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Evaluation of Technetium-99m Decay on *Escherichia coli* Inactivation: Effects of Physical or Chemical Agents

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Technetium-99m (^{99m}Tc) has been used in nuclear medicine and in biomedical research to label molecular and cellular structures employed as radiotracers. Here, we have evaluated, on a DNA repair proficient *Escherichia coli* strain, the ^{99m}Tc decay inactivation and the influence of the (i) pre-treatment with metal ion chelators or of the (ii) treatment with a free radical scavenger on the protection of the cells against the lethal effect of the ^{99m}Tc. As $SnCl_2$ is frequently used as a reducing agent in the ^{99m}Tc-labeling process, we have also studied the capability of $SnCl_2$ to alter the biological effects induced by the ^{99m}Tc decay. As we are exposed to either chemical or physical agents in the nature, we have decided to study a possible influence of the ultraviolet solar radiation in the biological phenomena induced by the ^{99m}Tc decay. Our data point out (i) a very important role of the Auger and/or conversion electrons in the cytotoxicity induced by the ^{99m}Tc decay; (ii) $SnCl_2$, the metal ion chelators and the free radical scavenger protect the cells against the lethal effect of the ^{99m}Tc; and (iii) near-UV does not alter the lethal effect of the ^{99m}Tc decay.

INTRODUCTION

Radionuclides play an important role in biomedical sciences and have contributed to the comprehension of many phenomena related to human beings. In nuclear medicine, they represent a powerful tool in diagnostic and therapy procedures [1, 2].

Since technetium-99m (^{99m}Tc)[c] was introduced in medical research, it has become one of the most employed radionuclide in nuclear medicine. ^{99m}Tc is utilized to label molecules and cells, utilized as radiopharmaceuticals and also to label biological species as cercariae, platyhelmint, red blood cells, leukocytes and bacteria [3-9]. ^{99m}Tc presents many desirable characteristics that justify its wide use: (i) its half-life of 6 hours is sufficient to allow different clinical and/or research evaluations; (ii) it is easily acquired from a molybdenum-99/technetium-99m (^{99}Mo/^{99m}Tc) generator; (iii) its chemical characteristics permit the labeling of various chemical agents; (iv) its photonic energy of 140 keV is suitable for gamma-camera images; (v) it presents a low cost; and finally, (vi) its

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[c] *Abbreviations:* ^{99m}Tc, technetium-99m; IC, internal conversion; ICE, internal conversion electrons; AU, Auger electrons; $SnCl_2$ stannous chloride; ROS, reactive oxygen species; EDTA, ethylenediaminetetraacetic acid disodium salt; LET, linear-energy transfer.

relatively simple handling of wastes disposables produces minimal environmental impact [1, 2, 10].

The primary path for nuclear decay of ^{99m}Tc is via two isometric transitions, beginning with decay of the metastable state by internal conversion (IC), which decays to the ground state predominantly by gamma (γ) ray emission. This two-step process results in the emission of internal conversion electrons (ICE) (128-138keV) with a yield of 1.1 per decay. The inner atomic shell vacancy, created by the internal conversion process, results in the emission of *Auger* electrons (AE) (15-21 keV) [10-14]. Several studies have demonstrated that the biological effects of radionuclides that emit AE can be severe and are primarily dependent on the cellular distribution of the radionuclide. These studies were reported with human leukocytes and spermatogonial cells of mouse, but have not been yet established with bacteria [13-16].

The chemical form of ^{99m}Tc available from $^{99}Mo/^{99m}Tc$ generator is sodium pertechnetate. The pertechnetate ion having the oxidation states +7 for ^{99m}Tc resembles permanganate ion and the perrhenate ion. Chemically, pertechnetate ion is a rather non-reactive species and does not label any compound by direct addition. In ^{99m}Tc-labeling of many compounds, prior reduction of ^{99m}Tc from the +7 state to lower oxidation state is required. In nuclear medicine, to get ^{99m}Tc-radiopharmaceuticals the reducing agent normally employed is the stannous chloride ($SnCl_2$) [2]. Although this reducing is widely used, some deleterious effects of this substance have been described [17-21].

Investigation of $SnCl_2$ toxicity indicate that it is highly irritant to the human skin and mucous membrane, although it presents a low systemic toxicity. In other animals, it can produce stimulation or depression of central nervous system. In bacterial assays, $SnCl_2$ appears to be capable of inducing and/or producing lesions in the DNA and can be a potential genotoxic agent. These effects may, at least in part, be attributed to the reactive oxygen species (ROS), generated during the $SnCl_2$ treatment, via the Haber-Weiss cycle [17, 19].

Ionizing radiations, as γ-rays, interact with matter originating activation and ionizing processes. The radiation energy may be directly transferred to DNA, modifying its structure, or to an intermediate molecule, like water, whose radiolysis gives the generation of the ROS, as hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet -}$). These ROS are important oxidants of lipids, proteins and nucleic acids and seem to play a relevant role in various biological phenomena as mutagenesis, carcinogenesis, aging, apoptosis and teratogenesis [22-26].

H_2O_2 and $O_2^{\bullet -}$ toxicity is thought to result from their conversion in presence of transition metal ion (Fenton reaction and the Haber-Weiss cycle) into •OH, which reacts with components of the cell. Transition metals, such as Fe^{+2} and Cu^{+1}, act as reducing agents in the formation of ROS in these reactions. In addition, the Fe^{+2} autoxidation to Fe^{+3} in Fenton reaction can be inhibited by pre-treatment with metal ion chelators, as ethylenediaminetetraacetic acid disodium salt (EDTA) and dipyridyl. Thiourea and sodium benzoate are well-known scavengers of •OH and can often protect the cells against damages caused for this ROS [17, 22, 26, 27].

Near-ultraviolet light (320-380 nm) is a component of solar light, which is known to induce deleterious effects such as killing and mutation. These effects may be related to the ROS generation by this non-ionizing radiation in the culture medium [21, 28, 29, 30]. As ^{99m}Tc has been chosen for employing in nuclear medicine and/or biological evaluations, the effects of AE and ICE must also be taken into account.

Escherichia coli was selected as an experimental organism because of its simple growth requirements, its ability to grow anaerobically or aerobically and the extensive knowledge available concerning its genetics and enzymology. The studies were carried

out with a widely and well-known cellular culture of *E. coli* K12S strain that is proficient in DNA repair mechanisms [31].

We have evaluated: (i) the effects of ^{99m}Tc decay on the survival of the cells; (ii) the capability of chemical agents ($SnCl_2$, EDTA, dipyridyl, thiourea and sodium benzoate) to alter the citotoxicity of the ^{99m}Tc decay; and (iii) the capability of physical agent (near-UV) to modify the deleterious effects of ^{99m}Tc decay.

MATERIAL AND METHODS

^{99m}Tc, as sodium pertechnetate, recently milked from a ^{99m}Mo/^{99m}Tc generator (Instituto de Pesquisas Energéticas e Nucleares, São Paulo, Brazil) (final activity per glass tube: 37 MBq/ml) was used in all treatments [32]. Due to the dilution of the aliquots to determine the numbers of the cells in the different experiments, the quantity of ^{99m}Tc in the plated medium was negligible. With the numbers of the cells, the survival fractions (N/N_0) were determined. N represents the number of cells after each treatment, and N_0 corresponds the number of cells in the beginning of the treatment.

In order to perform the experiments to evaluate the effects of ^{99m}Tc decay on *E. coli* K12S, an exponential culture growing on LB medium [33] was centrifuged (10 min, 7650 xg, 4°C) and suspended into 0.9 percent NaCl solution. The bacterial suspension (1-2 x 10^8 cells/ml) was treated with ^{99m}Tc as follows: (i) cells were exposed indirectly to the radionuclide when a glass tube, containing 1 ml of bacterial culture was put inside another one with ^{99m}Tc (0.5 ml); (ii) cells were exposed directly to the radionuclide when a glass tube, containing 1 ml of bacterial culture was mixed with ^{99m}Tc (0.5 ml); (iii) cells were exposed indirectly and directly to the radionuclide when a glass tube containing 1 ml of bacterial culture mixed with ^{99m}Tc (0.5 ml) was put inside to another one with ^{99m}Tc (0.5 ml). As control, 0.9 percent NaCl was employed instead of the ^{99m}Tc solution. All the experiment tubes were incubated at 37°C with shaking for 180 minutes. At 60-minute intervals, aliquots (0.1 ml) were withdrawn, conveniently diluted and plated for the determination of the survival fraction (Table 1) [32].

To analyze the capability of a metal ion chelator to alter the biological effects of ^{99m}Tc decay, exponential *E. coli* K12S cultures (1 ml) were mixed with 0.15 ml of EDTA or dipyridyl (final concentration: 10 mM) (both were purchased from Sigma Chemical Co., USA) or 0.9 percent NaCl solution as a control, following incubation at 37°C, with

Table 1. Survival fractions of *E. coli* K12S treated with ^{99m}Tc (activity final: 37 MBq/ml).

Incubation time (min)	Cells are exposed directly to ^{99m}Tc	Cells are exposed indirectly to ^{99m}Tc	Cells are exposed directly and indirectly to ^{99m}Tc	NaCl 0.9% solution (control)
0	1	1	1	1
60	4.4×10^{-1}	7.7×10^{-1}	4.8×10^{-1}	1
120	9.5×10^{-2}	7.1×10^{-1}	1×10^{-1}	1
180	5.6×10^{-2}	6.5×10^{-1}	4.9×10^{-2}	1

Exponentially growing cultures were centrifuged, washed in 0.9 percent NaCl and suspended in 0.9 percent NaCl solution. The bacterial suspension (1-2 x 10^8 cells/ml) was treated with ^{99m}Tc, as shown above, for 180 minutes at 37°C, with shaking. After each incubation time, aliquots (0.1 ml) were taken, diluted and plated onto Luria Broth medium for determination of the survival fractions (N/No). Values represent means of three independent determinations. Standard deviations did not exceed 15 percent.

shaking for 20 minutes. After this period of time, 0.5ml of ^{99m}Tc was added in each tube, and the incubation continued for another 180 minutes. At 60 minutes intervals, aliquots were withdrawn and plated for posterior survival fraction determination (Table 2) [23, 32].

In order to study the capability of the scavenger hydroxyl radical to protect the cells against the biological effects induced by the radionuclide, exponential *E. coli* K12S cultures (1 ml) were mixed with 0.15 ml of sodium benzoate or thiourea (final concentration: 10mM) (both were purchased from Sigma Chemical Co., USA) in presence of ^{99m}Tc (0.5 ml) or 0.9 percent NaCl solution (0.5 ml), following incubation at 37°C, with shaking for 180 minutes. At 60 minutes intervals, aliquots were withdrawn and plated for posterior survival fraction determination (Table 3) [23, 32].

To evaluate the effects of $SnCl_2$ on the survival of cultures exposed to ^{99m}Tc, exponential *E. coli* K12S cultures (1 ml) were mixed with 0.5ml ^{99m}Tc or 0.9 percent NaCl solution, following incubation at 37°C, with shaking for 60 minutes. After this period of

Table 2. Kinetics of inactivation of *E. coli* K12S pre-treated with metal ion chelator (EDTA or dipyridyl exposed to ^{99m}Tc (final activity: 37 MBq/ml).

Incubation time (min)	Cells treated with ^{99m}Tc	Cells pre-treated with EDTA and then exposed to ^{99m}Tc	Cells pre-treated with dipyridyl and then exposed to ^{99m}Tc	Cells treated with EDTA	Cells treated with dipyridyl	NaCl 0.9% solution (control)
0	1	1	1	1	1	1
60	4.4×10^{-1}	7.3×10^{-1}	6.2×10^{-1}	9.5×10^{-1}	9.6×10^{-1}	1
120	9.5×10^{-2}	4.8×10^{-1}	4.6×10^{-1}	9.2×10^{-1}	9.5×10^{-1}	1
180	5.6×10^{-2}	1.5×10^{-1}	3.1×10^{-1}	9.0×10^{-1}	9.5×10^{-1}	1

Exponentially growing cultures were centrifuged, washed in 0.9 percent NaCl and suspended in 0.9 percent NaCl solution. The bacterial suspension (1-2 x 10^8 cells/ml) was treated with metal ion chelator, EDTA (final concentration: 1 mM) or dipyridyl (final concentration: 10 mM) for 20 minutes. After this time, the bacterial suspension was treated with ^{99m}Tc, for 180 minutes, at 37°C, with shaking. The control not pre-treated was incubated, as shown above, for 180 minutes. After each incubation time, aliquots (0.1 ml) were taken, diluted and plated onto Luria Broth medium for determination of the survival fractions (N/N_0). Values represent means of three independent determinations. Standard deviations did not exceed 15 percent.

Table 3. Kinetics of inactivation of *E. coli* K12S treated simultaneously with free radical scavenger (sodium benzoate or thiorea and ^{99m}Tc (final activity: 37 MBq/ml).

Incubation time (min)	Cells treated with ^{99m}Tc	Cells treated with sodium benzoate and ^{99m}Tc	Cells treated with thiorea and ^{99m}Tc	Cells treated with sodium benzoate	Cells treated with thiourea	NaCl 0.9% solution (control)
0	1	1	1	1	1	1
60	4.4×10^{-1}	7.3×10^{-1}	6.5×10^{-1}	1	1	1
120	9.5×10^{-2}	6.5×10^{-1}	5.7×10^{-1}	1	9.5×10^{-1}	1
180	5.6×10^{-2}	5.7×10^{-1}	5.0×10^{-1}	9.5×10^{-1}	9.0×10^{-1}	1

Exponentially growing cultures were centrifuged, washed in 0.9% NaCl and suspended in 0.9% NaCl solution. The bacterial suspension (1-2 x 10^8 cells/ml) was treated with sodium benzoate (final concentration: 100 mM) or thiourea (final concentration: 100 mM)and ^{99m}Tc, for 180 minutes, at 37°C, with shaking. After each incubation time, aliquots (0.1 ml) were taken, diluted and plated onto Luria Broth medium for determination of the survival fractions (N/N_0). Values represent means of three independent determinations. Standard deviations did not exceed 15 percent.

time, stannous chloride, as $SnCl_2.2H_2O$ (Merck S.A, Brazil) (final concentration: 50 μg/ml) was added and the incubation continued for another 120 minutes. At 60 minutes intervals, aliquots (0.1 ml) were withdrawn and plated for the determination of the survival fraction (Table 4) [32].

To study the effect of the near-UV on cells exposed to ^{99m}Tc, exponentially growing *E. coli* K12S cultures were centrifuged, washed and suspended in 0.9 percent NaCl solution. The near-UV source, as described elsewhere [21], presents at least 90 percent emission lines at 365nm, and the fluency rate at the level of the preparation was determined with a VLX365 radiometer (Vilbert Lourmat, France). All monochromatic irradiations were performed in Petri dishes, diameter of 3.5 cm, containing 1.5 ml of the cell suspension. The dishes remained on ice and were shaken on a vibrator to allow a uniform distribution of cells within the irradiated volume for 60 minutes (90 kJ/m^{-2}). After this period of time, 0.5 ml of ^{99m}Tc or of 0.9 percent NaCl solution was added, following incubation at 37°C, with shaking for 180 minutes. At 60 minutes intervals, aliquots (0.1 ml) were withdrawn and plated for the determination of the survival fraction (Table 5) [21, 32].

RESULTS AND DISCUSSION

Table 1 shows that the survival fraction is higher in the experimental scheme where AE and ICE were eliminated by the glass-wall tube. When ^{99m}Tc solution was outside the culture tube, the glass wall blocked the passage of AE and ICE, but not the γ radiation. In the control, the culture was incubated in contact with ^{99m}Tc in the same glass tube [32]. The results indicate that *Auger* and conversion electrons are more important than gamma radiation to the ^{99m}Tc decay-induced inactivation effects. After an incubation time of 180 minutes, the survival fraction falls when the electrons are in contact with culture. We can speculate that this lethal effect can be attributed (i) directly to these electron emissions that present a higher ionization density when compared with that of γ rays and consequently a stronger damage potentiality and/or (ii) indirectly, by the generation of ROS. Those hypotheses are reinforced by the fact that the biological effects are almost totally abolished when ^{99m}Tc was not in contact with the culture, a situation in which the glass wall blocks the electrons emissions [32].

The results above are in accordance with other authors [11, 13, 14]. They have employed different methodologies and have reported effects of AE and ICE emitters as ^{99m}Tc. Several *in vitro* studies have demonstrated that the biological effects of *Auger* emitters can be severe and are primarily dependent on the subcellular distribution of the radionuclide. These electrons present high linear-energy transfer (LET) when the radionuclide is localized in the cells nucleus and/or is incorporated into the DNA. In contrast, when the electrons are localized in the cytoplasm, the biological effects are only as effective as low-LET radiations. Monte Carlo calculation indicates that an average of about four AE are emitted per decay of ^{99m}Tc [11, 13, 14].

The contact of ^{99m}Tc with cells can produce many kinds of damage especially on the DNA molecules, offering risks to the exposed specie. The AE and ICE emissions can generate ROS in the medium and/or can directly interact with cells causing lesive effects, like chromosome damages and cell division inhibition in leukocytes labeled with ^{99m}Tc [15, 16].

The most important mechanism of oxygen activation by transition metals involves Fenton/Haber-Weiss chemistry and autoxidation. The first allows for efficient conversion of H_2O_2 and $O_2^{\bullet -}$, two common metabolic products that do not react with DNA, into a powerful DNA-damaging •OH radical:

$$M^{n+} + H_2O^2 \rightarrow M^{(n+1)+} + {}^{-}OH + {}^{\bullet}OH \quad (1)\ \text{(Fenton)}$$

$$M^{(n+1)+} + O_2{}^{\bullet -} \rightarrow M^{n+} + O_2 \quad (2)$$

The balance of those two reactions is:

$$H_2O_2 + O_2{}^{\bullet -} \rightarrow O_2 + {}^{-}OH + {}^{\bullet}OH \quad (3)\ \text{(Haber-Weiss)}$$

Two oxidation states of the metal cation (M^{n+} and $M^{(n+1)+}$) form catalytic electron transfer (redox) couple. In the absence of chelators, the above reactions are driven by some ions, as Fe^{+2} and Cu^{+1} [22-26].

The results obtained by the prior treatment with metal ion chelators, EDTA or dipyridyl (Table 2), point to a very important role of ROS, as mediators on the inactivation effect induced by ^{99m}Tc decay. Prior treatment with both the metal ion chelators protect *E. coli* K12S cells against the lethal effects of ^{99m}Tc; these data indicate the participation of metals, mainly iron ions, in the generation of ROS by ^{99m}Tc through the Fenton reaction and Haber-Weiss cycle in bacterial cells [22-24]. This protection effect may be due to the capture of Fe^{+2} ions, therefore blocking the formation of ROS.

The simultaneous treatment with sodium benzoate or thiourea (Table 3) confers to the cells partial protection against the lethal effects of ^{99m}Tc, indicating •OH as the main damaging agent. These data indicate, probably, the participation of iron in the formation of •OH by H_2O_2 through the Fenton reaction [22-26].

The biological effects of $SnCl_2$ have been reported by many authors [17-21] and these effects have been attributed to the generation of ROS when the stannous ion is oxidized ($Sn^{+2} \rightarrow Sn^{+4}$). It has been shown that if ^{99m}Tc, originally obtained as pertechnetate, is in presence of a reducing agent like $SnCl_2$, it can bind to cells and molecules in lower valences [2]. The protection of the culture against the effect of $SnCl_2$ by the pre-treatment of the cells with ^{99m}Tc (Table 4) suggests that in the reducing process of ^{99m}Tc (TcO_4^-), $SnCl_2$ would be oxidized and lose part of its lethal effect.

Table 4. Kinetics of inactivation of *E. coli* K12S pre-treated with ^{99m}Tc (final activity: 37 MBq/ml) and exposed to $SnCl_2$ (final concentration: 50 μg/ml).

Incubation time (min)	Cells treated with ^{99m}Tc for 180 min	Cells pre-treated with ^{99m}Tc for 60 min and then exposed to $SnCl_2$ for 120 min	Cells treated with $SnCl_2$ for 180 min	NaCl 0.9% solution for 180 min (control)
0	1	1	1	1
60	4.4×10^{-1}	3.7×10^{-1}	9.0×10^{-1}	1
120	9.5×10^{-2}	5×10^{-4}	1.94×10^{-2}	1
180	5.6×10^{-2}	2×10^{-4}	9.6×10^{-6}	1

Exponentially growing cultures were centrifuged, washed in 0.9 percent NaCl and suspended in 0.9 percent NaCl solution. The bacterial suspension (1-2 x 10^8 cells/ml) was pre-treated with ^{99m}Tc for 60 minutes. After this time, the bacterial suspension was exposed to $SnCl_2$ for 120 minutes, at 37°C, with shaking. The control not pre-treated was incubated, as shown above, for 180 minutes. After each incubation time, aliquots (0.1 ml) were taken, diluted and plated onto Luria Broth medium for determination of the survival fractions (N/N_0). Values represent means of three independent determinations. Standard deviations did not exceed 15 percent.

Table 5. Kinetics of inactivation of *E. coli* K12S pre-treated with near-UV (90kJ/m^2) and exposed to ^{99m}Tc (final activity: 37 MBq/ml).

Incubation time (min)	Cells treated with ^{99m}Tc for 180 min	Cells pre-treated with near-UV for 60 min and then exposed to ^{99m}Tc for 180 min	Cells treated with near-UV for 180 min	NaCl 0.9% solution (control)
0	1	1	1	1
60	4.4×10^{-1}	3.2×10^{-1}	9.0×10^{-1}	1
120	9.5×10^{-2}	1×10^{-1}	8.6×10^{-1}	1
180	5.6×10^{-2}	5.9×10^{-2}	8.6×10^{-1}	1

Exponentially growing cultures were centrifuged, washed in 0.9 percent NaCl and suspended in 0.9 percent NaCl solution. The bacterial suspension (1-2 x 10^8 cells/ml) was pre-treated with a sublethal dose (90 kJ/m^2) of broad band near-UV light (365 nm) for 60 minutes. After this time, the bacterial suspension was treated with ^{99m}Tc for 180 minutes, at 37°C, with shaking. The control not pre-treated was incubated, as shown above, for 180 minutes. After each incubation time, aliquots (0.1 ml) were taken, diluted and plated onto Luria Broth medium for determination of the survival fractions (N/N_0). Values represent means of three independent determinations. Standard deviations did not exceed 15 percent.

In the literature we can find many studies reporting that the near-UV irradiation can generate ROS, as H_2O_2 and singlet oxygen [28, 29]. Another effect observed with the near-UV irradiated bacterial cultures is the growth delay [28]. In this case, the near-UV light exposition (concomitant treatment or prior treatment with physical agent followed ^{99m}Tc exposition) does not alter the citotoxicity caused for the radionuclide, at least, under our experimental conditions, as shown in Table 5.

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