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14. ABSTRACT

The increased health concern over embedded metal fragments within DoD was highlighted by the release of Health Affairs Policy Letter 07-029 where a framework for follow-up care for those with embedded fragment injuries was outlined, as well as a list of nine metals of concern. An area not addressed was the potential effect of these metals on the repair of the muscle wounds that inevitably result from these types of injuries. To address this, we have used an *in vitro* model system of cultured muscle cells to determine if metals of interest to DoD adversely affect the ability of the cells to repair wound damage and, if so, can pharmacological intervention mitigate the adverse effects induced by metal exposure. We have found that several metals including nickel, lead, and antimony inhibit wound repair. Soluble forms of iron, as well as insoluble forms of cobalt, also inhibited repair. In many cases, repair capacity could be restored with a variety of pharmacological agents including antioxidants, metal chelators, and chemokines.

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INTRODUCTION

The use of novel materials on the modern battlefield, both in military munitions as well as in Improvised Explosive Devices (IEDs), presents the possibility of wounds with embedded metal fragments. Because standard surgical guidelines recommend leaving embedded fragments in place except under certain circumstances, wounded personnel may carry these metals for decades.

The increased health concern over embedded metal fragments within DoD was highlighted by the release of Health Affairs Policy Letter 07-029 where a framework for follow-up care for those with embedded fragment injuries was outlined, as well as a list of nine metals of concern. An area not addressed was the potential effect of these metals on the repair of the muscle wounds that inevitably result from these types of injuries.

Therefore, in order to address this knowledge gap, we have proposed using an *in vitro* model system of cultured muscle cells to determine if metals of interest to DoD affect the ability of the cells to repair wound damage. For those metals that adversely affect muscle repair, we will assess the potential of pharmacological intervention in mitigating the adverse effects of metal exposure on repair or in accelerating the wound repair process. Classes of compounds that will be tested include antioxidants, chelators, steroids, and chemokines.

BODY

<u>Statement of Work</u>: The development of muniton systems utilizing previously unexploited metals and metal mixtures, as well as the continued use of Improvised Explosive Devices (IED's) by terrorist organizations, have raised concern within DoD of the health effects of embedded metal fragments. However, neither the fate of metals in embedded fragment wounds nor their effects on muscle wound repair have been extensively investigated. We hypothesize that metals, determined by DoD to likely be found in embedded fragment wounds, will inhibit muscle wound repair. Because of the extensive number of metals of concern designated by DoD, the most cost-effective manner to rapidly screen the effect of metals on muscle wound repair is with an *in vitro* model system. Our many years of experience investigating the biological effects of metals, as well as our expertise in cell culture models, puts us in a unique position to study this question.

We proposed three specific aims for this project.

Aim 1: Establish the L6 rat skeletal muscle cell line as an *in vitro* model and determine metal concentration ranges for testing. The commercially available L6 rat skeletal muscle cell line was propagated and characterized prior to testing. Suitable metal concentration ranges were determined to eliminate potential issues with acute metal toxicity.

Aim 2: Determine the effect of metal exposure on artificial wound repair in L6 muscle cells. Using metal concentration ranges determined in Aim 1, the ability of control and metal-treated L6 cells to repair an artificially inflicted wound was determined. Metals to be tested were derived from those listed in DoD Health Affairs Policy Letter 07-029 and included depleted uranium, lead, antimony, tungsten, nickel, cobalt, iron, tin, and copper.

Aim 3: Assess the effect of pharmacological intervention on the mitigation of adverse metal-induced disruption of muscle wound repair. For those metals that adversely affect wound repair, as determined in Aim 2, the ability of pharmacological

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intervention to mitigate the adverse effects was assessed. Several classes of compounds, already shown to be useful in metal detoxification or repair of skeletal muscle injury, were tested.

Progress to Date: All three proposed aims have been addressed and completed. The results for each Aim are described below.

General Research Design and Methods: This study utilized the L6 rat skeletal muscle cell line as a model system to assess metal exposure on wound repair. After growing cells to confluency and treating with sub-toxic concentrations of militarily-relevant metals, the cell monolayer was artificially "wounded" using established methods (1,2). The ability of the metal-exposed muscle cells to repair the wound was determined using light microscopy techniques. For those metals that adversely affected wound repair, pharmacological intervention using chelators, antioxidants, steroids, or chemokines were assessed to determine if normal wound repair capacity could be restored.

Both soluble and insoluble metal compounds were tested in order to more realistically approximate the environment around a muscularly-embedded metal fragment. All metals were obtained from Sigma-Aldrich (St. Louis, MO) with two exceptions. Uranyl nitrate was obtained from the Fluka Chemical Company (Ronkonkoma, NY) and uranium oxide was purchased from Alfa Aeser (Ward Hill, MA). Water-soluble test compounds included $UO_2(NO_3)_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $TaCl_5$, $NiCl_2 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, $Na_2WO_4 \cdot 2H_2O$, $PbCl_2$, $SbCl_3$, $SnCl_2 \cdot 2H_2O$, and $CuCl_2 \cdot 2H_2O$. Insoluble metals included powders of tungsten, nickel, cobalt, antimony, tantalum, lead, tin, iron, copper, and uranium oxide all with purities greater than 99%. All metals, except for tantalum, are listed in Health Affairs Policy Letter 07-029 (3) as being of concern to DoD. Tantalum served as a negative control metal since it is used for prostheses as well as embedded metal fragment studies with no reported adverse health effects (4-7).

Aim 1: Establish the L6 rat skeletal muscle cell line as an *in vitro* model and determine metal concentration ranges for testing: The rat myoblast cell line L6 (CRL #1754) was

purchased from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2. Cultures were routinely fed every 3-4 days and passaged weekly. All experiments were conducted using cells at 80-90% confluency as determined by microscopic observation.

All metal solutions were sterilely prepared in DMEM prior to use, with insoluble mixtures prepared as previously described (8,9). In order to avoid acute cytotoxicity, optimal concentration ranges of all test metals were determined prior to the initiation of wound repair assessments. Assessments of viability, including morphological, plasma membrane integrity (LDH release), lysosomal function (neutral read uptake), and metabolic viability (MTT assay), were conducted. For morphological assessment, L6 cells were plated in multichambered slides (Nalge Nunc International, Roskilde, Denmark). Upon reaching 80-90% confluency, cells were treated with soluble and insoluble forms of the test metals listed above at concentrations ranging from 0 to 100 µg/ml. After 24h incubation at 37°C, the medium was removed from the multichambered slide, the cells washed three times with Dulbecco's Phosphate-Buffered Saline (PBS, Invitrogen), and fixed with methanol. The slides were stained with Giemsa (Invitrogen) and viewed using an Olympus Model BX61 microscope (Olympus America, Inc., Melville, NY). Photomicrographs were taken with a Model DP70 Digital Camera (Olympus America, Inc.) attached to the microscope and processed with the DP70-associated software. Assessments of confluency and morphology (cell size and appearance, nuclear blebbing, formation of micronuclei) were conducted.

Plasma membrane integrity was assessed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega Corporation, Madison, WI). The assay for plasma membrane integrity is based on the quantitative colorimetric determination of lactate dehydrogenase (LDH). LDH, a stable cytosolic enzyme, is released into the culture medium when damage occurs to the plasma membrane of the cell. L6 cells were plated in 96-well tissue culture plates and treated with the appropriate metals. At various times after the initiation of metal treatment, the plates were removed from the incubator and centrifuged at 250 x g for 4 minutes. An aliquot (50 µl) of the resulting

supernatants was added to the wells of a fresh 96-well plate. "Substrate Mix" (50 µl, supplied in the kit) was added to each well and the plate incubated for 30 minutes in the dark at room temperature. The color reaction was terminated by the addition of 50 µl of "Stop Solution" (supplied in the kit) and the absorbance of the reaction mixtures determined at 490 nm in a microplate reader (SpectraMax Model 250 Microplate Spectrophotometer, Molecular Devices Corporation, Sunnyvale, CA). LDH release from metal-treated cells was compared to untreated cells.

The Neutral Red Assay was also used to determine cell viability after metal exposure and was purchased from Sigma-Aldrich. This assay measures the ability of viable cells to take up and concentrate neutral red into lysosomes. Non-viable cells do not take up the dye. Briefly, the cell cultures were grown in 96-well tissue culture plates and treated with metals as previously described. Two hours before the end of the incubation period, the neutral red dye solution is added to the cells. At the end of the incubation period, the cells are washed and then solubilized to release the internalized neutral red. The amount of dye is then determined at 540 nm using the SpectraMax Microplate Spectrophotometer.

Metabolic viability (MTT assay) was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega Corporation). The assay for metabolic viability is based upon the ability of dehydrogenase enzyme systems, located in the cell mitochondria, to reduce a tetrazolium compound to a colored formazan product. The water soluble nature of this product allows it to be easily detected colorimetrically. Cells were plated in 96-well tissue culture plates at a predetermined concentration to assure maximum response for the assay. After allowing time for the cells to adhere to the plate, the test metals were added and the plates returned to the incubator. One hour prior to termination of the incubation period, 10 µl of CellTiter 96® Aqueous One Solution Reagent was added to each well of the plate and the plate returned to the incubator for 1 h. After this time, the absorbance was determined at 490 nm using a SpectraMax Model 250 Microplate Spectrophotometer. Metabolic viability of metal-treated cells was compared to untreated control cells.

Aim 1 results:

Four different assessments of cellular viability were used in this study: morphological changes, lactate dehydrogenase release, neutral red retention, and enzymatic conversion of a tetrazolium salt compound. Although the original plan was to test concentrations up to 1000 µg/ml, these levels proved to be toxic to the cells in practically all metals tested. Therefore, we limited the investigation to three concentrations of metals, namely, 1, 10, and 100 µg, for both soluble and insoluble compounds.

Soluble metals: Results from the morphological examination section are shown in Figures 1-10 found in the Appendices. Treatment with Ta, U, W, or Sn resulted in no adverse morphological findings at any of the concentrations tested. Both Ni and Fe showed significant morphological effects but only when used at 10 and 100 µg/ml. L6 cells treated with 100 µg/ml of Co, Pb, Cu, or Sb also exhibited morphological changes as a result of the metal exposure.

The LDH assay measures the release of lactate dehydrogenase into the extracellular medium and is an indicator of plasma membrane integrity. Results from the LDH assay using soluble metals can be found in Figure 21 of the Appendices. Treatment times of 4 and 24 h were tested. Exposure to Ta, U, or W showed no effect on LDH release over untreated control cells at any of the concentrations tested. In many cases, elevated LDH release did not correlate with visible morphological changes indicating that cell death may have occurred early in the treatment time period or that a subset of cells was particularly sensitive to the metal. In other cases, such as with Fe and Sb, LDH release and morphological changes were correlated.

The neutral red retention assay assesses the ability of the cell to uptake the dye neutral red and concentrate it in the lysosomal vesicles. Except for several cases, neutral red retention was not greatly affected by metal exposure (Figure 23, Appendices). The exceptions include the high dose (100 µg/ml) of Ni, Co, Fe, Pb, Cu, and Sb, as well as both the low (1 µg/ml) and intermediate (10 µg/ml) concentrations of Fe and Sb for a 24 h treatment period.

The MTT assay measures the ability of mitochondria to uptake and enzymatically convert a soluble tetrazolium salt to an insoluble product. This assessment gives an indication of the metabolic viability of the cell. No adverse effects were seen at any time tested at the 1 µg/ml concentration (Figure 25, Appendices). At the 10 and 100 µg/ml

concentrations for the 24 h treatment time, Ni, Co, Fe, Cu, Sb, and Sn exposure resulted in decreased metabolic viability. These adverse effects were also observed in the 4 h treatment groups for Ni, Co, Cu, and Sb.

Based on these results, we selected both the 1 and 10 µg/ml concentrations for soluble metals for testing in the wound repair section (Aim 2) of this project.

Insoluble metals: The results of morphological examination of cells treated with insoluble metal compounds ranging in concentration from 1 to 100 µg are shown in Figures 11-20 in the Appendices. Only slight morphological changes were observed at 100 µg for Ni and Co exposure, while a 24 h exposure to insoluble Sb and Sn resulted in significant morphological effects. No other significant changes were observed.

Ni, Fe, and Sb, at 100 µg for 24 h, resulted in significant LDH release into the extracellular medium (Figure 22, Appendices). On the other hand, both Co and Cu at 100 µg for 4 h or 24 h, resulted in a significant decrease in LDH compared to control cells, indicating that these treatments may result in cell condensation followed by apoptotic death rather than necrosis.

The only significant metal effects for the neutral red retention assay occurred with Ni, Co, Pb, Cu, and Sb at the 100 µg concentration level (Figure 24, Appendices). The MTT assay was significantly affected at the 100 µg concentration level / 24 h time point by Ni, Co, Fe, Pb, Cu, Sb, and Sn exposure (Figure 26, Appendices). In addition, decreases in metabolic viability were seen at the 10 µg level for Co, Cu, and Sb and at the 1 µg level for Co.

Based on the results, both the 1 and 10 µg concentrations for insoluble metals were selected for further testing in Aim 2.

Aim 2: Determine the effect of metal exposure on artificial wound repair in L6 muscle cells. Rat L6 cells were plated on multichambered slides and grown to approximately 90% confluency as described in Aim 1. A wound was then inflicted by pressing a sterile plastic pipet tip through the cell monolayer and dragging it diagonally across the well of multichambered slide. This technique is a slight modification of those already described (1,2). Dragging the pipet tip across the multichambered slide also serves to "notch" the slide and provide a line-of-demarcation between undamaged cells and the wound area

that is visible microscopically. After wounding, the wells were washed with Dulbecco's PBS to remove cell debris and the various test metals, in DMEM, added to the wells. Appropriate metal concentrations were determined in Aim 1. Control cells were treated with DMEM without metals after wounding. After 24h incubation at 37ºC, the medium was removed from the slides, the cells washed three times with Dulbecco's PBS, and fixed with methanol. The slides were stained with Giemsa and viewed with an Olympus Model BX61 microscope as described above. The ability of the metal-treated cells to repair the artificial wound was assessed and compared to untreated cells.

Aim 2 results:

Soluble metals at 1 μg/ml did not affect wound repair in rat L6 cells for any of the metals tested. At 10 μg/ml, Fe, Pb, and Sn all affected repair capacity of the L6 cells, with the greatest effect seen for Fe (Figure 27, Appendices). All three of these metals, at 10 μg/ml concentration, were selected for testing in Aim 3.

The effect of insoluble metal exposure on wound repair in rat L6 cells in shown in Figure 28 in the Appendices. As seen, at a concentration of 1 μg, only Ni exposure affected wound repair. When the concentration was raised to 10 μg, in addition to Ni; Co, Pb, and Sb also affected the repair of wounded L6 cells. Therefore, Ni, Co, Pb, and Sb were selected for testing in Aim 3.

Aim 3: Assess the effect of pharmacological intervention on the mitigation of adverse metal-induced disruption of muscle wound repair.

Only those metals that adversely affect wound repair, as determined in Specific Aim 2, will be used in this section to assess the utility of pharmacological intervention in the mitigation of metal-induced alterations in muscle wound repair. The purpose of this specific aim is to screen several categories of pharmacological agents for the ability to mitigate adverse metal-induced effects on wound repair. It is not designed to be a comprehensive assessment of all potential therapies. The broad categories of compounds that will be assessed include antioxidants, chelators, steroids, and chemokines. Experiments will be performed as follows. Rat L6 cells will be plated on

multichambered slides, grown to 90% confluency, and wounded as described in Specific Aim 2. Metals, determined in Specific Aim 2 to adversely affect muscle wound repair, will be added to the slides and the cells incubated for 24h at 37°C. After this initial incubation, the pharmacological agent to be tested will be added to the cells and the slides returned to the incubator for an additional 24h. After this time, the medium will be removed and the cells washed and processed for microscopic examination and image analysis as described above. Total number of migrating cells, as well as total and mean migration distances will be determined for each pharmacological agent and compared to results obtained from metal treatments alone. The particular agents to be tested are as follows.

Antioxidants: Three antioxidant compounds will be tested: ascorbic acid (10), α-tocopherol succinate (11), and the soy isoflavanoid genistein (12). All three compounds will be purchased from Sigma-Aldrich. Ascorbic acid will be prepared in saline and added to a final concentration of 10 µM, while both α-tocopherol succinate and genistein will be prepared a stock solutions in polyethylene glycol MW 400 (PEG-400, Sigma-Aldrich) before addition to cells at a final concentration of 100 µg/ml.

Chelators: The chelators to be tested will depend upon the particular metals being screened and will be selected from the following: EDTA (for Pb, Fe, Ni, Co, Cu, Sn)(13); dimercaprol (for Pb, Ni)(14); desferrioxamine (for Fe)(15); D-penicillamine (for Cu, Pb, Ni)(16); and ethane-1-hydroxy-1,1-bisphosphonate (for U)(17,18). EDTA will be purchased from Invitrogen as a 0.5M stock solution in water and will be added to the cells to a final concentration of 10 µM. Dimercaprol, desferrioxamine, D-penicillamine, and ethane-1-hydroxy-1,1-bisphosphonate will all be obtained from Sigma-Aldrich and prepared in saline before addition to the cells at a final concentration of 10 µM.

Steroids: The 5-androstene steroid, 5-androstenediol (androst-5-ene-3β,17β-diol, Steraloids, Wilton, NH) (19) will be dissolved in PEG-400 before adding to the cells at a final concentration of 100 µg/ml.

Chemokines: Three rat-specific chemokines will be assessed (20): Monocyte Chemoattractant Protein-1 (MCP-1, CCL2, AbD Serotec, Raleigh, NC), Macrophage Inflammatory Protein 1-alpha (MIP1α, CCL3, AbD Serotec), and Macrophage Inflammatory Protein 1-beta (MIP1β, CCL4, Cell Sciences Inc., Canton, MA). The

compounds will be prepared in saline and added to the cells at a final concentration of 100 ng/ml.

Aim 3 results:

The results from Aim 3 can be found in Table 1 in the Appendices. As can seen, α-tocopherol succinate was successful in restoring the repair capacity of Fe-treated cells. Surprisingly, the iron chelator, desferrioxamine, had no effect. The chelators EDTA and D-penicillamine were successful in restoring repair capacity to L6 cells treated with soluble Pb. The repair capacity of Sn-treated L6 cells was restored by treatment with ascorbic acid, α-tocopherol succinate, EDTA, and the chemokine, MIP1β.

A wider variety of pharmacological agents were useful in restoring repair capacity to L6 cells after treatment with insoluble metals. For Ni exposure at a 1 µg concentration level, ascorbic acid, α-tocopherol succinate, genistein, 5-androstenediol, EDTA, and the chemokines, MCP-1, MIP1α, and MIP1β were all successful in restoring repair capacity. When the concentration of the insoluble Ni was raised to 10 µg, the same agents were successful with the exception of genistein and 5-androstenediol. Successful pharmacological agents for restoring repair capacity included ascorbic acid, α-tocopherol succinate, genistein, 5-androstenediol, EDTA, and the chemokines, MIP1α, and MIP1β for insoluble Co exposure; ascorbic acid, α-tocopherol succinate, genistein, 5-androstenediol, EDTA, and the chemokines, MCP-1, MIP1α, and MIP1β for insoluble Pb exposure; and ascorbic acid, α-tocopherol succinate, EDTA, and the chemokines, MIP1α, and MIP1β for insoluble Sb exposure.

KEY RESEARCH ACCOMPLISHMENTS

- Established the rat L6 cell line as a model for *in vitro* wound repair studies.

- Determined toxicity levels for both the soluble and insoluble forms of the nine metals determined by DoD to be of interest with respect to embedded fragments.

- Assessed the effect of these metals, at non-toxic doses, on the ability of rat L6 cells to repair a wound.

- Investigated the ability of pharmacological agents to restore wound repair capacity after metal exposure.

REPORTABLE OUTCOMES

None.

CONCLUSION

The long-term health effects of embedded metal fragments are now a critical concern for both the Department of Defense and the Department of Veterans Affairs. The use of novel materials on the modern battlefield, both in military munitions, as well as in improvised explosive devices, presents the possibility of embedded fragment wounds with metals whose toxicological properties may not be well understood. Since standard surgical guidelines recommend leaving embedded fragments in place except under certain circumstances, wounded personnel may carry these fragments for decades.

DoD Health Affairs Policy Letter 07-029 listed nine metals of interest with respect to embedded fragment injuries. The nine include depleted uranium, tungsten, nickel, cobalt, iron, lead, copper, antimony, and tin. This study focused on the effect of these metals, in both soluble and insoluble forms, on a cultured rat muscle cell line, as well as the effect of metal exposure on the ability of the cells to repair an artificial wound. For those metals that adversely affected wound repair, the ability of a variety of pharmacological agents to restore repair capacity was assessed. A number of pharmacological agents were tested including antioxidants, metal chelators, steroids, and chemokines.

As described above, several metals did affect wound repair, but that effect could be

mitigated through the use of pharmacological agents. While this project provided some valuable information on the effect of metals on muscle cells and wound repair, it is clearly just a first step in an issue that requires further scientific investigation. For example, while the metals were tested individually, in reality they will probably occur as mixtures or alloys in the case of embedded fragment wounds. The interaction between different metals can greatly affect the overall toxicity of the mixture as was seen in the case of heavy-metal tungsten alloy (8,9) where the mutagenic potential of a mixture of tungsten, nickel, and cobalt was far greater than the sum of the individual metals. This result points to a need to also test those mixtures and alloys that are likely to be found on the battlefield as well as testing the individual metals. In addition, a better understanding of the effects of metal exposure on gene expression and signal transduction is required, not only to determine toxicological effects, but also to aid in the development of more efficient therapeutics to mitigate the adverse effects of metal exposure.

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APPENDICES

Figure 1: Photomicrograph of Rat L6 Cells Treated with Soluble Tantalum (Ta) for 24h.

Figure 2: Photomicrograph of Rat L6 Cells Treated with Soluble Uranium (U) for 24h.

Figure 3: Photomicrograph of Rat L6 Cells Treated with Soluble Tungsten (W) for 24h.

Figure 4: Photomicrograph of Rat L6 Cells Treated with Soluble Nickel (Ni) for 24h.

Figure 5: Photomicrograph of Rat L6 Cells Treated with Soluble Cobalt (Co) for 24h.

Figure 6: Photomicrograph of Rat L6 Cells Treated with Soluble Iron (Fe) for 24h.

Figure 7: Photomicrograph of Rat L6 Cells Treated with Soluble Lead (Pb) for 24h.

Figure 8: Photomicrograph of Rat L6 Cells Treated with Soluble Copper (Cu) for 24h.

Figure 9: Photomicrograph of Rat L6 Cells Treated with Soluble Antimony (Sb) for 24h.

Figure 10: Photomicrograph of Rat L6 Cells Treated with Soluble Tin (Sn) for 24h.

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Figure 19: Photomicrograph of Rat L6 Cells Treated with Insoluble Antimony (Sb) for 24h.

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Figure 21: Effect of Soluble Metals on Lactate Dehydrogenase Release from L6 Cells.

Figure 22: Effect of Insoluble Metals on Lactate Dehydrogenase Release from L6 Cells.

Figure 23: Effect of Soluble Metals on Neutral Red Retention in L6 Cells.

Figure 24: Effect of Insoluble Metals on Neutral Red Retention in L6 Cells.

Figure 25: Effect of Soluble Metals on Metabolic Viability in L6 Cells.

Figure 26: Effect of Insoluble Metals on Metabolic Viability in L6 Cells.

Figure 27: Soluble Metal Effects on Wound Repair in Rat L6 Cells.

Figure 28: Insoluble Metal Effects on Wound Repair in Rat L6 Cells.

Table 1: Restoration of Wound Repair Capacity in Rat L6 Cells by Treatment with

Pharmacological Agents after Metal Exposure.