

## Usporedba sustavnih upalnih i hematoloških parametara kod zdravih C57Bl/6 i genetski dijabetičnih db/db miševa tijekom cijeljenja lokalne rane

### Comparison of systemic inflammatory and hematology parameters in normal C57Bl/6 and genetically diabetic db/db mice during local wound repair

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#### Sažetak

**Uvod:** Upala je početni odgovor domaćina na ozljedu. Ona nije ograničena samo na mjesto rane, nego izaziva sustavne promjene uključujući raznovrsne fiziološke i biokemijske promjene koje se skupno nazivaju odgovorom akutne faze. Ove se promjene nastavljaju tijekom rješavanja upale i procesa cijeljenja rane. U ovom ispitivanju smo usporedili serumski amiloid A protein (SAA), hematološke parametre (ukupna bijela krvna slika, postotak neutrofila i limfocita) te koncentracije interferona-gama (IFN- $\gamma$ ) u serumu tijekom cijeljenja neokludirane, ekscizijske kožne rane u punoj debljini kod genetski dijabetičnih db/db miševa i nedijabetičnih C57Bl/6 miševa iz istoga legla.

**Materijal i metode:** Područje rane izazvane „punch“ biopsijom (promjera 8 mm) kod svakog je miša analizirano planimetrijski uz računalnu potporu. Trećeg, 6., 9. i 13. dana od ranjavanja SAA i IFN-g mjereni su u plazmi testovima ELISA, a hematološki parametri u punoj krvi na automatskom hematološkom analizatoru Sysmex SF 3000.

**Rezultati:** Šestog i devetog dana jasno je zabilježeno kašnjenje u zatvaranju rane kod db/db miševa u usporedbi sa zdravim miševima. Ukupna bijela krvna slika bila je značajno viša u db/db miševa 9. i 13. dana. Kroz čitavo razdoblje obnove rane, diferencijalni broj neutrofila bio je viši, a broj limfocita niži kod db/db miševa u usporedbi s C57Bl/6 miševima. Vršne koncentracije SAA zabilježene su 3. dana kod C57Bl/6 miševa i db/db miševa (368,7 mg/L odnosno 173,5 mg/L), s težnjom prema nižim vrijednostima kod db/db miševa. Razine IFN- $\gamma$  bile su značajno više ( $P < 0,05$ ) 9. i 13. dana kod db/db miševa (75,3 pg/mL odnosno 89,9 pg/mL) u usporedbi s razinama kod C57Bl/6 miševa (66,6 pg/mL odnosno 57,2 pg/mL).

**Zaljučak:** Lokalni proces tkivne regeneracije kod miševa nakon lokalne kožne ozljede uzrokuje sustavne promjene u perifernoj krvi. Niti određivanje koncentracije SAA niti IFN- $\gamma$  nije se moglo rabiti za motrenje dinamike cijeljenja rane u ovim vremenskim točkama.

**Ključne riječi:** db/db miševi, serumski amiloid A protein, interferon-gama, upala

#### Abstract

**Introduction:** Inflammation is the initial host response to injury. It is not only localized to the wound site but also causes systemic changes, including a variety of physiological and biochemical changes collectively called the acute phase response. These changes continue during the resolution of inflammation and the wound healing process. In this study we compared serum amyloid A protein (SAA), hematological parameters (total white blood cell count, neutrophil and lymphocyte percentage) and interferon-gamma (IFN- $\gamma$ ) concentrations in serum during healing of non-occluded, excisional, full-thickness dermal wounds in genetically diabetic db/db mice and non-diabetic C57Bl/6 littermates.

**Materials and Methods:** Area of a punch biopsy (8 mm in diameter) wound in each mouse was analyzed by computer-assisted planimetry. On days 3, 6, 9 and 13 after wounding, SAA and IFN- $\gamma$  were measured in plasma by ELISA assays and hematological parameters in whole blood by Sysmex SF 3000 automatic hematology analyzer.

**Results:** A delay in the closure of wounds in db/db in comparison to normal mice was clearly seen on days 6 and 9. Total white blood cell count was significantly higher on days 9 and 13 in db/db mice. Differential neutrophil counts were higher and lymphocyte counts lower in db/db mice in comparison to C57Bl/6 mice throughout the wound repair period. Peak SAA concentrations were seen on day 3 in C57Bl/6 and db/db mice (368.7 mg/L and 173.5 mg/L, respectively), but tended to be lower in db/db mice. IFN- $\gamma$  levels were significantly higher ( $P < 0.05$ ) on days 9 and 13 in db/db (75.3 pg/mL and 89.9 pg/mL, respectively) in comparison to those in C57Bl/6 mice (66.6 pg/mL and 57.2 pg/mL, respectively).

**Conclusion.** The local tissue regeneration process in mice after local skin injury causes systemic changes in peripheral blood. Determination of neither SAA nor IFN- $\gamma$  concentrations could be used to monitor wound healing dynamics at these time points.

**Key words:** db/db mice, serum amyloid A protein, interferon-gamma, inflammation

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## Uvod

Akutna upala je presudna početna faza u procesu cijeljenja rane, koja dovodi do strukturnog i funkcijskog popravka oštećenog tkiva. Upalna kaskada nastupa smjesta, prvenstveno putem aktiviranih monocita u krvi i tkivnih makrofaga na mjestu ozljede, te oslobođenih upalnih posrednika kao što su IL-1 i IL-6, što također uzrokuje sustavne promjene (1). Brz porast serumskih koncentracija proteina akutne faze (APP) jedan je od najbolje istraženih sustavnih odgovora na akutni upalni poticaj (2,3). Serumski amiloid A protein (SAA) je APP niske molekularne težine koji se prvenstveno proizvodi u jetri u odgovoru na proupalne citokine. Razina SAA u krvi raste najsnažnije i najbrže među svim APP, pa je toga osjetljiv pokazatelj upale i koristan u motrenju učinkovitosti protumikrobne i protuupalne terapije (4-6). Interferoni pripadaju mreži citokina koji su uključeni u kontrolu stanične funkcije i imaju značajnu ulogu u aktiviranju makrofaga, poglavito interferona-gama (IFN- $\gamma$ ), važnog aktivatora imunog odgovora i modulatora cijeljenja rane (7,8). IFN- $\gamma$  je uključen u homeostazu proliferacije epidermalnih keratinocita (9), pa sustavno davanje IFN- $\gamma$  nakon ozljede smanjuje nakupljanje kolagena rane i očito snižava početni upalni odgovor (10). Poznato je da je šećerna bolest udružena s odgođenim cijeljenjem rana, a genetski dijabetični (db/db) miševi su koristan životinjski model za ovo stanje, jer je u ovih životinja cijeljenje rane znatno odgođeno u usporedbi s nedijabetičnim C57Bl/6 miševina iz istoga legla (11,12). Poremećaj cijeljenja obilježen je kašnjenjem u staničnoj infiltraciji i stvaranju granulacijskog tkiva, smanjenom angiogenezom, sniženim kolagenom i njegovom organizacijom (13,14). Kod db/db miševa je razvoj šećerne bolesti povezan s odsutnošću funkcijske izoforme receptora leptina (15,16). Leptin, proizvod ob gena, sintetizira se u masnom tkivu i njegove koncentracije koreliraju s količinom tjelesne masti (17). Leptin je uključen u regulaciju energetske ravnoteže, ali isto tako i u odgovor akutne faze na tkivnu ozljedu i sustavnu upalu, vjerojatno zato što transmembranski receptor leptina ima strukturnih sličnosti s porodicom citokina sličnih IL-6 (18,19).

U ovoj studiji ispitivali smo promjene u sustavnom upalnom odgovoru, odnosno njihov odraz na SAA, IFN- $\gamma$  te broj neutrofila i diferencijalni broj limfocita u perifernoj krvi tijekom oporavka kožne rane kod db/db miševa i njihovih kontrola iz istoga legla, kako bismo utvrdili je li sustavni upalni odgovor također odgođen u db/db miševa. Nadali smo se kako ćemo na taj način moći utvrditi mogu li se sustavni upalni parametri rabiti kao biološki biljezi za lokalno cijeljenje rane.

## Introduction

Acute inflammation is a crucial initial phase in the wound healing process that leads to structural and functional repair of injured tissue. Prompt initiation of the inflammatory cascade occurs primarily through activated blood monocytes and tissue macrophages at the wound site, and the released inflammatory mediators such as IL-1 and IL-6, also causing systemic changes (1). One of the most intensively studied systemic responses to an acute inflammatory stimulus is the rapid increase in serum concentrations of acute phase proteins (APPs) (2,3). Serum amyloid A protein (SAA) is a low molecular weight APP, which is produced primarily by the liver in response to pro-inflammatory cytokines. The SAA level in blood exhibits the most intense and rapid increase among all APPs and therefore is a sensitive indicator of inflammation and valuable in monitoring efficacy of antimicrobial and anti-inflammatory therapy (4-6). Interferons belong to the network of cytokines that are involved in the control of cellular function and they play an important role in macrophage activation, especially interferon-gamma (IFN- $\gamma$ ), an important activator of the immune response and modulator of wound healing (7,8). IFN- $\gamma$  has been implicated in the homeostasis of epidermal keratinocyte proliferation (9), and systemic administration of IFN- $\gamma$  upon injury decreased wound collagen deposition and clearly reduced the initial inflammatory response (10).

Diabetes is well known to be associated with delayed healing of wounds and genetically diabetic (db/db) mice are useful as an animal model for this condition, since wound healing in these animals is markedly delayed as compared to non-diabetic C57Bl/6 littermates (11,12). Healing impairment is characterized by delayed cellular infiltration and granulation tissue formation, reduced angiogenesis, decreased collagen and its organization (13,14). In db/db mice, development of diabetes is coupled with the absence of the functional isoform of the leptin receptor (15,16). Leptin, a product of the ob gene, is synthesized by adipose tissue and its level correlates with the amount of body fat (17). It is involved in energy balance regulation but also in the acute phase response to tissue injury and systemic inflammation, probably because the leptin transmembrane receptor has structural similarities to the IL-6-like cytokine family (18,19).

In this study, we investigated changes in the systemic inflammatory response, as reflected by SAA, IFN- $\gamma$  and neutrophil and lymphocyte differential counts in peripheral blood during cutaneous wound repair in db/db mice and their littermate controls to determine whether the systemic inflammatory response is also delayed in db/db mice. In this way, we hoped to be able to determine whether systemic inflammatory parameters could be used as biomarkers for local wound healing.

## Materijali i metode

### Životinje

Dvadeset genetski dijabetičnih ženka db+/db+ C57BL/KsJ miševa i dvadeset kontrolnih ženka C57Bl/6 miševa, svi stari 6 tjedana, pribavljeni su od Charles River Laboratories, Belgija. Životinje su ostavljene da se prilagode kroz 10 dana, obilježene i pojedinačno smještene za pokuse. Miševi su držani u standardnim laboratorijskim uvjetima, uz hranu *ad libitum*.

Svi postupci na životinjama provodili su se u skladu s a) Direktivom Savjeta EEC 86/609 od 24. studenoga 1986. o približavanju zakona, pravila i administrativnih propisa Zemačja članica glede zaštite životinja koje se rabe u eksperimentalne i druge znanstvene svrhe; i b) Ustavom Republike Hrvatske, Zakon o dobrobiti životinja, Narodne novine 081-99-266/1 od 9. veljače 1999.

### Eksperimentalni postupak

Dijabetični db/db miševi i nedijabetični C57Bl/6 miševi podijeljeni su u 4 skupine po 5 životinja. Dana -1. miševi su anestetizirani udisanjem izoflurana (Forane, izofluran, inhalacijski anestetik, Abbott Laboratories, Engleska) i 5%-tnog kisika u komori za indukciju anestezije (Stoelting Co., SAD). Upuhivanje plinova izvedeno je pomoću sustava Fluovac 240V (International Market Supply, Engleska). Potom se anestezija održavala pomoću maske (Stoelting Co., SAD) izofluranom i 4%-tnim kisikom, uz povremenu provjeru nožnog refleksa. Kad su životinje bile u potpunoj anesteziji, interskapularno područje se temeljito obrijalo (aparatus za šišanje na baterije Contura, International Market Supply, Engleska) i preostala dlaka je uklonjena četkicom. Nakon 24 sata (dan 0.) životinje su anestetizirane kao na dan -1., a obrijano područje je dezinficirano (Pursept-A, Merz Hygiene, Njemačka). Primjenjujući strogo aseptične postupke, načinjena je jedna ekscizijska rana u punoj debljini, promjera 8 mm, po sredini obrijanog područja kod svake životinje (20) sterilnim jednokratnim zaobljenim nožićem za biopsiju (Stiefel Laboratories Ltd., Irska), otkrivajući mišićnu fasciju. Životinje su vraćene u kaveze u koje je dodano ponešto poput komadića hrane da im odvratiti pozornost, te ostavljene da se povrate iz anestezije. Trećeg, 6., 9. i 13. dana je pet db/db miševa i pet C57Bl/6 miševa anestetizirano kao što je gore opisano, zatim im je ispuštena krv ubodom u jugularnu venu i zajedničku karotidnu arteriju i prikupljena u mikro-eprovete Becton Dickinson koje su sadržavale EDTA.

### Analiza zatvaranja rane

Napravljene su serijske standardne 2D fotografije svake rane digitalnim aparatom Olympus C-2040 Zoom (Olympus Optical Co., Ltd., Japan) odmah nakon ranjavanja (dan 0.) te 1., 3., 6. i 9. dana dok su životinje bile pod anestezijom izofluranom. Digitalne snimke su obrađene uz primjenu

## Materials and methods

### Animals

Twenty genetically diabetic female C57BL/KsJ db+/db+ mice and twenty female C57Bl/6 control mice, all 6 weeks old, were obtained from Charles River Laboratories, Belgium. Animals were allowed to acclimatize for 10 days, marked and individually housed for the experiments. Mice were kept under standard laboratory conditions. Food and water were provided *ad libitum*.

All procedures on animals were performed in accordance with the (a) EEC Council Directive 86/609 of November 24, 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes; and (b) Statute of Republic Croatia, Animal Welfare Law, Official Gazette 081-99-266/1 of February 9, 1999.

### Experimental procedure

Diabetic db/db mice and non-diabetic C57Bl/6 mice were divided into 4 groups of 5 animals. On day -1, mice were anesthetized by inhalation of isoflurane (Forane, isoflurane, inhalation anesthetic, Abbott Laboratories, England) and 5% oxygen, delivered in an anesthesia induction chamber (Stoelting Co., USA). Gas scavenging was provided using the Fluovac 240V system (International Market Supply, England). Subsequently, anesthesia was maintained, using a mask (Stoelting Co., USA), with isoflurane and 4% oxygen, the pedal reflex response being checked at intervals. In the fully anesthetized animal, the interscapular region was surgically close-shaved (Contura cordless clipper, International Market Supply, England), brushing off excess loose hair. Twenty-four hours later (day 0), animals were anesthetized as on day -1 and the shaved region was disinfected (Pursept-A, Merz Hygiene, Germany). Utilizing strictly aseptic procedures, a single full-thickness excisional wound 8 mm in diameter was made midline in the shaved region of each animal (20), with a sterile, disposable biopsy punch (Stiefel Laboratories Ltd., Ireland), exposing the underlying muscular fasciae. Animals were returned to their cages with some form of distractive enrichment like food pellet, and allowed to recover from the anesthesia. On days 3, 6, 9 and 13 five db/db and five C57Bl/6 mice were anesthetized as before and exsanguinated by puncturing the jugular vein and common carotid artery into Becton Dickinson EDTA containing microtainers.

### Analysis of wound closure

Serial standard 2D photographs of each wound were made with an Olympus C-2040 Zoom digital camera (Olympus Optical Co., Ltd., Japan) immediately after wounding (day 0) and on days 1, 3, 6 and 9 while animals were un-

sustava Leica QWin Image Processing and Analysis System (Leica Imaging System Ltd., Velika Britanija) s ručnim uređajem koji omogućava ručno praćenje granica rane. Za svaku snimku je izračunato područje rane.

### Priprava i analiza uzoraka

Alikvoti krvnih uzoraka prethodno su razrijeđeni otopinom Sysmex Cell-pack u omjeru 1:5 i analizirani na automatskom hematološkom analizatoru Sysmex SF 3000 unutar 4 sata od uzorkovanja. Potom je krv centrifugirana na 3500 okr/min kroz 15 minuta na sobnoj temperaturi. Alikvoti plazme smrznuti su na  $-20^{\circ}\text{C}$  i pohranjeni do analize. Kako bismo odredili razine SAA, uzorci su prethodno razrijeđeni 1:200. Koncentracije SAA određivale su se istraživačkim testom ELISA dostupnim na tržištu (Phase Serum Amyloid A Assay (Murine), Tridelta, Irska) (CV < 10%, analitička osjetljivost 0,03 mg/mL).

IFN- $\gamma$  se mjerio istraživačkim testom ELISA (Mouse IFN $\gamma$  Biotrak Assay, Amersham Biosciences, Velika Britanija) (CV < 10%, osjetljivost < 10 pg/mL).

### Statistička analiza i procjena

Statistički izračuni za sve parametre izvedeni su pomoću programa GrafPad. Kako bismo utvrdili razliku između db/db miševa i zdravih C57Bl/6 miševa, sirovi podaci su analizirani pomoću Mann-Whitneyevog testa. Razlika između vremenskih točaka unutar iste skupine analizirana je neparametrijskom jednosmjernom analizom varijance (ANOVA) uz uporabu Kruskal-Wallisova testa s Dunnovim testom višestruke usporedbe. Razina značajnosti postavljena je na  $P < 0,05$ . Svi parametri su prikazani kao medijani s 95%-tnom granicom pouzdanosti, osim za SAA (medijani s 1. i 3. kvartilom).

## Rezultati

### Zatvaranje rane

Izrezivanje kože dovelo je do uvlačenja rane u prvom danu kod svih životinja, ali je bilo značajno izraženije kod C57Bl/6 miševa (tablica 1.). Dana 0. su područja rane bila značajno veća kod db/db miševa negoli kod C57Bl/6 miševa iz istoga legla, ukazujući na smanjenu elastičnost rubova rane kod dijabetičnih pretilih životinja. Kod db/db miševa se uvlačenje rane nastavilo do 6. dana, dok je kod C57Bl/6 miševa u to vrijeme već započelo zatvaranje rane. Trećega dana je relativna veličina rane kod C57Bl/6 miševa bila jednaka kao i kod db/db miševa, dok je 9. dana veličina rane bila značajno manja kod C57Bl/6 miševa u usporedbi s db/db miševima, ukazujući na značajno brže zatvaranje rane kod nedijabetičnih C57Bl/6 miševa. Do 13. dana zacijelile su sve rane kod C57Bl/6 miševa i 70% rana kod db/db miševa (ovi podaci nisu prikazani).

der isoflurane anesthesia. Digital images were processed using the Leica QWin Image Processing and Analysis System (Leica Imaging System Ltd., UK), the manual tool bar allowing manual tracing of the wound margins. Wound area was calculated for each image.

### Sample preparation and analyses

Aliquots of blood samples were pre-diluted with Sysmex Cell-pack solution at a 1:5 ratio and analyzed on the Sysmex SF 3000 automatic hematology analyzer within 4 h of withdrawal. Afterward, blood was centrifuged at 3500 rpm for 15 min at room temperature. Plasma aliquots were stored frozen at  $-20^{\circ}\text{C}$  until analyzed.

In order to determine the levels of SAA, samples were pre-diluted 1:200. SAA concentrations were determined by commercial ELISA (Phase Serum Amyloid A Assay (Murine), Tridelta, Ireland) research kit (CV < 10%, analytical sensitivity 0.03  $\mu\text{g/mL}$ ).

IFN- $\gamma$  was measured by commercial ELISA (Mouse IFN $\gamma$  Biotrak Assay, Amersham Biosciences, UK) research kit (CV < 10%, sensitivity < 10 pg/mL).

### Statistical analysis and evaluation

Statistical calculations for all parameters were performed using GrafPad software. In order to determine the difference between db/db and healthy C57Bl/6 mice, raw data were analyzed by Mann-Whitney test. The difference between time points within the same group was analyzed by non-parametric one-way analysis of variance (ANOVA) using Kruskal-Wallis test with Dunn's multiple comparison test. The level of significance was set at  $P < 0.05$ . All parameters are presented as medians with 95% confidence limits, except for SAA (medians with 1<sup>st</sup> and 3<sup>rd</sup> quartiles).

## Results

### Wound closure

Excision of skin resulted in retraction of the wounds within the first day in all animals and was significantly greater in C57Bl/6 mice (Table 1). On day 0, wound areas in db/db mice were significantly greater than in C57Bl/6 littermates, indicating reduced elasticity in the wound margins of diabetic, obese animals. In db/db mice, wound retraction continued till day 6, while in C57Bl/6 mice at that time wound closure had already started. On day 3, relative wound size in C57Bl/6 was the same as in db/db mice, while on day 9 it was significantly smaller in C57Bl/6 mice than in db/db mice, indicating significantly faster wound closure in non-diabetic C57Bl/6 mice. By day 13, all wounds in C57Bl/6 and 70% of wounds in db/db mice were healed (data not shown).

**TABLICA 1.** Parametri mjereni kod C57Bl/6 i db/db miševa tijekom studije. Podaci su prikazani kao medijani uz 95%-tne granice vjerodostojnosti (N = 5)**TABLE 1.** Parameters measured in C57Bl/6 and db/db mice during the study period. Data are presented as medians with 95% confidence limits (N = 5)

Parameter	Time point (days)	C57Bl/6 mice	db/db mice	P (Mann-Whitney test)
Wound area (pixel)	0	18786 (14531–20849)	27671 (25382–29599)	0.008
	1	33421 (24107–41852)	36881 (31364–44432)	n.s.
	3	35973 (26269–43097)	36674 (31156–49157)	n.s.
	6	25724 (16283–33575)	34792 (23912–46593)	n.s.
	9	10107(3491–13726)	15935 (10379–28663)	0.016
WBC (x10 <sup>9</sup> /L)	3	4.6 (3.1–5.2)	3.7 (2.8–5.1)	n.s.
	6	5.0 (3.3–6.7)	5.7 (4.4–7.7)	n.s.
	9	3.9 (2.5–4.7)	8.4 (4.5–10.6)	0.032
	13	3.0 (2.5–3.7)	7.4 (4.1–9.1)	0.016
Neutrophil (%)	3	10.8 (4.8–17.8)	28.7 (17.3–49.8)	0.008
	6	11.6 (8.2–13.3)	21.3 (18.9–28.9)	0.008
	9	9.3 (5.0–11.9)	28.1 (19.7–39.0)	0.008
	13	7.3 (4.4–11.4)	34.1 (23.5–53.8)	0.008
Lymphocyte (%)	3	85.9 (80.0–91.6)	64.4 (41.4–74.4)	0.008
	6	86.9 (83.9–90.1)	74.0 (61.7–78.9)	0.008
	9	90.2 (88.2–93.0)	64.9 (53.3–74.3)	0.008
	13	91.3 (85.9–94.8)	62.1 (42.1–71.4)	0.008
SAA (mg/L)*	3	368.7 (170.5–599.2)	173.5 (107.1–437.2)	n.s.
	6	62.0 (24.9–77.7)	61.0 (49.9–137.7)	n.s.
	9	10.0 (10.0–10.0)	11.7 (10.0–13.8)	n.s.
	13	10.0 (10.0–10.0)	13.3 (10.6–22.2)	n.s.
IFN-γ (pg/mL)	3	62.7 (54.1–79.2)	67.6 (44.4–111.8)	n.s.
	6	69.1 (56.1–83.2)	74.3 (58.8–92.6)	n.s.
	9	66.6 (63.5–71.6)	75.3 (67.2–86.8)	0.032
	13	57.2 (39.3–90.7)	89.9 (61.6–122.4)	0.014

\*Data are presented as medians with 1<sup>st</sup> and 3<sup>rd</sup> quartiles (N = 5); n.s. – non significant

### Hematološki parametri

Kod C57Bl/6 miševa se ukupan broj bijelih stanica u krvi povećao i postigao vršnu vrijednost 6. dana, nakon čega se snižavao do 13. dana, uza statističku značajnost ( $P = 0,037$  (tablica 1.). Kod db/db miševa je ukupna bijela krvna slika postigla vršnu vrijednost kasnije (9. dana) nego kod nedijabetičnih miševa iz istoga legla, dostižući statističku značajnost prema 3. danu ( $P = 0,048$ ) i ostala je relativno visoka sve do kraja ispitivanja (13. dan). Devetog i 13. dana je ukupna bijela krvna slika bila značajno viša kod db/db miševa negoli kod C57Bl/6 miševa ( $P = 0,032$  odnosno  $P =$

### Hematology parameters

Total white blood cell (WBC) count in blood from C57Bl/6 mice increased and reached peak value on day 6, subsequently falling to day 13, achieving statistical significance ( $P = 0.037$ ) (Table 1). In db/db mice, total WBC count reached peak value later (day 9) in relation to non-diabetic littermates reaching statistical significance vs. day 3 ( $P = 0.048$ ), and remained relatively high until the end of the study (day 13). On days 9 and 13, total WBC count was significantly higher in db/db mice than in C57Bl/6 mice ( $P = 0.032$  and  $P = 0.016$ , respectively) (Table 1). The WBC refe-

0,016) (tablica 1.). Prema Charles River Laboratories, referentni raspon za bijelu krvnu sliku za ženske C57Bl/6 miševе je  $8,09 \pm 2,29 \times 10^9/L$ .

Diferencijalni broj neutrofila u krvi C57Bl/6 miševa postupno je opadao kroz čitavo vrijeme promatranja. Diferencijalni broj neutrofila u krvi db/db miševa bio je 3. dana gotovo trostruko viši od onoga kod zdravih miševa i taj se porast održavao tijekom čitavog razdoblja promatranja (tablica 1.). Šestoga dana se postotak neutrofila kod db/db miševa snizio, ali bez statističke značajnosti. Diferencijalni broj limfocita u krvi zdravih miševa pokazao je blagi porast prema kraju studije. Suprotno tome, broj limfocita u krvi db/db miševa povećavao se do 6. dana, nakon čega je uslijedio pad sve do kraja studije, ali bez statističke značajnosti (tablica 1.).

### SAA i IFN- $\gamma$

Referentni raspon za SAA kod miševa je 0,2–40 mg/L (21,22). Kod ranjenih C57Bl/6 miševa se koncentracija SAA snižavala od 3. do 6. dana, ali to nije bilo statistički značajno. Devetog i 13. dana od ranjavanja su razine SAA kod svih životinja bile ispod 40 mg/L, unutar normalnog raspona (tablica 1.). Kod ranjenih db/db miševa su 3. dana razine SAA bile više od referentnog raspona kod svih 5 životinja. Medijan vrijednosti (s 1. i 3. kvartilom) bio je niži negoli kod C57Bl/6 miševa u istoj vremenskoj točki, ali razlika nije dostigla statističku značajnost. Kao i kod nedijabetičnih miševa iz istoga legla, kod db/db miševa se koncentracija SAA vratila unutar referentnog raspona za SAA 9. i 13. dana (tablica 1.).

Koncentracije IFN- $\gamma$  bile su više u krvi ranjenih db/db miševa u usporedbi s C57Bl/6 miševima tijekom čitavog razdoblja ispitivanja, no ta je razlika bila statistički značajna samo 9. i 13. dana (tablica 1.).

### Rasprava

Primjenom prethodno razvijene, precizne i ponovljive kompjutorizirane metode za motrenje zatvaranja rane na koži (23,24) potvrdili smo kako je cijeljenje rane znatno odgođeno kod dijabetičnih db/db miševa. Naša smo zapažanja, uz to, dalje proširili kako bismo pokazali da je sustavni upalni odgovor i odgovor akutne faze u ovih miševa odgođen i duže traje nego kod nedijabetičnih miševa iz istoga legla.

Poznato je da je cijeljenje rane odgođeno kod ljudi sa šećernom bolešću i kod životinjskih dijabetičkih modela kao što je db/db miš (7,12). U našem ispitivanju bilo je jasno da učinak procesa šećerne bolesti ima najmanje dvije sastavnice. S jedne strane, mehaničko uvlačenje granice rane poremećeno je kod db/db miševa u usporedbi sa zdravim miševima. Koža glodavaca razlikuje se od ljudske kože i mehaničko uvlačenje je važan početni odgovor na tkivnu ozljedu kod glodavaca, ali ne i kod ljudi. Poremećeno uvla-

rence range for female C57Bl/6 mice, according to Charles River Laboratories, is  $8.09 \pm 2.29 \times 10^9/L$ .

Neutrophil differential counts in blood from C57Bl/6 mice decreased gradually throughout the observation period. Neutrophil differential counts in blood of db/db mice on day 3 were almost three-fold those in healthy mice, and this increase was sustained throughout the observation period (Table 1). On day 6, neutrophil percentage of db/db mice decreased, however, it did not reach statistical significance. Lymphocyte differential count in blood from healthy mice showed a slight increase towards the end of the study. In contrast, lymphocyte count in blood from db/db mice increased until day 6, followed by a decrease until the end of the study, however, without reaching statistical significance (Table 1).

### SAA and IFN- $\gamma$

In mice, the SAA reference range is 0.2–40 mg/L (21,22). In wounded C57Bl/6 mice, SAA concentration declined from day 3 to day 6, however, not reaching statistical significance. On days 9 and 13 after the wound incision, SAA levels in all animals were below 40 mg/L, within the normal range (Table 1). In wounded db/db mice, SAA levels were increased in all 5 animals on day 3 in relation to the reference range. Median values (with 1<sup>st</sup> and 3<sup>rd</sup> quartiles) were lower than in C57Bl/6 mice at the same time point, yet not achieving statistical significance. As in non-diabetic littermates, SAA concentration in db/db mice returned to the SAA reference range on days 9 and 13 (Table 1).

IFN- $\gamma$  concentrations in blood from wounded db/db mice tended to be higher in relation to C57Bl/6 mice during the study period, but this difference only reached statistical significance on days 9 and 13 (Table 1).

### Discussion

We have confirmed, using a previously developed, precise and reproducible computerized method for monitoring skin wound closure (23,24), that wound healing is markedly delayed in diabetic db/db mice. We have also extended these observations to show that the systemic inflammatory and acute phase response is delayed and more sustained than in non-diabetic littermates.

It is well known that in diabetic humans and animal models of diabetes, such as the db/db mouse, wound healing is delayed (7,12). From our study, it is clear that the effect of the diabetic disease process has at least two components. On the one hand, the mechanical retraction of the wound margin is impaired in db/db mice in comparison to normal mice. Rodent skin is different to human skin and mechanical retraction is an important initial response to tissue injury in the rodent and not in humans. The impaired wound retraction in db/db mice is probably attributable to the large amount of subcutaneous adipose

čenje rane kod db/db miševa vjerojatno se može pripisati velikoj količini potkožnog masnog tkiva kod ovih pretilih životinja, ali bi isto tako moglo biti povezano s promjenama elastičnosti vezivnog tkiva. S druge strane, ponovna epitelizacija i nakupljanje granulacijskog tkiva također su poremećeni u ranama kod ovih miševa, što dovodi do kašnjenja u zatvaranju rane, a to smo zapazili i u ovoj studiji. Unatoč mnogim studijama o učincima nedostatka receptora za leptin na lokalne promjene u cijeljenju rane kod db/db miševa, malo se zna o sustavnim upalnim promjenama u ovim uvjetima. Zabilježili smo povećan i ustrajan porast broja bijelih krvnih stanica kroz najmanje 13 dana od ranjavanja kod dijabetičnih db/db miševa u odnosu na zdrave miševe. To je poglavito bilo zbog porasta broja neutrofila u cirkulaciji. Ovi su nalazi sukladni onima iz prijašnje studije Wetzlera i sur. (25) koji su pokazali kako je kod ekscizijskih rana u punoj debljini kod db/db miševa lokalna upala mjerena ekspresijom IL-1 $\beta$  i TNF- $\alpha$ , kao i tkivna infiltracija neutrofilima i makrofazima znatno povećana i produžena u usporedbi sa zdravim C57Bl/KS miševima kroz najmanje 13 dana. Ovaj pojačan lokalni upalni odgovor bio je udružen s povećanom ekspresijom kemokina MIP-2 i MCP-2 u tkivu rane. Stoga se čini da zapažene sustavne hematološke promjene točno odražavaju lokalnu staničnu infiltraciju na mjestu rane kod db/db miševa. Štoviše, poremećen je razvoj limfocita u timusu db/db miševa (26), što je možda doprinijelo sniženju diferencijalnog broja limfocita zabilježenom u našem ispitivanju. Ustrajan upalni odgovor kod db/db miševa možda je pridonio kašnjenju u cijeljenju rane odgađajući promjene od upale do nakupljanja vezivnog tkiva.

Proteini akutne faze često se rabe kao biljezi sustavnog odgovora na akutnu i subakutnu upalu. U našem ispitivanju mjerenje koncentracija SAA (SAA je najvažniji protein akutne faze kod miševa) nije značajno odrazilo promjene koje nastaju na mjestu rane, jer se SAA povisuje rano u odgovoru na ozljedu i ima kratak život u cirkulaciji. To je bilo ograničenje naše studije, jer je prva vremenska točka bila 3. dan. Međutim, iako nije bilo statističke značajnosti, 3. dana su razine SAA bile niže kod db/db miševa nego kod zdravih miševa. Ovi nalazi koreliraju s onima drugih autora koji su pokazali kako su nakon intraartikularne injekcije zimosana A u zglobove ob/ob miševa (s pomanjkanjem leptina, a ne receptora leptina) rane razine SAA (27) u cirkulaciji snižene, što je bilo udruženo s pojačanim artritičnim odgovorom kod ovih dijabetičnih životinja (28).

IFN- $\gamma$  je veoma važan u procesu zatvaranja rane. Jedva ga se može otkriti u ekscizijskim ranama koje su u procesu cijeljenja kod db/db miševa, možda zbog visokih razina citokina TGF- $\beta$ 1 koji ga suzbija (29). To vjerojatno objašnjava izostanak razlike u koncentracijama IFN- $\gamma$  u cirkulaciji između db/db miševa i C57Bl/6 miševa 3. i 6. dana u našem ispitivanju. Međutim, razine ovoga citokina u cirkulaciji značajno su se povisile u db/db miševa tek 9. i 13. dana,

tissue in these obese animals, but may also be related to changes in the elasticity of the connective tissue. On the other hand, re-epithelialization and deposition of granulation tissue in the wounds of these diabetic mice are also impaired, resulting in a delay in wound closure also seen in our study.

Although extensive studies have been performed on the effects of the leptin receptor deficiency on local changes in healing wounds in db/db mice, little is known about systemic inflammatory changes under these conditions. We found an increased and sustained WBC count that lasted for up to at least 13 days after wounding in diabetic db/db as opposed to normal mice. This was predominantly accounted for by an increase in the circulating neutrophil count. These findings correlate very well with those of a previous study by Wetzler *et al.* (25) who showed that in full-thickness excision wounds in db/db mice, local inflammation, as measured by IL-1 $\beta$  and TNF- $\alpha$  expression, as well as the infiltration of the tissue by neutrophils and macrophages were markedly enhanced and prolonged in comparison to healthy C57Bl/KS mice for at least 13 days. This enhanced local inflammatory response was associated with increased MIP-2 and MCP-2 chemokine expression in the wound tissue. The systemic hematological changes we observed, therefore, seem to reflect closely the local cell infiltration occurring at the wound site in db/db mice. Moreover, lymphocyte development in the thymus of db/db mice is impaired (26), which may have contributed to the reduced lymphocyte differential cell count observed in our study. The sustained inflammatory response in db/db mice may contribute to the delay in wound healing by delaying the change from inflammation to connective tissue deposition.

Acute phase proteins are frequently used as markers of the systemic response to acute or sub-acute inflammation. Measurement of SAA concentrations (SAA is the most important acute phase protein in mice) in our study did not significantly reflect the changes occurring at the wound site since SAA increases in response to injury early and has a short lifetime in circulation. This was a limitation to our study since the first time point was on day 3. However, although there was no statistical significance, SAA levels were lower on day 3 in db/db than in normal mice. These findings correlate with those of other authors, showing that after the intra-articular injection of zymosan A into the joints of ob/ob (leptin-deficient rather than leptin receptor deficient) mice, early circulating levels of SAA (27) are decreased in association with the enhanced arthritic response in these diabetic animals (28).

IFN- $\gamma$  is very important in the process of wound closure. It is barely detectable in healing excisional wounds in db/db mice, possibly due to high levels of the suppressing cytokine TGF- $\beta$ 1 (29). This probably accounts for the lack of difference in circulating IFN- $\gamma$  concentrations between

odražavajući možda ustrajan upalni odgovor na mjestu rane i/ili sustavno upalno poticanje drugih organa. Doista, opisano je 13 do 30 puta veće stvaranje proupalnih citokina u srcu i aorti db/db miševa u odgovoru na lipopolisaharid u usporedbi sa zdravim miševima (29). Kasni porast IFN- $\gamma$  mogao bi pomoći u izazivanju promjena na mjestu rane od upale do popravka tkiva, s obzirom na uzajamno antagonistične učinke IFN- $\gamma$  (30) i TGF- $\beta$  (10,31,32).

Dok je korelacija koncentracija IFN gama u lokalnim tkivima i njegovim sistemskim koncentracijama u ranjenih životinja ograničena kao pokazatelj lokalnog cijeljenja rane, određivanje SAA treba dalje istražiti kako bi se utvrdilo može li ga se rabiti kao primjereniji biljeg lokalne upale tijekom procesa cijeljenja rane. Također naglašavamo primjenu ranijih vremenskih točaka kako bi se utvrdilo može li se na taj način predvidjeti dinamika lokalnog cijeljenja rane.

U zaključku, proces lokalne tkivne regeneracije kod miševa nakon lokalne kožne ozljede uzrokuje sustavne promjene u perifernoj krvi. Niti određivanje koncentracije SAA niti IFN- $\gamma$  ne može se rabiti za motrenje dinamike cijeljenja rane u navedenim vremenskim točkama.

## Zahvale

Zahvaljujemo Slavici Skender i Vedranu Vrbanu na izvrsnoj tehničkoj pomoći u provedbi ove studije.

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db/db and C57bl/6 mice on days 3 and 6 in our study. However, the circulating levels of this cytokine significantly increased in db/db mice only on days 9 and 13, possibly reflecting the sustained inflammatory response at the wound site and/or systemic inflammatory stimulation of other organs. Indeed, in response to lipopolysaccharide, the generation of pro-inflammatory cytokines by heart and aorta of db/db mice has been reported to be 13- to 30-fold that in normal mice (29). The late rise in IFN- $\gamma$  may help induce a change in the wound site from inflammation to tissue repair, in view of the mutually antagonistic effects of IFN- $\gamma$  (30) and TGF- $\beta$  (10,31,32).

While the concentrations of different tissue to systemic IFN- $\gamma$  concentrations in wounded animals may limit the use of this cytokine as a marker of local wound response, SAA measurement needs further investigation to find out whether it can be used as a more appropriate marker of local inflammation during the wound healing process. We also suggest using earlier time points to see whether it may predict the dynamics of local wound healing.

In conclusion, the local tissue regeneration process in mice after local skin injury causes systemic changes in peripheral blood. Determination of neither SAA nor IFN- $\gamma$  concentrations could be used to monitor wound healing dynamics at these time points.

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