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ORIGINAL RESEARCH REPORT

Contrasting mechanisms by which social isolation and restraint impair healing in male mice

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Abstract

Stress modulates vital aspects of immune functioning in both human and non-human animals, including tissue repair. For example, dermal wounds heal more slowly and are associated with prolonged inflammation and increased bacterial load in mice that experience chronic physical restraint. Social stressors also negatively affect healing; however, previous studies suggest that the affected healing mechanisms may be stress model-specific. Here, the effects of either social isolation or physical restraint on dermal wound healing (3.5 mm wounds on the dorsum) were compared in hairless male mice. Social isolation beginning 3 weeks prior to wounding delayed healing comparably to physical restraint (12 h/day for eight days), in spite of marked differences in metabolic and hormonal consequences (i.e. body mass) between the two stress models. Additionally, isolated mice exhibited reductions in wound bacterial load and inflammatory gene expression (interleukin-1beta [*IL-1β*], monocyte chemoattractant protein [*MCP*]), whereas restraint significantly increased both of these parameters relative to controls. Experimentally augmenting bacterial concentrations in wounds of isolated mice did not ameliorate healing, whereas this treatment accelerated healing in controls. This work indicates that social isolation and restraint stressors comparably impair healing, but do so through disparate mechanisms and at different phases of healing.

Keywords

Infection, inflammation, proliferation, stress, tissue repair, wound

History

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Introduction

A growing body of basic and clinical research indicates that social environment profoundly modulates mental and physical health in social species (Cacioppo et al., 2011; Karelina & DeVries, 2011). In general, social support or positive social interactions improve health and mood outcomes, whereas social isolation or negative social interactions lead to mental and physical health impairments. In humans, perceived social isolation (i.e. loneliness) is associated with a pro-inflammatory phenotype (Cole et al., 2007), increased blood pressure (Hawkley et al., 2010), and poor stroke outcomes (Boden-Albala et al., 2005). The behavioral and physiological consequences of social isolation in humans are remarkably similar to those observed following social isolation (single-housing) in laboratory rodents. For example, socially isolating rodents precipitates depressive-like behavior (Martin & Brown, 2010), autonomic and hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Grippo et al., 2007; Weiss et al., 2004; Williams et al., 2009), and altered heart rate (Grippo et al., 2007). These physiological effects translate into negative stroke (Craft et al., 2005) and cardiac arrest outcomes (Norman et al., 2011), and impaired dermal wound healing (Detillion et al., 2004). How isolation alters the kinetics of tissue repair was the focus of this study.

Wound healing is an intricately synchronized immune process characterized by three overlapping phases: inflammation, proliferation, and remodeling (Chen et al., 2010). During dermal healing in mice, the inflammatory phase (~first 3 days of healing) is characterized by neutrophil and macrophage recruitment to the wound site for bacterial clearance. The proliferative phase (~3–7 days post-wounding) consists of re-epithelialization of the wound, collagen formation, and angiogenesis. The final phase lasts weeks to months and is characterized by scar formation and extracellular matrix restructuring (Engeland & Marucha, 2009).

Many factors have been shown to modulate healing in humans including: sex, age, metabolic disease, nutrition,

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and psychological stress (Engeland et al., 2006; Engeland & Graham, 2011; Guo & Dipietro, 2010). For example, stress associated with the responsibility of caring for loved ones with Alzheimer's disease prolongs dermal wound closure by 24% compared to age-matched non-caregiver controls (Kiecolt-Glaser et al., 1995). Such negative modulators of healing can increase the risk of wound bacterial infection and post-operative complications (Robson, 1997). In mice, a repeated physical restraint stress paradigm similarly impairs dermal healing. In-depth characterization of this model has determined that restraint induces prolonged inflammation, increases wound bacterial load and impairs bacterial clearance (Mercado et al., 2002a; Padgett et al., 1998; Rojas et al., 2002). This increase in wound bacterial load following restraint is thought to be the primary mechanism by which stress delays healing. Social isolation stress also impairs dermal healing in rodents (Detillion et al., 2004; Glasper & Devries, 2005; Levine et al., 2008), although the healing mechanisms by which this less physical and more psychosocial stressor exerts its effects remain unclear. In addition, isolation of research animals is a standard practice used in many biomedical studies; therefore it is important to understand the consequences of isolation on immunity.

In this study, the mechanisms by which social isolation delays dermal wound healing in mice were examined and compared to those of the well-established physical restraint stress model. The influence of these stressors on wound closure, gene expression for factors necessary for the inflammatory (i.e. pro-inflammatory cytokines, chemokines) or the proliferative (i.e. wound contraction, re-epithelialization, and angiogenesis) phases of tissue repair, and wound bacterial load were determined. We predicted that social isolation would impair dermal tissue repair, albeit more modestly than physical restraint, through similar deficits in healing mechanisms. In contrast, the results indicate that social isolation impairs healing in mice to the same magnitude as a physical stressor (restraint) but through divergent healing mechanisms.

Methods

Animals

Virus-antibody-free SKH-1 male mice (6–8 weeks of age) were obtained from Charles River, Inc (Wilmington, MA). SKH-1 mice were chosen because their skin is largely hairless (similar to human skin) and their wound healing process has been well-characterized (Eijkelkamp et al., 2007; Mercado et al., 2002a, b; Padgett et al., 1998). Mice were housed in polypropylene cages ($27.8 \times 7.5 \times 13$ cm) with corncob bedding and microisolator lids in a vivarium at a temperature of $21 \pm 1\,^{\circ}$C under a 14:10 h light:dark cycle (lights off at 6 pm) and had access to food (Harlan 7912 rodent chow) and water *ad libitum* (unless otherwise noted). The vivarium is accredited by The American Association for the Accreditation of Laboratory Animal Care (AAALAC) and all procedures were approved by the Office of Animal Care and Institutional Biosafety at the University of Illinois at Chicago (UIC) and conform to the *NIH Guide for the Care and Use of Laboratory Animals*.

Experiment 1: Comparison between chronic social isolation and restraint on physiology and behavior

Forty mice were used for this experiment to compare the effects of social isolation and restraint on body mass, HPA axis reactivity, and behavior. Mice were either (1) isolated for three weeks before and throughout assessments (ISOLATION); (2) group-housed controls (GROUP; 5/cage); (3) physically restrained (12 h/day) for three days prior to the HPA axis assessment (RESTRAINT; see below); or (4) respective controls which were food- and water-deprived (but free to roam their cage) for the same 12-h period (FWD). Body mass was recorded prior to isolation, after three weeks of isolation (just before restraint for RESTRAINT group), and 3 days later (after 3 days of restraint for RESTRAINT group).

Chronic restraint group

Three days before HPA axis reactivity assessment, RESTRAINT mice were placed in well-ventilated 50 ml polypropylene tubes daily for 12–13 h per day within their homecages (18:00–06:00 h) (Padgett et al., 1998).

HPA axis reactivity to acute stressor

Circulating corticosterone concentrations were determined before and after an acute stressor (50-min bout of restraint) to compare HPA axis feedback function among treatment groups. Within two minutes of isoflurane anesthetization, retro-orbital blood samples (100 µl) were collected: (1) before a 50-min bout of physical restraint (baseline), (2) immediately after restraint (post-acute stressor), and (3) 55 minutes after the end of restraint (recovery). All mice in a cage were anesthetized simultaneously. Mice were returned to their homecage between the collection of the post-stressor and recovery blood samples. The acute stressor was the same as that described for the chronic RESTRAINT group, except the immobilized mice were placed on a counter under standard ambient lighting for 50 min. Corticosterone was measured in duplicate in all blood samples via EIA according to the manufacturer's instructions (Enzo Life Sciences, Plymouth Meeting, PA) after 1:30 dilution. Intrassay variation was 9% and interassay variation for the three plates was 2.4%.

Anxiety-like behavior

As a follow-up measure to the HPA assessment, total locomotor activity was measured using an open field test in isolated and group-housed mice. Increased total locomotor activity and velocity in an open field are indicative of anxiety-like behavior (Crawley, 2000). One week after HPA axis reactivity assessment and after 30 min of acclimation to the dark testing room, mice were individually placed in a clear 27×27 cm acrylic box inside a ventilated cabinet using dim red light (during active dark phase: 20:00–23:00 h). A frame at the base of the box consisting of 24 photobeams in a 12×12 arrangement detected the location of horizontal movement (Med Associates Inc, St. Albans, VT). Total locomotor activity and average velocity were tracked for 5 min.

Experiment 2: Comparison between social isolation and restraint on wound healing

Eighty mice were used for this experiment to determine the effects of chronic social isolation on dermal wound healing relative to group-housed controls and to compare these findings to those of a well-established model of chronic restraint stress. Mice were either (1) isolated for three weeks before and throughout healing (ISOLATION); (2) group-housed controls (GROUP; 5/cage); (3) physically restrained three days prior to wounding and five days after wounding for 12 h/day (RESTRAINT); or (4) respective controls which were food- and water-deprived (but free to roam their cage) for the same 12-h period (FWD). Three days before wounding and five days after wounding mice were restrained as described in Experiment 1 for chronic restraint group. Based on pilot studies in our lab, three weeks of isolation prior to wounding was determined to exert significant and repeatable healing impairments. In separate groups of mice, wounds were biopsied prior to sacrifice on Days 1, 3, or 5 post-wounding to measure gene expression for factors important for the inflammatory and proliferative phases of healing, and to quantify bacterial load.

Dermal wounding

Mice (at 8–9 weeks of age) were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine; i.p.), the skin was cleaned with alcohol, and two full-thickness 3.5 mm excisional wounds were placed on the dorsum using sterile biopsy punches (Miltex Instrument Company, Plainsboro, NJ) just caudal to the shoulder blades. In one cohort of mice, wound closure was captured by daily photographs (through Day 5 post-wounding) and images were analyzed by a single investigator blind to the treatments (L.Y.). Photographs of the biopsy sites were taken with a 3.5 mm standard-sized dot placed beside the wound to control for variations in photograph angle and distance. Wound size was measured using Canvas 9 software (ACD Systems, Seattle, WA; Horan et al., 2005) and expressed as the ratio of the wound area to the standard dot measurement, then as a ratio to the original wound size on Day 0 (Horan et al., 2005). In separate cohorts of mice, both wounds were harvested on Days 1, 3, or 5 post-wounding by sterile 6.0 mm punch biopsies (Miltex Instrument Company, Plainsboro, NJ) following deep anesthetization (ketamine/xylazine). One wound was used to quantify bacteria ($n = 4$–5/group) and the other wound was used for genomic comparison of factors known to regulate wound healing using quantitative real-time PCR (qRT-PCR; $n = 9$–12/group).

Bacterial quantification

Wounds harvested for bacterial quantification were weighed and then homogenized on ice in 1 ml chilled PBS. The homogenates were serially diluted 1:10 six times with PBS, and 100 µl of each dilution was plated in duplicate on brain-heart infusion agar (Becton Dickinson, Franklin Lakes, NJ). Following overnight incubation at 37 °C with 5% CO_2, colonies were counted to determine initial colony forming units (CFU) per gram of wound tissue (Rojas et al., 2002).

qRT-PCR

Gene expression for various factors involved in the inflammatory and proliferative phases of wound healing were determined (see Table 1 for gene function and primer/probe sequences). Wounds harvested for qRT-PCR were immediately placed in 1 ml Trizol (Invitrogen, Carlsbad, CA), flash frozen in liquid nitrogen, and stored at −80 °C. Total RNA was extracted from wound tissue, and reverse transcribed to create cDNA, as previously described (Horan et al., 2005;

Table 1. Primer and TaqMan probe sequences for qRT-PCR.

Gene	Function	Sequence (forward, reverse, probe)
Interleukin-1 beta (IL-1β)	Pro-inflammatory cytokine that recruits immune cells and induces growth factor release	TCGCTCAGGGTCACAAGAAA, CATCAGAGGCAAGGAGGAAAAC CATGGCACATTCTGTTCAAAGAGAGCCTG
Tumor necrosis factor alpha (TNF-α)	Pro-inflammatory cytokine that recruits immune cells and induces growth factor release	CCCCAAAGGGATGAGAAGTTC, TGTGAGGGTCTGGGCCATA AAATGGCCTCCCTCTCATCAGTT
Interleukin-6 (IL-6)	Pro-inflammatory cytokine that recruits immune cells and induces growth factor release	GAGGATACCACTCCCAACAGACC, AAGTGCATCATCGTTGTTCATACA AAGTGCATCATCGTTGTTCATACA
Monocyte chemoattractant protein-1 (MCP-1/CCL2)	Macrophage chemoattractant	CCACTCACCTGCTGCTACTCAT, TGGTGATCCTCTTGTAGCTCTCC CACCAGCAAGATGATCCCAATGAGTAGGC
Macrophage inhibitory protein-1 alpha (MIP-1α/CCL3)	Macrophage chemoattractant	ACAAGCAGCAGCGAGTACCA, TCATGATGTTGAGCAGGTGACA CCCTTTTCTGTTCTGCTGACAAGCTCACC
Keratinocyte chemoattractant (KC/CXCL1)	Neutrophil chemoattractant and stimulates keratinocyte proliferation and migration	TCCCCAAGTAACGGAGAAAGAA, TGTCAGAAGCCAGCGTTCAC AGACTGCTCTGATGGCACCGTCT
Alpha-smooth muscle actin (α -SMA)	Enhances wound contraction	AAACGAACGCTTCCGCTG, GATGCCCGCTGACTCCAT CCAGAGACTCTCTTCCAGCCATCTTTCATTG
Keratinocyte growth factor (KGF)	Stimulates keratinocyte migration and proliferation	Assay ID Mm00433291_m1 (Applied Biosystems)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Housekeeping gene	Cat# 4352339E (Applied Biosystems)

Mercado et al., 2002a). All TaqMan primers and probes (Biosearch Technologies, Navato, CA) were designed using Primer Express software (Applied Biosystems, Carlsbad, CA) with the exception of GAPDH and KGF (off-the-shelf primer/probe sets, Applied Biosystems). Using a 7000 Sequence Detection System (Applied Biosystems), relative gene expression of individual samples were calculated by the comparative C_T method ($2^{-\Delta CT}$) with GAPDH used as a housekeeping gene.

Experiment 3: Effects of social isolation on bacterial clearance

Based on the finding in Experiment 2, that wound bacterial burden was significantly lower in isolated mice compared with group-housed controls, 35 mice were used for a follow-up experiment to determine whether supplementation of wound bacteria would ameliorate healing in isolates. Appropriate skin bacterial concentrations and species promote dermal healing (Gutierrez-Garcia & Contreras, 2009; Hasen et al., 2010). Bacteria were harvested from intact dorsal skin of group-housed mice via skin swabs and grown in culture; such cultures primarily consist of aerobic proteobacteria species (Bikowski, 1999; Grice et al., 2008). Mice were either isolated for three weeks before and throughout wounding ($n = 15$) or group-housed (5/cage; $n = 10$). 1.6×10^8 bacteria (in 25 µl sterile PBS) or PBS for controls ($n = 5$–10/group) were applied topically once to freshly excised wounds and allowed to air-dry while mice recovered from the anesthesia. This quantity of bacteria corresponds to the average number of bacteria in wounds of restrained mice on Day 5 post-wounding as determined in Experiment 2. Wound size was recorded daily.

Statistical analysis

Differences in wound closure, bacterial quantification, and mRNA expression were analyzed using repeated measures and 2-way ANOVA using SPSS v.19.0 software (Chicago, IL). PCR data were square-root transformed to achieve a normal distribution. Data were determined to be statistically significant when $p < 0.05$. Error bars represent standard error of the mean (SEM).

Results

Experiment 1: Effects of social isolation and restraint on physiology and behavior

Body mass

Three weeks of social isolation increased body mass relative to group-housing (GROUP, RESTRAINT, and FWD were combined because these three groups were treated identically up to this point; $t_{1,38} = 2.7$, $p = 0.01$; Figure 1A). Three days of chronic restraint decreased body mass relative to all other groups ($F_{1,36} = 28.8$, $p < 0.001$; Figure 1A). Body mass of isolated mice remained higher than group-housed controls at this time ($t_{1,18} = 7.7$, $p < 0.001$).

Circulating corticosterone concentrations

Circulating corticosterone concentrations were higher in RESTRAINT mice than all other groups at baseline ($F_{3,32} = 5.8$, $p = 0.003$), post-challenge ($F_{3,34} = 3.6$, $p = 0.02$), and recovery ($F_{3,31} = 2.2$, $p = 0.1$; Figure 1B), except compared with group-housed controls at recovery due to high variability ($p = 0.2$). At recovery, isolated mice tended to have higher circulating corticosterone concentrations compared with group-housed controls ($t_{1,16} = 1.78$, $p = 0.09$).

Anxiety-like behavior

Isolation increased locomotor velocity in an open field relative to group-housing ($t_{1,18} = 3.2$, $p = 0.005$; Figure 1C). Similarly, total locomotor activity was increased in isolated mice, although this difference was not statistically significant ($t_{1,18} = 1.8$, $p = 0.08$; Figure 1C). While data were not shown for the restraint group because the daily restraint treatment had ceased for one week by the time of the behavioral testing, neither locomotor activity nor velocity was altered in this group relative to controls ($p < 0.05$).

Experiment 2: Effects of social isolation and restraint on wound healing

Both isolation and restraint delayed wound closure (Figure 2; $F_{1,28} = 10.451$, $p < 0.01$; $F_{1,28} = 30.590$, $p < 0.001$, respectively) and altered the pattern of healing over time ($F_{4,112} = 40.446$, $p < 0.001$; $F_{4,112} = 11.977$, $p < 0.001$, respectively) compared with respective controls. Specifically, isolation delayed wound healing from Days 3 to 5 compared with group-housed controls ($p < 0.001$ on each day) and restraint delayed wound healing from Days 1 to 5 relative to food- and water-deprived controls (FWD; $p < 0.05$ or better; Figure 2). Wound closure did not differ between the two control groups at any time point (group-housed and FWD; $p > 0.05$). Wounds in restrained mice were significantly larger than those of isolates on Day 1 post-wounding only ($p < 0.05$).

Bacterial quantification

Despite inducing comparable wound closure impairments, social isolation decreased wound bacterial load whereas restraint increased wound bacterial load relative to the appropriate control groups on Days 1, 3, and 5 post-wounding (Figure 3; $p < 0.05$ in all cases).

Wound gene expression of factors that regulate healing

Isolation decreased wound tissue gene expression of proinflammatory cytokine, interleukin-1β (IL-1β), and chemokine, monocyte chemoattractant protein-1 (MCP-1/CCL2), on Day 1 post-wounding (Figures 4A and B; $p < 0.05$), whereas restraint increased *IL-1β*, *TNF-α*, and *MIP-1α/CCL3* mRNA on Day 1 (Figure 4A; Table 2). Both social isolation and restraint decreased gene expression for keratinocyte growth factor (KGF) on Days 1 (isolation) and 3 (isolation and restraint), as well as, α-smooth muscle actin (α-SMA) on Days 3 (isolation and restraint) and 5 (restraint) (Figures 4C and D; $p < 0.05$). Additionally, isolation reduced keratinocyte chemoattractant (KC/CXCL1) on Day 3 post-wounding (Figure 4D; $p < 0.05$), whereas restraint had no effect on gene expression of this factor involved in both the inflammatory and proliferative phase. Conversely, restraint singularly decreased Day 5 mRNA expression of vascular

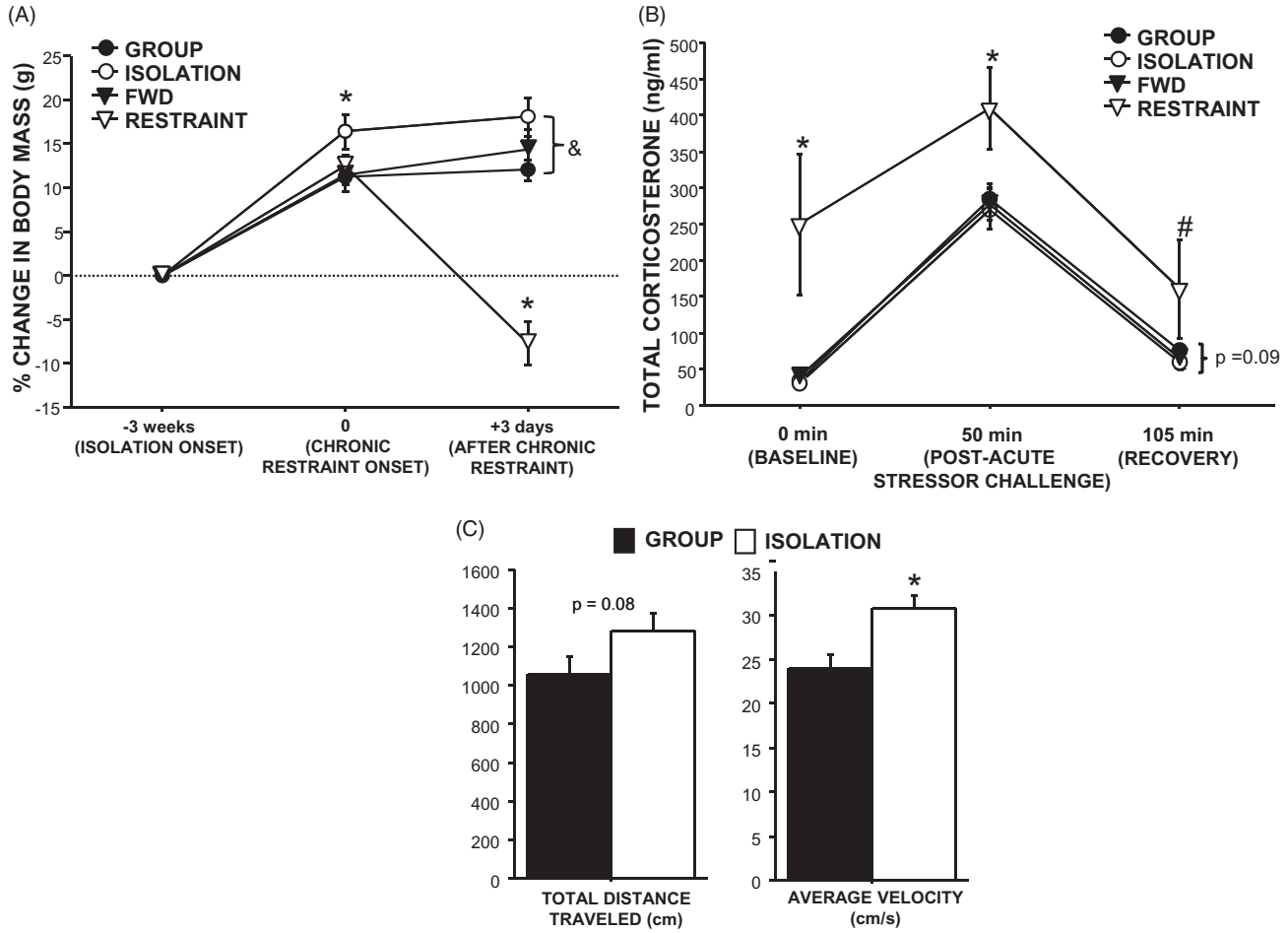

Figure 1. Effects of social isolation and chronic restraint on physiology and behavior. (A) Three weeks of social isolation (ISOLATION) increased body mass compared with group-housed controls (GROUP), whereas daily 12–13-h restraint for three days (RESTRAINT) decreased body mass relative to controls that were food- and water-deprived during the same time period (FWD). $*p<0.05$ compared with all other groups; $\&p<0.05$ between group and isolation treatments. (B) Three days of chronic restraint (12 h/day) increased circulating corticosterone at rest (baseline), immediately after a 30-min acute restraint challenge, and 55 min after the challenge (recovery) compared with FWD controls and three weeks of social isolation or group housing. Isolation tended to decrease recovery corticosterone concentrations relative to group-housed controls. $*p<0.05$ relative to all other groups, $\#p<0.05$ relative to all other groups except group-housed controls, $p=0.09$ between ISOLATION and GROUP. (C) Four weeks of social isolation increased total distance traveled and average locomotor velocity during a 5-min open field test compared with group-housed controls. $*p<0.05$. $n=10$/group.

endothelial growth factor (VEGF) relative to FWD controls (Table 2; $p<0.05$). No differences in *IL-6* were observed using either stress paradigm.

Experiment 3: Effects of social isolation on bacterial clearance

Based on the findings in Experiment 1, that wound bacterial burden was significantly lower in isolated mice compared with controls while wound closure was impaired, this experiment was designed to determine whether supplementation of wound bacteria would ameliorate healing in isolates, given that appropriate skin bacterial concentrations and species promote dermal healing by stimulating appropriate macrophage recruitment (Gutierrez-Garcia & Contreras, 2009; Hasen et al., 2010).

Similar to the previous experiment, isolation alone delayed wound closure ($F_{4,32}=39.4$, $p<0.001$; Figure 5). The addition of indigenous skin bacteria to wounds improved healing rates in group-housed control mice compared with PBS-treated group-housed controls ($F_{4,32}=12.6$, $p<0.001$), driven primarily by the immediate reduction in wound size on Day 1 post-wounding ($p<0.05$). In contrast, bacterial supplementation did not affect healing rates in isolated mice ($F_{4,52}=1.6$, $p>0.05$). As such, the wound sizes of isolated mice with bacterial treatment remained larger than those of bacteria-treated group-housed mice ($F_{4,52}=33.8$, $p<0.001$) on Days 1–5 post-wounding and non-treated group-housed mice on Days 1 and 3–5 ($p<0.05$ for each day).

Discussion

Previous studies demonstrate that both models of stress, social isolation (Detillion et al., 2004; Glasper & Devries, 2005; Levine et al., 2008) and restraint (Mercado et al., 2002a; Padgett et al., 1998; Tymen et al., 2013), independently impair healing, findings which were corroborated by the present experiments. However, different types of stressors (e.g. psychosocial versus physical) can have varied effects on physiological processes (Gutierrez-Garcia & Contreras, 2009; Santha et al., 2013). This is the first study to directly compare the magnitude of the healing impairment and the

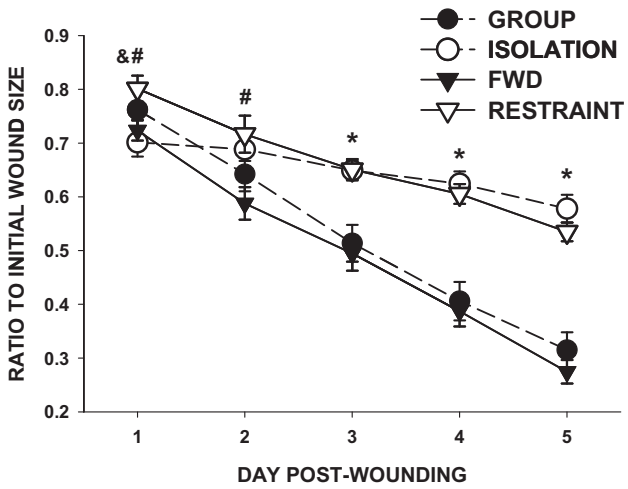

Figure 2. Social isolation and restraint comparably impair dermal wound closure. Three weeks of social isolation (open circles) reduced wound closure (relative to initial wound area) compared with group-housed controls (closed circles) on Days 3–5 post-wounding. Daily 12-h restraint (open triangles) 3 days prior and throughout wound healing decreased wound closure compared with food- and water-deprived controls (closed triangles) on Days 1–5 post-wounding. $n = 15$/group; #$p < 0.05$ for FWD versus RESTRAINT, *$p < 0.001$ for both stress groups versus respective controls, &$p < 0.05$ RESTRAINT versus ISOLATION.

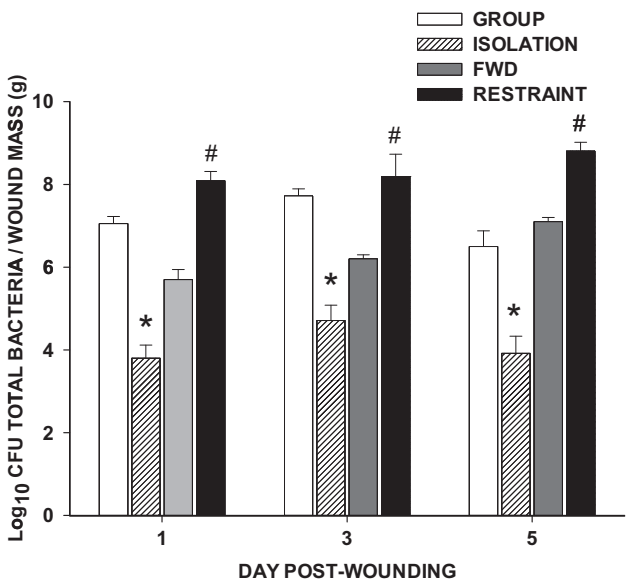

Figure 3. Social isolation reduces, and restraint increases, total bacterial load. Three weeks of social isolation (ISOLATION) decreased wound total bacterial load on Days 1, 3, and 5 post-wounding compared with group-housed controls (GROUP). Conversely, daily 12-h restraint 3 days prior and throughout wound healing (RESTRAINT) increased wound total bacterial load on Days 1, 3, and 5 post-wounding compared with food- and water-deprived controls (FWD). $n = 4$–5/group; *$p < 0.05$ compared with GROUP, #$p < 0.05$ compared with FWD.

underlying healing mechanisms affected by these two stressor paradigms. In contrast to our prediction, that isolation would have a more modest effect on wound closure than restraint, we observed that three weeks of social isolation was sufficient to induce healing deficits of a similar magnitude as those reported following eight daily sessions of 12-h restraint in mice. Thus the physiological consequences of social isolation on wound healing appear comparable to that of a more physical stressor.

The temporal dynamics of the observed healing deficits differed between the two stressors. Wound closure impairments began on Day 1 (inflammatory phase) in restrained mice and on Day 3 (proliferative phase) in isolated mice, relative to controls. This timing discrepancy suggests that the early differences in wound closure of restrained mice were initially driven by changes in bacterial/inflammatory mechanisms and later by effects on proliferative phase mechanisms, whereas the relatively later healing impairment observed in isolated mice were driven by effects on proliferative phase mechanisms only. In support of this, social isolation decreased gene expression for factors important in the proliferative phase of healing: keratinocyte growth factor (KGF) and α-smooth muscle actin (α-SMA). These decreases in *KGF* and α-*SMA* suggest that re-epithelialization (Raja et al., 2007) and wound contraction (Desmouliere et al., 2005) are negatively affected by isolation. Similar results were seen during restraint, and indeed wound contraction is impaired in restrained mice (Horan et al., 2005). Isolation also decreased keratinocyte chemoattractant (KC) gene expression (important for re-epithelialization), whereas restraint had no such effect. Conversely, gene expression for *VEGF* (critical for angiogenesis) was reduced with restraint but not with isolation. Taken together, these data suggest that isolation and restraint affect different phases of wound healing. A more detailed examination of the influence of isolation on tissue repair mechanisms through the later remodeling phase of healing is warranted.

Based on previous studies using the restraint stress model, high bacterial load has been hypothesized to mediate stress-impaired healing in rodents (Rojas et al., 2002). However, the present results in male mice support recent findings in female mice (Pyter et al., 2014) indicating that while the healing rates of isolated and restrained mice were similar, wounds from isolated mice had very low bacterial counts, whereas those of restrained mice were high compared to controls. This difference likely reflects, in part, the relatively higher frequency with which wounds from group-housed mice came into direct contact with surfaces containing microbes (e.g. littermates or restraint tubes). Isolated mice, in contrast, were not exposed to bacteria from the skin or feces of other mice. Alternatively, such differences in microbiology may be due to differences in the physiological consequences of these two stressors, with physical restraint causing the skin to be more amenable to bacterial growth. A definitive understanding of the causes underlying the disparate wound microbiology between these two stressors remains to be determined.

Predictably, inflammatory gene expression in the wound corresponded with the aforementioned wound bacterial burden. Compared to controls, wounds of restrained mice (high bacterial load) displayed increased proinflammatory gene expression (IL-1β, TNFα, MIP-1α), whereas wounds of isolated mice (low bacterial load) displayed decreased proinflammatory expression (IL-1β, MCP-1). This is consistent with previously reported increases in neutrophil activity and proinflammatory cytokine production in wounds of restrained mice (Mercado et al., 2002a; Rojas et al., 2002; Tymen et al., 2013). The combination of elevated proinflammatory responses and circulating glucocorticoid

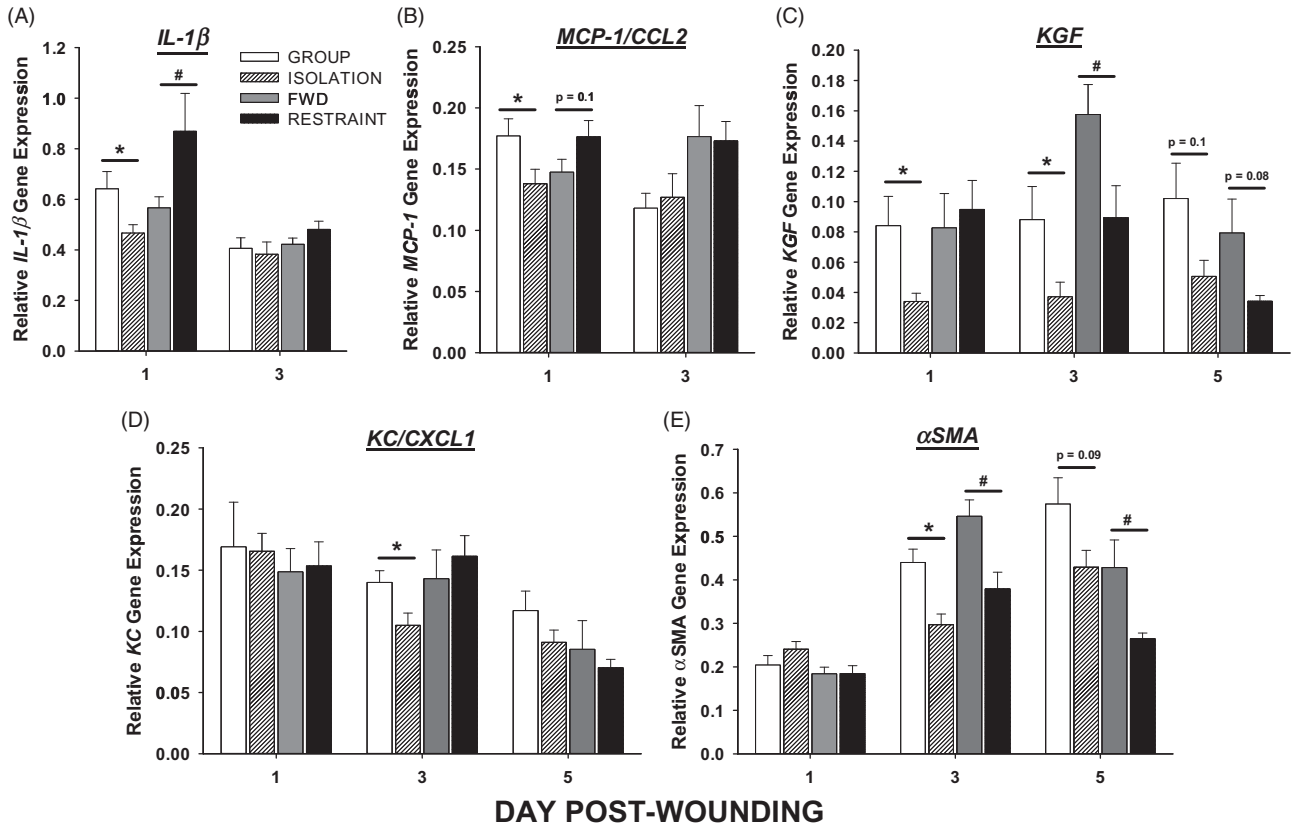

Figure 4. Social isolation alters healing gene expression differently than restraint. Three weeks of social isolation decreased wound (A) interleukin-1β (IL-1β) and (B) monocyte chemoattractant protein-1 (MCP-1) mRNA on Day 1, decreased (C) keratinocyte growth factor (KGF) mRNA on Days 1 and 3, and decreased (D) keratinocyte chemoattractant (KC) and (E) α-smooth muscle actin (αSMA) mRNA on Day 3 post-wounding as measured by qRT-PCR. Daily 12-h restraint 3 days prior and throughout wound healing increased *IL-1β* on Day 1, decreased *KGF* and *KC* on Day 3, and decreased α*SMA* on Days 3 and 5 post-wounding. $n = 9$–12/group; $^*p < 0.05$ compared with GROUP, $\#p < 0.05$ compared with FWD.

Table 2. Effects of social isolation and restraint stress on wound gene expression for factors that regulate healing.

Gene	Group	Isolation	FWD	Restraint	Group	Isolation	FWD	Restraint
Day Post-Wounding:			1				3	
IL-6	0.063 ± 0.01	0.040 ± 0.00	0.037 ± 0.01	0.027 ± 0.00	0.025 ± 0.00	0.017 ± 0.00	0.025 ± 0.01	0.023 ± 0.00
TNF-α	0.046 ± 0.01	0.043 ± 0.01	0.036 ± 0.01	0.134 ± 0.02*	0.054 ± 0.00	0.057 ± 0.01	0.056 ± 0.00	0.054 ± 0.01
MIP-lα/CCL3	0.300 ± 0.03	0.315 ± 0.02	0.353 ± 0.03	0.690 ± 0.08*	0.339 ± 0.04	0.331 ± 0.02	0.409 ± 0.01	0.413 ± 0.02
Day Post-Wounding:			3				5	
VEGF	0.108 ± 0.01	0.094 ± 0.01	0.142 ± 0.02	0.119 ± 0.01	0.132 ± 0.02	0.099 ± 0.02	0.155 ± 0.02	0.096 ± 0.01*

$^*p < 0.05$ between restraint and FWD

concentrations in restrained mice may reflect glucocorticoid insensitivity of immune cells, as is observed in other chronic stress models (O'Connor et al., 2003; Sheridan et al., 2000).

To determine the relevance of the reduced bacterial load observed in isolated mice on wound closure, wounds were supplemented with bacteria in a subset of isolated and control mice. The beneficial effects of bacteria on wound closure have been shown previously in unmanipulated mice (Levenson et al., 1983; Tenorio et al., 1976). In the present study, supplementing bacteria onto wounds resulted in healing improvements for group-housed control mice only. In contrast, isolated mice that had wounds treated with bacteria healed just as slowly as non-treated isolates, suggesting that lowered bacterial burden is not the primary mechanism by which slower healing occurs during isolation. Rather, increased wound bacteria may be responsible for the relatively earlier wound healing impairments observed in restrained mice, but is not seemingly related to the later healing impairments in isolated mice. This is consistent with findings in restrained mice, whose healing rates also remain unchanged when supplemented with bacteria (Mercado et al., 2002a; Padgett et al., 1998; Rojas et al., 2002). Together, these results indicate that bacterial load and wound closure rates can be dissociated.

Some potential confounding factors inherent to group-housing (e.g. grooming, huddling) that might modulate wound repair are lacking in social isolation paradigms. While grooming was not directly assessed in this study, previous work (Vegas et al., 2012) and our observations (unpublished) indicate that group-housed mice do not groom

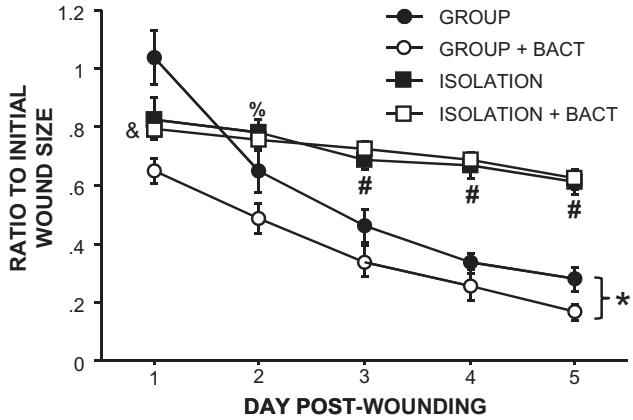

Figure 5. Supplementation of bacteria on wounds does not improve healing in socially isolated mice. Supplementation with indigenous bacteria to wounds at the time of wounding improved healing in group-housed but not isolated mice. $n = 5$–10/group; $^*p < 0.05$ repeated measures between GROUP and GROUP + BACT; $\#p < 0.05$ between GROUP, GROUP + BACT and ISOLATION, ISOLATION + BACT; $\&p < 0.05$ between ISOLATION + BACT and both GROUP and GROUP + BACT; $\%p < 0.05$ between ISOLATION + BACT and GROUP + BACT.

the wounds of cagemates. Single-housed mice also display compensatory thermoregulatory mechanisms (Himms-Hagen & Villemure, 1992) which make potential differences in body temperature unlikely to contribute to the observed effects on tissue repair. This work supports previous studies that demonstrate the negative effects of social isolation in rodents on other physical health outcomes (Advani et al., 2007; Hasen et al., 2010; Norman et al., 2011). The potential impact of isolation on immune processes, such as tissue repair, reinforces the need for thoughtful consideration and reporting of social housing conditions in biomedical studies.

The effects of two common rodent models of stress on wound healing were compared in this study. Stressors are generally validated by the physiological (e.g. HPA axis response), behavioral (e.g. anxiety-like behavior), and/or neurobiological (e.g. hippocampal damage) effects they elicit. For example, restraint is characterized as a physical stressor and reliably elicits elevated HPA axis output (glucocorticoid release) (Barlow et al., 1975; Glavin et al., 1994), whereas isolation is considered more of a psychological stressor and mixed results have been reported on HPA axis output following chronic isolation (Sanchez et al., 1998; Scaccianoce et al., 2006; Vegas et al., 2012). Using the present restraint and isolation paradigms, several physiological and behavioral effects were observed. Body mass decreased in restrained mice and increased in isolated mice as previously reported by our lab and others' (Hotchkiss et al., 2004; Jeong et al., 2013; Martin & Brown, 2010; Pyter et al., 2014). The reduction in body mass of restrained mice was presumably independent of food and water deprivation, as food- and water-deprived controls maintained their body mass.

Three days of chronic restraint resulted in consistently elevated circulating corticosterone concentrations over an assessment of HPA axis reactivity to an acute stressor. These elevations appear to conflict with other reports in which chronic restraint renders the HPA axis less sensitive to subsequent novel stressors (Buwalda et al., 1999; Deak et al., 1999). However, in the present study, they likely reflect (1) a sustained corticosterone release for several hours following this prolonged restraint paradigm (12–13 h) and (2) a corroboration of previous observations that mice do not habituate to repeated exposure to the same stressor (e.g. restraint) (Hotchkiss et al., 2004; Tuli et al., 1995). Elevated corticosterone has been associated with impaired wound healing in rodents and humans (Glaser et al., 1999; Padgett et al., 1998), although other stress-induced changes likely contribute as well (Eijkelkamp et al., 2007; Padgett et al., 1998).

In contrast, isolation had little influence on circulating corticosterone concentrations and responses to an acute stressor. Isolation has been reported to have mixed effects on HPA axis output ranging from corticosterone increases (Vegas et al., 2012), to corticosterone decreases (Boggiano et al., 2008; Martin & Brown, 2010), to eliciting no change (Arndt et al., 2009; Scaccianoce et al., 2006). These mixed results are likely due to differences in housing conditions, strain, sex and circadian timing of blood sampling. Based on a previous study from our lab, females of this same strain (SKH-1) exhibit decreased corticosterone concentrations both at baseline and during recovery from a similar acute stressor (Pyter et al., 2014). Although corticosterone has been shown to be partially responsible for restraint stress-induced delays in wound closure (Detillion et al., 2004; Mercado et al., 2002a; Padgett et al., 1998; Rojas et al., 2002) this is unlikely to be relevant to isolation-induced healing impairments in males given the observed lack of changes in corticosterone following isolation. Alternatively, endogenous oxytocin may play a key role in isolation-impaired healing, as central oxytocin levels are reduced during social isolation (Karelina & DeVries, 2011) and central treatment with an oxytocin agonist ameliorates wound healing in socially-isolated animals (shown in hamsters; Detillion et al., 2004).

To assess potential behavioral consequences of isolation, total locomotor activity was determined one week after HPA axis responses were measured. Isolation increased overall activity and speed, which are suggestive of altered psychomotor systems or a deficit in locomotor habituation, and is consistent with other studies of isolated rodents (Naert et al., 2011; Rilke et al., 1998; Voikar et al., 2005). Taken together, both restraint and isolation distinctly disrupted various physiological and behavioral processes.

Conclusions

These data are consistent with the growing evidence that social interactions garner significant health benefits for social animals, whereas social isolation is consistently detrimental (Karelina & DeVries, 2011). How social environment affects health has potential implications for decisions about standard rodent housing conditions in research and the value of social environment (e.g. social support) in the field of medicine. In addition, this work indicates that tissue repair is an excellent paradigm by which to understand how different stressors can negatively affect immunity through potentially disparate mechanisms. By identifying stressor-specific

mechanisms, there is a better potential for individualizing health treatments and thereby optimizing healing outcomes.

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Declaration of interest

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