Effect of calorie restriction and refeeding on skin wound healing in the rat

Nicole D. Hunt · Garrick D. Li · Min Zhu · Andrew Levette · Mark E. Chachich · Edward L. Spangler · Joanne S. Allard · Dong-Hoon Hyun · Donald K. Ingram · Rafael de Cabo

Received: 11 August 2010 / Accepted: 23 September 2011 / Published online: 27 October 2011
© American Aging Association (outside the USA) 2011

Abstract Calorie restriction (CR) is a reliable anti-aging intervention that attenuates the onset of a number of age-related diseases, reduces oxidative damage, and maintains function during aging. In the current study, we assessed the effects of CR and other feeding regimens on wound healing in 7-month-old Fischer-344 rats from a larger cohort of rats that had been fed either ad libitum (AL) or 40% calorie restricted based on AL consump-

tion. Rats were assigned to one of three diet groups that received three skin punch wounds along the dorsal interscapular region (12-mm diameter near the front limbs) of the back as follows: (1) CR ($n=8$) were wounded and maintained on CR until they healed, (2) AL ($n=5$) were wounded and maintained on AL until wound closure was completed, and (3) CR rats were refed (RF, $n=9$) AL for 48 h prior to wounding and maintained on AL until they healed. We observed that young rats on CR healed more slowly while CR rats refed for 48 h prior to wounding healed as fast as AL fed rats, similar to a study reported in aged CR and RF mice (Reed et al. 1996). Our data suggest that CR subjects, regardless of age, fail to heal well and that provision of increased nutrition to CR subjects prior to wounding enhances the healing process.

N. D. Hunt · G. D. Li · M. Zhu · A. Levette ·
E. L. Spangler · J. S. Allard · R. de Cabo (✉)
Laboratory of Experimental Gerontology, National Institute
on Aging, National Institutes of Health,
Baltimore, MD 21224, USA
e-mail: decabora@mail.nih.gov

M. E. Chachich
Department of Psychology,
Towson University,
Towson, MD 21252, USA

D.-H. Hyun
Department of Life Science,
Ewha Womans University,
11-1 Daehyeon-dong Seodaemun-gu,
Seoul 120-750, South Korea

D. K. Ingram
Nutritional Neuroscience and Aging Laboratory,
Pennington Biomedical Research Center,
Louisiana State University System,
Baton Rouge, LA 70808-412, USA

Keywords Aging · Calorie restriction · Refeeding · Wound healing

Abbreviations

AL	Ad libitum
CR	Calorie restriction
ECM	Extracellular matrix
EE	Energy expenditure
UCPs	Uncoupling proteins
PGC-1 α	Peroxisome proliferator-activated receptor gamma coactivator-1alpha

SIRT1	Silent information regulator 1
RF	Refeeding
PPARs	Peroxisome proliferator-activated receptor (α —alpha, β/Δ —beta/delta, γ —gamma)

Introduction

Adequate nutrition is essential for the complex processes involved in wound healing (Ayello et al. 1999; Lim et al. 2006; de Luca et al. 2007). In calorie restriction (CR) studies, most often performed in rodents, a level of nutrients, vitamins, and minerals is provided in the diet that is comparable to ad libitum (AL) consumption, but in CR there is a reduction in calories consumed, i.e., undernutrition without malnutrition. The CR model is a reliable anti-aging intervention with numerous health benefits that enable maintenance of function during aging (Chipalkatti et al. 1983; Masoro 1988; Weindruch and Walford 1988). However, its effects on wound healing have not appeared to be as beneficial (Reiser et al. 1995; Roth et al. 1997). Reed et al. (1996) performed an extensive study comparing the wound healing of AL fed young and middle-aged mice to aged mice that had been fed AL, CR, and CR that were refed AL for 1 month prior to wounding. Aged refed mice in that study healed at rates similar to the young AL fed mice. Further, in vitro studies of wound explants of the aged mice revealed enhanced cell proliferation and contractility in refed compared to aged AL and aged CR mice.

In the study reported here, we have compared the effects of different feeding regimens on wound healing in young, male Fischer-344 rats. Similar to the study performed by Reed et al. (1996) in aged mice, we evaluated wound healing in three different diet groups. The groupings were (1) CR rats consuming 40% less than AL fed rats prior to and throughout the wound healing study, (2) AL fed rats that were fed AL prior to and throughout the wound healing study, and (3) CR rats that consumed 40% less than AL until 48 h prior to wounding when they were refed AL and maintained on AL feeding throughout the study. Our hypothesis was that refeeding prior to wound healing in young rats on CR until 48 h prior to wounding would enhance recovery and closure of wounds in the RF group, similar to the results observed in the middle-aged and aged mice in the Reed et al. (1996) study.

Materials and methods

Subjects, diets, and experimental groups

Twenty-two male Fischer-344 rats 7 months old at the outset of the study served as subjects. The rats were part of a larger cohort of rats that were bred, reared, and housed in a vivarium maintained at the Gerontology Research Center, NIA, NIH, Baltimore, MD. A sentinel system was used to determine if the vivarium was free of the following pathogens: GDVII, MHV, MPul, Sendai, and HAI/MVM.

At weaning (4 weeks of age), rats were individually housed in plastic cages with wood chips for bedding in a large metal rack and with AL access to water via an automated watering system. Rats in the AL fed group had free access to food (NIH 31; Taconic) while rats in the calorie restricted group were fed the same diet but 40% less calories than a group of AL monitor rats from the same cohort. Food consumption by the AL monitors was measured weekly and the daily allotment of chow for the CR rats was calculated based on the AL monitor consumption for that week. The vivarium was maintained on a 12 h light/12 h dark cycle (lights on 6AM/off 6PM EST) with the vivarium maintained at 21°C.

At 7 months of age, rats ($n=22$) were assigned to one of the three following treatment groups that were later wounded as the experimental treatment: (1) CR rats ($n=8$) maintained on 40% CR throughout the study; (2) AL rats ($n=5$) maintained on AL throughout the study; and (3) a RF group (RF; $n=9$) that had been 40% CR since weaning that began an AL diet 48 h prior to wounding, and then maintained on an AL diet until the end of the study. All rats were monitored daily and body weights were recorded weekly. Food consumption of all groups was measured weekly.

Skin biopsy punch and wound closure measurements

The rats were first anesthetized with a ketamine/acepromazine solution (i.p. 1 ml/kg volume of injection) under aseptic conditions. Electric hair clippers were then used to shave the backs of rats. Fine hair was removed with Nair hair removal cream. The backs of rats were then washed with water and dried with a towel. The dorsal interscapular region (at the level of the front limbs) was cleaned with 70%

ethanol and blotted with betadine to reduce the risk of infection. Surgical tools were first autoclaved to assure wounding occurred under aseptic conditions. An Acu-Punch 12-mm-diameter sterile disposable skin biopsy punch (Acuderm, Inc., Fort Lauderdale, FL, USA) was used to create three separate wounds on the dorsal interscapular region at the following locations: left, right, and bottom left. Skin punches were separated by a 20–30-mm distance. Bleeding was attenuated using sterile gauze. Rats were returned to clean cages with a paper towel covering the bedding to prevent adherence to freshly inflicted wounds. The towel was taken out of the cage on the following day. Observations were made daily by lightly anesthetizing each rat with isoflurane gas (2%). Wounds were measured at the time of surgery (day 0) and every 2 days thereafter until complete closure. The area of the wounds was traced onto transparency film. A digital camera, Nikon Coolpix L6, was used to capture images which were then transferred to a computer for analysis. All digital images that were taken included fiduciary markers (penny and ruler). All procedures were carried out in

accordance with the guidelines and regulations of the Animal Care and Use Committee, NIA, NIH.

Dependent measures and statistical analysis

The area of the wounds was measured by NIH Image J 1.37v software. A one-way ANOVA was also computed on the number of days to 50% wound closure. Repeated measures ANOVAs were calculated for body weight and food intake.

Results

Data for different groups of rats are presented as follows: CR rats wounded and maintained on CR, AL rats wounded and maintained on AL, and CR rats refed AL for 48 h then wounded and maintained on AL for the duration of the study.

Food consumption

These rats were part of a larger cohort of animals that were either 40% CR or AL fed. The food consumption of the CR group was based on the daily food consumption of five AL monitors each week. As shown in Fig. 1, the daily consumption of the CR group did not change during the course of the

Fig. 1 Food consumption—food consumption was measured in CR, AL, and RF Fischer-344 rats. AL food intake was increased in comparison to RF initially after the skin biopsy punch. Significant differences between AL and RF were only observed in the first 4 days after punching. On the remaining days in the study, the food consumption patterns were nearly identical ($P < 0.0001$ AL vs. RF). CR animals consumed 40% of AL based on five AL monitors of the same age that were not part of this study but were part of a larger cohort of rats in the AL/CR colony

Fig. 2 Body weight—body weight was assessed in three groups: CR, AL, and RF. We observed a significantly reduced body weight in CR in comparison to AL and RF. RF rats steadily gained weight. AL rats maintained stable weight patterns ($P < 0.0001$ vs. other points and $P < 0.0001$ vs. CR)

Fig. 3 Panels (a)–(l) show the reduction in the area of wounds over time in representative Fischer-344 rats from each of the three groups: CR, AL, and RF. Each skin punch biopsy was approximately 12 mm in diameter and located at three positions on the dorsal interscapular region: left, right, and bottom left. Scale bars represent 10 mm in length. A penny, ruler, and blue sharpie sketching were used as fiduciary markers

experiment, and these data are not used for comparison following wound healing. After the skin biopsy punch procedure was performed, the food intake of the AL group significantly increased in comparison to RF ($P<0.0001$). However, this effect was only observed in the first 3–4 days after wound infliction. On the remaining days in the study, the food consumption patterns were nearly identical.

Body weight

As expected, body weights for the CR group are significantly reduced (30% less) compared to the AL ($P<0.0001$; approximately 360 g for AL vs. 250 g for CR). In Fig. 2, it can be observed that the RF rats gained weight on the AL diet becoming significantly heavier beginning at day 9 of the experiment ($P<0.001$) compared to CR.

Wound closure

Healing was measured as the reduction in the area of wounds over time (see Fig. 3a–l for representative sample from each group). Days after wound infliction are shown: day 1 (Fig. 3a–c), day 6 (Fig. 3d–f), day 12 (Fig. 3g–i), and day 18 (Fig. 3j–l). The images revealed that by day 6 wounds in the CR group had larger wounds than did the AL or RF groups and were noticeably larger than those in the RF group (Fig. 3d–f). Figure 3 represents the typical pattern of wound closure that we observed in the AL, CR, and RF groups.

Effects of calorie restriction, ad libitum, and refeeding on 50% wound closure

Wounds in AL fed rats were noticeably larger (14%) than those in RF, but this result was not significant ($P=0.1075$) (Fig. 3k, l). A one-way ANOVA calculated on the days to 50% wound closure revealed a significant effect of treatment. Fisher LSD post hoc tests revealed that both RF and AL healed faster

compared to the CR group (see Fig. 4, P 's <0.05). It is evident from Fig. 4 that the CR rats required an additional 1–2 days to reach 50% wound closure compared to AL and RF rats.

Discussion

Similar to data from a previous study in aged mice (Reed et al. 1996), we observed that refeeding young calorie-restricted rats for a 48-h period prior to wounding resulted in wound healing resembling that observed in AL rats and that was superior to that observed in CR rats, the group that had the poorest wound healing.

We cannot determine possible mechanisms that may be involved in this rapid recovery in the young re-fed CR rat since we did not evaluate the wounds histologically or perform any in vitro experiments. Such evaluation would have enabled comparisons of wounds for cell proliferation, growth factors, and gene expression. Further studies to demonstrate the beneficial effects of refeeding in both young and aged CR animals provided with adequate or enhanced

Fig. 4 Delay in CR wound reduction—the days it took for CR, AL, and RF rats to reach approximately 50% original wound area are shown. There was a significant 1–2-day delay in the days that it took for CR wounds to reduce wound area in comparison to those in AL and RF rats ($P<0.05$ vs. CR)

nutrition are warranted, specifically to evaluate the beneficial effects by cell culture (in vitro) studies to assess cell proliferation and contractility and western blotting to assess gene expression.

Based on studies to date, including the present data, it is clear that regardless of age at wounding, CR animals fare poorly compared to refed CR or AL animals, and that this phenomenon is most likely related to the nutrition provided. Moreover, since the healing data in the Reed et al. (1996) study with aged mice appear similar to our data, we suggest that wound healing in a CR model with young animals will have direct relevance to wound healing in the aged where other issues may confound interpretation, e.g., problems regulating body temperature under anesthesia, increased risk of infection, and the risk of loss of subjects due to the severe nature of this type of study.

Although our limited study precludes resolution regarding mechanisms responsible for the beneficial effects observed for wound healing in the RF animals, other investigations have provided plausible evidence for mechanisms involved. For instance, Reed and colleagues (1996) reported that the capacity for old (30–33 months) CR and AL mice to reduce wound area is diminished in comparison to young (4–6 months) and middle-aged (15–17 months) AL mice. The aged RF mice were fed a 40% CR diet for approximately 26 months and were subsequently fed AL for a 4-week period before wound infliction. These investigators observed that the wound area of aged RF mice was reduced at rates similar to those of young and middle-aged mice. They also found that old RF mice exhibited an enhanced capacity to synthesize type I collagen, increased expression of insulin-like binding protein 3, and more efficient contractile capacity compared to the old AL and CR fed mice.

The results of the present study then suggest that the CR wound healing paradigm may be a valuable tool for studying undernutrition and wound healing in the aged. The results achieved in the RF group suggest that increasing nutrition, i.e., supplementing calories, in undernourished elderly prior to events such as surgery

may be critical to recovery. Evaluation of this hypothesis will require further studies with this wound healing paradigm to evaluate potential mechanisms that may be involved in wound healing and that are impaired in the aged such as collagen remodeling and contractility (Ballas and Davidson 2001).

Acknowledgments This research was supported (in part) by the Intramural Research Program of the NIH, National Institute on Aging.

References

- Ayello EA, Thomas DR, Litchford MA (1999) Nutritional aspects of wound healing. *Home Health Nurse* 17:719–729
- Ballas CB, Davidson JM (2001) Delayed wound healing in aged rats is associated with increased collagen gel remodeling and contraction by skin fibroblasts, not with differences in apoptotic or myofibroblast cell populations. *Wound Repair Regen* 9(3):223–237
- Chipalkatti S, De AK, Alyar AS (1983) Effect of diet restriction on some biochemical parameters related to aging in mice. *J Nutr* 113:944–950
- de Luca C, Deeva I, Mikhal'Chik E, Korkina L (2007) Beneficial effects of pro-/antioxidant-based nutraceuticals in the skin rejuvenation techniques. *Cell Mol Biol* 53:94–101 (Noisy-le-grand)
- Lim Y, Levy MA, Bray TM (2006) Dietary supplementation of N-acetylcysteine enhances early inflammatory responses during cutaneous wound healing in protein malnourished mice. *J Nutr Biochem* 17:328–336
- Masoro EJ (1988) Life span extension and food restriction. *Compr Ther* 14(6):9–13
- Reed MJ, Penn PE, Li Y, Birnbaum R, Vernon RB, Johnson TS, Pendergrass WR, Sage EH, Abrass IB, Wolf NS (1996) Enhanced cell proliferation and biosynthesis mediate improved wound repair in refed, caloric-restricted mice. *Mech Ageing Dev* 89(1):21–43
- Reiser K, McGee C, Rucker R, McDonald R (1995) Effects of aging and caloric restriction on extracellular matrix biosynthesis in a model of injury repair in rats. *50A(1): B40–B47*
- Roth GS, Kowatch MA, Hengemihle J, Ingram DK, Spangler EL, Johnson LK, Lane MA (1997) Effect of age and caloric restriction on cutaneous wound closure in rats and monkeys. *J Gerontol A Biol Sci Med Sci* 52(2):B98–B102
- Weindruch R, Walford RL (1988) The retardation of aging and disease by dietary restriction. Thomas, Springfield