Effect of Progesterone Stimulated Fibroblast on Wounds Healing in Rats (Wister rat)

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Abstract:
Fibroblasts: One of the components of the connective tissue and an essential component of the skin that produces and organizes dermis cells. Fibroblasts play an important role in regulating skin physiology, repairing skin wound and an increasing role in bioengineering, Moreover, they play an important role in natural wound healing as they migrate to the location of the wound. Hormones stimulate a modification process, which is beneficial to wound healing and skin health. In our research, we intended to combine the effect of fibroblasts and progesterone in accelerating wound healing, as (40 adult male rats) were obtained and divided into four groups (A, B, C and D). Afterward, fibroblasts were isolated from the skin of 20 adult male rats, the two groups B and C. Pieces of dermal tissue were obtained from the dorsal area of the adult rats to isolate fibroblasts from them and they were cultured in DMEM and 15% FBS medium in a gelatin-coated CO2 cell culture incubator under the conditions of (5% CO2 -temperature 37°-humidity 75). Fibroblasts were identified under an inverted microscope on different days.
Afterwards, wounds were inflected on the back of the rats in the four groups and they were monitored until recovery after applying the treatment on them. Then, we performed a statistical analysis of the previous treatments and compared them with the control case, and we reached the important role of both fibroblasts and progesterone in wound healing, and the restoration of the function and tissue structure of the skin after injury. Moreover, through experiments, we have come to choose the most appropriate conditions for collecting samples, measuring wounds, and applying treatments, while making persistent and daily efforts to reach the desired goal.

Key Words: fibroblasts, progesterone, cell culture, DMEM culture medium, wound healing, wound measurement.

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Introduction

Living creatures in the course of life face all kinds of injuries and accidents. We must have ways to heal these damaged tissues in order to survive (Academy et al., 2017)because skin wounds greatly affect the global health care system, creating a huge burden on the economy and society (Raziyeva et al., 2021). It has been shown that the effect of non-healing wounds on mortality is comparable to the effect of cancer deaths (Ellis et al., 2018), for example, chronic wounds affect 4% of the UK population (Simpson et al., 2010), since the healing of skin wounds takes place according to a successive and organized cellular mechanism (Reinke & Sorg, 2012) therefore, the wound healing process is known as: a complex process that includes many immune and structural cells whose secretion of

cytokines, chemokines and growth factors lead to the regulation of recovery stages (Raziyeva et al., 2021)/(Wiegand et al., 2021)/(Cen et al., 2022). It is conventionally divided into four successive stages:

- Vascular response: hemostasis.
- Cellular response: inflammation.
- The proliferative stage and repair.
- Remodeling (Trumm, 2014)/(Archie, 2013)/(Liman et al., 2005)

Due to the large role of fibroblasts in wound healing (Abbasi et al., 2020)/(Aumari, 2012)/(Nilforoushzadeh et al., 2017), their high regenerative capacity (Gál et al., 2008)their use in cellular therapeutic applications in biological studies (Rittié & Fisher, 2005)/(Kisiel & Klar, 2019)/(Rittié & Fisher, 2005). and also in bioengineering of skin application(Kisiel & Klar, 2019)In recent years, numerous studies emerged on the effect of steroid hormones on wound healing, the application of topical hormone replacement therapy, the role of progesterone in wound healing (Philips & Devaney, 2003)and the fact that progesterone is one of the steroid hormones whose receptors have been identified on the skin (Scheid et al., 2000)/ ((Bychowski & Auger, 2012)/ (Bychowski & Auger, 2012)/(Routley & Ashcroft, 2009).
In our research, we aimed to combine fibroblast therapy and its effects with steroid hormones (progesterone) therapy in order to enhance wound healing within a short time. The fibroblasts were isolated from the skin of adult rats, cultured and used with the hormone progesterone in the treatment of wounds which was inflected on rats. A comparison was also made between the normal physiological state of wound healing and the treated cases. The following presents our study.

Methods

1) Materials:

- DMEM High Glucose.
- RPMI.
- (Fetal bovine serum) (FBS).
- Dulbecco´s PBS powder Dpbs.
- L-glutamine.
- Pencilium – streptomycin Simply and Amphoferieins B
- Progesterone.

2) Used Mediums:

Figure (1) The following table shows the components of the mediums used in our study.

Name of Medium	Components of Medium
(A) Transportation medium	1) RPMI 2) Antibiotic (penicillin-streptomycin)
(B) Washing medium	Dpbs (Dulbecco´s PBS powder)
(C) Digestion medium	1) Trypsin 2) Disepase
(D) Culture medium	1) DMEM H.G 2) L-glutamine 3) FBS(Fetal bovine serum) 4) Antibiotic (penicillin-streptomycin) 5) Amphotericin B 6) Gelatine 0.1%
Freezing medium	1) DMEM H.G 2) DMSO 3) FBS

3)Methods of Work:
The experiments were conducted in the laboratories of the Department of Animal Biology, Faculty of Science, and Damascus University.

Forty (40) adult male albino waster rats, two and a half months old and (200-150 g) each, were obtained from the Atomic Energy Authority in Damascus. They were randomly distributed in plastic cages, where each rat was placed in a separate cage and had free access to food and water. and lighting at an average rate. The lighting conditions throughout the day was 12 hours of light and 12 hours of darkness. These rats were left for 15 days to acclimatize to the surrounding conditions before starting the experiment.

The rats were divided into four groups, each group consisted of ten rats.
1) Rat Anesthesia: ether was used (Miranda et al., 2011)
2) Inflicting a wound on the back of the rats:
Sterilization with 70% ethanol (Escada, 2018)
Depilation of the area and sterilization of the wound with povidone-iodine 10% and ethanol 70% (Siengdee et al., 2018)
3) Obtaining fibroblasts: (primary culture)

- We took skin sample from the rat and placed

in the medium A (Fig. 1).
- We transferred the sample to Petri dishes containing medium B (Figure 1) on ice.
- We cut the sample into small pieces using a scalpel.
- We transferred the sample to 15 mL tubes with Dpbs (Figure 1) and was moved up and down.
- We applied centrifugation at 1000 rpm for 2 minutes (Kheirollah et al., 2016), and Dpbs was removed from the tubes (Figure 1).
- We added medium C to the tubes (Figure 1).
- We placed the tubes in a Shaking Incubator at 37°C for 10 minutes.
- We applied centrifugation at 1100 rpm for 2 minutes and the floating particles were removed.
- We poured medium D (Fig. 1) and the sample was moved up and down.
- We applied Centrifugation at 1100 rpm for 2 minutes and the floating particles were removed.
- We transferred the cells to a T25 culture tray coated with 1% gelatin (Aumari, 2012)
- We increased the volume to reach 5 ml by pouring culture medium D (Fig. 1)
- We incubated the culture tray in a CO2 incubator according to the conditions (5% CO2, temperature 37°, humidity 75%).
- We changed the culture medium 72 hours after the initial culture.

4) Subculture of fibroblast (Rittié & Fisher, 2005)
- We added trypsin 0.1% to the flask.
- We transferred the sample to two flasks and we written on both flask: name cell, number passage culture and date passage.
- We increased the volume to reach 5ml by pouring culture medium D.
- We in cubated the culture tray in a co2 in cubator according to the condition (5%co2, temperature 37c, humidity 75%)
- We changed the culture medium 48 hours after the intial culture.

5) Cell growth and vitality test(Campbell, 2015)
We calculated the number of cells according to the following formula:

No. of cells/mL = No. of cells x extension percentage x 104

We calculated the cell vitality according to the following formula:

Cell vitality = (1 - (number of dead cells / total number of cells)) x 100

6) Cryopreservation (Siengdee et al., 2018)
- We added trypsin 0.1% to the flask, and then we added 2ml of media.
- We transferred the sample to 15ml tube.
- We applied centrifugation at 1100 rpm for 2 minutes and the floating particles were removed.
- We added freezing medium to the tube.
- We transferred the sample to the tube om ice.
- We stored the tube in freezing comtainer 4c for 4 hours.
- We transferred the tube to -20c for 1 hours.
- We transferred its to -80c for 24 hours.
- We stored the tube in liquid nitrogen for long term.

7) Statistical processing:
We used the statistical program IBM SPSS 28 .
8) Wound measuring:
Wound measurements were taken according to (Nichols, 2015)

Results:

1) Weight of Rat:
We prepared a standard table for each rat, as shown in Figure (2), and measured weights of the rats as in Figure (3), which shows the rat's weight tray and the scale used.
It was monitored on a daily basis, the measurements were recorded and the weight of the rats was monitored. We calculated the average increase of weight of the rats in each group and studied the weight gain during the experiment in the four groups as in Figure (4) which shows the graphs of the average weights of the rats for each group during the experiment.

Mark: Rat:

Day	Weight	Measurements of the Wound			Wound surface area
		Length cm	Width cm	Depth cm	

Figure (2) :Standard table for each rat (photographed by the researcher)

Figure 3): Rat weighing tray and scale used (photographed by the researcher)

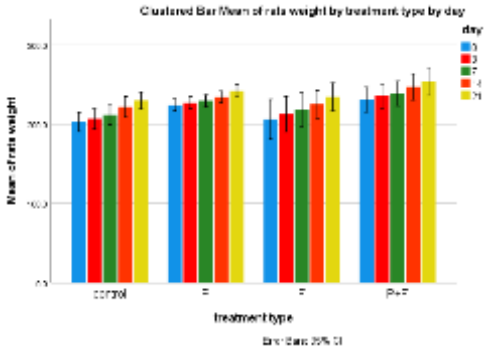

Figure 4: Graphs of the average weights of rats for each group during the experiment

We noticed an increase in the weight of the rats during the days of the experiment, and this is evidence of the rat's acclimatization during the experiment.

2) Wound measuring:

Measuring the wound is one of the basic elements in assessing the progress of wound healing. Wound measurements were taken as shown in Figure (5), which shows the steps of the wound measuring method.

Figure (5) Steps to measure the wound. (Photographed by the researche.

3) Primary culture of fibroblast

The cultured cells were examined by inverted microscope (optika) with magnification (200X & 400X).

We found cells with many elongations and branches, oval nucleus and cytoplasm, which is consistent with the morphology of the fibroblasts (Rittié & Fisher, 2005)The fibroblasts also showed clear adhesion at the bottom of the culture vessel, and the culture medium appeared transparent without any turbidity, bacterial or fungal contamination, and the medium maintained its pink color (Figure (6)).

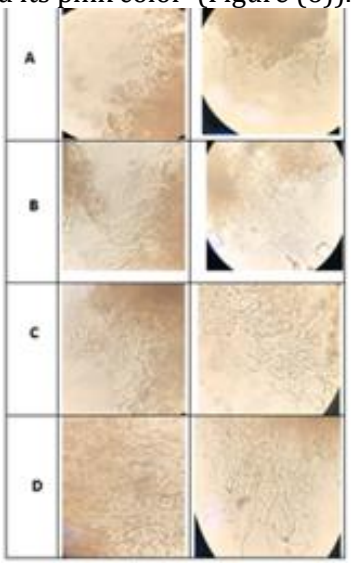

Figure (6):

A) Shows the crushed skin pieces on the first day of culture, using 200X magnification.

B) Shows cell migration and the onset of adhesion to the bottom of the culture vessel on day 6 of culture, using X200 magnification.

C) Shows clear cell migration and adhesion on day 10 of culture, using X200 magnification.

D) Shows how the culture vessel is crowded and the formation of a layer covering more than 75% of the surface of the dish on the 14th day, which necessitates the culture pass procedure, using 200X magnification. (Photographed by researcher)

4) Application of treatment and wound healing:

Group B and C received the same number of fibroblasts within the range (4 * 105 - 5 * 105) cell/1mL.

Group C and D received the same 15 mg/mL dose of progesterone.

The rats were given a daily dose of the painkiller (Flunxin) during the days of the experiment.

Group No.	Group A	Group B	Group C	Group D
Type of treatment	control case (natural wound healing mechanism)	Wound treatment with fibroblasts only.	Wound treatment with progesterone-activated fibroblasts.	Wound treatment with progesterone only.

Figure (7): Represents the topical treatment schedule for each group.

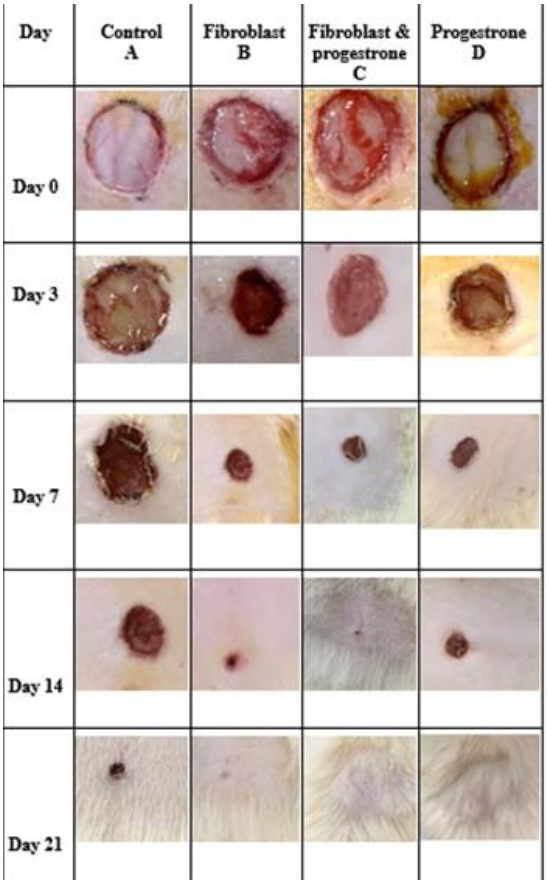

Figure (8)

Figure (8): Illustrative images representing wounds inflicted on rats during the experiment, and the progression of wound healing over time is noted.

Wound photos were taken for visual comparison of wound healing as in Figure (8), which represents illustrative pictures of wounds inflicted on rats during the experiment. The progress in wound healing was evaluated over time, and we noticed a continuous decrease in the area of the wound with the passage of days until full recovery was reached in the treated samples compared to the control case, which took longer to recover, as shown in Figure (8).

5) Statistical processing:

The statistical program IBM SPSS 25 was used, and measurements of wounds which were inflicted on rats were taken during five different time periods (days 0, 3, 7, 14 and 21).

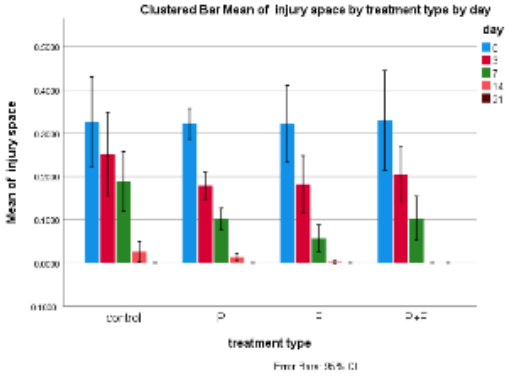

Figure (9): The graph shows the average surface area of wounds during the days of the experiment (Days (0, 3, 7, 14 and 21))

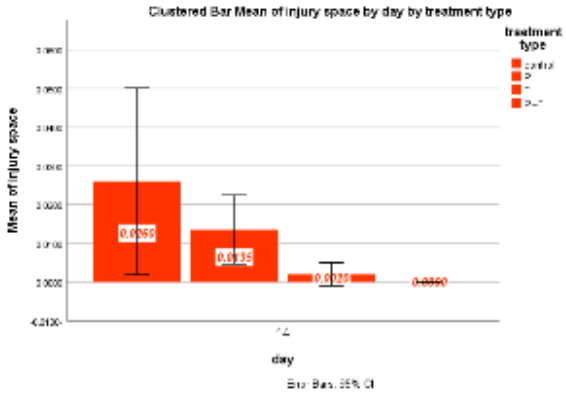

Figure (10): The graph shows the average surface area of wounds on the fourteenth day

Looking at the chart of the average surface area of the wounds in Figure (9), which shows the average surface area of wounds during the days of the experiment (Days (0, 3, 7, 14 and 21)), we noticed on the third day, represented in red, that the surface area of the wounds decreased in the treated groups (B, C and D) compared to the control group (A). On the seventh day, represented by the green color, the decrease continued in the average surface area of the wounds and on the fourteenth day, represented by the orange color, the surface area of the wounds in the treated samples (B, C and D) continued to decrease compared to the control sample (A). However, on the fourteenth day, represented in orange, we noticed that there were no trace of the wound in group (C) as shown in Figure (10) when compared to the two treated groups (B and D) and the control group (A).

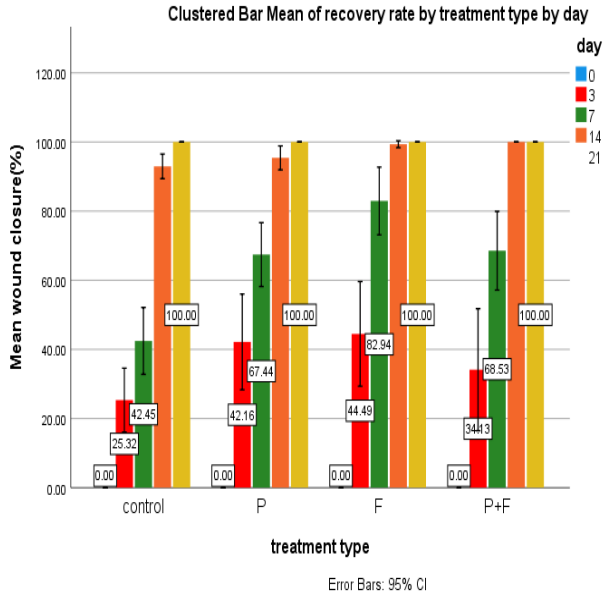

Figure (11): The chart shows the average rate of wound closure during the days of the experiment (Days (0, 3, 7, 14 and 21))

By looking at the chart of the average rate of wounds closure in Figure (11), which shows the average rate of wound closure during the days of the experiment (Days (0, 3, 7, 14 and 21)), we noticed on the third day, represented in red, an increase in the average rate of wound closure among the treated groups (B, C and D) compared to the control group (A).

On the seventh day, represented by the green color, it was followed by an increase in the average percentage of wounds closures. On the fourteenth day, represented by the orange color, the average percentage of wounds closures continued to increase in the treated samples (B, C and D) compared to the control sample (A), but in group (C) we noticed that the rate of wounds closure on the fourteenth day, represented in orange reached 100% as shown in Figure (11) compared to the two treated groups (B and D). Moreover, we noticed that the percentage of closure of wounds on the fourteenth day, represented in orange, in group B reached 99%, and we noticed that the percentage of wound closure on the fourteenth day, represented in orange, in group D reached 95.13% as shown in Figure (11). We noticed that the rate of closure of wounds on the fourteenth day, represented in orange, in the control group A reached 93.17% as shown in Figure (11). We noticed that a complete closure of the wounds was achieved in the entire groups on the twenty-first day by 100% as shown in Figure (11).

We conducted a statistical analysis of the data according to the following:

The Spearman's correlation

Correlation analysis between the data on the entire studied sample, regardless of the type of treatment, and we adopted the Spearman's correlation and found:

$$(\rho = -91.5\%,\ \text{sig} = 0.00)$$

There is a significant relationship between the samples, which is a strong inverse relationship by (ρ=-91.5%), and this means that the area of the wound decreases with time, i.e. the percentage of wound closure increases with the increase in the number of days from the day 0 of the experiment. Where it reached (sig = 0.00) <0.005 and this is evidence of the existence of significant differenc.

One-way ANOVA

Analysis of the test of significance of differences between the means through correlation One-way ANOVA

We noticed from the analysis of variance table represented in Figure (12) that shows the Sig differences between the wounds surface areas during the four treatments

Day	0	3	7	14	21
Sig	0.842	0.318	0.001	0.010	

Figure (12) Shows Sig differences between the wounds surface areas of the four treatments.

Looking at the variance analysis table represented in Figure (12):

At the beginning of the experiment (day zero), Sig = 0.842 is not significant differences, which is an expected and logical result.

On the third day, Sig=0.318. there were also non-significant differences between the wounds surface areas for the four treatments.

On the seventh day, Sig = 0.001, we notice significant differences where Sig < 0.05.

On the fourteenth day, Sig = 0.010, we notice significant differences where Sig < 0.05.

Tukey's post hoc test:

on the seventh day

In order to find out which of the treatments caused the differences in the mean surface areas of wounds on the seventh and fourteenth days, we conducted Tukey's post hoc test on the seventh day:

group	A	B	C	D
average	0.1885	0.05675	0.10285	0.10125

Figure (13) Shows differences between the mean surface areas of wounds for the four treatments on the seventh day.

In the result of Tukey's post hoc test which we conducted on the seventh day, Figure (13), we noticed that there were significant differences between the mean surface areas of wounds in the control sample and each of the three treated samples, where the mean surface area of wounds for the control sample was significantly greater than the means of the three other treated sample. The mean for the control sample was 0.1885 and did not exceed 0.102 in the other samples, which indicates a greater effectiveness of the three treatments in treating wounds compared to the control sample with non-significant differences appearing between the three treatments. We also noted that there were non-significant differences between the means of wounds for the three treated samples with similar values to the means in group D and C and a lower mean for group B, which indicates the possibility of a greater effectiveness of this treatment despite the non-significance of the apparent difference at this stage of the experiment.

on the fourteenth day

We conducted Tukey's post hoc test on the fourteenth day:

Group	A	B	C	D
Mean	0.026	0.002	0.0000	0.0135

Figure (14) Shows differences between the mean surface areas of wounds for the four treatments on the fourteenth day.

In the results of Tukey's post hoc test which we conducted on the fourteenth day, Figure (14), we noticed that there was no wound area for group C and almost no area for group B. A significantly greater mean was noticed for group D, and the largest value was for the control group A.

Therefore, we reached the results of Tukey's post hoc test that there were significant differences between the mean surface area of wounds for the control group (A), and each of the two treated groups (B and C), while group D was closer to the control sample so that the difference between it and the control sample was non-significant.

Discussion:

This work was carried out in three stages:

In the first stage:

fibroblasts were isolated from the skin of adult rats and cultured in DMEM culture medium and some of them were preserved in nitrogen.

In the second stage:

excisional wounds were inflicted on the back of adult rats, and different treatments were applied to the wounds, according to the following:

A. Treatment with fibroblasts which was isolated and cultured in the first stage.

B. Treatment with fibroblasts that were isolated and cultured in the first stage after being activated by progesterone.

C. Hormone treatment (progesterone)

In the third stage:

a statistical study was conducted for the measurements of the inflicted wounds during the days of the experiment, on days (0, 3, 7, 14 and 21), and the correlation of the mean surface areas of the wounds was analyzed, and the percentage of wound closure during the days of the experiment was studied.

Through the stages of work, our results showed the great role that fibroblasts play in natural wound healing through decreasing the wound surface area from the beginning of the injury until the fourteenth day and thus the increase in the rate of wound closure, moreover, the important role of steroid hormones in the process of wound healing and the application of topical hormonal replacement therapy.

When comparing these results with the results of other researches in the same field, we found considerable similarity as many researches confirmed the important role of fibroblasts in the treatment of wound healing. They proved the appearance of fibroblasts at the place of injury after 24-48 hours in a research conducted at Cardin University in the United Kingdom in 2013, which led to the activation of wound healing and consequently a decrease in the area of the wound from the day of injury until the fourteenth day (Bainbridge et al., 2013). We also found considerable similarity with research conducted on rats, where deep wounds were inflicted on the back of rats on the right side as a control group and the left side that was treated with fibroblasts. After seven days of treatment, it was noted that the left side healed faster than the right side, and this showed the importance of fibroblasts in

treating and accelerating wound healing (Zhao et al., 2016) ..

From the graph of average wound closure rates, we noticed an increase in the closure rates at the beginning of the experiment until the seventh day represented in green in the samples treated with fibroblasts (B and C) compared to the control case (A) and the sample treated with progesterone only (D). After the fourteenth day the fibroblasts declined to the normal limit and this was consistent with previous studies. By looking at the fourteenth day, represented in orange, for the treated samples, we notice a faster decrease in the wound area in the samples treated with progesterone (C) and (D) compared to the control case and the group treated with fibroblasts only (B). This is consistent with a 2014 study that showed the role of progesterone in stimulating high levels of progesterone Collagen type III, accelerated epithelization from day seven to day fourteen (Nicolau et al., 2014). It is also consistent with a study conducted at the University of Texas on cultured cells (in vitro) that showed the role of progesterone and its effectiveness in closing the wound in a faster time compared to other treated and control cases (GeorgeR.et al, 2019).

We found, then, an increase in closure rates at the beginning of the experiment until the seventh day, represented in green in Figure 9, for samples treated with fibroblasts compared to the control case. After the fourteenth day, the fibroblasts decline to the normal limit. We also noticed an increase in the rates of closure at the beginning of the experiment until the fourteenth day, represented by the orange color in Figure (9), for the groups treated with progesterone compared to the control group, and also an acceleration in wound closure from the seventh until the fourteenth day in the groups treated with progesterone compared to the control group and the group treated with fibroblasts only.

Conclusion:

Supporting the idea of cellular therapy to treat many cases, including: (treatment of chronic wounds, treatment of surgical scars, severe burns and diabetic foot patients)

Starting treatment with fibroblasts after injury and then applying topical progesterone therapy.

Expanding this study from a statistical point of view, building statistical models and using them to predict the surface area of wounds by knowing the time and type of treatment, or using them to predict wound closure through knowing the time and type of treatment.

Increasing the size of the studied sample to reduce the possibility of error, that is, the appearance of significant differences in the form of non-significant differences.

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