# Original Article

# Skin Wound Healing in Obese and Lean Male Adolescent Rats Submitted to Pre-Weaning Litter Size Manipulation

(obesity / wound healing / skin / metabolic syndrome)

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**Abstract. We investigated whether early postnatal over-nutrition affects normal course of skin wound healing. To induce over-nutrition the litter size was adjusted on the first day after birth to four pups/nest (small litters). In parallel, as a control, normal nests of 10 pups/nest (normal litters) were used. For the wound healing experiment 30 male Sprague-Dawley rats, 15 from normal nests and 15 from small nests, were used. Two parallel full-thickness skin incisions and two full-thickness excisions were performed on the back of each rat. Samples for histological examination (excisions) and wound tensile strength measurement (incisions) were collected on days 2, 6, and 14 after surgery. Our study demonstrates that rats from the small nests had enhanced plasma levels of insulin and enhanced body weight/fat parameters. Furthermore, in small nests, rats that expressed the above-mentioned symptoms displayed slight improvement of epidermis regeneration, accelerated demarcation line formation, and increased wound tensile strength. From this point of view the small nest model used in the present experiment is helpful for exploration whether these acquired changes might be considered as a sufficient essential factor involved in the regulation of metabolic homeostasis and wound repair in juvenile obese male rats. Nevertheless, further studies need to be performed to verify the present findings also on other animal models and humans and to describe the exact underlying mechanism.**

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Abbreviations: HDL – high-density lipoproteins, HE – haematoxylin-eosin, HFD – high-fat diet, LDL – low-density lipoproteins, NL – normal litter, SL – small litter, TAG – triglycerides, ZDF – Zucker diabetic fatty.

## Introduction

Sedentary behaviour and overeating represent the main factors frequently resulting in increased incidence

of obesity (Stubbs et al., 2004). Rodent models have long been used to explore the impact of litter size on obesity and health complications. Rats nursed in small nests (SL) in comparison with pups from normal nests (NL) during the lactation period displayed metabolic and hormonal disturbances and many of these syndromes, such as overweight, enhanced fat pad values (Plagemann et al., 1999; Mozeš at al., 2007, 2013; Šefčíková et al., 2011), elevated plasma levels of insulin, glucose, cholesterol, leptin (Plagemann et al., 1999; Hou et al., 2011; Habbout et al., 2012, 2013), persisted into the adulthood. It was also documented that rats raised in small litters ingested more milk than controls (Cunha et al., 2009). Moreover, litter size reduction was also closely related with significantly increased fat/energy concentrations in the milk of rat dams nursing small litter nests (Fiorotto et al., 1991; Mozeš at al., 2007, 2013; Šefčíková et al., 2011), leading to over-nutrition, enhanced adiposity and body weight in their pups. At present, however, information whether the litter size manipulation affects the normal course of skin wound healing is lacking.

It is well known that untreated obesity may lead to the development of metabolic syndrome and might finally escalate into diabetes. The main defects in diabetic wound healing are impaired re-epithelization, stagnation of granulation tissue formation as a result of loss of function of diverse growth factors that drive keratinocyte, fibroblast, and endothelial cells functions (Kao et al., 2011). In addition, this type of healing is characterized by increased inflammatory reaction. The phenotype in obese and diabetic animals is also related to their adipose tissue mass (Hotamisligil et al., 1993) where macrophages, activated by fat tissue, contribute to the development of insulin resistance (Weisberg et al., 2003).

Nevertheless, to our best knowledge no data are available in terms of early wound healing phenotype in 40-day-old obese rat pups submitted to postnatal over-nutrition. At this time period, it can be expected that the early developing metabolic syndrome has not yet caused irreversible changes leading to delayed wound healing as it occurs in diabetic individuals (Brem and Tomic-Canic, 2007). On the other hand, increased insulin expression without markedly increased glycaemia would likely improve the healing processes. To test this hypothesis we evaluated wound healing at the biomechanical and histological levels by using a new experimental model in which obesity was induced by early life litter size manipulation.

## Material and Methods

### *Animal model*

Sprague-Dawley virgin rat dams (Charles River Laboratories, Prague, Czech Republic) were mated at 10 weeks of age and individually housed in Plexiglas cages in a temperature-controlled environment of 22 ± 2 °C with a relative humidity of 60 ± 15 % and a 12 L : 12 D regime (light on 06:00–18:00 h). The mothers had free access to a standard laboratory diet (Laboratory diet M1, Řicmanice, Czech Republic; 3.2 kcal/g, with 26.3 % energy as protein, 9.5 % as fat and 64.2 % as carbohydrate) and tap water. To induce early postnatal over-nutrition and normal nutrition in their progeny, the litter size was adjusted on the first day after birth to four pups/nest (small litters, SL) and 10 pups/nest (normal litters, NL). All nests contained males and females, but only male rats were used for the wound healing study (Fiorotto et al., 1991; Mozeš et al., 2007, 2013).

All animal work was performed in compliance with the rules of the Animal Ethics Committee of the Institute of Animal Physiology SAS, Košice, Slovak Republic.

### *Wound model*

Fifteen rats from the SLs and 15 rats from the NLs (2 months of age) were anesthetized by a combination of ketamine (33 mg/kg; Narkamon a.u.v., Spofa a. s., Prague, Czech Republic), xylazine (11 mg/kg; Rometar a. u. v., Spofa a.s.) and tramadol (5 mg/kg; Tramadol-K, Krka d.d., Pharmacy Company, Ljubljana, Slovenia). Two parallel, 4 cm long, full-thickness skin incisions and two full-thickness excisions, 4 mm in diameter, were performed on the back of each rat (Fig. 1) and immediately sutured by intradermal running suture (Chiraflon 5/0, Chirmax a.s., Prague, Czech Republic). Samples for histological examination (excisions) and wound tensile strength measurement (incisions) were collected on days 2 (inflammatory phase), 6 (proliferation phase), and 14 (maturation phase) following surgery.

### *Body weight and fat weight measurement*

At the end of the experiment, total body weight as well as bilateral adipose tissue samples were collected from the epididymal and perirenal regions after cervical dislocation of the animals. The perirenal fat was obtained by dissection of fat pads located around the kidneys and adjacent fat along the back of the peritoneal cavity.

### *Wound tensile strength measurement*

The device for measuring wound-breaking strength was designed and constructed at the Institute of Experimental Physics, Slovak Academy of Science. Wound tensile strength measurement was described in detail in our previous study (Gál et al., 2009). Briefly, samples including skin incisional wounds were removed from the body and trimmed to optimal dimensions 40 × 10 mm. The skin strip was then lengthwise placed between the two clamps of the tensile strength testing device. Pulling

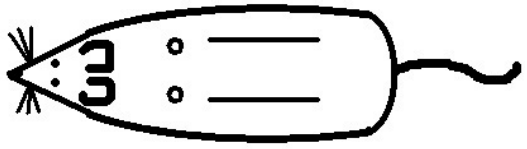

*Fig. 1.* Localization of incisional and excisional wounds on the back of each rat.

was performed perpendicularly to the original direction of the incision.

The maximal breaking strength was measured for each sample. Tensile strength was calculated by using the following formula: TS = MBS/A (TS = tensile strength [g/mm$^2$], MBS = maximal breaking strength [g], A = wound area [mm$^2$]).

### *Histological evaluation*

Skin wounds were processed routinely for light microscopy (fixation, dehydration, embedding, sectioning, and staining with haematoxylin-eosin (HE) – basic staining).

The histological structures and processes (PMNL, re-epithelization, fibroblasts, new luminized vessels, and collagen) were semi-quantitatively evaluated in coded slides according to the scale 0, 1, 2, 3, and 4 (Table 1) (Gál et al., 2008).

### *Measurement of blood serum parameters*

Blood samples were collected from 8:00 to 9:00 a.m. after over-night fasting from the heart of all rats immediately after rapid stress-less cervical dislocation. Subsequently, concentrations of serum glucose, cholesterol, insulin, triglycerides (TAG), high-density lipoproteins (HDL), and low-density lipoproteins (LDL) were determined in these animals. In addition, the presence of glucose was evaluated by using test strips (Hepta Phan, Erba Lachema, Brno, Czech Republic) in urine of all rats.

Aliquots of serum were stored at -70 °C until analysis. Serum insulin was determined with electrochemiluminescence immunoassay kits (Elecsys) in Roche Elecsys 1010/2010 and modular analytics E170 immunoassay analysers (Roche Diagnostics GmbH, Mannheim, Germany). Plasma glucose was measured by the glucose oxidase method in a Beckman autoanalyser (Beckman Instruments, Yellow Springs, OH). Cholesterol, TAG and HDL were measured by routine enzymatic methods. LDL was derived using the Friedewald equation.

### *Statistical evaluation*

Parametric data are presented as means ± SD, while semi-quantitative data (non-parametric) are expressed as median. Statistical significance was set at $P < 0.05$. Parametric data between groups were compared by using the one-way ANOVA followed by Tukey-Kramer post-hoc test, while non-parametric data by the Kruskal-Wallis test. All statistical analyses were performed using the software package Statistica 6.1 (StatSoftCR, Prague, Czech Republic).

## Results

### *Body weight, epididymal, and perirenal fat*

The weight parameters are summarized in Table 2. Overfed SL rats showed accelerated growth resulting in significantly higher mean body weight (g) and enhanced epididymal plus perirenal fat pads exceeding by about 20 %, 45 % and 78 % the values of NL pups, respectively.

### *Blood parameters*

The blood parameters are summarized in Table 3. The excess energy intake during early life of SL pups led to obesity and significantly increased serum levels of insulin, cholesterol, HDL, and LDL. On the other hand, no significant differences were observed in other evaluated parameters, such as glucose and TAG. No glucose was present in the urine of any rat from both groups.

### *Wound tensile strengths*

The wound tensile strengths are shown in Fig. 2. Approximately 20% higher wound tensile strengths were measured on days 6 and 14 in over-nourished rats when compared to the control. The differences were found significant on day 6 ($P < 0.05$).

*Table 1. Explanation of the scale used in semi-quantitative evaluation of histological sections (ST – surrounding tissue, i.e. tissue out of GT; DL – demarcation line; SCT – subcutaneous tissue; GT – granulation tissue)*

| Scale | Epithelization | PMNL | Fibroblasts | Vessels | Collagen |
|---|---|---|---|---|---|
| 0 | thickness of cut edges | absent | absent | absent | absent |
| 1 | migration of cells (< 50%) | mild - ST | mild - ST | mild - SCT | minimal - GT |
| 2 | migration of cells (≥ 50%) | mild - DL/GT | mild - GT | mild - GT | mild - GT |
| 3 | bridging the excision | moderate - DL/GT | moderate - GT | moderate - GT | moderate - GT |
| 4 | keratinization | marked - DL/GT | marked - GT | marked - GT | marked - GT |

*Table 2. Evaluated weight parameters in normal (NL) and obese (SL) rats (data are presented as mean ± SD; ANOVA followed by Tukey-Kramer post-hoc test was used for statistical comparison)*

| | NL | SL | P |
|---|---|---|---|
| **Body weight (g)** | 184.00 ± 15.03 | 201.57 ± 25.36 | < 0.05 |
| **Epididymal fat (g)** | 0.36 ± 0.04 | 0.47 ± 0.10 | < 0.01 |
| **Perirenal fat (g)** | 0.25 ± 0.08 | 0.41 ± 0.11 | < 0.01 |

*Table 3. Evaluated blood parameters in normal (N) and obese (O) rats (data are presented as mean ± SD; ANOVA followed by Tukey-Kramer post-hoc test was used for statistical comparison)*

| | NL | SL | P |
|---|---|---|---|
| **Insulin (pg/ml)** | 1633.90 ± 345.79 | 2524.73 ± 695.16 | < 0.05 |
| **Glucose (mmol/l)** | 8.39 ± 0.48 | 8.72 ± 0.55 | = 0.095 |
| **Cholesterol (mmol/l)** | 1.54 ± 0.24 | 1.82 ± 0.26 | < 0.01 |
| **TAG (mmol/l)** | 0.71 ± 0.31 | 0.77 ± 0.34 | = 0.6237 |
| **HDL (mmol/l)** | 0.88 ± 0.17 | 1.00 ± 0.11 | < 0.05 |
| **LDL (mmol/l)** | 0.39 ± 0.07 | 0.49 ± 0.09 | < 0.01 |

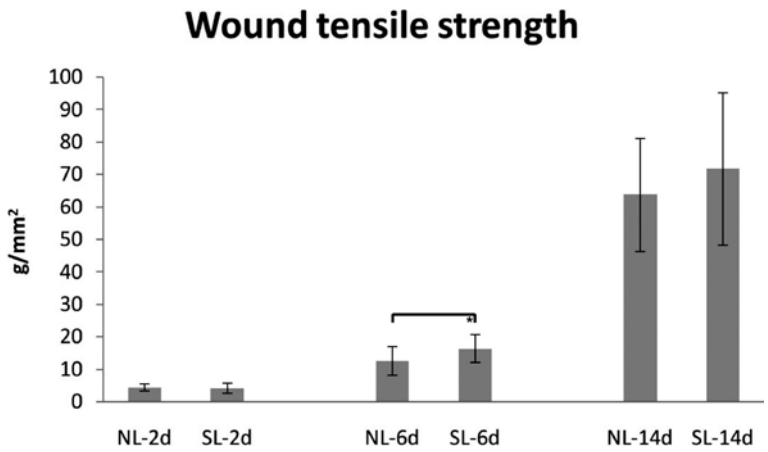


*Fig. 2.* Wound tensile strength on days 2, 6, and 14 post-surgery (data are presented as mean ± SD; one way ANOVA followed by Tukey-Kramer post-hoc test were used for statistical comparison; *P < 0.05).

### *Histology*

Summarized semi-quantitative analysis is shown in Table 4. Two days post-wounding the dermis near the excision in NL rats was rich in inflammatory cells (PMNL), and thus the demarcation line was formed and separated necrosis from vital tissue (Fig. 3). In contrast, the SL rats demonstrated almost finished acute inflammatory process since the demarcation line was completely formed and no PMNL were present in the dermis near the wound edges (Fig. 4). The epidermis was thickened at its cut edges and keratinocytes started to migrate beneath the scab with slight improvement in the SL group (Fig. 3). The number of fibroblasts slightly increased in the dermis near the wounded area.

Histological analysis on day 6 demonstrated complete remission of the inflammatory process in all wounds (Fig. 3). The keratinocytes migrated beneath the scab and almost completely bridged the excisions. A slight improvement of epidermis regeneration was observed in SL rats that had increased plasma levels of insulin. Furthermore, this time period showed a typical histological picture of the proliferative phase, with expressive representation of fibroblasts and new luminized vessels in all wounds, with no significant differences between wounds. Of note, the newly formed granulation tissue was poor in collagen in both groups.

Fourteen days post-surgery the regeneration of epidermis was finished. Improvement in hair follicle creation over the granulation tissues was seen in potentially obese rats when compared to their normally nourished controls. Moreover, remodelling and reorganization of extracellular matrix was characteristic; thus, the scar was formed (Fig. 3), but with no remarkable differences between the groups. Accordingly, polarized light reflecting collagen fibres was not seen either in NL or in SL rats (Fig. 4). In addition, a mild regress of luminized vessels in the granulation tissue was shown at this time interval when compared to wounds removed six days after surgery, but with no remarkable differences between the groups.

## Discussion

It has been shown that over-nutrition as a consequence of litter size reduction leads to an increased plas-

*Table 4. Semi-quantitative analysis of histological structures/changes 2, 6, and 14 d after surgery (data are presented as median; Kruskal-Wallis non-parametric test was used for statistical comparison; *P < 0.05)*

| Day | Epithelization | PMNL | Fibroblasts | Luminized vessels | Collagen |
|---|---|---|---|---|---|
| **2** | 1 / 1 | 3 / 4* | 1 / 1 | 0 / 0 | 0 / 0 |
| **6** | 3 / 3 | 1 / 1 | 2.5 / 3 | 3 / 3 | 2 / 2 |
| **14** | 4 / 4 | 0 / 0 | 3 / 3 | 2 / 2 | 3 / 3 |

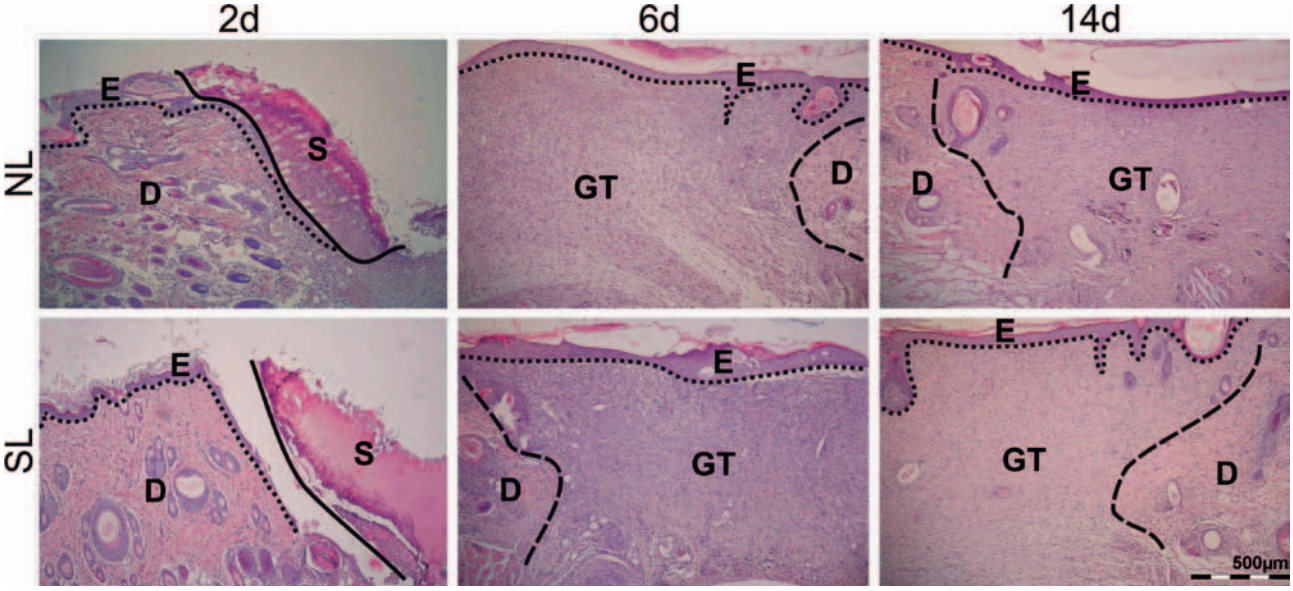


*Fig. 3.* Skin wounds on days 2, 6 and 14 post-surgery (HE, 100×). For orientation, dotted/broken lines are given separating distinct regions referred to by the following abbreviations: E – epidermis, D – dermis, GT – granulation tissue, S – scab. In detail, the dotted line sets epidermis from the dermis and/or granulation tissue; the broken line distinguishes dermis from granulation tissue.

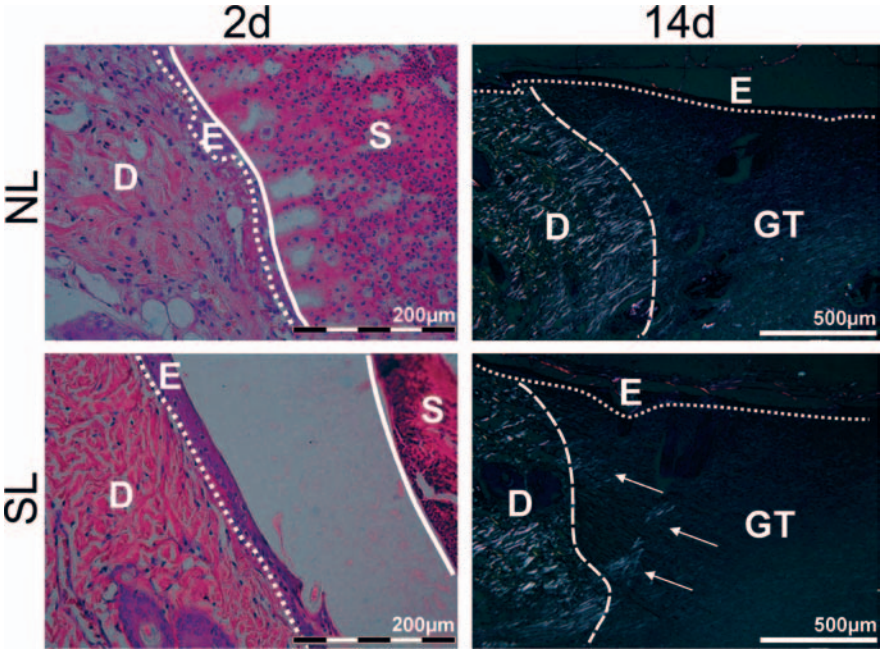


*Fig. 4.* Skin wounds on days 2 (HE, 400×, normal light) and 14 (100×, polarized light). Well-formed demarcation line with the finished process of acute inflammation may be seen on day 2 in obese rats, while in normal rats incomplete demarcation line formation and remaining presence of inflammatory cells in the dermis at the injury site may be observed. Evaluation under polarized light revealed still poor organization of collagen fibres in both groups on day 14; however, with slight improvement in obese rats (arrows). For orientation, dotted/broken lines are given separating distinct regions referred to by the following abbreviations: E – epidermis, D – dermis, GT – granulation tissue, S – scab. In detail, the dotted line sets epidermis from the dermis and/or granulation tissue; the broken line distinguishes dermis from granulation tissue.

ma level of insulin during early postnatal life that might program the development of obesity, associated with hyperinsulinaemia, and diabetes in later life (Plageman et al., 1999; Cunha et al., 2009; Hou et al., 2011; Habbout et al., 2012). In the present experiment we also showed that insulin secretion is increased in previously overfed rats. However, the diabetic symptoms in SL rats, such as glycosuria, have not yet been developed. From this point of view, our results revealed that dietary manipulation in SL suckling pups was associated with altera-

tions in their metabolism and body composition. In addition, we showed that this manipulation is also capable of modulating the levels of physiological processes regulating skin wound healing.

In genetically diabetic-obese and in obese mouse strains submitted to long-term high-fat diet (HFD), significantly increased serum insulin and glucose levels were documented. In contrast to impaired wound conditions in genetically obese animals, the wounds of HFD mice did not develop a chronic inflammatory state and were re-epithelized within 11 days of repair (Seitz et al., 2010). Despite enhanced blood levels of insulin and glucose, the skin wound healing process in Zucker diabetic fatty (ZDF) rats was associated with increased length of new epithelium when compared to their non-diabetic controls (Slavkovsky at al., 2011). Similarly, it has been shown that topical and/or systemic insulin wound treatment resulted in accelerated healing in diabetic animals by regulating the inflammatory response (Chen et al., 2012), wound closure, and stimulating angiogenesis (Liu et al., 2009). Therefore, one potentially interesting aspect of the present results is that they reveal the importance of the obese SL model used in the present experiment for the assessment of the wound healing process during development of the metabolic syndrome.

In summary, we demonstrated that postnatal overfeeding and obesity was associated with improved wound healing in adolescent male rats. Of note, the observed wound healing changes in SL rats may not exclusively be related to their increased insulin levels. Although the general molecular mechanisms of wound healing are similar, a direct extrapolation from this experimental to human situation is not possible due to the interspecies variability. Therefore, further studies need to be performed to verify the presented findings also on other animal models and humans. Moreover, the question whether these changes will persist or change in the adulthood needs to be answered as well.

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