

# Investigation of the wound healing effects of chitosan on FGFR3 and VEGF immunolocalization in experimentally diabetic rats

Z. Deniz Sahn Inan<sup>1,\*</sup>, Serpil Unver Saraydın<sup>1</sup>

<sup>1</sup> Department of Histology and Embryology, Faculty of Medicine, Cumhuriyet University, Sivas, Turkey

## Email address:

zeynepdnz@hotmail.com (Z. D. S. Inan)

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**Abstract:** Chitosan is a naturally occurring substance that stimulates correct deposition, assembly and orientation of collagen fibres in extracellular matrix components in wounds and promotes migration of inflammatory cells. Fibroblast growth factor (FGF) is one of the most important growth factors playing crucial roles in angiogenesis and wound healing. Biologically, it acts via binding to the cellular surface receptors. FGFR3 is one of the most important receptors. Therefore the aim of the present study was to investigate, histologically and histochemically, the effect of chitosan on wound healing in experimentally diabetic rats divided into four groups. When compared to the diabetic and the control groups, chitosan group had more inflammatory cells, endothelial cells, newly formed blood vessels and reticular – collagen fibres in the wound healing area from the third day of operation. Moreover, in Chitosan Group, stronger VEGF and FGFR3 immunolocalizations were evident and all steps of wound healing process were more regular. FGFR3 antibody used in this study had been tested only on diabetic wound healing. In conclusion, we have concluded that application of chitosan was essential to accelerate wound healing process in diabetic patients.

**Keywords:** Diabetes, Wound Healing, Chitosan, VEGF, FGFR3

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## 1. Introduction

Diabetes mellitus is a chronic hyperglycaemic disorder (1). The prevalence of diabetes has increased tremendously and diabetic complications have become a serious health concern worldwide. Impaired-wound healing is one of these complications (2, 3, 4). Lack of cellular and molecular signals required for normal wound-repair process such as angiogenesis, granulation tissue formation, epithelization, and remodelling may be the major factors in poor wound healing in diabetes mellitus (3-7). Animal models are useful in studying early changes in diabetes. It has been observed that streptozotocin or alloxan might destroy  $\beta$  cells (8).

The healing of a wound requires a well-orchestrated integration of complex biological and molecular events of cell migration, proliferation, and extracellular matrix (ECM) deposition (9). Each phase is modulated by a vast array of cytokines and growth factors, which form an elaborate communication network co-ordinating the healing process. New understandings in the complexities of wound healing,

and particularly the role of growth factors, are enabling clinicians to manage superficial wounds such as skin flaps and even the most difficult-to-heal wounds more effectively, but are deficient in diabetic wounds (3, 5-7). Multiple factors can lead to impaired wound healing in diabetic animals and patients. One of the important factors is that diabetic animals and patients do not produce enough growth factors (vascular endothelial growth factor; VEGF, fibroblast growth factor; FGF) and growth factor receptors (fibroblast growth factor receptor 3; FGFR3) (10-12).

Biomaterials are mostly polymers and are used in artificial organ production in contemporary medicine. The other areas for hydrogel usage are artificial tendon production, as bioadhesives in wound repair, as artificial kidney membranes, as artificial skin and biomaterial in plastic surgery. There are many monomers used in biomaterial production. In our previous studies, we investigated whether some monomers used in biomaterial production such as acrylamide, metacrylamide, N-isopropylacrylamide, acrylic acid, 2-hydroxyethyl methacrylate, 1-vinyl-2-pyrrolidone and

ethylene glycol had cytotoxic effects and induced apoptosis or not spinal cord. Immunolocalization of glial fibrillary acidic protein (GFAP) was also determined, and it was evaluated by using semi-quantitative morphometrical techniques. The cytotoxicity of monomers on cultured fibroblastic cell lines was also examined *in vitro* (13-16). Chitosan, which is a poly D-glucosamine, is a deacetylated derivative of chitin (17). Chitosan and its oligomers are well known for their interesting biological properties which have led to various applications (17-20). Chitosan stimulate correct deposition, assembly and orientation of collagen fibers in extracellular matrix components in wounds. Moreover, histological findings indicate that chitosan membrane stimulates migration of inflammatory cells such as polymorphonuclear leucocytes, macrophages and fibroblasts. Thus it promotes granulation and cellular organization (17, 21-23).

Vascular endothelial growth factor (VEGF) is a multi-functional growth factor produced by endothelial cells, fibroblasts, smooth muscle cells, trombocytes, neutrophils and macrophages. Its function is to elicit proliferation, migration and differentiation of the said cells (24-26). Studies on wound healing process in experimentally diabetic animals have shown that several growth factors, including VEGF, are dramatically decreased (27-29). It has been suggested that administration of VEGF-A *via* protein or gene transfer methods increases granulation tissue formation, angiogenesis and matrix deposition in experimentally diabetic mice (27).

Fibroblast growth factor (FGF) demonstrates strong mitogenic properties in fibroblasts, osteoblasts, smooth muscle cells, endothelial cells, chondrocytes and melanocytes (27-30). It is one of the most important growth factors playing crucial roles in embryonic development, angiogenesis and wound healing. Biologically, it acts via binding to the cellular surface receptors belonging to the tyrosine kinase receptor family. Four of such surface receptors (FGFR 1-4) have been identified up until now (31-33). It has been clearly demonstrated in previous studies that FGFR3 is localized in the suprabasal region of epidermis, in the inner epidermal root sheath of hair follicles, in the smooth muscle cells of blood vessels of the normal skin tissue whereas FGFR3 is immunolocalized in the suprabasal and basal layers of epidermis, around the blood vessels of the granulation tissue, in fibroblasts and inflammatory cells of the skin during wound healing process (33). However, the number of histopathological studies on VEGF immunolocalization is still limited while there exist no studies on FGFR-3 in wound healing process in diabetic subjects.

Therefore the aim of the present study was to evaluate immunolocalizations of vascular endothelial growth factor (VEGF) and fibroblast growth factor receptor-3 (FGFR-3) in chitosan treated normal and experimentally diabetic rat skin during wound healing process and to study contribution of chitosan on wound healing process in diabetic conditions.

## 2. Materials and Methods

### 2.1. Animals

In the present study, 51 Wistar albino rats reared in the Experimental Animal Laboratory, Cumhuriyet University, Sivas, Turkey and weighing 250-300 g were used. Animals were divided into four groups. While the control group had 6 animals, other groups had 15 animals all of which were fed with food and tap water *ad libitum*. All animals were treated under the guidance of Local Ethics Committee of Experimental Animals, Cumhuriyet University Sivas, Turkey. All the treatment procedures employed in the present study were approved by the same committee too.

### 2.2. Experimental Groups:

An incision was made on the back of each animal in all groups.

**Diabetic + Chitosan Group (DC) (n=15):** Chitosan (Sigma, USA) was applied on the incision area every day.

**Diabetic + Acetic Acid Group (DA) n=15):** Only acetic acid was applied on the incision area every day.

**Diabetic + Control Group (DO) (n=15):** Citrate buffer was applied on the incision area every day.

**Control Group (C) (n=6):** Diabetes was not induced and betadin was applied on the incision area every day.

### 2.3. Experimental Diabetes Induction Procedure

Rats were not fed for overnight and their blood glucose levels were measured in the following morning (Lever Check TD-4222). Those having a blood glucose level between 80-100 mg/dl were regarded as non-diabetic. In order to induce diabetes mellitus, rats of either sex were given a single dose of 60 mg/kg streptozotocin (STZ) in 0.1 M citrate buffer, pH: 4.5, (Sigma Chemical Co., St Louis Missouri, USA) intraperitoneally (34, 35). Forty eight hours after STZ injection, blood glucose levels were measured in samples obtained from the tail veins. Animals having a blood glucose levels above 250 mg/dl were regarded as diabetic. Rats had free access to food and water after STZ administration.

### 2.4. Preparation of Chitosan

In order to prepare 0.8 % Chitosan solution, 1 g Chitosan was dissolved in 100 cc 1% acetic acid and mixed for a few hours (36). The solution was calibrated to have a pH of 5.5. Solution was kept under UV light overnight for sterilization and to avoid bubbles. At the end of those processes, a sterile gel Chitosan was obtained to use in the experiments.

### 2.5. Surgical Procedures

All the animals received intramuscular injections of 90 mg/kg ketamine hydrochloride and 3 mg/kg xylazine hydrochloride into the left front leg muscles. The rats were anesthetized but allowed to breath spontaneously during the surgical procedure. In order to prevent postoperative

pain, 4 mg/kg rimadyl was injected subcutaneously for 3 days. Using a surgical blade No. 10, two cm long full thickness incisions (37) were made at the back of rats. The wounds were not closed throughout the experiments. Chitosan, acetic acid and betadine were applied to the incision area every day and on the 3rd, 7th and 14th days after operation. 5 animals from each group were sacrificed by injecting a high-dose (200 mg/kg) sodium pentobarbital intraperitoneally. Tissue samples were obtained from the incision area to conduct light microscope and immunohistochemical investigations.

### 2.6. Light Microscopy

Skin samples obtained from the wound area were fixed in 10% buffered neutral formaline for 48 hours and blocked in paraffin after routine histological dehydration procedures. For immunohistochemical investigations, two - three  $\mu\text{m}$  thick tissue sections were taken by a Leica RM 2125 RT microtome. Sections were stained immunohistochemically for VEGF and FGFR3 and convenient fields of views were photographed using Olympus BX51 (Tokyo, Japan) photomicroscope.

### 2.7. Immunohistochemistry

For immunohistochemical staining, the deparaffinized and rehydrated tissue sections were inactivated using endogenous peroxidase by incubation in 3%  $\text{H}_2\text{O}_2$  for 10 minutes. To recover antigen, these sections were put into EDTA solution (pH 8.5) and heated in microwave oven twice. Slides were then washed in PBS (pH 7.2-7.6) twice. Non-specific binding sites were blocked in Ultra V Block (Lab vision, USA) solution for 20 minutes. After the redundant liquid was discarded, sections were incubated in primary antibodies (VEGF Ab-1 and FGFR3, Lab Vision USA) at room temperature for 1 hour and washed in PBS. Slides were then incubated in biotinylated secondary antibody (Lab Vision, USA) for 20 minutes and washed in PBS which was followed by incubation in streptavidin-HRP (Lab Vision, USA) for 20 minutes and by washing in PBS. Antibody binding sites were visualized by incubation with an AEC chromogen (Lab Vision, USA) solution. Slides were counterstained for 1 minute in hematoxylin and then dehydrated in sequential ethanol series for sealing and microscopic observations.

## 3. Results

Histological features stained immunohistochemically, were evaluated semi-quantitatively and the results are shown in Tables 1 and 2. In Chitosan Group, immunolocalization of vascular endothelial growth factor (VEGF) (Table 1, Fig. 2,4,6) revealed a strong expression in the epithelium close to the wound region, in the healing area, in the sebaceous glands and around the blood vessels on the 3rd day; however, its immunolocalization decreased gradually on the 7th day and it was rather weak on the 14th day when

compared to the other groups. Immunohistochemical staining by FGFR3 (Table 2, Fig. 8,10,12) in Chitosan Group revealed a very strong expression on the 3rd day, a rather weak expression on the 7th day and a strong expression on the 14th day in the epithelium close to the wound region and around the hair follicles, sebaceous glands and blood vessels when compared to the other groups.

## 4. Discussion

Diabetes is a chronic metabolic disease affecting the majority of the world population. While the number of people having diabetes for several reasons has been increasing, complications observed in diabetes have been increasing too (1-4). One of the most important complications seen in diabetic patients is the impaired wound healing (38, 39).

At the present, several subsidiary biomaterials have been used for a better wound healing therapy and chitosan is one of them. Chitosan is a natural polymer made by chitin. Chitin is the most abundant polymer after cellulose and it is present in the cell wall of sea shells and mushrooms (17, 18, 40). Chitosan, a biodegradable and biocompatible polymer, is an important and indispensable biomaterial in pharmacology and in medicine since it is nontoxic and causes no allergy or irritation. At the same time, through accelerating the wound and bone healing, Chitosan is a haemostatic, antibacterial and antifungal immune system stimulant (19-21, 41).

Wound healing process occurs around three main events; haemostasis and inflammation, new tissue formation and remodelling (30, 42, 43). These events occur not in a particular order but in a complicated manner (30, 42-44). During wound healing process, angiogenic growth factors such as vascular endothelial growth factor (VEGF), placental growth factor (PGF), acidic and basic fibroblast growth factor 1 and 2 (FGF1 and 2), fibroblast growth factor 3 and 4 (FGF3 and 4), FGF receptors, transforming growth factor  $\alpha$ - $\beta$  (TGF $\alpha$ - $\beta$ ), epidermal growth factor (EGF), hepatocyte growth factor (HGF), angiogenin, platelet derived growth factor (PDGF), granulocyte colony stimulating factor (GCSF), interleukin 8 (IL 8), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and proliferin are important (45).

Vascular endothelial growth factor (VEGF) is a member of multifunctional growth factor family and has specific effects especially on endothelial cells. It binds to its receptors with a complex system thus regulates blood vessel formation (24-26, 45). During wound healing process, vascular endothelial growth factor (VEGF) is produced by several cells such as endothelial cells, fibroblasts, smooth muscle cells, trombocytes, neutrophils and macrophages. Its functions are proliferation, migration and differentiation of endothelial cells (23-25). In a previous study, VEGF immunoreactivity and its relationship with angiogenesis has been described in glioma cells (45-47). Recent studies have shown that VEGF may have effects on collagen deposition and epithelial formation (48). In the first phase of wound healing, VEGF stimulates the coagulant factors in

the endothelial cells. Therefore, the trombocyte accumulation and adhesion occur (46-48). For blood vessel formation, immunolocalization of vascular endothelial growth factor is increased in inflammatory cells during inflammation period of the wound healing while it is also observed in fibroblasts, endothelial cells and macrophages during the following phases of the wound healing process (46-48). Blood vessel formation (angiogenesis) occurs on the 3rd day of the wound healing process. In order to attract metabolites and oxygen to the healing region, the number of capillaries increases in the granulation tissue (48, 49). Previous studies have clearly shown that keratinocytes around the wound, fibroblasts and macrophages in the wound region, macrophages in the wound region and growing blood vessels start to produce VEGF whose secretion reaches its maximum level on the third and the seventh days (49, 50). In the present study, VEGF immunolocalization was strong on the 3rd day while it gradually decreased on the 7th and 14th days in the control group. Previous studies have demonstrated that VEGF production is rather high between the 3rd and 7th days in wound healing area (48-50). Formation of granulation tissue, which contains fibroblasts, macrophages and endothelial cells all of which are essential for VEGF secretion, occurs on the third and seventh days of the wound healing process (50). Findings of the present study revealed that VEGF expression was low on the 3rd day, moderate on the 7th day and strong on the 13th day in the diabetes+control and in the diabetes+acetic acid groups. Like all other growth factors, VEGF production is suppressed in diabetic conditions (27, 28). VEGF secretion from keratinocytes and fibroblasts has been shown to be decreased in diabetics (27). Altavilla *et al.* have suggested that oxydative stress causes impairments in VEGF secretion and regulation in diabetic patients (50). In a histopathological study on diabetic mice, Komesu *et al.* showed that the onset of inflammation phase was delayed in diabetic animals and chronic inflammation findings lasted longer in samples collected on the 1st, 3rd and 7th days (1). In our study, VEGF immunolocalization was low in the diabetes+control and in diabetic+acetic acid groups on the 3rd and the 7th days. However, VEGF immunolocalization was very strong on the 3rd day, strong on the 7th day and moderate on the 14th day in diabetic+chitosan group. Histopathological findings of previous studies have revealed that leukocytes and macrophages reach wound area faster in chitosan treated animals when compared to the controls (21-23, 51). Therefore, it can be suggested that chitosan might stimulate inflammatory cells and their growth factors in the wound area during the early phases of the wound healing process. In fact, chitosan attracts inflammatory cells and VEGF to the wound healing area during the early stages of the wound healing process (21, 51). Ueno *et al.* have suggested that chitosan accelerates granulation tissue formation (21). At the same time, chitosan activates fibroblasts in the granulation tissue, caused them to proliferate and accelerates extracellular matrix production (23). Therefore, the present study suggests that

chitosan might accelerate the wound healing by increasing VEGF secretion despite the negative effects of diabetes.

It has been suggested that acidic fibroblast growth factor (aFGF or FGF1) and basic fibroblast growth factor (bFGF or FGF2) are potential angiogenic factors in diabetics just like VEGF (22-24). Fibroblast growth factor receptors also play key roles in the wound healing process. Four types of FGF receptors have been found in epidermal layers, muscles, blood vessels, fibroblasts, hair follicles, granulation and inflammation tissues of normal and wounded tissues during the wound healing process (31-33, 52). Takenaka *et al.* have evaluated normal and burned human tissue samples and suggested that FGFR1 and FGFR3 have strong expressions in the first stage of the healing process while these receptors have weaker expressions following the granulation tissue formation (33). On the other hand, they have also suggested that when FGFR1 and FGFR3 have weak immunolocalizations, FGFR2 and FGFR4 have strong expressions along with moderate FGFR1 and FGFR3 expressions towards the final stage of the healing process (33). However, there are no data on the FGFR3 expression in diabetic wound healing process. In the present study, the FGFR3 immunolocalization in the basal layer of epidermis, in the epidermal root sheath of hair follicles, around the blood vessels, in sebaceous glands and in the healing region were very strong on the 3rd day, moderate on the 7th day and strong on the 14th day in Chitosan Group when compared to the control and the other groups. Chitosan is very affective on the secretion of growth factors from the cells in all stages of wound healing process (17-23, 51). A similar finding, namely strong expression of FGFR3 in the wound healing area, was found in our study too. The present study showed that the healing process of full thickness incisions made in diabetic rats was affected positively when chitosan was applied. Besides having antibacterial and antifungal properties, Chitosan is a cheap and easily obtained natural polymer. Wound healing is a complex process including number of cells and growth factors. Due to being very a expensive and time consuming process, treatment of wounds in diabetic patients is very difficult. By demonstrating VEGF and FGFR3 expressions in the wound healing regions, the present study has clearly shown that application of chitosan, with its positive effects on wound healing process, helps to overcome the above mentioned difficulties.

**Table 1.** Semi-quantitative comparison of VEGF expressions on the 3<sup>rd</sup>, 7<sup>th</sup> and 14 day in the Control (C), Diabetic Acetic Acid+Diabetic Control (DA+DO) and ChitosaGroups (DC).

VEGF	C	DA+DO	DC
3 <sup>rd</sup> Day	++++	++	++++
5 <sup>th</sup> Day	+++	+++	++++
14 <sup>th</sup> Day	++	++++	++

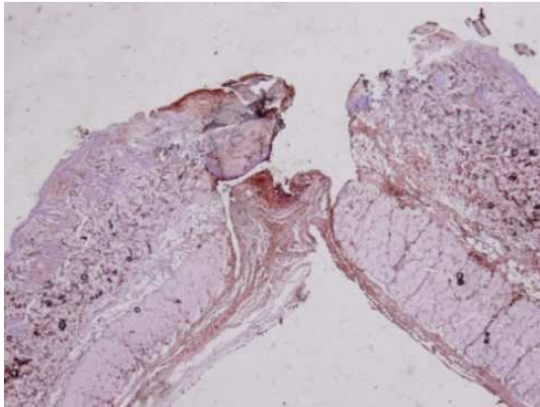
+++++very strong, ++++ strong, +++ moderate, ++ low, +very low



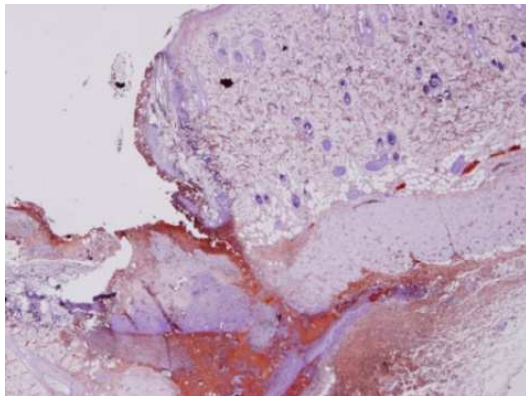
**Table 2.** Semi-quantitative comparison of FGFR3 expressions on the 3<sup>rd</sup>, 7<sup>th</sup> and 14<sup>th</sup> days in the Control (C), Diabetic Acetic Acid+Diabetic Control (DA+DO) and Chitosan Groups (DC).

FGFR3	C	DA+DO	DC
3rd Day	++++	+	+++++
5th Day	+++	+++	++
14th Day	++++	+++	++++

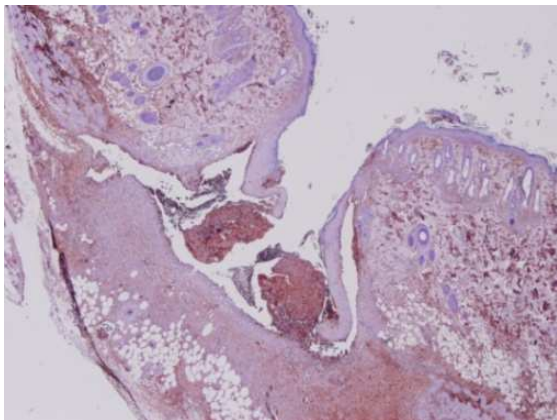
+++++very strong, ++++ strong, +++ moderate, ++ low, +very low



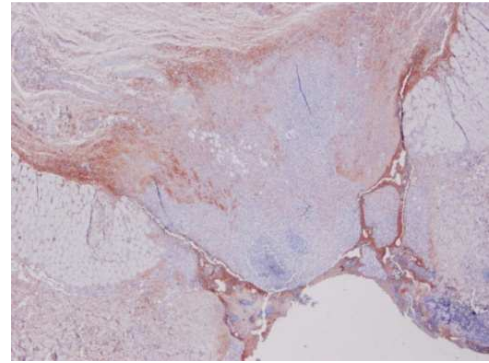
**Figure 1.** Control Group, on days 3 (X4), VEGF immunolocalization in the wound healing region.



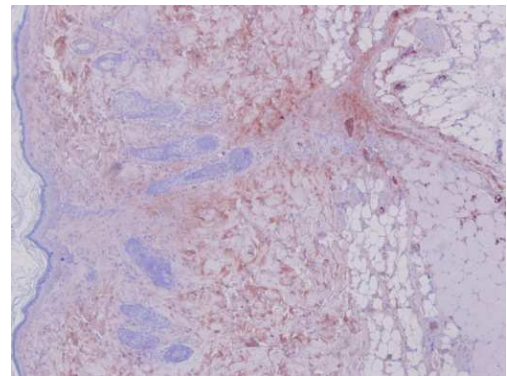
**Figure 2.** The VEGF immunolocalization on days 3 (X4), in the incision made and chitosan application group (DC).



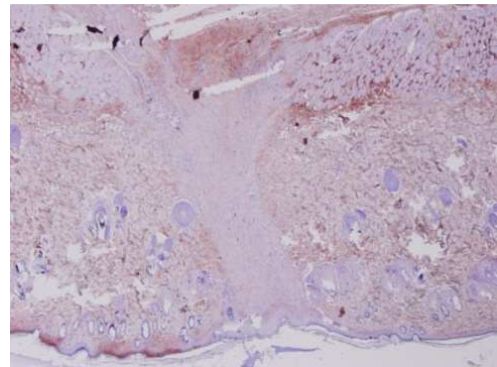
**Figure 3.** Control Group, on days 7 (X4), VEGF immunolocalization in the wound healing region.



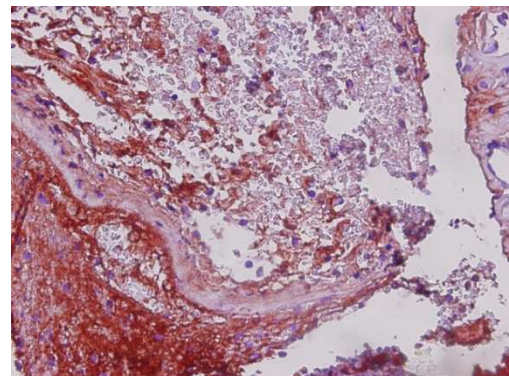
**Figure 4.** The VEGF immunolocalization on days 7 (X4), in the incision made and chitosan application group (DC).



**Figure 5.** Control Group, on days 14 (X4), VEGF immunolocalization in the wound healing region.

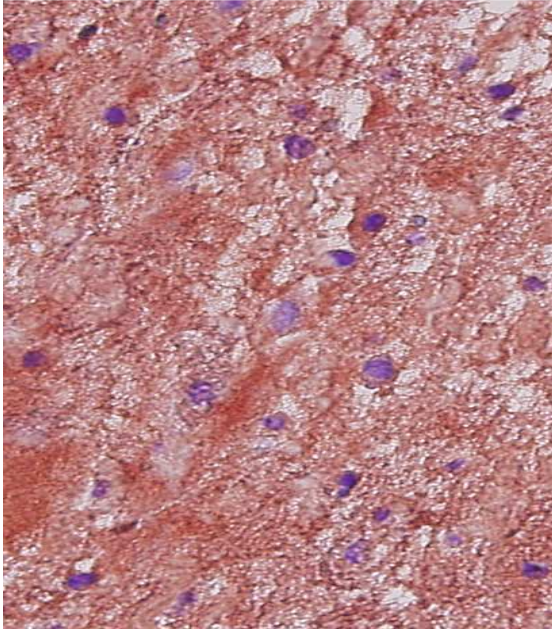


**Figure 6.** The VEGF immunolocalization on days 14 (X4), in the incision made and chitosan application group (DC).

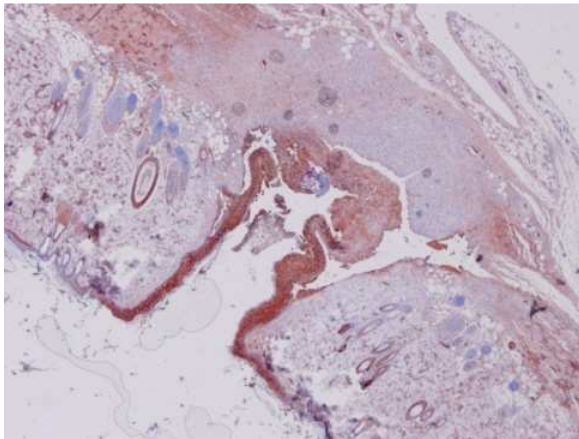


**Figure 7.** Control Group, on days 3 (X40), FGFR3 immunolocalization in the wound healing region.

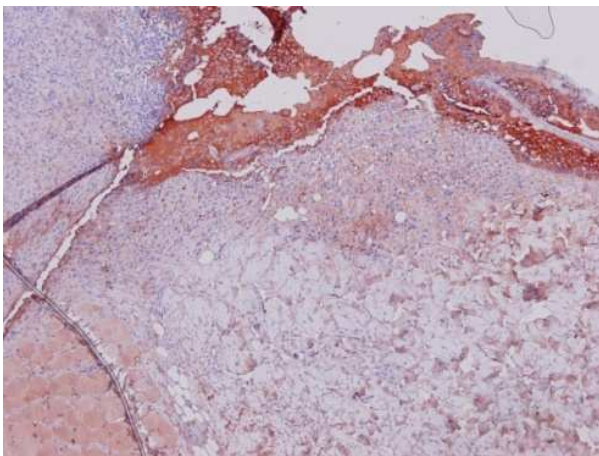




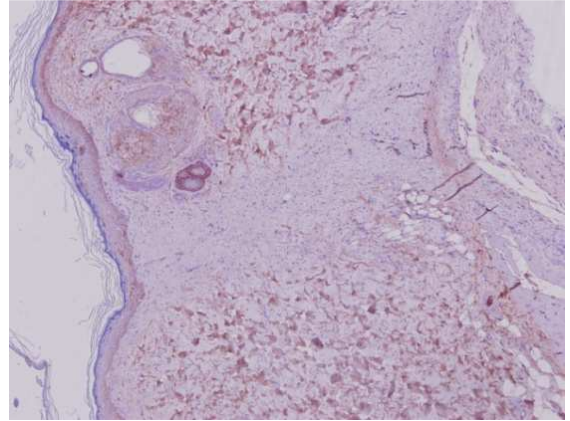
**Figure 8.** The FGFR3 immunolocalization on days 3 (X40), in the incision made and chitosan application group (DC).



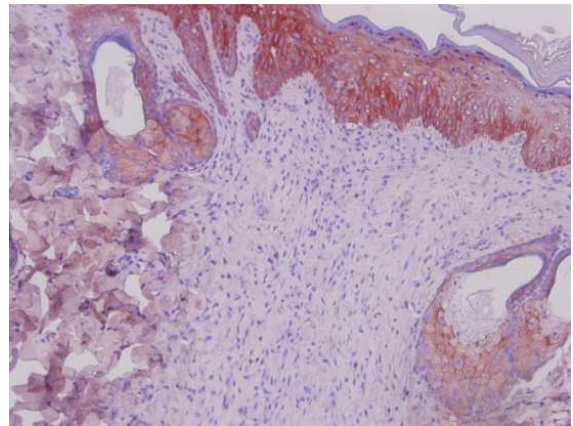
**Figure 9.** Control Group, on days 7 (X4), FGFR3 immunolocalization in the wound healing region.



**Figure 10.** The FGFR3 immunolocalization on days 7 (X10), in the incision made and chitosan application group (DC).



**Figure 11.** Control Group, on days 14 (X10), FGFR3 immunolocalization in the wound healing region.



**Figure 12.** The FGFR3 immunolocalization on days 14 (X20), in the incision made and chitosan application group (DC).

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