Short-term pre-operative dietary restriction in vascular surgery

Kip, P.

Chapter 2.

Preoperative Protein or Methionine Restriction Preserves Wound Healing and Reduces Hyperglycemia.

Kaspar Trocha MD[1,2*], Peter Kip MD[1,2*], Michael R. MacArthur MS[2], Sarah Mitchell PhD[2], Alban Longchamp MD PhD[1], J. Humberto Treviño-Villarreal MD PhD[2], Ming Tao MD[1], Miriam A. Bredella MD[3], Karen De Amorim Bernstein MD[4], James Mitchell PhD[2] & C. Keith Ozaki MD[1]

Asterisks indicate co-first authorship.

[1] Division of Vascular and Endovascular Surgery, Brigham & Women's Hospital, Boston, MA.

[2] Harvard T.H. Chan School of Public Health, Department of Genetics and Complex Diseases, Boston, MA.

[3] Department of Radiology, Massachusetts General Hospital, Boston MA,

[4] Department of Radiation Oncology, Massachusetts General Hospital, Boston MA.

Abstract.

Background: Dietary restriction (DR), defined as reduced nutrient intake without malnutrition, is associated with longevity extension, improved glucose metabolism and increased stress resistance, but also poor wound healing. Short-term preoperative DR followed by a return to normal feeding after surgery results in improved surgical outcomes in preclinical models. However, the effect of preoperative DR on wound healing and perioperative glucose homeostasis is currently unknown. Here we tested the effects of two different pre-operative DR regimens, protein-restriction (PR) and methionine-restriction (MR), on wound healing and perioperative glucose homeostasis using an established murine model of wound healing in both non-diabetic and diabetic mice.

Materials & methods: Surgical outcomes were tested using the McFarlane flap in non-diabetic and streptozotocin-induced diabetic mice. Short-term dietary preconditioning included one-week of PR or MR diet (1-2 weeks) vs. an isocaloric complete diet prior to surgery; all mice were returned to a complete diet post-operatively. Outcome measures of flap wound recovery included skin viability and laser Doppler imaging of flap perfusion, and assessment of CD45+ cell infiltration. Glucose homeostasis was assessed by glucose tolerance testing and by perioperative glucose levels in the diabetic cohort.

Results: No significant differences were observed in percentage of viable skin, perfusion or immune cell infiltration at 7-10 days after surgery in PR or MR mice compared to controls in healthy or diabetic mice. Pre-operative glucose tolerance and post-operative glucose levels were however significantly improved by both PR and MR in diabetic mice.

Conclusion: Short-term dietary preconditioning with PR or MR did not impair wound healing in non-diabetic or diabetic mice. However, both regimens reduced pre-operative hyperglycemia in diabetic mice. Thus, brief preoperative dietary manipulations stand as strategies to potentially improve perioperative hyperglycemia with no deleterious effects on wound healing in mice.

1. Introduction.

Pre-clinical studies have demonstrated that brief alterations in dietary intake prior to surgery, collectively known as dietary preconditioning, improve outcomes in a variety of surgical injury models, including in models of renal ischemia or hepatic ischemia reperfusion injury, stroke and vascular intimal hyperplasia.[1-6] The underpinnings of these studies lie in the phenomenon of dietary restriction (DR), defined as reduced calorie intake without malnutrition, and first popularized by its ability to extend longevity in rodents. [7]

While DR has been at the forefront of ageing research, it also confers numerous other benefits in experimental organisms with perhaps even greater translational relevance. These benefits range from improved metabolic fitness to increased stress resistance. Importantly, DR has recently been shown to improve glucose and lipid homeostasis in humans. [8-11]

Classic DR regimens associated with longevity extension in rodents involve enforced food restriction of up to 60% of normal calorie intake (calorie restriction, CR). Alternate DR regimens that do not enforce food restriction include dietary protein restriction (PR) [12] or restriction of essential amino acids such as methionine (methionine restriction, MR) [13], suggesting that energy restriction per se is not necessary for DR-mediated longevity benefits. While molecular changes responsible for CR, PR and MR benefits and thus the degree of mechanistic overlap remain unclear, there is considerable phenotypic overlap amongst regimens, including the ability to precondition against a variety of surgical injury models. [2, 4, 5] Furthermore, PR and MR may be more clinically relevant over CR due to difficulties inherent to food restriction.

Despite pleiotropic benefits including maintenance of innate and adaptive immunity with aging [14], DR is also associated with negative outcomes including poor wound healing. [15-18] Importantly, dietary preconditioning regimens differ from DR regimens associated with poor wound healing or susceptibility to infection in their return to normal feeding immediately after surgery. Nonetheless, increased risk of wound healing problems or infection would significantly dampen translational potential in surgery, but have not been rigorously assessed in this context.

Wound healing is also compromised in diabetic patients, an increasingly clinically prevalent population, secondary to hyperglycemia and chronic inflammation.[19, 20] Consequently it is important to assess both the metabolic effects and wound healing potential of PR and MR in diabetic as well as non-diabetic models. Here, we tested the effects of PR or MR on wound healing and glucose homeostasis in a McFarlane flap wound healing model in the context of both normoglycemic and hyperglycemia in an streptozotocin (STZ) induced diabetic mouse model.

2. Materials & Methods.

2.1 Experimental Animals.

All animal experiments were carried out with the approval of the Harvard Medical Area Institutional Animal Care and Use Committee. 10-12 week old male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME).

2.2 Diets.

For the non-diabetic cohort, after a 3-day acclimation period, mice were switched to a semi-purified nutritionally complete high-fat diet (HFD; 60% Kcal fat, 20% Kcal protein, 20% Kcal carbohydrate; Research Diets, New Brunswick, NJ) for two weeks. Cages were then randomized into the following isocaloric diet groups: control (complete HFD), PR (0% Kcal protein, 60% Kcal fat, 40% Kcal carbohydrates), MR (10% Kcal fat, 76% Kcal carbohydrate, 14% Kcal protein containing 0.05% methionine). Dietary preconditioning on PR lasted for 1 week, and MR for 2 weeks in the non-diabetic cohort and for 1 week in the diabetic cohort. A complete HFD was resumed immediately in all groups following surgery.

For the diabetic cohort, mice were injected with STZ (intraperitoneal, 50mg/kg) for 7 days, and after 10 days glucose measurements were obtained from venous tail blood. Mice were diagnosed as diabetic when fasting (4hr) glucose was >250mg/dl for 2 consecutive weeks. Mice were then randomized into same groups as above, and dietary preconditioning carried out for 1 week for all groups followed by complete HFD feeding immediately after surgery in all groups.

2.3 McFarlane Flap Surgery.

Briefly as previously described[21], mice were induced under 3% isoflurane and maintained under 1-2% isoflurane. The dorsal hairs were gently shaved and depiliated with Nair, and a 2.5 x 1.25cm area was measured on the dorsum just below the inter-scapular space. A scalpel was used to make the transverse incision and scissors for the remaining incisions to elevate a full-thickness peninsular flap. A sterile silicone sheet (0.14mm) was then placed inferior to the flap to prevent angiogenesis directly from below. The flap was approximated with interrupted 6-0 Vicryl sutures.

2.4 Laser Doppler Measures.

Laser Doppler (LDI, Moore Instruments, Axminster, United Kingdom) measures were performed pre-operatively, immediately post-operatively and then daily. Anesthetized mice were placed prone and maintained on continuous (1-1.5%) isoflurane anesthesia during scanning. Scan area was defined as only the area of the flap with approximately ~2mm of overlap with surrounding skin (flap scan area of X0:80, Y0:150, dX:35, dY:67) and a scan resolution of X:35 Y56 with scan speed of 4 ms/pixel. Data were analyzed using Moore LDI Review software V6.1.

2.5 Skin Viability Measures.

Daily digital photographs were taken while mice were under anesthesia for laser Doppler perfusion measurements. Flap viability/necrotic area was assessed and measured by a blinded surgeon with respect to gross appearance. The area of the flap was kept constant over all days assessed in the same mouse, the necrotic area was then traced and subtracted from the total surface area of the flap using Fiji software.

2.6 Glucose Measurements and Glucose Tolerance Test.

In the diabetic cohort, following the diagnosis of diabetes, daily non-fasting glucose was measured (AlphaTrak II) at the same time every morning during the week of dietary intervention. After 6 days of dietary precondition, mice were fasted for 4 hours and an oral glucose tolerance test performed (gavage, 1.5mg/kg glucose in 0.9% saline).

2.7 Harvest Tissue Processing and Histology.

Harvest of serum and tissues was performed at 7 or 10 days. Longitudinal sections of the flap were collected at the same position of the flap, fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E).

2.8 Immunohistochemistry and analysis.

Longitudinal flap sections of protein restricted and complete fed mice were stained with a CD45 antibody (rabbit anti-mouse, abcam10558) according to manufacturer's protocol. In short, slides were pre-incubated in a pressure chamber at 65ºC for 30 minutes and then deparaffinized. For antigen retrieval, slides were incubated in citrate buffer (abcam, ab94674) for 30 minutes at 95ºC and washed in PBS. Slides were pre-incubated with 10% goat serum (Vector, S-1000) for 45 minutes and stained overnight with the primary CD45 antibody at 4ºC. Next day slides were washed with PBS and stained with a biotinylated secondary antibody (goat anti-rabbit, Vector BA-1000) for two hours. After wash with PBS, slides were incubated in ABC-complex (Vector, PK6100) for 30 minutes, washed with PBS, stained with DAB peroxidase (Vector, SK4100) for 30 seconds, dehydrated and mounted with permount.

For Masson trichome histology staining were deparaffinized to 95% ethanol, stained in 5% picric acid (in 95% ethanol) for 3 minutes, washed with tap water, stained in working Harris Hematoxylin Solution (Fisher Scientific, cat# 245-678) 3 minutes, washed with tap water, stained with 1% Biebrich Scarlet in 1% acetic acid (Fisher Scientific, cat# A38S-500) for 3 minutes, rinsed in distilled water, 5% Phosphomolybdic/Phosphotungstic acid solution for 1 minute, stained with 2.5% light green SF yellowish in 2.5% acetic acid (Fisher Scientific, cat# A38S-500) for 4 minutes, rinsed in distilled water, then in 1% acetic acid solution (Fisher Scientific, cat# A38S-500) for 2 minutes. Slides were dehydrated with xylene and mounted with cover glass using permount

One cross section per mouse was imaged with a Zeiss Axio A1 microscope (Carl Zeiss) at 10x, and a flap overview was created by stitching photos with Photoshop (Adobe Photoshop 14.0). Resulting whole flap images were analyzed using the ImageJ (1.51p (Java 1.8.0_66) color deconvolution function. After thresholding each image, the percentage of CD45 positive area per whole flap was calculated or for collagen quantification the percentage of collagen area per whole flap was calculated and normalized to mm^2 of the flap.

2.9 Statistical Analysis.

All data are expressed as mean ± standard error of the mean. The Student T-test was performed for continuous variables and a p-value of less than 0.05 was considered significant. A 2-way ANOVA with multiple comparisons was employed when more than two groups were compared. All tests were performed with GraphPad Prism (7.0b).

3. Results.

3.1 Preservation of wound healing potential following short-term pre-operative protein or methionine restriction in non-diabetic mice.

Non-diabetic mice preconditioned on PR for 1week or MR for 2 weeks prior to surgery showed no impairment in percent viable skin compared to mice fed a complete diet (**Fig. 1**). Necrotic area was similar in both PR (n=8) and MR (n=8) mice compared to control mice fed a complete diet (n=8) (**Fig. 1A**). Laser Doppler perfusion imaging revealed a significant increase in mean flux intensity in PR mice on POD 1 (**Fig. 1A**, p=0.0189 vs. complete). The MR group also had a pronounced increase in skin perfusion pre-operatively, post-operatively and up to POD 5 (all p=<0.0001 vs. complete). However, no differences were observed at POD 7 in either PR or MR vs. complete diet controls (**Fig. 1A**).

Infiltration of leukocytes involved either in repair or in response to infection were also not significantly different between PR and complete diet fed controls (**Fig. 1G and I**). Also, there was no difference in collage content represented by collagen analysis after Masson trichrome staining of flaps (**Fig. 1H and J**). Thus, dietary preconditioning r preserved normal wound healing and immune infiltration in non-diabetic mice.

3.2 Preservation of wound healing potential following short-term pre-operative protein or methionine restriction in diabetic mice.

Similar results with regard to flap wound healing were observed in a cohort of STZ-induced diabetic mice (**Fig. 2**). No significant differences were observed in necrotic area between PR (n=11) or MR (n=11, one-week pre-op MR) and complete diet-fed diabetic mice (n=9). Interestingly, a significant increase in immediate post-operative flap perfusion (**Fig. 2B**, p=0.04) was observed in diabetic PR mice, consistent with the trend observed in non-diabetic PR mice. Importantly, there were no signs of infection that can accompany diabetic wound healing in any of the groups, nor any significant difference in leukocyte infiltration or collagen deposition between PR or complete fed mice (**Fig. 2F-I**).

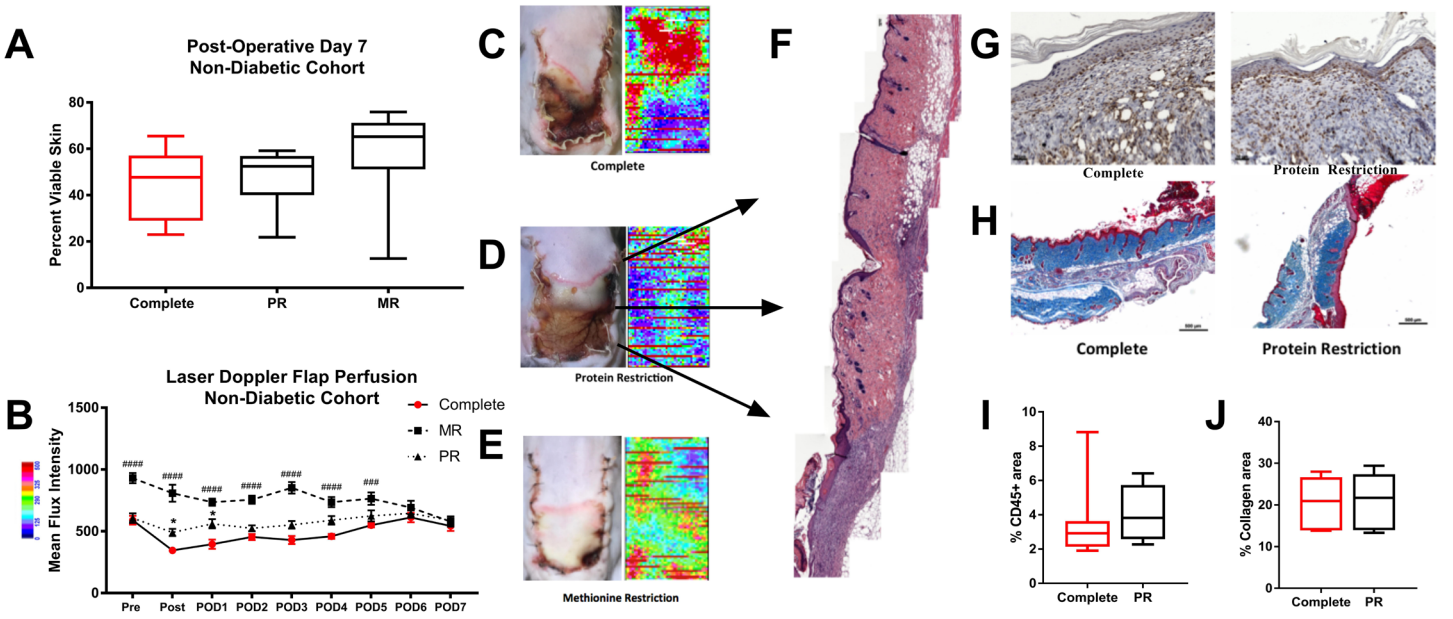

Fig. 1- Preservation of wound healing potential following short term pre-operative PR or MR.

(A) Percent viable skin in control (n=9), PR (n=8) and MR (n=10) mice revealing no significant differences on POD 7. (**B)** Laser Doppler flap perfusion measured pre (before surgery), post (immediately following surgery) and daily up to POD 7. Flap perfusion was increased in MR compared to complete group from pre-op up to POD 5 and in the PR group immediately post-surgery and on POD 1 compared to complete (2-way ANOVA) but was not significantly different amongst groups by day 6. **(C-E)** Representative images of flaps from complete, PR and MR mice on POD 7. Laser Doppler perfusion scans depict perfusion measurements. **(F)** Hematoxylin and eosin stained longitudinal flap section from a PR mouse with arrows depicting approximate location on flap section. **(G)** Representative images of skin flaps stained with CD45 (brown). **(H)** Representative images of skin flaps stained with Masson Trichrome for collagen. **(I)** Percent CD45 positive cells occupying area of flap (normalized to mm^2) in control (n=8) and PR mice (n=8). **(J)** Percent of area occupied by collagen (normalized to mm^2) in control (n=8) and PR mice (n=8). All data are represented as means ± standard error of the mean (* represents significant difference between complete and PR, # represents significant difference between complete and PR). (* P<0.05, *** P<0.001, **** P<0.0001)

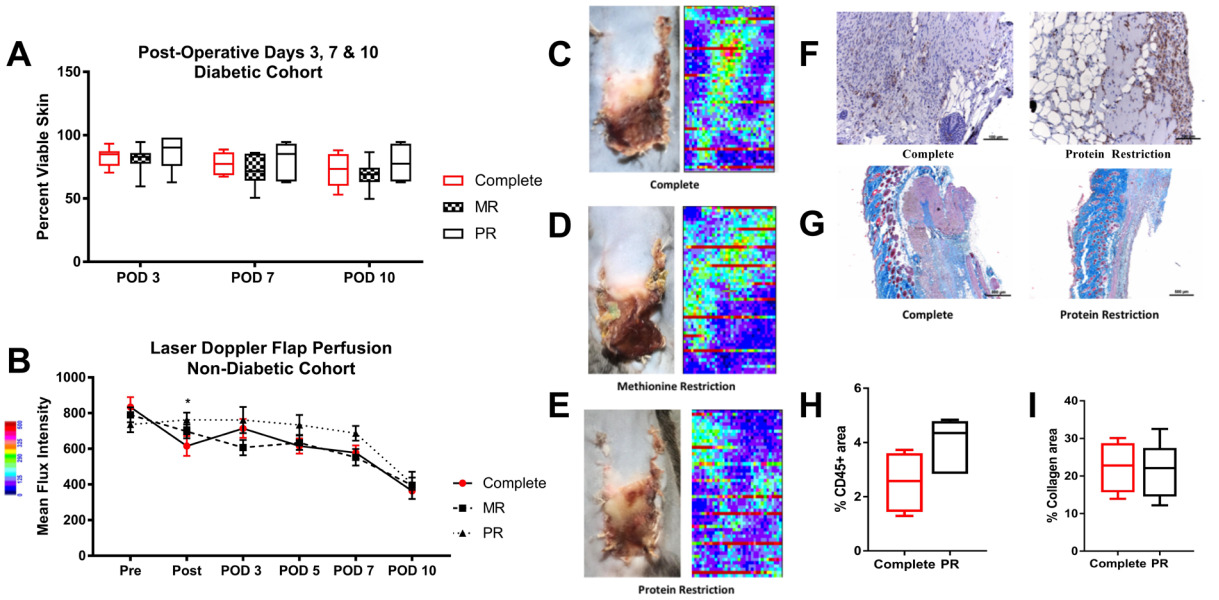

Fig. 2- Preservation of wound healing potential following short term pre-operative protein & methionine restriction in diabetic mice.

(A) Percent viable skin in control mice (n=9), PR (n=8) and MR (n=8) mice revealing no significant differences on days POD 3,7,10. (**B)** Laser Doppler imaging of flap perfusion measured pre (before surgery), post (immediately following surgery) and POD 3,5,7 and 10, showing increased perfusion in the PR group immediately post-surgery (2-way ANOVA). (**C-E**) Representative image of a control complete flap, a PR and MR flap at POD 7. **(F)** Representative images of skin flaps stained with CD45 (brown). **(G)** Representative images of skin flaps stained with Masson Trichrome for collagen. **(H)** Percent CD45 positive cells occupying area of flap (normalized to mm^2) in control (n=8) and PR mice (n=8). **(I)** Percent of area occupied by collagen (normalized to mm^2) in control (n=8) and PR mice (n=8). All data are represented as means ± SEM. (* represents significant difference between complete and PR) (*P<0.05)

3.3 Short-term preoperative protein or methionine restriction improves glucose tolerance and reduces perioperative hyperglycemia.

Although diabetes is a risk factor for impaired wound healing, it is also a concern for post-operative recovery. We thus monitored fed blood glucose levels and glucose tolerance as a function of dietary preconditioning during the perioperative period. In the diabetic cohort, PR rapidly and significantly reduced circulating glucose levels on days 1 and 3-7 of dietary preconditioning (**Fig. 3A**, p=0.009, p=0.006, p=0.049, p=0.006, p<0.0001, p=0.006) compared to control mice fed a complete diet. MR also significantly reduced circulating glucose levels on days 2-7 of dietary preconditioning (**Fig. 3A**, p=0.01, p=0.0004, p=0.001, p=0.001, p<0.0001, p=0.01 and p=0.048).

To further characterize glucose metabolism as a function of dietary preconditioning in diabetic mice, an oral glucose tolerance test was performed. PR improved glucose tolerance compared to control mice (**Fig. 3B**, 15 min. p=0.001, 30 min. p=0.005, 60 min. p=0.001, 120 min. p=0.002). MR also improved glucose tolerance at 60 and 120 minutes (**Fig. 3B**, p=0.01, p=0.002).

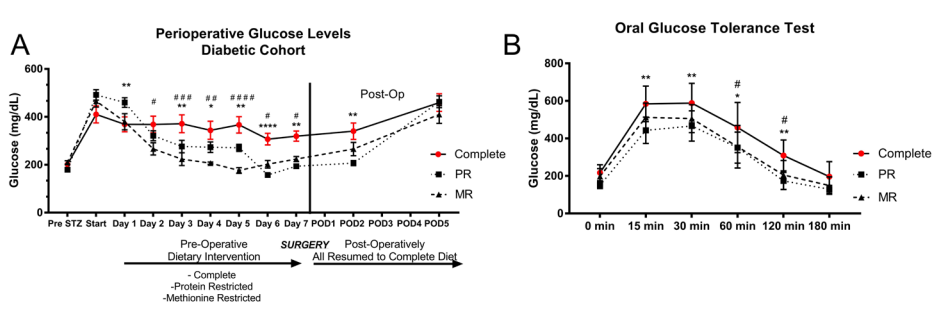

Fig. 3- Short term preoperative protein and methionine restriction improves perioperative circulating glucose and insulin levels.

(A) Non-fasted blood glucose in control, PR and MR mice during the perioperative period. Baseline (fasted, pre STZ) glucose measures are shown with rise in mean glucose levels in all groups (start) followed by glucose measures during the 7-day dietary intervention period as well as post-operatively up to POD 5. **(B)** Glucose tolerance test (1.5mg/kg) following one week of dietary intervention (2-way ANOVA). All data are represented as means ± SEM (* represents significant difference between complete and PR; # represents significant difference between complete and MR). (*/# P<0.05, **/## P<0.01, ### P<0.001, ****/#### P<0.0001)

Finally, because post-operative hyperglycemia is of concern, we monitored fed blood glucose level after resuming a complete diet. PR mice continued to have significantly lower blood glucose compared to controls at POD 2 (**Fig. 3A,** p=0.005).

4. Discussion.

There is currently a need for strategies to reduce risk of complications and improve outcomes in elective surgery. [22] Brief periods of DR prior to surgery followed by a return to normal food intake after surgery improve outcomes in a number of preclinical surgical models, thus representing a translational path forward to meet this demand. [4, 23, 24] Although anecdotal evidence based on multiple previous studies suggests that neither wound healing nor infection are increased in mice subject to dietary preconditioning, wound healing outcomes were never included as primary endpoints in the aforementioned studies. Here, we found no evidence of wound healing impairment in mice severely restricted in protein or the essential amino acid methionine before surgery however both diets significantly improved perioperative hyperglycemia. These data are also consistent with the results of clinical trials in humans subjected to various preoperative DR regimens that also show no apparent wound healing complications. [25, 26]

Previous preclinical studies of DR have shown impaired wound healing in part due to decreased collagen production *in vivo* and *in vitro*. [15-18] The discrepancies between this study and previous efforts to characterize the effects of DR on wound healing are likely due to the differences in dietary paradigms. In the dietary preconditioning paradigm, animals are refed a complete diet after surgery, while in previous studies DR was maintained both before and after surgery. [15-18] However, differences could also be due the models, as our pedicle flap model is a severe wound healing model relying predominately on angiogenesis for flap survival rather than collagen formation and wound contracture. Interestingly, the increase in perfusion measured by laser Doppler specifically in the MR group is consistent with our recent finding that MR increases angiogenesis.[27] Furthermore, the difference between MR and PR in the non-diabetic mice, as well as within MR groups between non-diabetic and diabetic cohorts, could be related to the duration of MR, which was 2 weeks only in the non-diabetic group in which increased perfusion was observed. Nonetheless, while this did not increase wound healing in this group, we conclude that neither MR nor PR worsen wound healing in the McFarlane flap model.

Various surgical subspecialties have shown a relationship with perioperative hyperglycemia in diabetic patients and wound complications.[28, 29] Although the relationship between perioperative hyperglycemia and wound complications is not yet clearly defined many studies have found positive correlations [30, 31], including a recent retrospective study pointing to a significant association between perioperative glucose measures above 200 mg/dl and increased rates of dehiscence.[29] Furthermore, current Enhanced Recovery After Surgery guidelines recommend consumption of carbohydrate-laden beverages in the perioperative period specifically to reduce post-operative hyperglycemia.[32] Here, we observed improved glucose tolerance and a reduction in perioperative glucose, including in the PR group on day 2 after a return to a complete diet, in diabetic mice made hyperglycemic by STZ pretreatment. Thus, in addition to the lack of negative effects on wound healing, dietary preconditioning improved glucose homeostasis and post-operative hyperglycemia in a diabetic mouse model.

5. Conclusion.

Here we show that dietary preconditioning regimens previously associated with improved surgical outcomes do not impair wound healing in non-diabetic or diabetic mice. Moreover, we found that these diets improved perioperative glucose tolerance and perioperative hyperglycemia in diabetic mice. In conclusion, brief dietary manipulations stand as simple strategies to potentially improve perioperative hyperglycemia with no deleterious effects on wound healing in mice, thereby further enhancing clinical applicability.

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5.2 Disclosures.

The authors declare no conflicts of interest.

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