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ORIGINAL ARTICLE

Gender-specific ischemic tissue tolerance in critically perfused skin

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Abstract
Purpose The purpose of this study is to determine gender-specific differences in the development of necrosis in persistent ischemic tissue and to analyze whether differences are due to gender-specific loss of vascular reactivity or change in ischemic tolerance.
Methods Hairless mice (skh-1) of both genders were assigned to three groups of adolescent, adult, and senescent age. Critical ischemia was induced by transection of the two distal pedicles of the animal's ear. Microcirculation was assessed over a 5-day period using intravital epifluorescence microscopy. Tissue necrosis, blood flow, functional capillary density (FCD), red blood cell (RBC) velocity, and capillary diameter were analyzed.
Results Induction of persistent ischemia caused an age-dependent demarcation of nonperfused flap tissue. Adult and senescent females developed markedly more necrosis than age-matched males (49±1% vs. 37±3% and 53±3% vs. 44±2%, respectively; $p<0.05$), whereas no gender-specific difference in flap necrosis was observed in adolescent animals (31±2% vs. 33±3%). Gender did not affect the amount of microcirculatory dysfunction in the flap. Thus, age-matched females and males exhibited a comparable decrease of FCD, RBC velocity, and capillary dilatory response.
Conclusions Both age and female gender may predispose for an increased susceptibility to develop ischemic tissue necrosis. The increased necrosis in female animals does not apply to an aggravated microvascular dysfunction, but rather to a reduced ischemic tissue tolerance.

Keywords Gender · Acute persistent ischemia · Ischemic tissue tolerance · Microcirculation · Necrosis

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Introduction

Preservation of the microcirculation and hence removal of by-products is a prerequisite to prevent tissue from ischemic necrosis. Insufficiency of the microcirculation on the other hand may lead to irreversible tissue damage due to persistent ischemia and hypoxia, which untreated will result in wound breakdown [1] and compromised tissue survival, including surgical accesses with extensive undermining or randomly perfused remote areas of the critically perfused flap tissue [2, 3].

Ischemic tissue damage generates increased health care costs [4], especially in the elder patients, which, in general, present with multiple comorbidities [5]. Recent data have demonstrated that critical microvascular perfusion is associated with an age-dependent manifestation of tissue necrosis which appears to be the consequence of an impaired vascular reactivity during aging, however independent of the expression of the chaperone heme oxygenase 1 (HO-1) [6].

In trauma and sepsis, growing evidence suggests that not only age, but also gender, plays a significant role for the outcome of the patients [7–9]. The incidence of atherosclerosis and the risk to develop cardiovascular diseases have been found higher in male patients [10, 11]. These gender-specific differences are thought to rely on various factors, including the levels of estrogen [12], the androgen-to-estrogen ratio [10], and the different regulation of estrogen receptor expression [8].

Despite the accumulation of comorbidity factors with aging, there is still a lack of knowledge on whether ischemia-associated defense mechanisms of the skin display a gender dimorphism. Under physiological conditions, tissue perfusion on a capillary level and oxygen supply match with tissue function and survival. Decreased microvascular perfusion may represent the cause for impaired tissue survival. However, tissue survival may potentially also be impaired without decreased microvascular perfusion and oxygen supply which would indicate a reduced ischemic tissue tolerance.

In general, it is assumed that, under physiological conditions, nutritive capillary perfusion and oxygen supply match with tissue survival and function. Accordingly, an increase in tissue tolerance to ischemia correlates with a reduction of oxygen demand by the surviving tissue despite a critical diminution of the nutritive perfusion and thus a limited oxygen supply. Contrariwise, a decrease in ischemic tolerance, i.e., ischemic intolerance, is associated with increased tissue necrosis despite maintained capillary perfusion.

The aim of the present study was, therefore, (1) to analyze whether gender has an impact on the development of necrosis in skin exposed to acute persistent ischemia and (2) to elaborate whether gender dimorphism of ischemic necrosis is due to differences in vascular reactivity and thus microvascular perfusion or due to differences in ischemic tolerance of the jeopardized tissue.

Materials and methods

Animals

All experiments were performed according to the guiding principles for research involving animals and the German legislation on the protection of animals. The experiments were approved by the local governmental animal care committee.

The ears of immunocompetent homozygous hairless mice (skh-1) of both genders (2–24 months; 26–42 g body weight (bw); Charles River Laboratories, Sulzfeld, Germany) were subjected to chronic ischemia utilizing a skin flap model described in detail previously [13]. The mouse ear consists of a single layer of cartilage sandwiched between two full layers of skin (overall thickness 300 μm) and is completely hairless in the skh-1 strain, thus ideal to serve for intravital repetitive microscopic studies. The blood supply is provided by three vascular pedicles, entering the ear at its base and forming an interconnecting capillary network in the periphery [13]. The animals were housed in single cages at a room temperature of 22–24°C and a relative humidity of 60–65% with a 12-h day–night cycle. The animals were allowed free access to drinking water and standard laboratory chow.

The life span of mice with one mouse year corresponding to ~30 to 40 human years allows investigating all life stages [14]. We, therefore, accounted for two distinct conditions associated with skh-1-mice: (1) 75% of immunocompetent homozygous hairless mice (skh-1) died of a natural death within a 72-week period [15]. Accordingly, 19-month-old animals (76 weeks old) have to be regarded as senescent, whereas 2- and 10-month-old animals were considered adolescent and adult, respectively [15]. (2) Furthermore, we rely on the fact that the absolute number and the general pattern of vessels within the defined tissue areas is fixed within the late juvenile growth phase [15].

Anesthesia

The animals were anesthetized using intraperitoneal injection of 90 mg/kg bw ketamine hydrochloride (Ketavet®, Parke Davis, Freiburg, Germany) and 25 mg/kg bw xylazine hydrochloride (Rompun®, Bayer, Leverkusen, Germany) for surgery, laser Doppler flowmetry, and repetitive intravital epifluorescence microscopy.

Surgical procedure

The right ear to be investigated was gently spread out on a Plexiglas pad and incised through four fifths of its base to create an anteriorly based axial-pattern skin flap. This included the transection of the posterior and central vascular bundle at the ear's base, i.e., the creation of an axially perfused skin flap which rendered the ear's perfusion exclusively dependent on the anterior, i.e., proximal pedicle (Fig. 1a). The preparation of this skin flap is associated with nutritive perfusion failure which results in the constant development of ~40% necrosis of the distal ear within a 5-day period [13].

Laser Doppler flowmetry

The ears were outlaid on a Plexiglas pad and covered with a transparent, flexible, and impermeable foil for in vivo microvascular analyses. Neither the laser Doppler

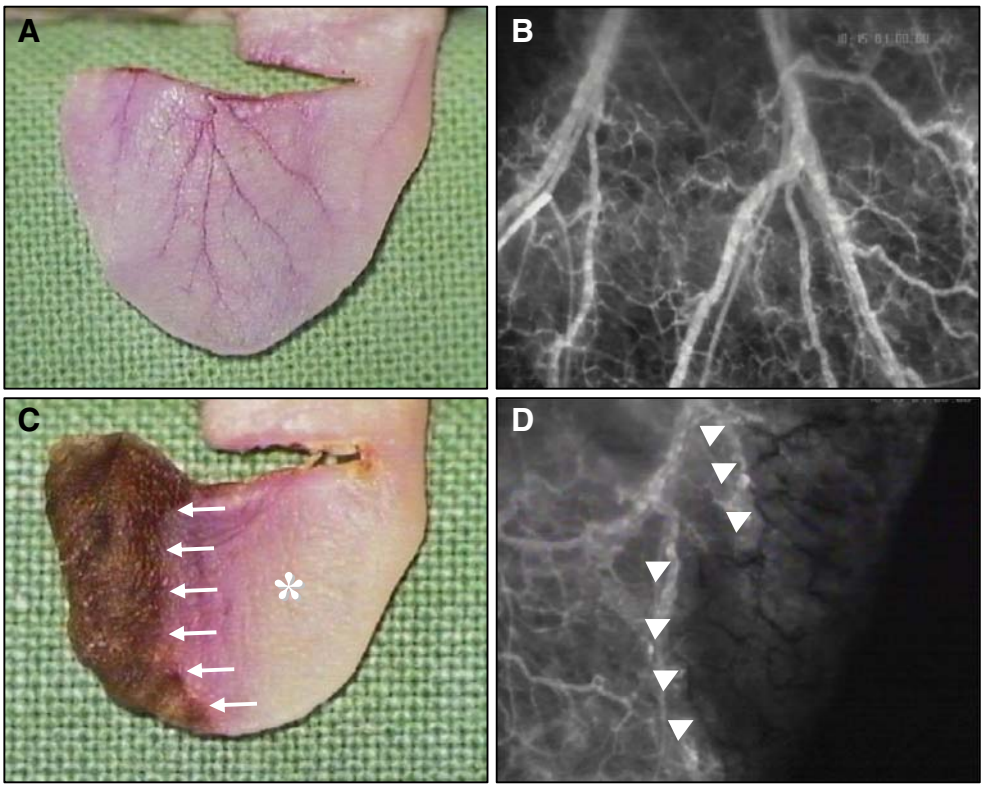

Fig. 1 Macroscopic view of an outlaid ear immediately after microvascular analyses (time point=30 min; **a**). High-resolution intravital microscopy of the proximal and central arteriovenular bundle with its distinct branching patterns and adjacent capillary structures (**b**). Macroscopic view of the ear of an adolescent hairless mouse at day 5 after flap creation (**c**). Note the distal flap necrosis (*left*) adjacent to the vital tissue proximally (*right*; *asterisk*), which is demarcated by a hyperemic fringe (*white arrows*). Intravital microscopy of the zone of demarcation (**d**) of the flap at day 3 after creation, demonstrating a tissue area with preserved capillary perfusion (*left*), directly neighbored (*arrowheads*) by a tissue area with complete shutdown of microvascular blood flow (*right*). Macroscopic and microscopic views at magnifications of ×16 and ×80, respectively

probe nor the objective of the microscope was in direct contact with the ear, thus affecting microcirculation of the ear.

Microvascular perfusion of the proximal (i.e., anterior), central, and distal pedicle of the flap was assessed separately using a two-channel laser Doppler perfusion monitor (PF3, Perimed, Stockholm, Sweden). The setup consisted of a laser diode (3 mW, 810 nm emission monochromatic wave length) and a fiber optic sensor which could be positioned manually over the tissue. The results were obtained by averaging five periods of measurements of 20 s of three different zones within each area of the ear, thus minimizing the spatial heterogeneity, a well-known phenomenon occurring even under conditions of normal tissue perfusion [16].

Intravital epifluorescence microscopy setup

Contrast enhancement for visualization of the microcirculation was achieved by intravenous injection of 0.1 ml 5% fluorescein isothiocyanate (FITC)-labeled dextran 150,000 (Sigma-Aldrich, Taufkirchen, Germany) into a tail vein. Subsequently, the ears were positioned under a Zeiss Axiotech microscope (Zeiss®, Oberkochen, Germany). The epi-illumination microscopic setup included a 100-W mercury lamp and a blue light filter set (450–490 nm excitation, >520 nm emission wavelength). Microscopic images were monitored by a charge-coupled device video camera (FK6990, Pieper, Schwerte, Germany) and recorded on video tape (Panasonic AG-7350-SVHS, Matsushita, Tokyo, Japan) for later off-line evaluation.

Microcirculatory parameters

The repetitive analyses of the microcirculation were performed at constant room temperature of ~23°C. Different objectives (×4, numerical aperture [NA]=0.16; ×10, NA=0.30; and ×20 long distance, NA=0.32) were used for recordings. At each observation time point, the surface of the ear was first scanned using the ×4 objective to determine the area of perfused and nonperfused tissue. Video printouts were made within the proximal, central, and distal area of the flap using the ×10 and ×20 objectives to localize exactly distinct arteriovenular branching patterns and capillary structures (Fig. 1b). This allowed repetitive measurements of functional capillary density (FCD), capillary red blood cell (RBC) velocity, and capillary diameter.

All parameters were analyzed off-line using a computer-assisted image system (CapImage®, Zeintl Software, Heidelberg, Germany) [17]. The flap area displaying microvascular shutdown and necrosis was quantified planimetrically and is given as tissue area with nonperfused capillaries in percent of the total tissue area of the ear [13]. FCD was defined as the length of RBC-perfused capillaries per observation field and is given in centimeters per square centimeter [18]. Capillary RBC velocity (in millimeters per

second) was analyzed using the line shift method [19]. The line shift method is based on the measurement of the shift (in millimeters) of an individual intravascular gray-level pattern over time (in seconds). Capillary diameters (in micrometers) were measured perpendicularly to the vessel path. Volumetric blood flow (in picoliters per second) was calculated in capillaries from RBC velocity and vessel cross-sectional area ($\pi \times r^2$) according to the equation of Gross and Aroesty, i.e., $Q = V \times \pi \times r^2$ assuming a cylindrical vessel shape [20]. At every time point of the investigation, three different fields were randomly selected in each zone of the flap in order to assess FCD, RBC velocity, and microvascular diameter. RBC velocity and diameters of capillaries were further measured in three capillaries of each field. Signs of an angiogenic response, including bud, sprout, and microvascular formation were looked for.

Experimental protocol

Thirty-six mice were assigned to six groups of six animals each. These included female and male adolescent (age=2±0 months), adult (age=10±1 months), and senescent animals (age=19±1 months). Baseline values were recorded before flap creation using laser Doppler flowmetry and intravital microscopy. The measurements of the microhemodynamics were repeated at 30 min as well as at 1, 3, and 5 days after flap creation. At the end of the experiments, the animals were euthanized by injection of an overdose of the anesthetic.

Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). For comparison between individual time points within each group, analysis of variance for repeated measures and an appropriate post hoc test were performed, including the correction of the alpha error according to Bonferroni probabilities. In accordance with the distribution of data, differences between the groups were assessed using the nonparametric Wilcoxon signed-rank test (SigmaStat®, Jandel, San Rafael, CA, USA). Statistical differences were considered significant at $p<0.05$.

Results

Tissue necrosis

The macroscopic demarcation of nonperfused and necrotic tissue of the ear could be reliably delineated from vital tissue during the 5-day observation period (Fig. 1c). Using intravital microscopy, the line of demarcation could be discerned by the presence of FITC-labeled dextran within perfused microvessels on one hand and by the absence of the dye within nonperfused microvessels on the other hand, indicating a complete shutdown of the microcirculation (Fig. 1d). The line of demarcation could be accurately determined and allowed for an accurate quantitative analysis of the area of skin necrosis (Fig. 2).

The zone of demarcation developed more proximally in adult and senescent mice when compared to adolescent mice (Fig. 2). Microvascular perfusion failure encompassed a tissue area of 12±2% of the total ear surface in adolescent mice 30 min after major interruption of blood supply to the ear, i.e., skin flap creation. Along with the persisting microcirculatory dysfunction, this resulted in 31% tissue necrosis at day 5. No significant difference was observed between adolescent female and male animals (31±2% vs. 33±3%; p=n.s.; Fig. 2a). The development of microvascu-

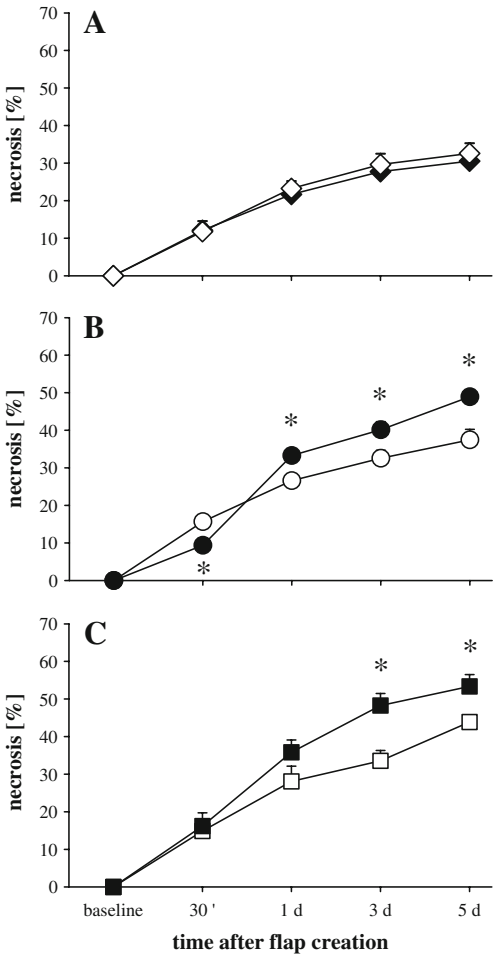

Fig. 2 Time course of necrosis (given in percent of the total area of the ear) in adolescent (*diamonds*; **a**), adult (*circles*; **b**), and senescent animals (*squares*; **c**). Females and males are represented by *closed* and *open symbols*, respectively. Note the age-dependent increase of flap necrosis in adult and in senescent animals when compared to adolescent mice. Adult and senescent females develop a significantly greater area of necrosis when compared to age-matched males. Mean ± SEM; *$p<0.05$ vs. male

lar perfusion failure displayed an age dependency, increasing up to 42±3% and 49±2% necrosis of the ear in adult and senescent animals, respectively ($p<0.05$ vs. adolescent mice). The development of tissue necrosis, however, showed gender specificity in elder animals when compared to adolescent mice. At day 5, we observed a significantly increased tissue necrosis of 49±1% and 53±3% in adult and senescent females, respectively, when compared to age-matched male animals (37±3% and 44±2%, $p<0.05$ vs. females; Fig. 2b, c).

Microvascular blood perfusion

Blood flow within the ear showed time-, site-, and age-specific alterations during the 5-day observation period. Blood flow revealed a general increase relative to baseline which reached almost 400% in the proximal pedicle of the ear of adolescent animals at day 5. A 20–35% decrease of baseline values of blood flow was observed immediately after flap creation in the central pedicle of the ear's flap in all three age groups (Fig. 3a–c). Though blood flow recovered to baseline values only in adolescent mice, adult and senescent animals showed an almost complete cessation of blood flow, which did not differ between male and female animals. Cessation of blood flow was also observed in the most distal pedicle of the ear with the exception of adolescent animals which were capable of maintaining a marginal residual perfusion of ~5% of baseline values, whereas in all adult and senescent mice, blood flow was found completely abolished . Despite time and site specificity, laser Doppler flowmetry revealed no gender-related differences of blood flow in age-matched flap tissue.

Capillary microcirculation

Analysis of FCD also showed time-, site-, and age-dependent alterations during the 5-day observation period which were similar to the overall blood flow. FCD within the most proximal area of the ear was only marginally affected over time, demonstrating a reduction to ~75% of baseline values at day 5 after surgery in all three age groups without any differences between male and female animals . Nutritive capillary perfusion revealed a more pronounced impairment in the critically perfused central area of the ear with FCD values decreased at ~50% of baseline at day 5 (Fig. 3d–f). Likewise, no gender-specific difference was found. Finally, marked microvascular perfusion failure was observed already 30 min after transection of the distal two pedicles within the remote areas of the ear. At day 5, adolescent and adult mice presented with residual nutritive perfusion of 20% and 8%, respectively, whereas complete breakdown of capillary perfusion was observed in senescent mice. These alterations of FCD were independent of the gender of the experimental animals as well (Fig. 3). The FCD value at baseline is found higher in the central and distal areas of the ear of females due to the thinner skin allowing for a clearer view of the tissues lacking edema.

Capillary RBC velocity showed a transient decrease which was comparable between the male and female gender in adolescent and adult groups of the proximal area of the ear. This decrease was followed by an almost full recovery at day 5 after surgery. Within the surviving proximal flap area of senescent animals, we observed a momentary decreased capillary RBC velocity only in females when compared to males.

In contrast, the initial decrease of RBC velocity recovered only incompletely in the central area of the ear until day 5 (Fig. 3g–i). Partial recovery of RBC velocity was observed in the distal flap areas of adolescent mice only, whereas the capillaries of the adult and senescent animals presented with an almost complete cessation of capillary RBC velocity at day 5 (data not shown). No significant difference in RBC velocity could be observed between age-matched female and male animals at any of the time points studied.

The analysis of diameters of perfused capillaries within the zone of demarcation revealed considerable microvascular dilation over the 5-day observation period. In this area, capillary diameters increased up to threefold to fourfold of that of the baseline (Fig. 4). The dilatory response of capillaries within the three distinct zones of the ear did not reveal a gender-specific difference in age-matched animals.

Discussion

The major finding of the present study is that aged female gender is associated with an increased necrosis of critically perfused skin when compared to age-matched male gender. This appears to be the consequence of a decrease in ischemic tissue tolerance rather than an enhancement of microvascular dysfunction within the tissue suffering from acute persistent ischemia.

Received opinion is that women normally tolerate better ischemic stress. Literature on that subject is, however, controversial, including clinical studies that have demonstrated an improved patient outcome in men or in women subsequent to atherosclerosis [21], namely, ischemia [22]. Unfortunately, there is only little information about gender differences in ischemically challenged skin.

This gender-specific difference of tissue survival subjected to critical ischemia was observed in adult and senescent animals, whereas adolescent female and male mice did not show any differences, neither in microcirculatory dysfunction nor in tissue survival. These insights are complementary to the one recently published by the same

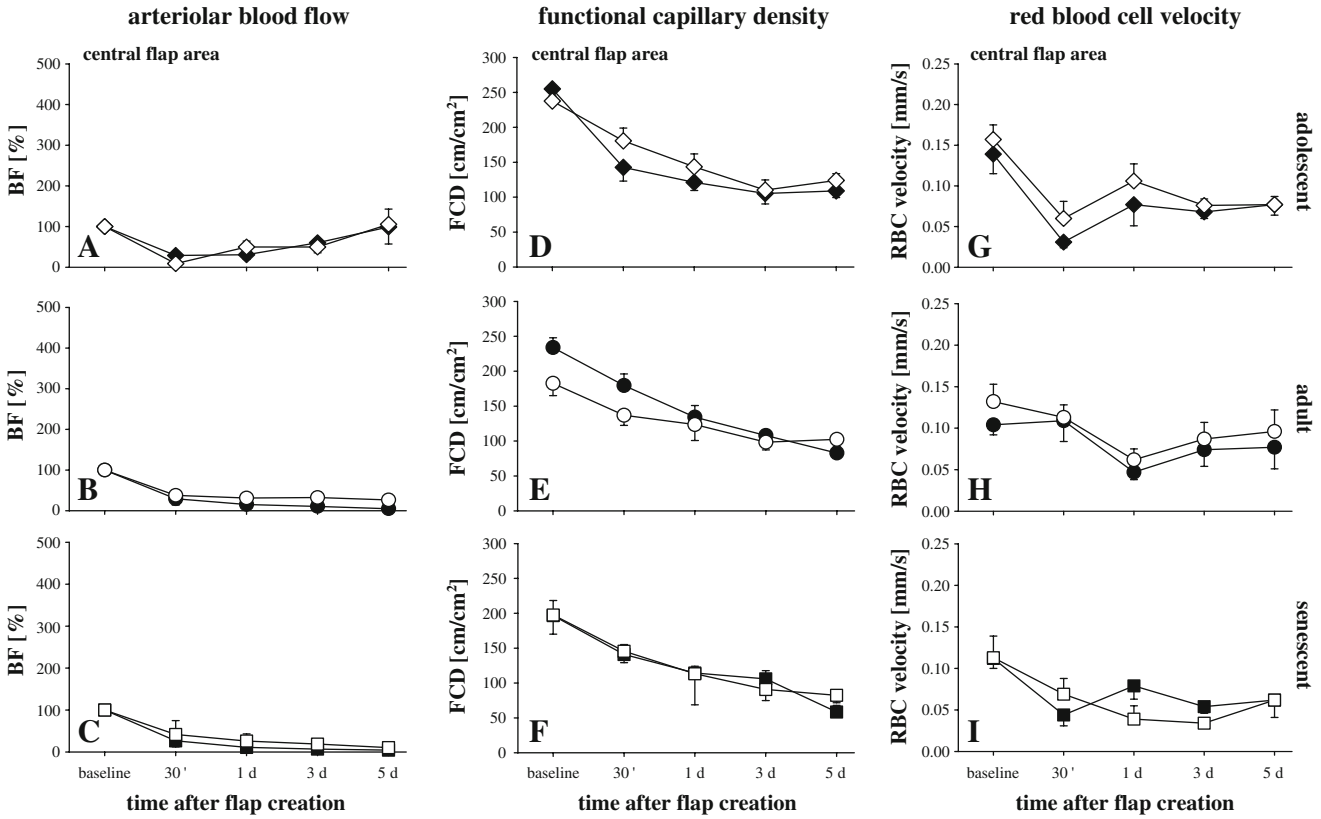

Fig. 3 Blood flow measured by LDF (**a**–**c**), FCD (**d**–**f**), which is defined as the length of all perfused capillaries per area of observation, and RBC (**g**–**i**) velocity of perfused capillaries over the 5-day observation period within the critically perfused central pedicle and area of the flap in adolescent (*diamonds*: **a**, **d**, **g**), adult (*circles*: **b**, **e**, **h**), and senescent (*squares*: **c**, **f**, **i**) animals. Females and males are represented by *closed* and *open symbols*, respectively. Immediate decrease of blood flow is observed within the pedicle of the central flap area in all three age groups (**a**–**c**) with recovery to baseline in the adolescent animals only (**a**). Note the absence of gender-associated differences in blood flow (**a**–**c**). Note the decrease of FCD by ~50% within the central flap area (**d**–**f**) of all animals at day 5 after onset of persistent ischemia without gender-specific differences. Capillary RBC velocity shows an initial decrease of RBC velocity in the central flap area of all animals with only partial recovery until day 5 (**g**–**i**). Of interest, no gender-specific differences are detected between age-matched female and male animals (**a**–**i**)

group using the ear model in the hairless mouse. The study demonstrated that increasing age correlated well with decreased microvascular reactivity, i.e., reduced post-ischemic reactive hyperemia in "in-flow" arterioles and a decreased number of perfused capillaries with limited dilatory capacity. Accordingly, this loss of vasoreactive properties of the cutaneous tissue, which is no longer capable to compensate persistent ischemia during the process of aging, resulted in an age-related increased necrosis of the skin. It has to be emphasized that all animals used for those experiments were raised and housed under conditions devoid of pathogens and exposed to identical pellet food. Furthermore, the mice did not show any clinical signs of manifest diseases, such as myocardial insufficiency, hypertension, or arteriosclerosis. Accordingly, we concluded that the physiological process of aging per se would increase the susceptibility for ischemic tissue injury [6].

The comparison of adult and senescent mice of both gender in the present study revealed the following. Male animals presented with decreased FCD and RBC velocity as well as limited dilation in perfused capillaries, which were comparable to that of females. Nevertheless, this comparable microvascular dysfunction resulted in a gender-specific tissue survival in favor of the male gender, indicating that disparate survival depends on tolerance to ischemia more than on maintenance of microvascular parameters. Of interest, FCD at baseline was seen slightly higher by ~15% especially in the distal area of the three age groups which results from a better visualization of the honeycomb-like-arranged capillary network within the very thin posterior area of the animals' ear.

The critically perfused zone of demarcation of a flap consists of an area with a rarefaction of perfused capillaries that dilate in order to increase the exchange surface of O_2 between vessels and interstitial tissue, i.e., to increase O_2 conductance [2]. These scarce microvessels may not serve as an adequate nutritional supply to the tissue but only function as "thoroughfare" channels, as described for

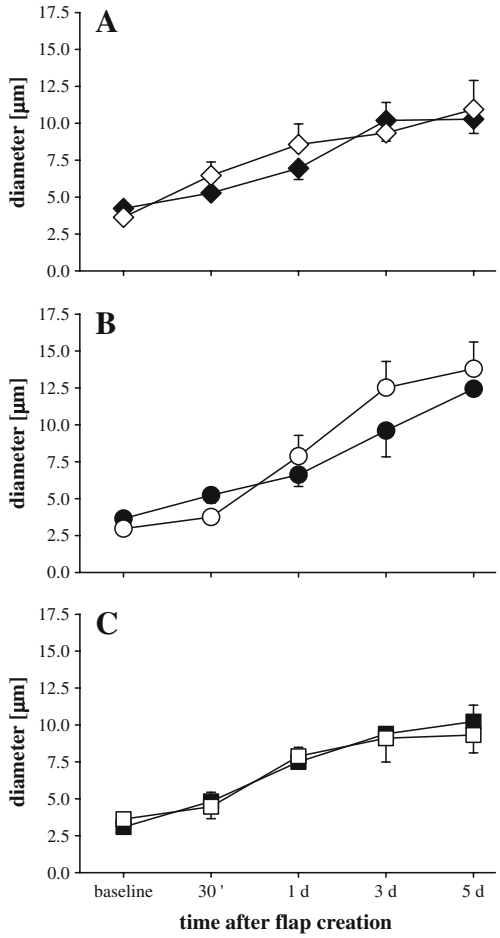

Fig. 4 Diameters of perfused capillaries over the 5-day observation period within the zone of demarcation in adolescent (*diamonds*; **a**), adult (*circles*; **b**), and senescent animals (*squares*; **c**). Females and males are represented by *closed* and *open symbols*, respectively. Note the marked capillary dilation over the 5-day observation period after flap creation. Adult animals show the most pronounced dilatory response. No gender-specific difference among the age groups is observed

arteriovenular shunts [23]. Tissue oxygen tension and so oxidative energy metabolism are dependent on the O_2 supply to the tissue, which is determined by the O_2 content of the blood entering the critically perfused tissue [24].

Because these remaining capillaries within the critically perfused tissue dilate inadequately in aged animals, one must assume that O_2 conductance and O_2 diffusion to the tissue are considerably reduced. Yet it is interesting to observe that aged male animals display an improved tissue survival despite a similar microvascular perfusion and a comparable morphological change of the angioarchitecture of the ear when compared to that of age-matched females. Despite the standardized conditions of housing and conduction of the experiments, this indicates two theories that ultimately are not provable with the current methods of investigation, i.e., laser Doppler flowmetry and intravital fluorescence microscopy: (1) The tolerance to ischemia decreases in female animals, i.e., disparate extension of tissue survival despite comparable microcirculatory parameters or (ii) perfused capillaries within the zone of demarcation are not able to deliver sufficient oxygen to the ischemically challenged tissue. This phenomenon occurring in female animals appears to be age-dependent because adolescent females develop both an ischemia-associated dysfunction of the microcirculation and an extent of tissue necrosis which is comparable to that observed in adolescent males.

Kamler et al. were the only ones to investigate the effect of ischemia on tissue oxygenation and healing of experimental defect wounds in this ear model. They ligated two of three main nutritional arteries to the ear that corresponds to the transection of the distal two pedicles in the present study. Tissue ischemia was verified by measurement of transcutaneous pO_2. They could thereby show that decreased pO_2 values correlated with significantly delayed wound closure time when compared to animals treated with the vasoactive drug buflomedil. Of interest, microhemodynamics, i.e., FCD, RBC velocity, and capillary diameter, were not different between buflomedil-treated and control animals, indicating a possible role for modulation of the ischemic tolerance by buflomedil [25].

On a molecular base, hormonal changes and imbalances could explain these gender-specific differences. In fact, Albrecht and coworkers have provided evidence for decreased hormonal secretion in aged mice that show a declined ovarian steroidogenesis as reproductive cycling ceases around 1 year of age [26]. Likewise, these mice exhibit an increased androgen-to-estrogen ratio, peaking in senescence, as also demonstrated by Kubota et al. [27] Araneo and coworkers demonstrated that systemic injection of dehydroepiandrosterone (DHEA), a metabolic intermediate product of testosterone, could prevent the progressive dermal destruction caused by persistent ischemia in thermal injury [28]. DHEA, whose endogenous production is known to decline with advancing age [29], acts on the androgen receptor [30], which is more commonly represented in the male gender when compared to the female gender.

In summary, the present study demonstrates that, during aging, the male gender compared to the female gender exhibits an improved survival of critically perfused skin. The absence of gender-specific differences in nutritive capillary perfusion indicates that improved tissue survival in males is not due to better microvascular reactivity to persistent ischemic stress but rather due to higher ischemic tolerance of the cutaneous tissue. Although these results emanate from experiments investigating murine skin, we feel that this message might be of importance facing a population that is growing older resulting in an increased life span where surgery is meanwhile performed routinely.

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Conflict of interest None of the authors has any conflict of interest with the present study.

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