Award Number: W81XWH-11-1-0629

TITLE:
Stabilized hemoglobin wound healing development

PRINCIPAL INVESTIGATOR:
Ross Tye

CONTRACTING ORGANIZATION:
IKOR Inc., Aberdeen, South Dakota 57401-2062

REPORT DATE:
October 2012

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and
should not be construed as an official Department of the Army position, policy or decision
unless so designated by other documentation.
**Wound Healing** is a highly complex orchestrated sequential process, which consists of inflammation, angiogenesis, and tissue formation and remodeling. An important participant, oxygen plays a multifaceted role in wound healing. Tissue oxygenation of the skin depends mainly on local blood supply and arterial oxygen tension as delivery of oxygen to wounds occurs by diffusion. Both clinical observations and animal studies have been shown that wound healing is delayed under hypoxia and cyclic reperfusion condition. Delivery of oxygen and the subsequent reversal of hypoxia may alleviate the decreased oxygen availability in cells thereby allow for wound repair in ischemic and ischemia-reperfusion (I-R) wounds. Oxygen is increasingly transported by the plasma and less by the red blood cells in wounds. IKOR-488, chemically-modified bovine hemoglobin increases the oxygen content of plasma and delivers oxygen selectively to hypoxic cells. This proposed project would establish dosing and treatment validation of IKOR-488 to prevent injury progression using our established animal (rabbit dermal wound) models. We expect that IKOR-488 will be effective in promoting wound healing in ischemic and cyclic reperfusion wounds by delivering oxygen and reversing hypoxia and its adverse effects. Currently there is a compelling, but unmet need for the development of oxygen therapeutic agents able to increase delivery of oxygen to ischemic and I-R wounds. Efficient delivery of oxygen using IKOR-488 in wounds as proposed in these studies can be a valuable therapeutic for promoting healing of combat injuries, acute as well as chronic wounds.

**15. SUBJECT TERMS**

wound healing, oxygen, hypoxia, ischemia-reperfusion, IKOR-488, therapeutic, rabbit dermal wound models
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Body</td>
<td>1</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>7</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>7</td>
</tr>
<tr>
<td>Conclusion</td>
<td>7</td>
</tr>
<tr>
<td>References</td>
<td>8</td>
</tr>
<tr>
<td>Appendices</td>
<td>8</td>
</tr>
</tbody>
</table>
**Introduction**

Combat wounds incurred on the battlefield are particularly challenging. These are due to: (a) the nature of the wounding (high-energy projectile wounding gives rise to significant devitalized tissue, hematoma, and tissue ischemia); (b) heavy contamination of battlefield wounding agents; and (c) in some cases, there are delays in casualty evacuation. The sufficient supply of oxygen to the wounds is one of the key factors for the successful healing of wounds. Combat wound can result in amputation and subsequent long-term disability among our returning soldiers. The success of amputation is in part dependent upon the oxygen levels present at the amputation. By increasing tissue oxygen level, we believe that IKOR-488 can improve the probability of wound healing and thereby reduce the possibility that amputation will be required. In those cases where amputation is unavoidable, IKOR-488 may allow for more distal amputation sites (e.g., below the knee versus above the knee) and improve the quality of life of service member amputees. Poor oxygen delivery is one of causes in delaying acute and chronic wounds. Combat wound care despite many efforts require continued improvement of the management of traumatic injuries.

Currently there is a compelling, but unmet need for the development of oxygen therapeutic agents able to increase delivery of oxygen to ischemic and I-R wounds.

**Body**

**IKOR 488 effect in rabbit ischemic ear model**

Oxygen plays critical role in cell metabolism as an essential molecule for energy production. During the wound healing process its role is even more important due to increased cell proliferation, migration, angiogenesis and other reparative processes such as protein synthesis, especially collagen synthesis. Low levels of pO2 are characteristic of wound environment and it has been shown that the wound healing is delayed in hypoxic condition. Hypoxia is one of the major causes of impaired wound healing through various pathways. Therefore ischemic rabbit ear model represent a good animal model to test the efficacy of supplemental oxygen and determining the exact role oxygen plays in wound healing which is complex and not fully understood.

Forty two animals have been used to complete experiments described in Specific Aim 1. Determine optimal condition, dosage and treatment time for IKOR-488 to enhance wound healing in the rabbit ear ischemic model.

To evaluate the efficacy of IKOR488 in ischemic wound healing and optimize the dosing dose range 100-400mg/kg was used. Histologic evaluation of re-epithelialization and granulation tissue formation is presented in the Figures 1-4.

To determine optimal dosage and timing of IKOR-488 systemic delivery in ischemic ear wound model 100-400mg/kg IKOR was delivered on postsurgical days 0,1,2,4 or later on days 3-7. The tissue was harvested at day 10 post-wounding.
Figure 1. Wound epithelialization in the ischemic injury rabbit ear model upon IKOR-488 treatment. The bars represent the average morphometric measurement of the distance between the two intruding epithelial edges of the 7mm wound which were collected 10 days after surgery. Marked decrease in epithelial gap was evident after 10 days following multiple treatments on days 3-7 in a 400mg/ml dose group. N-number of wounds. p>0.05  n=12 saline vs. IKOR-400

Figure 2. Granulation tissue formation in the ischemic injury rabbit ear model upon IKOR-488 treatment. The bars represent the average measurement of the granulation tissue area around two edges of the 7mm wound collected 10 days after surgery. The IKOR 400mg/kg dose showed statistically significant increase in granulation tissue area after delayed treatment on days 4-7. N-number of wounds (12). p<0.05, saline vs. IKOR
Figure 3. Wound epithelialization in the ischemic injury rabbit ear model upon IKOR-488 treatment. The bars represent the average morphometric measurement of the distance between the two intruding epithelial edges of the 7mm wound which were collected 8 days after surgery. N-number of wounds analyzed, saline n=22, IKOR100 n=22, IKOR200 n=122, IKOR400 n=21. IKOR-100 represents 100mg/kg treatment group, IKOR-200 is 200mg/kg and IKOR-400 is 400mg/kg treatment group. A completely epithelialized wounds would have epithelial gap value 0, whereas in the delayed wound healing majority of wounds are not covered with the newly formed epithelium and the epithelial gap is close to the size of the original wound of 7mm. Single factor ANOVA analysis confirmed that there was no statistically significant difference in wound re-epithelialization between all four treatment groups, p<0.3.

One of the characteristics of the ischemic wound model is the delay in wound healing and re-epithelialization caused by hypoxic condition. In comparison to non-ischemic model where 50% of the wounds are usually fully epithelialized by day 7, ischemic wounds are not covered with the new epithelium at the same time point. This set of experiments provided model validation, 95% of the control wounds of the rabbits receiving saline treatment only were not epithelialized, and average epithelial gap was 6.3mm. Multiple treatments with IKOR488 at any of the three concentrations tested early on at the time of ischemic injury and during the inflammatory stage of the wound healing, failed to improve epithelial coverage of the wounds which would indicate arrested healing.

The delay of the IKOR 488 delivery for 3 days had better healing effect when evaluated for re-epithelialization and granulation tissue formation as represented in Figure 1-2.

Figures 3-4 represent more complete data of the initial experiments to test the effect and dose response when administered immediately after surgery on days 0, 1, 2, 4. Additional experiments confirmed the initial observation of the arrested healing.
Figure 4. Granulation tissue formation in the ischemic injury rabbit ear model upon IKOR-488 treatment. The bars represent the average measurement of the granulation tissue area around two edges of the 7mm wound collected 8 days after surgery. N-number of wounds analyzed, saline n=22, IKOR-100 n=22, IKOR-200 n=22, IKOR-400 n=21. IKOR-100 represents 100mg/kg treatment, IKOR-200 is 200mg/kg and IKOR-400 is 400mg/kg treatment. ANOVA analysis confirmed that there was statistically significant difference in granulation area between all four treatment groups, p<0.01. T-test analysis between control and IKOR-100 p< 0.1 (4 rabbits each group).

Figure 5. Subcutaneous oxygen tension in the ischemia rabbit ear before and after injection of 400mg/kg IKOR-488

The tissue oxygen level was measured using Oxylite instrument in two animals after surgery by inserting the fiber optic probe in the back side of the ischemic ear, 30min and 3h after IKOR488 administration. The increase in oxygen delivery was evident after 3h in both animals as presented in Figure 5.
Figure 6. Synergistic effect of IKOR and growth factor treatment PDGF on epithelialization. PDGF is applied topically 5µg in fibrin sealant days 4-7 post-surgery. No statistically significant synergistic effect is observed with PDGF on wound healing.

Figure 7. Synergistic effect of IKOR and growth factor treatment PDGF on granulation tissue development. PDGF is applied topically 5µg in fibrin sealant days 4-7 post-surgery. No statistically significant synergistic effect is observed with PDGF on wound healing.
Figure 8. Staining for Ki67 proliferation marker in control and IKOR 400mg (4-7 days) treated wounds indicate increased granulation tissue proliferation after IKOR treatment.

Figure 9. Sirius red staining for collagen fibers. Saline (left) and IKOR 400mg treatment (4-7 days) (right)

Figure 10. CD31 expression in saline (left) and IKOR 400mg treated (right) wounds (4-7 days post surgery). Marker of angiogenesis is obvious in the dermal tissue.

Histologic evaluation of the new granulation tissue formation indicated that there was a dose dependent inhibition of granulation tissue development upon IKOR 488 treatment at 200 and 400mg/kg dose. There was no statistically significant difference between IKOR wounds and control wounds upon treatment. 100mg/kg dose revealed trend toward enhanced granulation tissue formation while higher concentration clearly indicated wound healing inhibition.

Hemoglobin levels were measured within one hour of infusion of IKOR488 400mg/kg in two rabbits. The results are: 12g/dl and 13.9g/dl with HCT 41% which is within reference range 10-15g/dl for rabbits. Control values in two rabbits treated with saline solution were: 10.5g/dl and 12g/dl, HCT 36%.

Supplemental oxygen is important in wound healing and has been given as a therapeutic modality to enhance healing. In the initial phase of wound healing the clotting cascade and the function of PDGF depend on reactive oxygen species activity which is induced by hypoxia. Hypoxia is a prerequisite for induction of other cytokines such as TGF-beta, VEGF, TNF alpha and endothelin-1 all affecting important processes in wound healing such as cell proliferation, chemotaxis or vascular permeability. These initial steps stimulated by hypoxia trigger startup of re-epithelialization. It has been reported that under in vitro conditions, hypoxia increases keratinocyte motility and cytoskeletal proteins levels involved in cell motility, however low levels of ROS inhibited migration and proliferation of keratinocytes (6-8).

Additional oxygenation at the early stages of wound healing can obviously affect these processes. The IKOR was given at the time of injury for 3 consecutive days with additional dose on day 4 post-surgery, it is possible that the cascades of events in the early stage of wound healing, hypoxia and re-oxygenation are counter regulated or under regulated by supplemental hemoglobin/oxygen which led to arrested healing.

When given later, 3-7 days post-wounding the highest dose of IKOR488 efficiently supported re-epithelialization and granulation tissue formation.
**Key Research Accomplishments:**

1.1 Determine optimal dosage of IKOR - 488 for systemic delivery

IKOR488 effect on ischemic wound re-epithelialization can be achieved by systemic treatment with IKOR488 in the rabbit ear model within 100-400mg/kg dose range. Granulation tissue formation was stimulated at 100mg/kg although not statistically significant while higher doses 200-400mg/kg indicated dose dependent inhibition of granulation tissue development. Statistically significant effect can be achieved at 400mg/ml.

1.2 Determine optimal treatment time of IKOR-488.

Multiple doses of 400mg/ml IKOR 488, administered systemically days 3 through 7 post-wounding significantly improved wound healing. Re-epithelialization was markedly increased comparing to saline control and the granulation tissue was significantly developed. Measurement of the ischemic tissue oxygen tension before and after IKOR488 400mg/ml injection confirmed that there is a detectable increase of the tissue oxygen 3h upon IKOR treatment, indicating that the oxygen can be delivered to the ischemic ear.

1.3 Determine synergistic effect of IKOR 488 with growth factor treatment.

Co-treatment of 400mg/ml IKOR488 with growth factor PDGF (5µg) topically had no effect on wound healing.

Immunohistochemical tissue evaluation indicates increased expression of proliferation marker Ki 67, marker of angiogenesis CD31 in the granulation area and collagen expression in IKOR 400mg treated wounds.

Measurement of the ischemic tissue oxygen tension before and after IKOR488 400mg/ml injection confirmed that there is a detectable increase of the tissue oxygen 3h upon IKOR treatment, indicating that the oxygen can be delivered to the ischemic ear.

**Reportable outcome**

None

**Conclusions**

In conclusion, the effect of IKOR488 in the ischemic ear wound model is dependent on the timing of administration with respect to the concentration and the phase of wound healing. IKOR 400mg is the most effective dose when applied on days 3-7 post surgery. There is a detectable increase of the tissue oxygen 3h upon IKOR treatment, indicating that the oxygen can be delivered to the ischemic ear.

**References**


**Appendices**

none