

Carbon Monoxide and Their Donor (CORM-2) Change the Healing Rate of Skin Wound Healing in Mice Through Reduced Expression of Aquaporin-3

Serhii BESCHASNYI*, Olena HASIUK**

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SUMMARY

Carbon monoxide (CO) is a toxic gas, has a beneficial effect on cells in low doses. Low concentrations of this gas are produced in the body during the decay of heme-containing proteins and have pro-apoptotic, anti-inflammatory, anti-allergic and vasodilator effects, stimulating angiogenesis. Danger of using this gas is the difficulty of its dosage. CO donors control the amount and gradual release of carbon monoxide. Main aim – studied the effect of treatment with CO and donor tricarbonyldichlororuthenium (II) dimer (CORM-2) on wound healing processes in laboratory mice. Most significant delay in healing was observed in animals whose wounds were treated with CO. In this group, aquaporin-3 (AQP3) mRNA expression was decreased to the most minor degree among all other animals. Most likely it caused the appearance of crusts. CORM-2 treatment also led to a decrease in AQP3 mRNA expression, but no crusts have appeared. Received data can be explained by the fact that CO is released slowly. Dry crust on the wound increases the healing time. Formation of a dry crust is helpful in healing of burns, because with a dry scab, pus cannot appear. With some degrees of burns it prevents supuration and creates a protective barrier. Study confirmed the hypothesis that CO and CORM-2 reduce AQP3 expression after treatment of damaged skin.

Key Words: Aquaporin-3, CO-releasing molecule, CORM-2, carbon monoxide, skin wound

Karbon Monoksit ve Donörünün (CORM-2), Aquaporin-3'ün Ekspresyonunu Azaltarak Farelerde Cilt Yara İyileşmesinin İyileşme Hızını Değiştirmesi

ÖZ

Karbon monoksit (CO) düşük dozlarda hücreler üzerinde yararlı bir etkiye sahip toksik bir gazdır. Bu gazın düşük konsantrasyonları, hem-içeren proteinlerin çürümesi sırasında vücutta üretilir ve anjiyogenezi uyaran pro-apoptotik, anti-enflamatuar, anti-alerjik ve vazodilatör etkilere sahiptir. Bu gazı kullanmanın tehlikesi, dozajını ayarlamamanın zorluğudur. CO donörleri, karbon monoksitin miktarını ve kademeli olarak salınımını kontrol eder. Ana amaç - CO ve donör trikarbonildiklororutenyum (II) dimer (CORM-2) ile tedavinin laboratuvar farelerinde yara iyileşme süreçleri üzerindeki etkisini incelemektir. İyileşmede en önemli gecikme, yaraları CO ile tedavi edilen hayvanlarda gözlenmiştir. Bu grupta, aquaporin-3 (AQP3) mRNA ekspresyonu diğer tüm hayvanlar arasında en az seviyeye kadar azalmıştır. Büyük olasılıkla skar dokusunun ortaya çıkmasına neden olmuştur. CORM-2 tedavisi ayrıca AQP3 mRNA ekspresyonunda bir azalmaya yol açmıştır, ancak skar dokusu ortaya çıkmamıştır. Alınan veriler, CO'nun yavaş yavaş serbest bırakılması gerçeğiyle açıklanabilir. Yara üzerindeki skar dokusu iyileşme süresini artırır. Skar dokusunun oluşumu yanıkların iyileşmesinde yardımcı olur, skar dokusu ile inflamasyon oluşumu engellenebilir ve irin görünmez. Bazı yanıklarda inflamasyonu önler ve koruyucu bir bariyer oluşturur. Çalışma, CO ve CORM-2'nin hasarlı cildin tedavisinden sonra AQP3 ekspresyonunu azalttığı hipotezini doğrulamaktadır.

Anahtar Kelimeler: Aquaporin-3, CO salan molekül, CORM-2, karbon monoksit, cilt yarası

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* ORCID: 0000-0002-7423-4112, Department of Human Biology and Immunology, Kherson State University, Ukraine

** ORCID: 0000-0003-1055-2848, Department of Human Biology and Immunology, Kherson State University, Ukraine

INTRODUCTION

Skin is the outer protective shell of the body, which supports the integrity of the organism. Skin damage can lead to pathological conditions and death. Once a wound is formed, complex processes associated with death of the damaged cells begin.

The repair of the defect is carried out through reparative regeneration and is completed by the scarring process. Continuing high level of domestic and industrial injuries, growth of purulent and inflammatory diseases determine the relevance of finding new means to ensure the effectiveness of treatment of this pathology. There are many known regional therapies (Chang, 2019; Kirsner, 2019; Patel, 2019; Jones, 2020), that can significantly improve treatment effectiveness. On the other hand, studies show that each of these methods has certain shortcomings, and it is very essential to make a differentiated choice without causing increased tissue injury (Jones, 2020).

Depending on the localization of the wound, the issue of not only closing wound defect but also cosmetic result is relevant. Restoration of the structure of intact skin does not occur in the case of full-layer wounds; only tissue regeneration is possible. A zone of tissue replenishment in the area of the wound defect, the changed variant of tissues forming the skin is determined (Singer, 1999). The following groups of drugs are mainly used: vitamins, steroid and non-steroidal anabolic and biogenic stimulants. Among them, there are no gases or gaseous drugs whose effects are less harmful to the body.

Recently, much attention has been paid to the studying of gases that are formed endogenously. These gases are called gas transmitters because they can influence the functions of cells and tissues in picomolar quantities (Wang, 2004; Kolupaev, 2019). Carbon monoxide (CO) is singled out among these gas transmitters. CO is a toxic gas that binds to hemoglobin and forms methemoglobin, enters the mitochondria, and blocks the respiratory chain (Her-

mann, 2012). At the same time, CO has been shown to form in the body as a result of the breakdown of heme in the spleen and liver, as well as other organs. The formation of CO is regulated by the enzyme heme oxygenase-1 (HO-1), which has anti-inflammatory properties (Olszanecki, 2007).

There are known cases of experimental use of CO or CO drugs-donors (CO-releasing molecules, CORMs) to treat colitis and stomach ulcers (Magierowski, 2017). At the same time, the authors noted the difficulty of using gaseous CO. All studies of regenerative and anti-inflammatory properties of CO were aimed at studying the production of pro-inflammatory cytokines, calcium-dependent ion channels and transcription factors (Brouard, 2002; Abdel-Magied, 2019).

Among donors of carbon monoxide are tricarbonyldichlororuthenium (II) dimer or CORM-2 (Magierowska, 2019). CORM-2 is commonly used in experiments to control the production of small quantities of CO without significant changes in carboxy-hemoglobin levels in the blood (Motterlini, 2003). CORM-2 also showed anti-inflammatory action (Qureshi, 2016; Liu, 2019). The drug suppresses lipopolysaccharide-induced inflammation of the respiratory tract (Lin, 2019), enhances plasma coagulation, and weakens fibrinolysis in plasma *in vitro* (Johnson, 2019). Preliminary studies revealed that CORM-2 affects the Ca²⁺-activated K⁺-channels and aquaporin 3 transmembrane channels (AQP3) of red blood cells (Beschasnyi, 2020).

Attempts to use CO for experimental wound healing have shown positive results. Hemin was used to enhance the expression of HO-1, which promotes CO release. The iron-containing porphyrin significantly increases cytokine interleukin 10 and decreases tumour necrosis factor alpha in the granulation tissue (Ahanger, 2021). Recent studies have shown that carbon monoxide accelerates wound healing gastrointestinal trauma (Takagi, 2021). CORM-2 after intravenous injection was a promoter for precision wound

healing and stimulated enhanced vascular growth in a chick chorion-allantoic envelope model (Ahanger, 2011).

However, an essential role in the healing process is played by the relatively recently discovered water and glycerin-aquaporin transporters. These are important channels that take part in the transport of water and glycerol into cells, in processes of embryogenesis, angiogenesis and oncogenesis (Ma, 2002; Ikarashi, 2019). Aquaporin transporters are present in the membrane of almost all cells (Verkman, 2014). More recently, AQP3 has been singled out among the aquaporin channels, which, besides water, is responsible for transporting glycerin and hydrogen peroxide (Bollag, 2020). How aquaporins respond to CO remains unknown.

The purpose of the study is to determine how CO and CORM-2 affect the expression of aquaporin-3 in the dermis and the wound healing rate in laboratory mice.

MATERIALS AND METHODS

The experiment was carried out on 30 white non-breeding male mice weighing 17-19 g. Conditions of the animals were per the standard conditions of the vivarium. Temperatures to be maintained within the range 20-24°C, humidity – 45-65%, light mode – 12 h light/12 h darkness.

Mice were in individual cells with free access to food and water. Animal experiments were carried out in full compliance with the European Council Directive on ethical principles in the management of laboratory animals (The European Council Directive (86/609/EEC)) and Directive 2010/63/EU of the European Parliament and the Council of the European Union. Study protocol was reviewed and approved by the Kherson State University Ethical Committee (ethical committee no: 2020/6).

The induction of pathology (surgical intervention to inflict skin wounds) was performed under anesthesia. “Zoletil 100” (Virbac, France) (30 mg/kg of body weight, intramuscular) was used as an anesthetic and anesthetic substance. Before inflicting a full-skin wound on the animal, the fur on its back was cut with scissors. Remains of the hair were removed with the help of depilation cream (Eveline Cosmetics, Poland). Operating field was then sequentially treated with 70° ethyl alcohol once. Using of a sterile dermo-punch skin biopsy stylet (5 mm in diameter, Sterylab, Italy), mice have applied two full-layer skin wounds through the pulled-back skin fold between the shoulder blades as deep as the surface muscle fascia (Galiano, 2004). The presence of the wound did not affect the motor activity and appetite of the animals.

Two experimental groups of mouse were formed. The case of the members of the first group, after developing skin wounds, two sterile polyethylene bladders (unrelated) were glued to the wound with medical adhesive (BF-6 Lubnypharm, Ukraine). Pure CO (Sciencegases, Ukraine) was injected into the cavity of the left polyethylene bubble, and atmospheric air was injected into the cavity of the right one. Indices obtained during wound healing in the right bubble were taken as control ones.

In the case of the second group (wounds were completely open, without polyethylene bubbles), one injury treated with a solution containing carbon monoxide donor CORM-2 (50 µm/l, Sigma-Aldrich) dissolved in saline. To improve the dissolution of CORM-2 in saline, it was initially dissolved in dimethylsulfoxide (DMSO), the resulting solution had a DMSO concentration not exceeding 0.1%, and this concentration didn't affect the experiment's performance. DMSO was added to saline to treat the control wound (Figure 1).

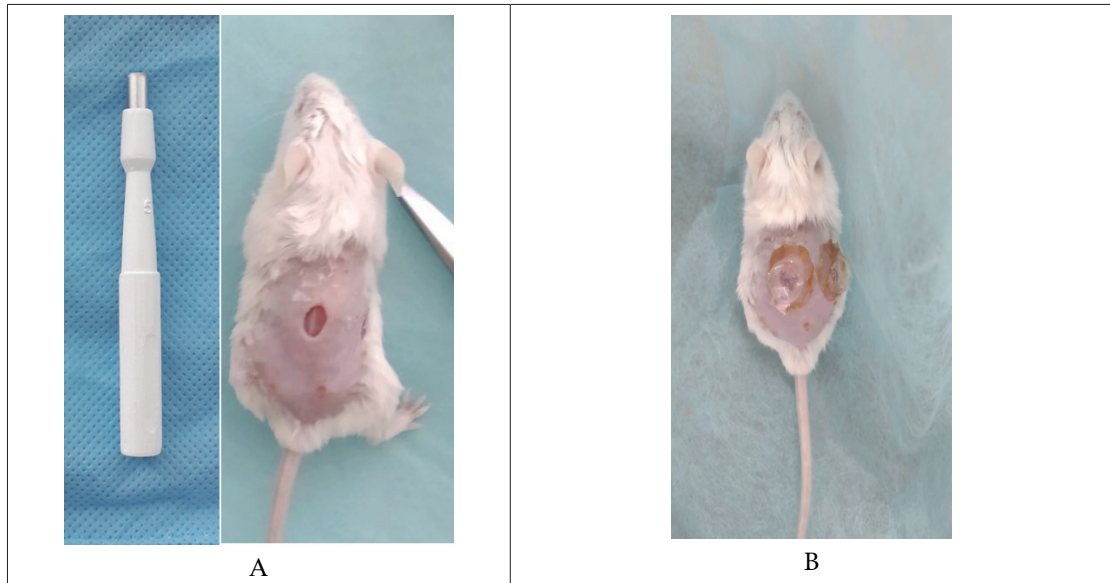


Figure 1. Mouse with a wound made with Dermo Punch (left) (A) and with polyethylene bubbles glued into which CO and atmospheric air were injected (B)

Throughout the experiment, the wounds were photographed daily. The area of wound injury was calibrated and measured using the Image J software (USA). Results were expressed in % of the original size. Every animal was weighed prior to the experiment and the following days.

At the end of the experiment, a skin biopsy was taken for further histological examination. Collected skin samples of mice were fixed in 10% formalin and placed in paraffin. The 4 μ m thick skin incisions were cut using a Leica RM 2125 RTS microtome (Wetzlar, Germany) and stained with hematoxylin and eosin (Sigma-Aldrich); the Van Gieson's trichrome stain (Diagnostic Biosystems, Pleasanton, USA) was used to identify collagen fibers.

On the 5th and 21st days, a skin biopsy was taken from animals for further PCR research into the quantitative expression of mRNA AQP-3. RNA was extracted, and complementary DNA was obtained by reverse transcription. Expression levels of AQP3 mRNA were measured by quantitative PCR using specific primers, (Forward: 5'-GTCAACCCTGCCGTGACTTTG, Reverse: 5'-CGAAGACACCAG-

CGATGGAACC, GenBank ID NM_016689.2) (Metabion, Germany). PCR reactions were performed using 30 μ l reaction mixtures containing 1.0 μ l of PrimeScript enzymes, 1.0 μ l of each primer (30 μ M), 4.0 μ l 5 \times buffer 2 PrimerScript, 14 μ l of sterile distilled water, and 10 μ l of DNA matrix (100 ng / μ l). Each sample was analyzed in three repeats, and the average Ct value was determined based on three experiments. Relative expression of mRNA was expressed as Δ Ct = Ct (target gene) - Ct (calibrator). Expression of mRNA β -actin was used as internal control, and the relative expression of mRNA was calculated as $\Delta\Delta$ Ct = Δ Ct (treatment) - Δ Ct (control). Relative levels of gene expression were transformed and expressed as a multiple of the difference ($2^{-\Delta\Delta$ Ct).

Statistical analysis

All data of measurements are presented as means \pm standard error of the mean (SEM). Comparison between independent samples was made using Mann-Whitney U-test and Wilcoxon Signed Ranks Test. Significance was considered at $P < 0.05$. Statistical analysis was performed using SPSS (SPSS-17, Chicago, IL, USA).

RESULTS

The general condition of mice with control and experimental wounds was satisfactory. The animals had a good appetite and high motor activity. No complications such as a wound infection or fluid collection occurred. During the experiment, the weight of the mice did not differ significantly from the baseline.

In the group of animals with wounds treated

with CO and air, observed the healing process of the wound from the 5th day in case of air treatment. Injuries treated by CO on the same animals began to heal after the 15th day. It is necessary to indicate that a massive crust was formed which covered the wound. From the 19th day on, the injuries treated with CO began to decrease sharply in size (by $45 \pm 2.25\%$) (Figure 2, 3).

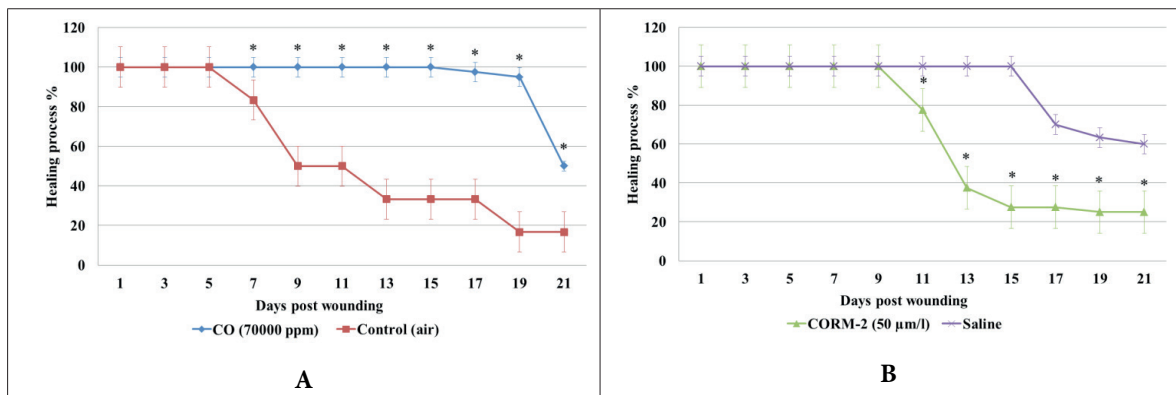


Figure 2. Diagram of the kinetics of wound closure in mice treated with different conditions. Each point represents the mean percentage of reducing the size of a wound. Data are expressed as mean \pm SEM. A - CORM-2 & saline (n=15), B - CO & control (n=15).

* - significant difference, $p \leq 0,05$ (U-test)

In the animal group (one wound was washed with CORM-2 and the second with saline) we found a positive effect of CORM-2 on wound healing.

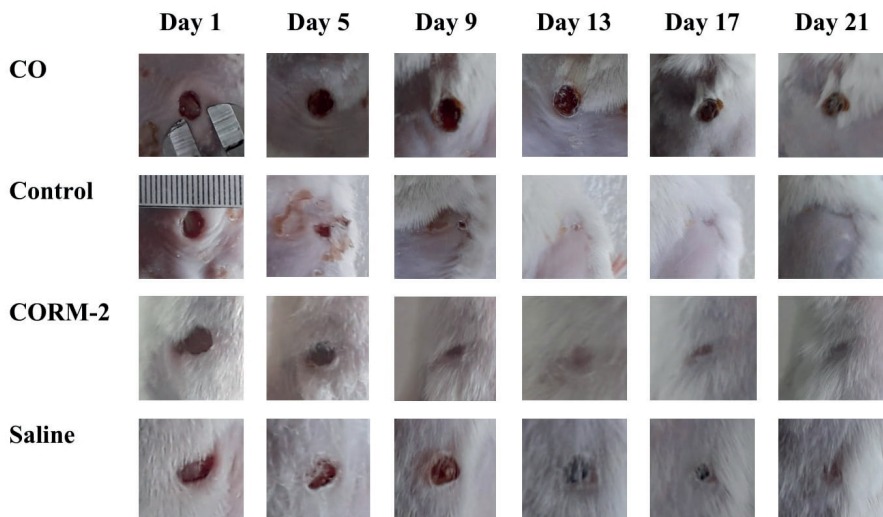


Figure 3. Representative photographs showing wound closure of wounds treated with CORM-2 (50 µm/l), CO (70,000 ppm), and saline immediately after an injury is inflicted (compared to the control).

Wounds washed with saline began to heal after the 15th day, and were treated with CORM-2 – from the 9th. In this case, from the 9th to the 11th day, the wound decreased by $22.5 \pm 1.1\%$. Then the healing rate decreased – the size of the injury decreased by

$40 \pm 1.25\%$ from day 11 to day 13, from day 13 to day 15 by $10 \pm 1.25\%$, from day 15 to 17 it didn't change, and from day 17 to day 19 it decreased by $2 \pm 0.1\%$. No complications, such as a wound infection or fluid collection, occurred in either group (Figure 4).

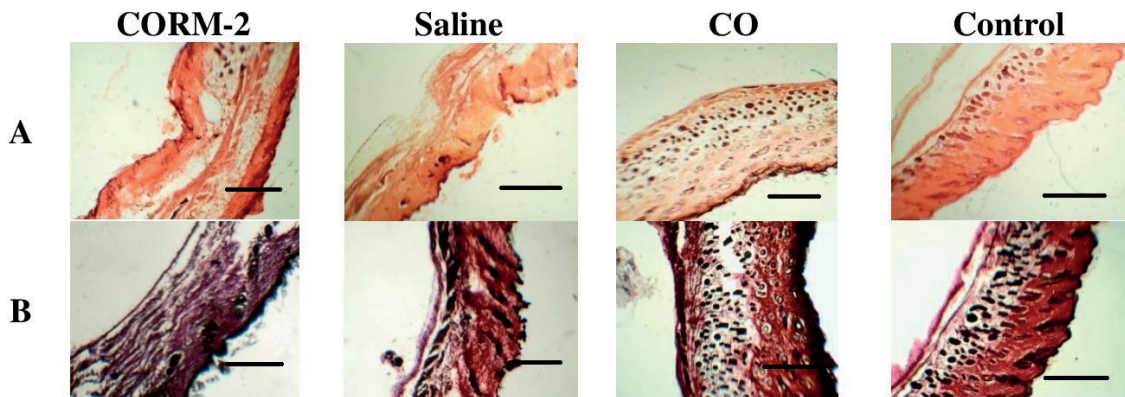


Figure 4. Histological sections of skin with hematoxylin-eosin (A) and Van-Gieson's stain (B), original magnification $\times 100$ (overview photo). Skin samples were taken on the 21st day of the experiment, 500µm scale bar.

The expression of AQP3 in epidermis cells differed in different groups. Compared with control, AQP3 expression in the epidermis of the group treated with

CORM-2 decreased on the 5th day and increased on the 21st day (Figure 5).

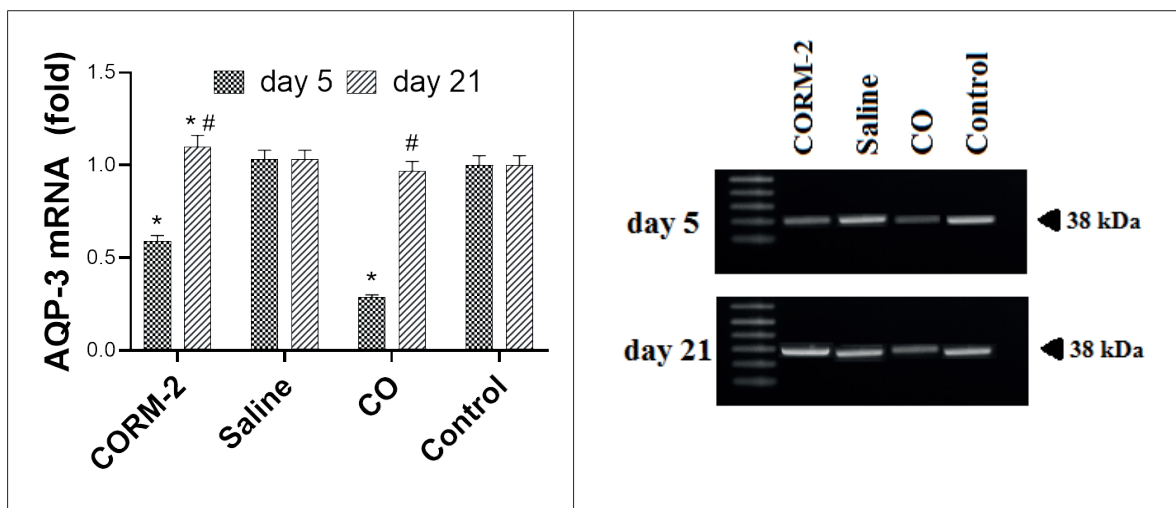


Figure 5. Effect of CORM-2, saline, CO on AQP3 mRNA expression level in the mouse skin
 * - significant difference compared with control, $p \leq 0,05$ (U-test)
 # - significant difference in comparison with the initial indicator, $p \leq 0,05$ (Wilcoxon Test)
 CORM-2 (50 µm/l) & saline (n=15), CO (70000 ppm) & control (air) (n=15)

After CO treatment, AQP3 (and protein) expression was also maximally reduced and increased at the end of healing. In wounds treated with saline, AQP3 expression, to the contrary, was raised, at the end of the experiment - decreased, but was at the level of the control group. The control group, the expression was initially increased but slightly reduced at the end of healing.

DISCUSSION

It is known that AQP3 transports water, glycerin, and hydrogen peroxide (Verkman, 2008; Hara-Chikuma, 2015), which in the epidermis promotes the proliferation and differentiation of keratinocytes (Bollag, 2007). Studies of AQP3 knockout mice suggest that this aquaglyceroporin is also necessary for normal skin hydration, barrier restoration, wound healing, and elasticity (Choudhary, 2015). Activity of AQP3 has also been associated with various human skin diseases (Boury-Jamot, 2006; Olsson, 2006; Qin, 2011; Ikarashi, 2012). Some AQP3 studies indicate that excessive AQP3 expression is associated with skin flat cell carcinoma (Hara-Chikuma, 2008).

CO was previously considered only in terms of toxicity. But after it was found that CO is produced in the body under normal conditions (when heme-containing proteins decompose), it was added to the group of gas transmitters (Wu, 2005). In addition, CO is a signal molecule with a potential therapeutic effect (Wang, 2020).

One of the effects on cells is the activation or inactivation of ion channels, due to which CO is involved in the regulation of the cardiovascular system: it relaxes the smooth muscles of blood vessels and stimulates angiogenesis (Kapetanaki, 2018), regulates apoptosis (Olas, 2014). However, the problem is that the therapeutic use of gas-like CO is very complicated. Therapy with CO is complex because this substance is very tough to dose. Promising way is to use carbon monoxide donor substances (Motterlini, 2017).

The findings confirm that CO (or donor of CO) can influence the healing process of the wound. Results showed that all effects (compared to control) reduced the rate of wound healing. Most significant

healing delay was observed in animals whose injuries were treated with CO. In animals of this group, the expression of aquaporins mRNA was the least reduced among all others. That was probably the source of the scabs. CORM-2 treatment also resulted in a decrease in AQP3 mRNA expression, but not a crust has been formed. It can be attributed to the fact that the CO from CORM-2 was released slowly.

According to modern views, the presence of dry crust on the injury worsens its healing: the time and traces of the wound increase. But, at the same time, the formation of a dry crust is helpful in healing burns (under a dry scab, the construction of pus is impossible, this is achieved in available treatment). Question is debatable, whether healing under a scab should be classified as a primary or secondary type of healing. Usually, the opinion is that it occupies an intermediate position and represents a particular type of healing of surface wounds. However, the importance of the scab in the open treatment of burn disease cannot be underestimated: at some degrees of burn, it prevents festering and creates a protective barrier. The study confirmed the hypothesis that CO (like CORM-2) after treatment of injured skin reduces AQP3 expression. Promising thing is that reducing AQP3 expression with CO or CORM can reduce cancer cell invasiveness, which is increased in malignant cells.

CONCLUSION

Wound treatment with CO results in the dry crust. Treatment with CORM-2 did not lead to the formation of a crust. Acting on wounds with CO or CORM-2 resulted in a decrease in AQP-3 mRNA expression early in the wound healing process. At the end of healing, expression increased in a compensatory way. After treatment with CORM-2 on the 11th day, we observed a sharp increase in the wound healing rate (compared with the saline-treated wounds). Changes in the wound healing rate are associated with changes in AQP-3 mRNA expression under the influence of CO.

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CONFLICT OF INTEREST

The authors declare that no conflict of interest exists

AUTHOR CONTRIBUTION STATEMENT

Idea (BS, HO), manuscript design (BS), performing experiments (BS, HO), data analysis (HO), data interpretation (BS, HO), literature review (BS), writing article (BS).

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