Charles University in Prague
Faculty of Medicine

WOUND REPAIR AND DIABETIC WOUND DEFECTS.

Rastislav Slavkovský

Abstract of the dissertation

Doctoral study program
Medical Chemistry and Biochemistry

Hradec Králové

2013

Dissertation thesis was written during combined doctoral study (PhD) study program Medical Chemistry and Biochemistry at the Department of Medical Biochemistry, Faculty of Medicine in Hradec Králové, Charles University in Prague.

Author: Rastislav Slavkovský, MSc.
Department of Medical Biochemistry, Faculty of Medicine in Hradec Králové, Charles University in Prague,
Contipro Biotech, Dolní Dobrouč.

Supervisor: Assoc. prof. Dr. Jiří Kanta, PhD, Department of Medical Biochemistry, Faculty of Medicine in Hradec Králové, Charles University in Prague.

Opponents: Assoc. prof. Lenka Veverková, MD, PhD, Clinic of Surgery, Faculty Hospital U Svaté Anny v Brně, Brno.

Dr. Barbora Dvořánková, PhD
Department of Anatomy, First Faculty of Medicine, Charles University in Prague

The defense will take place in the presence of the Committee for Dissertation Defense of Doctoral Board for Medical Chemistry and Biochemistry on June 18th, 2013, at 13:30 in the meeting room of Dean's Office (1st floor), Faculty of Medicine in Hradec Králové, Šimkova 870, Hradec Králové.

This work was supported by funding from Contipro Biotech, Grant Agency of Charles University GAUK 40207/2007/C and The Ministry of Education, Youth and Sports of Czech Republic MSM 0021620820.

The dissertation is available for inspection at the Study Department of the Dean's Office, Faculty of Medicine in Hradec Králové, Charles University in Prague, Šimkova street 870, 500 38 Hradec Králové (phone 495 816 131).

Assoc. prof. Jaroslav Cerman, MD, PhD
Chairperson of the Committee for Dissertation Defenses
in doctoral study program Medical Chemistry and Biochemistry

Table of Contents

Summary - Czech

HOJENÍ RAN SE ZAMĚŘENÍM NA DIABETICKÉ DEFEKTY.

Zhoršené hojení diabetických ran je důležitý problém aktuální medicíny, týkající se zejména starších pacientů zotavujících se po komplikovaných operacích anebo pacientů s ulceracemi na nohách.

Hyaluronan (HA) podporuje hojení poškozené pokožky a proto je používán na léčbu komplikovaných ran. Směs hyaluronanu o vysoké molekulové hmotnosti s jódovým komplexem KI_3 (Hyiodine) urychluje hojení ran u pacientů s diabetem a pacientů po chirurgických zákrocích. V této práci jsme zkoumali jak Hyiodine ovlivňuje kontrakci rány, parametry granulační tkáně (GT) a epitelizaci okrajů ran u potkanů. Hyiodine byl aplikován na excizní rány vytvořené na zádech potkanů. Zkoumali jsme plochu ran a histologii migrujících okrajů ran v průběhu hojení. Vlastnosti GT byly studovány v ránách, které kvůli vloženému plastikovému kroužku nekontrahovaly. Efekt Hyiodinu byl porovnán s efektem vysokomolekulárního a nízkomolekulárního HA, a s KI_3. Z našich výsledků vyplývá, že Hyiodine signifikantně zrychluje kontrakci rány v prvních 5 dnech hojení. Zatímco kontrolní rány ošetřené fyziologickým roztokem se ve třetím dni zmenšily na 75 % své původní velikosti, Hyiodinem léčené rány vykazovaly 63 % původní velikost. Zjistili jsme rovněž, že v sedmý den je proliferující epidermis ran ošetřených Hyiodinem byla silnější. V nekontrahujících ranách Hyiodine způsobil malé změny v GT, ale hmotnost krusty s exudátem vytvořené na povrchu rány byla vyšší o 351% ve srovnání s kontrolou. Samotné komponenty Hyiodinu neměli vliv na hmotnost exudátu. Zjistili jsme, že Hyiodine může podporovat hojení ran stimulací kontrakce a epidermální proliferace.

Obézní potkani Zucker Diabetic Fatty (ZDF) s mutací v leptinovém receptoru mohou být vhodným modelem ke studiu zhoršeného hojení ran. Samci a samice potkanů byli krmeni diabetogenní dietou s vysokým obsahem tuků. Změny ve velikosti nekrytých rán o poloměru 2 cm byly sledovány do desátého dne, u bandážovaných rán do zahojení. Tkáň byla analyzována morfologicky, histologicky a imunohistochemicky. Byl stanoven obsah hydroxyprolinu v GT. Exprese mRNA byla sledována pomocí DNA-array analýzy a pomocí kvantitativní PCR. Hojení ran bylo opožděné u diabetických potkanů a lišilo se mezi pohlavími. Pro diabetické rány byla charakteristická snížená kontrakce, zvýšená produkce krusty a zvýšený zánět s tvorbou hnisu. GT u diabetické skupiny obsahovala desátý den signifikantně více vmezeřené tukové tkáně a přítomná kolagenní vlákna byla neuspořádaná. Překvapivě délka nově-vytvořeného epitelu byla zvýšená v diabetických ranách. Koncentrace hydroxyprolinu byla snížená na polovinu vůči kontrole. Exprese interleukinu-6, myeloperoxidázy, stromelyzinu-1, kolagenzy-3 byla v ranách diabetických potkanů zvýšena, naopak exprese kolagenu typu I a elastinu byla snížená. Zjistili jsme, že potkan ZDF vykazoval zpomalené hojení rány, intenzivní zánětlivou odpověď a narušení organizace a obratu pojivové tkáně. Z našich výsledků vyplývá, že potkan ZDF je vhodným modelem pro studium zhoršeného hojení ran.

Summary - English

WOUND REPAIR AND DIABETIC WOUND DEFECTS.

Impaired diabetic wound healing is an important current medical issue, mainly concerning patients recovering from complicated operations or patients with ulcers on their feet.

Hyaluronan (HA) plays an important role in the repair of damaged skin and has been used for the treatment of wounds. A mixture of high molecular weight HA with the antiseptic iodine complex KI_3 (Hyiodine) was reported to accelerate wound healing in patients with diabetes and patients after surgery. We investigated how this mixture affects wound contraction, granulation tissue (GT) and wound edges in excision skin wounds in rats. Hyiodine was applied to full-thickness wounds made on the back of rats. The areas of the contracting wounds were calculated from digital photographs. The migrating edges of the wound were studied by histological methods. The properties of GT were studied in wounds in which contraction was prevented by the insertion of plastic rings. The effects of Hyiodine were compared with those of high molecular weight HA, low molecular weight HA and KI_3 solution. Hyiodine accelerated wound contraction significantly in the first 5 days of healing. On day 3, Hyiodine-treated wounds had reduced to 63% of the original area, whereas the wound area in saline-treated animals was 75% of the original size. The proliferating epidermis was thicker in Hyiodine-treated animals on day 7. In the wounds with inserted rings, Hyiodine caused little change in GT, but the weight of the exudate with crust formed on the top of the wound was increased by 351% compared with only minor changes caused by the Hyiodine components alone. Hyiodine may support wound healing by stimulating wound contraction and epidermal proliferation.

Obese Zucker Diabetic Fatty rat, with a mutation in leptin receptors, may be a good choice for studying impaired wound healing. Male and female rats were fed a diabetogenic high-fat diet. Wound size changes of excisional 2 cm circular wounds, were measured until sampling on day 10 in air-exposed wounds and until complete wound closure in bandaged wounds Wound tissue was analyzed morphologically, histologically, immunohistochemically. Hydroxyproline content in the granulation tissue (GT) was determined. mRNA expression was assayed by DNA-array analysis and realtime RT-PCR. Wound size changes were retarded in diabetic rats and differed between the sexes. Diabetic wounds were characterized by impaired contraction, abundant crust production, increased inflammation and pus formation. On day 10, the GT contained significantly increased amount of intercalated fat tissue and showed irregular arrangement of collagen fibres. Interestingly, the length of new epithelium was increased in diabetic wounds. The concentration of hydroxyproline in the GT of diabetic animals was significantly decreased to about one half when compared with non-diabetic controls. The expression of interleukin-6, myeloperoxidase, stromelysin-1, collagenase-3 was increased in the GT of diabetic rats on day 10, while the expression of type I collagen and elastin was decreased. Taken together, Zucker Diabetic Fatty rats exhibited impairments in wound size reduction, inflammatory response, tissue organization and connective tissue turnover and thus are proposed as a new model for studying impaired repair.

Background

Diabetes mellitus is the most common metabolic disorder characterized by the hyperglycemia and general metabolic disruption of regulatory and signaling pathways in the body. Diabetes mellitus (DM) is a medical problem with increasing importance: 246 million adults worldwide were suffering from DM in 2007 and it is estimated to increase to 380 million in 2025 (Gan 2007). Pathophysiological changes may include neuropathy, vasculopathy, micro- and macroangiopathy, especially in the legs. These disorders cause tissue hypoxia and ischemia of peripheral tissues. The condition may result in poorly healing chronic wounds, venous ulcers, bedsores, diabetic foot syndrome, and eventually in tissue necrosis. Approximately 4-10% of patients with diabetes suffer from diabetic foot ulcers and diabetic patients are often required surgery potentially causing chronic wounds. Impaired wound repair is connected to increased morbidity, mortality and quality of life (Bakker 1999). The pathogenesis of a diabetic wound is complicated. The formation of wounds is connected to neuropathies with the loss of sensibility, microangiopathies, macroangiopathies, decreased blood flow and impaired ability to fight infection (Brem et al. 2007). Recently, new procedures have been developed for treating complicated wounds. One possibility is to use hyaluronic acid. This glycosaminoglycan has a clinically beneficial effect on wound healing and is a component of various products used for wound treatment. Hyaluronic acid supports tissue hydration, interacts with several receptors, and affects the cells, extracellular matrix and factors regulating wound healing (Chen et al. 1999; Jiang et al. 2007).

The balance between extracellular matrix (ECM) synthesis and its degradation is important for normal wound healing, as is the induction of formation of fibrous granulation tissue (GT) and blood vessels. So far, several cellular and molecular factors were identified as contributors to wound healing deficiencies. These include impaired keratinocyte, fibroblast and macrophage function, as well as aberrant angiogenic response and growth factor production (Brem et al. 2007). The presence of diabetes can cause unfavorable proteolytic wound environment due to increased production of several matrix metalloproteases along with lowered levels of tissue inhibitor of matrix metalloproteases (TIMP) (Lobmann et al. 2002). Nonetheless, the exact cellular and molecular mechanisms underlying the pathogenesis of impaired wound repair are not fully understood. One way to clarify impaired wound healing during DM is to study wound repair in animals. Investigators need a variety of well described models to choose from in order to closely mimic the issue they are planning to study.

There are more than 10 different rodent models of experimental DM (Rees et al. 2005). However, researchers are still asking themselves which model(s) is the most suitable for a new insight into the mechanisms of impaired wound repair (Michaels et al. 2007). The obese male ZDF rat has been an important model for studying the mechanism of onset and treatment of type 2 diabetes (Peterson 2007), (Finegood et al. 2001), (Leonard et al. 2005), thus we have anticipated that obese ZDF rat could be suitable model to mimic certain aspects that are present in diabetic patients suffering complicated wounds. The ZDF rat is an inbred strain and rats with fa/fa genotype develop a genetically dependent obesity and subsequently diabetes due to a point missense mutation in extracellular domain of the leptin receptor (Peterson 2007). Lean ZDF animals, with fa/+ or +/+ genotype do not develop obesity or diabetes, and serve as controls. ZDF rats are characterized by non-insulin dependent DM accompanied by hyperglycemia, neuropathies, nephropathies, insulin

resistance, mild hypertension, hypertriglyceridemia, hypercholesterolemia, polyuria, polydipsy (Peterson et al. 1990), and microvascular damage (Danis et al. 1993). Animals gradually evolve from hyperinsulin-euglycemic state to hyperglycemic state with relative insulin deficiency (Finegood et al. 2001; Leonard et al. 2005) and the development of DM is connected with pancreatic beta cells dysfunction (Finegood et al. 2001). Whereas in ZDF rats DM starts to develop in early age, in humans, the manifestation of type II diabetes is usually after the age of 40 or later. In spite of the usefulness of obese ZDF rats as a model for studying experimental type II DM and metabolic syndrome, an extensive characterization of skin wound repair has not been published before this study.

Objectives

The aim of this work was to study mechanism of the action of hyaluronic acid-iodine complex in the models of experimental rat wounds and also to study molecular changes associated with wound repair occurring in the model of rat diabetes. Details of impairments in wound repair in ZDF rats and the molecular-biological aspect of our work could be helpful in wound therapeutic research and development.

Materials and methodology

Animals

All the projects with animals were acknowledged by the Ethics Committee of Faculty of Medicine in Hradec Králové, Charles University (FMHK-CU) and a Committee for Animal Protection of Ministry of Education, Youth and Sport of Czech Republic. Animals were maintained in an air-conditioned room in vivarium of FMHK-CU.

Experiments with permanent wounds in Wistar rats

Wistar rats (Biotest, Konárovice) weighing about 350 g, and 9 - 10 weeks old were used. The work was divided into 3 experiments with 25 animals. In each of 3 experiments, the animals were divided into 5 groups with n = 5 animal (SALINE; HA11 - 1.5% HA with 11 kDa molecular weight (m.w.); HA1200 - 1.5% HA with 1200 kDa m.w.;KI3 - 0.1% I_2/0.15% KI; HYIO – 1200 kDa HA with addition of 0.1% I_2 and 0.15% KI). In each group 0.6 ml of the solution was applied on the wound with a syringe.

Experiments with contractile wounds

Male Wistar rats (Biotest), 9 - 10 weeks old and weighing 300 – 380 g were used. 16 rats were observed until full wound closure (2 groups, n = 8), 24 rats were used for the histological assessment of wound tissue (2 groups, 4 time intervals – 3, 7, 11, 15 days, n = 3). In desired time interval the rats were euthanized and the wound tissue was taken for the histological analysis. 1 ml of saline or Hyiodine (see above) was applied on the gauze with a syringe and then applied on the wound.

ZDF animals and experiments with ZDF animals

Male and female ZDF rats originated from Charles River (USA). For the purposes of experiment the offsprings were bred by mating non-obese heterozygous (fa/+) carrier parents or by mating fa/fa males with fa/+ females. This resulted in approximately 25% and 50% obesity rates, respectively. All animals were fed standard laboratory diet ST-1 (8% kcal fat, Velaz, Lysá nad

Labem, Czech Republic) until the age of 6 - 8 weeks. Diabetes was then induced by feeding high fat diet (Peterson 2007) *ad libitum* : PURINA 5008 (17% kcal fat, IPS supplies) to males and C13004 (48% kcal fat, Research Diets, New Brunswick, NJ, USA) to females. Animals were considered to be diabetic when their non-fasting glucose levels reached 14 mmol/l in males and 9 mmol/l in females and when they possessed obese phenotype. Portable glucose meter, Accu-Chek Go was used for glucose measurement using a drop of blood collected from the tail vein. As ZDF animals were difficult to breed and were bred continuously in smaller numbers, there were many small experiments that were combined. In one experiment, animals with similar age and diabetic status were used. Totally 24 experiments with 2 – 12 animals in each experiment were done without bandage (air exposed wound) and 30 experiments with 2 – 16 animals in each experiment were done with bandage (bandaged wound).For both sexes lean non-diabetic (genotype +/+ or fa/+), and obese diabetic (fa/fa) groups were created. Animals were fed high fat diet from 6 - 8 week of life. Males were fed Purina 5008 (16% of kcal fat). Females were fed with RD 13004 (48% kcal of fat), (Peterson 2007).

Genotypization of ZDF animals

ZDF animals were genotyped by using a PCR followed by a cleavage by restriction endonuclease MspI (Sudre et al. 2002). The DNA was obtained by using Viagen Direct PCR solution or QIAGEN DNeasy kit. Bands for the +/+ (wild type) genotype were 118 bp in size, for +/fa genotype had size 118, 79 and 39 bp and for +/+ genotype had size 79 and 39 bp.

Creation of a permanent wound and granulation tissue collection

The permanent wounds (Fig. 1) were created as described by (Rudas 1960). Every day 0.6 ml of the solution was applied for seven days. Application was made with a syringe and a needle piercing through a nylon cap. After the biopsy whole tissue was weighted, part of it was put used for histological analysis, for hydroxyproline determination and for RNA isolation.

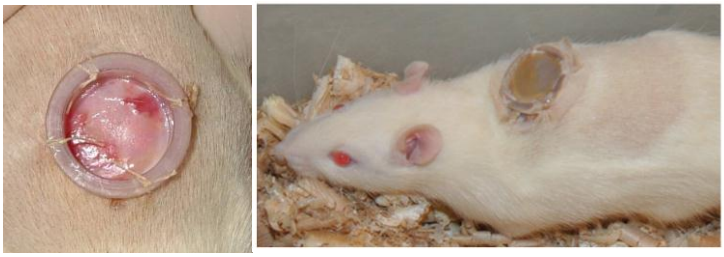

Fig. 1: The picture of permanent wound. In the upper part an implanted plastic ring without the cover is shown.

Induction of contractile wounds, tissue sampling

Full-thickness, 2 cm large in diameter, excisional wounds were made on the back of experimental animals and bandaged (Fig. 2). Every one to three days (according to plan of experiment) the treatment was performed and bandage was changed. The wound was photographed during the bandage change and the wound size was measured using ImageJ.

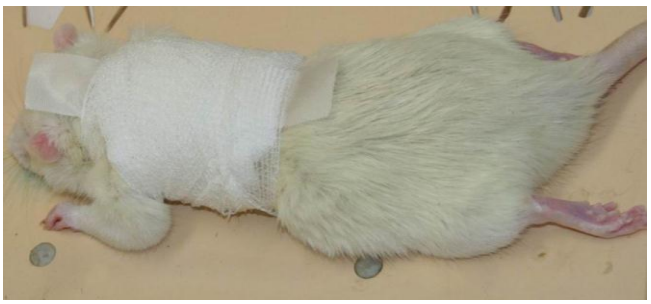

Fig. 2: Picture of the treated rat with a gauze pad, gauze and tape.

Hydroxyproline and uronic acids determination

Hydroxyproline content was determined by the method of (Hurych et al. 1962). In short, neutralized HCl hydrolysate of tissue was analyzed using oxidation reaction with Chloramine-T and reaction with p-dimethylaminobenzaldehyd. The crust with exudate was extracted with 0.5 mol ⁄ L NaOH at 60 °C for 2 h. The mixture was neutralized and ethanol was added to the final concentration of 80% (v ⁄ v). After centrifugation, the precipitate was re-dissolved in 0.5 mol ⁄ L NaOH and used for protein and uronic acid determinations. Protein was measured using a commercial protein assay (DC Protein Assay; Bio-Rad,) with bovine serum albumin (Sigma) as a standard. The carbazole method was used to determine uronic acid levels.

Determination of leptin, insulin, PAI-1, IL-6 and CRP in plasma

The blood was taken from the orbital plexus. Plasma made of heparinized blood was analyzed using ELISA kits (Insulin, Mercodia; IL-6, Alpco, and Bender MedSystem; PAI-1, American Diagnostica Imunoclone; Leptin and CRP, Biovendor, Brno).

Histological analysis of uninjured skin and granulation tissue

The skin that was removed during the wound induction was used for the histological analysis of uninjured skin. Wound biopsies that included wound tissue and approximately 3 mm of surrounding uninjured skin were taken. Tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. Six μm sections from the center of the wound were stained with blue trichrome for collagen and also with haematoxylin and eosin at the Department of Embryology and Histology of FMHK-CU. For the image analysis the samples were scanned with a scanner Epson Perfection V700 Photo. Samples were analyzed for: length of epithelization tongue, fat content in tissue (wound, skin), granulation tissue content, and thickness of crust (wound), fat layer and dermis (skin) using software ImageJ (NIH).

Processing and statistical evaluation of data

Data were accumulated into MS Excel. Graphs were made in this program and where suitable, the differences between groups were evaluated using unpaired t-statistical test. The significance levels are shown in the captions of graphs or in tables.

Microarray analysis and isolation of RNA

The software OligoArray 2.1 was used to design in average 50 bp oligonucleotide probes for microarray. Whole genome *Rattus norvegicus* mRNA database was obtained from the ftp server of The National Center for Biotechnology Information (NCBI). 92 genes were used in the first part of this study (effect of Hyiodine and hyaluronic acid in Wistar rats). 115 genes were used in the second

part of this work, in the studies of wound repair on ZDF rats. Genes were selected from these groups: hyaluronan metabolism, apoptosis/cell cycle, cytoskeleton, NO metabolism, ECM/ligamentous proteins, protease and their inhibitors, proteoglycans. The procedure of cDNA synthesis, biotin-dUTP labeling and hybridization was based on the recommendation of the manufacturer (Bioscience, Jena – previously Clondiag).

A slice of granulation tissue (without surrounding intact skin, without wound crust) was homogenized in the lysis buffer (Qiagen) with Ultra-turrax (IKA). Homogenate was extracted using the RNeasy Fibrous Tissue Mini-Kit (Qiagen). With use of random primers, RNA was transcribed to the cDNA using reverse transcriptase MuMLV (Fermentas) and deoxyribonucleotides (Abgene) plus biotin labeled deoxyuridintriphosfate (Fermentas). cDNA was then hybridized overnight with the probes on the array tube (AT) chip surface, which contained immobilized 50 bp DNA oligonucleotides probes specific for selected genes. After the washing and the peroxidase produced spots of the array was captured in AT reader. The signal was analyzed using IconoClust-AT array analysis software.

Analysis of mRNA gene expression using quantitative RT-PCR

For the real-time reverse-transcription PCR, total RNA (1 μg) was transcribed to cDNA using the cDNA archive kit and quantified with TaqMan Gene Expression Assays (both Applied Biosystems) in ABI 7500 Real-Time PCR System or ABI 7900 (Applied Biosystems). The results were normalized to 18S RNA and GAPDH expression. Data were analyzed using SDS software with absolute quantification module. Calibration curve was calculated from the signal of three dilutions of a calibration sample.

Immunohistochemistry

For immunohistochemical staining, the epitopes were de-masked thermally (98-100°C) in citrate buffer, pH = 6. Polyclonal antibodies against IL-6 (interleukin-6, Abcam), MPO (myeloperoxidase, Abcam), polyclonal antibody against MMP-3 (Santa Cruz Biotechnology) and polyclonal antibody against MMP-13 (Santa Cruz) were used at concentrations of 1:400, 1:50, 1:200 and 1:100, respectively. LSAB plus system-HRP was used as secondary antibody, samples were stained with DAB Chromogen (Dako) and counterstained with Mayer's haematoxylin.

Results

Part 1: Effects of hyaluronan and iodine on wound contraction and granulation tissue formation in rat skin wounds

Determination of wound contraction and histological analysis

Wound contraction in control rats was rapid in the first week of the experiment and it was almost complete by day 15. The contraction was significantly accelerated in the first few days by Hyiodine treatment. The wound area in control rats on day 5 was 60% of the original size. When the wounds were treated with Hyiodine, this percentage was reached almost 2 days earlier (Fig. 3). The wounds treated with Hyiodine did not adhere to the bandage and bleeding was little compared to control group.

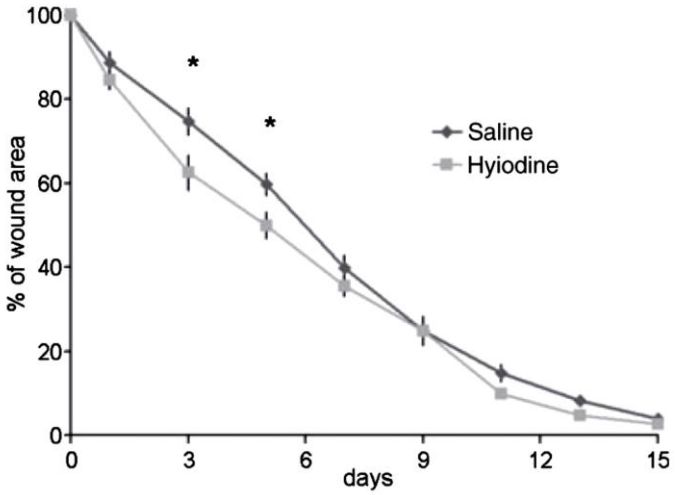

Fig. 3: Wound area as a percentage of the original area measured immediately after wounding. Data are means ± SEM, * significant results, p<0.05.

Fig. 4 shows the thickening of the epithelium in the wound on day 7 of Hyiodine treatment. The thickness of the epithelial layer measured immediately after wounding was 30 µm. It increased to 102 µm in both saline and Hyiodine treated wounds on day 3. However, it was 109 µm and 146 µm, respectively, in saline and in Hyiodine treated wounds on day 7. The difference was statistically significant.

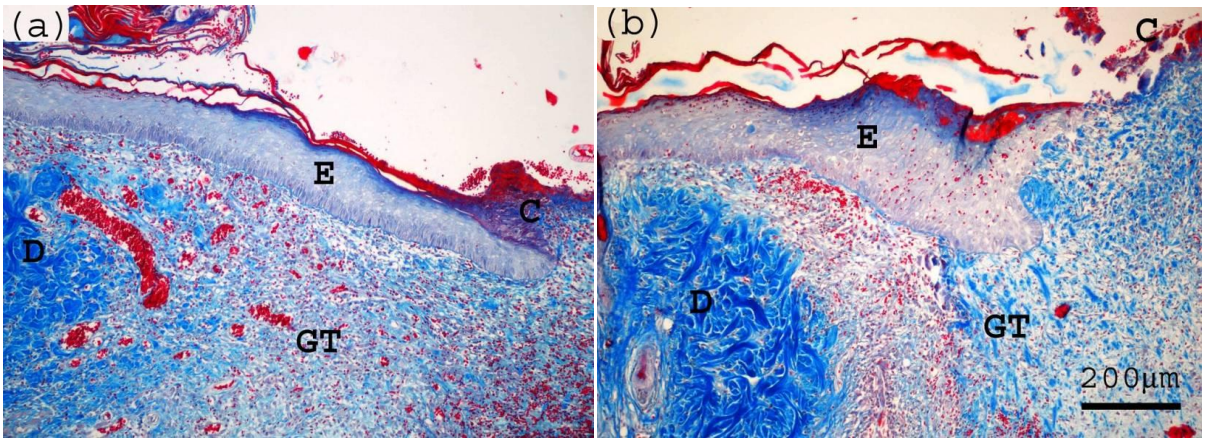

Fig. 4: Histological sections of saline (a) and Hyiodine (b) treated wounds. Blue trichrome; E epithelium, C crust, GT granulation tissue, D dermis.

Analysis of the granulation tissue from permanent wound

Seven-day treatment of GT with the mixture of HA1200 and KI_3 (Hyiodine) resulted in a 18% increase in the wet weight of the GT compared to saline treatment. The changes caused by HA1200 and by KI_3 alone were smaller and were not statistically significant. The concentration of hydroxyproline, the index of collagen, was decreased by Hyiodine treatment when compared to saline or other solutions but little changes were found in total hydroxyproline content (Tab. 1).

Granulation tissue	Saline	HA11	HA1200	HA1200 plus KI_3 (Hyiodine)	KI_3
Weight (mg)	363 ± 18	410 ± 16	375 ± 14	428 ± 17 [a]	409 ± 27
% dry weight	16.0 ± 0.9	16.1 ± 0.7	16.1 ± 0.6	15.9 ± 0.8	18.3 ± 1.2
Hyp concentration (mg/g)	4.03 ± 0.22	3.99 ± 0.19	4.25 ± 0.24	3.66 ± 0.14 [b]	3.88 ± 0.22
Hyp content (mg)	1.45 ± 0.09	1.63 ± 0.09	1.58 ± 0.09	1.56 ± 0.08	1.56 ± 0.11

Tab. 1: Changes in granulation tissue weight, dry weight and hydroxyproline (hyp) content after treatment of the wounds with saline, hyaluronan, iodine or Hyiodine. Means ± SEM. Statistical significance ($p<0.05$). a Hyiodine vs. saline, b Hyiodine vs. HA1200.

Analysis of the crust and exudate of permanent wound

The crust was formed on the top of the granulation tissue. It could not be separated from the exudate gel that was abundant especially after Hyiodine treatment. HA1200 caused a 37% increase in the crust/exudate weight and KI_3 alone did not have any effect. When applied together, these substances increased the weight of the layer by 351% (Tab. 2). Proteins and uronic acids were extracted from the crust/exudate with hot alkali. Protein concentration was similar in all groups but the total amount of protein was by far the highest in the Hyiodine group, 349% when compared with saline. The important source of the protein was blood plasma. SDS-PAGE showed that the protein pattern of 4 different exudate samples from Hyiodine group resembled that of plasma and serum. The band at 66 kDa corresponding to albumin was prominent. Total uronic acid content in dried crust/exudate was increased about 3fold by HA1200 and 14fold by Hyiodine when compared to saline (Tab. 2).

Crust/exudate	Saline	HA11	HA1200	HA1200 plus KI_3 (Hyiodine)	KI_3
Weight (mg)	95.0 ± 19.9	153.3 ± 22.3	130.0 ± 31.0	428.0 ± 81.2 [a,c,d]	96.1 ± 25.1
Protein concentration (% dry weight)	90.6 ± 9.2	91.4 ± 5.9	93.8 ± 6.8	92.5 ± 9.5	85.3 ± 8.9
Protein content (mg)	36.8 ± 7.1	38.7 ± 5.6	36.6 ± 9.0	128.3 ± 27.0 [a,b,c]	48.2 ± 11.3
Uronic acid concentration (% dry weight)	1.38 ± 0.22	1.25 ± 0.14	2.83 ± 0.52	4.07 ± 0.64 [a,b,d]	1.42 ± 0.20
Uronic acid content (mg)	0.37 ± 0.06	0.49 ± 0.05	1.08 ± 0.34	5.10 ± 0.94 [a,b,c,d]	0.53 ± 0.10

Tab. 2: Changes in the weight, protein and uronic acids content in the crust and exudate after treatment with saline, hyaluronan, iodine or Hyiodine. Means ± SEM. Statistical significance ($p<0.05$). [a] Hyiodine vs. saline, [b] Hyiodine vs. HA11, [c] Hyiodine vs. HA1200, [d] Hyiodine vs. KI_3.

Gene expression analysis in granulation tissue by DNA arrays

The mean expression of 92 genes studied was made equal to 1. Studied genes were cell cycle regulators, cytokines and growth factors, part of ECM, proteinases and their inhibitors, and receptors. No statistically significant differences were found by DNA arrays when Hyiodine- and saline-treated tissues were compared. Nonetheless, it was observed that many ECM proteins and proteoglycans mRNAs were highly expressed in the granulation tissue (collagens 1, 3 and 5, fibrilin-1, fibronectin, osteonectin, osteopontin, thrombospondin-1, vitronectin, biglycan, decorin, lumican, perlecan).

Gene expression analysis in granulation tissue by quantitative RT-PCR

Selected genes expression was also analyzed by qPCR. The results are shown in Tab. 3. None of the studied genes were significantly differentially expressed in none of the studied groups. MMP-7 and osteopontin were non-significantly elevated in HA1200 group and MMP-13 was slightly elevated in Hyiodine group.

GENE	Saline	HA11	HA1200	HYIO	KI3
MMP-2	0.89±0.06	0.99±0.09	0.837±0.07	0.83±0.07	0.969±0.09
MMP-7	4.32±1.22	4.9±1.53	7.27±1.83	2.65±0.54	2.14±0.53
MMP-13	0.37±0.08	0.24±0.06	0.37±0.10	0.58±0.20	0.41±0.14
Osteopontin	0.58±0.08	0.56±0.15	0.84±0.18	0.50±0.09	0.42±0.07
COL1A2	0.39±0.06	0.42±0.08	0.34±0.13	0.33±0.10	0.34±0.11
VEGF	1.27±0.18	1.39±0.17	1.32±0.24	1.33±0.25	1.27±0.17
HYAL-2	1.13±0.08	1.24±0.10	1.11±0.06	1.04±0.06	1.11±0.06

Tab. 3: Relative gene expression of selected genes in granulation tissue on day 7 as assayed by qPCR. Means ± SEM are shown. N=14-15.

Part 2: Zucker Diabetic Fatty rat - a new model of impaired cutaneous wound repair with type II diabetes mellitus and obesity

The rate of progeny genotype from carriers breeding (fa/+) was expected at 1:2:1 and this was confirmed by PCR-RFLP genotypization. Offsprings were: 26.5% with +/+ genotype, 47% with fa/+ genotype and 26.5% with fa/fa genotype.

Development of diabetes in ZDF rats

Non-fasting glycemia was increased in diabetic males and usually began to increase significantly at the age of 12-14 weeks. It was 3fold higher at the age of 20 weeks (Fig. 5).

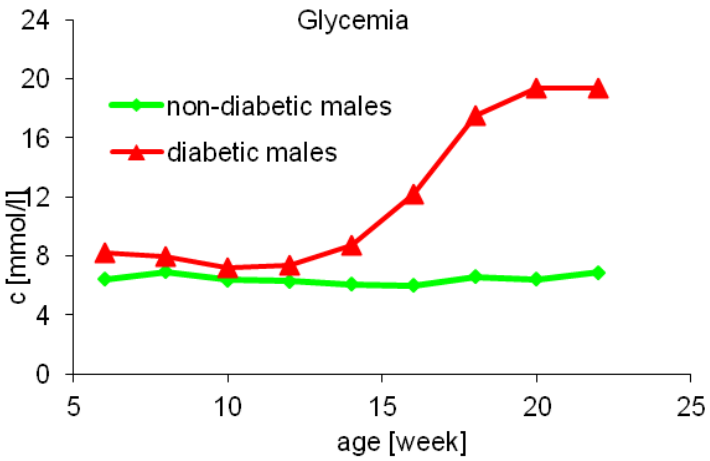

Fig. 5: Changes in blood glycemia in ZDF males fed by Purina 5008. Average of five animals is shown.

Non-fasting glycemia was increased in diabetic females during the diabetes development, however, it did not increase to such an extent as in males. It reached the level of approximately 50% higher compared to controls at the age of 20 weeks (Fig. 6).

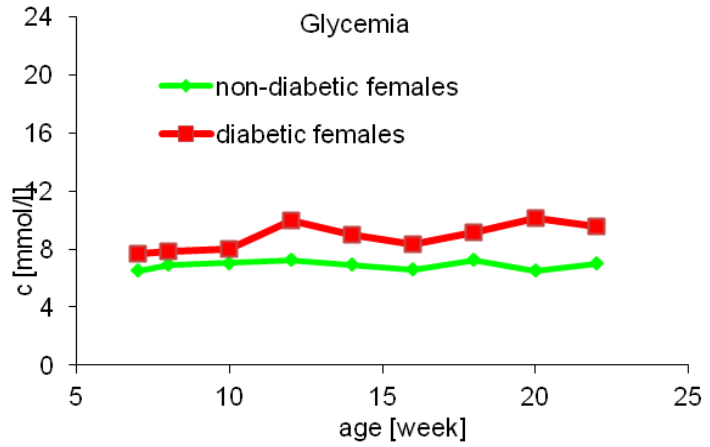

Fig. 6: Development of blood glycemia in ZDF females fed with C13004. The mean of five animals is shown.

Physiology of ZDF rats

As shown in Fig. 7 there was an expected difference in the growth curves of diabetic and non-diabetic animals.

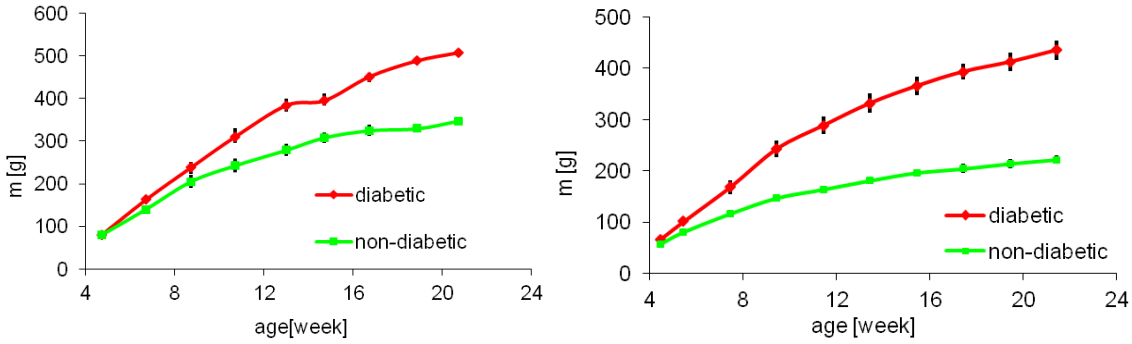

Fig. 7: Typical weight curve of ZDF males (left) and females (right). n=4, means ± SEM

The difference in weight increased at a steady pace during whole period of observation. Females did not show signs of metabolic deterioration at such an extent that was observed in males. At the start of the experiment with wounds, the body weight of diabetic animals was increased in both sexes and it was more pronounced in females, doubling that of control (Tab. 4). Elevated glucose levels, on the other hand, were more pronounced in diabetic males. Diabetes in the ZDF rats was associated with higher levels of plasma insulin, PAI-1, leptin and CRP (Tab. 4) compared to controls. Levels of MDA were elevated in males (Tab. 4) and correlated with glycemia (R=0.82, not shown). Levels of interleukin-6 were not elevated in diabetic groups (Tab. 4).

	Non-diabetic males	Diabetic males	Ratio	Non-diabetic females	Diabetic females	Ratio
Weight (g) n=13-18	363 ± 7	466 ± 13 **	1.3	218 ± 4	436 ± 7 **	2.0
Glucose (mmol/l) n=13-18	6.7 ± 0.2	17.8 ± 0.7 **	2.7	7.3 ± 0.3	11.4 ± 0.3 **	1.6
Insulin (ng/ml) n=11-14	1.28 ± 0.08	10.8 ± 1.48 **	8.4	1.63 ± 0.15	17.57 ± 1.64**	10.8
PAI-1 (ng/ml) n=9-11	2.45 ± 0.17	4.89 ± 0.75 **	2	2.62 ± 0.21	5.06 ± 0.71 **	1.93
Interleukin-6 (pg/ml) n=10-12	62.7 ± 3.0	64.0 ± 5.1	1.02	64.4 ± 6.3	62.5 ± 4.6	0.97
C - reactive protein (μg/ml) n=7-10	45.5± 5	62.5 ± 10.2	1.37	30.4 ± 3.3	47.4 ± 5.3 *	1.57
Leptin (ng/ml) n=12-16	5.05± 0.44	39.1 ± 3.29 **	7.7	2.85±0.39	51.39± 5.13 **	18.0

Tab. 4: Parameters of ZDF animals at the age of 18-20 weeks. Data are expressed as mean ± SEM, *: p<0.05, **: p<0.01 (diabetic animals compared to non-diabetic controls).

Wound closure of bandaged wounds in ZDF rats.

The appearance of bandaged wounds is shown in Fig. 9. On day 0 there was visible underlying fat tissue in diabetic wounds. On day 5 and day 10 wounds were larger in diabetic animals than in the control ones. When the wounds were covered with gauze dressing, the crust formation was suppressed (Fig. 9) as the exudate was absorbed by the gauze.

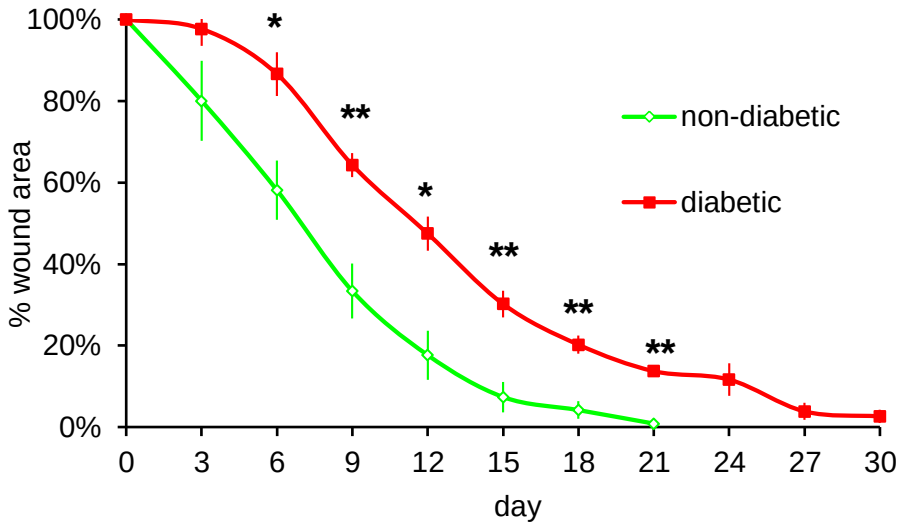

Fig. 8: Wound healing of bandaged wounds - ZDF females. n=5, mean ± SEM. *: p<0.05, **: p<0.01

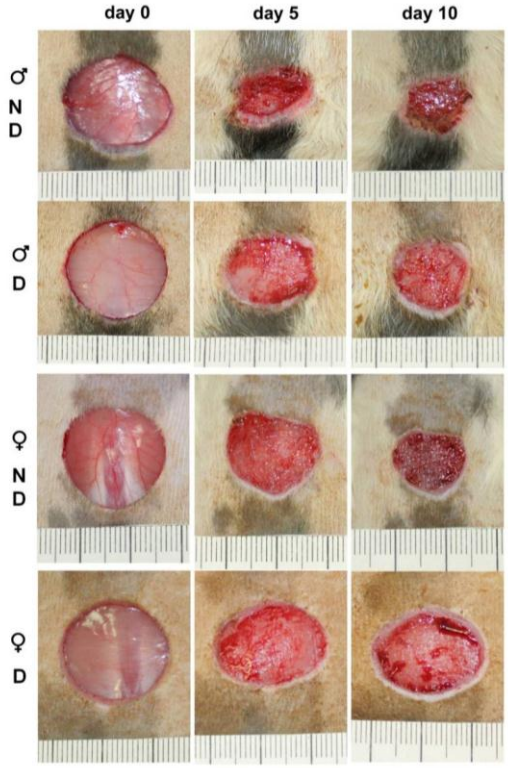

Fig. 9: Healing of excisional wounds in ZDF males and females. A: Photographs of bandaged wounds on day 0, day 5, and day 10. ND stands for non-diabetic animals, D stands for diabetic animals.

Fig. 8 and Fig. 10 show that the healing progress of diabetic wounds was retarded in both sexes, resulting in healing times about 80% longer in males and 60% in females.

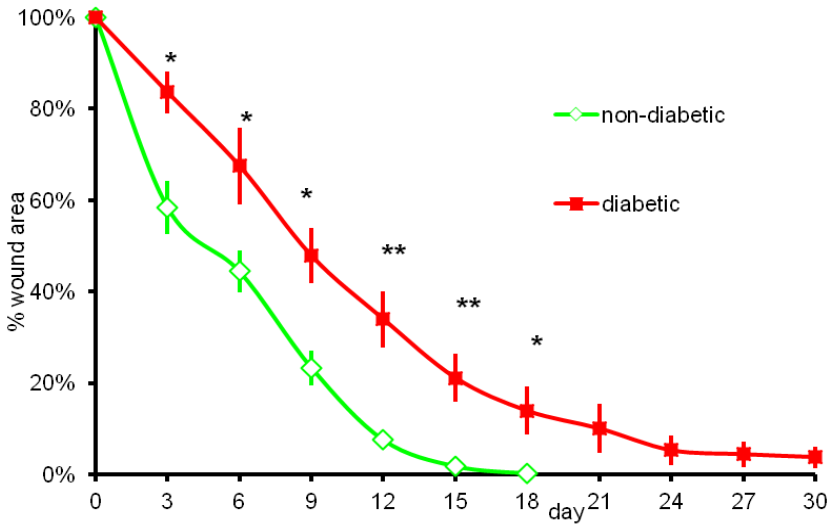

Fig. 10: Healing of bandaged wounds - ZDF males. n=6-7, mean ± SEM. *: p<0.05, **: p<0.01

Wound closure of non-bandaged wounds in ZDF rats.

Fig. 11 illustrates the macroscopic appearance of non-bandaged wounds. Wound size diminution in diabetic males was significantly retarded during 10 days after wounding (Fig. 12). The wounds were covered with a crust that was more pronounced in diabetic rats (Fig. 11).

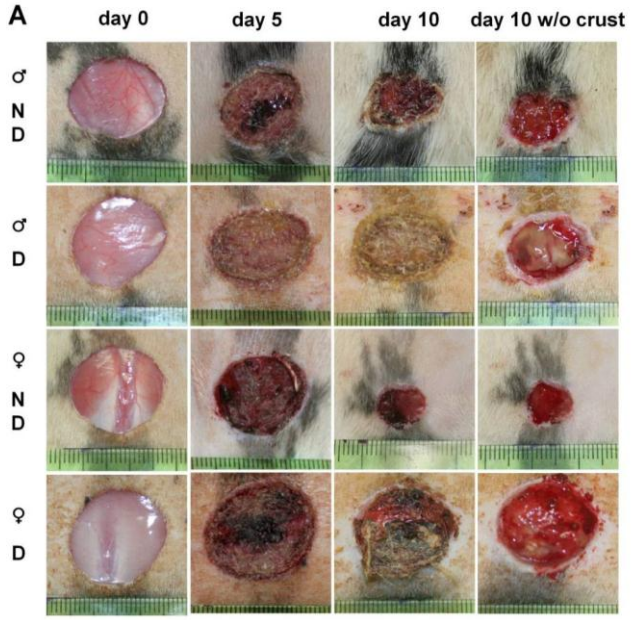

Fig. 11: Healing of excisional wounds. Photographs of wounds on day 0, day 5, on day 10 and on day 10 without crust that was removed. Note large crusts over diabetic wounds on day 10. ND stands for non-diabetic, D stands for diabetic.

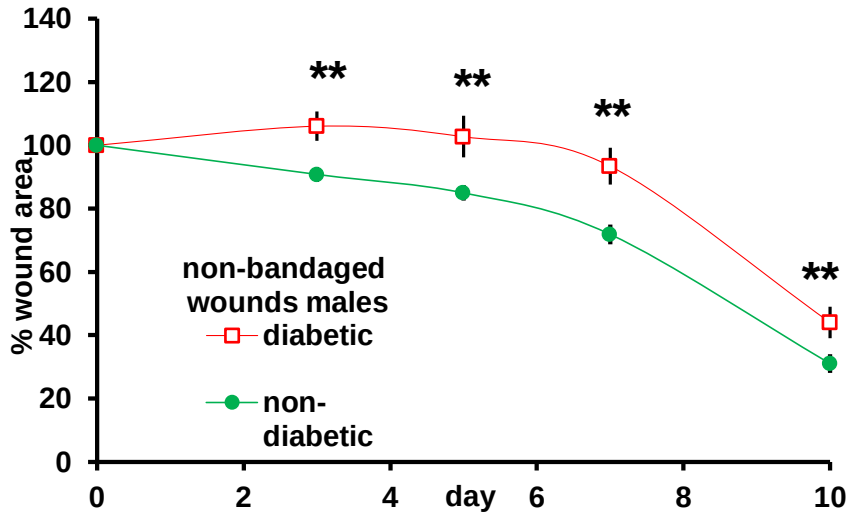

Fig. 12: Wound size changes of excisional wounds - males. Data are shown as means ± SEM. n=9-12. **: p<0.01 (diabetic animals compared to non-diabetic controls)

In diabetic females, the wound size diminution was even more profoundly retarded. The wounds tended to increase in size (peak on day 5, 128% of original size) diminishing below to their original size by day 8, whereas non-diabetic wounds were already half of their original size at that time (Fig. 13).

At the end of the experiment, on day 10, the male diabetic wounds were about 50% larger and female diabetic wounds were about 150% larger than control non-diabetic wounds.

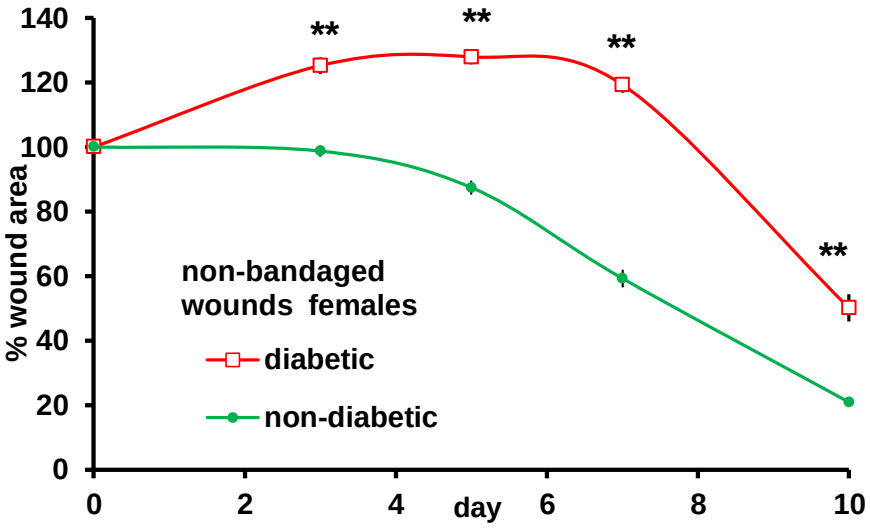

Fig. 13: Wound size changes of excisional wounds – females. Data are shown as means ± SEM. B: n=8-10, **: p<0.01 (diabetic animals compared to non-diabetic controls)

Histological analysis of adipose, epithelial, granulation and dermal tissue, and crust.

The unwounded dermis on the back of animals was slightly thinner in diabetic animals, notably in males by 30% (Tab. 5). Diabetic skin contained a thick layer of underlying adipose tissue, most notably in females (Tab. 5, Fig. 14A). The wound tissue retrieved on day 5 contained a significant amount of adipose tissue in both non-diabetic (37±5%) and diabetic animals

(70%±3%) with significantly ($p<0.01$) greater amount in diabetic animals (Fig. 14B). Fibrous granulation tissue was thicker in non-diabetic animals and it covered the entire surface of the wound, whereas in diabetic animals the granulation tissue had been infiltrated by adipose tissue and covered the wound only partially, mainly at the periphery (Fig. 14B). The wound tissue retrieved on day 10 (Fig. 14C) generally contained more granulation and fibrous tissue, and less adipose tissue compared to day 5. The granulation tissue in diabetic animals was poorly developed and irregular (tissue was not organized in a single continuous fibrous layer parallel to wound surface), and was infiltrated by adipose tissue (Fig. 14C). Diabetic wounds were characterized by an increased amount of crust (red in Fig. 14, Tab. 5). Large amount of polymorphonuclear infiltration was found under and inside the crust of diabetic animals. The proportion of adipose tissue in wound tissue was 4 to 5 times higher in diabetic than in non-diabetic animals (Tab. 5). The crust was 2 and 3 times thicker in diabetic animals in males and females, respectively (Tab. 5). The total length of the epithelial tongue over the wound was significantly increased in diabetic animals by 50% (Tab. 5).

	non-diabetic males	diabetic males	non-diabetic females	diabetic females
Dermis thickness in unwounded skin[μm]	1668±52	1159±44**	758±23	683±27
Adipose tissue thickness in unwounded skin[μm]	58±19	509±104**	45±6	1204±213**
Adipose tissue in wound tissue [%]	7.4±2.6	32.1±10*	7.2±2.4	34.2±4.2**
Crust thickness [μm]	421±47	806±59**	280±29	915±176**
Epithelial tongues [mm]	2.1±0.2	3.2±0.2*	2.3±0.1	3.4±0.2**

Tab. 5: Histological assessment of uninjured skin and wound tissue on day 10 in ZDF rats. Data are shown as means ± SEM. *: $p<0.05$, **: $p<0.01$ (diabetic animals compared to non-diabetic controls, n = 5). Length of epithelial tongues is a sum of epithelial tongues lengths from both sides of a histological sample.

Analysis of scar tissue

The wounds were closing by secondary intention, with significant contraction and resulted in the formation of scar tissue. The healed diabetic scars were thinner (Fig. 14D). Scars formed only 26% and 15% of original wound surface in non-diabetic males and females respectively. Scar size in diabetic animals was significantly increased: by 40% in males and by 140% in females. Epithelization made significantly increased contribution to wound repair in diabetic animals most notably in diabetic females.

Hydroxyproline

Hydroxyproline content per gram of wet tissue was lowered significantly in the skin of diabetic females. Accumulation of hydroxyproline during diabetic wound healing on days 5 and 10 was lowered to approximately half that of healthy controls. Lowered accumulation could be caused

by lower collagen production or more likely by partial expulsion/substitution of fibrous tissue by adipose tissue in wounds (Fig. 14 B, C).

mRNA analysis: DNA-arrays and real-time RT-PCR.

Using DNA-array analysis, simultaneously studying expression of 115 genes, it was shown that gene expression differed between diabetic and non-diabetic animals in wound tissue on day 10. In diabetic males the most up-regulated mRNAs were those of IL-6 and PAI-1, while tropoelastin mRNA was down-regulated (Tab. 6) when compared to controls. In female wounds the expression pattern was different. The mRNA levels of MMP-3 and MMP-13 were notably up-regulated in the diabetic group (Tab. 7). Significantly changed gene expressions are shown in Tab. 6 and Tab. 7.

Gene – males	mRNA expression ratio of diabetic to non-diabetic animals
Interleukin-6 (NM_012589)	1.38 *
Plasminogen activator inhibitor-1 (NM_012620)	1.23
MMP-3 (stromelysin-1, NM_133523)	1
MMP-13 (collagenase-3, M60616)	0.94
Tropoelastin (NM_012722)	0.71*
MMP-12 (macrophage metalloelastase, NM_053963)	0.68*
Interleukin-10 (NM_012854)	1.15*

Tab. 6: Relative gene expression of selected genes in granulation tissue in males on day 10 as assayed by DNA array (totally assayed 115 genes), ratio = gene expression in diabetic animals/ gene expression in non-diabetic animals, n = 7-8, *: p<0.05, **: p<0.01 (diabetic animals compared to non-diabetic controls).

Gene – females	mRNA expression ratio of diabetic to non-diabetic animals
Interleukin-6 (NM_012589)	0.93
Plasminogen activator inhibitor-1 (NM_012620)	1.20
MMP-3 (stromelysin-1, NM_133523)	2.52**
MMP-13 (collagenase-3, M60616)	1.5**
Tropoelastin (NM_012722)	0.96
iNOS (nitric oxide synthase 2, inducible, NM_01261)	0.69 *
Laminin gama 2 (XM_213902.4)	0.4 **
calgranulin B (MRP14, NM_053587)	0.79*
Plasminogen (NM_053491.1)	0.73 *
Tumor necrosis factor alpha (NM_012675)	0.72 *

Tab. 7: Relative gene expression of selected genes in granulation tissue on day 10 in females as assayed by DNA array (totally assayed 115 genes), ratio = gene expression in diabetic animals/ gene expression in non-diabetic animals, n = 7-8, *: p<0.05, **: p<0.01 (diabetic animals compared to non-diabetic controls).

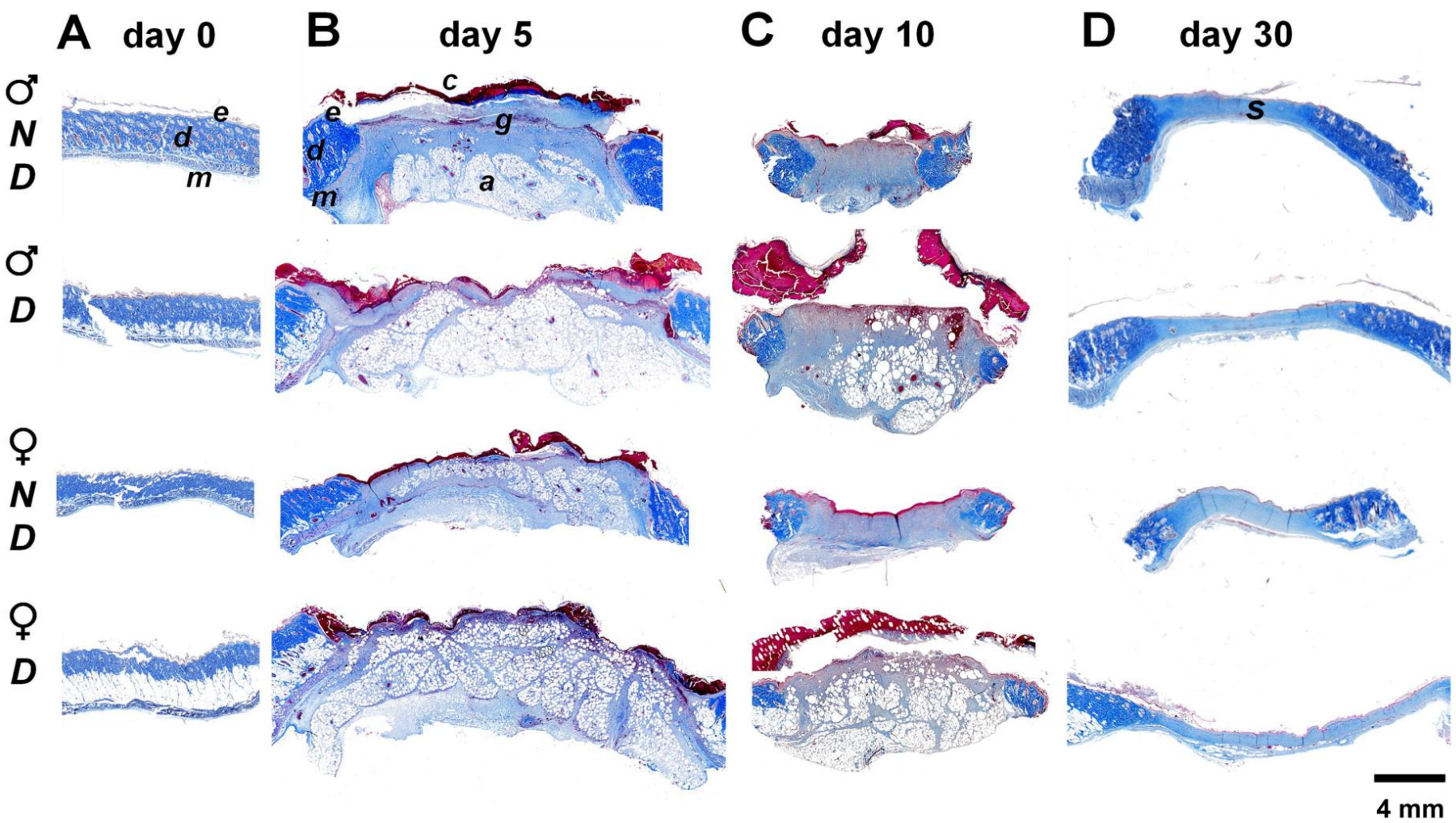

Fig. 14: Histological and immunohistochemical image of skin and wound tissue, blue trichrome-staining (1x magnification), A: skin on day 0, B: wound tissue on day 5, C: wound tissue on day 10, D: healed wound - scar on day 30. *D* - diabetic, *ND* - non-diabetic, *e* - epithelium, *d* - dermis, *m* - muscle layer, *c* -crust, *g* - granulation tissue, *a* - adipose tissue, *s* - scar.

As shown by real-time PCR analysis, the mRNA levels of IL-6, myeloperoxidase (MPO), p22-phox and rac2 subunits of NADPH-oxidase were up-regulated in males on day 10. The expression of tropoelastin and type I procollagen was down-regulated (Tab. 8). In females, the levels of IL-6, MPO, MMP-3 and MMP-13 were substantially up-regulated in diabetic wound tissue (Tab. 8).

mRNA expression ratio - diabetic to non-diabetic animals		
Gene	Males	Females
Inflammation		
Interleukin-6 (NM_012589)	3.30 *	2.76 *
Interleukin-1β (NM_031512)	1.54	1.15
Connective tissue metabolisms		
Plasminogen activator inhibitor-1 (NM_012620)	1.17	1.17
MMP-3(stromelysin-1, NM_133523)	1.33	5.70 **
MMP-13 (collagenase-3, M60616)	1.31	1.99 *
Tropoelastin (NM_012722)	0.39 *	0.83
Type I procollagen alpha II (NM_53356)	0.7*	0.85
Metabolism of reactive oxygen species		
Myeloperoxidase (XM_220830)	2.40 **	4.10 **
P22-phox –NADPH oxidase (NM_024160)	1.39 *	1.04
Rac2 - NADPH oxidase (NM_001008384)	1.64 **	1.11

Tab. 8: Relative gene expression of selected genes in granulation tissue on day 10 as assayed by quantitative RT-PCR, ratio = gene expression in diabetic / gene expression in non-diabetic animals, n = 10-14. *: p<0.05, **: p<0.01 (diabetic animals compared to non-diabetic controls).

Immunohistochemistry

Myeloperoxidase and interleukin-6

Immunohistochemical staining showed increased MPO and IL-6 positive signals at the very top inflammatory layer of diabetic wounds. This layer was composed mainly of pus and wound exudate. IL-6 positive signal was found in immune cells (Fig. 15A) and in granulation tissue fibroblasts. MPO was found in PMN cells and macrophage-like cells (Fig. 15B, C).

Matrix metalloproteinase 3 and 13

MMP-3 staining was more intense in wound tissue of diabetic females compared to control. Wound fibroblasts located on the top of granulation tissue under inflammatory layer were MMP-3 positive (Fig. 16D). Some immune cells and giant cells were also positive (Fig. 16C). The tip of the epithelial tongue was MMP-3 negative or very weak positive. MMP-13 staining was elevated in granulation tissue of diabetic females compared to control. MMP-13 was strongly positive in the epithelial cells at the bottom of the new epidermis leading edge (Fig. 16B). These positive epithelial cells were in contact with granulation tissue and were present in all studied groups. Fibroblastic cells inside the top layer of the granulation tissue were also MMP-13 positive and were parallel to would surface (Fig. 16A). This layer was located under the very top inflammatory layer.

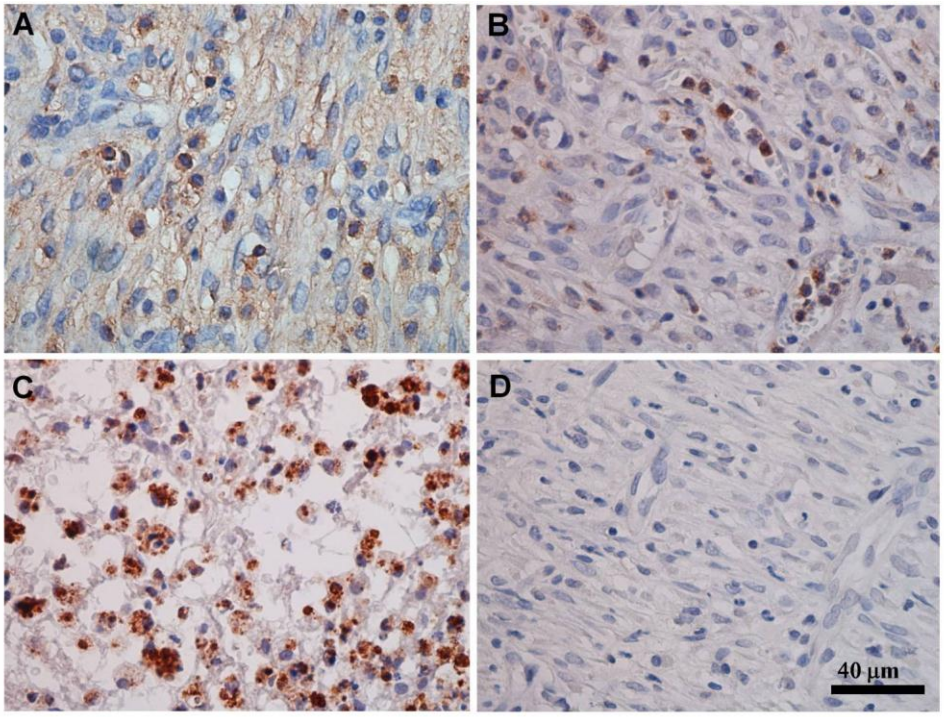

Fig. 15: Immunohistochemical image of wound tissue, peroxidase staining, wound on day 10 (60x magnification). A: Interleukin-6 in immune cells, B: myeloperoxidase in PMN cells, C: myeloperoxidase in macrophage-like cells in the wound pus, D: granulation tissue - negative control without antibody. Representative illustrative images from four to five stained tissues are shown.

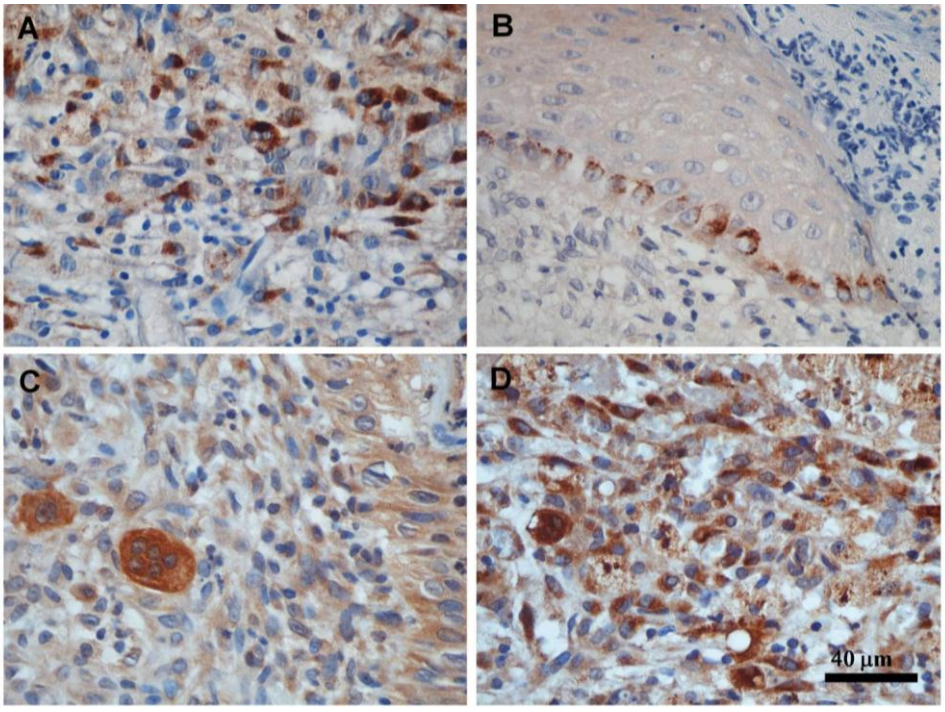

Fig. 16: Immunohistochemical image of wound tissue, peroxidase staining, wound on day 10 (60x magnification). A: MMP-13 in fibroblast-like cells on the top of granulation tissue, B: MMP-13 in epithelial cells at the tip of newly forming epithelial tongue, C: MMP-3 in multi-nucleated giant cell in the granulation tissue, D: MMP-3 in fibroblastic cell on the top of granulation tissue. Representative illustrative images from four to five stained tissues are shown.

Discussion

Part 1: Hyaluronan, Hyiodine - a potential for wound healing

Hyiodine, the combination of hyaluronate and iodine, is a novel product combining high m.w. HA and iodine. It was reported that the complex of hyaluronate-iodine had beneficial effect on wound repair in diabetic foot ulcers and hard-to-heal wound of different etiology (Sreenan et al. 1996; Sobotka et al. 2006; Sobotka et al. 2007). However, the exact mechanism of its action on wound repair is unknown. High m.w. HA has beneficial effects on wound healing (Balazs et al. 2000). It is highly viscous and when it was applied on rat skin wounds in our experiments, it made wound redressing easier because Hyiodine soaked gauze did not stick to the wound. Hyiodine applied on the wounds immediately after skin excision accelerated wound contraction in the first days of healing. Later on the course of wound closure was similar in the Hyiodine-treated and saline-treated group. The influence of added HA may be greatest in the proliferative phase of healing. The antiseptic properties of iodine may be more important in humans than in rats. Wounds treated with Hyiodine showed thickened epithelium on day 7. HA is a component of granulation tissue but its synthesis is not limited to mesenchymal cells. HA is contained in normal epidermis and it is synthesized by epidermal keratinocytes. Epidermal injury activates hyaluronan synthases in keratinocytes and causes an increase in epidermal HA. Keratinocyte migration is retarded when hyaluronan synthesis is blocked (Tsuboi et al. 1992). Exogenous HA may support epithelial hyperplasia (Arnold et al. 1995). The role of iodine in wound healing is not clear (Selvaggi et al. 2003) but PVP-iodine hydrogel was reported to improve epithelialization (Vogt et al. 2006). Hyiodine application did not change collagen accumulation in the granulation tissue. The expression of other ECM components, proteinases and cytokines was not changed when studied on mRNA level. Together, expression of 92 genes were studied, however it is now known that thousands of genes are differentially expressed during the wound repair in comparison to uninjured skin (Roy et al. 2008; Greco et al. 2010). It is possible that several important targets had been missed in our selection. Therefore, for the future clarification of the mode of action, the whole-genome wide approach is more advisable. Iodine greatly potentiated the ability of HA1200 to stimulate exudate formation. The protein composition of the exudate reminded of that of rat plasma with a prominent albumin band suggesting that a large part of exudate came from the plasma. Uronic acid content in the exudate was also increased. HA is a normal component of wound fluid but plausibly some HA applied on the wound have been retained on its surface. The nature of the interaction between iodine and HA is not clear. Iodine may bind to the glycosaminoglycan or it may oxidize it. The action of HA may be more powerful or its absorption may be slowed down and the effect of HA may be protracted in the presence of iodine.

Part 2: Characteristics of cutaneous healing of diabetic and obese ZDF rats

Diabetic wounds were accompanied by inflammation and were covered by larger crusts. During the healing, diabetic wounds were filled with a large amount of adipose tissue, poorly filled with granulation tissue and their contraction was impaired. Whereas non-diabetic wounds were almost entirely closed by contraction, in diabetic wounds, the epithelization had a more pronounced contribution to repair. Consequently, diabetic animals developed larger scars. Different fibroblast phenotype in adipose as opposed to dermal tissue could provide different

paracrine stimulation of epithelium. Adipose tissue could influence wound healing by limiting cell migration or by its adipokine production. The retardation in contraction could be mediated through physical changes such as tension in skin due to obesity. The organization of fibroblasts and myofibroblasts network could be impaired by abundant adipose tissue (Goodson et al. 1986; Bauer et al. 2004). Lower hydroxyproline concentrations and slightly lower type I collagen mRNA expression in granulation tissue was most probably caused by a displacement of collagen producing fibroblastic cells by adipocytes. The study of obese insulin resistant JCR rat showed impaired wound repair, contraction and wound collagen content (Bauer et al. 2004) comparable to our results. In our study, diabetic males had very high increase in blood glucose and relatively low increase in body weight. On the other hand, diabetic females showed a high increase in body weight and a relatively low increase in blood glucose compared to non-diabetics. We observed impaired wound repair in both sexes and therefore we propose that both factors (high obesity and high glucose) had negative effect on wound repair. Most probably the combination of high obesity and high glycemia would result in even higher impairment of wound repair due to cumulating effect. Together their study (Bauer et al. 2004) and our study suggest that obesity significantly impairs wound healing and that this parameter is as least as important as hyperglycemia.

In our work, epithelization played an important role in diabetic rats, as wound contraction was impaired. The length of epithelization tongue was increased. Increased epithelization could be caused by high levels of insulin (Apikoglu-Rabus et al. 2010) present in diabetic ZDF rats. Therefore the use of the present model for studying impaired epithelization is limited. The model we report could be modified for further studies by lowering rat insulin levels. Also increased leptin levels in diabetic rats could play a role in this phenomena, as leptin influences epithelial cells *in vitro* and *in vivo*, and epithelial tongue in wounds expresses leptin receptors (Frank et al. 2000). In db/db mice, epithelization was severely disturbed (Wall et al. 2002), and leptin levels were reported to be increased (Sahai et al. 2004) alike in ZDF rats. This difference in epithelization could be explained by different mutations of leptin receptor in ZDF fa/fa rats and in C57BL/KS-Leprdb/J db/db mice (Phillips et al. 1996).

Our study showed different patterns of the expression of several genes, some of them connected to inflammation, thus supporting observed abundant inflammation in diabetic animals. Wounds were possibly inflamed due to deregulated endogenous inflammation control or higher susceptibility to microbial infection. We have shown increased expression of IL-6 in diabetic wounds and established its spatial distribution on the very top of the wound. The role of IL-6 is complex. IL-6 is essential in the regulation of early immune response to trauma that is necessary for proper wound repair (McFarland-Mancini et al. 2010). It is produced by fibroblasts, keratinocytes, neutrophils and macrophages (Mateo et al. 1994) and its deficiency leads to impaired healing (Lin et al. 2003; McFarland-Mancini et al. 2010). However, a surfeit of IL-6 can slow down tissue repair (Gallucci et al. 2001) and decrease fibroblast proliferation (Mateo et al. 1994). IL-6 levels were elevated in patients with diabetic ulcers (Fu et al. 1999). We have observed higher levels of MMP-3 and MMP-13 mRNA, and immunohistochemical signal in diabetic females. Abundant expression of metalloproteinases could be partially responsible for lower collagen (hydroxyproline) content in diabetic wounds. Over-expression of wound MMPs can lead to proteolytic tissue damage (Lobmann et al. 2002). In chronic wounds, MMP-3 was expressed by

keratinocytes located above the basement membrane behind the leading edge of the hyperproliferative epithelium and was also detected in the granulation tissue (Saarialho-Kere et al. 1994). In our experiments, MMP-3 was not present in the epithelium, but we have detected MMP-3 in the granulation tissue in rats. MMP-3 has a role in wound contraction and activation of the contraction phenotype in fibroblasts (Bullard et al. 1999). *In vitro*, MMP-1 and MMP-3 can be induced by a shock via IL-6 autocrine stimulation in dermal fibroblasts (Park et al. 2004) and IL-6 can increase the MMP-13 gene expression in rat fibroblasts (de la Torre et al. 2005). We hypothesize that the abundant presence of IL-6 in diabetic wounds could cause the increased expression of MMP-3 and MMP-13. As MMP-1 is not present in rat tissues, rat MMP-13 is considered to play a predominant role in matrix degradation, comparable to the role of MMP-1 in humans (Okazaki et al. 2001). MMP-1 is produced by migrating epithelial cells (Parks 1999). In ZDF rats, MMP-13, playing similar role as human MMP-1, was clearly detected in keratinocytes located near wound edge and in upper layers wound fibroblasts. The limitation of our study is that only one time interval (day 10) was used for the analysis of mRNA and proteins using imunohistochemistry.

Conclusion

Positive action of hyaluronan-iodine complex observed on hard-to-heal wounds in diabetic patients was supported by our animal model study. Hyiodine speeded up the process of wound closure in the early phase of healing. The positive effect could be mediated by an effect on wound epithelium. Accentuated exudation keeps the wound moist and makes wound redressing easier. The influence of HA is supported by iodine that is not only a mere disinfectant but potentiates some effects of HA. The formation of granulation tissue and its main component, collagen, is not changed as was not the mRNA expression of the set of studied genes.

The present work revealed an impaired mode of cutaneous healing, and the structure of wound tissue in obese diabetic and lean non-diabetic ZDF rats. This model could be useful for further studies of molecular changes in wound repair caused by obesity and diabetes. The reported results warrant further research into the regulation and resolution of inflammation response, the role of adipose tissue, the deregulation of MMPs during wound repair in ZDF rats. Also the role of leptin and its receptor in epithelization could be studied on molecular level. Obese ZDF rats seem to be a suitable model for the testing substances designed to influence tissue repair and are proposed as a novel model of impaired cutaneous wound repair with type II diabetes mellitus and obesity.

References

Apikoglu-Rabus, S., et al. (2010). "*Effect of topical insulin on cutaneous wound healing in rats with or without acute diabetes.*" Clin Exp Dermatol **35**(2): 180-185.

Arnold, F., et al. (1995). "*Hyaluronan, heterogeneity, and healing: the effects of ultrapure hyaluronan of defined molecular size on the repair of full-thickness pig skin wounds.*" Wound Repair Regen **3**(3): 299-310.

Bakker, K. (1999). *International Consensus on the Diabetic Foot*. Brussels, International Working Group on the Diabetic Foot.

Balazs, E. A., et al. (2000). *Hyaluronan : Aiming for Perfect Skin Regeneration*. Scarless Wound Healing. G. H. Garg and M. T. Longaker. New York, Marcell Dekker**:** 143-160.

Bauer, B. S., et al. (2004). "*The JCR:LA-cp rat: a novel model for impaired wound healing.*" Wound Repair Regen. **12**(1): 86-92.
Brem, H., et al. (2007). "*Cellular and molecular basis of wound healing in diabetes.*" J Clin Invest **117**(5): 1219-1222.
Bullard, K. M., et al. (1999). "*Stromelysin-1-deficient fibroblasts display impaired contraction in vitro.*" J Surg Res **84**(1): 31-34.
Danis, R. P., et al. (1993). "*Microvascular retinopathy in the Zucker diabetic fatty rat.*" Invest Ophthalmol Vis Sci **34**(7): 2367-2371.
de la Torre, P., et al. (2005). "*Interleukin-6 increases rat metalloproteinase-13 gene expression through Janus kinase-2-mediated inhibition of serine/threonine phosphatase-2A.*" Cell Signal **17**(4): 427-435.
Finegood, D. T., et al. (2001). "*Beta-cell mass dynamics in Zucker diabetic fatty rats. Rosiglitazone prevents the rise in net cell death.*" Diabetes **50**(5): 1021-1029.
Frank, S., et al. (2000). "*Leptin enhances wound re-epithelialization and constitutes a direct function of leptin in skin repair.*" J Clin Invest **106**(4): 501-509.
Fu, X. B., et al. (1999). "*Transforming growth factor beta 1 and interleukin 6 mRNA expression in wound tissues of patients with diabetic ulcers.*" Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi **13**(5): 259-262.
Gallucci, R. M., et al. (2001). "*Interleukin-6 treatment augments cutaneous wound healing in immunosuppressed mice.*" J Interferon Cytokine Res **21**(8): 603-609.
Gan, D. (2007). *Diabetes Atlas, third edition*. Brussels, International Diabetic Federation.
Goodson, W. H., 3rd, et al. (1986). "*Wound collagen accumulation in obese hyperglycemic mice.*" Diabetes **35**(4): 491-495.
Greco, J. A., 3rd, et al. (2010). "*A microarray analysis of temporal gene expression profiles in thermally injured human skin.*" Burns **36**(2): 192-204.
Hurych, J., et al. (1962). "*Methods of hydroxyproline determination.*" Kozarstvi **12**: 317-323.
Chen, W. Y., et al. (1999). "*Functions of hyaluronan in wound repair.*" Wound Repair Regen **7**(2): 79-89.
Jiang, D., et al. (2007). "*Hyaluronan in tissue injury and repair.*" Annu Rev Cell Dev Biol **23**: 435-461.
Leonard, B. L., et al. (2005). "*Insulin resistance in the Zucker diabetic fatty rat: a metabolic characterisation of obese and lean phenotypes.*" Acta Diabetol **42**(4): 162-170.
Lin, Z. Q., et al. (2003). "*Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice.*" J Leukoc Biol **73**(6): 713-721.
Lobmann, R., et al. (2002). "*Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients.*" Diabetologia **45**(7): 1011-1016.
Mateo, R. B., et al. (1994). "*Interleukin-6 activity in wounds.*" Am J Physiol **266**(6 Pt 2): R1840-1844.
McFarland-Mancini, M. M., et al. (2010). "*Differences in wound healing in mice with deficiency of IL-6 versus IL-6 receptor.*" J Immunol **184**(12): 7219-7228.
Michaels, J., et al. (2007). "*db/db mice exhibit severe wound-healing impairments compared with other murine diabetic strains in a silicone-splinted excisional wound model.*" Wound Repair Regen **15**(5): 665-670.
Okazaki, I., et al. (2001). "*Reversibility of hepatic fibrosis: from the first report of collagenase in the liver to the possibility of gene therapy for recovery.*" Keio J Med **50**(2): 58-65.
Park, C. H., et al. (2004). "*Heat shock-induced matrix metalloproteinase (MMP)-1 and MMP-3 are mediated through ERK and JNK activation and via an autocrine interleukin-6 loop.*" J Invest Dermatol **123**(6): 1012-1019.
Parks, W. C. (1999). "*Matrix metalloproteinases in repair.*" Wound Repair Regen **7**(6): 423-432.
Peterson, R. G. (2007). *The Zucker Diabetic Fatty (ZDF) Rat - Lessons from a Leptin Receptor Defect Diabetic Model*. Animal models of diabetes: frontiers in research. E. Shafrir. Boca Raton, CRC Press**:** 103-118.
Peterson, R. G., et al. (1990). "*Zucker diabetic fatty as a model for non-insulindependent diabetes mellitus.*" ILAR News **32**: 16-19.
Phillips, M. S., et al. (1996). "*Leptin receptor missense mutation in the fatty Zucker rat.*" Nat Genet **13**(1): 18-19.
Rees, D. A., et al. (2005). "*Animal models of diabetes mellitus.*" Diabet Med **22**(4): 359-370.

Roy, S., et al. (2008). "*Characterization of the acute temporal changes in excisional murine cutaneous wound inflammation by screening of the wound-edge transcriptome.*" Physiol Genomics **34**(2): 162-184.
Rudas, B. (1960). "*On the quantitative determination of granulation tissue in experimentally produced wounds.*" Arzneimittelforschung **10**: 226-229.
Saarialho-Kere, U. K., et al. (1994). "*Distinct populations of basal keratinocytes express stromelysin-1 and stromelysin-2 in chronic wounds.*" J Clin Invest **94**(1): 79-88.
Sahai, A., et al. (2004). "*Obese and diabetic db/db mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: role of short-form leptin receptors and osteopontin.*" Am J Physiol Gastrointest Liver Physiol **287**(5): G1035-1043.
Selvaggi, G., et al. (2003). "*The role of iodine in antisepsis and wound management: a reappraisal.*" Acta Chir Belg **103**(3): 241-247.
Sobotka, L., et al. (2007). "*A case report of the treatment of diabetic foot ulcers using a sodium hyaluronate and iodine complex.*" Int J Low Extrem Wounds **6**(3): 143-147.
Sobotka, L., et al. (2006). "*Sodium hyaluronate and an iodine complex--Hyiodine--new method of diabetic defects treatment.*" Vnitr Lek **52**(5): 417-422.
Sreenan, S., et al. (1996). "*Prevention of hyperglycemia in the Zucker diabetic fatty rat by treatment with metformin or troglitazone.*" Am J Physiol **271**(4 Pt 1): E742-747.
Sudre, B., et al. (2002). "*Chronic inhibition of circulating dipeptidyl peptidase IV by FE 999011 delays the occurrence of diabetes in male zucker diabetic fatty rats.*" Diabetes **51**(5): 1461-1469.
Tsuboi, R., et al. (1992). "*A wound healing model using healing-impaired diabetic mice.*" J Dermatol **19**(11): 673-675.
Vaalamo, M., et al. (1997). "*Distinct populations of stromal cells express collagenase-3 (MMP-13) and collagenase-1 (MMP-1) in chronic ulcers but not in normally healing wounds.*" J Invest Dermatol **109**(1): 96-101.
Vogt, P. M., et al. (2006). "*PVP-iodine in hydrosomes and hydrogel--a novel concept in wound therapy leads to enhanced epithelialization and reduced loss of skin grafts.*" Burns **32**(6): 698-705.
Wall, S. J., et al. (2002). "*Differential expression of matrix metalloproteinases during impaired wound healing of the diabetes mouse.*" J Invest Dermatol **119**(1): 91-98.

Overview of publications

Original articles

- **Slavkovsky R**, Kohlerova R, Tkacova V, Jiroutova A, Tahmazoglu B, Velebny V, Rezačová M, Sobotka L, Kanta J. *Zucker diabetic fatty rat: a new model of impaired cutaneous wound repair with type II diabetes mellitus and obesity*. Wound Repair Regen. 2011 Jul-Aug;19(4):515-25. **IF = 2.91 (2011**)
- **Slavkovsky R**, Kohlerova R, Jiroutova A, Hajzlerova M, Sobotka L, Cermakova E, Kanta J. *Effects of hyaluronan and iodine on wound contraction and granulation tissue formation in rat skin wounds*. Clin Exp Dermatol. 2010 Jun;35(4):373-9. **IF = 1.279 (2010**).
- Jiroutova A, Peterova E, Bittnerova L, **Slavkovsky R**, Cevelova P, Rezacova M, Cerman J, Micuda S, Kanta J. *Collagenolytic potential of rat liver myofibroblasts*. Physiol Res. 2012 Nov 22. **IF = 2.053 (2011)**
- Jiroutova A, **Slavkovsky R**, Cermakova M, Majdiakova L, Hanovcova I, Bolehovska R, Hajzlerova M, Radilova H, Ruszova E, Kanta J. *Expression of mRNAs related to connective tissue metabolism in rat hepatic stellate cells and myofibroblasts*. Exp Toxicol Pathol. 2007 Jan;58(4):263-73. **IF = 1.555 (2011)**

Lectures and posters at professional meetings

- **Slavkovský R.**, Pavlík V, Stejskalová J, Kučera J, Klein P, Velebný V. *Profilování transkriptómu v průběhu hojení prasečí rány. Možnosti použití pro vývoj prostředků na hojení ran.* Conference RANK (Rutinní analýza nukleových kyselin molekulárně-biologickými technikami). Pardubice, 1 - 2. 2. 2012
- **Slavkovský R.**, Sojka M, Vištejnová L, Novotná M, Chládková D, Velebný V. *JÓD je efektivní volba v boji proti bakteriálnímu biofilmu.* 26 - 27. 1. 2012, X. celostátní kongres s mezinárodní účastí, ČSLR (Česká společnost pro léčbu rány), Pardubice.
- **Slavkovský R,** Tkáčová V, Köhlerová R, Jiroutová A, Sobotka L, Velebný V, Kanta. *Potkan ZDF - nový experimentální model narušeného hojení kožních ran vlivem diabetu typu II a obezity.* VII. Celostátní kongres s mezinárodní účastí na téma : Mezioborová spolupráce při léčbé ran a kožních defektů. Pardubice 29 - 30. 1. 2009.
- **Slavkovský R** et al. *ZDF rat – a novel model of impaired cutaneous wound repair under conditions of type II diabetes mellitus and obesity.* 5th International Postgraduate Medical Students Conference, Hradec Králové, 27 - 29. 11. 2008.
- Muthný T, **Slavkovský R**, Vištejnová L, Hašová M, Velebný V. Whole genome microarray assay of young and elderly human skin fibroblasts. ANTI-AGEING SKIN CARE CONFERENCE 2012. 12-13 June 2012, Royal College of Physicians, London.

Posters

- **Slavkovský R,** Pavlík V, Kučera J, Klein P, Velebný V. *Expression analysis of minipig wounds –potential markers of wound healing.* XXIII. biochemický sjezd, Brno, 26. – 29. 8. 2012. Presented by Pavlík V.
- **Slavkovský R**, Novotná M, Niedoba K, Klein P, Velebný V. *Iodine antiseptics eliminate biofilm formed in vitro by chronic wound bacteria.* Poster EWMA, Brussels, 25-27. 5. 2011.
- **Slavkovský R**, Köhlerová R, Hajzlerova M, Jiroutová A, Sobotka L, Kanta J : *Potkan Zucker Diabetic Fatty (ZDF) - experimentálny model k štúdiu zhoršeného hojenia kožných rán za prítomnosti diabetes mellitus typu II* – Konference Rány a Defekty 2007, Ostrava, 7. 9. 2007.
- **Slavkovský R**, Ruszová E, Hájková V, Dvořáková J , Velebný V. *Hyaluronan is an active molecule in the angiogenesis. In vitro study of the effect of hyaluronan on human dermal microvascular endothelial cells (HDMVEC).* Poster, 3rd World conference on regenerative medicine, Leipzig 18 - 20. 10. 2007.
- Muthný T, **Slavkovský R,** Velebný V. Aged fibroblast gene array – looking for new affected transcripts. Presented at 2012 Society for Investigative Dermatology Annual Meeting, 9 – 12. 5. 2012, Raleigh, North Carolina, USA. Abstract in Journal of Investigative Dermatology (2012) 132, S65–S74.