A Novel Countermeasure Against Ionizing Radiation-Induced Bone Loss

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Background – Exposure of Bone to Ionizing Radiation

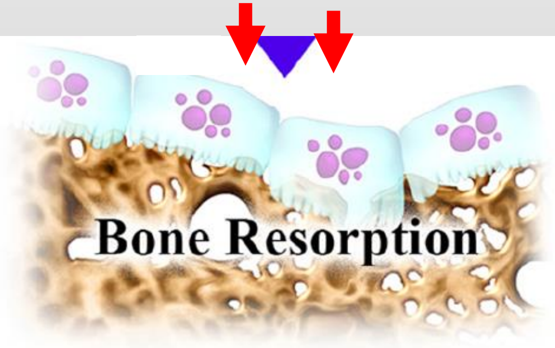
High Ca content → 30 - 40% more ionizing radiation (IR) absorption in bone[1]

✗ IR-induced bone toxicity:

- ↑ Bone Pain
- ↑ Bone Atrophy
- ↓ BMD
- ↑ Osteoporosis
- ↑ Fracture incidence[2]
- ↑ Bone repair delayed & incomplete

Major health concern with no effective prophylactic

✓ Macrophage (M1)
✓ Collagen dysregulation
✓ Microvessel necrosis
✓ Vascular thrombosis
✓ Osteoblast downregulation
✓ Osteoclast upregulation



[1]Curi et al. J Oral Max Surg, 2016, [2]Yaprak et al. BMC Cancer, 2018, [3]Baxter et al. *2005 Nov 23; 294(20):2587-93*

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Reactive Oxygen Species (ROS)

In presence of oxygen many radicals form during IR-exposure
e.g., $OH^{\bullet}$, $H^{\bullet}$, $H_{2,}$ H^{+}, $H_2O_{2,}$ $O_2^{\bullet -}/HO_2^{\bullet}$, organic radicals

✓ $OH^{\bullet}$ hydroxyl radical → most damaging. ⅔ DNA damage to cells, reacts with almost every organic biomolecule including DNA

✓ $O_2^{\bullet -}$ easily generated, major culprit, precursor to most other harmful ROS

✓ H_2O_2 – more toxic than $O_2^{\bullet -}$, directly generates $OH^{\bullet}$ (Fenton reaction)

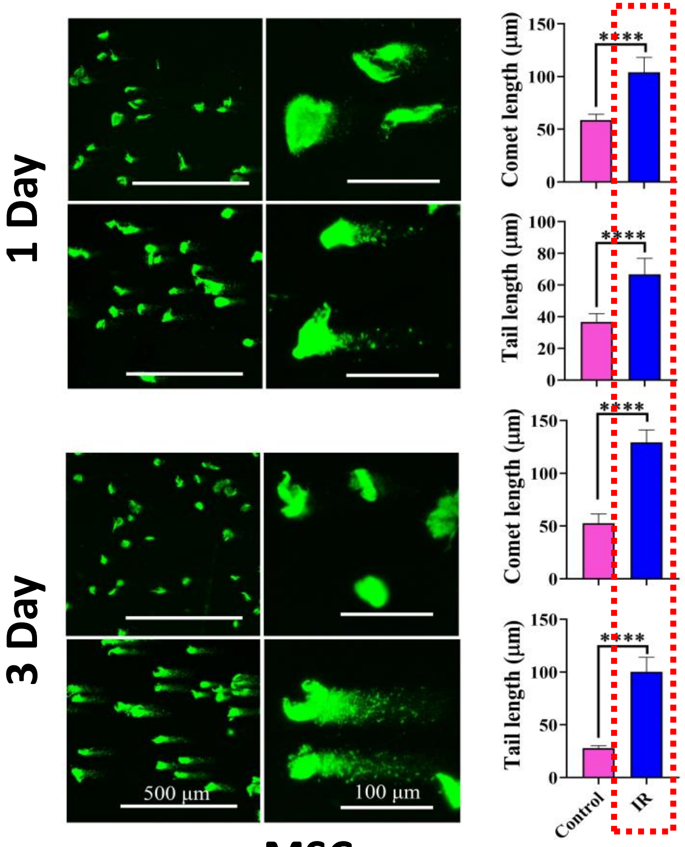
ROS micro-distribute known as "bystander effect"

- ✗ Protein carbonylation
- ✗ Lipid peroxidation
- ✗ Spontaneous gene mutations
- ✗ Neoplastic transformation

Ionizing radiation damages hBMSCs and macrophages

hBMSCs

DNA damage

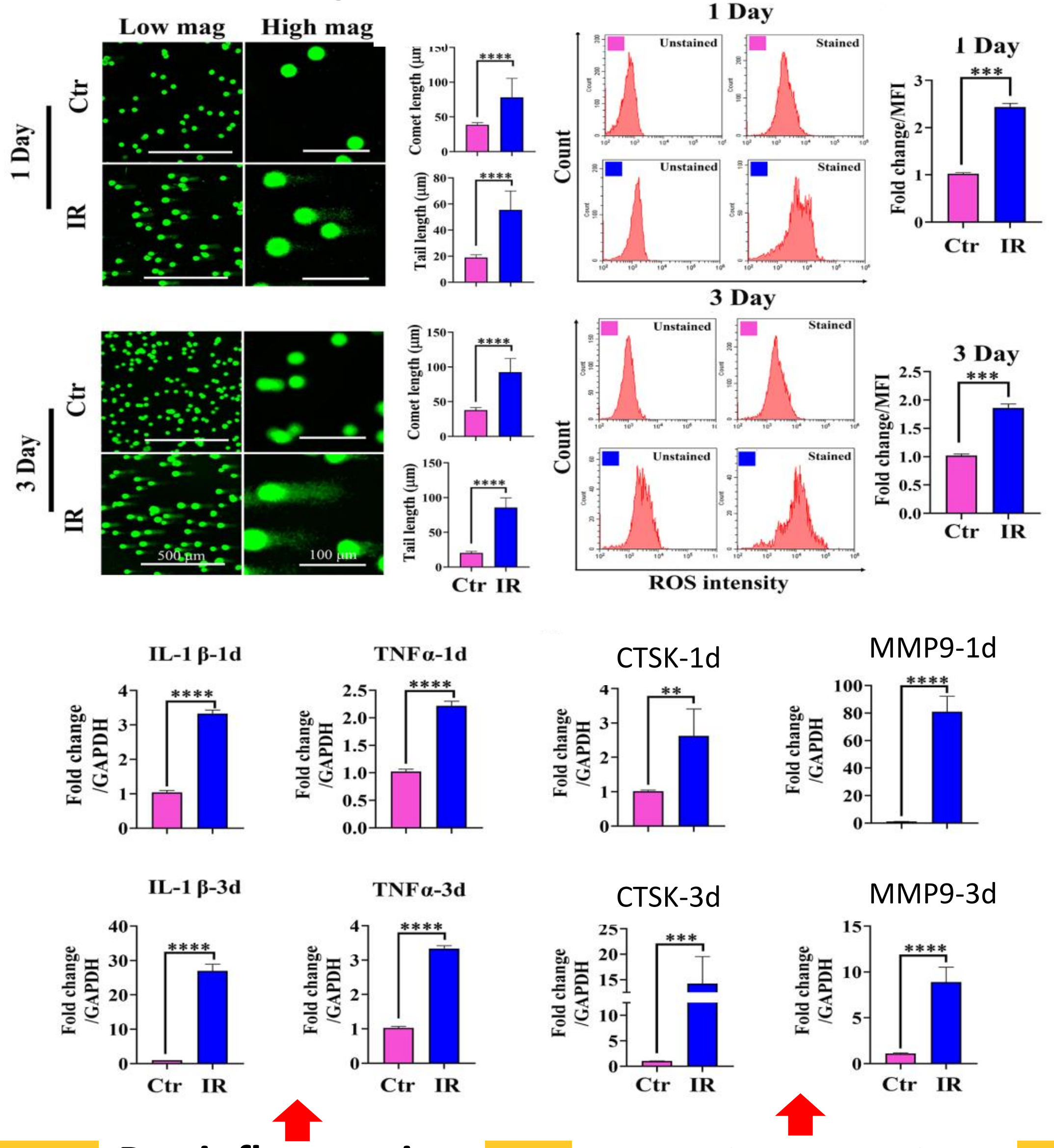


MSCs

Macrophages

DNA damage

Free Radicals

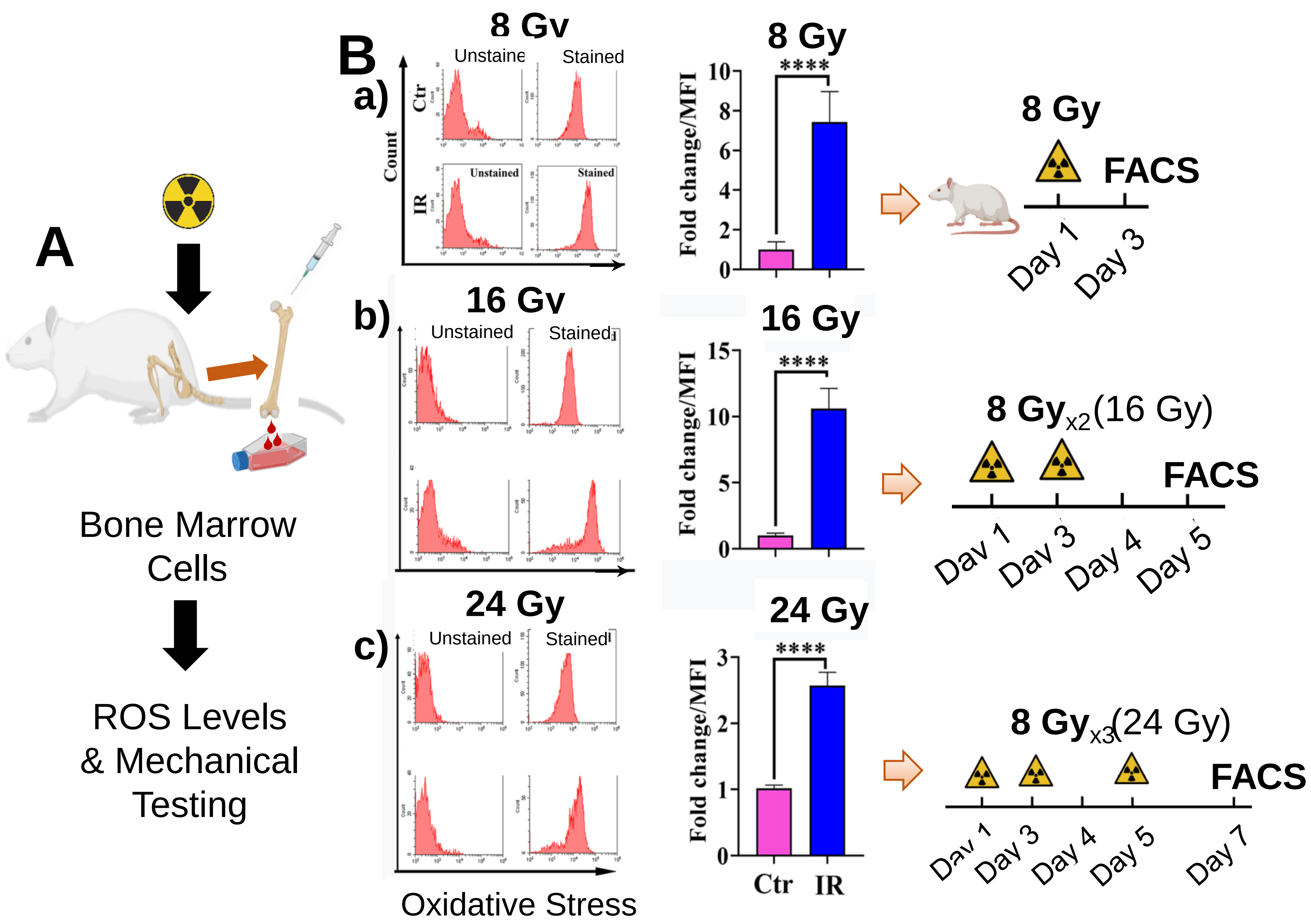


Pro-inflammation

Osteoclastic markers

$*p < 0.05$, $**p < 0.01$, $****p < 0.0001$

Effect of Incremental IR Doses on Bone



Ionizing radiation causes immediate ROS formation

$*p < 0.05$, $**p < 0.01$, $****p < 0.0001$

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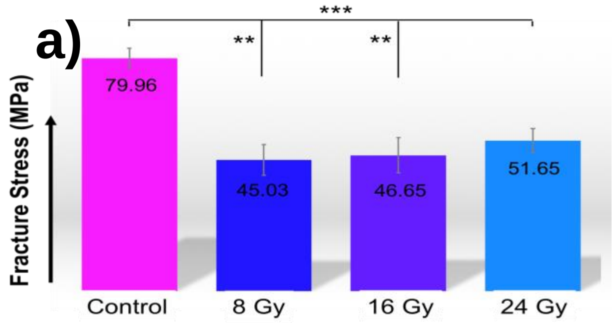
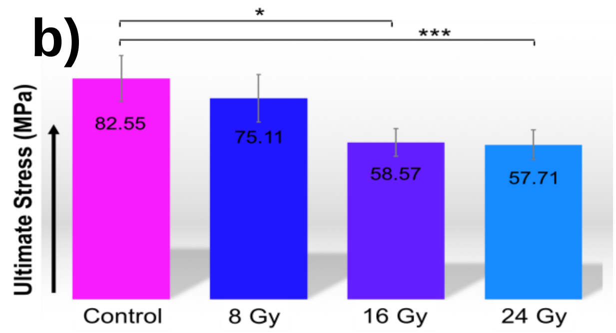
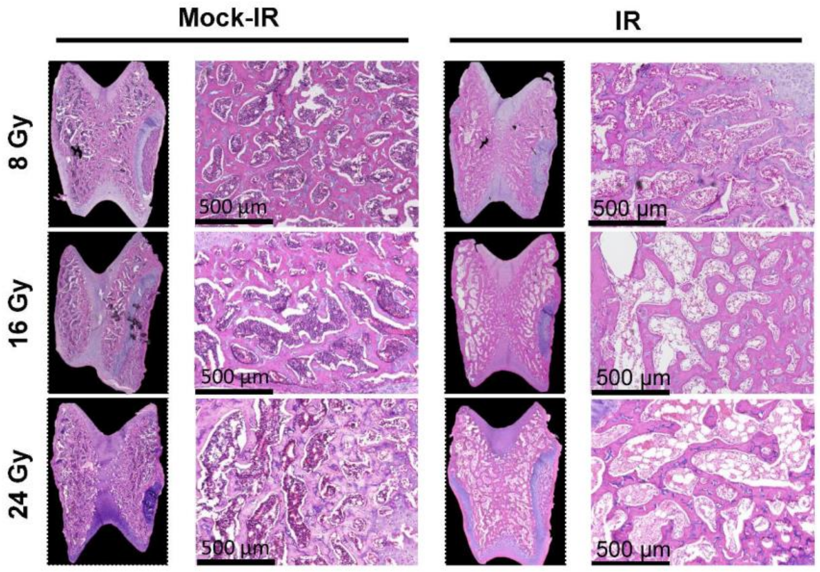


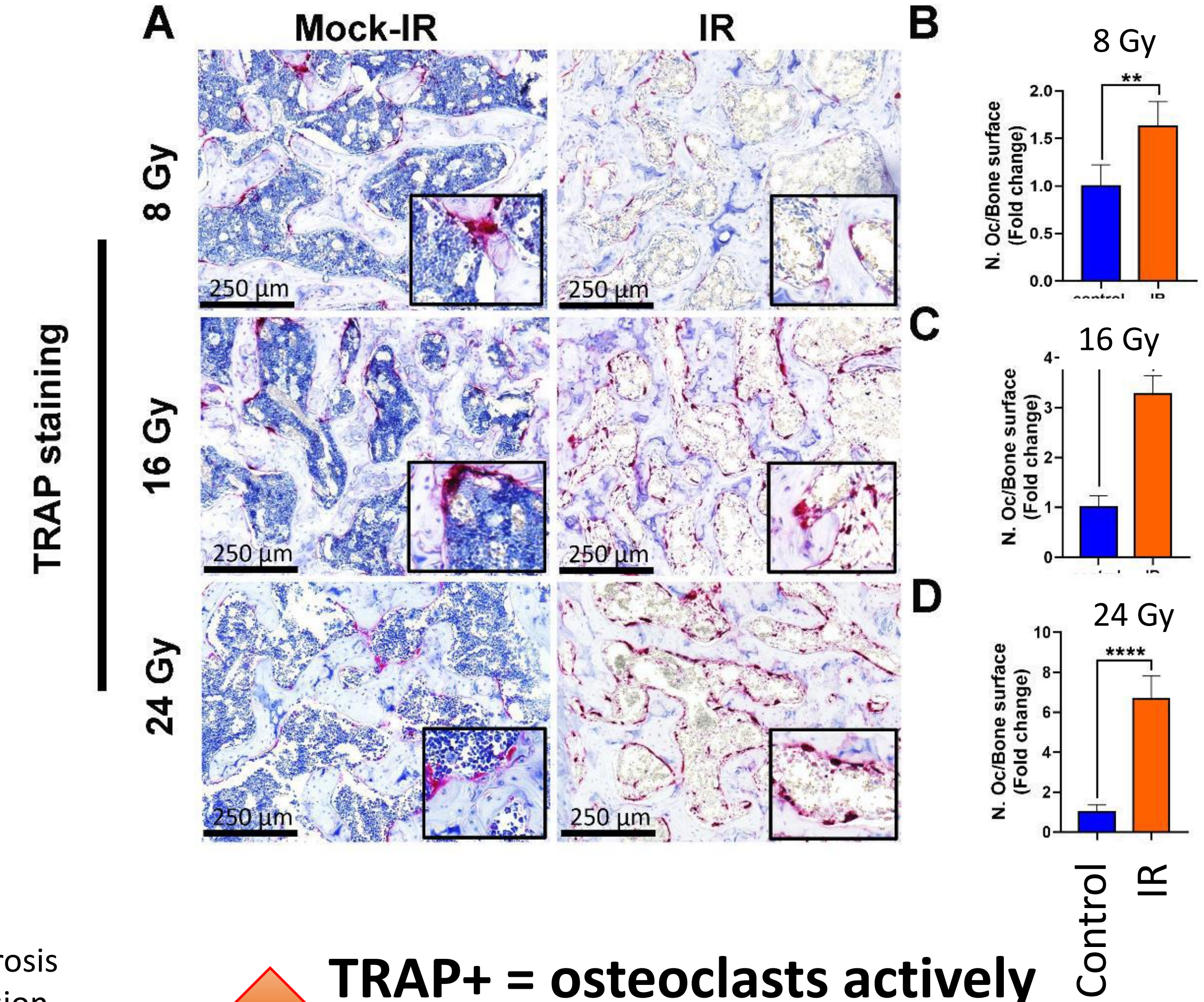
✓ Significant reduction in bone strength after first dose.





Osteoporosis progression with IR dose

Healthy Bone



TRAP+ = osteoclasts actively resorbing bone

Antioxidant Enzymes

- Cells counteract oxidative stress via radical scavenging by endogenous antioxidant systems *in situ* or by exogenous sources supplied through our diet

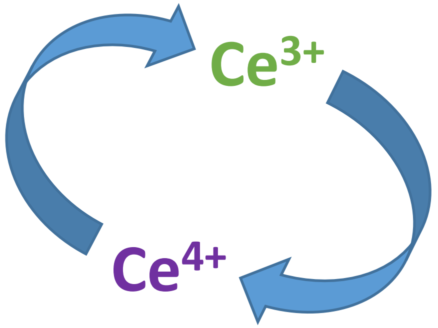
Antioxidants
- Endogenous
  - Enzymatic →
    - **Superoxide Dismutase**
    - **Catalase**
    - **Glutathione Peroxidase**
    - Peroxiredoxin
    - Thioredoxin
  - Non-Enzymatic →
    - Glutathione
    - Ubiquinol (CoQ10)
    - Uric Acid
    - Bilirubin
- Exogenous →
  - Vitamin A, Vitamin C, Vitamin E
  - Selenium, Polyphenols, Phytochemicals

✓ System becomes overwhelmed during oxidative stress
✓ Antioxidant biomaterials able to scavenge harmful free radicals and restore a healthy cellular redox balance are of growing interest

Cerium Oxide Nanoparticles (CeONPs)

- Rare earth metals are a promising strategic resource
- 4f orbitals imparts unique catalytic, magnetic and electronic properties that are not possible with transition and main group metals
- Cerium oxide nanoparticles (CeONPs) new generation of Nanozyme – "artificial enzyme"
- Mimics multiple endogenous antioxidants – able to scavenge almost all types of noxious reactive species
- Confirmed to outperform endogenous antioxidants

Ce^{3+} / Ce^{4+}
Autotransition



Cerium Oxide Nanoparticles (CeONPs) mimic activity of ***multiple*** endogenous antioxidant enzymes

Superoxide Dismutase + $0_2^{\bullet -}$ → H_2O_2

Ce^{3+} → Ce^{4+}

(Ce_2O_3) (CeO_2)

- ✗ Size
- ✗ Shape
- ✗ Surface area
- ✗ Valence state
- ✗ Bond lengths
- ✗ Zeta potential

Catalase
Glutathione peroxidase + H_2O_2 → H_2O + O_2

Ce^{4+} → Ce^{3+}

Free radical scavenging

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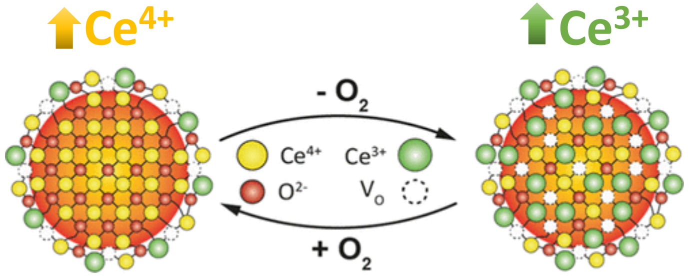
Q1: Will a nanozyme designed to target H_2O_2 relative to $O_2^{\bullet -}$ further increase the radioprotective effect of CeONPs to cells *in vitro*?

Q2: When administered into a rat model *in vivo*, is IR-induced DNA damage and subsequent bone loss prevented?



Study Aim

To investigate the radioprotective effectiveness of two nanozymes: designed for greater relative (i) CAT (Ce^{4+}) or (ii) SOD (Ce^{3+}) activity following irradiation-induced damage *in vitro* and *in vivo.*

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Study Hypotheses

H1: Pre-treatment of cells with CeONPs prior to IR, will protect primary human bone marrow derived stem cells (hBMSCs) and RAW 264.7 macrophages by targeted scavenging of H_2O_2 (and $OH^{\bullet}$) and will:

- Reduce DNA damage and senescence.
- Increase proliferation, osteogenic differentiation and bone mineral deposition of hBMSCs.
- Reduce inflammatory and osteoclastic marker expression in macrophages.

H2: Following IR-induced damage and when administered to rats, bone will maintain its volume, architecture and strength.

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Methods - CeONP synthesis & characterization

CeONP Synthesis

(1) Wet Chemical technique[6]: lower fraction of Ce^{4+} surface sites relative to Ce^{3+}
(2) Forced Hydrolysis[7]: higher fraction of Ce^{4+} surface sites relative to Ce^{3+}

CeONP characterization

- High-resolution transmission electron microscopy (HRTEM) (particle size)
- Dynamic Light Scattering (DLS) (hydrodynamic radius)
- Zeta sizer (surface charge)
- X-Ray photoelectron spectroscopy (XPS) (quantify Ce^{3+} & Ce^{4+} fractions)
- SOD ($O_2^{\bullet -}$) and Catalase (H_2O_2) assays
- Electron paramagnetic resonance (specificity of Ce^{3+} & Ce^{4+} to $O_2^{\bullet -}$)
- Density Functional Theory (specificity of Ce^{3+} & Ce^{4+} to H_2O_2)

CeONP Cellular Uptake

- FITC-labelled CeONPs: imaged using confocal microscopy at 24h
- Quantified cellular internalization using flow cytometry

→ In vitro & In vivo Analyses

[6]Hirst et al. Small. 2009 Dec 18;5(24)
[7]Das et al. Biomaterials. 2012 Nov;33(31)

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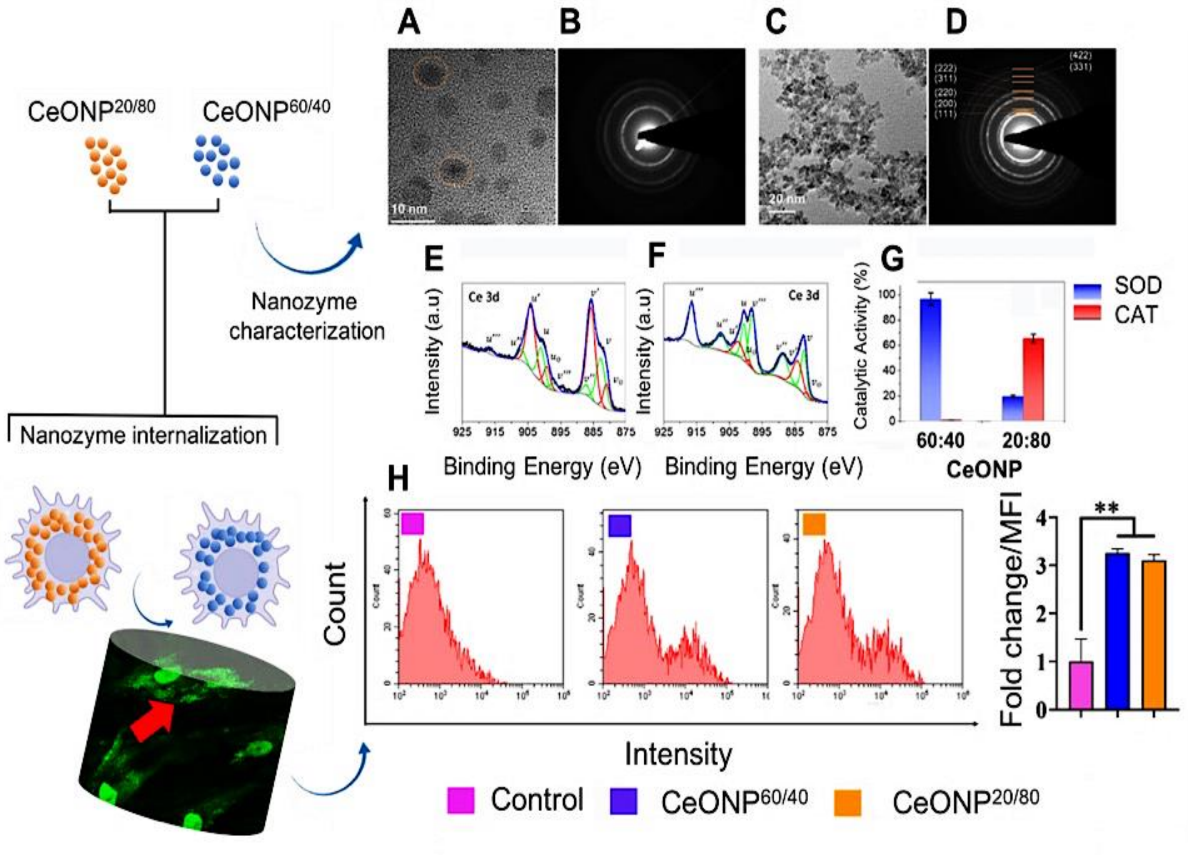
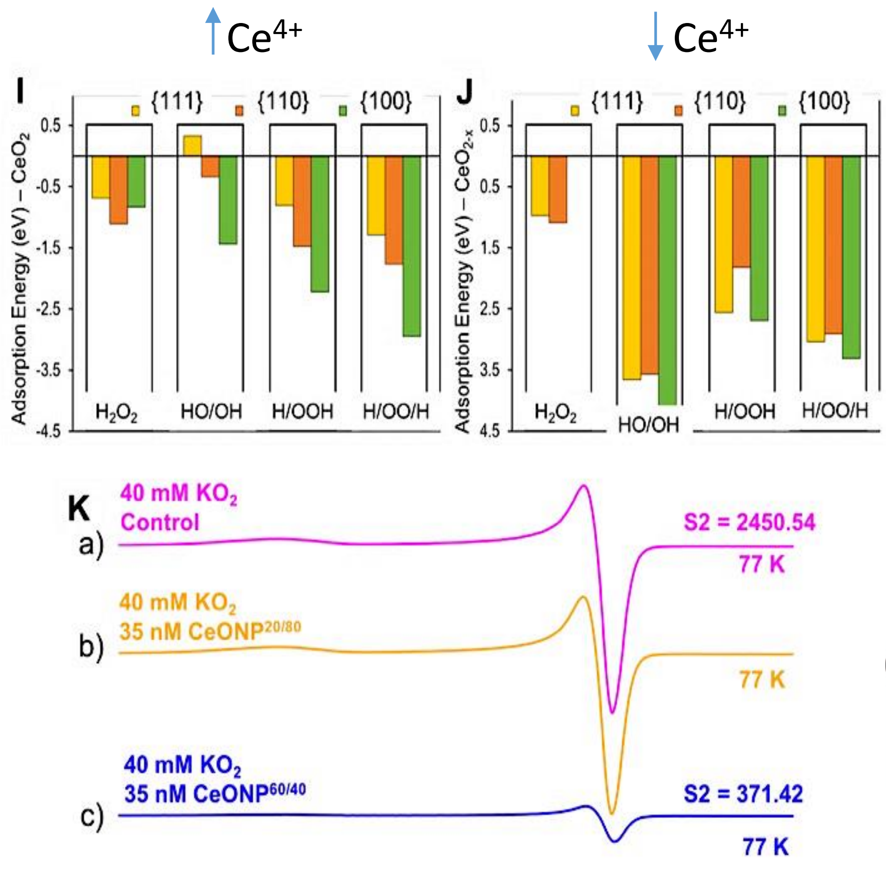
Nanoparticle characterization





Spherical NPs

✓ EPR: Ce^{3+} surface sites selectively neutralize $O_2^{\bullet-}$

✓ ↑ Ce^{3+} increases ROS interaction with surfaces

✓ ↑ Ce^{3+} has greater scavenging activity than Ce^{4+}

✓ $OH^{\bullet}$ (HO/OH) only form on CeO_2 surfaces but are scavenged on CeO_{2-x} surfaces Through $Ce^{3+} \rightarrow Ce^{4+}$

In vitro Methods – *in vitro* analyses of $CeONP^{60/40}$ and $CeONP^{20/80}$

(↑Ce^{3+}) (↑Ce^{4+})

- Material characterization (HRTEM, XPS, etc)
- DNA damage to hBMSCs and RAW 264.7 macrophages (7 Gy)
  - ✓ Alkaline Comet Assay®, 3d post-IR
- Intracellular ROS generation in hBMSCs (7 Gy)
  - ✓ Cellular ROS Assay Kit counter-stained with MitoSpy®, 24h post-IR
- Intracellular $O_2^{\bullet -}$ levels (7 Gy)
  - ✓ MitoSOX® Red mitochondrial superoxide indicator kit
- Gene expression (qRT-PCR), and protein release (ELISA) (7 Gy)
  - ✓ CAT, SOD and GPX in hBMSCs, 24h post-IR
  - ✓ Pro-inflammatory cytokine expression (IL-1β and IL-6) in macrophages
  - ✓ Bone-resorbing osteoclastic differentiation markers (RANKL and CTSK) in macrophages
- IR-induced cellular senescence (7 Gy)
  - ✓ *β*-galactosidase (*β*-gal) staining kit, hBMSCs, 28-days post-IR
- Bone forming osteogenic differentiation in hBMSCs and bone mineral deposition (7 Gy)
  - ✓ Alizarin Red assay, 28 days post-IR

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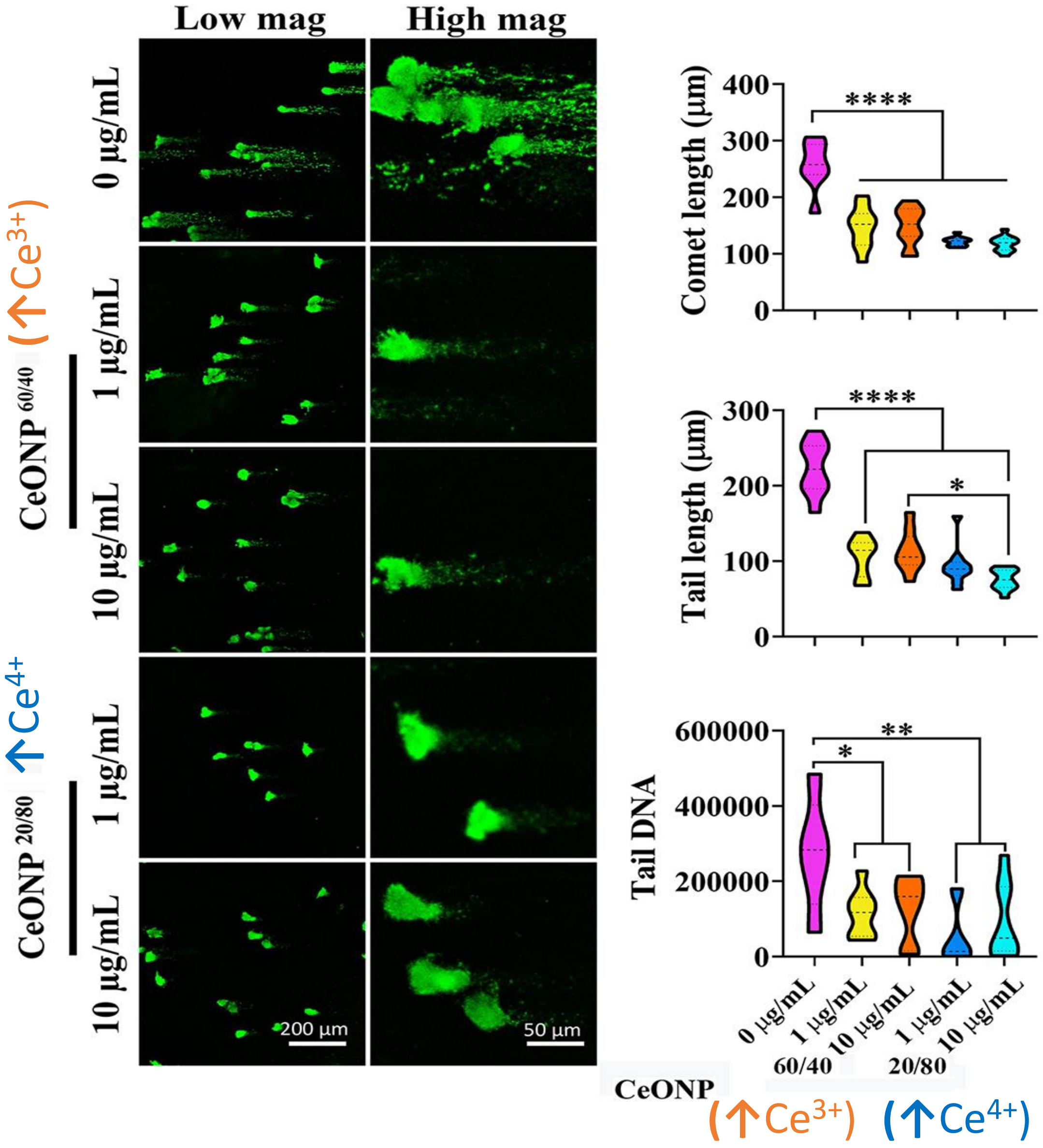
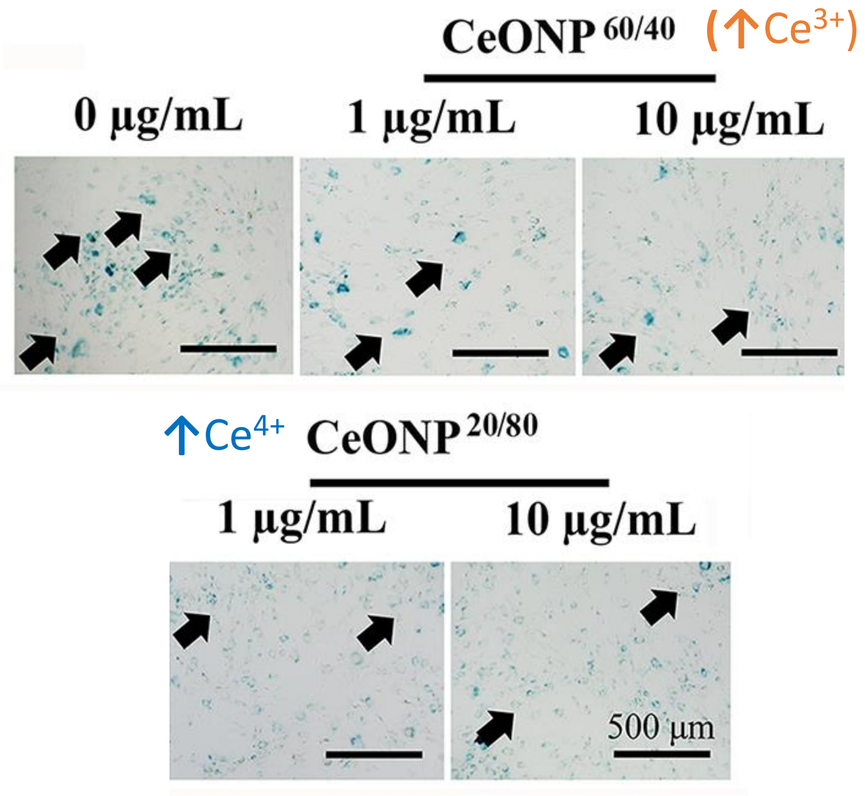
CeONP60/40 & CeONP20/80 reduces IR-induced DNA damage and cell senescence in hBMSCs

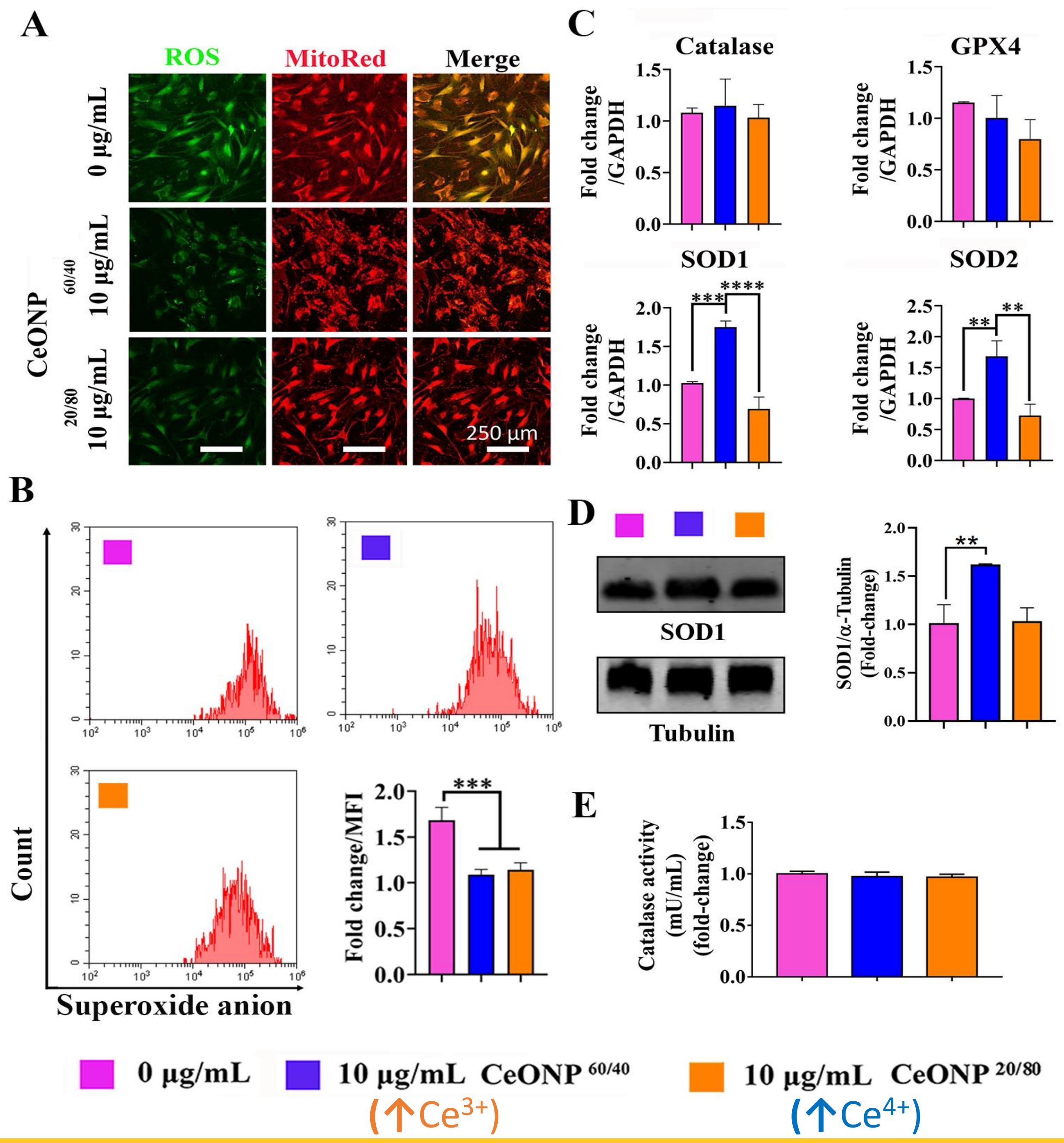




Cellular senescence

$*p < 0.05$, $**p < 0.01$, $****p < 0.0001$

CeONP$^{60/40}$ and CeONP$^{20/80}$ reduce radiation-induced intracellular ROS/superoxide anion generation



✓ ↑Ce^{3+} increases *SOD* but not *Catalase* or *GPX* gene expression in hBMSCs

✓ Both formulations scavenge $O_2^{\bullet-}$ to a similar degree

*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$

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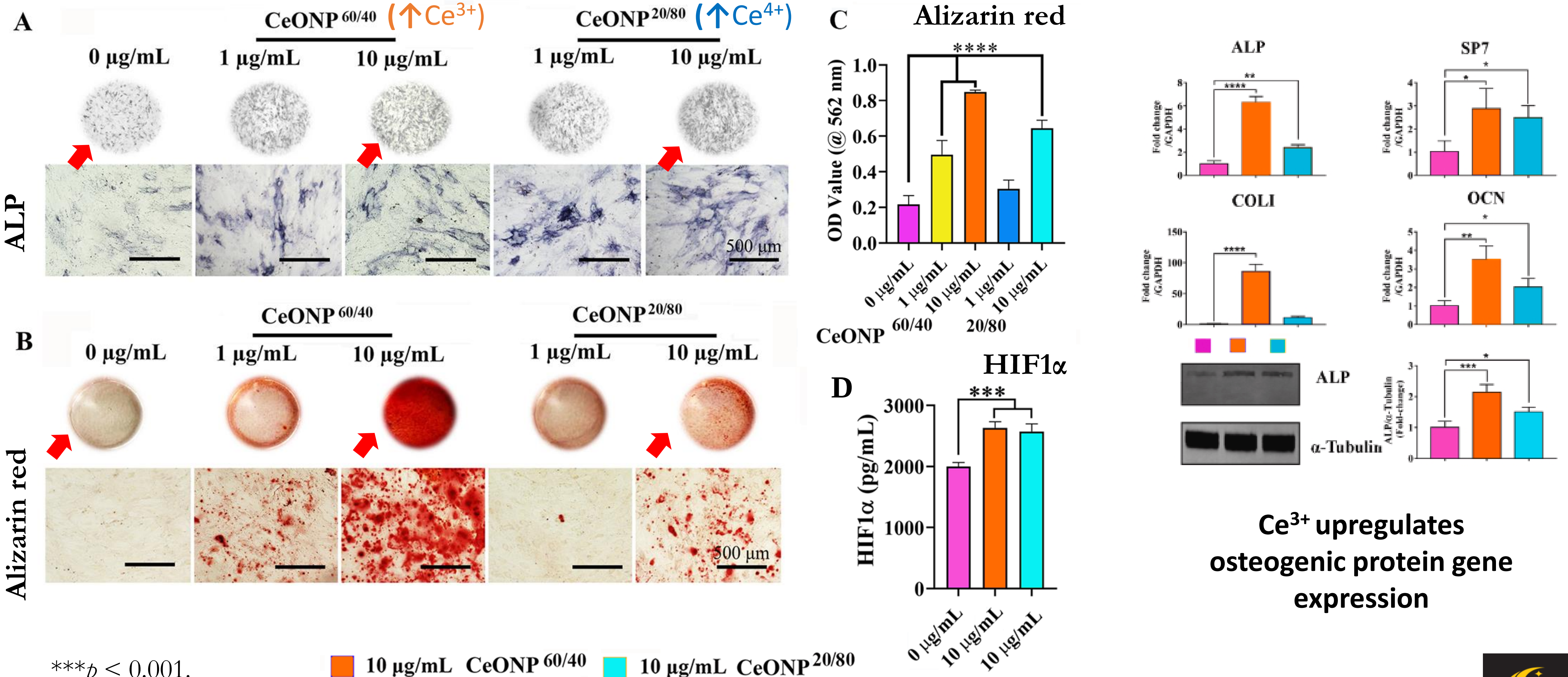
Both CeONP$^{60/40}$ and CeONP$^{20/80}$ liberates osteoblastogenesis following irradiation, but ↑Ce^{3+} promotes a greater response

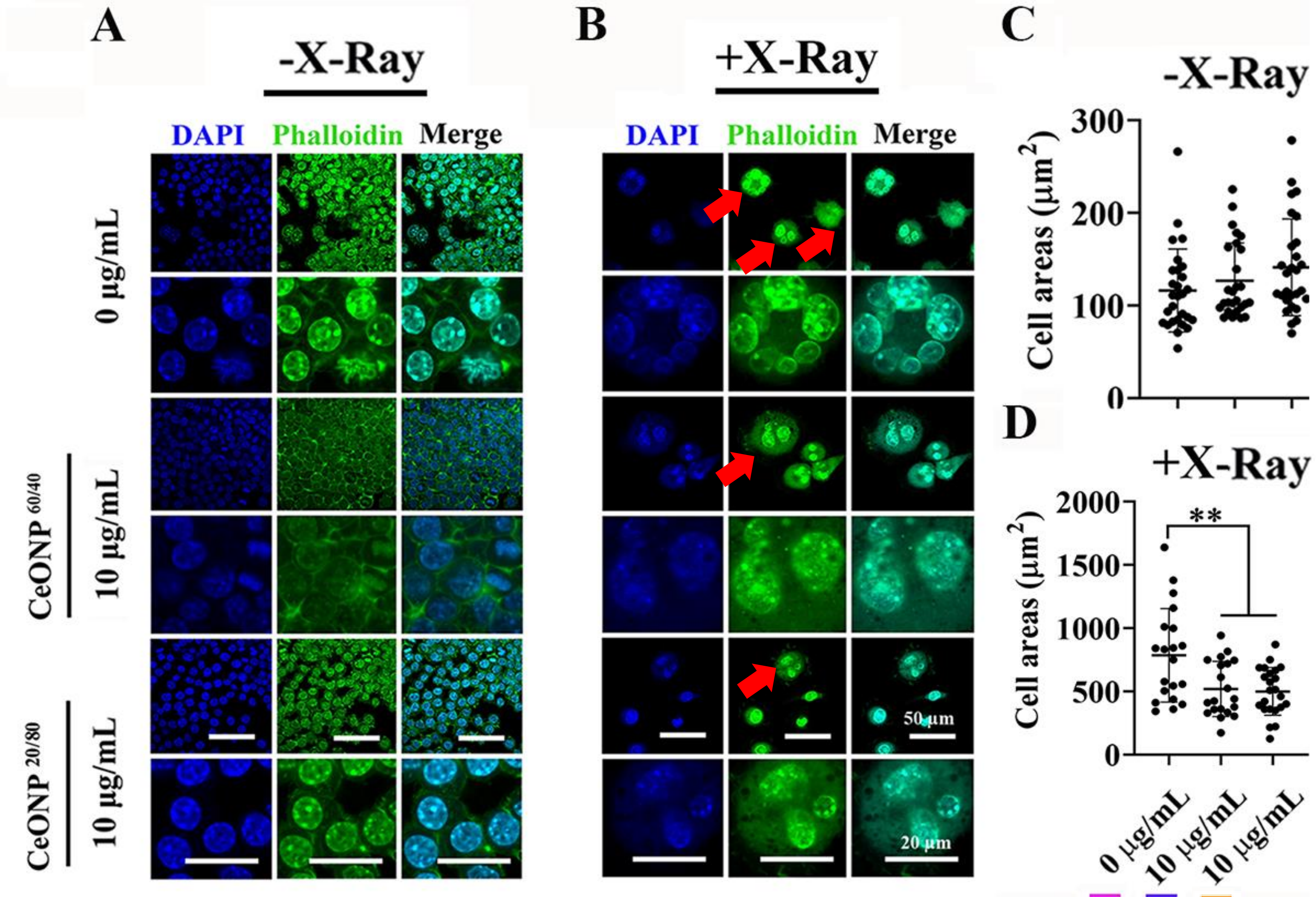


Ce^{3+} upregulates osteogenic protein gene expression

***$p < 0.001$.

10 µg/mL CeONP 60/40 (↑Ce^{3+})   10 µg/mL CeONP 20/80 (↑Ce^{4+})

CeONP$^{60/40}$ and CeONP$^{20/80}$ repress osteoclast-like giant cell formation following irradiation-induced cell damage to the macrophage



Phalloidin and DAPI staining

**$p < 0.01$.

Control | CeONP 60/40 | CeONP 20/80

(↑Ce^{3+}) (↑Ce^{4+})

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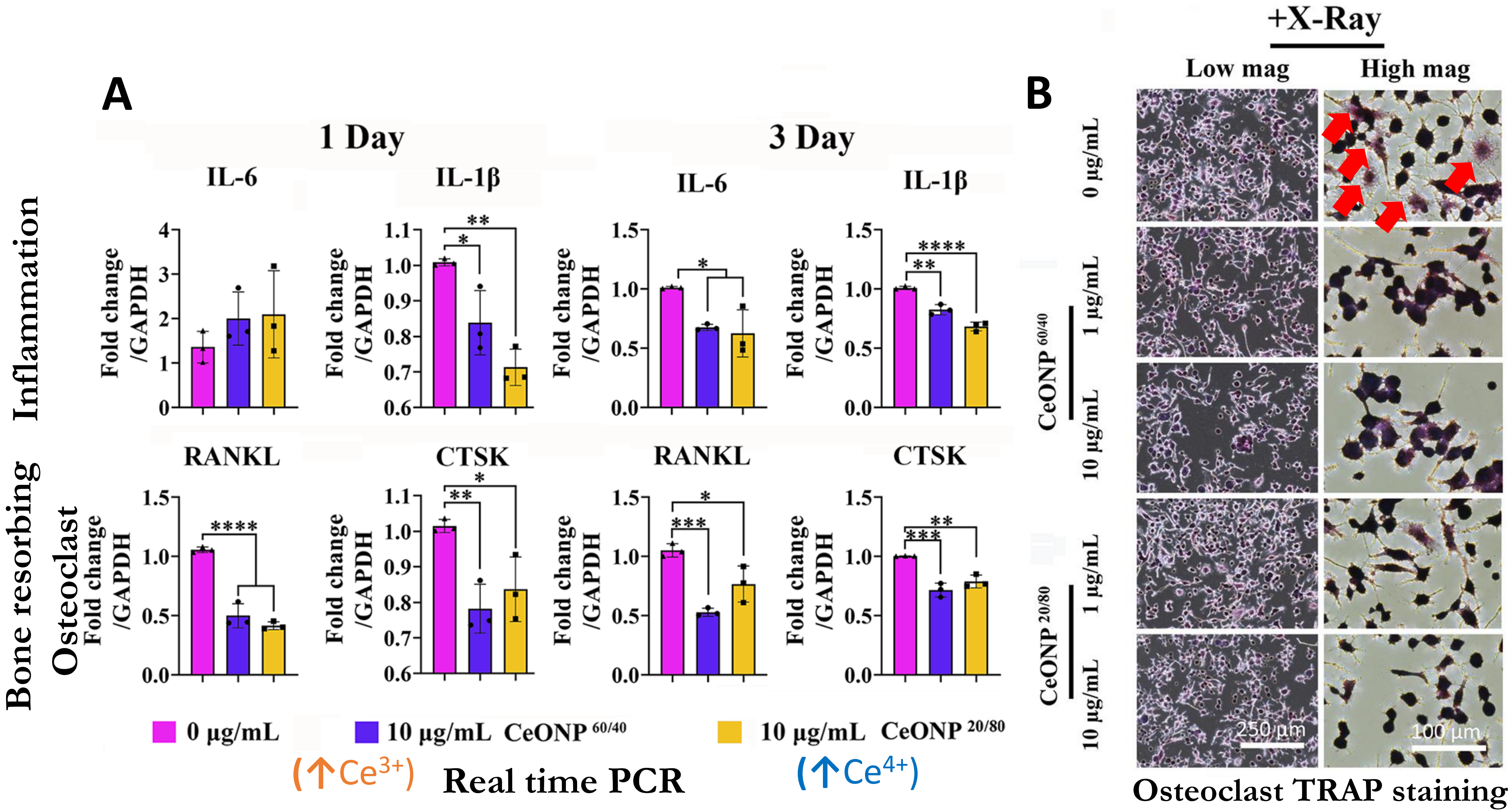
$CeONP^{60/40}$ and $CeONP^{20/80}$ repress inflammation and osteoclast markers following irradiation-induced cell damage to the macrophage



*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

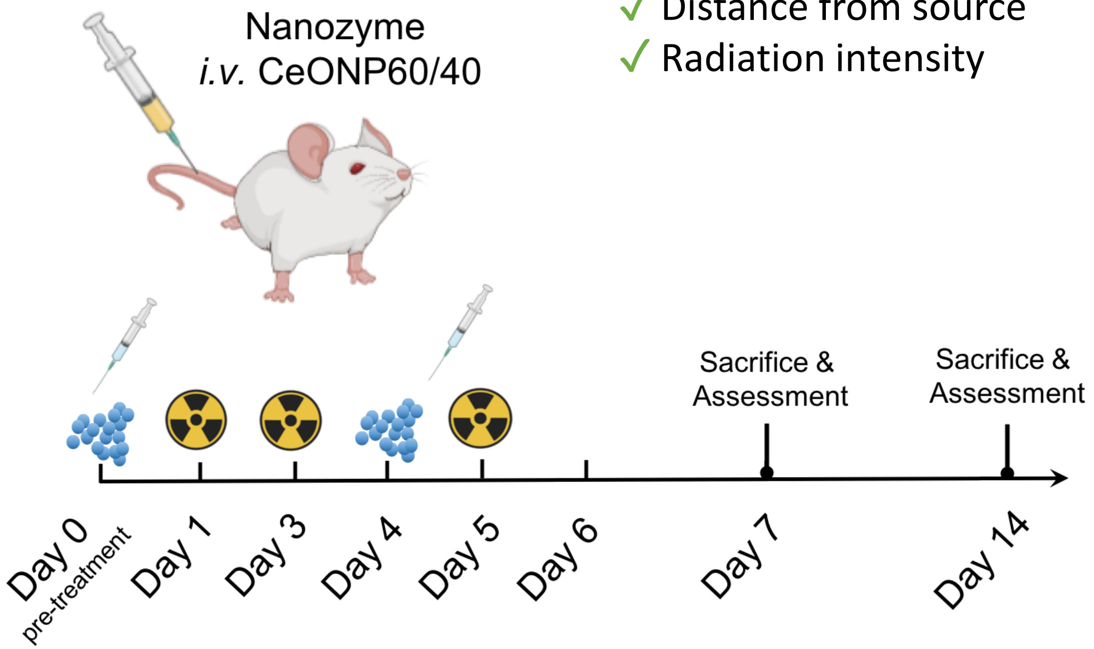
In vivo studies and analysis of ↑Ce^{3+}-CeONP in 9-week-old SAS Sprague-Dawley rats and following IR-induced tissue damage

Experimental groups (*n*=6/group; 48 rats in total, two-time points):

1) Control
2) Control + CeONPs
3) X-ray only
4) X-ray + CeONPs

✓ Histological analysis of kidney, spleen and liver.
✓ Complete blood count / blood chemistry.
✓ DNA damage (cells in bone marrow niche).
✓ Immunohistochemistry (RANKL, senescence).
✓ TRAP staining (osteoclastic activity).
✓ MicroCT.
✓ 3-point bending (fracture stress, ultimate stress).

Kimtron biological irradiator
Lead shielding
✓ Dose rate
✓ Distance from source
✓ Radiation intensity



8 Gy (total 24 Gy)

In rats 7 Gy/day for 5 days (35 Gy) - human equivalent of 70 Gy.
Hypofractionated total dose – human equivalent of 48 Gy

Results: *In vivo*

✓ Significantly reduced DNA damage to cells within the bone marrow niche.

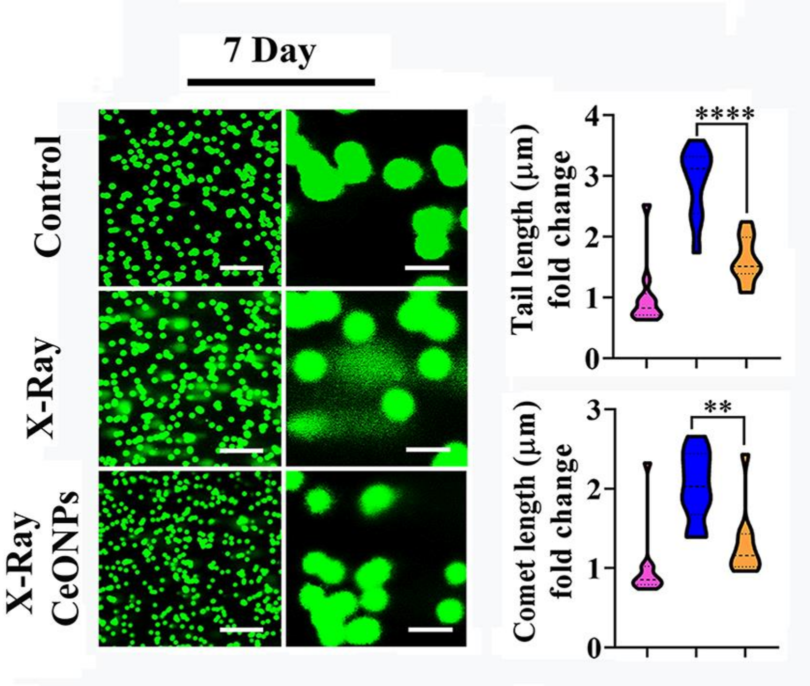
✓ Significantly reduced RANKL from cells within bone.

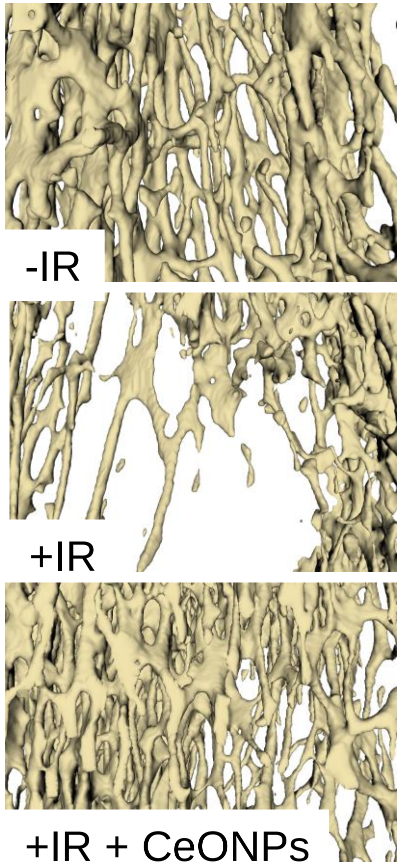
✓ Significantly reduced osteoclastic activity and CTX-1.

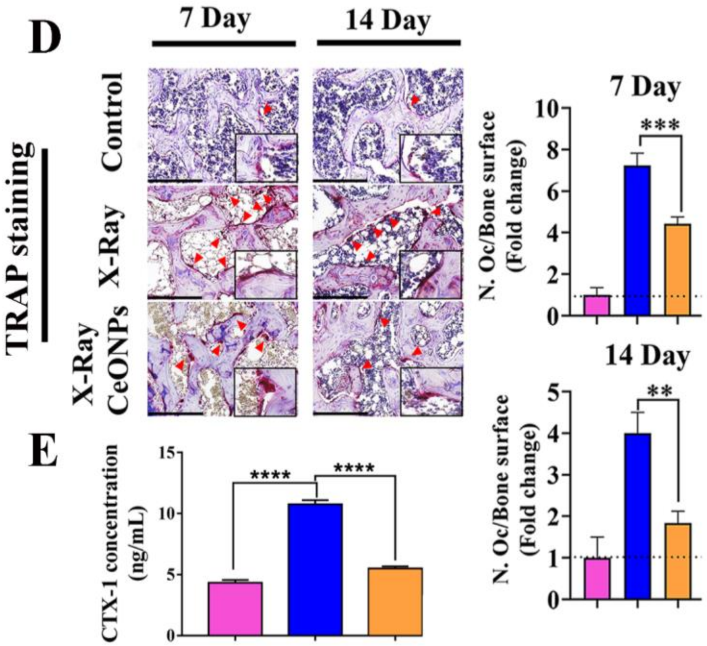
✓ Reduced cell senescence.

✓ Preservation of microarchitecture.

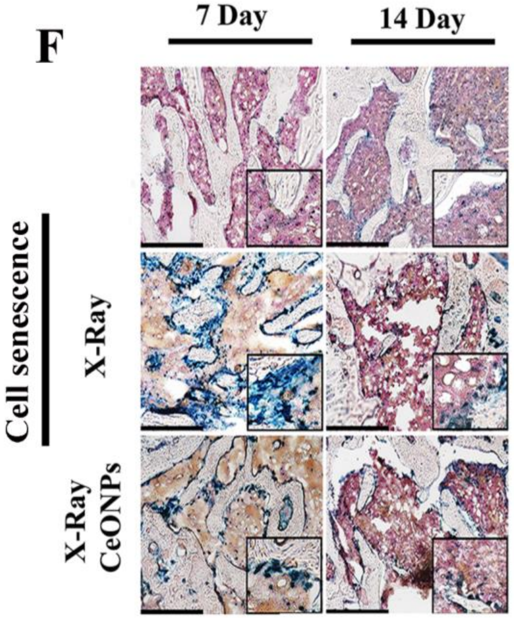
✓ Significant increase in fracture stress and ultimate stress.





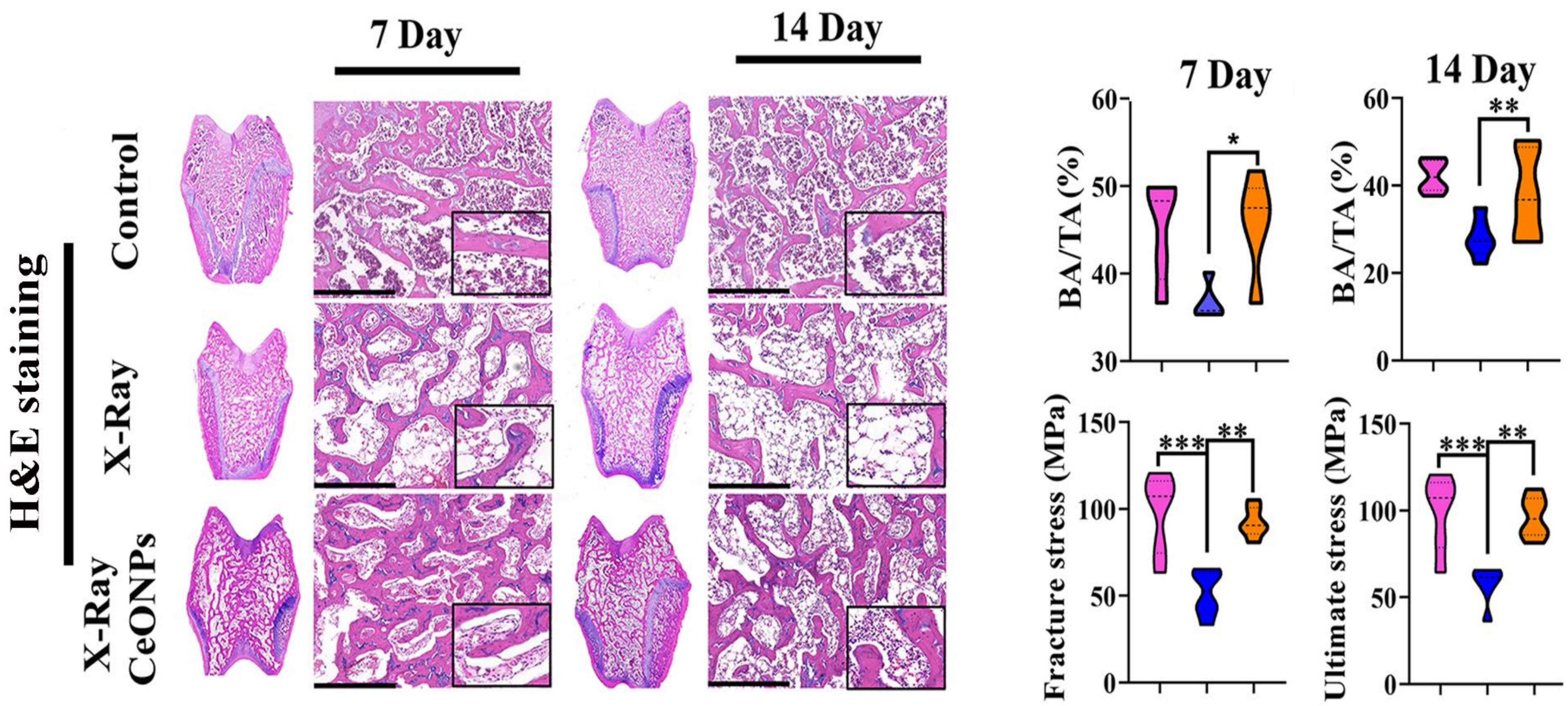


Collagen Release



Cell Senescence

**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$



Bone Area/Total Area

✓ **CeONPs maintained bone strength despite exposure to harmful IR.**

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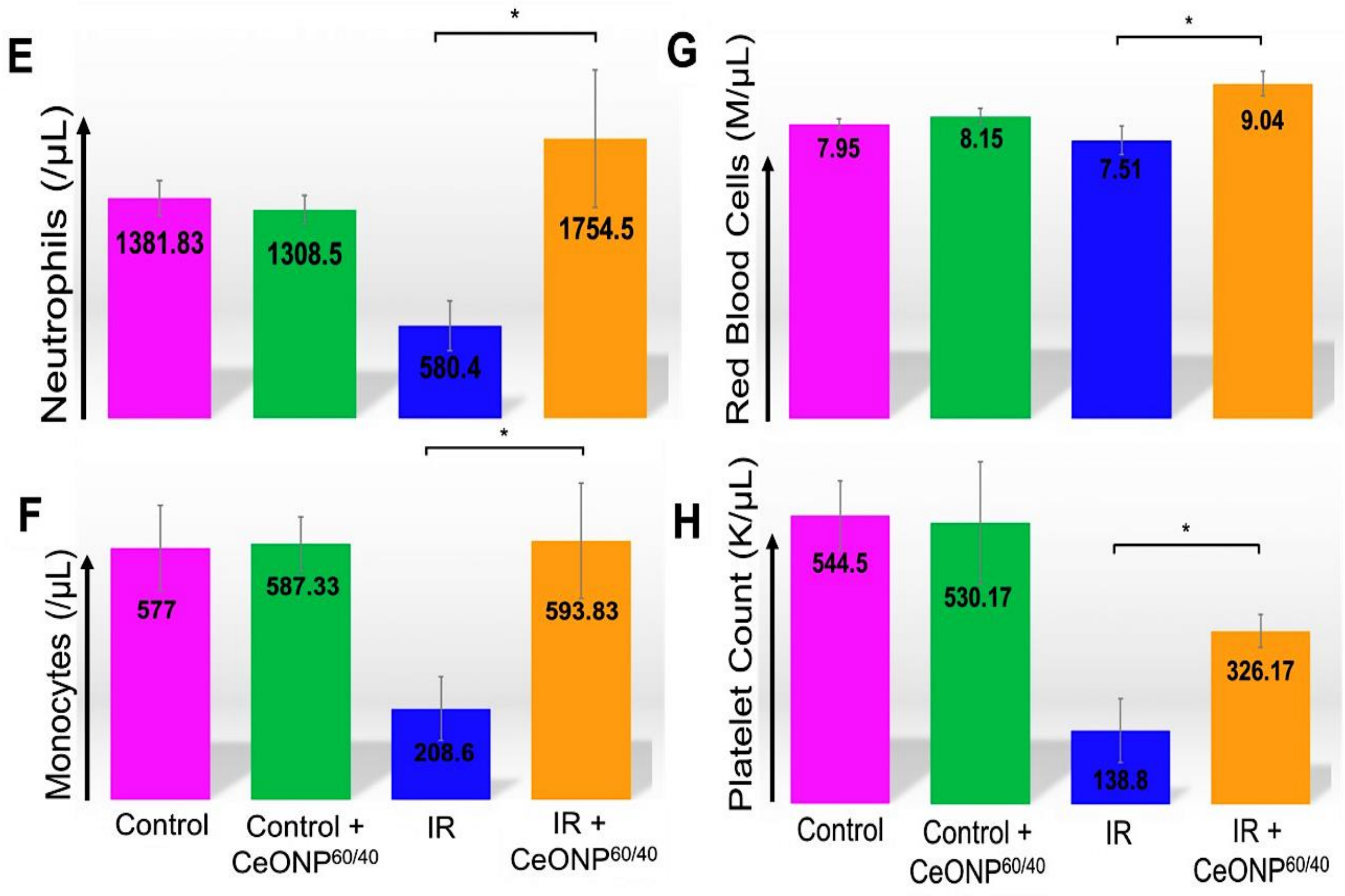
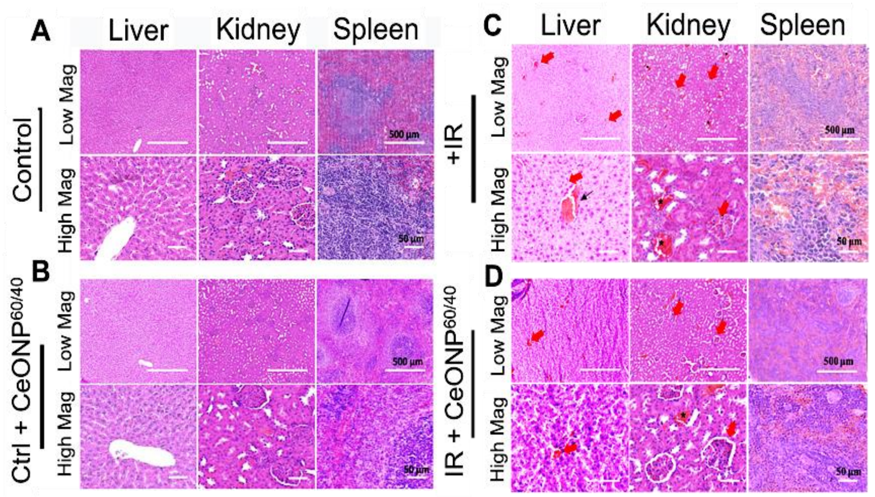
CeONP$^{60/40}$ showed no damage to healthy tissue and protected organs from IR damage

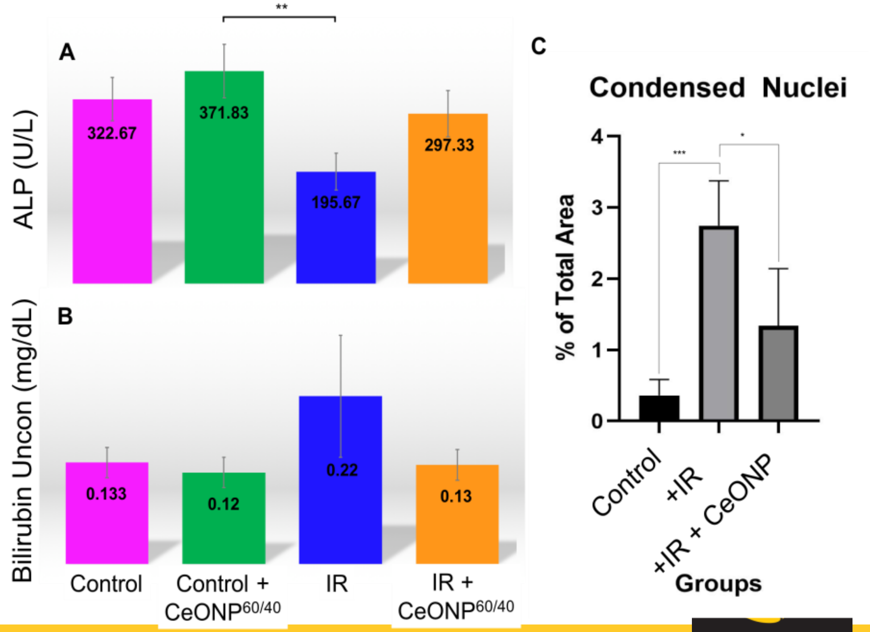


Key cells in the blood

Histological analysis



Liver damage



*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

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Conclusions

- CeONPs were well-tolerated and exhibited a multifunctional protective effect against ionizing radiation-induced damage while augmenting osteogenesis, reducing osteoclastic activity and preventing bone loss
- *Longer term studies are needed*

CeONPs hold promise as a novel multifunctional therapeutic strategy for irradiation-induced bone loss

To the best of our knowledge, this is ***first evidence*** of CeONPs role in:

✓ Bone regeneration + IR
✓ The critical role of increased Ce^{3+} surface sites
✓ *Potentially* non-redox related enhanced bone formation

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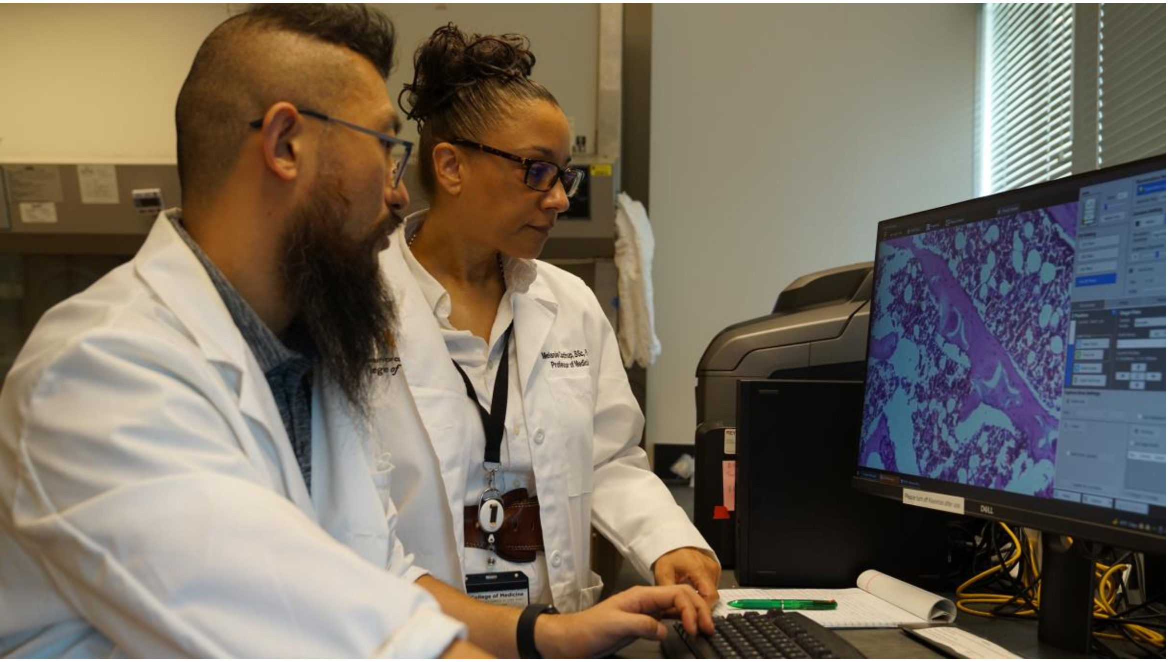
thank you

Dr. Fei Wei
Dr. Abinaya Pugazhendhi
Dr. Mahmoud Omer
Christopher Ngo
Raven Pascua



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