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ORIGINAL ARTICLE

Regulation of MMP 2 and MMP 9 expressions modulated by AP-1 (c-jun) in wound healing: improving role of *Lucilia sericata* in diabetic rats

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Abstract

Aims *Lucilia sericata* larvae have been successfully used on healing of wounds in the diabetics. However, the involvement of the extraction/secretion (ES) products of larvae in the treatment of diabetic wounds is still unknown. Activator protein-1 (AP-1) transcription, composed of c-jun and c-Fos proteins, has been shown to be the principal regulator of multiple MMP transcriptions under a variety of conditions, also in diabetic wounds. Specifically, MMP-2 and MMP-9's transcriptions are known to be modulated by AP-1. c-jun has been demonstrated to be a repressor of p53 in immortalized fibroblasts. The aim of the present study is to investigate the effects of *L. sericata* ES on the expression of AP-1 (c-jun), p53, MMP-2, and MMP-9 in wound biopsies dissected from streptozotocin induced diabetic rats.

Methods The expression levels of MMP-2, MMP-9, c-jun and p53 in dermal tissues were determined at days 0, 3, 7 and 14 after wounding, using immunohistochemical analysis and quantitative real-time PCR.

Results The treatment with ES significantly decreased through inflammation-based induction of MMP-2 and MMP-9 expression levels in the wounds of diabetic groups, compared to control groups at the third day of wound healing. At the 14th day, there were dramatic decreases in expression of c-jun, MMP-9, and p53 in ES-treated groups, compared to the diabetic group ($P < 0.001$, $P < 0.05$ and $P < 0.01$, respectively).

Conclusion ES products of *L. sericata* may enhance the process of wound healing in phases of inflammation, proliferation, and re-epithelization, essentially via regulating c-jun expression and modulating MMP-2 and MMP-9 expressions.

Keywords STZ diabetes · Maggot debridement therapy · *Lucilia sericata* · MMP · P53 · AP-1

Introduction

Wound healing, irrespective of the injury type, proceeds through events broadly classified as inflammation, formation of granulation tissue, angiogenesis and tissue remodeling.

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A diabetic mouse is an animal model of type II diabetes that shows impaired dermal wound healing and has been proposed as a model of human impaired wound healing in diabetics [1].

Matrix metalloproteinases (MMPs) which are subdivided into five main classes according to their structures and substrates: namely, collagenases, gelatinases, stromelysins, membrane-type MMPs and others [2] are involved in numerous biologic and pathologic processes as well as many essential roles in wound healing. So far, a number of studies have shown the elevated [MMP] expressions in chronic non-healing wounds [1]. However, the precise roles of MMPs during wound healing still remains to be elucidated. MMP-2 and MMP-9 are gelatinases which are able to cleave many different targets [extracellular matrix, cytokines, growth factors, chemokines, and cytokine/growth factor receptors] that in turn regulate key signaling pathways in cell growth, migration, invasion, inflammation, and angiogenesis [3]. Recently, it has been shown that MMP-2 is essential for angiogenesis and prolonged matrix remodeling. In wound healing, MMP-9 is suggested to be involved in keratinocyte migration and granulation tissue remodeling [4].

During wound healing, MMPs are secreted by different cell types such as keratinocytes, fibroblasts, and the inflammatory cells at different stages and locations, thereby regulating this process in a very coordinated and controlled way. In this process, a variety of transcription factors regulate the protease and cytokine expression [2]. Activator protein 1 (AP-1) and nuclear factor kappa B (NF-κB) are of particular interest due to their promiscuous distribution among MMP genes and cytokines. Several studies have highlighted the absolute requirement for a conserved proximal AP-1 binding site in the induction of MMP-9 [5–10], which is a distal stimulatory region encompasses an NF-κB element and an additional AP-1 binding site [5, 7, 9].

AP-1 is a protein complex that includes c-jun and c-fos proto-oncogenes that are held together by a leucine "zipper", in which residues of leucine interact with the homologous region on its partner. Hetero- and homodimers of the jun and fos families interact with the specific binding sites in the upstream regulatory regions of genes [6]. AP-1 proteins, mostly those that belong to the Jun group, control cell life, and death through their ability to regulate the expression and function of cell cycle regulators such as p53 and p16. p53 gives rise to the cellular apoptosis in case of infeasible repair. Because tissue repair involves a rapid but reversible increase in the proliferation of different cells involved in tissue repair, it is likely that alterations in the expression in p53 are involved in the healing process [11]. AP-1 modulates the transcription of several pro-inflammatory genes, in particular, that of MMP-2 and MMP-9 [6]. MMP-9 and MMP-2 preferentially degrade denatured collagens during cell migration and granulation events of wound healing.

It is known that the diabetic skin has deficient wound-healing properties and characterized by disturbances in collagen metabolism at the site of the non-healing wound [12]. Nowadays, *L. sericata* larvae are successfully used as medical maggots in the healing of wounds since it helps the healing process through proteolytic digestion of necrotic tissue disinfection and stimulation of granulation tissue formation [8–13]. Moreover, a treatment with ES products has been considered to effectively debride wounds and help the healing process [12]. However, effective mechanisms of ES treatment induced wound healing are not exactly understood, yet. Our study will help to clarify the molecular mechanisms of ES therapy in case of diabetic wound healing. The present study determines the effect of ES treatment on MMP-2 and MMP-9 expressions and their modulatory role on expression patterns of AP-1 and p53 in cutaneous wounds of diabetic and non-diabetic rats, based on different phases of wound healing.

Materials and methods

Experimental protocol and wound creation

Twenty healthy male Wistar rats aged 5–6 weeks and weighing 300–350 g were purchased from the Experimental Animal Center of Istanbul University [approval no. 2013/39]. Rats were randomly divided into four groups with five rats in each one. The first group consisted of control animals normal control group: (NC), the second group was the diabetic control group (DC), the third and the fourth groups comprised of non-diabetic and diabetic animals treated with topical ES (namely, normal treated: NT) and diabetic treated (DT) groups. To establish the diabetic models, a single dose of 60 mg/kg streptozotocin (STZ; Sigma, St. Louis, MO, USA) prepared in freshly and dissolved in 0.9% saline solution was injected intraperitoneally (within 10 min). Three days later, blood samples were collected from the tail of rats to determine fasting blood glucose levels. Plasma glucose levels more than 220 mg/dl were accepted as diabetic and were enrolled in the experiment. Three full thicknesses of wounds (approximately 12 mm^2) were excised from the dorsum of each rat with sterile punch biopsy needles. The wounds were treated topically with ES according to their respective groups, once daily for a period of 14 days. The equal concentrations of ES (protein concentration of 50 µg/ml) were used and the animals in group I were treated by 50 µl ES for each wound location [13, 14]. Treatment procedures were freshly prepared daily for 14 days, and the dressings were renewed. On the third day, the wound was removed using sterile scissors and pens. As done on the first day (day 0), treatment groups were treated with physiological saline for the larval secretion control group. The same

procedures were repeated for day 7 and day 14. However, no treatment was applied to the animals after the biopsy was taken on the 14th day. Control groups were treated topically with 150 µl of physiological saline-impregnated surgical sponges.

The wounded areas were photographed and the diameters of each wound at wounding days of 0, 3, 7 and 14 were measured and evaluated. Then the wounds of each day were collected. When the experiment was terminated, the animals were killed. A half part of wounds was stored at − 80 °C for RT-PCR. The other side of the wound was dissected and immersed in 10% formalin for immunohistochemistry studies. On 14th days, the rats were killed by cervical dislocation under anesthesia.

Preparation of ES [Maggot secretion]

ES was extracted in milli-O ultrapure water from sterile second and third instar larvae of *L. sericata*. ES was collected after incubating 2000 larvae/sterile beaker in 1 ml water for 60 min in ambient temperature. Then, 4 ml of water was added for 4 h with 1 h intervals since the larvae left their secretions into water [14].

Immunohistochemical staining and assessment

For immunohistochemical analysis, tissues were fixed in 10% buffered formalin and embedded in paraffin and cut into 4 µm thick sections. These sections were deparaffinized in toluene and rehydrated in graded alcohol series. Commercially available monoclonal antibodies against p53, MMP-9, and MMP-2 (Santa Cruz Biotechnology, California) were used. Histostain-Plus™ Broad Spectrum Kit (95-9943-B Zymed Lab. Ins. San Francisco CA, USA) was used for immunoperoxidase staining. The whole ELISA procedure was performed using a combination of microwave oven heating for antigen retrieval and standard in-direct streptavidin–biotin–peroxidase method. Endogenous peroxidase activity was blocked by hydrogen peroxide (3%). Each section was then incubated for 15 min at room temperature with blocking solution to stop cellular peroxidase activity. The sections were incubated with anti-p53, anti-MMP-2, and anti-MMP-9 monoclonal antibodies overnight at 4 °C, and then washed with PBS. Specific staining was performed with the biotinylated universal secondary antibody, horseradish peroxidase–streptavidin complex, and amino-ethyl-carbazole (AEC) as the chromogen in the kit. Immunohistochemical staining was evaluated semi-quantitatively using a modified H-SCORE analysis that assigned numerical values of 0–300 to the staining intensity. Each slide was assigned H-SCORE values for ten different areas by two investigators at different times. The investigators were blinded to the tissue type and source. These scores were averaged to generate the data presented by Karipcin et al. [15].

Total RNA isolation and cDNA synthesis

The remaining wound tissues stored in a − 80 °C freezer were used for total RNA extraction using Ambion PureLink RNA Mini Kit according to the manufacturer's protocol. RNA concentrations were determined by Nanodrop. The isolated RNAs were then reverse-transcribed to gain cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the routine procedure in a total volume of 20 µl. c-jun gene primers for the expression studies were designed using the RealTime ready configurator (Roche-Config. no: 100077513).

Quantitative real-time PCR

The expression of c-jun mRNA analysis was carried out using a Power SYBR Green PCR Master Mix (Applied Biosystems by Life Technologies). Two-microliter aliquots of diluted cDNA (approximately 100 ng) were used for RT-PCR amplification.

The final reaction volume was 20 µl. Each sample was analyzed in triplicate. A non-template control (RNase free water) was included on every plate. In the first instance, a standard curve and validation experiment was performed for each primer/probe set. PCR was performed in a Lightcycler 480 II system. Cycle-to-cycle fluorescence emission readings were analyzed with Lightcycler 480 Software Release 1.5.0 SP4. Relative mRNA concentrations were calculated using the comparative CT method. All quantifications were normalized to the housekeeping gene, namely β-actin.

Statistical analysis

All data were subjected to statistical analysis using GraphPad InStat Software (version 3.06). The findings were given as mean ± SEM and compared by ANOVA followed by Tukey–Kramer multiple comparisons test. The p value of less than 0.05, 0.01 and 0.001 was considered statistically significant.

Results

The rate of wound contraction of control and diabetic wounds is shown in Fig. 1 and Table 1. Wound contraction in diabetic rats was delayed compared to that in normal rats in a statistically significant manner. After ES treatment, diabetic rats showed a significantly faster rate of wound contraction compared to untreated ones on the macroscopic scale.

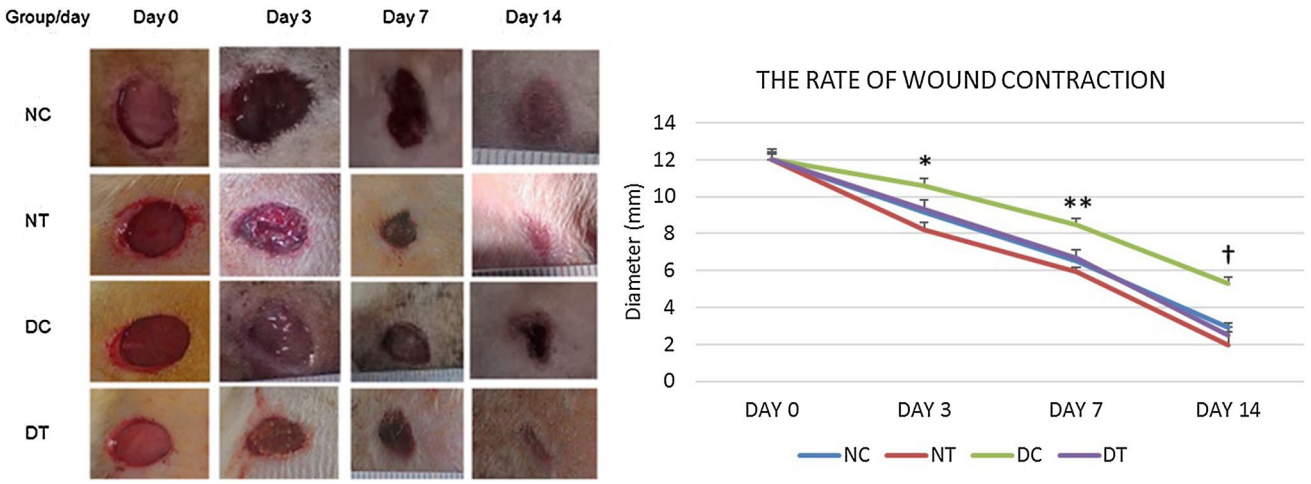

Fig. 1 Photographs of wound area on different days of control and ES-treated control, diabetic and ES-treated diabetic wounds. A total number of 20 animals ($n=5$) were used. *ES* excretion/secretion, *STZ* streptozotocin

Table 1 The rate of wound contraction of normal control (NC), normal ES-treated (NT), diabetic control (DC) and diabetic ES-treated (DT) rats

	DAY 0	DAY 3	DAY 7	DAY 14
NC	12 ± 0	9.16 ± 0.4	6.49 ± 0.58	2.92 ± 0.31
NT	12 ± 0	8.22 ± 0.39	5.92 ± 0.36	1.96 ± 0.51
DC	12 ± 0	10.6 ± 0.23*	8.46 ± 0.35**	5.32 ± 0.46†
DT	12 ± 0	9.3 ± 0.7	6.68 ± 0.32	2.5 ± 0.65

*$P < 0.05$ vs NT group

**$P < 0.01$ vs all groups

†$P < 0.01$ vs all groups

Table 2 AP-1 (cJun) expression levels ($2^{-\Delta Ct}$) in wound healing of normal control (NC), normal ES-treated (NT), diabetic control (DC) and diabetic ES-treated (DT) rats

AP-1*	NC	NT	DC	DT
Day 0	2.46 ± 0.71	2.51 ± 0.69	406.30 ± 59.60[a]	404.15 ± 58.70[a]
Day 3	9.30 ± 6.39	2.36 ± 1.81	42.67 ± 28.96	1.83 ± 0.85
Day 7	0.28 ± 0.08	0.47 ± 0.17	150.5 ± 76.25[b]	11.85 ± 7.36[c]
Day 14	0.29 ± 0.12	0.86 ± 0.67	13.98 ± 3.57[d]	2.71 ± 1.78[e]

Values are given as mean ± SEM

*All quantifications were normalized to the housekeeping β-actin gene

[a]$P < 0.0043$ vs untreated control day 0

[b]$P < 0.05$ vs untreated control day 7

[c]$P < 0.05$ vs untreated diabetic day 7

[d]$P < 0.05$ vs untreated control day 14

[e]$P < 0.05$ vs untreated diabetic day 14

Expression of Jun is transiently upregulated during wound healing process of diabetic rats

The differences in expression levels of c-jun in wound healing were compared between control and diabetic groups, beginning from 0 to 14 days prior to and after treatment with ES (See Table 2). In DC group, c-jun expressions increased gradually on 0th, 3th and 7th days while expression in 14th day decreased dramatically, compared to the levels on the 7th day ($P < 0.05$). In the DT group, c-jun expression decreased on all days in comparison with DC group but the significant findings were noticed only at 14th day of wound healing ($P < 0.001$).

The expression of MMP-2 and MMP-9 are indirectly correlated with AP-1 elevation

Figure 2 gives the photomicrographs and Fig. 3 gives the graphical presentation for MMP-2 immunoreactivities in wounds of all groups, especially with a reactivity on the connective tissue cells of the dermis. In diabetic tissues, an initial higher expression in MMP-2 was observed at the beginning of the experiment, compared to control groups ($P < 0.01$). However, MMP-2 expression of both diabetic groups elevated significantly at third day ($P < 0.001$ for DC and $P < 0.01$ for DT) while NT group had the lowest level of MMP-2 ($P < 0.05$). There were gradual decreases in MMP-2 reactivities between the very first day and the 14th day in all groups except NT group ($P < 0.05$ for NC, $P < 0.01$ for DC and $P < 0.001$ for DT) (see Figs. 2, 3).

In DC group, MMP-2 immunopositivity was elevated compared to that of all groups but after ES treatment on diabetic wounds, MMP-2 immunoreactivities were linearly decreased through day 0 to day 14, in a similar pattern with the control rats ($P < 0.001$).

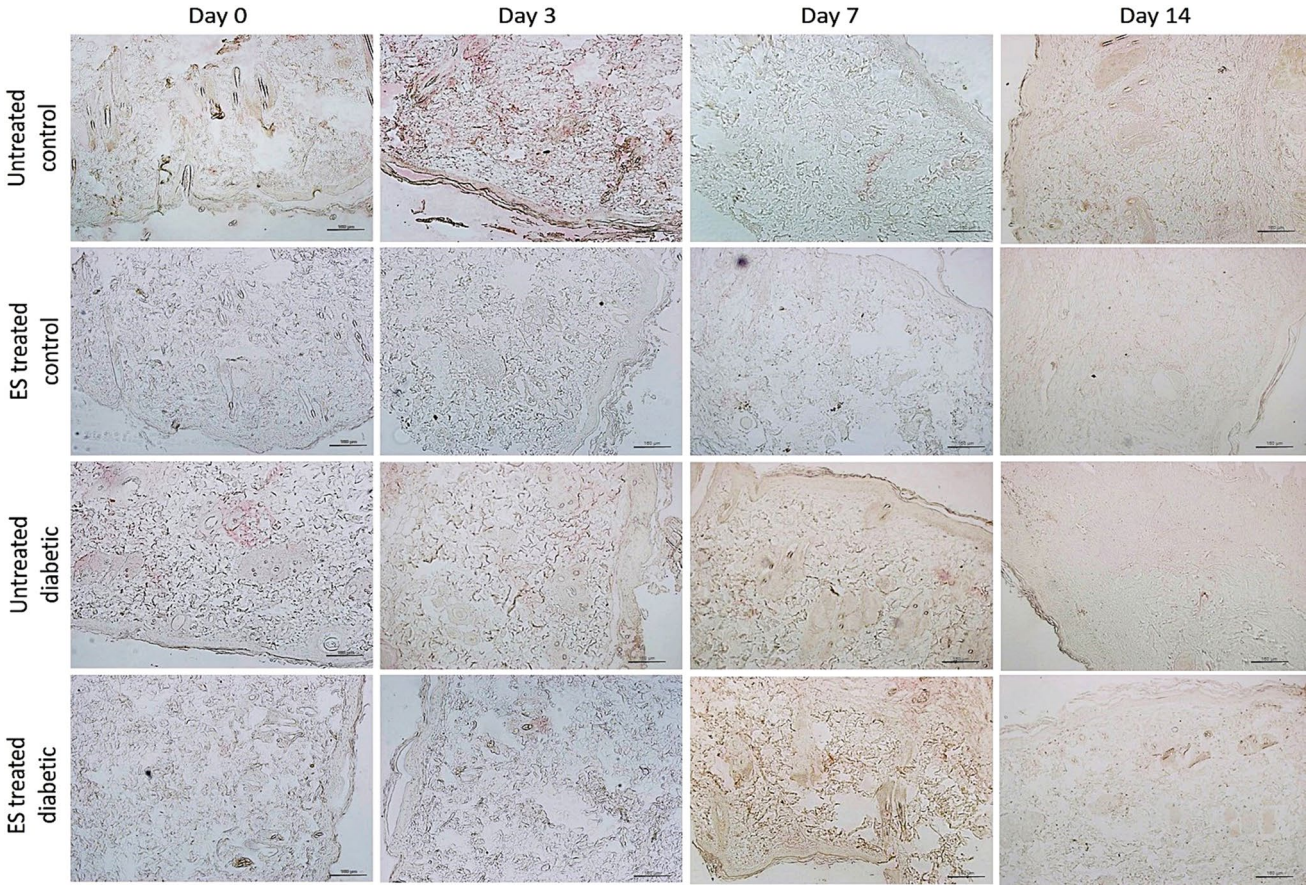

Fig. 2 Immunohistochemical analysis of MMP2 in wound healing process on 0, 3rd, 7th, and 14th days after treatment with excretion/secretion (ES), bar 160 µm. A total number of 20 animals [$n=5$] were used

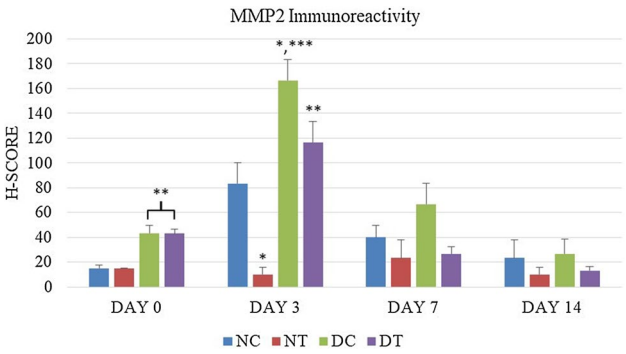

Fig. 3 A graphic for MMP2 immunoreactivity in wound healing of normal control (NC), normal ES-treated (NT), diabetic control (DC) and diabetic ES-treated (DT) rats. A total number of 20 animals ($n=5$) were used: Day 0; $P=0.0002$, $^{**}P<0.01$ NC, NT vs DC, DT, Day 3; $P=0.0005$, $^{*}P<0.05$ NC vs NT and DC, $^{***}P<0.001$ NT vs DC, $^{**}P<0.01$ NT vs DT, Day 7; $P=0.1257$, Day 14; $P=0.6683$

Figure 4 gives the photomicrographs and Fig. 5 gives the graphical presentation for MMP-9 immunoreactivities in wounds for all groups. A linear decrease in MMP-9 immunopositivity was detected between day 0 and day 14 in the wound of control tissue. In the DC group, MMP-9 reactivity was higher than the NC group on 3rd and 14th days ($P<0.001$ and $P<0.01$, respectively). In the ES-treated diabetic wounds, however, MMP-9 reactivity was lower than the DC group on the 3rd day ($P<0.05$). Reactivity of ES-treated control wounds was significantly lower than ES-treated diabetic wounds on 3rd day ($P<0.05$) but not on 7th and 14th days.

The p53 downregulation needs AP-1 involvement in wound-healing in diabetic animal model

The signal for p53 showed a peak in the epithelium at the post-wounding third day when the inflammatory response was also literally higher (Figs. 6, 7). The reactivity decreased at 7th day and 14th day in all groups but the only statistical significance was in the DT group ($P<0.05$). The immunopositivity gradually decreased at the 14th day and remained only in the top layers of the differentiated epithelium. The p53 immunoreactivity in ES-treated diabetic wounds was lower than the DC group especially on the 14th day ($P=0.017$) (See Fig. 7).

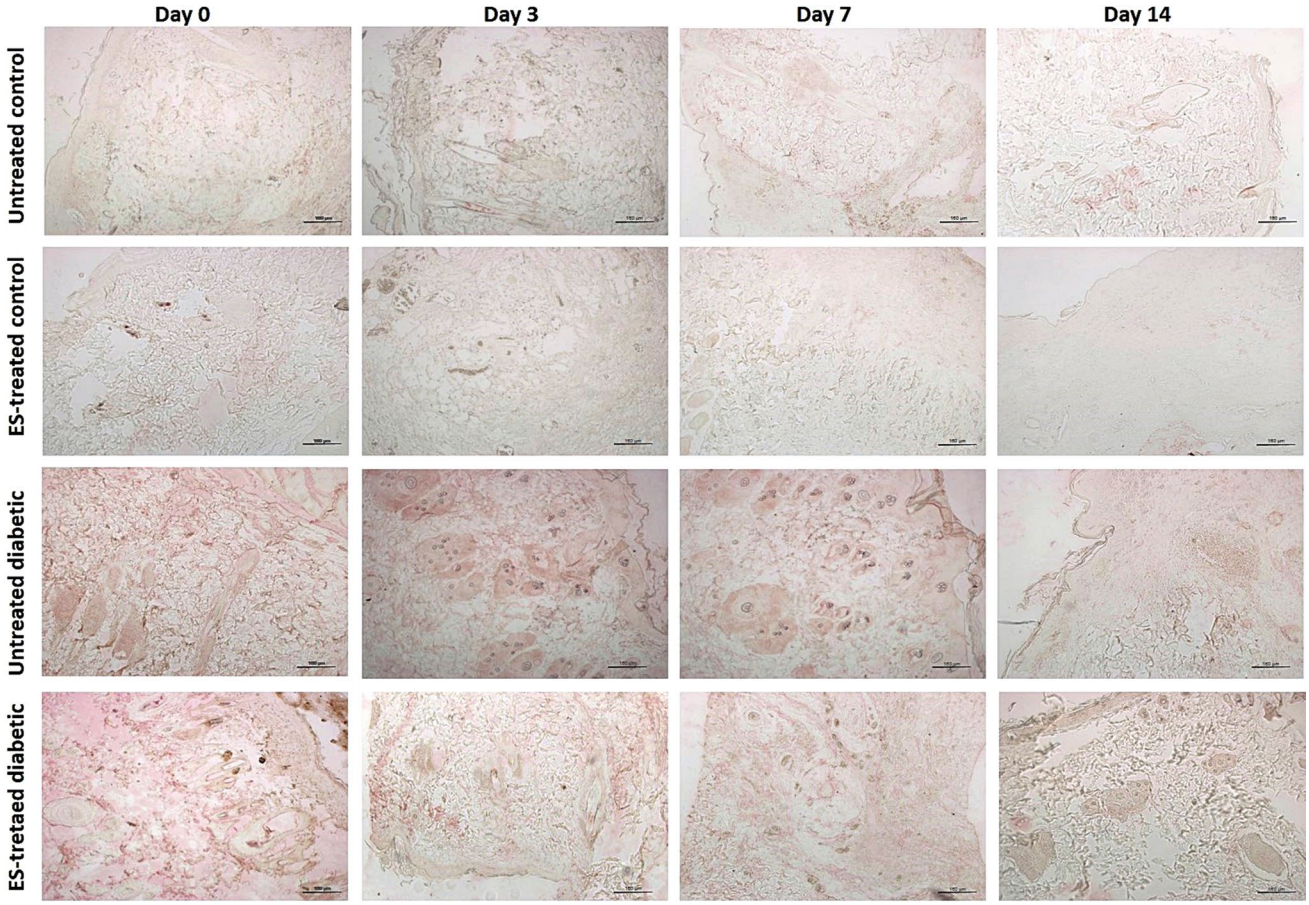

Fig. 4 Immunohistochemical analysis of MMP9 in wound healing process on 0, 3rd, 7th, and 14th days after treatment with excretion/secretion (ES), bar 160 µm. A total number of 20 animals [$n=5$] were used

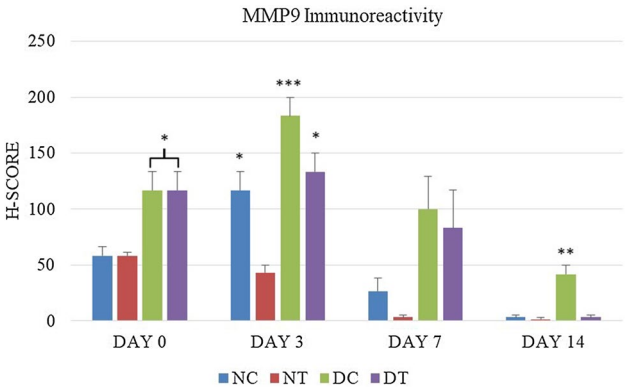

Fig. 5 A graphic for MMP9 immunoreactivity in wound healing of normal control (NC), normal ES-treated (NT), diabetic control (DC) and diabetic ES-treated (DT) rats. A total number of 20 animals ($n=5$) were used: Day 0; $P=0.0007$ *$P<0.05$ NC vs all groups, Day 3; $P=0.0011$ *$P<0.05$ NT vs all groups, ***$P<0.001$ DC vs NT, Day 7 $P=0.0516$, Day 14 $P=0.0005$ **$P<0.01$ DC vs all groups

Discussion

There is a large volume of published studies describing the role of larva treatments on non-healed wounds [13, 14]. Although some research has been carried out on animal models, there is very little scientific understanding of larva treatment, namely *Lucilia sericata* larvae, mode of action on wound healing. The larva has biochemical and mechanical actions such as debridement of necrotic tissue, reducing inflammation and stimulating granulation tissue in wounds. *Lucilia sericata* has mostly been accepted as debridement therapy. Disinfection in debridement and the acceleration of wound healing are improved by maggot ES products which include various proteases such as chymotrypsin—serine protease, which is a candidate factor for wound healing [16]. The STZ-induced diabetic rat is an excellent animal model of type II diabetes that shows impaired dermal wound healing and has been proposed as a model of human impaired wound healing. A number of MMP expression studies have reported in chronic wounds compared to an acute wound [2, 4]; however, little data have been studied to characterize

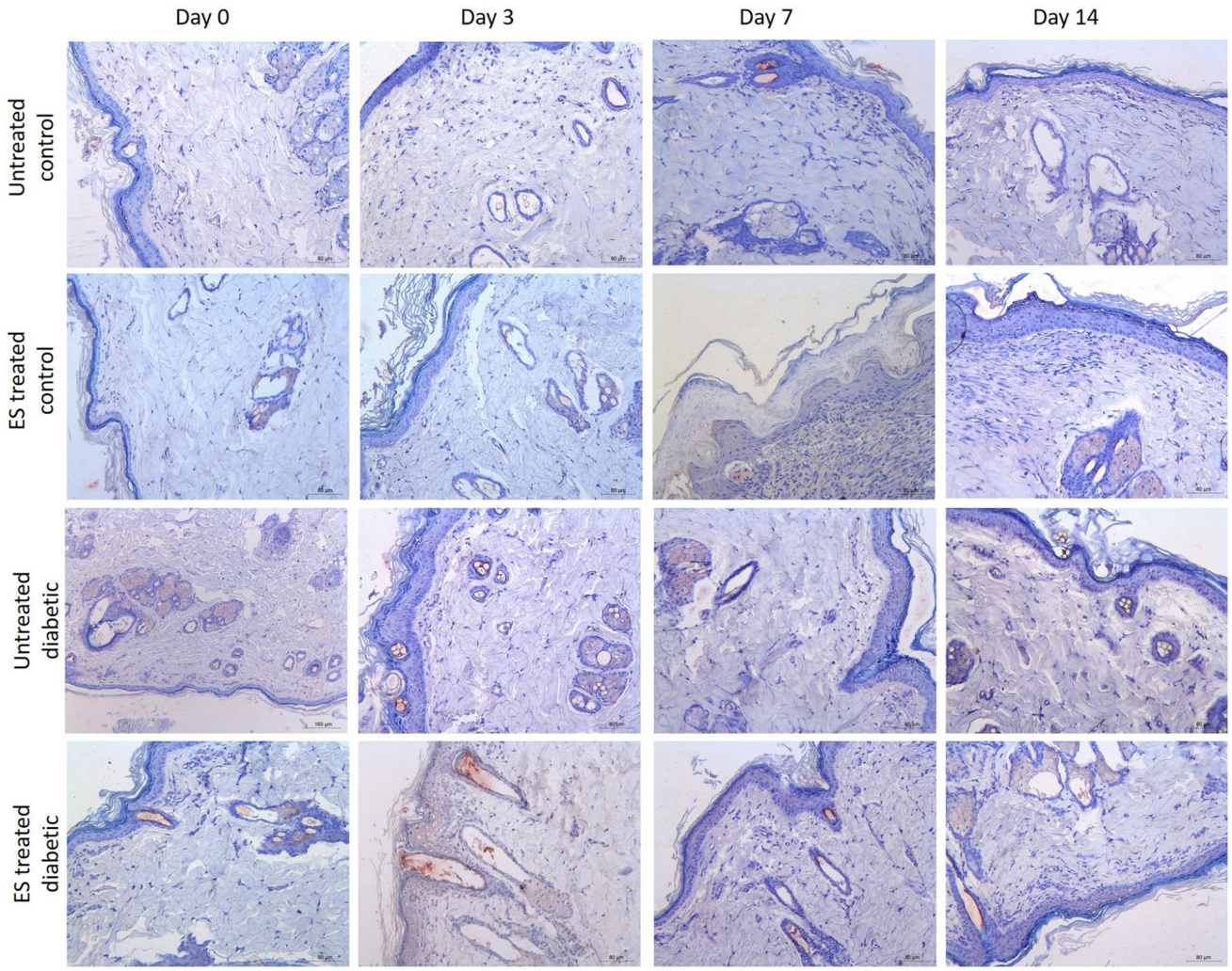

Fig. 6 Immunohistochemical analysis of p53 in wound healing process on 0, 3rd, 7th, and 14th days after treatment with excretion/secretion (ES). A total number of 20 animals [$n=5$] were used

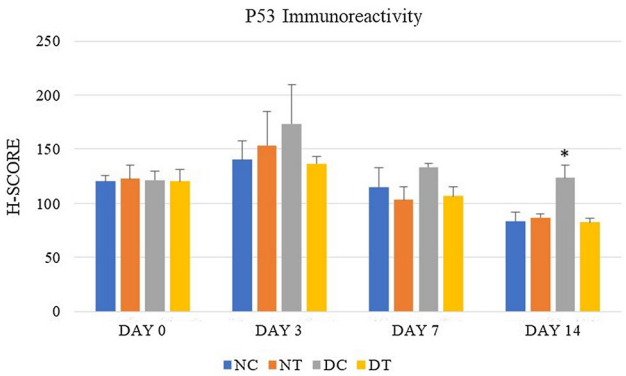

Fig. 7 A graphic for p53 immunoreactivity in wound healing of normal control (NC), normal ES-treated (NT), diabetic control (DC) and diabetic ES-treated (DT) rats compared with days and groups. A total number of 20 animals ($n=5$) were used: Day 0; $P=0.685$, Day 3; $P=0.641$, Day 7; $P=0.343$, Day 14; $P=0.017$ *$P<0.05$ vs all groups

animal models in a similar manner and to determine their usefulness. Most reports either do not include diabetic wounds in animal models, or do have limited numbers ($n=3$, 16), or focus on other types of MMPs [17]. In this study, we have determined the pro-inflammatory effect of the transcription factor AP-1 and the expression of two enzymes MMP-2 and MMP-9, whose transcriptions are known to be modulated by AP-1, in wound healing in case of STZ diabetic rats. AP-1 expression in the diabetic group significantly elevated at the beginning of wound when compared to non-diabetic control rats. In STZ diabetic wounds, AP-1 expression declined at 3rd, 7th and 14th days when compared to day 0. The induction of AP-1 elevates MMP expression, including MMP-1 (collagenase), MMP-3 (stromelysin-1), and MMP-9, and results in the degradation of extracellular matrix components in human skin in vivo [1, 18]. This degradation results in the accumulation of fragmented,

disorganized collagen fibrils, and these damaged collagen products downregulate new collagen synthesis, which suggests that collagen synthesis is negatively regulated by collagen breakdown [19, 20]. The combined actions of MMP-1, MMP-3, and MMP-9 degrade most of types I and III dermal collagen. Furthermore, AP-1 inhibits procollagen biosynthesis by suppressing gene expression of types I and III procollagen in the dermis, which results in a reduced collagen content [21]. AP-1 complexes are heterodimers of proteins of the two proto-oncogene families (jun and fos), bind to the promoter region of each inducible MMP gene. The overexpression of jun and fos proteins is known to enhance MMPs promoter activity [8]. In addition, the promoter of MMP-2 but not MMP-9 is known to contain a p53-binding element that mediates activation of MMP-2 gene transcription [22]. However, Cohen et al. reported that whereas exogenous p53 is able to downregulate MMP-9 promoter activity in cytotrophoblastic cells, endogenous p53 is not able to regulate MMP-9 expression [23].

In diabetic rats, MMP-2 and -9 immunoreactivities were significantly higher on day 0, in comparison with the control group, and MMP-2 and MMP-9 immunopositivities decreased on 3rd, 7th and 14th days in STZ diabetic wounds. It appears that elevated MMP-2 protein results in excessive matrix degradation preventing the normal matrix formation and remodeling and may result in the formation of a chronic wound, especially in diabetics. However, the findings of the current study do not support the previous researches of Chung et al. [24] and Polat et al. [14]. Chung concluded that MMP-9 but not MMP-2 was overexpressed in the torn tendons of the patients with diabetes when compared with those of the controls, among various genes of interest, both in gene and protein levels [24] while Polat et al. claims that MMP-9 was downregulated in the ES-treated wounds [14]. Even though MMP activity might be beneficial in the acute wounds, it likely leads to disrupted extracellular matrix ultrastructure and tissue function in the chronic wounds. Thus, in chronic wounds, the MMP overexpression would be harmful in wound healing. According to our results, MMP-9 expression increased in diabetic wounds compared with the control groups, while this increase was reduced at 7th and 14th days, probably due to decreased levels of AP-1. However, in other studies, hyperglycemia, either alone or in conjunction with matrix glycation, has previously been shown to reduce MMP-9 levels in mesangial cells in vitro [25, 26]. Additionally, other complications, such as varied cytokine expression [27], may also alter MMP expression within the diabetic wound. Therefore, the fact that our MMP9 findings differ from previously published data of other types of wounds may be due to diabetic complications not seen in the non-diabetic patients with chronic wounds.

In normal wound healing process and progressive tissue degeneration, the increased MMP-9 expression might significantly compromise the integrity of skin with disruptions, resulting in unhealing wounds. Several more studies revealed the relationship of diabetes with the upregulated MMP expressions in serum or tissue [28, 29]. However, much uncertainty still exists about the relation between MMPs and diabetes. One explanation is that enhanced monocyte and macrophage activation by increased circulating and tissue glucose level may induce inflammatory cytokines, leading to increased activation and expression of MMPs, particularly MMP9. In addition, chronic hyperglycemia-induced oxidative stress may result in increased activation and expression of MMP-9 via the ROS sensitivity pathway. A longitudinal study of diabetes by Wu et al. reports that MMP-9 is related to poor healing in diabetic foot ulcers [30]. As a support to these reports, we showed that diabetic untreated rats had higher MMP-9 reactivity than normal control rats on 3rd and 14th days of wound healing process [8].

Since tissue repair involves rapid but controlled increases in cell proliferation, it makes sense that p53 and AP-1 would be involved. With wounding, p53 levels would be expected to decrease, allowing the sudden increase in proliferation required to increase cellularity during the inflammatory phase of healing. At the completion of inflammation, proliferation should decrease along with the elimination of inflammatory cells. This period is likely to be associated with apoptosis to remove leukocytes. In the next phase, there is an increase in fibroblasts and one would expect another dip in p53 expression [31]. Kane et al. have suggested that there is a delay in the apoptosis pattern observed in the diabetic wounds that appears to be related to the marked prolongation of the inflammatory stage of healing but could not explain why the diabetic animals lose the apparent opposing levels of p53 and bcl-2 that was observed for nondiabetic animals [32]. The delays, as also seen in our study, refer to the fact that prolonged exposure leads to a continuous inflammatory response that lasts until complete wound closure. Kane et al. concluded that the loss of the usual controls of cellular proliferation may also contribute to the altered healing that is so commonly observed in the wounds of diabetic patients. In this study, the expressions of p53 showed a peak at post-wounding third day when inflammatory response was also literally higher. The reactivity decreased at 7th day and 14th day in diabetic groups but the only statistical significance was in DC group, compared to the other groups. The immunopositivity gradually decreased at 14th day and remained only in the top layers of the differentiated epithelium. The suppositions of the involvement of p53 in apoptosis phase of healing were supported by our findings that p53 initially increases and finally decreases in the healing process of rat wounds, showing the delay in apoptosis.

The content of the larvae secretion involves at least 400 protein spots with the majority of five proteins, namely fatty

acyl-CoA reductase, 60S ribosomal protein large subunit, a 38 kDa of larval protein, Golgi complex subunit-2 and Nipped-B. As mentioned in our previous paper, the larvae secretion can give rise to the actual pathway of wound healing in case of diabetic animal model [13]. In the present study, we revealed that the expression of MMP-2 and MMP-9 was progressively decreased after treatment with *L. sericata* extract in diabetic wounds after the third day of the healing process, which compromises the inflammatory phase in rats. The reason for this increase specifically in the third day may be due to elevated levels of inflammation modulated by NF-κB activity [13] and related genes such as MMPs.

On the other hand, the diabetic rats may not show similar alterations in MMP expression to those of the human condition, with a number of potential reasons such as different species, wound positions, and blood glucose regulation. Additionally, wound duration is a major factor for chronic wounds, often being over a year, although it is shorter in the diabetic animals [1]. Moreover, bacterial content is relevant in diabetic wounds: any infection must be omitted from the study, whereas the majority of human chronic wounds is infected [33]. This would be a major factor contributing to the differences between animal and human as bacterial products to induce and activate MMP-2 [34, 35]. Therefore, a diabetic rat is better considered as an animal model of impaired acute wound healing in diabetics rather than of chronic wounds. Although the animal model may be an unfavorable choice for testing wound therapies involving MMP modulation, it can be used for a number of other studies. These would include investigating the potential factors that cause elevated levels of MMPs within human wounds by modulating the bacterial content and glucose levels of the animals [1]. Thus, our study is one of the rare animal model research to compare altered MMP expressions in chronic wounds of diabetics.

The data of the present study indicated that AP-1 expression in wound healing of diabetic animal model has a similar pattern with MMP2 and MMP9 expressions, thereby it would appear that ES products of *L. sericata* may enhance the process of wound healing via the regulatory effect of AP-1 expression on MMP-2 and 9 expressions. As stated in our previous study, ES products of *L. sericata* are able to increase the synthesis of ECM elements such as collagen I and III which are degraded by MMPs in wound healing process. In this study, it is meaningful to suggest that decreased MMP2 and MMP9 expressions may be related to improvement of wound healing process.

Conclusion

As a summary, the proliferation, inflammation, and differentiation play important roles in wound healing process [10]. The process also involves a series of genes such as p53, AP-1 and MMPs [15], as confirmed by this study. The induction or inhibition of mentioned genes has role in the acceleration of wound healing [16].

Knowing the expression of MMP-2 and MMP-9 requires modulatory effect of AP-1 and p53, our results establish that the inhibitory effect of ES extract on the MMP expression in STZ diabetic wounds might be through the inhibition of AP-1 and p53 expressions. Understanding as-yet-unknown mechanisms of wound healing in diabetics may provide many opportunities in the treatment of non-healing chronic wounds, allowing alternative therapeutic intervention such as maggot therapy.

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Compliance with ethical standards

Conflict of interest All authors stated that there is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

Ethical standard statement All procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Ethical Committee of Istanbul University (Approval no. 2013/39).

Informed consent For this type of study, formal consent is not required.

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