Effects of Topical Tetracycline in Wound Healing on Experimental Diabetes in Rats

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Abstract: A common complication of diabetes is impaired wound healing. Systemic tetracycline improves healing in diabetics, but causes adverse side affects. There are no informations regarding topical tetracyclin use. OBJECTIVES: The objective of this study was to evaluate the effects of topical tetracycline on wound healing. METHODS: Diabetes was induced in Wistar rats by alloxan use. The control group comprised age-matched animals not submitted to alloxan injection. Diabetic state was confirmed by glycosuria and hyperglycemia. Under tribromoethanol anesthesia, four skin wounds (4mm diameter), were performed on shaved dorsal area (2 each side of median line). Topical tetracycline was applied daily only on both wounds on right side of median line. Animals were sacrificed on day 3 or 7 after surgery and tissue samples were prepared and observed under light microscopy. Histological, histometric and stereological methods were used for analysis. RESULTS: Topical tetracycline accelerated wound closure in diabetic compared to nondiabetic rats. No expressive effects were observed in controls. CONCLUSION: Topical tetracycline could be helpful in diabetics, in order to improve the wound healing process avoiding possible adverse effects from systemic medication. Furthermore, there was no indication that tetracycline improoves wound healing on controls.

Keywords: Wound healing, tetracycline, tetracyclin, diabetic rats, experimental diabetes.

INTRODUCTION

Diabetes Mellitus is a syndrome more than a disease, and affects about 150 million people worldwide [1-4]. Metabolic disorders and higher infection susceptibility are its clinical signs. Studies have shown delayed wound healing in diabetics due to cell proliferation deficiency, infection, decreased cell surviving, and reduced wound contraction [5-8]. The initial inflammatory response on healing areas is also delayed or deficient, mostly in uncontrolled diabetes [3, 9].

Diabetes can be experimentally produced in animals by Streptozocin or alloxan i.v. (intravascular) and injection of alloxan monohydrate produces insulin decreasing and hyperglycemia in a few days [10, 11]. Many human characteristics of diabetes was observed in alloxan diabetic rats [12].

Collagen is also an essential part of wound healing [13]. Diabetic rats show non-enzymatic collagen glucosylation twice that as much than normal rats, associated to deficiency in collagen synthesis during wound healing [2, 11, 14].

Tetracycline has been used as antimicrobial agent for a long time, but more than that, its ability to inhibit mammalian collagenase activity has been observed. Studies have shown decreased collagenase activity and increased repair by systemic tetracycline [15]. MOSKOW and TANNENBAUM (1991) also observed repair and regeneration improvement in a group of diabetics that was given systemic tetracycline [15].

So, in general, wound healing deficiency is a very common complication in diabetics and it has been observed that: 1) decreased collagen concentration [16]; 2) tetracycline may inhibit collagenase [14]; and 3) insulin may also have some beneficial effect on wound healing [2].

Our study uses topical tetracycline, in diabetic and control animals, and evaluate the wound healing process. This procedure is used to avoid the adverse effects of systemic tetracyclin, such as increased skin pigmentation, discoloration of nails and teeth, uremia, headache, enteritis and inappetence, among other effects [17].

The general objective of this work is to evaluate the effects of topical tetracycline on surgical wound healing process. The specific objectives are: 1) Evaluate the initial phases of wound healing in the skin of normal and diabetic animals; and 2) Compare wound healing areas in diabetics and their controls after local tetracycline use.

MATERIALS AND METODS

White Wistar rats (*Rattus norvegicus)* were separated into groups as follows: Group I - 10 control animals - Group Ia - 5 animals sacrificed 3 days after surgery; and Group Ib - 5 animals sacrificed 7 days after surgery. Group II - 10 diabetic animals without insulin therapy - Group IIa - 5 animals sacrificed 3 days after surgery; and Group IIb - 5 animals sacrificed 7 days after surgery. Group III - 10 diabetic animals on insulin therapy - Group IIa - 5 animals sacrificed 3 days after surgery; and Group IIb - 5 animals sacrificed 7 days after surgery.All animals were kept, during the whole experimental period in plastic boxes (40 x 32 x 17cm) under controlled light conditions (12 hours of light;

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12 hours of darkness) and temperature (21-25°C), at the Ribeirão Preto school of Dentistry (FORP-USP).

Diabetes Production

After 36 hours of food deprivation, the diabetic group were i.v. Alloxan injected (40 mg/kg of body weight). 30 minutes after injection, food and water were offered again. The injected animals were controlled 4 days after injection to verify the presence of glicosuria(test tape). All animals with comproved glucosuria were considered diabetic. Animals with similar age and weight were controls. The animals with diabetes were separated into 2 groups - without insulin (Group II) and on insulin therapy (Group III).

Insulin Therapy

Subcutaneous injection of insulin (0.1U long duration insulin Humulin-Lilly™), 100g body weight every two days. The Group III animals were kept under insulin therapy for 15 days before wound surgery.

Experimental Wound

All the animals suffered dorsal surgery as described: after anesthesia (2,2,2-Tribromoethanol Aldrich, 25mg/100g body weight - intraperitoneal injection) and after removed the hair from the dorsal skin, 4 skin wounds were prepared (4 mm diameter) approximately 2cm from each other (modification from MUSTOE *et al.*, 1987 [18], WHITBY & FERGUSON, 1991 [19], and MOST *et al.*, 1996 [20]). Povidone-iodine was used as topical anti-infective. Half of the wounds received topical application of tetracycline (EMS®) daily until sacrifice; iodine solution was applied immediately after surgery in the remainder. All the animals also received veterinary pentabiotic, (0.4ml intra-muscular -Fort Dodge®), immediately after surgery.

Sacrifice and Collection of Material

Animals were sacrificed by cardiac and respiratory breakdown during surgery, when anesthetized. Healing tissues were removed and fixed (formalin 10% - 24 hours). Glycemia were measured at the moment of sacrifice using a Glucometer Elite (Bayer®).

Histological Technique

The tissues were dehydrated and paraffin embedded. Slides were prepared (6µm thickness) and placed on glass lamina to be properly staining.

Morphometric and Statistical Analysis

HE were used for routine histological evaluation of tissues in healing area. A tricromic stain (for platelets and fibrin, according to BEHMER *et al.*, 1986) were used in the evaluation of collagen formation, using the color differences - blue for collagen and platelets; red for fibrin; yellow for erytrocytes. The epithelial neoformation is calculated (in percent of area) based on percent of points using an 100 points grade. The healing periods observed were 3 and 7 days after surgery, and 10 different healing areas are observed, as a modification of Chalkley's method [21]. An image analiser software (KS200) were used for collagen evaluation [22]. The collected data is presented as mean values and Mann-Whitney test was used to compare groups. P values <0.05 are considered statistically significant.

RESULTS

Evaluation of Diabetes

Animals with glycemia >200mg/dl were considered diabetic, according to NAGY *et al.*, 1961 [23]. Hiperglycemia were observed in all diabetic animals. Mean blood glucose levels (360ml/dl on day 3 and 426mg/dl on day 7 after surgery) compared with non-diabetic animals (151mg/dl on day 3 and 139mg/dl on day 7 after surgery) are shown in Fig. (**1**) and Table **1**.

Macroscopic Results

Initial macroscopic evaluation is shown in Figs. (**2**, **3**). Wound healing process in diabetic animals with (right side) and without (left side) topical tetracycline.

Macroscopic evaluation of healing area (days 3 and 7 after surgery) shows a very clear beneficial effect of topical tetracycline on the healing process in diabetics. A well marked difference in the epithelial neoformation (days 3 and 7 after surgery) can be observed following use of tetracycline.

Histopathological Results

Evaluation of Epithelial Neoformation

The epithelial neoformation was evaluated taking as parameters: a) diabetics, without tetracycline treatment (Fig. **4**); b) topical tetracycline in controls (Fig. **5**); and c) topical tetracycline in diabetics (Figs. **6**, **7**) and Table **2**.

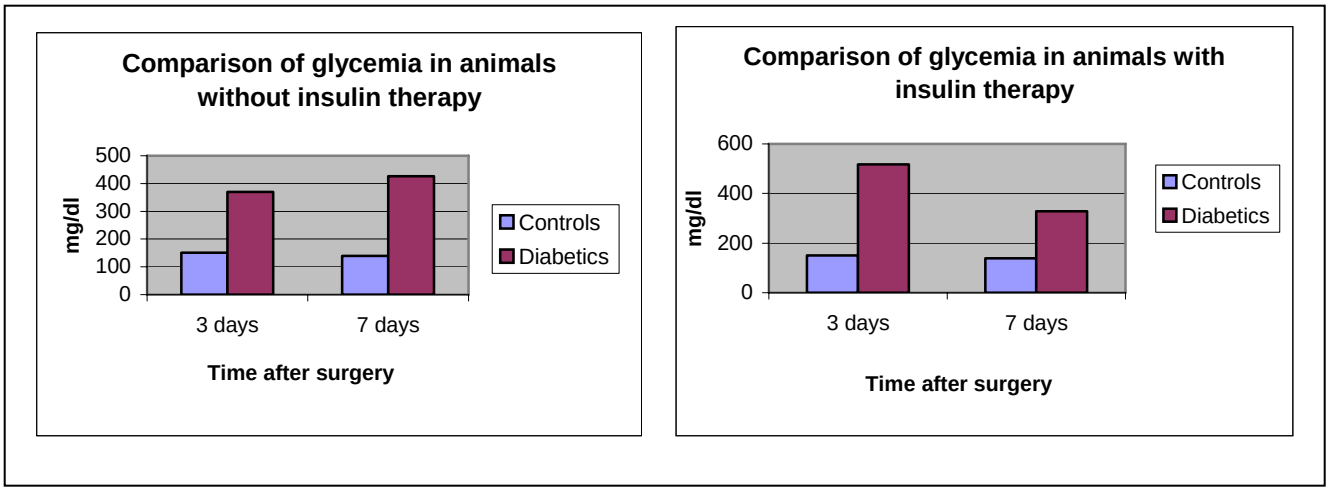

Fig. (1). Comparison of control and diabetic animals, on days 3 and 7 after surgery.

The results show that diabetes caused a delay in the epithelial neoformation in the healing area. Figs. (**5-7**) show the epithelial neoformation in control (Fig. **5**) and diabetic animals (Figs. **6**, **7**) after topical tetracycline.

Table 1. Blood Glucose Evaluation (mg/dl)

Animals without Insulintherapy		
Controls	**Day 3 After Surgery**	**Day 7 After Surgery**
105	406	464
177	445	251
134	518	441
155	214	509
186	268	465
151 mg/dl	370 mg/dl	426 mg/dl
TEST U - MANN WHITNEY	U Calc.= 0 * P[U]= 0.004	U calc.= 0 * P[U]= 0.004
Animals With Insulintherapy		
Controls	**Day 3 After Surgery**	**Day 7 After Surgery**
105	579	453
177	600	251
134	385	233
155	481	495
186	542	209
151 mg/dl	517 mg/dl	328 mg/dl
TEST U - MANN WHITNEY	**U Calc.= 0 * P[U]= 0.004**	**U calc.= 0 * P[U]= 0.004**

* Statistically significant.

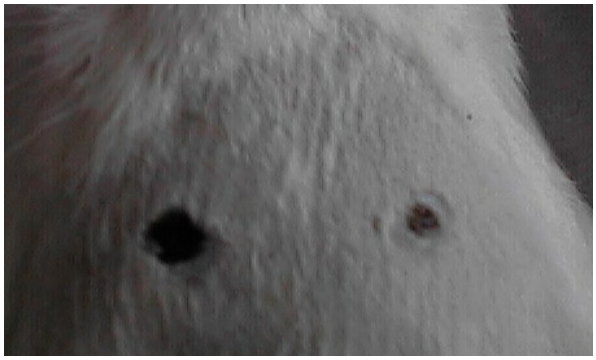

Fig. (2). Healing area in diabetic animal, day 3 after surgery, observe the improved cicatrization at right side wound.

Fig. (3). Healing area in diabetic animal, day 7 after surgery. Observe the difference on surgical areas at the right side.

The results showed that:

1. diabetic animals presented a delayed epithelial neoformation;
2. topical tetracycline use is associated with an improvement in the wound healing of diabetic animals; and
3. there was no evidence indicating an effect of topical tetracycline in controls.

Evaluation of Collagen Formation

The collagen formation in healing area was evaluated woth special stains (Trichromic for platelet and fibrin, according to described in Behmer *et al*., 1976). The medium values obtained by evaluating the quantity of collagen appeared in healing areas are expressed in next Figures and Tables.

Observe that diabetic animals present a remarkable deficiency of collagen at 7th day after surgery (Figs. **8**, **9**). The obtained results allow us to observe that:

1. the collagen deficiency at day 3 after surgery is statistically significant among animals with initial diabetes (without insulin therapy) as shown in Table **3**;
2. in animals on insulinthera the diffeerence of collagen content in areas of healing tend to be less expressive as the one observed in controls.

DISCUSSION AND CONCLUSION

Studies have shown that many factors are associated to the deficiency in healing process in diabetics, and the deficiency in collagen synthesis is one of them. Deficiency in collagen deposition in healing areas was observed in our study when comparing control and diabetic animals. Deficiency in epithelial neoformation was also observed.

Hyperglycemia, a clinical marker of diabetes, was evident in our experimental diabetic animals, with and without insulin therapy every two days, and one important point to be ovserved is the that despite insulin therapy, the blood glucose level in many diabetic patients do not match the controlled levels achieved in normal individuals (as observed in our animals). In our animals it was possible to observe healing and epithelial neoformation delay (Fig. **4**).

Tetracycline, by beiong liberated in oral fluids and by its anticollagenolytic effects, is important for periodontal tissue repair. Studies showed improvement in repairing conditions of periodontal tissues, but as inconvenient, some collateral effects, due to systemic administration such as some gastrointestinal discomfort, diarrhea, increased skin and tooth pigmentation and uremia [12, 15, 17, 24, 25].

It was possible to observe that tetracycline, and insulin, present benefic effects in collagen formation in healing area. Comparing the wound healing in diabetic and control animals, we observed that topical tetracycline resulted in faster healing in diabetic animals and this was most evident during the initial phases of the healing process - day 3 after surgery.

Table 2. Mean Value of the Percent of Points on Epithelium and Pseudomembrane ub Wounded Areas in Diaberic Animals and Their Controls were Evaluated Wounded Areas with (w/tcl) and wihtout (out/tcl) Tetracyclin Topical Treatment

Control		*DIAB.W/TCL*		*DIAB.OUT/TCL*	
Epithelium	**Pseudomembrane**	**Epithelium**	**Pseudomembrane**	**Epithelium**	**Pseudomembrane**
3th day after surgery					
14.17%	85.83%	6.58%	93.41%	10.96%	89.04%
14.85%	85.15%	7.13%	92.57%	15.97%	84.03%
16.39%	83.61%	6.38%	93.62%	8.99%	91.01%
16.73%	83.27%	8.28%	91.72%	8.74%	91.26%
11.48%	88.52%	7.93%	92.07%	14.46%	85.54%
14.72%	85.28%	7.26% ↓	92.74% ↑	11.82%	88.18%
MANN WHITNEY TEST					
		U calc.= 0* **P[U]= 0.004**		U calc.= 5 P[U]= 0.075	
7th day after surgery					
69.98%	30.02%	48.73%	51.26%	50.23%	49.76%
100%	0%	43.36%	56.63%	89.82%	10.18%
62.85%	37.50%	35.86%	64.14%	94.92%	5.08%
100%	0%	50.81%	49.18%	100%	0%
100%	0%	31.85%	68.15%	100%	0%
86.57%	13.43%	43.32%	57.88%	86.99%	13.01%
MANN WHITNEY TEST (compared with control)					
		U calc.= 0* **P[U]= 0.004** ↓	↑	U calc.= 11 P[U]= 0.421	
MANN WHITNEY TEST (comparing W/TCL and OUT/TCL)					
				U calc.= 1* P[U]= 0.008 ↑	↓

W/TCL = With Tetracyclin.
OUT/TCL = Without Tetracyclin.
* = Statistically significant.

Our results showed that topical tetracycline rendered the healing in diabetic animals near to that observed in controls. It was very interesting to observe that, in spite of tetracycline re-establishing the velocity of healing in diabetic animals we did not observe any significant effects of topical tetracycline in the controls. Probably tetracyclin effects are exactly the one necessary for diabetic healing deficiency.

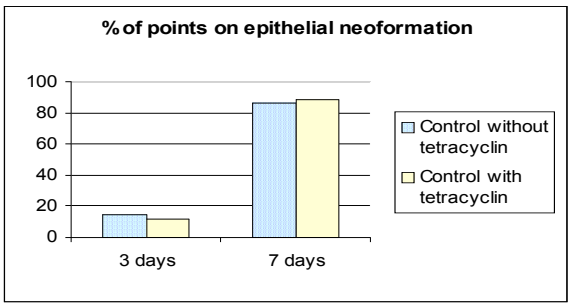

Fig. (4). Evaluation of relative percentage of points in neoformed epithelium surgical areas of control animals with and without topical application of tetracycline.

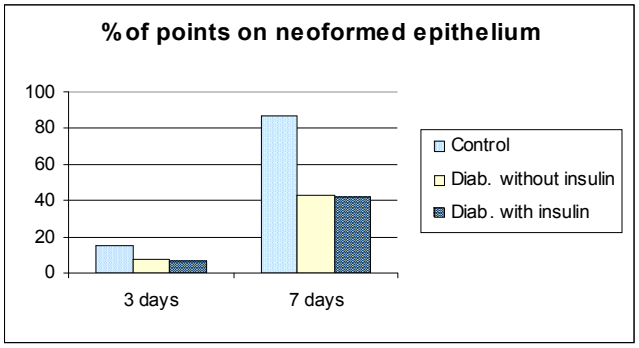

Fig. (5). Evaluation of relative percentage of points observed on neoformed epithelium on on surgery area of control and diabetic animals, with and without insulintherapy.

Another important aspect is to observe the role of insulin in wound healing. The insulin is very important in controlling the glycemia of mammalian, however has also an important role in the healing process (as observed in Tables **3** and **4**). The results of wound healing showed the important

role of insulin as a promoter of healing, even in when the animals are still hyperglycemic.

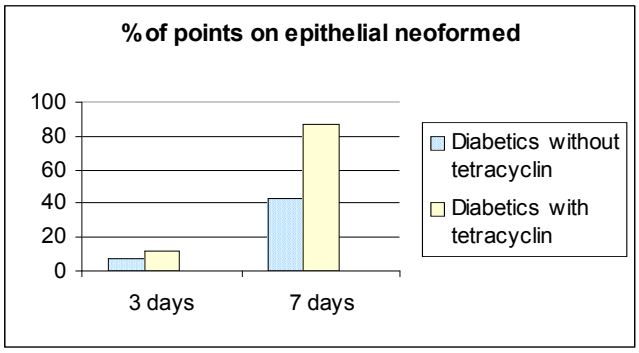

Fig. (6). Evaluation of points on epithelial neoformation on surgical areas of diabetic animals (without insulin therapy) with and without topical tetracycline.

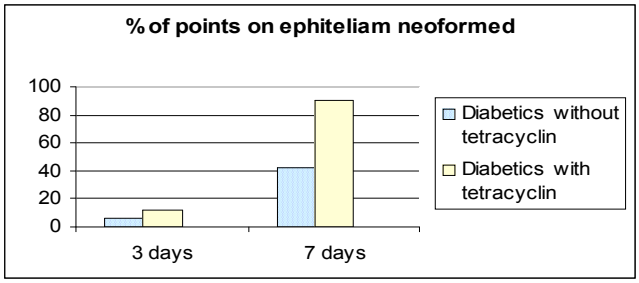

Fig. (7). Evaluation of points on epithelial neoformation on surgical areas of diabetic (on insulin therapy) with and without topical tetracycline.

Our results allow to conclude that:

1. diabetic animals, as soon short duration diabete as long duration diabetes, really show a deficiency of healing, compared to controls;

Table 3. Collagen Evaluation in Healing Areas with Tetracyclin (W/TCL) and without Tetracyclin (OUT/TCL) on Day 3 After Surgery

OUT/TCL			W/TCL		
Density	***Area***	***% of Area***	***Density***	***Area***	***% of Area***
Controls					
2510x10³	11.7x10³	6.47%	3724x10³	17.4x10³	9.60%
3394x10³	15.9x10³	8.75%	3227x10³	15.1x10³	8.32%
3092x10³	14.5x10³	7.97%	3027x10³	14.2x10³	7.80%
2965x10³	13.9x10³	7.65%	3981x10³	18.7x10³	10.26%
3619x10³	17.0x10³	9.34%	4433x10³	20.8x10³	11.43%
3116x10³	14.6x10³	8.03%	3678x10³	17.2x10³	9.48%
Diabetic without Insulintherapy					
1243x10³	5.8x10³	3.20%	2236x10³	10.5x10³	5.76%
2236x10³	10.5x10³	5.76%	2359x10³	11.0x10³	6.08%
2150x10³	10.1x10³	5.54%	2440x10³	11.4x10³	6.29%
2251x10³	10.6x10³	5.81%	2208x10³	10.3x10³	5.69%
1975x10³	9.2x10³	5.09%	2956x10³	13.8x10³	7.62%
1971x10³	9.2x10³	5.08%	2439x10³	11.4x10³	6.28
U TEST - MANN WHITEY					
U calc= 0* ↓ **P[U]= 0.004**			**U calc= 0 * ↓** **P[U]= 0.004**		
Diabetic with Insulintherapy					
2526x10³	11.8x10³	6.51%	4849x10³	22.7x10³	12.50%
2946x10³	13.8x10³	7.59%	6560x10³	30.8x10³	16.92%
3090x10³	14.5x10³	7.97%	6817x10³	32.0x10³	17.58%
2400x10³	11.3x10³	6.19%	6362x10³	29.8x10³	16.41%
2956x10³	13.8x10³	7.62%	5323x10³	25.0x10³	13.73%
2783x10³	13.0x10³	7.77%	5982x10³	28.0x10³	15.43
U TEST- MANN WHITNEY (Compared to controls)					
U calc= 5 ns P[U]= 0.075			**U calc= 0 * ↑** **P[U]= 0.004**		
U TEST- MANN WHITNEY (Compared to diabetic without insulintherapy)					
U calc= 0* ↑ **P[U]= 0.004**			**U calc= 0* ↑** **P[U]= 0.004**		

* = Satistically sighificant.

Table 4. Collagen Evaluation in Healing Areas. Without Tetracyclin (OUT/TCL) and with Tetracyclin (W/TCL) on Day 7 After Surgery

OUT/TCL			W/TCL		
Density	***Area***	***% of Area***	***Density***	***Area***	***% of Area***
Controls					
9557x10³	51.20x10³	28.12%	11720x10³	63.5x10³	34.88%
12680x10³	68.80x10³	37.7%	13195x10³	98.7x10³	54.22%
23609x10³	103.3x10³	56.73%	12050x10³	42.6x10³	23.73%
9011x10³	47.00x10³	25.82%	23373x10³	106.1x10³	58.23%
26209x10³	111.4x10³	61.20%	26279x10³	120.7x10³	66.27%
16213x10³	76.34x10³	41.91%	17323x10³	86.32x10³	47.46%
Diabetic without Insulintherapy					
9737x10³	62.2x10³	34.20%	9780x10³	66.2x10³	36.30%
6218x10³	40.4x10³	22.23%	7749x10³	50.8x10³	27.90%
6862x10³	38.9x10³	21.38%	7929x10³	57.2x10³	31.44%
10645x10³	68.1x10³	37.44%	11855x10³	46.8x10³	25.74%
11312x10³	74.4x10³	40.89%	9738x10³	62.2x10³	34.21%
8954x10³	56.8x10³	31.22%	9410x10³	56.64x10³	31.11%
U TEST - MANN WHITEY					
U calc= 6 ns P[U]= 0.111			**U calc= 1 * ↓** **P[U]= 0.008**		
Diabetic with Insulintherapy					
12094x10³	65.0x10³	35.71%	12898x10³	87.9x10³	48.31%
10742x10³	46.0x10³	26.24%	10703x10³	58.1x10³	31.90%
11011x10³	69.3x10³	38.08%	12552x10³	75.9x10³	41.72%
11430x10³	69.3x10³	38.08%	11248x10³	70.5x10³	38.75%
10906x10³	46.1x10³	25.90%	13196x10³	98.7x10³	54.22%
11236x10³	59.14x10³	32.80%	12119x10³	78.22x10³	42.98%
U TEST - MANN WHITNEY (compared to controls)					
U calc= 10 ns P[U]= 0.345			U calc= 7 ns P[U]= 0.155		
U TEST - MANN WHITNEY (compared to diabetic without insulintherapy)					
U calc= 3* ↑ **P[U]= 0.028**			**U calc= 2 * ↑** **P[U]= 0.016**		

* = Statistically significant.

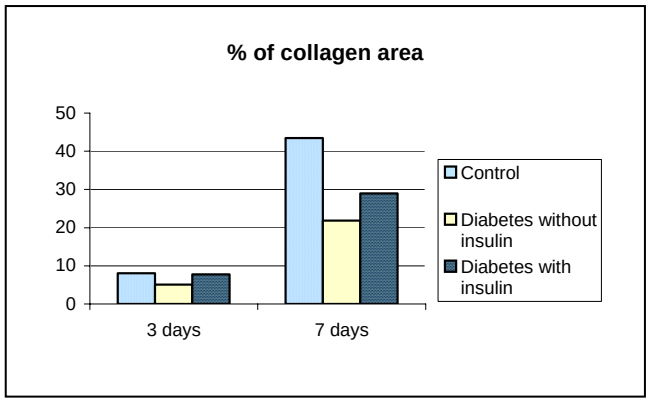

Fig. (8). Graphic representation of collagen presence in healing area of control animals, initial diabetes and long duration diabetes.

2. the use of topical tetracycline could be a helper way to healing process in diabetics without inconvenient of adverse side effects when used in a systemic way; and

3. although the benefic effect of tetracycline on the healing process of diabetic, it wasn't observed benefits that could justify the use of tetracyclin in normal rats, or be it, those that didn't present deficiency of healing.

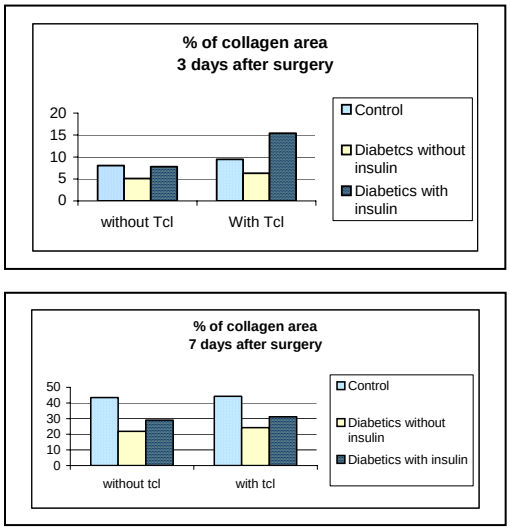

Fig. (9). Comparison of the effects of topical tetracycline (Tcl) through the percentage of collagen in healing area in control animals, diabetics without insulin therapy and diabetics with insulin therapy.

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