

Nanotamoxifen Delivery System: Toxicity Assessment After Oral Administration and Biodistribution Study After Intravenous Delivery of Radiolabeled Nanotamoxifen

Jaya Shukla, Amit Kumar Dinda¹, Abhay Krishna Srivastava², Kamna Srivastava³, Bhagwant Rai Mittal, Guru Pad Bandopadhyaya⁴

Department of Nuclear Medicine and PET, Post Graduate Institute of Medical Education and Research, Chandigarh, ¹Departments of Pathology, ²Lab Medicine and ⁴Nuclear Medicine and PET, All India Institute of Medical Sciences, New Delhi, ³Molecular Cardiology, Dr. BR Ambedkar Center for Biomedical Research, University of Delhi, Delhi, India

Abstract

Tamoxifen is the most prescribed anticancer oral drug for increasing overall survival and decreasing recurrence and the risk of contralateral disease. However, some side effects, such as endometrial and liver tumors, thromboembolic disorders, and drug resistance, are associated with long-term tamoxifen treatment. We assessed the hematologic and organ toxicity after oral administration of three different doses of nanotamoxifen formulations. We also performed biodistribution studies of Technetium-99m (^{99m}Tc)-nanotamoxifen after intravenous administration. The results demonstrated that nanotamoxifen was well-tolerated, with no adverse effect on biochemical parameters of blood and at the cellular level. Nitric oxide (NO) levels indicated no free radical formation. Oral nanotamoxifen is well-tolerated, with no hepatic or renal toxicity. Intravenous nanotamoxifen has potential to escape the liver, and is known for producing the harmful metabolite 4-hydroxytamoxifen (4OH-tamoxifen), which can cause uterine cancer.

Keywords: Biodistribution, delivery system, free radical formation, nanotamoxifen, oral dose, radiolabeling, toxicity

Introduction

Tamoxifen is a potent anticancer agent known to interrupt the enhanced estrogen activity of malignant mammary gland cells and approved by the U.S. Food and Drug Administration (FDA) as an adjuvant therapy for breast cancer. Tamoxifen is the most widely prescribed anticancer drug across the globe. Tamoxifen benefits the breast cancer patients with an increase in overall survival,

decreases in recurrence and the risk of contralateral disease.^[1] Tamoxifen has beneficial effects on bone density and lipid profiles. It displays estrogen-like effects in the relevant organs and tissues, and decreases the incidents of cardiovascular mortality.^[2-4] Tamoxifen antagonizes estrogen (E2)-regulated gene expression, and can promote the reexpression of E2-repressed genes and regulate the expression of E2-independent genes.^[5] Tamoxifen is most beneficial for younger women with an elevated risk of breast cancer. It decreases the incidence of invasive and noninvasive breast cancer, and is also involved in the reduction of the incidence of fractures.^[6-8] In various meta-analysis of tamoxifen therapy, the incidents of contralateral breast cancers decreased to 50% in women receiving postoperative adjuvant tamoxifen therapy, as compared to untreated women in the control group.^[9-15] Following long-term

Access this article online

Quick Response Code:



Website:
www.wjnm.org

DOI:
10.4103/1450-1147.167594

Address for correspondence:

Dr. Jaya Shukla, Department of Nuclear Medicine and PET, Post Graduate Institute of Medical Education and Research, Chandigarh - 160 012, Haryana and Punjab, India. E-mail: shuklajaya@gmail.com

therapy, tamoxifen can induce endometrial and liver cancer, thromboembolic disorders, the drug resistance of tumors and other disorders.^[1,16,17]

Solid tumors possess unique pathophysiological characteristics, such as extensive angiogenesis, defective vascular architecture, impaired lymphatic drainage and recovery system, and increased production of a number of permeability mediators. These result in leaky tumor vasculature with enhanced capacity for the uptake of macromolecules, including colloidal drug carriers, and are collectively known as the enhanced permeability and retention (EPR) effect.^[18,19] In an attempt to increase the local concentration of tamoxifen in estrogen receptor (ER)-positive breast cancer cells, a nanotamoxifen was formulated and characterized.^[20] The anticancer efficacy of such a system was increased many folds. It could be predicted that such a delivery system might provide better therapeutic benefits by delivering the drug locally for a longer period of time and reducing the systemic side effects.

Nitric oxide (NO) decreases the activity of platelets and neutralizes free radicals, and thus helps in preventing atherosclerosis. NO is produced by the action of endothelial NO synthase (eNOS/NOS3) and neuronal NO synthase. Neuronal NO synthase (nNOS/NOS1) is a heme protein that exists in its inactive form as a monomer, but dimerizes before action. Inducible NO synthase (iNOS/NOS2) is induced mostly during inflammation, and is expressed on macrophages, smooth muscle cells, and hepatocytes; it is responsible for pathological vasorelaxation. The eNOS is membrane-bound, while nNOS and iNOS are present in soluble form. NO is removed from circulation mostly by reaction with free radicals such as superoxides. Reduction of NO levels is the indicator of free radical formation.^[21]

We have undertaken this study in rats to determine the subacute toxicity in rats after daily oral dosing with nanotamoxifen for 15 days and 30 days, with a focus on endpoints commonly affected by tamoxifen. These include hematological aspects, organ histopathology, and NO levels.

Materials and Methods

Materials

Tamoxifen and 2-hydroxypropyl- β -cyclodextrin (HP β CD) were purchased from Sigma-Aldrich (Sigma-Aldrich, Co, USA) and nanotamoxifen was formulated in our lab. Animal experiments were done after taking permission from the institutional Ethics Committee for animals. The animals used, Wistar albino rats (200-250 g), were housed in cages. Forty female albino Wistar rats of 200-250 g body weight and aged around 7 weeks were obtained from the institute's

animal house and were housed in steel mesh cages under standard environmental conditions at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, in a 12-h light: 12-h dark cycle; a conventional rodent diet and water were allowed *ad libitum*; acidic Griess reagent (pH 2), glycine buffer (100 μM , glycine; 100 μM , NaCl; and 40 μM , HCl), and NaNO₂ were used for NO estimation.

Methods

Oral dosing and toxicity studies of nanotamoxifen

Wistar albino rats approximately 7 weeks old were marked and weighed before the start of oral treatment. A total of 30 female rats were divided into four groups: one control ($n = 6$) and three dose groups ($n = 8$ each) for nanotamoxifen. Nanotamoxifen was administered orally as an aqueous solution. Throughout the study, all animals were inspected twice daily. Nanotamoxifen was administered daily by gavage to all dose groups of for 15 days and 30 days at dose levels 5 $\mu\text{g}/\text{kg}$, 30 $\mu\text{g}/\text{kg}$, and 200 $\mu\text{g}/\text{kg}$ body weight. As previously described, 200 $\mu\text{g}/\text{kg}/\text{day}$ was taken as the maximum tolerated dose (MTD).^[22] Control rats were not given any treatment. After 15 days, four rats from each group were sacrificed and their organs (liver, kidney, breast, uterus, ovary, pancreas) were removed for histopathology. Blood was collected by cardiac puncture for plasma chemistry. Body weight loss/gain and the plasma chemistry of all groups including the control group were observed. Serum aspartate aminotransferase (AST) alanine transaminase (ALT), and alkaline phosphatase (ALP) levels were selected as parameters to measure hepatotoxicity. A standard curve was generated using different concentrations (50 μM , 25 μM , 12.5 μM , 6.25 μM , 3.125 μM , and 1.565 μM) of NaNO₂ and absorbance values were recorded by photometer at 546 nm. Blood samples were centrifuged at 500 rpm for 5 min and serum was collected. Equal volume of Griess reagent (pH 2) and 40 μL of glycine buffer were added and incubated for 15 min at 37°C , and the absorbance was recorded at 546 nm. NaNO₂ concentrations were extrapolated using standard curves.^[21]

Radiolabeling and biodistribution of nanotamoxifen

Nanotamoxifen was radiolabeled with Technetium-99m pertechnetate (^{99m}TcO₄⁻) by the stannous chloride reduction method, in which 100 μg nanotamoxifen was dissolved in sterile water for injection, stannous chloride in dil. HCl was added, and the pH was adjusted to 6.5. Labeling efficiency was assessed by instant thin-layer chromatography (ITLC) using methyl ethyl ketone (MEK) as a solvent front. For biodistribution studies, radiolabeled nanotamoxifen was injected in normal Wistar albino rats ($n = 6$) and the images were acquired under gamma camera (Mill VG, G E Electronics,

Hyfa Israel) 15 min, 1 h, and 3 h after intravenous injection ($n = 3$). The animals were sacrificed after the images were acquired. The organs were removed and counted by using a scintillation well counter (Biodex, Biodex Medical Systems, Inc. USA). The percentage of injected dose per gram of the tissues (%ID/g) was calculated.

Statistical analysis

Quantitative variables have been expressed as the mean and standard deviations have been used to summarize qualitative data. Two-sample Wilcoxon rank-sum (Mann-Whitney) tests were used to assess treatment-related toxicity at various time points. $P < 0.05$ was considered to indicate a significant difference at 15 days and 1 month.

Results

General condition and body weight

The rats were in good condition throughout the study period. The doses were well-tolerated. All animals in all dose groups showed normal feed consumption. The body weight was independent of the amount of doses, and there was no effect on body weight during the study period ($P > 0.05$).

Blood biochemical parameters

The nanotamoxifen particles were spherical and nanometer-sized, ranging 60-180 nm.^[20] The nanotamoxifen was administered orally to normal Wistar albino rats. Their serum creatinine (cre) levels after 15 days for control, 5- μ g, 30- μ g, and 200- μ g doses were 0.40 ± 0.14 , 0.80 ± 0.14 ; 0.88 ± 0.05 ; and 0.60 ± 0.24 , respectively. The results demonstrated an increase in cre after 15 days of daily administration, but the levels were decreased to the baseline level after 1 month of daily dosing [Figure 1a]. No morphological nephrocellular

injuries were observed in the histopathology of kidneys of the rats after 15 days or 30 days from oral nanotamoxifen administration.

AST (360 ± 111.13 , 552.50 ± 67.66 , 604 ± 46.71), ALT (443.25 ± 6.65 , 811 ± 14.83), and ALP (590.25 ± 56.21 , 617.75 ± 83.64 , 704.5 ± 27.29) levels in blood were increased from baseline after 15 days of oral dosage of nanotamoxifen [Figure 1b]. However, the levels of these enzymes were restored within 30 days of oral administration. Hepatocellular necrosis, fatty changes, and fibrosis were not observed even after 1 month of oral dosing in all dose groups. The levels of bilirubin (bil), albumin (alb), globulin (glob), total protein (tp), uric acid (ua), total cholesterol (tc), and urea remained unaltered throughout the study period [Figure 1]. No significant difference was observed in the nitrite level, an index of NO, with either of the three nanotamoxifen doses (5 μ g, 30 μ g, and 200 μ g) even after 1 month of oral administration [Table 1].

On histopathological studies, ovary showed no unusual maturation with all three doses up to 1 month. In the uterus, the epithelium, subepithelium, and muscles were normal. Overall, the endometrium mucosa and cervix were normal with 5- μ g and 30- μ g doses. Mild increases in cellular proliferation in the endometrium and mucus accumulation in cervical cells, with 200- μ g/kg doses, was observed at 30 days (1 month). Breast tissue showed no abnormal change throughout the study period.

Biodistribution study

Nanotamoxifen was labeled with ^{99m}Tc , with $>98\%$ labeling efficiency. ^{99m}Tc -nanotamoxifen imaging after 15 min showed the heart, kidney, and bladder activities. The 3hr image [Figure 2] show high concentration of ^{99m}Tc -nanotamoxifen in urinary bladder and kidneys. The rats were sacrificed after imaging and the organs

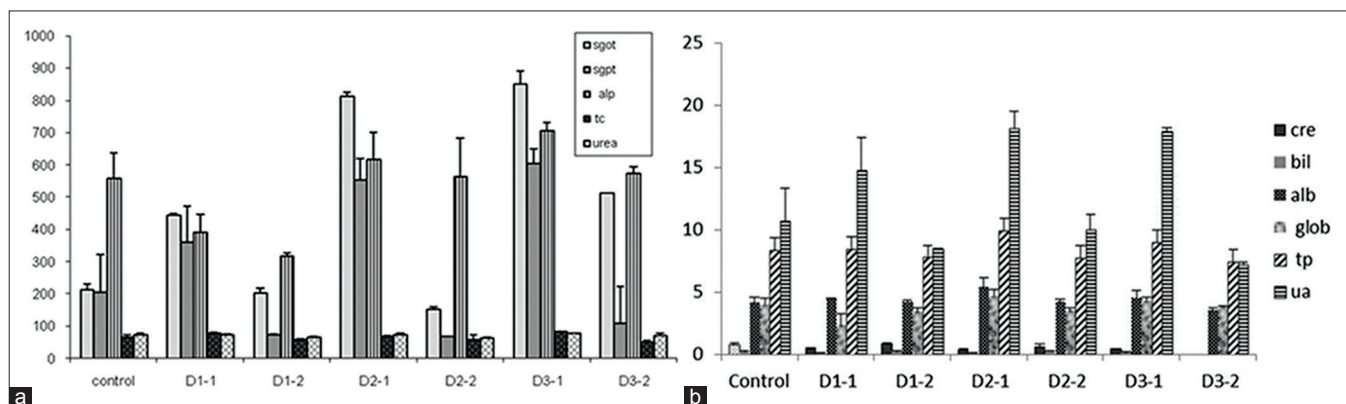


Figure 1: (a) Histogram showing average \pm S.D ($n = 3$) of cre, bil, alb, glob, tp, ua per dose group (D1 = 5 μ g, D2 = 30 μ g, D3 = 200 μ g) for 15 days (-1) and 30 days (-2) of exposure to oral nanotamoxifen (b) Histogram showing average \pm S.D ($n = 3$) of serum glutamic oxaloacetic transaminase (SGOT) or AST, serum glutamic-pyruvic transaminase (SGPT) or ALT, ALP, tc, urea per dose group D1 = 5 μ g, D2 = 30 μ g, D3 = 200 μ g for 15 days (-1) and 30 days (-2) exposure to oral nanotamoxifen

were counted in a well counter. The histogram represents the average percentage of injected dose per gram [Figure 3]. Approximately 14% ID/g in blood, ~31% ID/g in kidney, ~1.3% ID/g in heart, ~2.2% ID/g in spleen, ~3% ID/g in lung ~7% ID/g in liver, and ~2% ID/g in bone were observed. Ovary, uterus, and breast showed 1.66% ID/g, 1.54% ID/g, and 1.45% ID/g activity, respectively. Intestine and thyroid exhibited negligible ^{99m}Tc -nanotamoxifen uptake.

Discussion

The oral tamoxifen administration has no significant effect on the body weight of the rats at 15 days and at 1 month. The levels of bil, alb, glob, tp, ua, tc, urea remained unaltered throughout the study period. The results showed an increase in cre after 15 days of administration, but the cre levels were decreased to the baseline level, the same as that of control, after 1 month of administration. No morphological nephrocellular injuries were observed on histopathology of the kidneys of the rats after 15 days as well as 30 days of oral nanotamoxifen administration. AST, ALT, and ALP levels in blood were also increased after 15 days of daily oral dosing, but were reduced to baseline levels within 30 days with continuing oral administration. One month of oral dosing showed no cellular toxicity in histopathology. These results demonstrated that nanotamoxifen was well-tolerated in the liver and also that the nanotamoxifen had no adverse effect on the biochemistry of blood.

The biodistribution studies of nanotamoxifen demonstrated predominantly renal excretion [Figure 2]

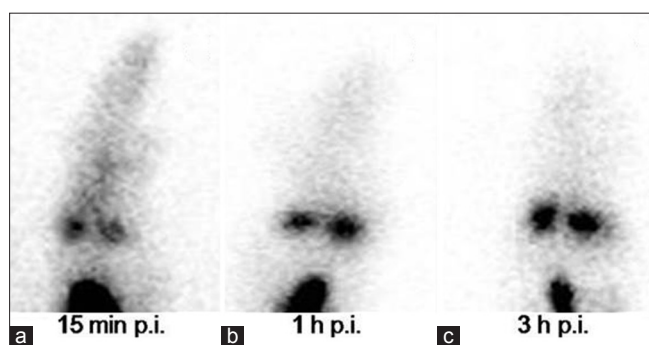


Figure 2: Gamma camera images of Albino wistar rats after Tc-99m-nanotamoxifen injection from tail vein. (a) 15 min, (b) 1hr, (c) 3hr post intravenous injection. B-blood, Bo-bone, M-muscle

and low uptake in the rest of the tissues. Nanotamoxifen showed excellent *in vivo* stability, as no uptake, at any point of time, was visualized in the thyroid. ^{99m}Tc -nanotamoxifen remained in the circulation at high levels after intravenous injection (14% after 3 h). The liver uptake was only ~7% ID/g after 3 h [Figure 3]. Normally, nanoparticles are recognized as foreign and removed by the reticuloendothelial system (RES), the defence mechanism of the body. Our nanotamoxifen particles were prepared with HP β CD, rich in hydroxyl moiety (i.e. it is hydrophilic), and helped to escape liver (RES). The circulating nanoparticles will concentrate in the tumor due to the property of EPR of tumor vasculature.^[18,19] Tamoxifen is metabolized in the liver to 4-hydroxytamoxifen (4OH-tamoxifen) and N-desmethyltamoxifen. 4OH-tamoxifen is an active metabolite and responsible for cellular proliferation in uterus.^[23] NO is short-lived, diffusible, and cytotoxic. The nitrite level, an index of NO, was unaltered in the treated rats and the controls, with no pathophysiological alterations in liver tissue, indicating that there was no free-radical production during nanotamoxifen treatment.^[21]

Conclusion

Nanotamoxifen is safe as an oral formulation. Radiolabeled nanotamoxifen when given via intravenous route could escape the liver and may have further application as a theranostic tool for ER-positive breast cancer.

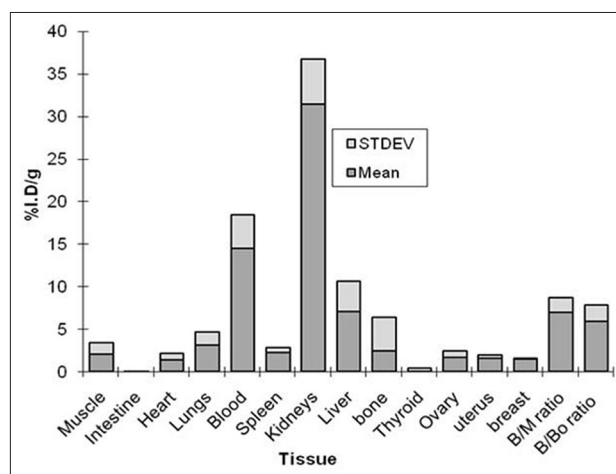


Figure 3: ^{99m}Tc -nanotamoxifen-organ distribution (3 h post injection) in albino Wistar rats

Table 1: Mean serum concentration of NaNO_2 after 15 days and 30 days of daily oral administration of nanotamoxifen, 5 μg , 30 μg , and 200 μg , in albino Wistar rats

	Control group	Nanotamoxifen 5 μg	Nanotamoxifen 30 μg	Nanotamoxifen 200 μg
15 days μM of NaNO_2	17.86 \pm 1.08	18.88 \pm 1.26	17.19 \pm 1.48	22.65 \pm 1.78
30 days μM of NaNO_2	21.38 \pm 0.98	20.03 \pm 1.11	18.88 \pm 1.56	21.60 \pm 2.60

Acknowledgments

This work is funded by the Department of Science and Technology, the Ministry of Science and Technology, India. The authors are thankful to the Department of Pathology, Department of Lab Medicine and the animal house, All India Institute of Medical Sciences, New Delhi for their support in completing the work.

References

- Day R; National Surgical Adjuvant Breast and Bowel Project P-1 study (NSABP-1). Quality of life and tamoxifen in a breast cancer prevention trial: A summary of findings from the NSABP P-1 study. National Surgical Adjuvant Breast and Bowel Project. *Ann N Y Acad Sci* 2001;949:143-50.
- Bilimoria MM, Assikis VJ, Jordan VC. Should adjuvant tamoxifen therapy be stopped at 5 years? *Cancer J Sci Am* 1996;2:140-50.
- McDonald CC, Stewart HJ. Fatal myocardial infarction in the Scottish adjuvant tamoxifen trial. The Scottish Breast Cancer Committee. *BMJ* 1991;303:435-7.
- McDonald CC, Alexander FE, Whyte BW, Forrest AP, Stewart HJ. Cardiac and vascular morbidity in women receiving adjuvant tamoxifen for breast cancer in a randomized trial. The Scottish Cancer Trials Breast Group. *BMJ* 1995;311:977-80.
- Frasor J, Stossi F, Danes JM, Komm B, Lyttle CR, Katzenellenbogen BS. Selective estrogen receptor modulators: Discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. *Cancer Res* 2004;64:1522-33.
- Gail MH, Costantino JP, Bryant J, Croyle R, Freedman L, Helzlsouer K, *et al.* Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst* 1999;91:1829-46.
- Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, *et al.* Tamoxifen for prevention of breast cancer: Report of the national surgical adjuvant breast and bowel project P-1 study. *J Natl Cancer Inst* 1998;90:1371-88.
- Jahanzeb M. Reducing the risk for breast cancer recurrence after completion of tamoxifen treatment in postmenopausal women. *Clin Ther* 2007;29:1535-47.
- Tamoxifen for early breast cancer: An overview of the randomized trials. Early Breast Cancer Trialists' Collaborative Group. *Lancet* 1998;351:1451-67.
- Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer. *Cochrane Database Syst Rev* 2001;CD000486.
- Clarke MJ. WITHDRAWN: Tamoxifen for early breast cancer. *Cochrane Database Syst Rev* 2008;CD000486.
- Fisher B, Costantino J, Redmond C, Poisson R, Bowman D, Couture J, *et al.* A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. *N Engl J Med* 1989;320:479-84.
- Raloff J. Tamoxifen quandary. *Science News* 1992;141:266-9.
- Diver MJ, Jackson IM, Fitzgerald JD. Tamoxifen and non-malignant indications. *Lancet* 1986;1:733.
- Fentiman IS, Powles TJ. Tamoxifen and benign breast problems. *Lancet* 1987;2:1070-2.
- Killackey MA, Hakes TB, Pierce VK. Endometrial adenocarcinoma in breast cancer patients receiving antiestrogens. *Cancer Treat Rep* 1985;69:237-8.
- Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfverswärd C, *et al.* Adjuvant tamoxifen in early breast cancer: Occurrence of new primary cancers. *Lancet* 1989;1:117-20.
- Duncan R, Sat YN. Tumor targeting by enhanced permeability and retention (EPR) effect. *Ann Oncol* 1998;9(Suppl 2):39-50.
- Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: A review. *J Control Release* 2000;65:271-84.
- Shukla J, Sharma U, Kar R, Varma IK, Juyal S, Jaganathan NR, *et al.* Tamoxifen-2-Hydroxylpropyl-beta-cyclodextrin-aggregated nanoassembly for non breast er-positive cancer therapy. *Nanomedicine (Lond)* 2009;4:895-902.
- Galea E, Feinstein DL, Reis DJ. Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures. *Proc Natl Acad Sci U S A* 1992;89:10945-9.
- Kennel P, Pallen C, Barale-Thomas E, Espuña G, Bars R. Tamoxifen: 28-day oral toxicity study in the rat based on enhanced OECD test Guideline 407 to detect endocrine effects. *Arch Toxicol* 2003;77:487-99.
- Reed CA, Berndtson AK, Nephew KP. Dose-dependent effects of 4-hydroxytamoxifen, the active metabolite of tamoxifen, on estrogen receptor-alpha expression in the rat uterus. *Anticancer Drugs* 2005;16:559-67.

How to cite this article: Shukla J, Dinda AK, Srivastava AK, Srivastava K, Mittal BR, Bandopadhyaya GP. Nanotamoxifen delivery system: Toxicity assessment after oral administration and biodistribution study after intravenous delivery of radiolabeled nanotamoxifen. *World J Nucl Med* 2016;15:7-11.

Source of Support: Department of Science and Technology, India.
Conflict of Interest: None declared.