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Effects of psychological stress and housing conditions on the delay of wound healing

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This study explores the effects of stress and housing conditions on the healing of cutaneous wounds and its relationship with circulating levels of corticosterone. Specifically, we set out to examine the effect of combined physical (restraint stress and ultrasound) and psychological (predator scent) stressors on the cutaneous wound healing of female mice that had been housed either in groups (with social support; n= 16) or individually (without social support; n= 16). In contrast with other studies, the model of multiple ethological mild stressors utilized in this study significantly increased the levels of corticosterone, but failed to dramatically alter the healing of skin wounds. However, the results of this study provide evidence of the importance of housing conditions, suggesting that positive social interactions in females accelerate the rate of wound healing, and reduce levels of anxiety and circulating corticosterone. The level of anxiety, as well as the basal levels of corticosterone, proved to be valid predictors of the healing rates during different stages of cutaneous wound healing.

Efectos del estrés psicológico y de las condiciones de alojamiento en el retraso en la cicatrización de heridas. Este trabajo explora los efectos del estrés y de las condiciones de alojamiento, en el proceso de cicatrización de heridas cutáneas, y su relación con los niveles de corticosterona circulante. Concretamente, proponemos examinar el efecto combinado de estresores físicos (estrés por inmovilización y ultrasonidos) y psicológicos (olor de un depredador), en la cicatrización de heridas cutáneas en ratones hembras alojados en grupo (con apoyo social; n= 16) o individualmente (sin apoyo social; n= 16). Frente a otros estudios, el modelo etológico de estresores medios múltiples utilizado en este trabajo incrementó los niveles de corticosterona, pero no alteró de manera significativa el proceso de cicatrización de las heridas en la piel. Sin embargo, los resultados de este estudio muestran la importancia de las condiciones de alojamiento, sugiriendo que las interacciones sociales positivas en hembras aceleran el proceso de cicatrización de las heridas y reducen los niveles de ansiedad y de corticosterona circulante. Tanto el nivel de ansiedad como los niveles de corticosterona basal mostraron ser predictores válidos del nivel de cicatrización en diferentes momentos del proceso de curación de heridas cutáneas.

The proper healing of cutaneous wounds is critical for an animal's survival. A break in the integrity of the integument turns on a complex series of mechanisms, including the coagulation cascade, inflammatory cell influx, chemokine and growth factor secretion, angiogenesis, cellular motility and proliferation, as well as granulation. Although one can didactically divide the process of wound healing into three stages: inflammation, tissue formation and remodelling, many of their aspects tend to occur simultaneously.

For more than 50 years, glucocorticoids have been known to exert deleterious effects on the rate of wound healing. Following these initial observations, various studies showed that physical and/or psychological stressors also prolonged healing of tissue wounds. Studies utilizing prolonged restraint of rodents showed that this stressor significantly delayed the process of cutaneous wound healing by inhibiting the early influx of inflammatory cells and the secretion of cytokines such as interleukin (IL)-1β (Gallucci et al., 2000), and attributed these effects to elevated levels of corticosteroids (Li, Liege, Moze, & Neveu, 2000; Padgett, Marucha, & Sheridan, 1998; Rojas, Padgett, Sheridan, & Marucha, 2002). Furthermore, previous studies have shown that glucocorticoid receptor blockade attenuate stress-impaired wound healing (Padgett, 1998). However, the administration of phentolamine, a non-specific α-adrenergic antagonist, also restores stress-impaired healing (Eijkelkamp, Engeland, Gajendrareddy, & Marucha, 2007), suggesting that the inhibitory effect exerted by stressors over the wound healing process does not depend exclusively on adrenal corticosteroids (Eijkelkamp, 2007; Saito, Tazawa, Yokoyama, & Saito, 1997).

Fifty years ago, Toivanen et al., (Toivanen, Hulkko, & Naatanen, 1960) reported that psychological stress delayed wound

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healing in the skin of group housed male, but not female rats. Subsequently, Cohen (Cohen, 1979) observed similar delays in mice, however, without specifying genders. Similar findings were reported for healing of jejunal anastomoses in male rats (Derr, 1981). More recently, investigators showed that restraint stress prolonged healing of skin wounds in group housed female SKH and male C57BL/6 mice; however, social stressors did not affect the healing in the latter (Padgett, 1998; Sheridan, Padgett, Avitsur, & Marucha, 2004). Detillion et al., (2004), showed that a positive social intervention based on housing conditions (pair vs. single housed) had a significant influence in the rate of wound healing following restraint stress in female hamsters, which appeared to be mediated through the release of oxytocin (Detillion, Craft, Glasper, Prendergast, & DeVries, 2004). In the latter study, housing conditions had no effects on wound healing if not preceded by stress.

In order to analyse how social support may impact on the negative effects of stress on wound healing, we set out to examine the effect of combined physical (restraint) and psychological (predator scent) stressors on the cutaneous wound healing of female mice that had been housed in groups or in isolation.

Previous studies have supported the notion that individual behavioural characteristics (i.e. coping strategies) expressed by subjects in stressful situations could account for different immune responses to a stressor (Bartolomucci et al., 2001; Bohus, Koolhaas, Heijnen, & de Boer, 1993; Gasparotto, Ignacio, Lin, & Goncalves, 2002; Marsland, Bachen, Cohen, Rabin, & Manuck, 2002; Vegas, Fano, Brain, Alonso, & Azpiroz, 2006). The current study was performed to determine the effect of a model of a mild ethological stressor or housing conditions on the healing of cutaneous wounds, the possible impact of differential individual coping (as determined by latency to emerge from a tube) and its relation to levels of circulating corticosterone.

Methods

Subjects and husbandry

Six week-old female hairless SKH1 mice (SKH:H-1-hrBr) were obtained from Charles River Laboratories (Wilmington, MA), reared in isolator cages, and acclimated for two weeks prior to beginning the experiment. Upon arrival they were randomly assigned to their social conditions and housed in an AAALAC-accredited (Association for Assessment and Accreditation of Laboratory Animal Care), climate-controlled facility (20 °C±1 °C at 50% humidity), with a 12 h light/dark cycle under fluorescent lights, and fed *ad libitum* with a commercial diet and water. All procedures were approved by the University Committee of Animal Resources of the University of Rochester (UCAR). Animals were housed individually (n= 16) or in groups of 4 per cage (n= 16) for 14 days in standard transparent plastic cages.

Extensive studies have established that female mice have regular ovarian cycles when living alone, but in groups exchange pheromones that prolong the lifespan of the corpus luteum, resulting in long progesterone dominant cycles, if not pseudopregnancies (Lee-Boot Effect; Lee & van der Boot, 1955). We chose not to perform vaginal lavage to evaluate the phase of the cycle because the necessary handling reduces reactivity of the adrenal axis, and could potentially attenuate the stress caused by predator odors and restraint.

Stress paradigm

After 14 days of housing, half of the mice in each housing group were assigned to either the stressed (S) or the non-stressed control (NS) sub-groups.

Mice assigned to the S group were restrained for 120 minutes in an apparatus that consisted of modified 50 ml centrifuge tubes. These were adequately ventilated, and the caps contained a compartment that allowed the placement of a gauze pad impregnated with 1 ml of predator urine as source of multiple predators' scent (Funk & Amir, 2000); a wire mesh prevented direct contact between the animals and the gauze. The mice were exposed to the odors of grey fox urine, bobcat urine, coyote urine, red fox urine and raccoon urine (Kishel's, East Aurora, NY). During the two hours of restraint the mice were also exposed to ultrasound emitted by a unit with a pressure level of 96dB at 0.5 meter and a variable frequency ranging between 3 and 40 kHz (Transonic Pro, Bird-X, Chicago, IL). Mice were exposed to this stress paradigm for 12 days prior to and 12 days post-wounding, for a total of 24 days of stressor exposure.

Wounding and wound measurement

After 12 days of exposure to stressors, two 3.5 mm round wounds were placed on the shoulder blades of all mice. Mice were briefly anesthetized with isoflurane in oxygen-enriched air. Using a sterile 3.5 mm biopsy instrument (Miltex Instrument, Bethpage, NY, USA), two symmetrical, 3.5 mm cutaneous wounds were created between the shoulder blades of each mouse, to prevent them from reaching and grooming their wounds. Additionally, mice that were group housed were filmed for 2 hours during the dark period, utilizing video-tracking software, to rule out that they groomed each other's wounds, which could account for differential healing rates.

Digital images of the wounds were obtained daily until healing was complete. Each photograph included a standard-sized reference circle (3.5 mm ID). The wound size of each mouse was determined using imaging software (NIH, Bethesda, MD) and expressed as the percentage of wound area (in pixels) in relation to the area of the standard circle in the photograph.

Defensive Withdrawal Test (DWT)

The defensive withdrawal test has been validated as a test of anxiety in rats (Yang, Gorman, & Dunn, 1990) and mice (Bhatnagar, Nowak, Babich, & Bok, 2004). The defensive withdrawal procedure was carried out as described by McElroy et al., (2002), with some minor modifications (McElroy et al., 2002). Immediately following the initial exposure to stressors, we determined the time to emerge from the restraint tube for each individual animal. Following restraint, the tubes were carefully placed in an open field (40×40×30), the caps removed, and the latency to emerge from the restraint tube was measured, with a maximum cut-off of 5 minutes. The latency to exit the chamber, defined by the placement of all four paws into the open field, was recorded (in seconds).

Corticosterone assays

Blood was obtained for corticosterone measurements at baseline (prior to the initial stress exposure), and after 11 and 21 days after initiation of stress. Stressed animals and controls were bled after 45 minutes of stressor exposure. Blood samples (50-100μl) were

collected from the submandibular vein of un-anesthetized animals (Golde, Gollobin, & Rodríguez, 2005) into heparinized tubes. All samples were collected between 9:00-10:00 AM. Following centrifugation at 4 ºC for 20 min at 2,400 g, plasma was collected and stored at -70 ºC. Plasma corticosterone concentration (ng/ml) was determined using commercially available ELISA kits (Assay Designs, Ann Arbor, MI, USA), employing an Opsys MR Microplate Reader (ThermoLabsystems, Chantily, VA, USA) and Revelation QuickLink Software at 490 nm. Assay sensitivity was 5 pg/tube and intra- and inter-assay variation coefficients were 7 and 8%, respectively.

Data analysis

Two-way analysis of variance (ANOVA) for repeated measures was conducted to examine stress and housing effects on wound closure and on corticosterone levels. Mauchly's test was used to assess sphericity, and non-sphericity was corrected for using the Lower-bound correction. Post hoc comparisons were conducted using Fisher's PLSD test.

The relationships between the scores for percentage wound remaining and corticosterone levels, and the latency to escape from the restraint tube were examined using Pearson's correlation coefficients. Finally, a multiple regression analysis (stepwise) was carried out using the basal corticosterone levels and the latency measurements as independent variables, and each day of the wound healing was assessed as dependent variables. This analysis enabled us to determine the impact of each of the variables considered on the wound closure.

Effects were considered statistically significant if $p<0.05$.

Results

Effects of stress and housing on wound healing

There was a significant effect of day ($F_{(12,336)}= 414.6$; $p<0.0001$) and housing ($F_{(1,28)}= 20.8$; $p= 0.0001$) on the percentage of wounds remaining over time. Wounds were significantly larger in the individually-housed mice compared to group-housed mice on days 1-11 post-wounding (Fig. 1). There was no significant effect of stressor exposure ($F_{(1,28)}= 2.8$; $p= 0.104$) on wound healing.

Effects of stress and housing on plasma corticosterone levels

There was a significant effect of day ($F_{(2,56)}= 38.6$; $p<0.0001$) and stress exposure ($F_{(1,28)}= 25.4$; $p<0.0001$) on plasma corticosterone level. Stressed mice showed higher corticosterone levels relative to non stressed mice on both 11 ($p<0.0001$) and 21 days ($p= 0.0002$) after the start of stress (Fig. 2). Post hoc comparisons revealed no differences between the stress group-housed mice and the control groups on day 21. The effect of housing approached significance ($F_{(1,28)}= 2.7$; $p= 0.078$); orthogonal planned comparisons indicated that individually-housed mice had significantly higher basal corticosterone levels than the animals housed in groups ($p= 0.001$). At no other time point were there significant differences in corticosterone levels between the grouped and isolated animals.

Corticosterone levels, Defensive Withdrawal Test and wound healing

Pearson's correlation coefficients were calculated for corticosterone levels and the % of wound remaining prior to initiation of stress,

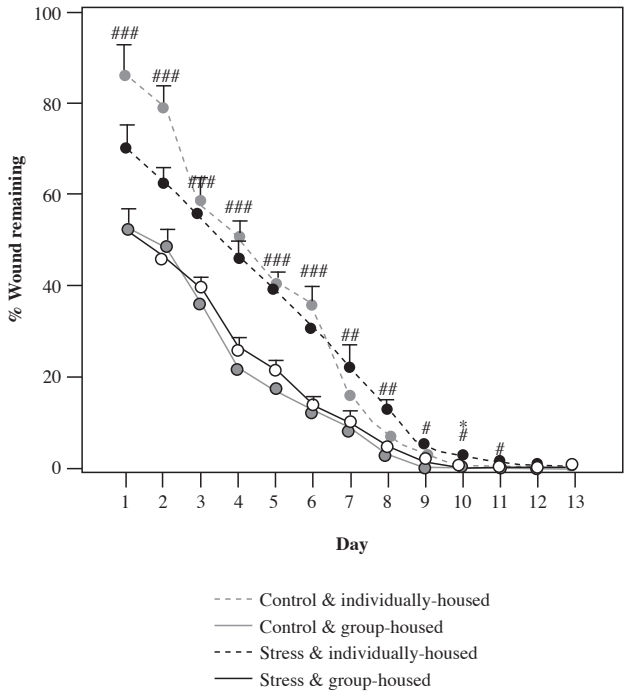

Figure 1. *Effects of stress and housing on wound healing. Effects of the multiple stress paradigm and housing conditions on wound healing. Data represent mean ± SEM, n= 8/group. # comparison between individually- and group housed mice (# p<0.05, ## p<0.01, ### p<0.001); * comparison between all stressed animals compared to all control mice, p<0.05*

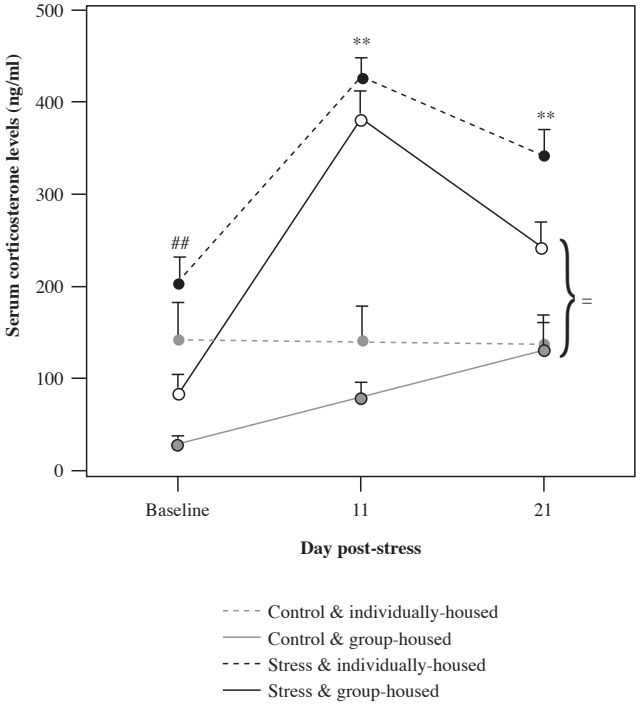

Figure 2. *Effects of stress and housing on corticosterone levels. Effects of the multiple stress paradigm and housing conditions on plasma corticosterone levels. Data represent mean ± SEM, n= 8/group. ## comparison between individually- and group housed mice, p<0.01; ** comparison between all stressed animals compared to all control mice, p<0.01*

and following 11 and 21 days of stress. The baseline corticosterone levels and the percentage of wound remaining were significantly and positively correlated on days 3 to 12 following post-wounding (12 days after initiation of stress, Table 1). Corticosterone levels on day 11 following stress initiation were positively associated with the percentage of wounds remaining on days 8, 10 and 12; corticosterone levels 21 days after the start of stress were positively correlated with percentage of wound remaining on days 10 and 12.

Stressed and individually-housed mice showed significantly longer latency to escape from the restraint tube in the DWT than stressed and group-housed mice ($F_{(1,15)}$= 4,86; p= 0.045). Latency and the percentage of wound remaining showed a positive significant correlation on days 1-6 (Table 1). There were no significant correlations between latency and corticosterone levels.

Basal corticosterone levels and latency behavior: predictive power for time to wound healing

Based on the relationship observed between the basal levels of corticosterone and the healing of the wounds, and the correlation between the latter and escape latency, we hypothesized that these two measurements (one biological and the other behavioral) could predict the percentage of wound healing on a given day. To address this hypothesis, we performed stepwise multiple regression analyses (Table 2).

For the first six days after wounding, latency to emerge from the tube following the initial stress exposure was the best predictor of wound healing, accounting for 20-50% of the variance on days 1-6. Interestingly, on day 6, basal corticosterone and latency both predicted percentage of wound remaining, together accounting for 60% of the variance. For the last six days, the low levels of basal corticosterone were the sole predictors of wound healing, accounting for 27-56% of the variance the days 7-12.

Discussion

The model of multiple mild stressors utilized in this study (ultrasounds, predator odor and restraint) significantly increased levels of corticosterone, but failed to significantly alter healing of skin wounds, in contrast with other studies (Detillion 2004).

Table 1
Pearson correlations between corticosterone levels (prior to initiation of stress, and day 11 and day 21 of stress exposure) or latency and the percentage of wound remaining on each day post-wounding

		% Wound remaining (days post-wounding)											
		Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**
Corticosterone													
Basal	*r*	0.144	0.185	**0.475****	**0.558****	**0.390***	**0.355***	**0.512****	**0.615****	**0.436***	**0.487****	**0.461****	**0.435***
	P	0.433	0.311	0.006	0.001	0.027	0.046	0.003	0.000	0.013	0.005	0.008	0.013
Day 11	*r*	-0.239	-0.213	0.198	0.181	0.174	0.032	0.276	**0.408***	0.139	**0.368***	0.286	**0.370***
	P	0.188	0.241	0.277	0.322	0.342	0.862	0.127	0.020	0.450	0.038	0.112	0.037
Day 21	*r*	-0.234	-0.133	0.082	0.091	0.017	-0.038	0.238	0.279	0.125	**0.384***	0.236	**0.445***
	P	0.197	0.468	0.654	0.619	0.924	0.838	0.190	0.122	0.496	0.030	0.193	0.011
Latency	*r*	**0.532***	**0.556***	**0.563***	**0.608***	**0.499***	**0.728****	0.436	0.472	0.158	0.278	0.462	0.021
	P	0.034	0.025	0.023	0.012	0.049	0.001	0.091	0.065	0.559	0.298	0.071	0.939

** p<0.01; * p<0.05

Table 2
Stepwise multiple regression analysis of the basal corticosterone levels and latency to emerge from the restraint tube measurements for twelve days post wounding

					Basal corticosterone levels			**Latency to emerge from tube**		
Days	R^2	*df*	F	*P*	Beta	*t*	*P*	Beta	*t*	*P*
1	0.232	15	5.54	0.034		Excluded		**0.532**	**2.35**	**0.034***
2	0.260	15	6.26	0.025		Excluded		**0.556**	**2.50**	**0.025***
3	0.269	15	6.51	0.023		Excluded		**0.563**	**2.55**	**0.023***
4	0.473	15	7.72	0.006	**0.444**	**2.22**	**0.045***	**0.453**	**2.27**	**0.041***
5	0.195	15	4.64	0.049	Excluded			**2.15**	**2.15**	**0.049***
6	0.607	15	12.61	0.001	**0.384**	**2.22**	**0.045***	**0.594**	**3.44**	**0.004****
7	0.476	15	14.61	0.002	**0.715**	**3.82**	**0.002****	Excluded		
8	0.495	15	15.70	0.001	**0.727**	**3.96**	**0.001****	Excluded		
9	0.559	15	19.99	0.001	**0.767**	**4.47**	**0.001****	Excluded		
10	0.411	15	11.45	0.004	**0.671**	**3.38**	**0.004****	Excluded		
11	0.408	15	11.35	0.005	**0.669**	**3.37**	**0.005****	Excluded		
12	0.273	15	6.62	0.022	**0.567**	**2.57**	**0.022***	Excluded		

To date, most of the animal studies that have shown a negative effect of stress on wound healing utilized models of prolonged restraint stressor for periods ranging between 12 (Padgett, 1998; Sheridan, 2004) and 15 hours per day (Horan et al., 2005; Rojas 2002), disrupting the circadian corticosteroid rhythm. Similar stress applied daily for 2 hours did not affect wound healing in male hamsters (Kinsey, Prendergast, & Nelson, 2003). However, one study of female hamsters demonstrated a negative effect following 2 hours of restraint for 14 days (Detillion, 2004). Studies utilizing other types of stressors did not always show results in the same direction (Sheridan, 2004; Toivanen, 1960); this highlights the importance of other factors on wound healing and the need to account for the timing, acuteness or chronicity, as well as the quality of the stressors (physical, psychological), individual characteristics of the subject (species, sex, age, behaviour) or the general environmental characteristics (housing, light cycle). Therefore, although the results of this study lacked a significant effect of stress on the wound healing time, we cannot rule out that the continuous exposure to stressors for 12 days prior to wounding resulted in habituation, and therefore a decrease in the HPA axis response (Grissom & Bhatnagar, 2009).

The results of this study provide evidence of the importance of housing conditions which, in agreement with others (Detillion, 2004; Glasper & Devries, 2005), show that positive social interactions accelerate the rate of wound healing. Prior to exposure to stress, the baseline levels of corticosterone were already lower in subjects exposed to social stimulation, suggesting that social housing provides a buffer against stress-induced activation of the HPA axis (DeVries, Glasper, & Detillion, 2003; Glasper & Devries, 2005), and retarded cutaneous wound healing (Detillion, 2004; Martin, Glasper, Nelson, & Devries, 2006). In this same direction, we found that those animals with lower basal levels of corticosterone were the ones that healed wounds faster. Further studies showed that wound healing is accelerated in those animals that are exposed to physical contact, which is associated with enhanced secretion of oxytocin, which in turn suppresses the HPA axis and facilitates wound healing (Amico, Mantella, Vollmer, & Li, 2004; Detillion, 2004; DeVries, Craft, Glasper, Neigh, & Alexander, 2007; Herman & Cullinan, 1997; Ring et al., 2006).

Taylor et al., (2000) have proposed that social support plays a particularly significant role in females (Taylor et al., 2000). Although fight-or-flight may characterize the primary physiological responses to stress for both males and females, these researchers suggest that behaviourally, females' responses are more marked by a pattern of "tend-and-befriend," mediated by oxytocin, female reproductive hormones and endogenous opioids. The results of our study are in support of this model, suggesting that female responses to stress may build on attachment-caregiving processes that downregulate sympathetic and HPA responses to stress. In a similar manner, we can consider that the lack of social support represents an additional social stressor that enhances the physiologic stress response and it's associated detrimental effects.

Although some researchers have already suggested that the effect of stressors on immunity and health in general depend on behavioural coping strategies, (Bohus 1993; Cacho Fernández, Garmendia Rezola, Vegas Moreno, & Azpiroz Sánchez, 2008; Sklar & Anisman, 1979; Vegas, 2006), this study is the first to analyze the effects of individual coping with stress on wound closure. Animals housed in groups showed less "immobilized" reactive conduct following the stressor; this may reflect a lower state of anxiety in subjects exposed to social stimulation (Fanselow & Helmstetter, 1988). This study provides evidence of the inverse relationship between prolonged latency escape conduct and wound healing. In contrast to previous studies (De Boer, Koopmans, Slangen, & Van der Gugten, 1990; Korte, Bouws, Koolhaas, & Bohus, 1992), this study failed to find a relationship between this conduct and levels of corticosterone. However, latency conduct and corticosterone levels were found to be valid predictors of the rate of healing of wounds.

We found that immobility following a stressor correlates with a delay in the early inflammatory phase of wound healing, whereas corticosterone levels correlate with impairment in the late proliferative phase. Elevated levels of glucocorticosteroids have been shown to participate in the impairment of wound healing observed during stress (Padgett, 1998). However, our findings support the notion that stress inhibits healing through more than this pathway, suggesting that while basal HPA axis reactivity affects the later phase of wound healing, other mechanisms are involved in prolonging the early phase.

In conclusion, the data obtained in our study support the notion that social interactions improve wound healing, reduce the level of anxiety and circulating corticosterone. Furthermore, the level of anxiety as measured by reactive behavior of immobilility, as well as the basal levels of corticosterone, are valid predictors of different stages of wound healing.

These findings may have clinical relevance by shedding light on social isolation as a potent risk factor for wound healing and underscores the role played by social support in the maintenance of health.

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