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Silver Nanoparticles Attenuate Inflammation aggravation in Hepatocellular Carcinoma in Rats

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ABSTRACT

Using silver nanoparticles is a promising tactic in healthcare and cancer therapy, either alone or as an adjuvant in traditional chemotherapy. In addition, using gamma radiation in nanoparticles preparation is simple, powerful, and environmentally friendly. The present study aims at assessing the impact of silver nanoparticles (AgNPs) prepared by gamma radiation (*γ*-radiation) on hepatocellular carcinoma (HCC) induced by diethyl-nitrosamine (DEN). MTT Cytotoxicity assay of AgNPs has been detected *in vitro on* Wi38 normal fibroblast and HepG2 hepatocellular carcinoma cell lines. Twenty-eight adult male albino rats were divided into four groups. After HCC development, rats received (10µg/g b. wt.) of AgNPs, three times a week for four weeks. Liver enzymes were investigated. TNF-*α*, TGF-*β*, and IL-1*β* inflammatory markers were detected. Furthermore, the transcriptional factor (NF-*κ*B) was assayed. The obtained results confirmed the selective influence of AgNPs on the HCC cell line in a time and dose-dependent manner. Moreover, the role of AgNPs is documented in diminishing the inflammation accompanied by HCC development through decreased inflammatory markers and increased apoptosis.

INTRODUCTION

Cancer is a challenging disease. Conventional therapeutic strategies have countless problems and side effects. Therefore, finding alternative treatment modalities is a fundamental goal. Using nanotechnology may have been considered a promising method that helps. Nano-size materials display unique properties; they have a higher surface area-to-volume ratio, which makes them potentially more reactive [1].

According to many reports, silver nanoparticles (AgNPs) are well known for their extraordinary physical and biochemical properties, making them suitable for many biological applications such as wound healing, contraception, cosmetics, and food technology [2].

Silver nanoparticles (AgNPs) exhibit low toxicity in humans and have diverse in *vitro* and in *vivo* applications **[3].** In addition, they have been widely used as antibacterial agents in food storage and the environment [4]. Recently, silver nanoparticles have been shown to provide a novel approach to overcoming tumors, especially those of hepatocarcinoma [5]. Silver nanoparticles (AgNPs) have fundamental antibacterial and anticancer properties, through several mechanisms, for example, due to silver ions or the formation of radical species [6].

Gamma radiation is a powerful and simple technique to synthesize nanoparticles of controlled size and shape in a solution as well as in heterogeneous media such as hydrogels [7]. AgNPs characteristics, including morphology, size, distribution, surface area, surface charge, stability, and agglomeration, have a significant effect on their interaction with biological systems. The current study aims at examining the influence of silver nanoparticles prepared by gamma radiation on the aggravation of inflammation associated with hepatocellular carcinoma induced by the chemical carcinogen.

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MATERIALS AND METHODS

Chemicals

Sliver nitrate ($AgNO_3$; 99.0%) was purchased from Gamma, Laboratory Chemical, Polyvinyl pyrrolidone (PVP) with an average molecular weight of 40 kDa and isopropyl alcohol ($(CH_3)_2CHOH$; 99.7%) were purchased from Sigma-Aldrich (Germany). Diethyl-nitrosamine (DEN) was acquired from Sigma-Aldrich Corporation (USA). Normal human fibroblast cells (WI-38) and human liver cancer cells (HepG2) were obtained from the National Research Center. (The tissue culture unit of the Holding Company for Biological Products and Vaccines (VACSERA), Giza, Egypt.

Gamma Irradiation Source

Irradiation of samples was carried out using a Co^{60} gamma source installed at the National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority, Egypt, at a dose rate of 1.06 Gy/h .

Animals

Twenty-eight male albino rats weighing 110 – 130 g, were obtained from NCCRT, Cairo, Egypt. Rats were housed in an environmentally controlled clean-air room with standard temperature and maintained a 12 h light/ dark cycle. They were kept on standard food pellets containing all nutritive elements and liberal water *ad libitum*, according to previously reported method **[8,9]**. All animal procedures were carried out in compliance with the guidelines of the NCCRT, Egypt, and with guidance for the proper treatment and use of laboratory animals.

Synthesis and Characterization of the silver nanoparticles (AgNPs)

Ag NPs were synthesized using ^{60}Co-gamma irradiation (25kGy) as a clean tool for synthesis and characterized according to Sheikh et al 2009, and Dhayagude et al. [10,11].

Cytotoxicity of AgNPs

In *vitro* cytotoxicity assay (MTT) was performed for AgNPs, where MTT reagent [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] was used. Wi38 normal fibroblast and HepG2 cell line was obtained from VACSERA. MTT assay was assessed according to van de Loosdrecht, et al. [12]. Briefly, in 100 µl of phosphate-buffered saline (PBS, 0.2 M, PH=7.4), the cells ($2.5x\ 10^6$) were seeded and mixed with 100 µl of various concentrations of AgNPs (1000 - 7.81 µg/mL) in a 96 wells plate and incubated for 24 h at 37◦C.Wi38, and HepG2 cells in PBS without treatment were considered as control (100% viable). From (5 mg/ml) MTT stock solution,0.5mg/ml MTT was added into each sample. The plate was incubated for an additional four hours in the dark to develop color. To dissolve MTT, 200 µl of DMSO/well was added and absorbance measured at 570 nm [12]. The percentages of the cell cytotoxicity and viability were calculated as follows:

Viability % = sample abs /control abs x 100 and Cytotoxicity % =viability % -100

Experimental design

Rats were randomly categorized into 4 groups, 7 rats each as follows:

(1) Control group (C); Normal male rats received intraperitoneal injections (i.p.) with 0.9% saline solution. (2) Silver nanoparticles group (Ag); animals were i.p. injected with AgNPs (10 µg/g) body weight 3 times a week, for 4 weeks [14]. (3) HCC-rat group (T); rats were orally administrated with 20 mg/kg b.wt. of DEN (dissolved in 0.9% normal saline), five times a week for eight weeks, followed by 10 mg/kg for another two consecutive weeks according to a reported modified method **[15,16]**. (4) HCC-AgNPs treated rat group (TAg)(rats developed HCC as group 2 and were injected with AgNPs as group 3).

Samples collection and preparation

Blood was drawn from the heart and separated into two parts; EDTA whole blood for flow cytometric analysis and serum for other biochemical investigations. Liver tissues were excised and dissected for further assessments using RT-qPCR and ELISA techniques.

Biochemical investigations

The liver enzymes (ALT, AST, and ALP) activities were determined *via* diagnostic colorimetric kits as described using Biomed diagnostic kits according to the manufacturer's instructions. Liver tissue homogenates were assayed for TGF-β *via* Rat ELISA Kit Cat #BMS623-3 purchased from Invitrogen, TNF-α (ELISA Kit, eBioscience, San Diego, CA, USA), and Invitrogen IL-1β ELISA Kit Cat #BMS630, respectively.

Real-time polymerase chain reaction (RT-PCR) analysis

Extraction of RNA from liver tissue homogenate was performed *via* the RNeasy plus mini kit (Qiagen, Venlo, the Netherlands), as per the constructer's prescriptions. DNase-on-column control eliminated genomic DNA. RNA perfection was assessed by electrophoresis on 2%

agarose gels. Consequently, 1 µg of RNA was used in the cDNA synthesis reaction, performed using the Reverse Transcription System (Promega, Leiden, The Netherlands). The RNA concentration was determined by spectrophotometer at 260 nm *via* the Nano-Drop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and the pureness of RNA was reviewed through the absorbance ratio at 260/280 nm. To prevent secondary structures, total RNA was incubated at 70°C for 10 min., supplemented with $MgCl_2$ (25mM), RNase inhibitor (20 U), oligo (t) primers, dNTP mixture (10mM), RTase buffer (10X), and AMV reverse transcriptase (20 U/µl). This mixture was incubated at 42°C for 1h.

Quantitative real-time PCR: In an optical 96-well plate, qRT-PCR was implemented with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and international cycling conditions of 40 cycles of 15 sec. The initial denaturation step occurred at 95°C for 60 sec followed by 60°C for 10 min, then 10 µl reaction mixture containing [(5 µl SYBR Green Master Mix (Applied Biosystems), 1.9 µl nuclease-free water, 0.3 µl gene-specific reverse and forward primers (10 µM), and 2.5 µl cDNA)] was added. The sequence of the PCR primer pair used for NF-*κ*B is shown in Table (1). Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of the studied gene was calculated using the comparative threshold cycle method. All values were normalized to the endogenous control Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) [17].

Table (1): Primer used for qRT-PCR

Primer	Sequence
NF-κB	Forward: 5'- ACGGCATGG ATCTCAAAGAC-3' Reverse: 5'- CGGACTCCGCAAAGTCTAAG -3′
GAPDH	Forward: 5'- CTCCCATTCTTCCACCTTTG-3′ Reverse: 5′- CTTGCTCTCAGTATCCTTGC-3′

GAPDH* as internal reference gene

Statistical analysis

A statistical analysis of the results was executed *via* SPSS version 20.0. All data are given as means ± standard error (SE). Differences were considered significant at $p<0.05$, and Data were analyzed statistically using one-way ANOVA followed by *Tukey as Post Hoc and LSD* were detected.

RESULTS

In vitro MTT assay of AgNPs

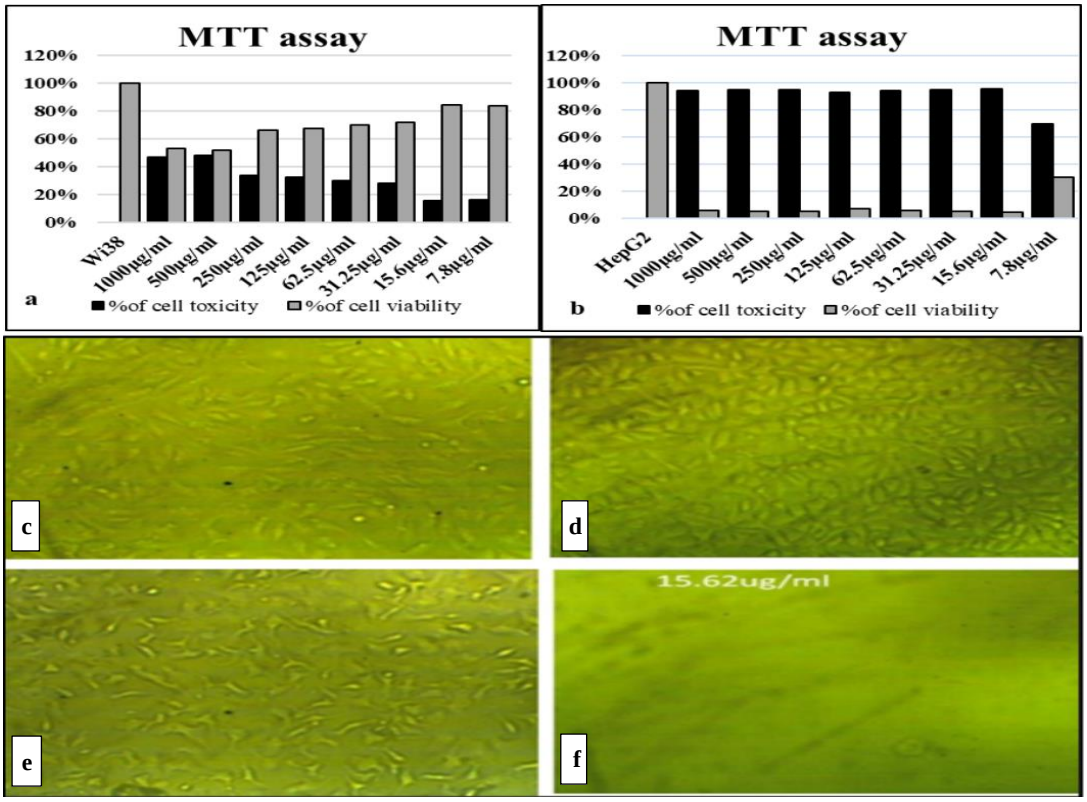

Fig. (1): MTT cytotoxicity assay (% of cell viability and % of cell toxicity) of AgNPs on both (a)Wi38 and (b)HepG2 cells with different AgNPs concentration (the value displayed as means ± SE (n=3) of two independent experiments. (c)control Wi38 cells. (d)control HepG2 cell. (e)The effect of AgNPs onWi38. (f) AgNPs effect on HepG2

MTT in vitro assay was performed to measure the impact of AgNPs on Wi38 normal fibroblast cells and HepG2 hepatocellular carcinoma cell lines. The results are illustrated in Figures (1a & b). Wi38 and HepG2 cells were considered as the control (100% viability and 0 %toxicity). AgNPs wide concentrations (1000µg/ml-7.8µg/ml) range was used. The results indicated a potent decline in HCC cell line and cell viability when incubated with AgNPs (anticancer activity) in dose and time-dependent means from 100% in control to 4.4% in 15.6µl/ml in 24hr. However, the cell viability in Wi38 normal fibroblast recorded 84% in the lowest AgNPs concentration (7.8µl/ml). Figure 1(c, d, e, and f) presents the wi38, HepG2, AgNPs-Wi38, and AgNPs-HepG2 respectively, under the microscope which reveals the selective effect of AgNPs on cancer cells other than the normal ones.

Liver function enzymes

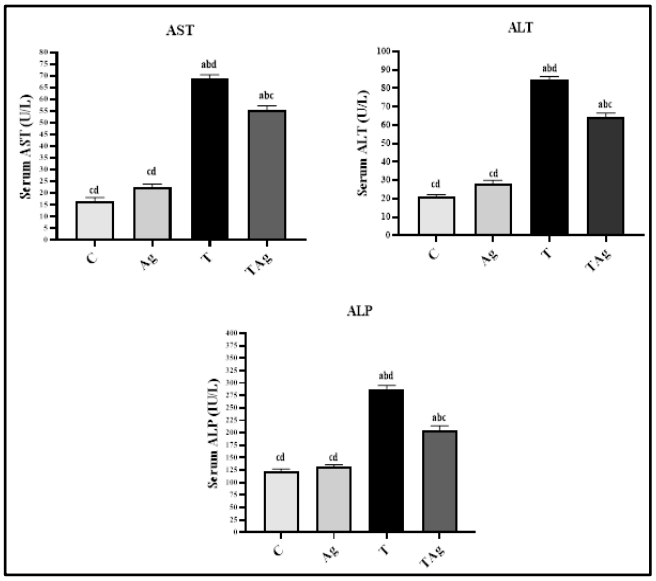

Fig. (2): Liver enzyme activities of serum (AST, ALT, and ALP (U/L)) in different male rat groups. Each value represents the mean ± SE (n=7). Mean values represent at (p<0.05) significant differences. a: different from control, b: different from Ag, and c: different from T

The liver enzymes activities were examined in the serum of all the tested groups. AST, ALT, and ALP exhibit a significant elevation (P < 0.05) in the HCC group compared to their equivalent values in control rats. However, in HCC rats treated by AgNPs (TAg) significant ameliorations (P<0.05) in the activities of the three enzymes were observed when compared to the HCC group.

Anti-inflammatory Markers

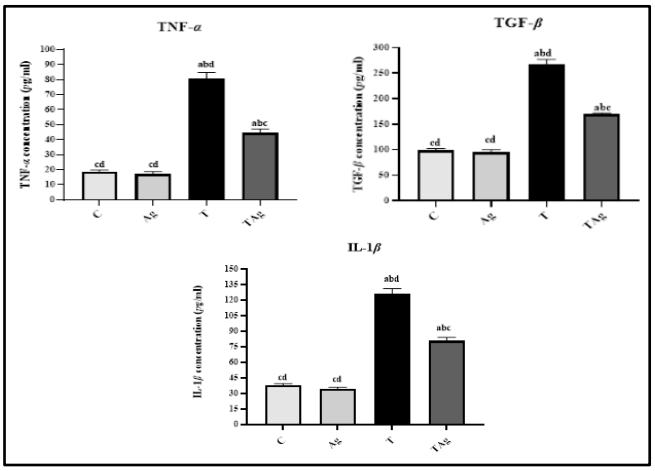

Fig. (3): The inflammatory markers (TNF-*α*, TGF-*β*, and IL-1*β*) levels (ng/ml) of different rats. Each value represents mean ± SE (n=7). Columns over headed with different letters are considered significantly different (p<0.05) .a: different from control, b: different from Ag, and c: different from T

The inflammatory markers; TNF-α, TGF-β, and IL-6 levels in liver tissue were assayed in the current study. As illustrated in Figure (3), animals that have developed HCC showed significant elevations (p<0.05) in liver tissues (80.70 ± 4.13), (266.72 ±10.08), and (126.27±5.07), respectively compared to the control animals. On the other hand, AgNPs treatment ameliorates the three inflammatory markers levels (44.49±2.69), (169.46±2.57), and (80.86±3.53), respectively when compared to HCC or to the control groups.

NF-κB gene expression

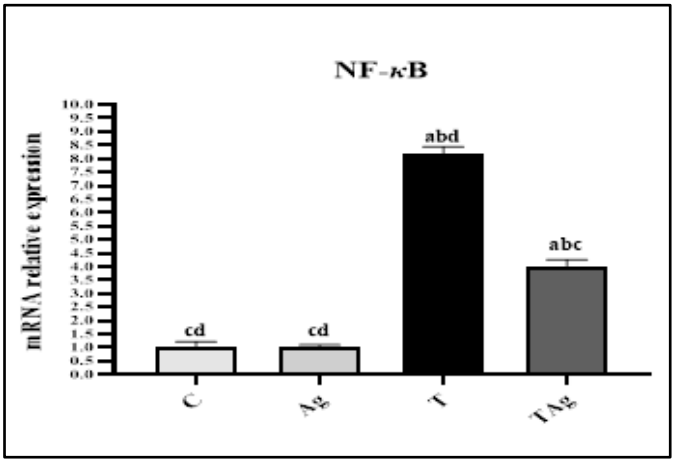

Fig. (4): NF-κB mRNA relative expression of different rat groups. Each value represents the mean ± SE (n=7). Columns over headed with different letters are considered significantly different (p<0.05). a: different from control, b: different from Ag, and c: different from T

Figure (4) displays a significant boost of NF-κB expression in tumor liver tissues (T group) (8.19 ± 0.25) in comparison to the control (1.04 ± 0.18). Meanwhile, there was a significant decline in its expression in TAg (4.03 ± 0.24) compared to T (8.19 ± 0.25**)**.

DISCUSSION

Targeting the inflammatory tactic resulting from HCC has a strong biological validation in tumor therapy. In an attempt to improve cancer therapeutic protocols, the present study was undertaken to evaluate the mechanism of the anti-inflammatory effect of silver nanoparticles (AgNPs) synthesized via γ-radiation against HCC inflammatory consequences.

The size of the nanoparticles is considered a vital factor in their cytotoxicity and cellular uptake [18], and it also influences their interaction with mammalian cells and where the NPs accumulated [19]. In the present work, AgNPs exhibited a diameter of 7-10nm. Many reports documented that decreasing the size leads to an increase in the surface area of the particles which facilitates the diffusion of the particles into the cell [19].

The polymeric materials coating AgNPs enhances their stability and reduces the Ag + ions dissolution from AgNPs surface [20].

Using several coating materials affected AgNPs toxicity. Anderson et.al. documented that PVP-coated AgNPs directed to the lung get cleared faster than citrate-coated AgNPs, so surface coating has an important role in the internal clearance and translocation of AgNPs [20]. Therefore, the authors prepared PVP-coated AgNPs to sidestep any coating-dependent disparity in cellular response. Fehaid et al. [22] recommended PVP for coating AgNPs for in vitro studies because of their higher stability with less aggregation and dissolution changes.

The efficiency of AgNPs on HCC- induced rats was studied by measuring liver function enzymes (ALT, AST, and ALP) activities as they are indicators of hepatocellular injury. In healthy (control) liver organs, homeostasis occurs through cell death and tissue regeneration. Therefore, ALT and AST levels were maintained within a normal range [23]. However, a significant increase in liver enzyme activities was documented as a result of HCC development, due to hepatocytes injuries and enzymes release to the blood circulation [24]. ALT is a more specific biomarker for acute liver injury at its early stages, and some types of cancers such as HCC [25], but both enzymes' activities were decreased after AgNPs treatment (TAg), which might be due to the probable role of AgNPs in regeneration of hepatocytes and possible healing of the hepatic parenchyma [26].

MTT assay was carried out in the present work to evaluate the cytotoxic effect of AgNPs on normal fibroblastic cells (Wi38) and (HepG2) HCC cell lines. The results verify the dose-dependent inhibitory ability of AgNPs on HCC cell proliferation. Moreover, the cytotoxicity curves revealed the selective effect of AgNPs on HCC cells, but not on the normal cells (Fig. 1, a & b). This marked effect was probably attributed to the effect of AgNPs on mitochondrial metabolism and finally the toxic effect of these nanoparticles on the cellular protein contents and their considerable influence on the antioxidant enzymes (GSH and SOD). Given that ROS plays a vital role in activating many cellular pathways which can cause cellular death and DNA damage. This occurs via changing nucleotide structure, consequently, the ROS generated by AgNPs, when getting into the cell, is the key step that begins several cell death mechanisms. It is important to highlight the different cell lines' sensitivity in terms of their natural antioxidant levels [27]. These results are in agreement with those of Yang et al. [28] who revealed that AgNPs lead to mitochondrial damage and rush ROS production in a dose-dependent manner. Furthermore, Lee et al. [29] showed that AgNPs enhance the cell cycle arrest in the G2/M phase possibly because of damaged DNA repair which increases apoptosis.

Cytokines are signaling molecules that facilitate and regulate cellular activities. Pro-inflammatory cytokines IL-1β and TNF-α regulate immunity, inflammation, and apoptosis [30]. TNFα participates in many signal transduction pathways, such as NF-κB activation, MAPK activation, and cell death signaling, as a result of cellular responses such as inflammation, DNA damage, proliferation, differentiation, and cell death [18]. TNF-α and TGF-β are supposed to be central inflammatory mediators of pro-inflammatory cytokines and growth factors activated by monocytes and macrophages during inflammation. They seem to potentiate the expression of IL-1β R29. R118. In this work, TNF -α is significantly elevated in HCC rats, and this may be due to its role as NF-κB - light- chain-activator which plays a vigorous role in inflammation and carcinogenesis [22]. On the other hand, the treatment of AgNPs significantly decreases TNF-α levels. This might be because of the influence of AgNps in reducing the TNFR1 binding on the cell membrane, which reduces the signal transduction

of TNF- α including its apoptotic and DNA damage effect [22]. Additionally, the obtained results followed those of Wong et al. and Seung et al.[31,32] who found that a decrease in inflammation markers such as TNF-α in animals treated with AgNPs. Meanwhile, Aparna Mani et al. [33] claimed that synthesized AgNPs were able to reduce the expression levels of TNF-α, IL-1β, and IL-6 in human PBMC cells [34]. In the normal state, NF-κB dimers are sequestered in the cytoplasm by IκBs as an inactive form [35]. By tumor development, NF-κB activation is initiated by phosphorylation, immediate degradation of IκB protein by activated IκB kinase (IKK) [36]. The liberated NF-κB translocates to the nucleus and binds as a transcription factor to κB motifs in the promoters of target genes, leading to their transcription.[37]. The obtained results are in agreement with those of Crisan et al. [38] who revealed that AgNPs continued pro-inflammatory cytokines suppression from activated macrophages in vitro by decreasing phosphorylation of IκBα. As mentioned before, NF-kB is an impressive agent in different steps of cancer occurrence and it can activate the production of many factors such as VEGF and TGF- β [39], and these findings agree with present results.

CONCLUSION

In conclusion, this study has revealed that AgNPs prepared by gamma radiation can reduce the inflammation resulting from HCC development in rats by diminishing IL-1β, TGF-β, TNF-α, and NF-κB inflammatory markers.

ETHICAL DECLARATION

The present study followed the recommended guidelines for the use and care of laboratory animals of the National Institute of Health (NIH publication no. 85-23, revised 1996) and regulations of the Ethical Committee (REC) of the NCRRT, Atomic Energy Authority, Cairo, Egypt (Approval No: 30A/21). REC has approved this research protocol, following the 3Rs principles for animal investigation (Replace, Reduce, and Refine), and is operated and organized as per the CIOMS and ICLAS International Guiding Principles for Biomedical Research Involving Animals 2012.

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