ANALYSIS OF THE EFFECT OF SUBCUTANEOUS INJECTION OF OMENTAL-DERIVED CELLS ON THE HEALING OF THIRD DEGREE BURNS IN RATS: A PRELIMINARY STUDY

EFFET DE L'INJECTION SOUS-CUTANÉE DE CELLULES ÉPIPLOÏQUES SUR LA CICATRISATION DE BRÛLURES DU TROISIÈME DEGRÉ CHEZ LE RAT : ÉTUDE PRÉLIMINAIRE

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SUMMARY. Burn injury is considered a global health issue. Third degree burn wounds do not heal spontaneously and require skin grafts. Some factors could contribute to wound healing. In this study we assessed the effect of non-fatty omental cells in burn wound healing. Similar third degree burn wounds were induced on the back of 192 rats. Forty-eight of these rats were put in a control group that did not receive any treatment. The rest of the rats were put in 3 groups, each receiving a different treatment regime. Rats in group 2 had a daily application of silver sulfadiazine; group 3 rats were injected with omental cells, and group 4 rats were injected with phosphate buffer saline (PBS) once, followed by daily application of Vaseline to the burned region. Parameters such as open epidermis length, number of epidermal cell layers, granulation tissue thickness (GTT) and neutrophil density were evaluated in each group. The average open epidermis length in the omental cell group was less than in the other groups on days 10 and 20 ($P<0.05$). The thickness of epidermal cell layers in the group receiving cells was greater than in the other groups on all days. On the 20th day, there was a significant difference in GTT between the four groups ($P<0.05$). The injection of non-fatty omental cells has a positive effect on third degree burn wounds in rats.

Keywords: third degree burn, wound, omental cells

RÉSUMÉ. *Les brûlures sont un problème de santé publique. Celles du 3ème ne peuvent guérir spontanément et requièrent des greffes cutanées. Certains facteurs pourraient contribuer à la cicatrisation. Nous avons évalué l'effet des cellules épiploïques non adipocytaires sur la cicatrisation des brûlures. Des brûlures similaires, du 3ème degré au niveau du dos ont été infligées à 192 rats. Quatre vingt huit d'entre eux, contrôles (groupe 1), n'ont reçu aucun traitement. Les autres ont été répartis en 3 groupes recevant chacun un type de traitement. Le groupe 2 a reçu chaque jour une application de sulfadiazine argentique ; le groupe 3 a reçu une injection de cellules épiploïques ; le groupe 4 une injection de sérum salé suivis d'application journalière de vaseline. La longueur non épidermisée, le nombre de couches de cellules épidermiques, l'épaisseur du tissu de granulation et la densité de neutrophiles ont été évalués. La longueur non épidermisée à J10 et J20 était plus courte dans le groupe 3 ($p<0,05$). L'épaisseur des couches épidermiques était constamment supérieure dans ce groupe. À J20, les différences d'épaisseur du tissu de granulation étaient significatives entre tous les groupes. L'injection de cellules épiploïques non adipocytaires a un effet favorable sur l'évolution de brûlures du 3ème degré chez le rat.*

Mots-clés: *brûlure, 3ème degré, cicatrisation, cellules épiploïques*

Introduction

Burn injuries are one of the leading causes of death in the developing countries of Southeast Asia and the Middle East.[1,2] In Iran, burns are the eighth most common cause of death and the thirteenth most frequent cause of disabilities (DALY).[3]

Severe third-degree burns can lead to secondary infections, which can be a major cause of morbidity and mortality in patients.[4] Burns damage the skin, which is the body's first defence, and in the event of third degree burns, its entire thickness is completely damaged.

Vascular thrombosis also occurs in the areas surrounding the burn. As a result, there is increased edema and dehydration.[5] Skin loss leads to underlying tissues being exposed and a subsequent greater risk of infection, septicaemia and other life-threatening complications.

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Hence, re-epithelialization of damaged tissue and the re-establishment of tissue perfusion are the priorities in burn patient care.

Extensive third degree burns require treatment, including skin grafts, in order to recover. Recovery needs to be fast in order to reduce the lasting effects of these burns. It is important to develop treatments that stimulate and speed up the healing process. Given the positive results of using omental cells in other healing applications,[6,7,8,9,10,11] we decided to study the effects of these cells on the recovery time of third degree burn wounds.

The positive effects of using omental cells are attributed to different factors, such as angiogenesis, chemotactic and progenitor cells derived from the omentum that act as stem cells and can convert to other tissues.[12,13,14] Therefore, we investigated the effects of adipose derived nucleated cells (ADNC) from the omentum on tissue reconstruction and repair of third degree burns. This study opens the way for further studies on this relatively new application of ADNC.

Materials and methods

In this study, we used 192 white, male Wistar rats, 2-3 months old, weighing on average 200-240 grams. They were bred at the animal-breeding centre of Urmia veterinary school. After initial health checks, the rats were placed in individual plastic boxes for at least 14 days for stress elimination and adjustment to the new environment. Sawdust was used to cover the rat-beds. The room temperature was 25±4, and humidity level was 45-55%, in 12 hours light and 12 hours darkness. Air-conditioning was on from 9.00 am to 9.00 pm. Food and water were replenished every morning, and sawdust was replaced every 2-3 days.

After 14 days, the rats were anesthetized using a mixture of Ketamine, Xylazine and Acepromazine, injected intraperitoneally. Their backs were shaved and sterilized and two regions in the shaved area were burned. The burning technique was as follows: a brass hammer was submerged in boiling water for 3 minutes until it reached the water temperature (100 C). Then, the hammer was placed, with no pressure exerted, on the shaved skin for 30 seconds.[15,16,17,18] The contact surface was a circle of 1cm diameter. Immediately after the burn, 2-4 ml of normal saline solution was injected intraperitoneally in order to replenish lost water and electrolytes.

All the burn wounds were full thickness. This was confirmed clinically by an experienced burn surgeon and histologically with biopsies.

The 192 rats were randomly divided into 4 groups of 48:

Group 1 received no treatment after the burn (the control group);

Group 2 were treated with silver sulfadiazine ointment daily;

Group 3 were injected with omental cells once, and Vaseline was applied daily to the burned area;

Group 4 were injected with phosphate buffer saline (PBS) once, and Vaseline was applied daily to the burned area.

After the burn procedure, the rats in the control group were placed in their boxes without any treatment. Silver sulfadiazine ointment was applied to the group 2 rats precisely 1 hour after the burn, and was repeated every day. The group 3 rats received omental cells 5 h after the burn, by subcutaneous injection of around 0.8mL of cell suspension into each burned region. Vaseline ointment was applied to the burned area for 30 days. Each rat received about 3,500,000 cells subcutaneously, based on published resources.[19] The group 4 rats received a subcutaneous injection of PBS five hours after the burn, and Vaseline was applied every day for 30 days.

On days 3, 10, 20 and 30, 12 rats from each group, chosen at random (for the first 3 sampling periods), were sacrificed and burned areas were examined histologically.

Preparing the omentum suspension

The entire abdomen of donor rats (i.e. not the rats in the 4 study groups) was prepared aseptically and after l midline incision, 4-5 gr of omentum were harvested. We then used the corrected Schmidt et al. method for preparing the omentum suspension.[20]

The omentum was washed with Hanks balanced salt (HBS) solution, and cut into very small pieces using a scalpel. They were then transferred to a 50mL Erlenmeyer flask containing 1500 units/ml of type II collagenase (Sigma Germany). The ratio of omentum to type II collagenase was 1 gr to 2 mL. The omental tissue and collagenase suspension were placed in a hot water bath for 40 min, at 37° C, with 100 movements per minute. The homogenized and digested tissue was transferred to 15ml tubes and centrifuged twice for 5 minutes, with an acceleration of 100 rmp. The supernatant portion contained mainly fatty cells and collagenase solution. A suspension was prepared from the pellet cells using 10 mL of PBS.

This suspension was passed through a 250-micrometer mesh, so that the larger, un-digested parts were removed. The remaining solution was washed twice with HBS. The solutions containing the cells were then transferred to insulin syringes. This experiment was designed in such a way that in every 0.1 mL of the syringe there were 4 to 4.5 million cells. In other words, every full syringe contained about 45 million cells. The cellular population count was achieved using a hemocytometer and 45000000 nucleated cells per millilitre of adipose tissue were counted.

Results

In this study, we assessed the restoration of burn-related tissue damage in the 4 different groups from a quantitative perspective, using histopathological methods. Using microscopic analysis, the ANOVA test was performed to measure parameters such as open epidermal length, number of epidermal cell layers, granulation tissue thickness (GTT) and neutrophil density.

Open epidermal length

This parameter shows the length of epidermis that is needed to fill the gap after new epidermis is formed following tissue necrosis. It was measured using biopsies of whole wounds. Analysis of this parameter on days 10 and 20 showed a significant difference among the groups being tested ($P<0.05$).

The average open epidermis length in group 3 was shorter than in the other groups on all the days it was assessed; for instance, on the 10th day of the experiment, this parameter was 3082 micrometres (μm) in the control group, 3005μm in the PBS group, 1903μm in the silver sulfadiazine group, and 1802μm in the omental cell group. On the 20th day, this parameter was 500μm in the control group, 1166μm in the PBS

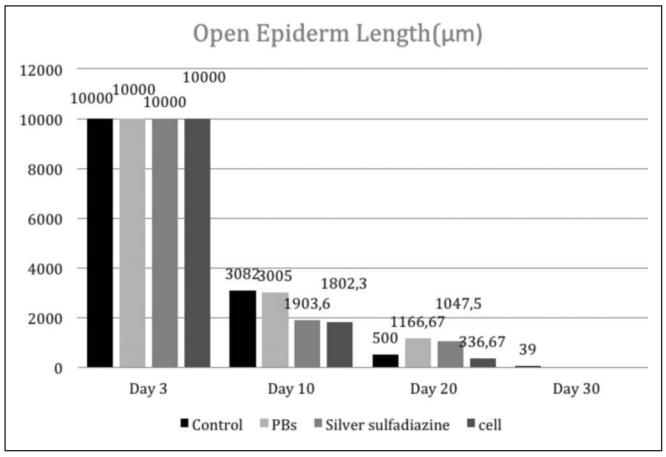

Diagram 1 - Open epidermal length in the four groups (μm).

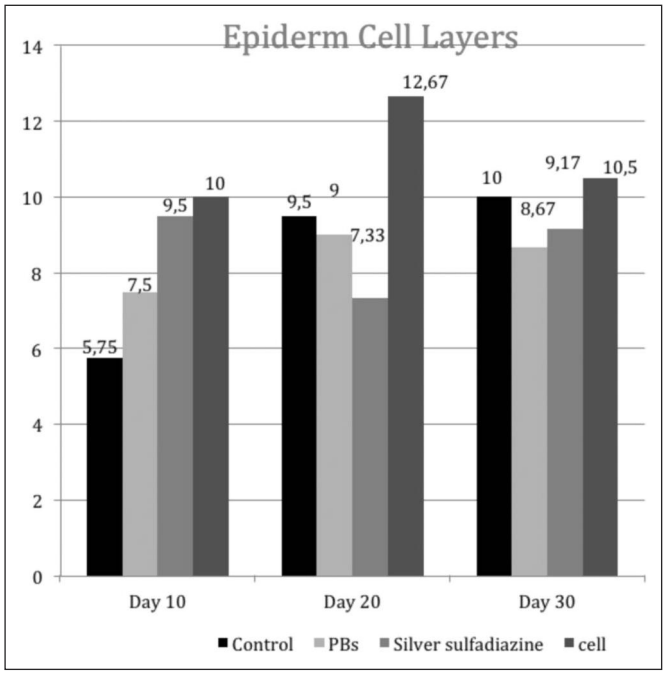

Diagram 2 - Average epidermis cell layers.

group, 1047μm in the silver sulfadiazine group and 336μm in the cell group (*Diagram 1*).

Number of epidermal cell layers

This parameter shows the number of cell layers formed in the new epidermis in the burn area. The tissue samples were taken from the periphery of the wound along with normal skin.

On the 10th and 20th days, there was a significant difference between the groups ($P<0.05$), but on the 30th day, this difference was insignificant ($P>0.05$).

We also noticed that the highest number of cell layers was formed and, hence, the thickest epidermis was seen in group 3 on all the days assessed. For instance, on the 10th day, the average number of cell layers formed in the epidermis was 5.75 in the control group, 7.5 in the PBS group, 9.5 in the silver sulfadiazine group, and 10 in the omental cell group. On the 20th day, the average number of cell layers formed in the epidermis was 9.5 in the control group, 9 in the PBS group, 7.3 in the silver sulfadiazine group, and 12.67 in the omental cell group.

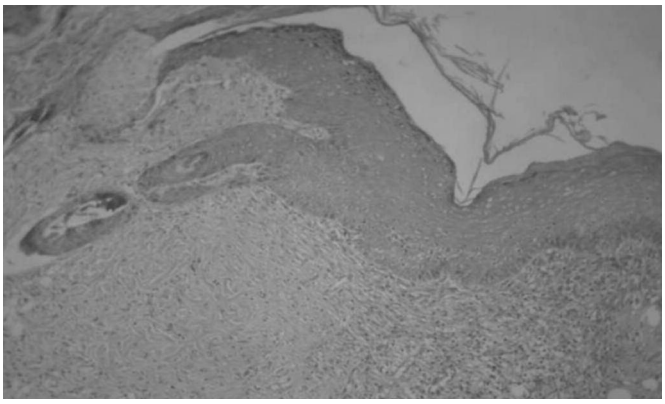

Fig. 1 - Multiple layers of epidermis cells in the healed region, the cell group, day 10, magnification x100.

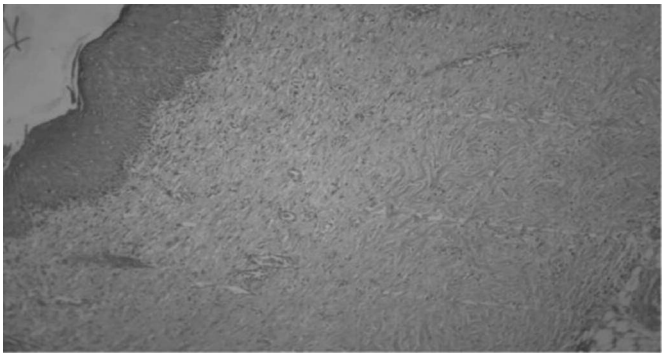

Fig. 2 - Average GT thickness and epidermis layers, silver sulfadiazine group, day 10, magnification x100.

On the 30th day, this number was 10 in the control group, 8.67 in the PBS group, 9.17 in the silver sulfadiazine group, and 10.5 in the omental cell group (*Diagram 2*) (*Fig. 1*).

Granulation tissue thickness (GTT)

We measured GTT using tissue samples taken from the periphery of the wound, as mentioned above. GTT shows the thickness of the tissue granulation formed on various days in the burn region. In this analysis, on the 10th and 30th days no significant difference was seen between the four groups ($P>0.05$). However, on the 20th day there was a significant difference between the four groups examined ($P<0.05$). On the 10th day, the average GTT was 701μm in the control group, 560μm in the PBS group, 719μm in the silver sulfadiazine group, and 660μm in the cell group. On the 20th day, when we expected to see maximum GTT growth in the damaged tissue, there was a significant difference between GTT in the cell group compared with the other groups. In fact, GTT was 1010μm in the control group, 1053μm in the PBS group, 975μm in the silver sulfadiazine group, and 1283μm in the cell group. On the 30th day, we expected a gradual reduction of GTT, and this was the case in all groups except the silver sulfadiazine group. On the 30th day, GTT was 780μm in the control group, 911.6μm in the PBS group, 1086.6μm in the silver sulfadiazine group, and 1040μm in the cell group (*Diagram 3*) (*Fig. 2*). Day 3 is not shown in this diagram because GTT had still not formed.

Neutrophil density (ND)

Due to the fact that infection rate impacts both inflamma-

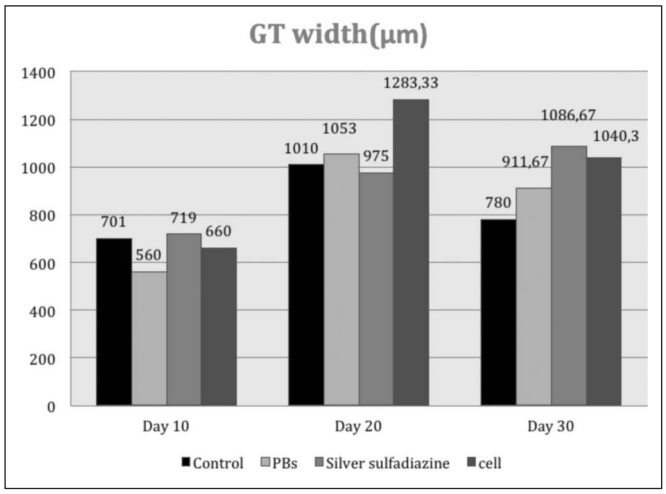

Diagram 3 - Average GTT**.**

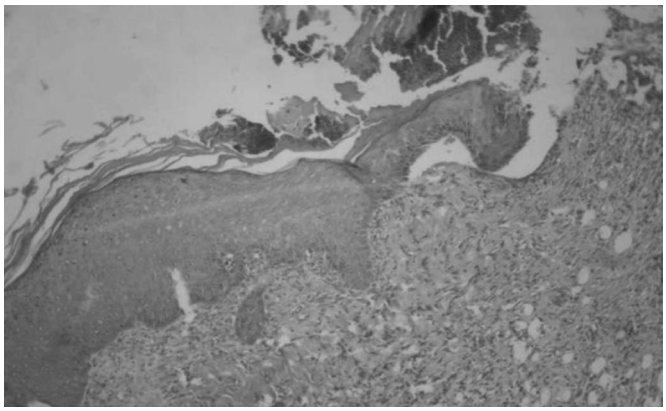

Fig. 3 - Newly-formed epidermal layers in the dense neutrophil area, the PBS group, day 10, magnification x100.

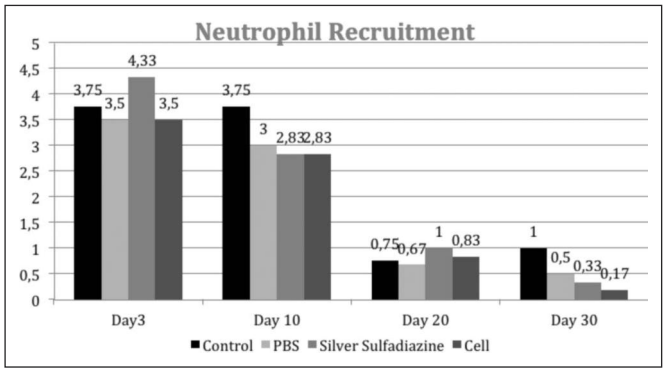

Diagram 4 - Average neutrophil density.

tion rate as well as number of neutrophils, the wounds were examined clinically and experimentally with wound cultures. No clinical infections were found.

Neutrophil density is the number of neutrophils in the burn tissue. It was measured by assigning values of 1 to 5, where:

1 = ND of less than 20 neutrophils;
2 = ND between 21 to 50 neutrophils;
3 = ND of 51 to 100 neutrophils;
4 = ND of 101 to 200 neutrophils;
5 = ND of more than 200 neutrophils in the tissue.

The cell group showed the lowest density compared to the other groups. This indicated that the new epidermis had almost completely covered the injured area, tissue perfusion had established, local inflammation had resolved, and the tissue was completely healed and viable.

The ND average on the days of experiment was as follows:

On the third day, ND was 3.75 in the control group, 3.5 in the PBS group, 4.33 in the silver sulfadiazine group, and 3.5 in the cell group. On the 10th day, this number remained unchanged at 3.75 in the control group, but fell to 3 in the PBS group, and to 2.83 in both the silver sulfadiazine and the cell groups (*Fig. 3*).

On the 20th day, the ND average fell to 0.75 in the control group, 0.67 in the PBS group, 1 in the silver sulfadiazine group, and 0.83 in the cell group. On day 30, apart from the control group, which showed an increase in number of neutrophils from 0.75 to 1, there was a decrease in density. This dropped to 0.5 in the PBS group, 0.33 in the silver sulfadiazine group, and the lowest density, 0.17, in the cell group (*Diagram 4*).

Discussion

Research on tissue and wound healing using omentum has mainly focused on utilizing omental flaps around the damaged tissue rather than merely using the omental cells. Applying omental cells for this purpose is rare, and research has mostly focused on secretory factors and cellular features. Using omental cells in the wound healing process is a very novel area of practice and research needs to be better elucidated. Some omental cells have the ability to express stem cell markers, and they can be counted as natural sources of growth factors. Therefore they can be used in damaged tissues.[13] One study observed that new cells with the ability to produce insulin were formed in the pancreas of diabetic mice when omental cells were transferred to their pancreas.[21]

Furthermore, the role of these cells in liver tissue regeneration has been demonstrated.[12]

Sub-conjunctival injection of activated omental cells showed satisfactory beneficial effects on corneal injuries in mice. There was a reduction in neutrophil number in the damaged tissue.[22] In another study, stem cells derived from human fatty tissues were bonded to the cornea of rabbits by sub-conjunctival injection. The cornea was previously chemically burnt by sodium hydroxide (NaOH). Surprisingly, the healing process was accelerated.[23]

Activated omentum contains immunity-modulating cells as well as multipurpose stem cells, which cooperate in regenerating damaged tissues.[24]

Omental suspensions are also used for blocking gaps in gastrointestinal anastomosis, assisting homeostasis after hepatectomy and filling hepatic liver cyst space. Moreover, they are used to protect unshielded carotid arteries, as a free vascularized link in head and neck surgeries, etc.[25]

Omentum tissue has been successfully used for revascularization of the ischemic brain[26] and myocardium.[27] There are also trials underway to control Alzheimer's disease by injecting omentum suspensions.[28]

Analyzing the charts showed that quick epidermis growth and development took place in the cell group, because after 10 days the control group had the longest open epidermis and the cell group had the shortest. This trend of epidermis growth and maturation continued to the 20th day, with the cell group having

the shortest open epidermis. Interestingly, the longest open epidermis was seen for the PBS group on the 20th day. Injection of omentum cells encourages topical growth and maturation of the epidermis and results in rapid regeneration and completion of the epidermis to cover the burned area. Therefore, this reduces the risk of infection and expedites healing. Omentum cells produce large amounts of vascular endothelial growth factors (VEGF) when activated by traumatic tissues.[18] Human +CD34 omental cells produce angiogenic basic Fibroblast Growth Factor (bFGF) and VEGFs and they can extensively strengthen neovascularization in rats.[29]

One study showed that human omentum expresses large amounts of pre-inflammatory and anti-inflammatory cytokines and anti-microbial peptides.[30] This is consistent with previous investigations on bovine omentum, where it was shown that omentum contained acidic fibroblast which had an outstanding mitogenic activity on fibroblast replication and beta cell endothelial growth factors that play significant roles in vascularization.[31]

The trend of granular maturation and development in the control, PBS and cell groups is more similar. We indicated that on the 10th day it started thin; on the 20th day thickness reached its peak point, and on the 30th day its thickness decreased due to contraction and shrinkage of the tissue. This may indicate the return of the tissue to its natural activity and previous size. On the 10th day, the thickness of the granulation tissue was similar to the other groups. The rate of GTT reduction, between days 20 and 30, was 77% in the control group and 81% in the cell group. This shows faster contraction and shrinkage of granulation tissue in the cell group. In other words, despite the granulation tissue being thicker between the 20th and 30th day, it matured and developed faster than in the control group. Among these three groups, on the day we expected the thickest granulation tissue, i.e. on the 20th day, the cell group had the thickest granulation tissue. In the silver sulfadiazine group, no particular pattern in contraction and shrinkage of granulation tissue was observed and GTT increased.

This was probably due to the substances in this ointment. As we know, a large part of granulation tissue is formed by new micro-vessels and tissue regeneration cells (fibroblasts). As mentioned above, omentum cells have strong vascularization characteristics, and due to their bFGF secretion they cause fibroblast growth. As a result, these cells possess both factors involved in the formation of granulation tissue, and cause early activation and maturation. Therefore, we can expect a significant difference in the thickness of granulation tissue in the cell group.

Development of newly-formed epidermis will lead to maturation of the epidermal cells and a decrease in the cellular population. In turn, this causes gradual thinning of the epidermis to its natural thickness. This process seems to take longer in the control group. Therefore, the time required for healing is greater. In the cell group, the increase and decrease trend of epidermis cell layers is different to that in the control group. On the 10th day, the number of cell layers started from an average of 10, and reached its peak with an average of 12.6 layers on the 20th day. On day 30, this number fell to 10.5 layers.

Hence, there were two findings regarding the cell group: first, the number of epidermal cell layers was greatest upon cell suspension injection. Second, the development and maturation of epidermis layers occurred faster. The chart for the PBS group is similar to the one for the cell group. However, the difference lies in the number of cell layers, which is lower during all 3 time intervals. Furthermore, there is an evident difference in the silver sulfadiazine group, with 9.5 layers on the 10th day, 7.4 layers on the 20th day, and 9 layers on the 30th day. In this group, development and maturation of the epidermis did not occur as we expected. There were differences in the number of cell layers, which was most likely due to substances in the ointment. This will require further research.

In this study, we showed that the greatest neutrophil recruitment was seen in the silver sulfadiazine group, with an average of 4.33 on the 3rd day. Subsequently, with the completion of the epidermis in all groups, neutrophil density was reduced. The greatest reduction occurred in the cell group, with a density of 0.17 on the 30th day. IL-1β and IL-8 are well-known neutrophil chemo-attractants produced by infected or damaged tissues.[32] A hypothesis would be that omental immune adjuster cells suppress the expression of inflammatory cytokines, such as IL-1β and IL-8. Consequently, the enclosure of the scar, contact between the tissue and the outside environment, the presence of infectious factors and recruitment of inflammatory cells are obviously reduced upon the development of epidermis; whereas the burn area remains exposed to secondary contamination in the control group on the 30th day.

After the control group, the PBS and the silver sulfadiazine groups have the highest neutrophil density on the 30th day. Although the scar was completely covered in the PBS and the silver sulfadiazine groups, there were still some inflammatory cells present and the inflammation had not yet contracted. Therefore, we propose that cells derived from the omentum may be effective in improving subcutaneous tissue damage in burned mice. A study by Mehrtash et al. demonstrated the effect of adipose derived nucleated cells on wound healing.[19]

Study limitations

One of the drawbacks of this study was that due to the rats only being sampled once, no rat was compared with itself on different days.

The results of this study may have been more reliable if all the types of wound treatment had been used on the same rat.

A major disadvantage of this study is that the contraction of the wound was not assessed. Another limitation is that the immunological ramifications of ADNC have not been demonstrated.

Furthermore, allograft cells were not used, and in further research it would be best to use autologous ADNC.

In conclusion, the findings of this study indicate that the injection of non-fatty cells derived from omentum have a positive effect on wound healing in burned mice. The current data highlight the importance of omentum in clinical applications. However, more experiments are required to determine the immune-modulating mechanisms attributed to omentum-derived cells and optimize procedures to harvest the most effective outcomes.

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