

Gamma-Irradiated *Trichoderma harzianum* Mutants: Safer for human and animals and More Effective Biocontrol against Root-Knot Nematodes

Running title: Gamma-Irradiated *Trichoderma harzianum* is safer for human and animals

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ABSTRACT

Introduction: Mutant microorganisms offer effective approach for enhancing the efficacy and safety of biological control.

Materials & Methods: *Trichoderma harzianum* isolate NAS120 was exposed to gamma irradiation for generating the mutant strain NAS120-M44 with improved antagonistic activity against the root-knot nematode *Meloidogyne javanica*.

Twenty Wistar rats were divided as follow: Four animals as control received gavage of distilled water. Four Animals as S-A received bio-formulation without spores, (sucrose, starch, Arabic gum and Kaolin). Six animals as TC received 0.20% per test of washed spores of *Trichoderma* plus bioformulation, six animals as TM received 0.2% per test of washed spores of mutant *Trichoderma* plus bioformulation for three weeks. Washed spore suspensions were prepared from seven-day-old slant cultures using sterile saline solution. Suspensions were irradiated in a cobalt-60 γ -irradiator at a dose rate of 0.18 Gy s⁻¹. Tissue sampling were done from liver, kidney and intestine.

Results: Molecular identification of the wild-type and mutant isolates were confirmed by amplification and sequencing by MEGA software. Mutant isolate was more powerful against the nematode.

Histopathological evaluation indicated minimal adverse effects on liver, kidney, and intestine in rats fed with mutant strains. Changes in wild strain were as follow: fatty change in liver and scattered necrosis in kidney, while the intestine was normal. Animals treated with the mutant isolate (TM) showed no pathological alterations in the liver and intestine. Renal lesions were still evident.

Conclusion: Gamma-irradiated *T. harzianum* mutants can be a better biocontrol agent, providing a sustainable alternative to chemical nematicides in crop protection.

Keywords: *Meloidogyne javanica*, Mutant strain, Rat pathology, Safety assessment, *Trichoderma*.

1. Introduction

Advances in genetic engineering caused making of new strains of microorganisms with good capabilities of biocontrol and improved traits specifically tailored to control plant pathogens, including nematodes [1, 2].

Exposure of *Trichoderma* and *Bacillus* strains to gamma radiation enhances their biocontrol potential while maintaining stability and safety within agricultural ecosystems [3, 4].

We wanted to compare the mutant strain of *Trichoderma harzianum* (*T. harzianum*) pathologic effects on kidney, Liver and intestine of the rats with wild strain beside assessment of their efficacy in reducing nematode infestation and to determine optimal application methods by in vitro assays and greenhouse experiments, and molecular identification techniques. this research aims to demonstrate the potential of these mutant strains instead of nematicides.

2. Materials and Methods

2.1. Fungal isolate and culture conditions

The *Trichoderma harzianum* isolate NAS120 was obtained from the Plant Protection Collection of the Nuclear Agriculture Research School, Nuclear Science and Technology Research Institute (NSTRI), Alborz, Iran [5].

NAS120 was cultured in 250 mL flasks containing Potato Dextrose Broth (PDB) and incubated for one week at 28 ± 2 °C under continuous shaking [6].

2.2. Gamma irradiation and mutant induction

Washed spore suspensions were prepared from seven-day-old slant cultures using sterile saline solution (9 g L⁻¹ NaCl in distilled water). Suspensions were irradiated in a cobalt-60 γ -irradiator at a dose rate of 0.18 Gy s⁻¹ at NSTRI, Alborz, Iran. A dose of 250 Gy was applied in triplicate. The optimal irradiation dose for mutation induction was selected based on approximately 40–50% inhibition of spore germination on PDA medium (Soufi et al., 2021).

2.3. Effect of *T. harzianum* NAS120 on Egg Hatching and Larval Mortality of Root-Knot Nematode

To evaluate the effect of *T. harzianum* NAS120 on egg hatching and larval mortality of the root-knot nematode (*Meloidogyne javanica*), 100 eggs or 100 second-stage juveniles (J2) were separately placed in 6 cm Petri dishes containing 1 mL of sterile water. Five milliliters of fungal suspension (1 × 10⁷ CFU mL⁻¹) or cell-free culture were added to each dish. Distilled water served as the control. Larval mortality controlled after 48 h, and unhatched eggs were counted after 72 h. Experiments had got four replications [1].

For the greenhouse assay, tomato seeds (cv. Early Urbana) were sown in 3 kg plastic pots containing a soil: sand mixture (1:2, v/v). At the four-leaf stage, seedlings were inoculated with 20 mL of fungal suspension or other treatments. Three days later, each plant received 6000 *M. javanica* eggs received no treatment.

Sixty days after nematode inoculation, plant growth parameters and nematode indices were recorded. Among the biocontrol agents tested, *T. harzianum* NAS120 significantly reduced nematode reproduction, with efficacy comparable to the chemical nematicide abamectin. This indicates that the metabolites produced by this isolate effectively control nematode damage. The mutant isolate NAS120-M44 was selected based on enhanced antagonistic activity against *M. javanica* compared with the wild-type isolate [1].

2.4. Molecular Identification of *Trichoderma*

2.4.1. DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from 100 mg of lyophilized mycelium of both parental and mutant isolates following Soufi et al. [7]. DNA quality and concentration were assessed using spectrophotometry and 1% agarose gel electrophoresis.

Table 1. The bio-material code in experiments in treatments on Mouse.

Bio-material	The bio-material code in experiments	Isolate code in NCBI	Isolate code in NSTRI
(wild-type) <i>Trichoderma harzianum</i>	T Group Tc, group S	MW719878.1	NAS120
(Mutant) <i>Trichoderma harzianum</i>	M group TM	PP316640.1	NAS120-M44
(Control) Water	C	-	-

The ITS-rDNA (internal transcribed spacer) and TEF-1 α (translation elongation factor 1 α) regions were amplified for molecular identification. PCR reactions (20 μ L) contained 0.2 U/ μ L Taq DNA polymerase, 1.5 mM MgCl₂, 0.2 μ M of each primer, and 10 ng genomic DNA. Primer sequences were as follows:

- ITS: ITS1 5'-CGTAGGTGAACCTGCGG-3',
- ITS4 5'-TCCTCCGCTTATTGATATGC-3'
- TEF-1 α : EF1-728F 5'-CATCGAGAAGTTTCGAGAAGG-3',
- EF1-986R 5'-TACTTGAAGGAACCCTTACC-3'

PCR conditions: initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 15 s, annealing at 55 °C (ITS) or 50 °C (TEF-1 α) for 20 s, and extension at 72 °C for 1 min; final extension at 72 °C for 5 min (Shahbazi et al., 2025). (Shahbazi et al., 2025). PCR products were visualized on 1.2% agarose gel and sequenced.

2.4.2. Sequence alignment and phylogenetic analysis

This was done using MEGA software. Evolutionary relationships were inferred using the Neighbor-Joining method with 1000 bootstrap replicates. Branch lengths were drawn to scale, representing base substitutions number per site. Evolutionary distances were computed using the Maximum Composite Likelihood method. The analysis included 23 nucleotide sequences with a total of 2515 positions; ambiguous positions were removed using pairwise deletion. Phylogenetic analyses were conducted in MEGA software [6].

2.4. Spore preparation for experiments

All isolates (*T. harzianum* NAS120 and NAS120-M44) were cultured in sterile PDB at 28 °C for 72 h. Mycelial masses were incubated at the same temperature for 7 days in PDA. Spores were harvested with the usage sterile saline solution, and concentrations were adjusted to 1×10^6 spores mL⁻¹ using a Neubauer hemocytometer. Spores were pelleted by centrifugation at 4500×g for 10 min and washed twice with sterile saline solution [4].

Table 2. The components of bio-material (mix of Wild and Mutant Isolates of *Trichoderma*) used for treatments

Components	Bioformulation	Effective material (bio material)
<i>Trichoderma</i> spore mix (pure)	0.2%	2%
Sucrose	4.8%	48%
Starch	4.8%	48%

Arabic gum	0.2%	2%
(Al ₂ Si ₂ O ₅) Kaolin	90%	-

2.6. Histopathology (Acute oral pathogenicity)

Twenty healthy Wistar albino rats (ten of each sex, 180 - 200 g weight and 10 weeks old) were obtained. The groups were as follow: Control: four animals (two of each sex) received gavage of distilled water. Four Animals as S-A received bio-formulation without spores, (sucrose, starch, Arabic gum and Kaolin) with distilled water with gavage. Six animals (equal number from both sex) as TC group received 0.20% (1.00×10^6 CFU) per test of washed spores of *Trichoderma* with other ingredients of bioformulation with gavage. Six animals (equal number from both sex) as TM received 1.00×10^6 CFU per test of washed spores of mutant *Trichoderma* with other ingredients of bioformulation with gavage. Volume of suspension was 2.00 mL per 100 g body weight. The animals had no access to food overnight before the test and after administration of the substance they were fasted for 4hrs. The animals were under control for 21 days and then were euthanized by decapitation under deep anesthesia [1]. Tissue specimens (kidney, liver and small intestine) were collected from them. The specimens were put in to neutral 10% buffered formalin (Dr Mojallali, Iran). After one week, the tissues processed with automatic tissue processor (Did Sabz, Iran) and were cut to 5 micrometer slices using a rotary microtome (Did Sabz, Iran). Built microscopic slides were stained with hematoxylin and eosin (Sigma-Aldrich, USA) and studied with a light microscope (A.Crusser ML2100, Germany)." The United States environmental protection agency preventing pesticide and toxic substances (7101) EPA712-c-96-315 February 1996. Microbial pesticide test guidelines OPPTS885-3050- Acute Oral Toxicity /pathogenicity" was followed.

3. Results

3.1. Effect of *Trichoderma harzianum* on *Meloidogyne javanica*

The antagonistic activity of *Trichoderma harzianum* NAS120 and its gamma-irradiated mutant NAS120-M44 against *Meloidogyne javanica* was evaluated in vitro and in greenhouse conditions. In vitro assays showed that NAS120 significantly inhibited egg hatching and increased second-stage juveniles' mortality (J2) compared to the control indicating a marked suppression of nematode development in treated groups.

Under greenhouse conditions, tomato plants treated with NAS120 or the mutant isolate NAS120-M44 exhibited a significant reduction (73% for wild strain and 77% in mutant strain) in nematode infestation relative to the untreated control.

3.2. Molecular Identification and Phylogenetic Analysis

Molecular identification of the wild-type and mutant isolates was confirmed by amplification and sequencing of the ITS-rDNA and TEF-1 α regions. Phylogenetic analysis using the Neighbor-Joining method with 1000 bootstrap replications demonstrated that both NAS120 and NAS120-M44 clustered within the *Trichoderma harzianum* clade (Fig. 1). Minor nucleotide variations were detected in the mutant isolate, consistent with genetic changes induced by gamma irradiation. The phylogenetic tree constructed using MEGA software and the Maximum Composite Likelihood model confirmed the close genetic relationship between the parental and mutant strains.

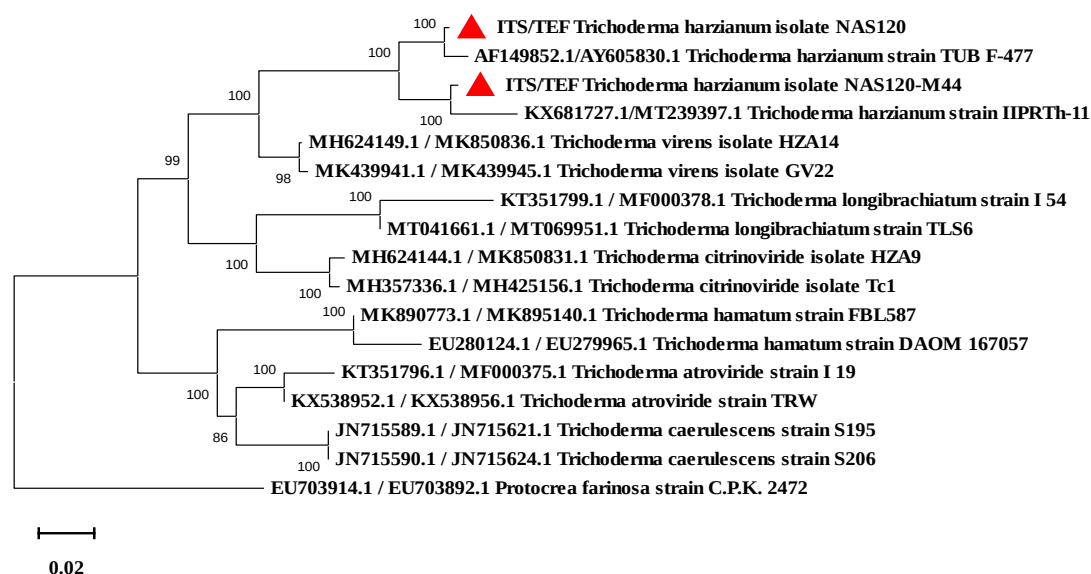


Fig. 1. Phylogenetic relationship of *Trichoderma harzianum* NAS120 and the mutant isolate *T. harzianum* NAS120-M44 inferred from combined ITS and TEF-1 α gene sequence alignments using the maximum likelihood method with 1000 bootstrap replicates in MEGA 10.0. Red triangles indicate the isolates obtained in this study.

3.3. Morphological Characteristics

Colony morphology of the mutant isolate NAS120-M44 exhibited typical *Trichoderma* characteristics, comparable to the wild-type isolate (Fig. 2). No abnormal morphological traits were observed.

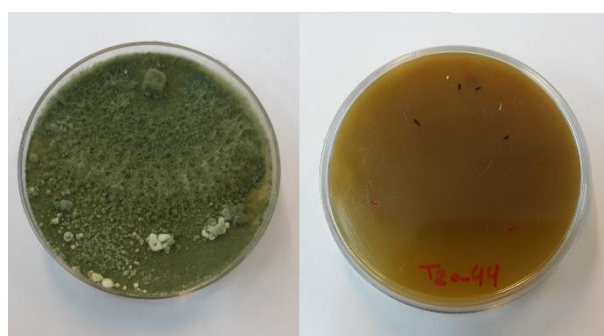


Fig 2. Culture plate (Top and back) characteristics of *Trichoderma harzianum* NAS120-M44

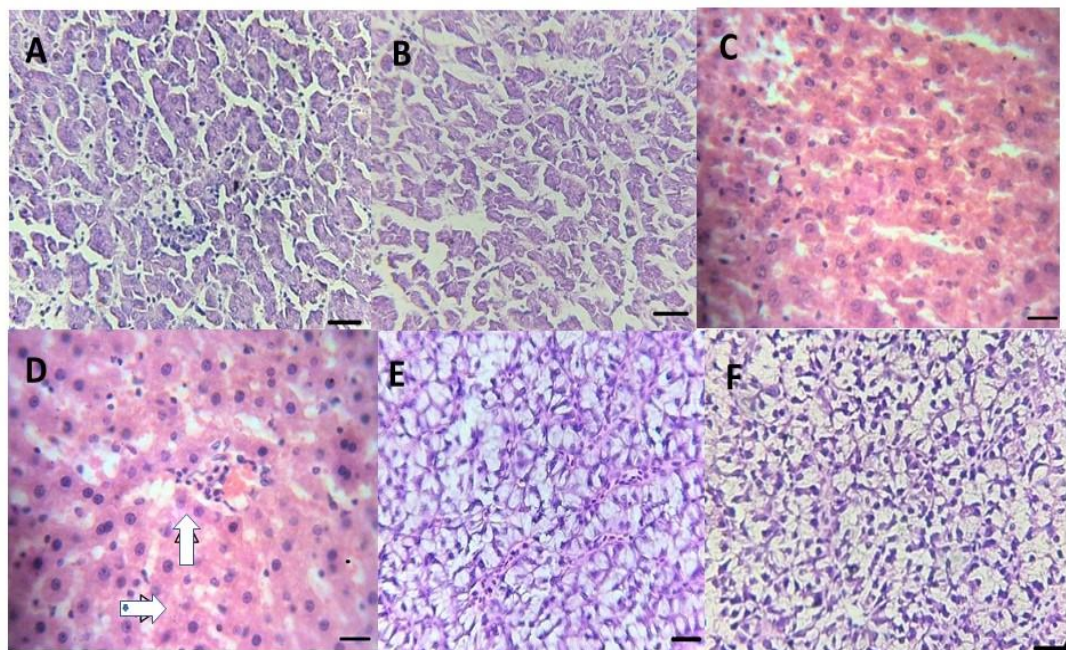
3.4. Histopathological Findings

Microscopic examination of tissue sections revealed a time- and treatment-dependent histopathological changes in the liver, kidney, and no that much effect on intestine.

In the control groups, normal histological structure was observed in all examined organs at both 3 and 6 weeks. Although the SA group was not treatment group, mild fatty changes in the liver were noted, suggesting minimal background or dietary effects (Fig3B). Kidney was normal. After 3 weeks of oral exposure to *Trichoderma*. Animals in the wild-type treatment groups (TC) showed slight fatty changes in the liver (Fig3D). Renal examination revealed scattered necrosis of tubular epithelial cells accompanied by cellular swelling, (Fig4D) while the intestine exhibited normal condition. In contrast, animals treated with the mutant isolate (TM) showed no pathological alterations in the liver (Fig3C). Renal lesions were very slight hyperemia and degeneration (Fig4C), Again intestinal changes were not observed

After 6 weeks, control group (CL) display normal histological features (Fig3A, 4A). In animals treated with the wild-type isolate (TC), marked fatty degeneration was evident in the liver (Fig3F). The kidney showed tubular epithelial fatty degeneration, tubular epithelial necrosis, cell swelling, indicating notable renal impairment (Fig4F). Intestine was normal. In the mutant-treated group (TM), the liver exhibited fatty change (Fig3E). Renal lesions included mild tubular fatty degeneration, and epithelial cell swelling (Fig4E). Intestinal alterations were not observed.

Overall, histopathological assessment demonstrated that the mutant strain NAS120-M44 caused fewer and less severe lesions, indicating a reduced systemic toxicity and a safer biological profile relative to the wild-type strain.



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Fig3. Liver.A.Group Control(CL)6weeks:Normal hepatic architecture Hepatocytes are regularly arranged with no visible vacuolation or fatty change. Sinusoids appear clear, and nuclei are intact.

B.GoupS-A 6 weeks: Slight fatty change in hepatocytes. Small vacuoles are scattered throughout the cytoplasm, indicating mild lipid accumulation. No necrosis or inflammatory infiltration observed.**C. Group TM (mutant strain) 3 weeks:** Normal hepatic architecture.

D.GroupTC (wild strain) 3 weeks: Very slight fatty change(arrows).

E . Group TM (mutant strain), 6 weeks: Marked fatty change in liver. Hepatocytes show multiple large vacuoles. No significant necrosis observed. **F.GroupTC(wild strain) 6**

weeks: Marked fatty change in liver. Hepatocytes display large lipid vacuoles. H&E staining. Bar:200 micrometer.

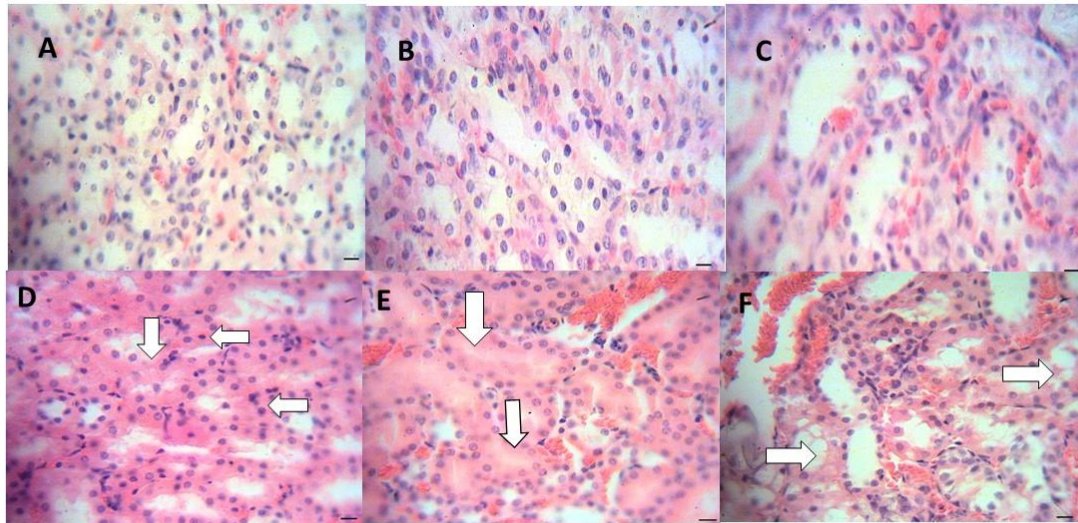


Fig4. Kidney. **A. Group Control (CL)6weeks:** Normal kidney. Tubular structures are intact, no signs of necrosis or swelling. Tubular epithelial cells appear healthy. **B. Group S-A 6 weeks:** Normal kidney Tubular structures are intact, no signs of necrosis or swelling. Tubular epithelial cells appear healthy. **C. Group TM (mutant strain) 3 weeks:** Some degeneration and hyperemia. **D. Group TC (wild strain) 3 weeks:** Some renal tubular degeneration and cell swelling (arrows). **E. Group TM (mutant strain), 6 weeks:** Vast renal tubular degeneration and scattered necrosis(arrows) **F. Group TC (wild strain) 6 weeks:** Vast renal tubular degeneration and necrosis. Necrosis is not scattered and is in more progressed condition than the mutant group(arrows), H&E staining. Bar:200 micrometer

4.Discussion

The present study demonstrates that gamma irradiation can enhance the efficacy and safety profile of *Trichoderma harzianum* (a biological control agent) [8]. Biological control using antagonistic fungi such as *Trichoderma spp.* has emerged as a sustainable alternative [9]. In vitro assays in this study showed significant reduction in egg hatching and elevated larval mortality of *M. javanica*, confirming that *Trichoderma spp.* produce nematocidal compounds and hydrolytic enzymes that disrupt nematode development [10].

Greenhouse experiments further demonstrated that both wild-type and gamma-irradiated mutant *T. harzianum* isolates effectively suppressed nematode infestation. The mutant isolate NAS120-M44 achieved a 77% reduction in the reproduction factor, surpassing the wild-type strain (73%). This improvement aligns with previous reports showing that gamma irradiation induces genetic variability that can enhance antagonistic traits without altering the species identity [7].

Molecular analyses indicated that gamma irradiation caused minor sequence variations in the ITS-rDNA and TEF-1 α regions while maintaining the taxonomic placement within *T. harzianum*. This is consistent with earlier findings that irradiation-induced mutants remain genetically stable and do not compromise species identity [11, 12]. Preservation of species identity is crucial for ecological safety and regulatory approval of biocontrol agents.

Histopathological examination revealed differential effects between wild-type and mutant strains. Control animals showed normal liver, kidney, and intestinal structures, whereas the wild-type strain caused fatty liver degeneration and renal tubular necrosis, especially after prolonged exposure. Similar observations have been reported in mammals exposed to high concentrations of fungal spores or certain microbial metabolites [13]. In contrast, the mutant NAS120-M44 induced milder histopathological changes, with limited fatty degeneration, minimal tubular epithelial necrosis in kidney, suggesting a reduced systemic impact and higher safety profile.

The reduced toxicity of the mutant strain may be associated with changes in secondary metabolite production or cell wall components that interact with host tissues [14]. *Trichoderma* cell wall proteins and small metabolites can influence host immune responses, and irradiation-induced modifications may reduce cytotoxic or inflammatory effects without affecting nematode antagonism [15].

From an agronomic perspective, the mutant *Trichoderma* strain offers considerable benefits. Its nematode suppression efficacy is comparable to chemical nematicides, supporting integration into nematode management strategies that reduce chemical inputs and promote sustainable agriculture [16].

Future research should focus on elucidating the molecular mechanisms underlying enhanced antagonism and reduced toxicity in gamma-irradiated mutants. Metabolomic and transcriptomic analyses could identify key genes and metabolites responsible for nematocidal activity and safety, while multi-location field trials are necessary to validate greenhouse results under diverse environmental conditions.

Conclusion

This study showed that *T. harzianum* NAS120-M44 the gamma irradiated mutant of *Trichoderma harzianum* wild strain exhibited improved antagonistic activity against the root-

261 knot nematode *Meloidogyne javanica*. More than that histopathological alterations in non-
262 target tissues compared to the wild-type strain, indicated its potential as an effective and safer
263 biocontrol agent.

264 **Ethical Consideration**

265 **Compliance with ethical guidelines**

266 This research was approved by the Research Ethics
267 Committee (REC) Nuclear Science and Technology Research Institute. Tehran. Iran in
268 accordance with institutional guidelines for animal experimentation.

269 **Data availability**

270 The data used and/or analyzed during the current study
271 are available from the corresponding author upon reasonable
272 request

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276 **Authors' contributions**

277 Conceptualization and study design: M Heidarieh and S.Shahbazi .Data Analysis and
278 interpretation: H.Askari and AA Shahbazfar. Investigation: A A Shahbazfar , M. Heidarieh
279 and S Shahbazi, Writing the original draft: M Heidarieh A A Shahbazfar , S Shahbazi and H
280 Askari

281 **Conflict of interest**

282 The authors declared no conflict of interest.

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