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Research Article



Preparation and biodistribution study of ⁶⁷Ga-rosemary (Salvia rosmarinus Spenn.) extract nanoparticles and its SPECT imaging in healthy rainbow trout (Oncorhynchus mykiss)

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Abstract

The aim of this study was to evaluate the labeling of rosemary extract nanoparticles (RE-NPs) using ⁶⁷Ga radioisotope, and their biodistribution in healthy rainbow trout (Oncorhynchus mykiss) tissues. RE-NPs were prepared by gamma irradiation (irradiated at a dose of 30 kGy of gamma-ray by ⁶⁰Co (PX-30 IssIedovapel, Russia, dose rate of 0.02 Gy sec⁻¹) and ultra-sonication methods at two concentrations of 0.5% and 1% and labeled by ⁶⁷Ga radioisotope. Quality control studies were done using the RTLC method. The radiolabeling RE-NPs at 1% concentration showed 97% efficiency using a mixture of sodium acetate and acetic acid buffer at pH=6.5 after 30 min at room temperature. In the biodistribution study, 54 healthy fish were randomly distributed into two treatment groups (n=18 fish/treatment group) and one group served as healthy control. Fish received 3.7-7.4 MBq (300 µL) of radiolabeled RE-NPs at 1% concentration and free ⁶⁷GaCl₃, intraperitoneally. The ⁶⁷Ga-RE-NPs single-photon emission computed tomography (SPECT) demonstrated the highest tracer accumulation in the kidney and the least uptake in the brain at 24 h after injection of ⁶⁷Ga-RE-NPs. However, 48 h after injection of ⁶⁷Ga-RE-NPs, the uptake was negligible in all organs as compared to free ⁶⁷GaCl₃. The obtained results revealed that administration of ⁶⁷Ga-RE-NPs, as a natural tracer, by IP injection can be a good imaging method to visualize and understand the whole-body, especially the kidney and spleen tissues distribution and pharmacokinetics in healthy rainbow trout.

Keywords: Rosemary, ⁶⁷Ga, radiolabeling, Biodistribution studies, Rainbow trout, Gamma irradiation, SPECT

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Introduction

Antioxidants are extensively used to prevent the negative consequences of lipid oxidation (Shahidi, 1997). Natural antioxidants are being extensively studied, and spices and herbs are identified as important sources of these compounds. Diverse studies evaluated the antioxidant properties of rosemary, Salvia rosmarinus Spenn. (Madsen and Bertelsen, 1995; Xie et al., 2017; Rezanejad et al., 2019a). Rosemary contains certain compounds with high antioxidant activity like carnosic acid and carnosol (Offord et al., 1997; Tironi et al., 2010). Rosemary is the spice with the highest antioxidant activity and a member of the Lamiaceae family which is an attractive evergreen shrub with pine needle-like leaves that grows wild in most Mediterranean countries (Pintore et al., 2002). Radiolabeling is a process that is frequently used in medicine, drug and development research and environmental case studies (Saha, 2004). Additionally, isotopic labeling is a technique used to track the passage of an isotope through a reaction in metabolic pathways or cells (Kitson et al., 2014). Among them, gallium 67 (67Ga) is increasingly utilized in a great number of fields. The 78.3 h physical half-life and a rather good detectability of its photon emission make gallium 67 one of the suitable nuclides for most radiopharmaceutical research (Heidarieh et al., 2014). The focus of the present work was to study the labeling of gamma-irradiated (at 30 kGy) rosemary extract nanoparticles (RE-NPs) using ⁶⁷Ga radioisotope, and their

biodistribution in healthy rainbow trout (*Oncorhynchus mykiss*) tissues.

Materials and methods

Fish

54 healthy rainbow trout (*Oncorhynchus mykiss*) weighing 400–450 g were raised from a commercial fish farm in Karaj, Iran, and kept in running water (flow rate 0.4 l/s) in polypropylene tanks (300 L) with a water temperature of 15±2°C, dissolved oxygen of 8.2 ppm, and natural photoperiod of 10 L:14 D.

Preparation of gamma-irradiated rosemary extract nanoparticles (RE-NPs)

Rosemary (Salvia rosmarinus Spenn) extract nanoparticles were prepared using ultrasonic and gamma irradiation methods which were described by Rezanejad et al. (2019a). Rosemary powder was suspended in phosphatebuffered saline (PBS) (0.2 M, sterile, pH=7.2). Samples were sonicated for 30 min ultrasonic water bath (Jencons, England) and centrifuged at 6000 rpm for 20 min. Subsequently, precipitated in 98% ethanol, ground rosemary powder was dried at 45°C and milled to the micromesh sieves in aperture sizes from 53 to 96 µm. Then, Rosemary extract powder was irradiated at a dose of 30 kGy by 60Co (PX-30 IssIedovapel, Russia, dose rate of 0.02 Gy sec⁻¹). RE-NPs solutions of 0.5% and 1% were prepared in PBS buffer (0.1 M, pH=6.5) (Rezanejad et al. 2019a).

Transmission electron microscopy

A transmission electron microscope (TEM) was used to analyze the morphology and size of RE-NPs (Amelinckx and Van Landuyt, 2003). The nanoparticles were immobilized on a coated copper grid and were allowed to dry at room temperature. The particle size and shape were observed using an FEI/Philips EM 208S TEM.

Labelling of RE-NPs using ⁶⁷GaCl₃

For the radiolabeling experiment, ⁶⁷Ga was received from a cyclotron, in Karaj, Iran (Jalilian et al., 2007). RE-NPs were labeled using an optimization protocol according to Orlando et al. (1994), with modifications. minor Α fraction containing ⁶⁷GaCl₃ (37-110 MBq) in a volume of 100 µL was dried by air flow and mild heat was used. Radiolabeling was performed by mixing 100 µl ⁶⁷GaCl₃ and 1 mL of phosphate buffer and 50 µL of RE-NPs and incubating at 90°C for 30 min. The resulting solution was stirred at room temperature for 30 min.

Quality control of ⁶⁷Ga-RE-NPs

The radiolabeling yield was determined by thin layer chromatography analysis (TLC), for the purity of the radio-labeled sample, small drop one of radiopharmaceutical mixture of RE-NPs labeled with ⁶⁷GaCl₃ (0.5% and 1%) was dropped on the lower side chromatography paper strips (Whatman No. 1. Whatman, Maidstone, UK). A mixture of sodium acetate and acetic acid buffer (1 M) was used as a mobile phase which moved up and passed through the droplet and carried on free ⁶⁷GaCl₃. The strip was dried after the mobile liquid reached the top and it was immediately scanned using RTLC (Bioscan AR-2000). ⁶⁷Ga-RE-NPs remained at the application point, while unbound ⁶⁷Ga ions migrated with the solvent front. The labeling efficiency of ⁶⁷Ga-RE-NPs was determined from the upper and lower-end counts. A control test was also carried out, in the absence of RE-NPs (Jalilian *et al.*, 2007).

Ex vivo biodistribution studies

All studies were approved by the University of Tehran, Biomedical **Ethics** Research Committee (IR.UT.VETMED.REC.). The 45 healthy rainbow trout specimens were acclimated for 7 days and randomly divided into two treatment groups and control (n=18 fish/each group). Each fish was IP injected with 300 ul free 67GaCl3 and the radiolabeled nanoparticles. The total amount of radioactivity injected into each rainbow trout was measured by a 1-ml syringe before and after injection in a dose (CRC-15R, calibrator Capintec Company) with fixed geometry.

SPECT imaging of ⁶⁷Ga- RE-NPs in rainbow trout

The biodynamic routes of the ⁶⁷Ga-RE-NPs inside the fish were studied using a SPECT camera. The rainbow trout was selected randomly and transferred to a small plastic aquarium and euthanized with an overdose of MS222 (200 mg/L) (Sigma-Aldrich, Denmark) at specified times and the radioactivity was quantitated in tissues. Fish were scanned

by a dual-head gamma camera system (model: DST-XL made by SMV company). SPECT images of the whole-body were obtained 24 h and 48 h after injections of free ⁶⁷GaCl₃ and ⁶⁷Ga-RE-NPs. The Medium-Energy All Purpose (MEAP) collimator was used at the head of the static planar acquisition with no gantry movement.

Biodistribution of ⁶⁷Ga- RE-NPs in rainbow trout

Anatomical biodistribution of ⁶⁷Ga- RE-NPs was determined by administering nanoparticles to healthy rainbow trout. For preventing any aggregation of these nanoparticles, the labeled nanoparticles were sonicated before IP injecting (Boyer et al., 2010). At times of 24 h and 48 h, the fishes were injected with rosemary extract nanoparticles and were euthanized and the major organs were collected (3 fish/time/tank). Then, the extracted organs (liver, kidney, heart, spleen, intestine, skin, muscle, stomach, brain and gill) were washed with normal saline, dried on filter paper, weighed, and their radioactivity of them measured using High Purity Germanium (HPGe) radiation detectors (Canberra, GC1020-7500SL). Accumulation of ⁶⁷Ga-RE-NPs in each sample was expressed as the percentage injected activity per gram of tissue (% ID/g±Standard Deviation) and calculated compared to activities of the standard dose of the injected solution (Jalilian et al., 2011).

Results

The TEM images, as shown in Figure 1, reveal the morphology of RE-NPs. The

average diameter of the particles was about 55-70 nm. The best labeling results were obtained at **RE-NPs** solution (1% concentration, irradiated at a dose of 30 kGy of gamma ray) after 30 min at pH=6.5 (Fig. 2). The measured labeling efficiency of RE-NPs labeled with ⁶⁷Ga was ~97%, as determined by RTLC method, which could be used without further purification for later studies. Rainbow trout showed uptake of free ⁶⁷GaCl₃ and ⁶⁷Ga-RE-NPs in the skin, muscle, heart, brain, gill, stomach, spleen, liver and kidney at 24 h and 48 h following IP injection (Fig. 3). The organ with the highest concentration of ⁶⁷Ga-RE-NPs was kidney (10.12±3.21 %ID/g), followed by spleen (7.83±1.32 % ID/g), liver (4.32 \pm 0.54 %ID/g), and muscle $(3.91\pm0.26 \% ID/g)$ at 24 h. There was low-level tracer uptake into the brain (0.11±0.02 %ID/g) and heart $(0.83\pm0.01 \text{ \% ID/g})$ with minimal uptake into the other organs at 24 h. After 48 h, the accumulation level of ⁶⁷Ga-RE-NPs was negligible for all of the organs except the liver and skin. Specifically, accumulation of 67Ga-RE-NPs in the liver was increased with time (4.32±0.54 % ID/g at 24 h, and 6.71±0.23 % ID/g at 48 h, after injections). On the contrary, in the heart, stomach, spleen, intestine, gill, muscle and skin the uptake showed the opposite behavior. The small percentage of uptake in the brain can be attributed to the blood-brain barrier (BBB) that prevents molecules from reaching the brain (Shakeri et al., 2020). Free ⁶⁷GaCl₃ showed a much more general biodistribution to most organs, while also showing elevated uptake in

the liver $(8.51\pm1.45 \% \text{ ID/g} \text{ at } 24 \text{ h}, 11.8\pm3.02 \% \text{ ID/g} \text{ at } 48 \text{ h} \text{ after injections})$, kidney $(6.01\pm2.47 \% \text{ ID/g} \text{ at } 24 \text{ h}, 5.38 \pm1.15 \% \text{ ID/g} \text{ at } 48 \text{ h} \text{ post injection})$, and spleen $(6.53\pm1.99 \% \text{ ID/g})$

at 24 h after injections) in rainbow trout (Fig. 4).

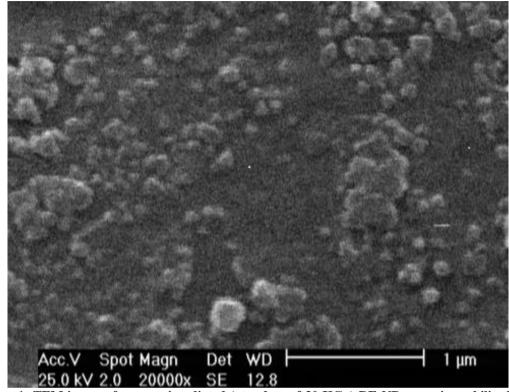


Figure 1: TEM image of gamma-irradiated (at a dose of 30 KGy) RE-NPs were immobilized on a coated copper grid at room temperature (n=9).

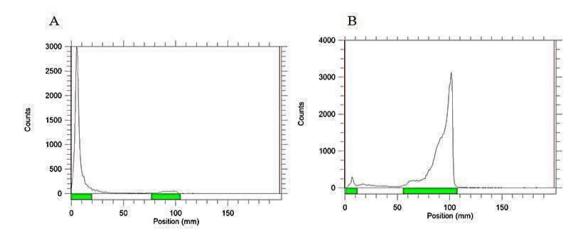


Figure 2: RTLC of (A) ⁶⁷Ga- RE-NPs (1%, 30 KGy) and (B) Free ⁶⁷GaCl₃ in Sodium acetate and acetic acid as mobile phase (n=9).

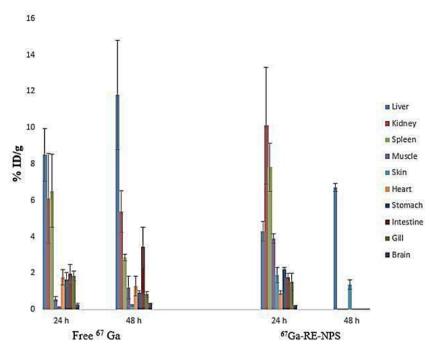


Figure 3: Organ concentrations of radioactivity (presented as SUV, mean \pm SD) obtained at 24 h and 48 h after the injections of 67 Ga- RE-NPs and free 67 GaCl₃ (n = 9), error bars represent standard deviation.

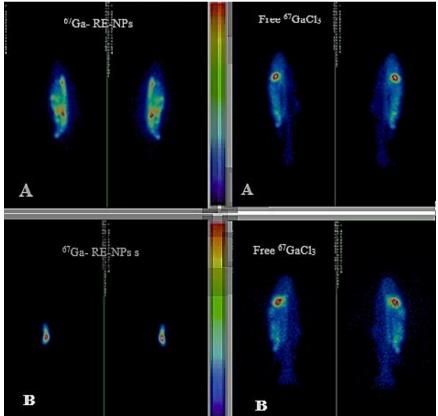


Figure 4: Representative SPECT whole-body images of ⁶⁷Ga-RE-NPs and free ⁶⁷GaCl₃ biodistribution in healthy rainbow trout 24 h (A) and 48 h (B) after intraperitoneal injection (n=9). Organs with significant uptake are labeled.

Discussion

This study demonstrated the feasibility and validity of SPECT imaging to investigate tissue accumulation of RE-NPs in healthy rainbow trout. SPECT is a powerful and non-invasive imaging technique to visualize the biodistribution of molecules labeled with radioactive isotopes such as ⁶⁷Ga. ^{99m}Tc and ¹²³I (Knight et al., 2019). Different parameters affect the labeling reaction; such as the pH of the reaction medium, Rosmarinic acid concentration, reaction time and temperature (El-Sharawy et al., 2022). This current study shows the radiolabeled RE-NPs at 1% concentration were prepared using ⁶⁷Ga with good labeling at about 97% efficiency (RTLC method). El-Sharawy et al. (2022) also reported radiolabeling yield of Rosmarinic acid with 99mTc radioisotope was 92.2% in pH=4 for 15 min.

To the best of our knowledge, this is the first report of SPECT imaging in rainbow trout demonstrating feasibility of tracking of RE-NPs uptake. Heidarieh et al. (2014) injected ⁶⁷Gaalginic acid nanoparticles into rainbow trout through a peritoneum to determine the whole-body distribution of alginic acid nanoparticles. In this study, the biodistribution of ⁶⁷Ga-RE-NPs was performed to assess their behavior as potential natural antioxidant agents. The imaging technique using SPECT camera detected higher net uptake in the kidney after 24 h post injection of ⁶⁷Ga-RE-NPs in healthy rainbow trout.

Cells have crucial antioxidant defense mechanisms to protect themselves against toxic injury by free-radicals. The enzymatic antioxidants are SOD, CAT, and GPx (Pisoschi and Pop, 2015; Mirończuk-Chodakowska et al., 2018). for the SOD_1 accounts highest proportion of SOD activity in the kidney (Schieber and Chandel, 2014), SOD₂ is also expressed in most tissue cells, such as the stomach, skeletal muscle, spleen, heart, liver, and the brain (Van Remmen et al., 1999). SOD3 is highly expressed in blood vessels, kidneys and the heart (Wang et al., 2018). CAT is abundantly present in the liver and kidneys (Ho et al., 2004). The kidney is a major source of plasma GPx (Avissar et al., 1994). Based on recent published data, plant extracts enriched with polyphenols could improve the enzymatic antioxidant defense system in salmon (Santana et al., 2021). Among natural antioxidant plants, rosemary was known for the presence of phenolic compounds (Collins and Charles, 1987; Moreno et al., 2006). Based on recently research findings, biodistribution data increased renal and splenic uptaking of ⁶⁷Ga-RE-NPs in fish after administration can indicate that RE-NPs can improve the renal and splenic enzymatic antioxidant defense system in rainbow trout. The present results also were confirmed by the work presented by Hernández et al. (2014) and Rezanejad et al. (2019b) indicating that using natural antioxidant RE-NPs in the diet can improve the resistance of fish muscle against oxidation and also fish fillet quality. Thus, based on the above investigation it is evident firstly that the potential of ⁶⁷Ga-RE-NPs to determine RE-NPs pharmacokinetics, elimination pathway, metabolism, and potential degradation, shows *in vivo* safety in rainbow trout. Secondly, the supplemented feed with gamma irradiated RE-NPs can be a good candidate for modulating the rainbow trout antioxidant system activities.

The applications of nanoparticles are widely studied for both treatment applications and molecular imaging. In this study, the radiolabeled RE-NPs at 1% concentration were prepared using ⁶⁷Ga with good labeling at about 97% efficiency (RTLC method). Biodistribution of the radiolabeled RE-NPs was checked in healthy rainbow trout up to 24 h compared to free ⁶⁷GaCl₃. The data showed that the tracer accumulation was high in kidney and spleen tissues. Based on the current study, the radiolabeling of RE-NPs as natural tracers can be a true and sensitive method to image and understand their whole-body distribution and pharmacokinetics in fish models.

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