Efficacy of extracts from cryopreserved placenta on third-degree burns in rats


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Introduction

The use of low temperatures, on the one hand, promotes more efficient release of biologically active substances into the extraction solution, and on the other it ensures the preservation of high viability of various cryopreservation objects, including cells and tissues of fetoplacental origin (Ananian et al., 2019; Nikulina et al., 2019; Prokopyuk et al., 2020). Central to such objects is the placenta itself, because as a non-classical but highly active endocrine gland, it purposefully influences the development and formation of all human organs and systems before birth. The placenta is a rich source of biologically active substances used in the treatment of various diseases (Adani et al., 2019; Nagae et al., 2020; Ghoneum & Lombardi, 2021). Burn wound healing is a complex process consisting of several continuous stages (inflammation, proliferation, tissue remodeling) (Guo et al., 2020). Excessive activation of lipid peroxidation processes, increased activity of free radical processes, which leads to the formation of oxidative stress in the burn wound and nearby areas of skin (Bhatia et al., 2016; Babu & Babu, 2018) is essential. The development of oxidative stress is accompanied by a decrease in the body's natural antioxidant defenses. The process of lipid peroxidation in the body is counteracted by the antioxidant system, the most important enzyme components of which are superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6), the combined action of which provides detoxification of potentially harmful oxygen reactive substances, i.e. anion-radical superoxide and peroxide. However, not only the imbalance of antioxidants and prooxidants, but also the violation of the ratio of enzymes to antioxidants can lead to increased oxidative stress due to the additional ge-
peration of reactive oxygen species. Their excess promotes the development of necrosis in the burn area and increases the level of lipid peroxidation (Surmazzeznan et al., 2020). Therefore, special attention should be paid to treatments aimed at balancing redox homeostasis and antioxidant status in skin burn injuries. Promising in this regard is the use of preparations of human placenta, in particular its extracts (HPE) (Pogozhykh et al., 2018). The aim of this work was to study the regenerative properties of extracts from cryopreserved placenta in the treatment of full-thickness burns in rats.

Materials and methods

The studies were performed on Wistar white male rats weighing 150–170 g in the vivarium of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. All the experimental interventions and slaughter of the animals were performed in accordance with the requirements of the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine, agreed with the Directive of the European Parliament and the Council of the European Union from 22.09.2010.

HPE obtained from placentas weighing 400–600 g (40 weeks gestational age, n = 6) were examined. Fragments of freshly obtained placentas were frozen in plastic bags to –196 °C by immersion in liquid nitrogen and stored for 6 months. Rewarming was performed in a water bath at 37 °C. Fragments of cryopreserved placenta were thoroughly washed from mucus and blood with isotonic NaCl solution, which was changed several times. The amniotic and chorionic membranes were separated from the washed placenta with scissors, following which the placenta was ground into 3 x 2 cm fragments, which were immersed in a vessel with saline in 1:5 ratio of placenta and solution (v/v) and stirred for 2–3 minutes. The supernatant was drained and a fresh portion of saline was added, and the procedure was repeated 3–4 times. Saline was added to the washed pieces of placenta in a volume ratio of 1:1 and homogenized with a high-speed homogenizer RT-1 U4.2 (Odessa Experimental Factory of Laboratory Medical Equipment, Ukraine) for 3 minutes. The homogenates were kept for 12 hours at +4 °C, centrifuged at 3000 g for 15 minutes. The supernatant was collected and passed through a 0.45 μm membrane filter (Millipore Corp. Carrigtwohill, Ireland). The filtrate is an aqueous-salt extract of the placenta (Rozanova et al., 2011).

The effect of placental cryopreservation on the regenerative properties of its extracts was studied by their effect on the wound healing process in the model of thermal burn of III B degree in rats (Komienko et al., 2019). To inflict burns on the outer surface of the thigh, the skin was trimmed and epilated in advance. After anesthesia, thermal contact (local) burns were performed with a device that contained a thermostat and a metal plate with an area of 2.5 x 2.5 cm, which was heated to 200 °C. The time of skin contact with the device was 10 seconds.

Treatment of burns was carried out by applying HPE (0.5 mL) to the wound two times a day, the wound surface was not isolated. The effectiveness of HPE was monitored by planimetric and histological methods on days 1, 3, 7, 14, 21 and 28 after the burn, the process of wound healing was assessed photographically (Chang et al., 2011). A ruler with a minimum scale of 1 mm was placed on the burn site and photographed with a Nico D 5100 digital camera, the expansion of the obtained images was accounted Bonferroni correction).

Results

Severe burn injury caused necrosis of all layers of the injured skin area with the attachment of adjacent tissues, which led to the formation of burns of III B degree (deep dermal burn, long-term healing by type of scarring, Fig. 1). The burns were uneven and spotty. In all rats, the injured skin was dry with a thin whitish-yellow scab, the epidermis did not exfoliate, underwent coagulation necrosis, and the animals were lethargic. At the end of the first day, the animals had a thickening and compaction of the scab.

On day 3, loose inhomogeneous surfaces with convex and drooping areas of yellowish-brown colour, cracks with serous exudate and blood impurities were observed in the group of control animals. Some wounds had areas with a pronounced serous-purulent exudate. In animals after treatment with HPE there were relatively clear contours of the wound edges. The surface of the damaged area was dry (excluding two animals, which had wet white areas of the wound), partially covered with a bumpy dark brown crust, there was the formation of a scab of dark purple colour.

In the burn area (Fig. 1) namely, the wound, in the upper part of the wound is a mass of necrosis with coagulation of all layers of the epidermis, papillary layer of the dermis, in the deep layers of the dermis a typical inflammation develops with a pronounced vascular reaction and leukocyte infiltration, in addition the zone of secondary lysis of fabric comes to light. On the periphery of the wound there are folded epithelial layer, leukocyte infiltration, vascular reactions (Fig. 2). There are also areas with purulent inflammation complicated by the microflora.

When calculating the surface area of the wounds, it was found that three days after the thermal burn in the control group and the group of animals treated with HPE, there were some increases in the area of the burn wound (Table 1). This is due to the fact that areas of the skin surface, which on the first day after the burn were bluish, then became a zone of necrosis.

The results of visual studies showed that on the 7th day after the burn the edge of the wounds in experimental animals were not in contact. In all rats, areas with microscopically defined necrosis turned into a necrotic scab of brownish-brown colour, firmly fixed with adjacent tissues, but there were differences in the appearance of the wound and the condition of the scab in groups. In 70% of animals in the control group, the wound surface was covered with a thick scab with protruding hair shafts, and in

rats with a thin scab on the wounds areas of purulent colour were observed with bloody exudate on the edge of the wound. When using HPE for the treatment of animals, the wound surface was mainly covered with a well-organized dense scab, fairly separated from the surrounding tissues, which protruded above the surface of the intact tissue. In 20% of animals, the onset of scab rejection was observed.

On the 7th day of the experiment, there was an acute inflammation with fibrous exudation that permeates all layers of the dermis (Fig. 3). At the border of the wound, we found hyperplasia of the marginal epithelium and its derivatives. There was healing by secondary tension. When HPE treated animals, the wound surface was mainly covered with a well-organized dense scab, fairly separated from the surrounding tissues, which protruded above the surface of the intact tissue. In 20% of animals, the onset of scab rejection was observed.

On the 14th day after the burn, the difference in the healing of burn wounds between the control and the experimental group of rats was more pronounced. The size of the wound surface generally decreased (Table 1), but a thick scab partially remained. Analysis of macrophotographic images showed that 60% of control rats had a thick scab with protruding hair shafts. In two rats, the scab was higher located over the level of intact tissue, the edges of which were uneven and wet with the smell of manure. Wounds

<table>
<thead>
<tr>
<th>Groups of animals</th>
<th>Number of days after burns</th>
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<tbody>
<tr>
<td>Control</td>
<td>319 ± 23</td>
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<tr>
<td></td>
<td>338 ± 26</td>
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<tr>
<td></td>
<td>316 ± 25</td>
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<td>269 ± 14</td>
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<td>75.7 ± 3.4</td>
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<td></td>
<td>10.8 ± 1.5</td>
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<tr>
<td>HPE</td>
<td>312 ± 22</td>
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<tr>
<td></td>
<td>326 ± 27</td>
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<tr>
<td></td>
<td>300 ± 24</td>
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<td>219 ± 13*</td>
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<td>28.0 ± 3.1**</td>
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<td>0.9 ± 0.3**</td>
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Note: significant differences compared to the control. * – P < 0.05, ** – P < 0.01.

The nature of changes in these indices was reflected in their balance. As can be seen from the results of the assessment of the balance of antioxidant enzymes in the blood of animals. A day after the thermal burn the activity of SOD, the main enzyme of specific antioxidant protection of the body, decreased relative to baseline by 1.3 times. The activity of CAT being an enzyme containing heme and leads to the decomposition of hydrogen peroxide into oxygen and water, on the first day after the burn decreased relative to baseline by 2.9 times, which reflects the development of oxidative stress (Fig. 7). On the 3rd, 7th, and 14th days of the study, CAT activity increased, but did not reach the level of baseline values. On the 7th day after the burn, the growth of CAT activity was more intense in the group of animals treated with HPE (77.4% vs. 63.3%).

On the 14th day after the burn, blood analyses showed that 60% of control rats had a thick scab with protruding hair shafts. In two rats, the scab was higher located over the level of intact tissue, the edges of which were uneven and wet with the smell of manure. Wounds with a partially absent scab and wet or dry granulation tissue of bright red colour were also observed. Histological research revealed places of formation of a scar with focal hyalinization (Fig. 5). After treatment with HPE in two rats on the 14th day, on the periphery of the burn wound granulation processes developed with a partially rejected scab (without clear boundaries); two wounds were covered with moist granulation tissue with minor remnants of scab; 50% of rats had a wet pink or bright red surface after scab rejection. The absence of a solid thick scab facilitated the migration of cells on the surface of the wound. Scar, mature granulation tissue formed and the phenomenon of active epithelialization with foci of fibroblast proliferation developed after HPE treatment (Fig. 6) in contrast to the control group.

The antioxidant system of rats was assessed up to 14 days after the burn. Modeling of grade III B thermal burns led to a decrease in the activity of antioxidant enzymes in the blood of animals. A day after the thermal burn the activity of SOD, the main enzyme of specific antioxidant protection of the body, decreased relative to baseline by 1.3 times. The activity of CAT being an enzyme containing heme and leads to the decomposition of hydrogen peroxide into oxygen and water, on the first day after the burn decreased relative to baseline by 2.9 times, which reflects the development of oxidative stress (Fig. 7). On the 3rd, 7th, and 14th days of the study, CAT activity increased, but did not reach the level of baseline values. On the 7th day after the burn, the growth of CAT activity was more intense in the group of animals treated with HPE (77.4% vs. 63.3%).
Fig. 3. Histological changes in skin of rats (control, without treatment) for 7 days after burn of III B degree:
1 – severe leukocyte infiltration, 2 – impregnation of exudate masses of necrosis, formation of fibrinous tissue scab; hematoxylin and eosin stain

Fig. 4. Histological changes in skin of rats (after treatment with extracts from cryopreserved human placenta) on the 7th day after burn of III B degree:
1 – proliferation of granulation tissue, epidermal combs, 2 – boundaries of fibrinous tissue scab, 3 – hyperplasia of epidermis on wound edge, wound epithelization; hematoxylin and eosin stain

Fig. 5. Histological changes in skin of rats (control, without treatment) for 14 days after burn of III B degree:
1 – proliferation of granulation tissue, 2 – organization of necrosis masses with scar formation, 3 – scar with hyalinosis development, 4 – organization of necrosis masses, proliferation of macrophages, fibroblasts, hyperemia and paravascular edema; hematoxylin and eosin stain
Histological changes in skin of rats (after treatment with extracts from cryopreserved human placenta) on the 7th day after burn of III B degree:
1 – thickening of epithelium, formed granulation tissue, foci of proliferation of fibroblasts and histiocytes,
2 – processes of active epithelialization due to marginal hyperplasia epidermis, with formation of epidermal combs,
3 – hyperplasia of epithelium of the basal layer of epidermis, epidermal combs; hematoxylin and eosin stain

On the 7th day after thermal burns, the CAT/SOD ratio increases, but this occurs against the background of decreased SOD activity (44.9%) and increased CAT activity in the control group and the group of animals treated with HPE, relative to previous indicators (63.3% and 77.4%, respectively). This imbalance between the components of enzymatic antioxidant protection indicates the development of oxidative stress mainly due to the depletion of the ability of SOD to neutralize superoxide radicals.

On the 14th day after the burn (the first and second phases of the wound healing process), there was a continued increase in the activity of antioxidant enzymes of both groups of animals and a tendency to restore the balance of the studied enzymes (Fig. 7).

Visual observation showed that on the 16th day after the burn, only in the group of rats treated with HPE, was complete clearance of the wound from necrotic scab observed. On the surface of the wound after the rejection of the scab a thin, shiny, pink granulation surface appeared.

On the 21st day, there was an independent cleansing of the burn wound from necrotic tissues in the control group, revealed hyperplasia of the epidermis and the phenomenon of epithelialization of the edge of the scar tissue (Fig. 8). In the presence of mature granulation tissue, foci of hair follicle proliferation were not detected. In the group treated with HPE, there was an active regeneration of all skin derivatives, manifested in dry, smooth granulation tissue, signs of epithelialization and the formation of different sizes and colours of scars. We can say that the healing of a burn wound on the 21st day after the burn occurs in the third phase of the wound process, which is reorganization of the scar and epithelialization of the wound. The reduction in the size of the defects is due to marginal epithelialization or wound contraction. The analysis of the area of the wound related to the total surface area of the animals revealed probable differences between the experimental and control groups of animals starting from the 14th day of the experiment (Fig. 9).
cell growth and differentiation. Thus, at the stage of granulation there is an
increase in the activity of cellular metabolism and the growth of fibro-
blasts, which begin to synthesize and produce a significant amount of
extracellular collagen. The collagen content in the wound is regulated by
many factors. Control of these processes provides a therapeutic opportuni-
ty to intervene in the process of wound healing and prevent pathological
scar formation. Expanded necrosis can be reduced by general or local
action on the wound, both HPE and other drugs from placenta.

Conclusions

Treatment of burn wounds with extracts from cryopreserved placenta
helped to obtain a higher rate of healing, which was manifested in the
acceleration of changes in the phases of the regeneration process: short-
tened period of cell infiltration and accelerated rate of granulation forma-
tion. Macroscopic differences in the acceleration of the wound healing
process were observed by the 7th day after the burn in the form of rejec-
tion of the necrotic scab and the formation of granulation tissue. Thermal
damage to the skin of rats was accompanied by a decrease in the activity
of SOD and CAT in the blood, which indicates a decrease in antioxidan

Fig. 9. Rate of wound healing in dynamics relative to entire body
surface of rats after burns of III B degree in control (red line) and after
treatment with extracts from cryopreserved human placenta (blue line):
* – significant difference compared to control (P < 0.05, n = 8)

In the control group of rats on day 28 after the burn, the wound sur-
face was covered with granulation tissue, scarring was observed. In the
experimental group (HPE), the wound was completely healed and hair
was restored. Epithelial hyperplasia was revealed (Fig. 10a) along the edge
of dense scar tissue, along with proliferation of granulation tissue, in
some – epithelialization with the formation of epidermal combs. For the
group treated with HPE (Fig. 10b) there was a restoration of the skin, the
formation of a group of follicles and sebaceous glands.

Discussion

In the area of damage from deep thermal burns on the first day, the
wound healing process is in the first phase (Stone et al., 2018), which is
accompanied by blood stasis in the capillaries and ischemia, resulting in
weakened tissue respiration in cells of this area with subsequent death of
epithelial cells of demarcation zone (skin necrosis) (Bhatia et al., 2016).

In HPE, vasculoendothelial growth factor is manifested as a potential
mitogen for vascular epithelial cells and has a significant effect on their
permeability. It is known that epidermal growth factor accelerates the
growth and regeneration of keratinocytes. Wound healing can also be the
result of the activity of a peptide enzyme similar to fibronectin of the third
type. This type of peptide has also been found in HPE (Nath & Bhattacha-
ryya et al., 2007). Fibronectin forms a bridgehead in the wound, to which
fibroblasts then migrate. Successful wound healing requires the presence
of cytokines, the restoration of deficiency of which contributes to the
effective treatment of chronic wounds. Cytokine TGF (transforming
growth factor) is an immunoregulatory cytokine that has a great influence
on intercellular connections and is actively involved in the regulation of
cell growth and differentiation. Thus, at the stage of granulation there is an
increase in the activity of cellular metabolism and the growth of fibro-
blasts, which begin to synthesize and produce a significant amount of
extracellular collagen. The collagen content in the wound is regulated by

Anaerobic glycolysis leads to acidosis, which promotes the appear-
ance of inflammatory mediators and rapid inactivation of antioxidan
t enzymes (Korać & Buzadžić, 2003). The primary source of cytokines and
anti-inflammatory mediators in burns is the area of damage with increased
migration of neutrophils (Baskaran & Yarmouch, 2000), which clean the
damaged skin from the products of thermal decomposition of biomole-
cules with oxygen reactive substances. In the second phase (proliferation
phase) of the wound process, when cleaning the wound from purulent-
reparative secretion and the formation of granulation tissue in it, extracellular
and cellular proteolytic enzymes are of great importance. Therefore con-
sidering the cell elements involved into proliferation phase, it is advisable to
accelerate angiogenesis, activate the synthetic function of fibroblasts to
form granulation tissue, activate epithelialization of intact skin derivatives
(Okur et al., 2020). To do this, it is advisable to use biologically active
substances of natural origin (Vaghdoost et al., 2018; Thanapaul et al.,
2020). Effective tissue stimulants are HPE. In vitro experiments in cell
models have shown that HPE exhibits antioxidant and anti-inflammatory
properties (Pogozhykh et al., 2008; Bobrova et al., 2018), and therefore
can stimulate neangiogenesis. The low-temperature effect does not have a
negative effect on the biologically active components of the placenta and
extracts from it have antioxidant, anti-radical and anti-inflammatory ef-
effects (Bobrova et al., 2018; Narozhnyi et al., 2018). The anti-inflammatory
effect of HPE is accompanied by a decrease in platelet aggregation (Go-
vorova et al., 2017) and therefore an increase in capillary permeability,
which allows serum enriched with proteins to penetrate into the interstitial
space. At the last stage of the wound healing process (third phase), when
the epithelization of the wound and the reorganization of the scar begins,
the drugs with a pronounced effect of stimulating cell growth are used.
That is why HPE is most effective in the second and third phases of
wound healing and eliminates skin defects. The wound-healing effect of
HPE is due to numerous regulatory components and the fact that the pla-
centa contains a number of growth factors (Sharma & Bhattacharyya,
2015; Pogozhykh et al., 2018). They cause immunomodulatory, anti-
inflammatory, wound healing effect, promote cell proliferation and rege-
neration.

Fig. 10. Histological changes in skin of rats on the 28th day after burns of III B degree in the control (a) and after treatment with extracts
from cryopreserved human placenta (b): 1 – skin epithelization, 2 – sebaceous glands, 3 – epidermis restoration; hematoxylin and eosin stain
protection of the body. The positive effect of HPE in the treatment of burns was manifested in a more intense increase in the activity of CAT by the 7th day of treatment.

The study was carried out within the framework of the state theme “Influence of cryopreservation of placenta and its water-salt extracts on antioxidant and anti-inflammatory action of extracts” (state registration No. 0118U003491).

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

References


