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Delayed Wound Healing in Aged Mice is Associated with Traumatic Stress, not with EGFR Block

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Abstract

Background and Aims: Studies evidenced no wound healing risks related with EGFR blockade but it is unknown whether anti-epidermal growth factor receptor therapy poses an additional risk for the wound healing process in aged patients. The objective was to evaluate the effect of EGF block in the wound healing of aged mice.

Methods: BALB/c mice were used for this study, a half with 11-12 weeks of age and the others with 53 or more weeks of age. EGF was blocked with a monoclonal Antibody specific to the murine EGFR (7A7 mAb) on days 6, 4 and 2 before and days 2 and 5 after the skin wound on the back of each animal while Phosphate Buffer Solution injection was used as control. Two groups were maintained as wound healing control, without treatment. Wound closure dynamics were measured and the planimetry study was carried out. Also, histological score was determined after skin injury.

Results: No significant differences in wound healing between different ages, without treatment, were detected. Surprisingly, a delayed wound healing in all treated aged mice was evidenced. As conclusion, aging or EGF block *per se* had not a deleterious effect on the healing process in adult mice but stress and aging combination can significantly affect wound healing.

Keywords: Age; EGF; Stress; Mice; Wound healing.

Introduction

In many human epithelial cancers there is an increased expression of Epithelial Growth Factor (EGF) and its receptor (EGFR). Therefore, EGF/EGFR system is an interesting target for novel anti-tumor therapy [1]. Passive immunotherapies with anti-EGFR antibodies (mAb) are in clinical trials along or combined with conventional treatments, showing anti-tumor activities [2-4].

The epidermal growth factor ligand plays a very important role in both, normal and pathological wound healing process; it promotes dermal wound healing stimulating proliferation and migration of keratinocytes. EGF also stimulates formation of granulation tissue and fibroblast motility [5].

Life expectancy is progressively increasing worldwide; advancing age is a very important factor for develops many types of can-

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cers over the world. Thus, the demand of cancer cares, surgeries, chemo-radiotherapies and immunotherapies are also increasing [6,7].

The relationship between ageing and wound healing had been examined previously but there are contradictory criteria concerning the effect of age in wound healing; some authors conclude that healthy aging people have a delayed cutaneous wound healing [8-10]. However others have evidenced a normal wound healing process in elderly people [11].

In the other hand, previous studies evidenced no impaired wound healing process related to the EGF/EGFR blockade [12-14]. It is unknown whether anti-epidermal growth factor receptor therapy possesses an additional risk for the wound healing process in aged patients. Thus, search to evaluate the effect of EGFR block on the wound healing process of aged mice using the 7A7 mAb, a specific antibody against the murine EGFR [15].

Materials and methods

Ethics statement

All studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee from the National Center for Laboratory Animal Breeding, with permit number 17/17.

7A7 mAb

7A7 mAb is an anti-murine EGFR extracellular domain monoclonal antibody (IgG1). It was generated by immunization of BALB/c mice with the recombinant extracellular domain of murine EGFR as a valuable tool for EGFR-based therapeutic pre-clinical studies in mice, in order to allow a more effective extrapolation of the pre-clinical data to the clinical setting [15]. This mAb was also described to prolong survival and show antimetastatic effects in a D122 mouse tumor model [16].

Mice and immunization protocols

Female BALB/c/Cenp mice with 11-12 weeks and 53 or more weeks of age were obtained from the National Center for Laboratory Animal Breeding (CENPALAB, Havana, Cuba) and maintained in standard racks (Tecniplast, Varese, Italy). Autoclaved food EAO 1004 (CENPALAB, Havana, Cuba) and water were offered *ad libitum*. Room temperature (20–23°C), humidity (65 ± 10%) and the photoperiod cycles (12 h per day), were automatically controlled.

Mice were treated with 2.8 mg/kg of 7A7 mAbor with Phosphate Buffered Saline (PBS) by intraperitoneal way on days 6, 4 and 2 before and days 2 and 5 after the skin wound. A group of each age was maintained as wound healing control, without treatment.

Wound healing model

All animals were anesthetized with intramuscular ketamine chloride (50 mg/kg) (AICA, Havana, Cuba) and their dorsal regions were depilated and washed with sodium chloride (NaCl) 0.9% (LABIOFAM, Havana, Cuba) and ethanol 70%. Then, 8 mm diameter, full-thickness skin wound was performed on the back of each animal with a biotome (Acu Punch, Acuderm Inc., Fort Lauderdale, FL) in aseptic conditions.

In-life observations

The animals were monitored twice a day by an experienced technician for any abnormal reactions, health problems or complications, and to determine if significant clinical abnormalities were present in animals from any of the treatment groups. All animals were weighed weekly using a precision balance (Sartorius, Germany).

Wound closure dynamics were measured with a caliper (Mitutoyo, Japan) at days 0, 2, 5, 8 and 12. Area was calculated by: $\pi*a*b$ (*a* and *b* are radius of ellipse)

Digital photographs of the wounds were taken on days 8 and 13th after skin wound and the planimetry study was carried out on skin image (2 images/animal). Digitalized images were treated with the DIGIPAT IBM/PC computer system [17] and the following parameters were determined:

1. Percent of total re-epithelized area, the percentage of wound closure was calculated as: (area of original wound – area of actual wound)/area of original wound × 100.

2. Percent of reduction in wound perimeter, was calculated as (perimeter of original wound – perimeter of actual wound)/ perimeter of original wound × 100.

Histological preparation

A half of mice were euthanized at 8th day post-surgery and the rest on the 13th day. Ulcer area and a portion of surrounding tissue were excised using surgical scissors. The samples were fixed in 10% buffered formalin and paraffin-embedded sections were stained with hematoxylin/eosin. Samples were blindly evaluated by two pathologist for determining the extent of the healing process. Also, histological score for wound healing was determined by two independent observers under an optical microscope using semi-qualitatively graded as follow [18]:

Epidermis

Grade 1: Incomplete reepithelialization, scanty projection of the epidermal edges with thin thickness.

Grade 2: Complete reepithelialization with thin epidermal thickness and permanence of the desiccated clot.

Grade 3: Complete reepithelialization with moderate thickness of the regenerated epidermis. Absence of the desiccated clot.

Dermis

Grade 1: Some collagen fibers in the neomatrix with no organization and focally distributed. The infiltration of macrophages and angiogenesis is evident.

Grade 2: More presence of collagen fibers, partially orientated in location parallelly to the epidermis. Persistence of some dilated blood vessels.

Grade 3: Complete restitution of the new matrix with collagen fibers horizontally orientated. Absence of macrophages and scanty collapsed blood vessels.

Statistical analysis

All statistical analyses were carried out using Minitab Data

Analysis Program version 14 (Minitab Inc for Windows, 2003). Statistical evaluation was performed by a randomized complete Analysis of Variance (ANOVA) design with significance assessed at $p<0.05$ level or by the unpaired t-test. When data did not have a normal distribution, the Kruskall–Wallis test and the two-tailed Mann Whitney test were used. The statistical evaluation of histological semi-qualitative analysis was performed by two-way ANOVA.

Results

Ageing per se does not affect skin wound repair

Non-treated young and aging BALB/c mice with wounds in their backs were observed daily and wound areas and planimetry studies were performed at previously determined days. Figure 1 shows that wound closure dynamics between ages was similar. Additionally, Table 1 shows there were no significant differences between young and ageing mice according to the studied parameters.

7A7 mAb was well tolerated in aged mice

No clinical signs were evidenced in mice during the whole experiment observational time period. No differences were seen in body weight of immunized mice with full-thickness skin wound respect to control animals, independently of age (Figure 2). Additionally, no changes appeared at the inoculation site in mice.

Skin wound repair in aged mice is not affected by EGFR block

A group of aged mice was immunized with 7A7 mAb or PBS as control and a full-thickness skin wound was performed. As shown in Figure 3, no significant differences were evidenced in wound closure dynamics within age groups ($p>0.05$, Mann Whitney no parametrical test).

During the hold study and after surgery no wound healing complications were observed in mice. In the histopathological study of the resected skin displayed no complications of wound healing, such as hyperplasia or changes in pigmentation in any animal.

Wound healing is delayed in all aged treated mice compared to young mice

Clinical observation of mice skin (Figure 4) suggests a delayed wound healing in all aged mice, compared to young mice.

Planimetry study, using DIGIPAT software, on days 0, 8 and 13 after skin wound evidenced a significant reduction of studied parameters: percent of total re-epithelized area and percent of reduction in wound perimeter in all aged mice in comparison with young mice, independently of the treatment (Figure 5).

Histopathological study on day 13 showed, in all young mice, a corrected skin healing, independently of the received treatment; with a completed wound re-epithelization, moderate thickness epidermis, without scabs and a complete restitution of neo matrix in dermis, characterized by abundant horizontally oriented without macrophages and sanguineous vessels. On the contrary, in the 13th day after surgery, several aged animals showed scab persistency in epidermis, collagen fibres with partial horizontal orientation and abundant dilated sanguineous vessels in dermis.

Histological semi-qualitative analysis of wound repair (Table 2) and statistical analysis by two-way ANOVA corroborate no significant differences in wound healing between treatment inside each age groups whereas the comparison between age according therapy.

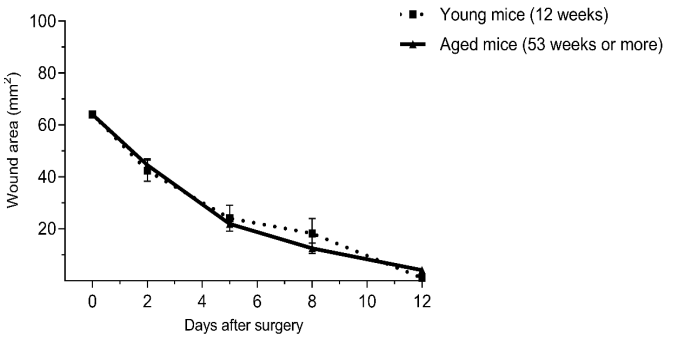

Figure 1: Wound closure dynamic in young and aged BALB/c mice without treatment. Full-thickness skin wound, 8 mm diameter, was performed on the back of each animal. Wound size was measured with a caliper at days 0, 2, 5, 8 and 12. The values represent mean and SEM in each group. No significant differences were evidenced (two-tailed Student test, $p > 0.05$).2 experiments, n = 10.

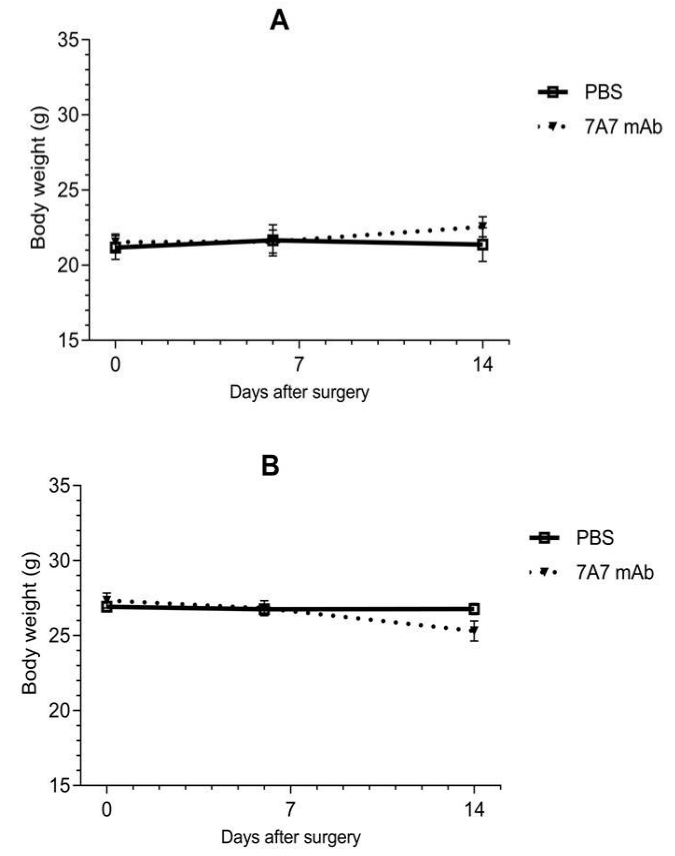

Figure 2: Body weight in young and aged BALB/c mice with full-thickness skin wound (8 mm diameter) on the back immunized with 7A7 mAb or maintained as control (PBS). The values represent mean and SEM in each group. No significant differences were evidenced (two-tailed Student test, $p > 0.05$). **(A)** Young mice. **(B)** Aged mice. n = 10.

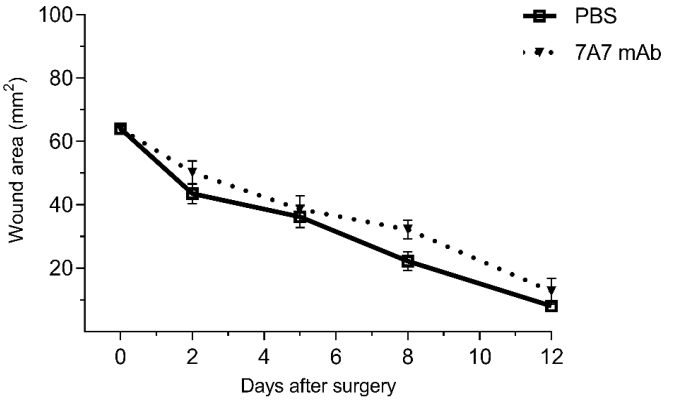

Figure 3: Wound closure dynamic in aged BALB/c mice treated with PBS or 7A7 mAb. Full-thickness skin wound, 8 mm diameter, was performed on the back of each animal. Wound size was measured with a caliper at days 0, 2, 5, 8 and 12. The values represent mean and SEM in each group. No significant differences were evidenced within age groups (two-tailed Student test, $p > 0.05$).n = 10.

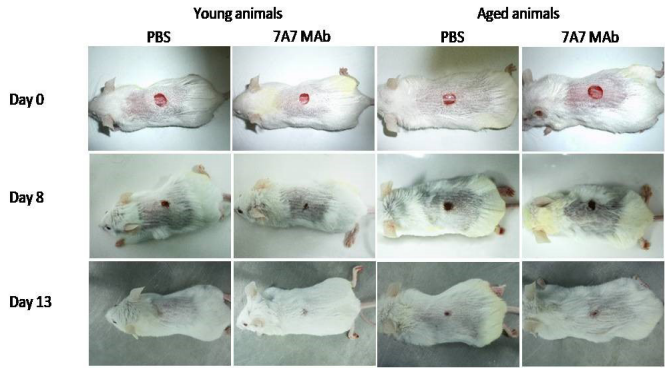

Figure 4: Skin deep wound closing dynamics in young and aged BALB/c mice immunized with 7A7 mAb or maintained as control (PBS).

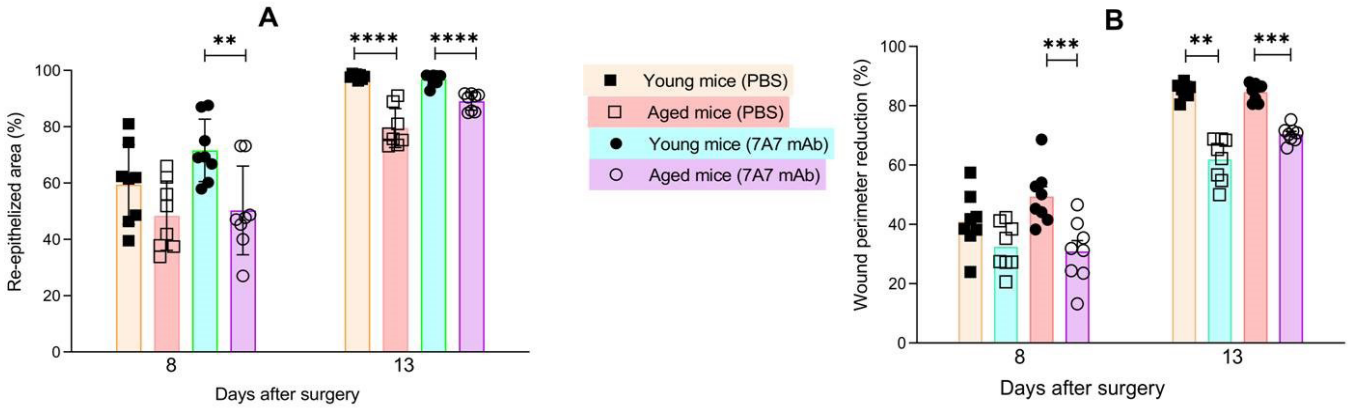

Figure 5: Wound planimetry values 8 and 13 days after surgery in young and aged BALB/c mice treated with 7A7 mAb or PBS. **(A)** Re-epithelized area (%), **(B)** Wound perimeter reduction (%). The values represent mean and SEM in each group. Mann Whitney no parametrical test (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). n = 8.

Table 1: Wound planimetry values 8 and 13 days after surgery in young and aged BALB/c mice without treatment.

Day	Age	Re-epithelized area (%)		Wound perimeter reduction (%)	
		Median	Range	Median	Range
8th	Young	49,00	37,10	28,60	31,50
	Aged	51,90	62,32	42,50	23,50
13th	Young	98,075	0,585	86,98	0,76
	Aged	93,54	3,58	75,19	5,15

Discussion

In response to tissue damage, the innate immune system responds trying to repair the tissue damaged integrity and its normal physiological functions [19]. During the aging process also some intrinsic and extrinsic factors produce alterations, e.g. hormonal change levels, sun and contaminants exposition [20].

In the present study, no differences were evidenced in wound healing between young and aged mice without treatment. This

Table 2: Histological semi-qualitative analysis of wound repair in young and aged BALB/c mice with full-thickness skin wound (8 mm diameter) on the back, immunized with 7A7 mAb or maintained as control (PBS).

Day	Age	Treatment	Epidermis (% of animals)			Dermis (% of animals)		
			1	2	3	1	2	3
8th	Young mice	PBS	0	67	33	0	33	67
		AcM 7A7	67	0	33	33	33	33
	Aged mice	PBS	100	0	0	0	100	0
		AcM 7A7	100	0	0	0	100	0
13th	Young mice	PBS	0	0	100	0	0	100
		AcM 7A7	0	0	100	0	0	100
	Aged mice	PBS	0	60	40	0	60	40
		AcM 7A7	33	0	67	33	0	67

result agrees with the current thinking that the effect of age after controlling known associated factors is not marked and that wound healing in healthy older people is essentially normal [11,21] despite alterations to individual processes.

It is known that EGF ligand regulates many aspects of wound healing, including inflammation, wound contraction, proliferation, migration, and angiogenesis. Previous studies about the effects of anti-EGF/EGFR therapeutic anti-cancer drugs on the wound healing process concluding that apparently, this kind of treatment do not affect wound healing [12]. The present research demonstrated no impact of EGFR block in skin wound repair, in aged mice. A similar result was obtained by Fernández et al., who in a retrospective study elucidated that old patients previously depleted of EGF and receiving surgical procedures were without post-surgical wound healing complications [14].

In spite of aged people has alterations in the normal skin process, it is not well documented that advanced age impairs wound healing *per se*. Nevertheless, there is some inevitable injures in old people that could produce local and systemic problems impairing wound healing [22].

There are many factors associated with the wound healing process such as pain and stress [23]. Contemporary evidences of different types of stress indicate an important impact on human and animal wound healing [24,25]. Animals submitted to stress heal a wound slow than control animals [26,27].

Neuro-hormonal mechanisms of stress originated from the hypothalamus release hormones from the pituitary gland which stimulates glucocorticoid hormone production, mainly cortisol in the adrenal cortex [28]. Normal inflammatory and immune responses can be modified by stress due to a maintained release of cortisol [29]. Therefore, a depression of some inflammatory cytokines (IL-1α, IL-1β, TNFα) and loss of matrix metalloproteinase regulation interrupt the normal wound healing cascade [30,31].

Some authors had evidenced age-related changes in immune cell populations and chemokine production during wound healing [32-34]. The results showed here evidenced the negative effect of repeated injections (PBS or 7A7 mAb) on aged mice wound healing. It corroborates the harmful effect of stress and age which should be related with reduction in the IL-1 producing capacity by cells of old mice associated with immunosenescence [35]; this factor, joined to age-related alterations of collagen fibrils, impaired skin structure and function [36] and delayed angiogenesis [32,37] creates a tissue microenvironment that promotes delayed wound healing in stressed aged individual. However, specific mechanisms related with delayed wound healing in aged stressed individuals were not evaluated in the present study, which is a limitation for this study. Further research need to be conducted to investigate the relationship between physiological mechanisms underlying delayed wound healing in aged individuals and its relation with stress.

Conclusion

In summary, our data showed that aging or EGF block *per se* had not a deleterious effect on the healing process in adult mice but stress and aging combination can affect significantly wound healing.

Declarations

Data availability: The data that support the findings of this study are available from the corresponding author on request.

Author contributions: All authors contributed to the study conception and design:

Conceptualization: Dasha Fuentes, Angel Casacó

Data Curation: Daniel Jay, Nidia Fernández

Investigation: Dasha Fuentes, Daniel Jay, Nidia Fernández, Belinda Sánchez

Methodology: Dasha Fuentes, Angel Casacó, Nidia Fernández

Supervision: Angel Casacó

Writing – Original Draft Preparation: Dasha Fuentes

Writing – Review & Editing: Angel Casacó, Belinda Sánchez

All authors read and approved the final manuscript.

Conflict of interest: The authors declare that they have no conflicts of interest.

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