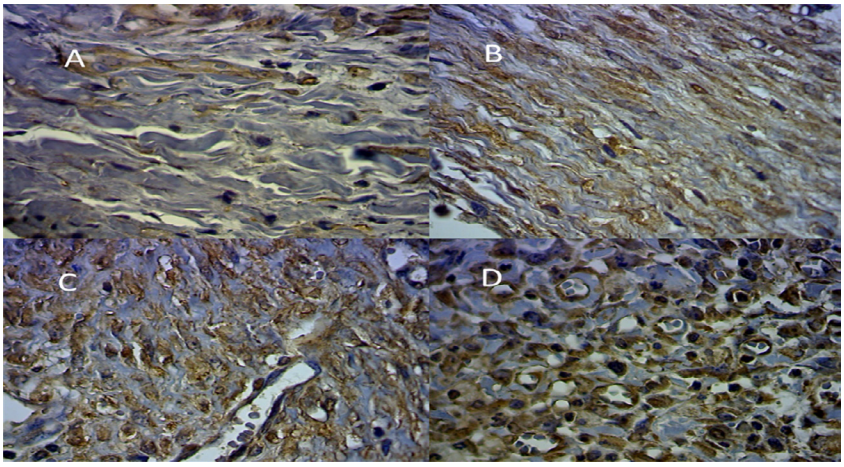

Figure 7 - Average tissue immunostaining (in square micrometers per CGA) of TGF-β in relation to the CBS and UHS groups, on the 3rd, 7th,14th and 21st days.

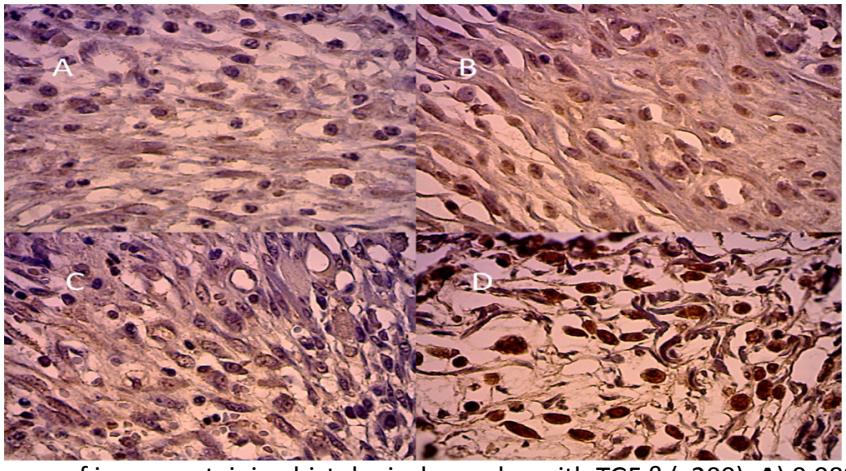

Figura 8 - Average area of immunostaining histological samples with TGF β (x200): **A**) 0.08% CBS 14; **B**) 0.64% UHS 7; **C**) 1.85% CBS 3; **D**) 2.92% CBS 3.

Alpha smooth muscle actin (α-AML)

α-AML expression showed levels slightly variable in the series where incision with CBS was carried out, with a slight increase from 3rd to 7th days, discreet decrease from 7th to 14th days and a slight increase up to 21st, but remained constant. When using UHS, there was increase from 3rd to 7th days, repeated from 7th to 14th day, and slight decrease after 21 days.

α-AML statistical analysis comparing the two scalpel types after 14 days is seen in Table 3 and Figure 9.

α-AML expression was observed in all cases (Figure 10) varying the tissue immunostaining between 0.88% and 3.23%.

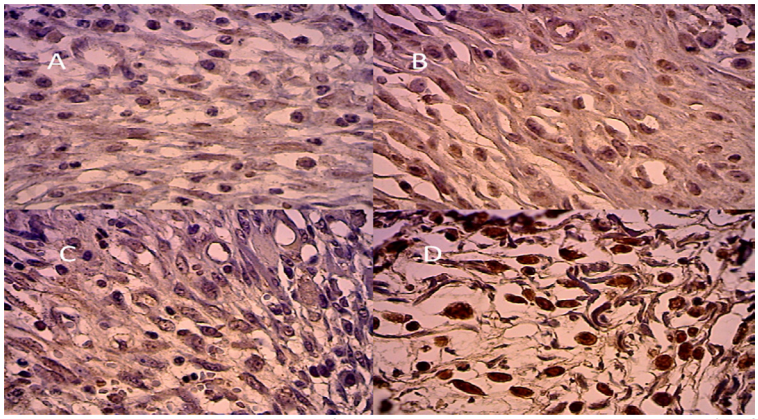

Figure 9 - α-AML average tissue immunostaining (in square micrometers per CGA) of in relation to CBS and UHS groups on the 3rd, 7th,14th and 21 days.

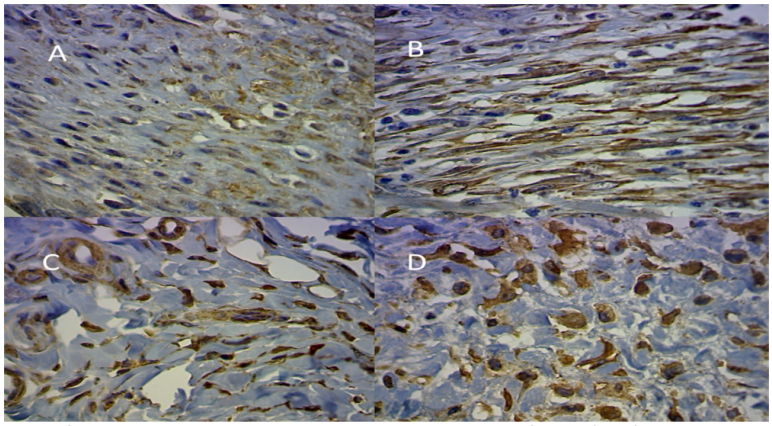

Figure 10 - Average area of immunostaining samples with α-AML (x200): **A**) 1.39% CBS 21; **B**) 1.45% CBS 7; **C**) 2.04% CBS 14; **D**) 3.23% UHS 3.

Picrosirius color red

Collagen dosage analysis showed that the mature type I, showed no statistic significant variation in the samples; the immature collagen type III, on the 3rd day was higher in UHS group; on the 7th day was higher in the CBS group; in 14th was equivalent in both groups; and on 21st day the deposit was higher in the group UHS. As a general score, there was higher deposition of collagen type III in UHS group than in the CBS with $p<0.001$ or 0.01%, with statistical significance (Figure 11).

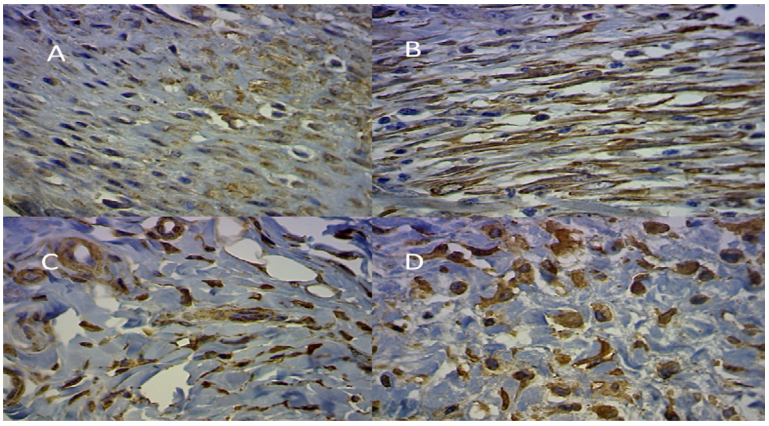

Figure 11 – Quantification of mature (type I) and immature (type III) collagen in both groups.

■ Discussion

Dieresis and synthesis are routine surgical procedures and the proper understanding of the healing process is of great importance, since disturbances in the normal healing course can pose severe clinical problem with morbidity and mortality[5-8].

The objective of increasing the safety and quality of a surgical procedure aimed in time reduction and complications. New tools were created, as electrocautery, laser radiation, cryosurgery and currently the harmonic scalpel.

The UHS is being used over 15 years in various surgical specialties with advantages. It was here applied because less energy is transduced to tissue compared to other devices, and do not pass electricity through the body[5,9]. Another benefit is reduction in analgesics administration in the postoperative period[10-15].

CBS is the most used in surgical incisions despite bleeding, need for sutures and other disadvantages, but still promotes less trauma to adjacent tissues[16]. This statement was also confirmed in this study. Other authors argue that conventional scalpel produces leakage of blood and lymph causing greater swelling and scarring[11,17-19].

A major problem attributed to UHS is the high cost of the equipment. Irfan *et al*.[20] believe that all the advantages mentioned about the harmonic scalpel justify the costs. The clash of these conflicting opinions draws attention to the important aspect of cost/ benefit ratio: the level of the surgeon's ability to manipulate the equipment can decrease hospital costs.

Tissue microarray technique (microarray-Tma tissue)

As the conventional method of analyzing samples one by one is relatively expensive, this technique has been used to reduce costs. It is based on the construction of a paraffin block with cylindrical pieces of tissue samples obtained from tens or hundreds of original paraffin blocks. Thus, the use of immunohistochemistry to know the expression of a particular marker, may occur in a TMA slide with hundreds of samples at the cost of a single reaction[21].

The TMA compared to conventional methods has: satisfactory assay areas at different shear levels; possibility of experiment repetition; large number of samples simultaneously analyzed; great savings in time; lower costs; and reactions standardization. The fidelity of the results regarding the use of conventional cuts was studied during method validation and is demonstrated in several papers in the literature.

Microscopy analysis

Costa Filho[22] in the same procedure revealed that microscopic examination with hematoxylin-eosin and Gomori trichrome revealed that the cut with UHS delays the healing, has more prolonged inflammatory process, greater tissue necrosis and fibrogenesis delay. Microscopic evaluation proved more intense inflammatory phase in the incisions made by the UHS compared with CBS; this difference was most evident in the 3rd and 7th days, and after 14 days the parameters were similar. Garcia *et al*.[23], performing similar experiment, but using electrocautery, found no significant changes in healing in both groups.

The expression of MMP-9 in incision with the CBS, showed a progressive increase in concentration, more evident from 7th to 14th days, and less in 21st. In the group where was used UHS, on the 3rd day had higher MMP-9 up until the 7th day, decreasing on 14th and maintained until 21th day, as expected. The presence of MMP-9 throughout the study

period, showed high inflammatory phase in remodeling process, more intense and prolonged in UHS[22].

Navarro *et al*.[24] reported that MMP-9 participates in many physiological processes in the human metabolism, also involved in pathological processes such as tissue destruction. Perches *et al*.[25] said that MMPs may be observed in any inflamed tissue and cell cultures varying according to the disease, since every tissue has extracellular matrix needing MMPs to tissue remodeling. Oliveira[26], in liver healing, demonstrated the importance of TGF-β in the fibrogenesis process in combination to levels in collagen type I.

Martinez[27] in dental pulp cells and human gingival obtained results showing that TGF-β 1 induces α-AML expression suggesting myofibroblastic stimulation, which was not confirmed in this study.

The α-AML expression levels showed be more constant in the series where incision with cold blade was performed. In UHS group wound remodeling was more intense.

The simplest method for quantification of collagen is the use of the dye Sirius Red F3BA[28]. Collagen types I, II, III discloses various colors and intensities of birefringence in the same histological section. This is due to the fact that different interstitial collagens show different patterns of physical aggregation. The type I has thick collagen fibers composed of fibrils densely packed and therefore exhibits birefringence active in yellow or reddish. The type III comprises fine reticular fibers, composed of fine fibrils, loosely arranged presenting weak greenish birefringence. The fiber with green color, compatible with type III collagen, is predominant in the initial period declining in later ones. Already, thick and red fibers, compatible with type I collagen, have opposite behavior. In this study there was predominance of collagen type III in UHS compared with CBS.

■ Conclusions

The use of cold blade performed better than the harmonic ultrasonic scalpel in healing process of abdominal aponeurosis in rats. The ultrasonic showed greater lesion area at the time of incision that promoted delay on regeneration and maturation of the scar.

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