Purified Exosome Product as a Therapy for Sulfur Dioxide Lung Injury: Insights into Mechanism and Sex-Specific Gene Expression

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RESEARCH & DEVELOPMENT ®

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The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

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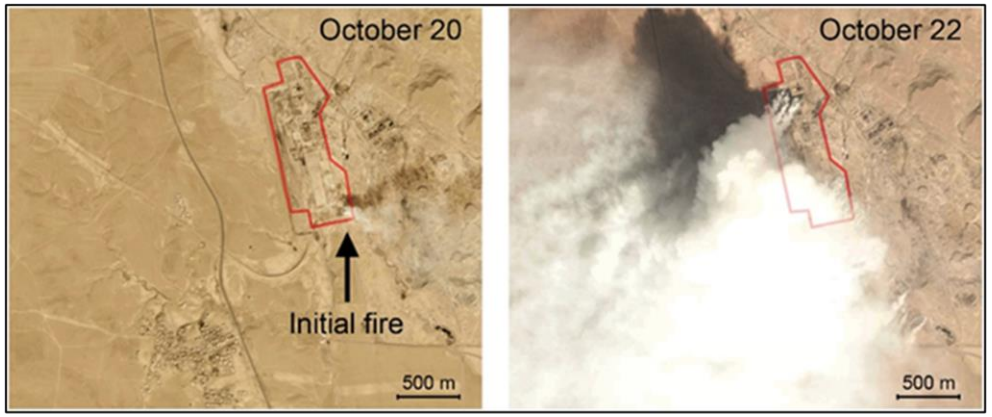
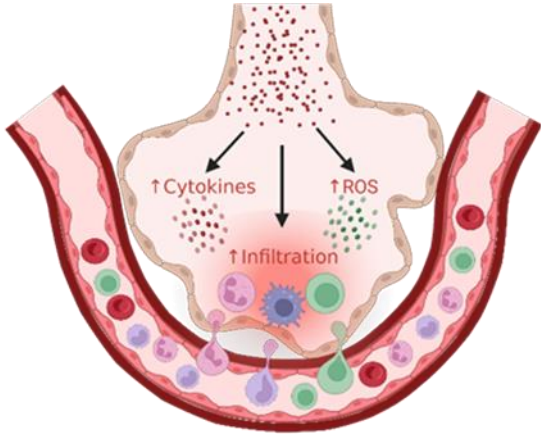
Mishraq Sulfur Mine in Iraq



From Bjornham et al. 2017

Exposure Event 1: A 2003 fire at the Mishraq Sulfur Mine in Iraq exposed deployed US Military personnel to massive, toxic concentrations of sulfur dioxide (SO_2). An estimated **600,000 tons of SO_2** was released by the fire, averaging 21,000 tons per day. At the time of the fire, **191 Army firefighters and 6,341 soldiers** were located within a 50 km radius of the plant.

Exposure Event 2: In October 2016, the Al-Mishraq Plant was once again set on fire, this time by **Daesh in a scorched earth tactic**, putting nearby US military members and civilian populations at risk. **Two civilian deaths and over 1000 respiratory complaints** were reported from the 2016 fire.



Clinical Consequences: US service members experienced acute symptoms and many sustained numerous long-term injuries, including unexplained **dyspnea, sarcoidosis, interstitial lung disease, hypersensitivity pneumonitis, respiratory bronchiolitis, and constrictive bronchiolitis.**

Purified Exosome Product (PEP)

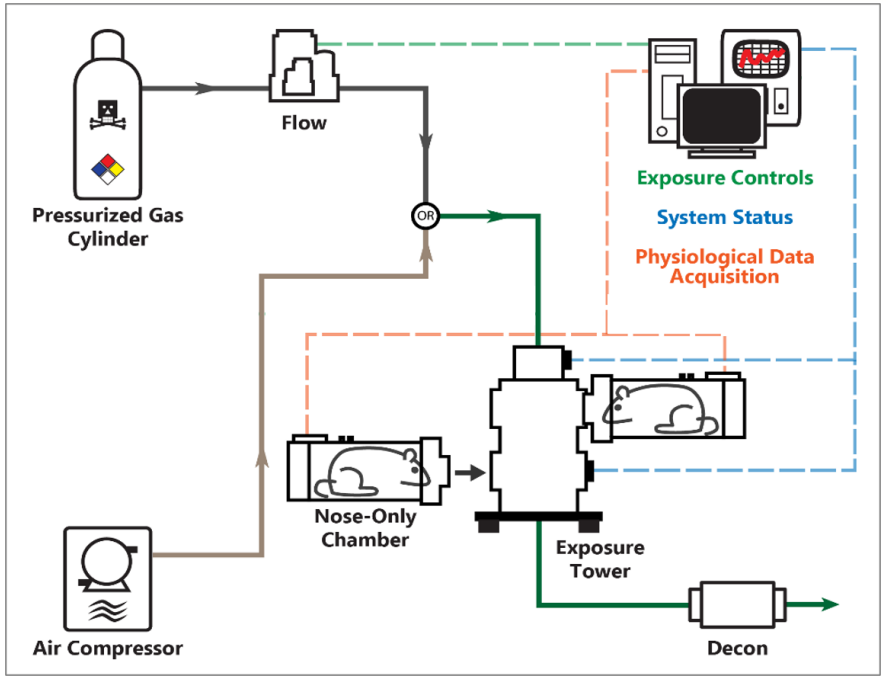
Exosomes:

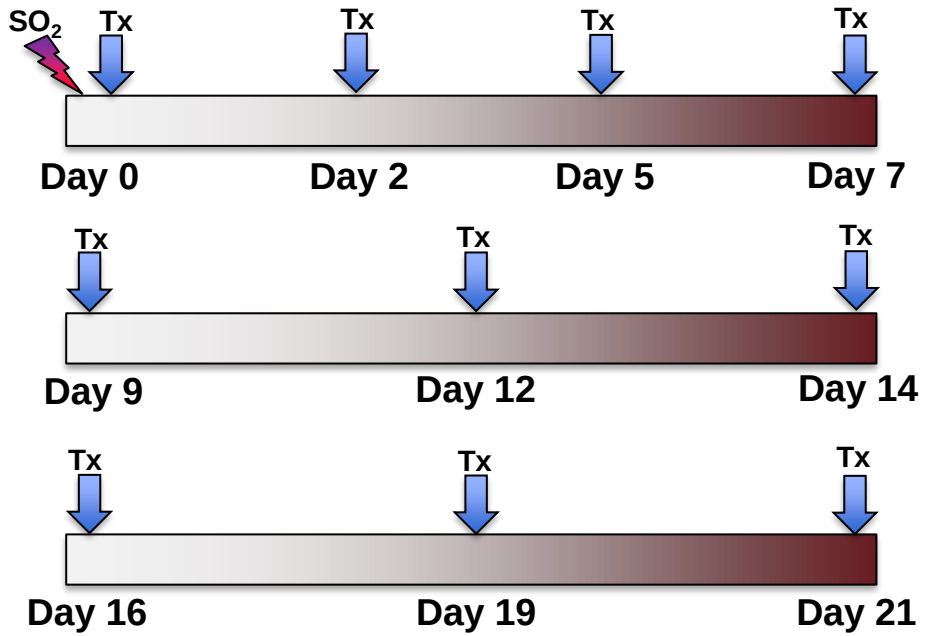
- Lipid bilayer-delimited vesicles that serve as the body's native long-range communication network.
- Naturally structured to encapsulate, protect, and transport vital molecular cargos (including amino acids, nucleic acids, and small signaling molecules) throughout biological systems.
- Promotes highly efficient, cell-specific binding and uptake and triggers downstream intracellular signaling pathways to execute targeted therapeutic instructions.

PEP (Rion Inc.) Inhalation Therapy:

- A standardized, scalable exosome-based platform designed to drive tissue regeneration and resolve severe inflammation.
- Room-Temperature Stable: Eliminates complex cold-chain logistics, enabling field storage and stockpiling.
- Ready-to-Use Reconstitution: Quickly reconstituted at the point of care/time of use.
- Direct-to-Lung Inhalation: Uniquely suited for aerosolized/nebulized inhalation, delivering protective exosomes directly to the injured alveolar-capillary barrier.

Experimental Design



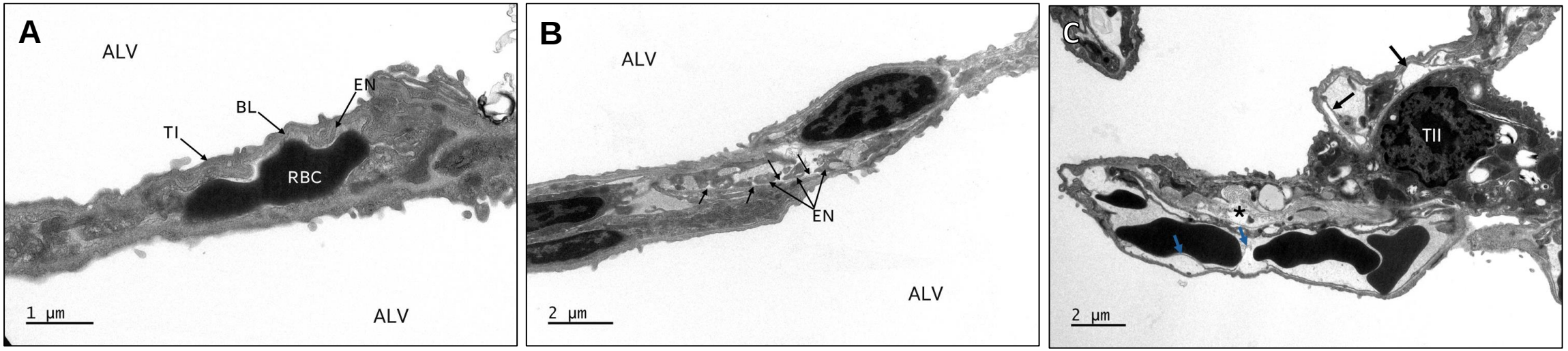


Exposure System: Male and Female C57BL/6 mice were exposed to 3200 ppm of gaseous sulfur dioxide for 26.5 minutes using a nose-only exposure system (DSI exposure tower).

PEP Treatment: Following SO_2 exposure, the cohort received nebulized PEP at a concentration of 15 mg/ml (or saline control) for 30 minutes, treatment was repeated three times weekly for 21 days

Longitudinal Monitoring: Weight, clinical assessments, and repeat respiratory function measurements using whole-body plethysmography for 1 hour, on treatment days. At the study endpoint, all animals were euthanized, and tissues and biosamples were collected for downstream analysis.

Sulfur Dioxide Injury

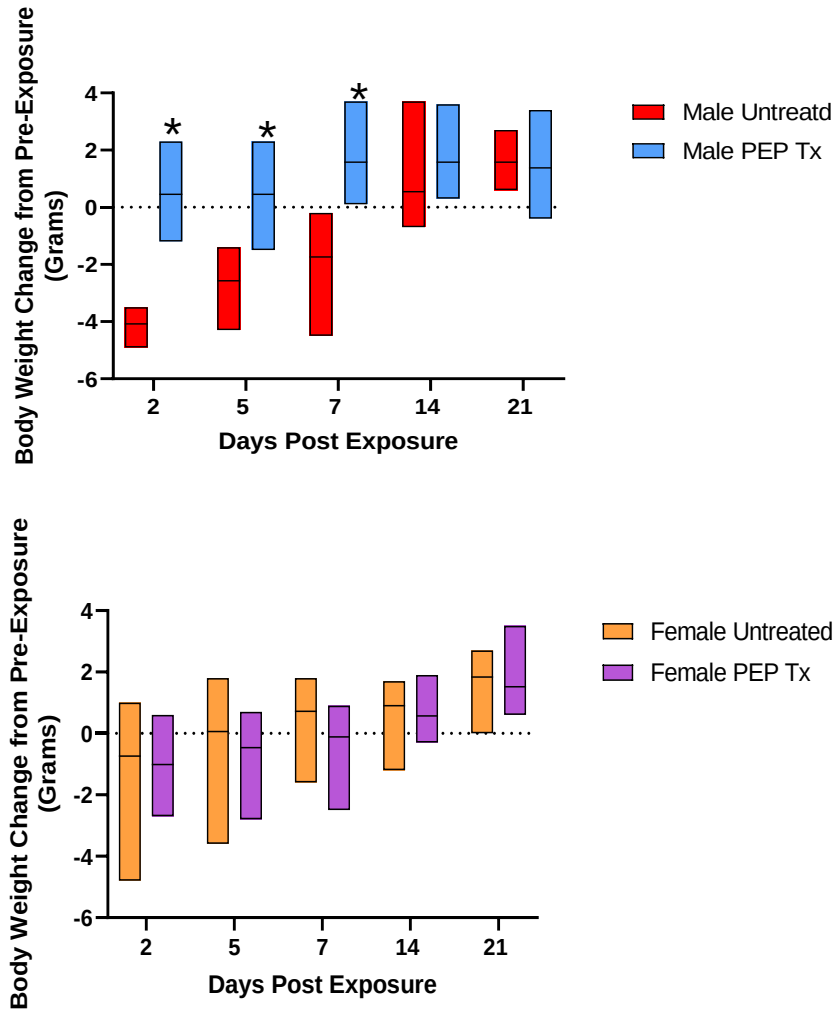


A) **Air Control:** Alveolar septum showing normal blood-air-barrier cell structure and tissue architecture. Squamous capillary endothelium (EN), basal lamina (BL), Type I pneumocyte (TI), alveolar lumen (ALV).

B) **SO_2 Exposed 24 hours post exposure:** Basal lamina (arrows) is left exposed to the capillary lumen by an incomplete endothelial cell barrier (EN). The gap in these fenestra left the basal lamina denuded (arrows) which severely compromises the integrity of the blood-air-barrier.

C) **SO2 Exposed 21 days post exposure:** The frequency of endothelial blebs increased in comparison to those observed in Naïve and air exposed. Unique to the 21-day tissues was the increase of Type I pneumocyte edema. Alterations to Type II pneumocytes were exclusive to exposed tissues at 21 days post exposure. Specifically noted were early signs of necrosis; swollen mitochondria and organelles.

Physiological Changes



*p < 0.05, multiple student's T-tests.

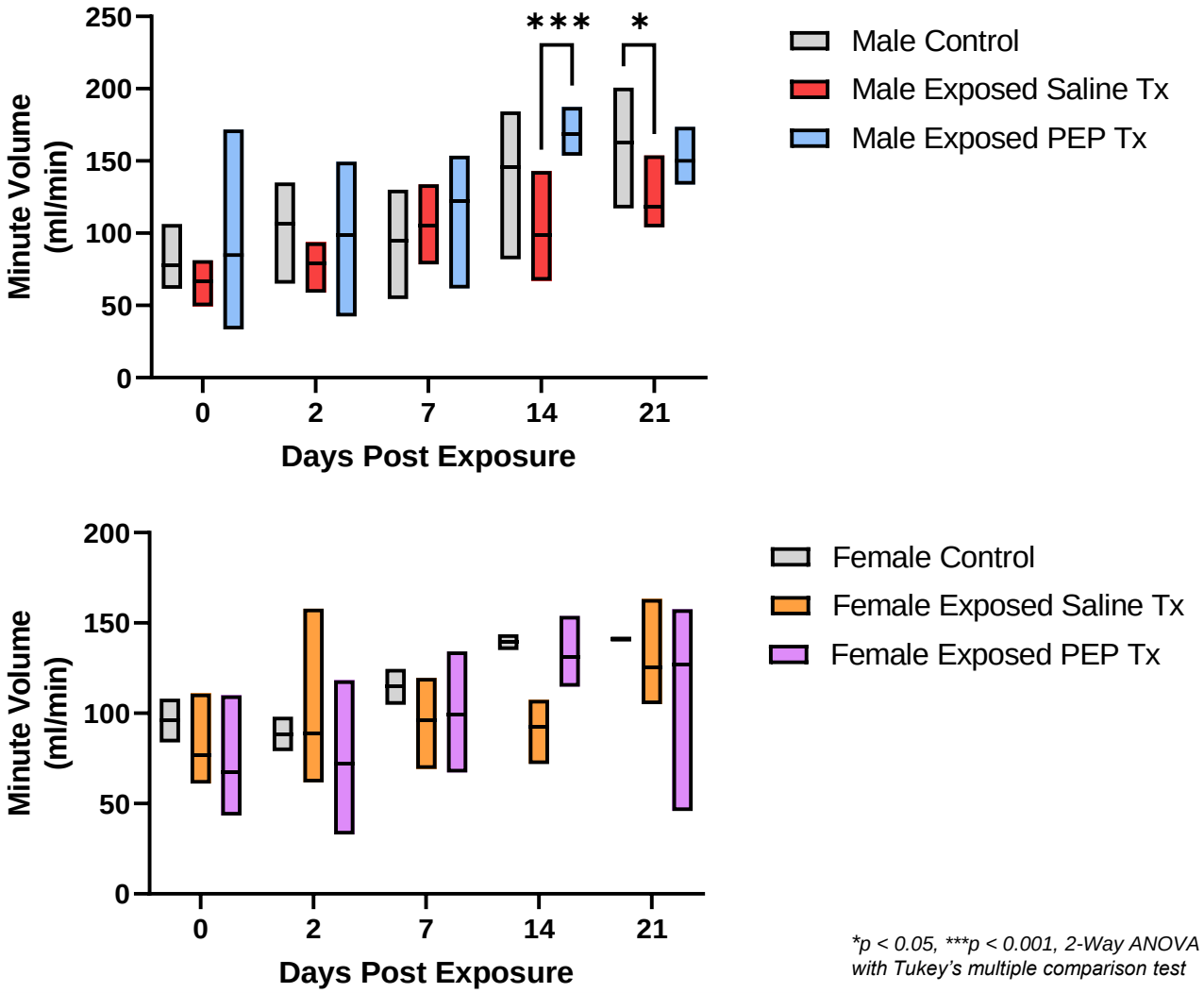
PEP Protects Against Acute Systemic Wasting:

- **Sustained Prevention of Mass Loss:** Untreated males suffer severe and rapid weight loss following SO_2 exposure. PEP treatment significantly prevents this acute wasting, maintaining significantly higher body weights throughout the first week (Days 2, 5, and 7; $*p<0.05$).
- **Mitigation of Systemic Cachexia:** The dramatic mass loss in untreated males (~17% on Day 2) is indicative of systemic cachexia and cytokine-driven inflammatory stress. PEP's protective effect correlates with its ability to suppress systemic and local inflammatory signaling.
- **Sex-Divergent Wasting Profiles:** Unlike males, female mice did not exhibit a significant weight drop post-exposure. This may relate to baseline physiological differences or lower starting body masses (Average male starting weight = 23.1 g, average female starting weight = 19.3 g).

Physiological Changes

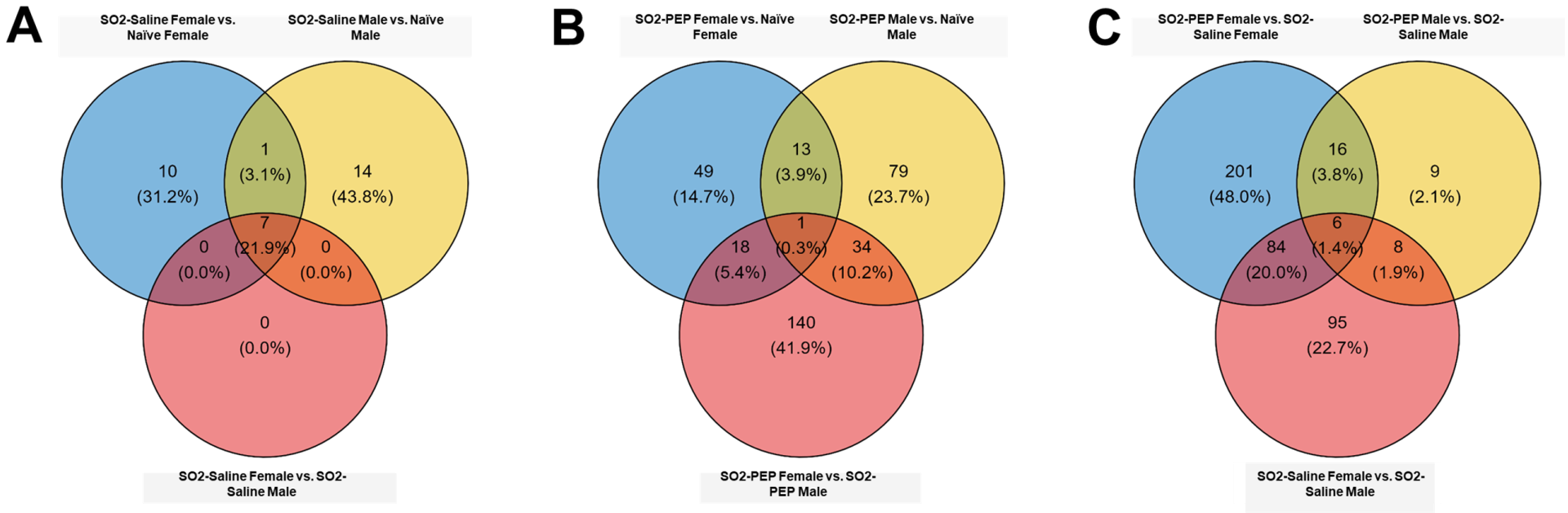
PEP Rescues SO_2-Induced Ventilatory Depression:

- **Day 14 (Therapeutic Peak):** PEP treatment dramatically rescues SO_2-suppressed Minute Volume in males, achieving a highly significant increase compared to the untreated Saline cohort (***p<0.001).
- **Day 21 (Chronic Resolution):** Untreated males continue to suffer from significant, chronic respiratory depression compared to Air Controls (*p<0.05). Conversely, PEP-treated animals successfully maintain normal respiratory function, showing no significant deficit from baseline.
- **Conservation Across Sexes:** Females exhibit a visually consistent therapeutic trend toward respiratory rescue, though high baseline variability prevented it from reaching statistical significance.



*p < 0.05, ***p < 0.001, 2-Way ANOVA with Tukey's multiple comparison test

Transcriptomic Shift – Broad View



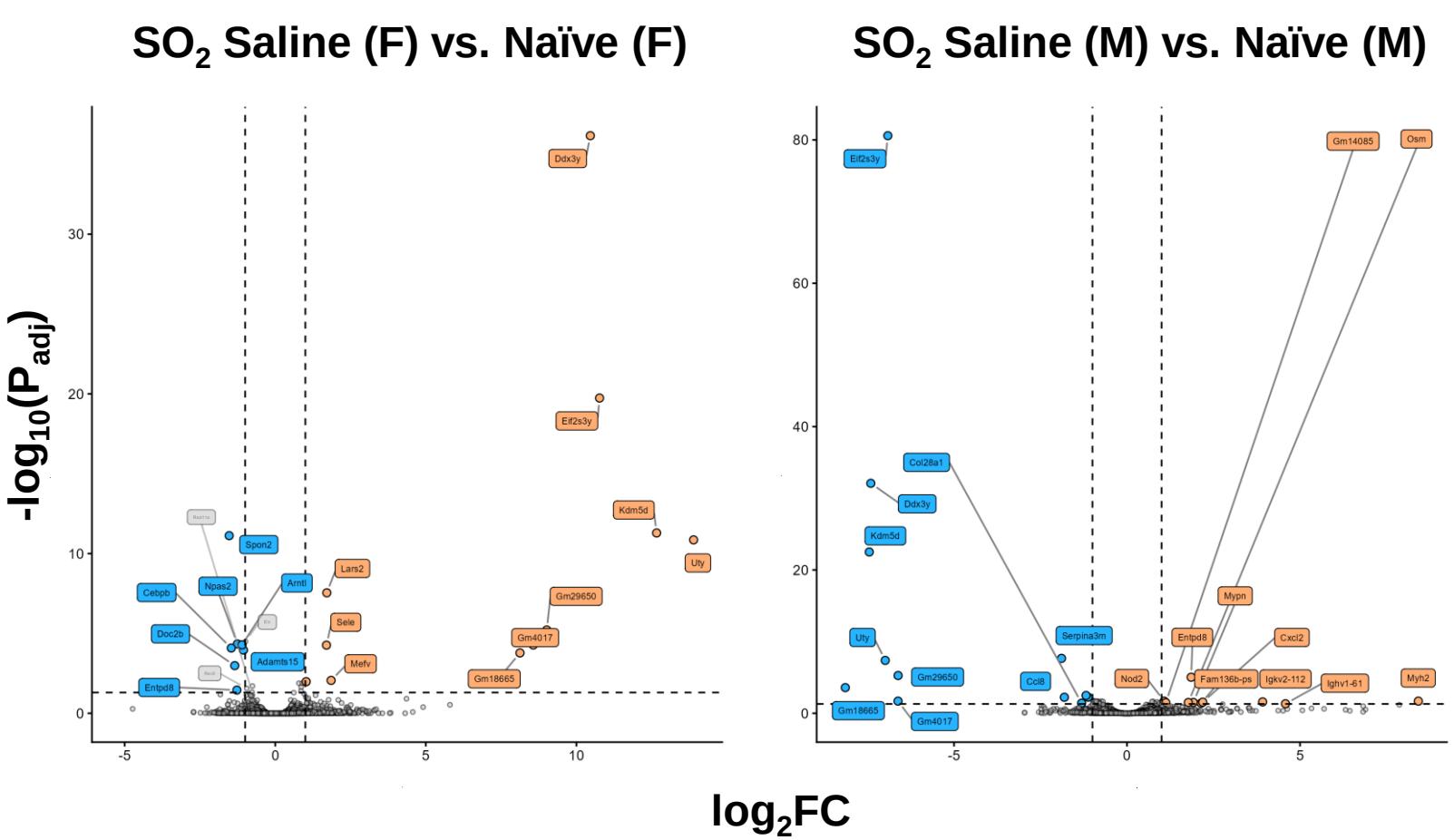
Unique and Overlapping Differentially Expressed Genes (DEGs) Across Treatment Groups and Sex:

A) In saline-treated animals, SO2 exposure induces largely distinct transcriptional responses in females (10 unique DEGs) and males (14 unique DEGs) when compared to their respective naïve controls, with only 7 genes shared between the sexes.

B) In PEP-treated animals, there is an increased number of shared DEGs (13 genes) between males and females relative to naïve controls.

C) The direct effect of PEP treatment (PEP vs. Saline) is highly divergent between the sexes. Females show a large response with 201 unique DEGs, whereas males show only 9 unique DEGs.

Transcriptomic Shift – Broad View

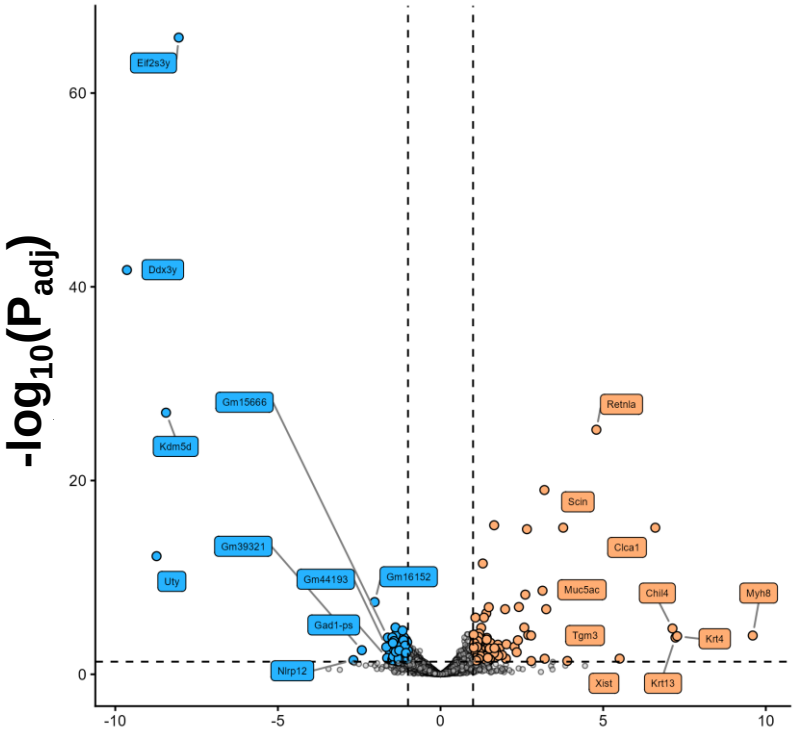


THE UNTREATED STATE: Stagnation and Failure to Initiate Repair

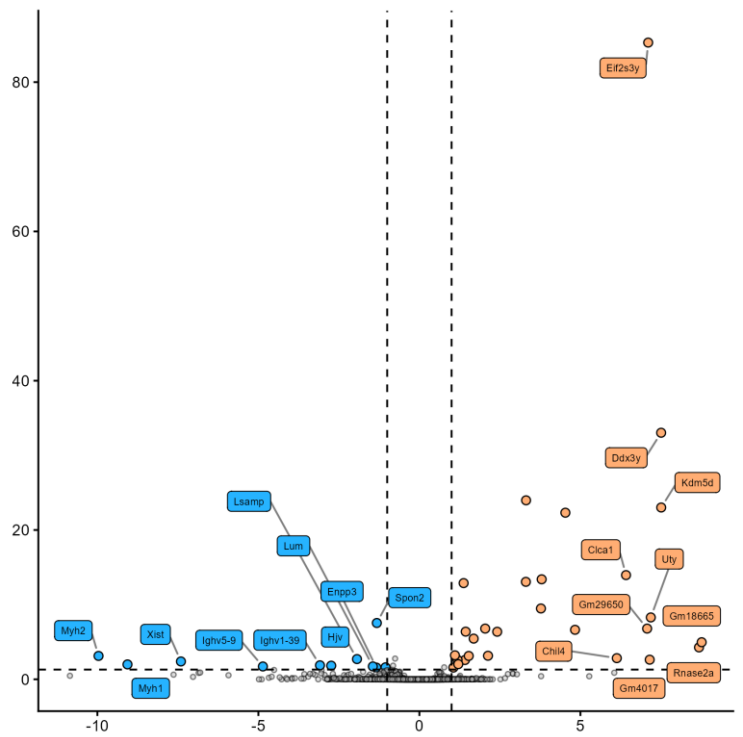
- This extremely low number of DEGs indicates a transcriptional stagnation. The untreated lung fails to coordinate a tissue-repair program. Instead, it remains trapped in a chronic, passive wound-healing arrest state, characterized by persistent cellular necrosis, alveolar-capillary leakage, and chronic alveolar wall degradation (as shown by TEM).

Transcriptomic Shift – Broad View

SO_2 PEP (F) vs. SO_2 Saline (F)



SO_2 PEP (M) vs. SO_2 Saline (M)

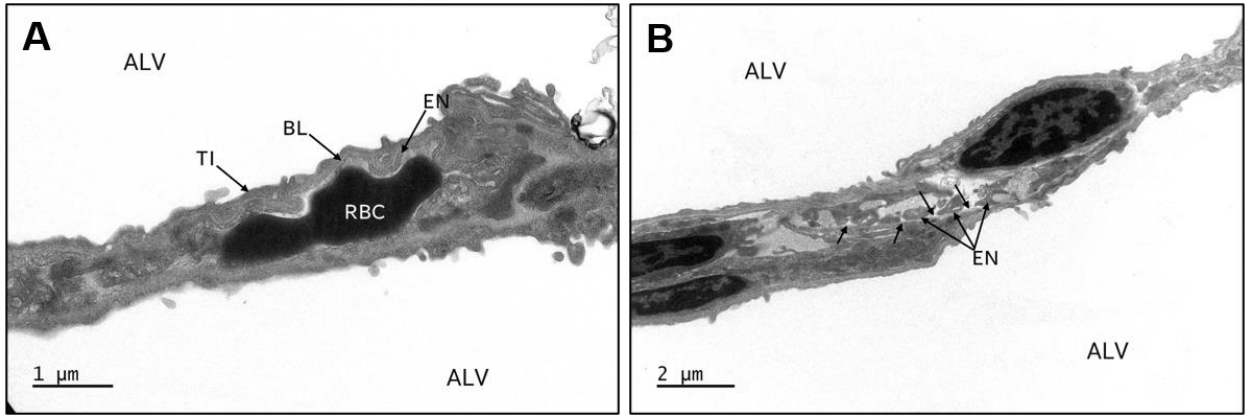


log_2FC

THE PEP STATE: Resolving the Injury

- PEP activates a massive wave of 419 total DEGs acting as a genomic "switch," waking up the tissue from untreated transcriptional stagnation (only 32 DEGs) and driving it toward active cellular clearing and repair.

Gene Signatures – Acute Defense



Control **24 Hrs Post SO_2**

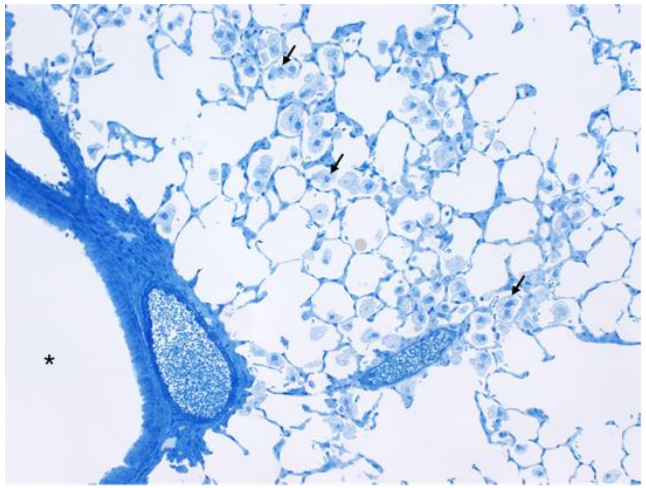
Acute SO_2 Pathology: SO_2 gas causes severe, immediate chemical burns to the respiratory epithelium, **destroying the blood-air barrier.** This triggers a pathological "acute defense" alarm state, characterized by the hyper-recruitment of neutrophils (PMNs) and the massive upregulation of destructive antimicrobial peptides and acute-phase proteins.

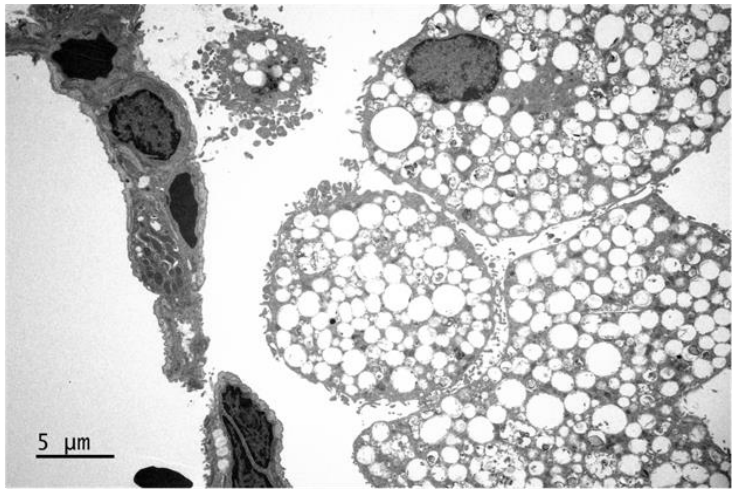
PEP Treatment Effect: When PEP is administered, the transcriptomic profile reveals a profound, highly significant **downregulation of the primary mediators of this acute defense system**. This represents a complete shutdown of the active neutrophil-driven inflammatory cascade:

Gene Symbol	Female (Log_2FC)	Male (Log_2FC)	Biological Role in PEP-Mediated Rescue
Ltf (Lactoferrin)	-4.5	-4.2	Lactoferrin chelates iron to starve bacteria but also recruits inflammatory cells and promotes oxidative stress.
Lcn2 (Lipocalin-2)	-3.9	-3.8	Lipocalin-2 is an acute-phase protein that acts as a potent neutrophil chemoattractant. Downregulating *Lcn2* by nearly 4-fold breaks the positive-feedback loop of continuous neutrophil recruitment.
Camp (Cathelicidin)	-3.6	-3.5	While cathelicidin is an effective antimicrobial peptide, in sterile chemical injuries, high concentrations are cytotoxic to host epithelial cells. PEP-mediated downregulation of *Camp* prevents this "friendly fire" epithelial necrosis.
Slpi (Secretory Leukocyte Peptidase Inhibitor)	-2.0	-1.8	PEP has halted neutrophil influx and stopped elastase release, the tissue no longer requires high levels of *Slpi* to protect itself, allowing antiprotease levels to return to baseline.

Gene Signatures - Repair

M2 Macrophage Recruitment & Phagocytic Debris Clearance: Light microscopy at 21 days post-exposure reveals PEP treatment induces **clusters of activated alveolar macrophages** migrating adjacent to the respiratory bronchioles (*). TEM ROI analysis reveals that these cells are **densely packed with debris-filled phagolysosomes.**





Gene Symbol	Functional Program	Female (Log_2FC)	Male (Log_2FC)	Biological Role in PEP-Mediated Rescue
Chil4	M2 Macrophage/Clearing	6.5	6.0	Canonical M2 alternative-activation marker. *Chil4* reprograms macrophages away from the destructive M1 state into a repairing M2 state.
Ccl8	M2 Macrophage/Clearing	4.8	4.0	Establishes chemokine gradient to recruit fresh monocytes to injured alveoli.
Retnla	M2 Macrophage/Clearing	5.0	3.2	Sustains alternative M2 anti-inflammatory and tissue repair programs.
Mmp12	M2 Macrophage/Clearing	4.2	3.8	M2 cells upregulate Mmp12 to digest damaged elastin and clear the alveolar spaces (structurally validated by TEM)
Muc5ac	Mucofluid Shielding	4.0	2.5	Synthesizes the major gel mucin polymer to physically shield raw epithelium.
Fkbp5	Stress Alleviation	-2.8	-2.5	Downregulated; resolves glucocorticoid receptor stress and hyperactivation.

Gene Signature: PEP treatment initiates an active, cellular 'cleanup program' driven by **alternative M2 macrophage polarization.** These cells express matrix metalloproteinase *Mmp12* to surgically **clear damaged tissue debris** from gas exchange surfaces. PEP downregulates the stress co-chaperone *Fkbp5* to alleviate intracellular glucocorticoid receptor resistance, **successfully driving the lung toward homeostatic resolution**.

Conclusions

Therapeutic Modulation: Nebulized Purified Exosome Product (PEP) effectively modulates the pulmonary immune response following acute, high-dose SO2 exposure, acting as a potent biological countermeasure.

Shift to Tissue Repair: Transcriptomic profiling reveals that continuous PEP treatment downregulates acute innate immune and antimicrobial responses, driving a robust shift toward barrier restoration (mucin regulation) and M2-macrophage-mediated tissue repair.

Morphological Confirmation: Microscopy directly corroborates the genetic data, visualizing dense clusters of highly active alveolar macrophages packed with phagolysosomes in PEP-treated tissue, indicative of active clearance and remodeling.

Significant Sex Differences: RNA-sequencing uncovered a divergence in treatment response between sexes. Female mice exhibited a broader transcriptional remodeling response to PEP (201 unique DEGs) compared to males (9 unique DEGs). Conversely, male mice exhibited more pronounced, statistically significant physiological rescue, which correlates directly with their heightened baseline susceptibility to acute systemic wasting and ventilatory depression.

Clinical Relevance: These pre-clinical findings highlight PEP as a highly promising, non-invasive regenerative therapy for Service Members suffering from chronic, deployment-related lung injuries

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Questions