not find any bullous or purpuric or necrotic lesions with eschar formation, which are known to occur with warfarin-induced skin necrosis. Because the patient also had been receiving long-term subcutaneous heparin therapy, especially in the anterior abdominal wall bilaterally, we felt that the most plausible explanation was skin necrosis or local changes in subcutaneous fat induced by the heparin administration, leading to the deposition of ^{99m}Tc-MDP. Lipoatrophy caused by long-term insulin therapy was excluded because the patient had been receiving the insulin predominantly in the thighs and the whole-body bone scan did not reveal soft-tissue uptake of ^{99m}Tc-MDP anywhere except in the abdomen.

In our patient, the incidental finding of extraskeletal uptake of ^{99m}Tc-MDP was thought to be most likely due to subcutaneous heparin injections. These caused either skin necrosis and microscopic dystrophic calcification in the anterior abdominal wall or an altered biochemical milieu in subcutaneous fat, leading to dissociation of the technetium from the radiopharmaceutical.

CONCLUSION

Interpretation of the soft-tissue uptake of bone scanning agents is always an interesting academic exercise, presenting the nuclear medicine physician and radiologist with a wide range of possibilities. Although in most instances the cause is straightforward, physicians are sometimes confronted with a more complex clinical situation in which any one of many agents might have caused the extraskeletal uptake of the bone scanning agent in a patient. In our patient, the incidental finding of the extraskeletal uptake of ^{99m}Tc-MDP was thought to be most likely attributable to subcutaneous heparin injections, causing either skin necrosis and microscopic dystrophic calcification in the anterior abdominal wall.

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Transferrin-Dependent Uptake and Dosimetry of Auger-Emitting Diagnostic Radionuclides in Human Spermatozoa

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Localization of Auger-emitting radionuclides within spermatozoa could lead to the induction of transmissible genetic damage. We have quantified in vitro uptake of the widely used diagnostic Auger-emitters, ^{111}In and ^{99m}Tc, by ejaculated human spermatozoa and investigated the role of transferrin in their cellular localization. The resultant dose to sperm heads, including cellular dosimetry for Auger emissions, has been calculated for each radionuclide and compared with that achieved using conventional macrodosimetry. **Methods:** Freshly isolated human spermatozoa were incubated in a physiological salt solution containing ^{111}In-chloride, ^{99m}Tc-pertechnetate or the transferrin-binding isotope ^{59}Fe-citrate as a positive control. Cellular uptake mechanisms were investigated with transferrin competition and temperature dependence studies. The percentage uptake of each radionuclide was determined, and the dose to individual sperm heads was calculated using both conventional macrodosimetric methods and by consideration of radionuclide localization and energy deposition at the cellular level, including Auger electron emissions from ^{111}In and ^{99m}Tc. **Results:** On in vitro incubation, human spermatozoa were found to accumulate ^{111}In and ^{59}Fe but not ^{99m}Tc. Cell uptake of ^{111}In and ^{59}Fe was transferrin-mediated; however, an alternative transferrin-independent uptake pathway was also present for ^{111}In. The dose to sperm heads from ^{111}In, calculated using measured uptake and cellular dosimetry, was found to be larger than that calculated using conventional dosimetry by a factor of more than 100. In contrast, conventional dosimetry was adequate for ^{99m}Tc and ^{59}Fe. **Conclusion:** Isolated human spermatozoa appear to accumulate transferrin-binding isotopes, such as the Auger-emitter ^{111}In. If this uptake mechanism operates in the male reproductive tract, the resultant high dose to the sperm head could indicate that contraception may be advisable after large diagnostic doses of ^{111}In and, possibly, other transferrin-binding radionuclides. Such precautions could prevent transmission of any genetic damage from irradiated spermatozoa.

Key Words: spermatozoa; transferrin; radionuclides; Auger emissions; dosimetry

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Transferrin-binding radionuclides, such as ^{59}Fe and ^{111}In, are commonly used in diagnostic nuclear medicine. Laboratory and

clinical studies have reported localization of such radionuclides within the groin and, possibly, testes of male subjects during imaging procedures (*1,2*). Our recent studies in rodents have suggested that some radionuclides may use the physiological iron-transferrin pathway to breach the blood-testis barrier and thus gain access to developing germ cells within the sanctuary of the seminiferous tubule (*3–5*). Although it is always difficult to extrapolate directly from rodents to humans, the transferrin pathway is ubiquitous among mammalian species (*6*) and is well-documented in the testes (*7*). Thus, it is possible that transferrin-binding nuclides may be deposited in the human seminiferous epithelium by similar mechanisms. Such localization could result in isotope uptake by developing germ cells. Once mature germ cells have left the testis and are in the reproductive tract, they may also be subject to uptake radionuclides from the circulating blood pool.

The most radiologically important target in mature spermatozoa is the head, which forms a flattened ellipsoid of semiaxes approximately 2.5 μm × 1.25 μm × 0.75 μm (*8*). The head consists almost entirely of nucleus, containing highly condensed DNA and chromatin. Although spermatozoa are not as radiosensitive as earlier germ cell types (*9*), they have little capacity for DNA repair. Consequently, although radiation damage to spermatogonia may subsequently cause cell death, direct irradiation of spermatozoa may result in the induction of mutations that may not be expressed until after fertilization (*10*). Accurate dosimetry for such cells is necessary to determine whether contraception would be advisable for a relatively short period of time after clinical administration of the radiopharmaceutical.

Indium-111 is an emitter of Auger and Coster-Kronig electrons (*11*). The short range of such emissions (micrometers and below) leads to dose heterogeneity at cellular level; thus, the Medical Internal Radiation Dose (MIRD) system currently used to calculate absorbed doses to entire organs, assuming homogeneous source distributions and energy deposition, may be inadequate for such radionuclides (*12–15*).

In these studies, both quantitative and mechanistic aspects of radionuclide uptake by spermatozoa were investigated. The quantitative data have been further used to calculate the cellular dose arising from Auger emissions, where applicable. The dose thus obtained has been compared with that calculated using conventional MIRD dosimetry.

MATERIALS AND METHODS

Radionuclides and Dosimetry

The transferrin-binding radionuclides ^{59}Fe-citrate (185–370 MBq/mg) and ^{111}In-chloride (500–1850 MBq/μg) were investigated. Technetium-99m-pertechnetate (10–200 GBq/μg) does not bind to transferrin and was used as a control. Radiopharmaceuticals and specific activities were chosen to approach typical diagnostic values, although it is recognized that a wide variety of ^{111}In-labeled pharmaceuticals are used in diagnostic nuclear medicine.

The radiation dose to spermatozoa after intravenous radionuclide administration could arise from four possible sources:

1. Penetrating radiations from activity elsewhere in the body;
2. Activity in testicular tissue;
3. Activity in the fluids of the male reproductive tract, such as seminal fluid; and
4. Activity localized within spermatozoa themselves.

The conventional MIRD approach to macroscopic radiation dosimetry assumes a uniform activity distribution within each source organ. This methodology deals adequately with the first two sources and will also take account of the third, provided the activity concentration in the fluids of the reproductive tract is the same as in testicular tissue. However, the MIRD calculation does not take the fourth source of radiation dose, that arising from intracellular activity, into account. Therefore, we have adopted two distinct approaches in calculating the dose to spermatozoa (in milligray/MBq injected).

First, we have taken the dose to the testes as a whole from the International Commission on Radiological Protection (ICRP) Publication No. 53, which uses the MIRD methodology as a macroscopic estimate of the dose to sperm (*16*). Because the decay schemes used for the MIRD calculations involve all penetrating radiations and internal conversion electrons but only the more prominent Auger emissions, we have further calculated the additional dose due to the remaining Auger electron spectrum of ^{111}In and ^{99m}Tc (*11*), which could arise from activity in reproductive tract fluid. This appears to make a negligible correction to MIRD figures (approximately 1% for ^{111}In and 0.2% for ^{99m}Tc).

Second, we have determined the dose attributable to intracellular radionuclide localization within spermatozoa. This dose is dominated by contributions from short-range Auger electrons and has been calculated using a previously described technique to compute the dose per unit cumulated activity from Auger-emitters located within an ellipsoid of sperm head dimensions (*17*). The S factors were calculated, assuming that the radionuclide was either homogeneously distributed throughout the spermatozoa head (3.8×10^{-2} Gy/Bq) or localized within a 0.1-μm-thick outer shell (3.0×10^{-2} Gy/Bq). Cumulated activity per cell was calculated using the fractional uptake per cell determined from our in vitro uptake studies and an activity concentration in reproductive tract fluid equal only to nonspecific average body concentration.

The total dose to the spermatozoa in mGy/MBq injected activity was then determined from the sum of the conventional MIRD figure for ICRP Publication No. 53 and our calculation of the dose that is attributable to intracellular Auger electrons.

Cell Preparation

Semen samples were collected by masturbation from normal donors after 2 days of sexual abstinence. Spermatozoa were sedimented by centrifugation (400 × *g* for 8 min) and then resuspended in sperm culture fluid (SCF) and recentrifuged. This washing procedure was repeated, and then spermatozoa were resuspended in SCF at a concentration of $2–4 \times 10^6$/ml, except for the quantification studies in which cells were at 4×10^6/ml or 29×10^6/ml. Ejaculates containing less than 40×10^6 spermatozoa in total were not used.

Sperm culture fluid consisted of Earle's balanced salt solution supplemented with 4 m*M* $NaHCO_3$, 14 m*M* Na-lactate, penicillin and streptomycin. Osmolarity was 284 mOsm. SCF was prepared up to 1 mo before use and stored at 4°C. Human serum albumin (10%) was added immediately before use.

Transferrin Preparation

Human apotransferrin was purchased from Sigma Chemical Co., Poole, UK. Iron-saturated transferrin was prepared using iron-nitrilotriacetate according to methods described previously (*18*). Full saturation was confirmed by an $A_{465\ nm}$-to-$A_{280\ nm}$ ratio of 0.046. Samples with a ratio of less than 0.04 were discarded.

Experimental Protocols

The percentage of radionuclide taken up per million cells was determined by incubating known numbers of cells with 20 kBq of radionuclide within 1 ml of SCF. Ejaculates were diluted with SCF to a final concentration of 4×10^6 cells/ml or 29×10^6 cells/ml. The same quantities of radionuclide and SCF were dispensed into tubes without cells to act as controls. Cells and controls were incubated for 2 hr at 34°C, and then all samples were washed twice

TABLE 1
Uptake of Iron-59 and Indium-111 by Human Spermatozoa After a 2-hr Incubation

No. of cells/ml	^{59}Fe-citrate	^{111}In-chloride	^{99m}Tc-pertechnetate
4×10^6	0.48% ± 0.03%	11.2% ± 0.5%	No uptake above background
29×10^6	0.17% ± 0.09%	2.8% ± 0.2%	No uptake above background

Values show the percentage of total activity in 1 ml of incubation fluid taken up per 10^6 cells. Data are expressed as the mean ± s.e.m. for three separate ejaculates at each cell concentration.

at 4°C with Hanks' Balanced Salts Solution (HBSS). Sample volume was reconstituted to 1 ml in all tubes, including controls, and radioactivity was measured using a gamma counter. The results are expressed as percentage uptake per million cells.

To determine the effect of excess transferrin on cell radionuclide uptake, spermatozoa were incubated with 20–25 kBq of ^{59}Fe or ^{111}In in the presence of increasing concentrations of apotransferrin or diferric transferrin for 2 hr at 34°C. Concurrent controls were prepared for each experiment as described above. At the end of the experiment, samples were washed twice and treated with 0.5 mg/ml proteinase K on ice for 45 min to remove surface-bound transferrin. Samples were then washed again, and radioactivity was counted.

Radionuclide retention was monitored by incubating spermatozoa with the radionuclides overnight at 34°C to ensure a steady state was attained and then washing with HBSS. Radionuclide-loaded spermatozoa were reincubated in fresh (radionuclide-free) SCF, and aliquots of cells were removed at regular intervals and treated as described above.

The effects of temperature on radionuclide uptake were investigated by incubating spermatozoa with the radionuclides at 4°C or 34°C for 2 hr. Cells were then washed twice with ice-cold HBSS and treated for 45 min at 4°C with 0.25 mg/ml proteinase K in phosphate-buffered saline (PBS) or with PBS alone to distinguish between surface-bound and internalized radionuclides.

RESULTS

Radionuclide Uptake Studies and Dosimetry

The percentage uptakes of ^{111}In, ^{59}Fe and ^{99m}Tc by spermatozoa over a 2-hr incubation period are shown in Table 1. Data were obtained for samples containing both 4×10^6 spermatozoa/ml and 29×10^6 spermatozoa/ml, to reflect the wide range of ejaculate concentrations encountered in the general population (*19*). Although there was no significant uptake of ^{99m}Tc, both the indium and iron radionuclides were readily accumulated at each cell concentration; however, uptake of ^{111}In was markedly higher than that of ^{59}Fe. Radionuclide uptake per million cells was considerably higher in samples containing 4×10^6 cells than for samples containing 29×10^6 cells. This may be attributable to the higher radionuclide-to-cell ratio at low cell concentrations.

The dose to the sperm heads was calculated using the MIRD method supplemented by our own calculation of the additional dose due to Auger emissions from intracellular uptake. In the case of ^{111}In, where a value for percentage uptake was required for the calculation of the dose contributed by intracellular Auger electrons, the uptake value obtained for 29×10^6 cells was used because this sperm concentration is closer to the population average (*19*). As shown in Table 2, consideration of specific uptake and cellular dosimetry of Auger electrons significantly increases the dose attributable to ^{111}In, from 5.3×10^{-2} mGy/MBq injected to as much as 8.5 mGy/MBq injected. In contrast, intracellular localization has no effect on the dosimetry of ^{59}Fe because this isotope has no Auger component to its decay scheme (*11*).

Effect of Excess Transferrin on Spermatozoa Radionuclide Accumulation

To determine the role of transferrin in spermatozoa accumulation of ^{111}In and ^{59}Fe, competition studies were performed with both apotransferrin and diferric transferrin. Excess transferrin inhibited isotope uptake in a dose-dependent manner (Fig. 1). However, although apotransferrin and diferric transferrin had similar effects on ^{59}Fe uptake, with an IC_{50} around 30 μg/ml (Fig. 1A), apotransferrin was a far more potent inhibitor of ^{111}In uptake, with an IC_{50} of around 3 μg/ml compared to approximately 20 μg/ml for diferric transferrin (Fig. 1B).

Temperature Dependence of Radionuclide Uptake

Temperature dependence studies are shown in Table 3, in which data are expressed both as uptake per million cells in counts per minute and as a percentage of uptake at 34°C without proteinase K treatment. At 34°C, virtually all cell-associated ^{59}Fe or ^{111}In was proteinase K-resistant, suggesting that the majority of activity was internalized within the cell. At 4°C, uptake of ^{59}Fe was reduced by more than 80%, and proteinase K treatment did not alter the percentage of cell-associated radionuclide. In contrast, ^{111}In uptake was reduced by only 25% before proteinase K treatment and by a further 25% after proteinase K treatment to remove surface-bound radionuclide.

Retention of Radionuclides by Human Spermatozoa

Figure 2 shows the retention of ^{59}Fe and ^{111}In by spermatozoa over a 4-hr reincubation period. Cell-associated activity remained at around 100% for the duration of the reincubation, suggesting that uptake is unidirectional, and both radionuclides are retained within the cells.

DISCUSSION

Previously, we reported transferrin-mediated deposition of various transition metal and actinide radionuclides in the rodent testis (*3–5*). We have now extended these investigations to

TABLE 2
Calculated Sperm Head Doses

	^{59}Fe-citrate	^{111}In-chloride	^{99m}Tc-pertechnetate
MIRD (ICRP Publication No. 53)	5.0	5.3×10^{-2}	2.7×10^{-3}
From intracellular Auger electrons*	No Auger emissions	8.4†, 6.7‡	No intracellular uptake
Total dose	5.0	8.5†, 6.8‡	2.7×10^{-3}

* The lowest estimate of percentage uptake per million cells, determined for 29×10^6 cells/ml, was used for all calculations (see Table 1).
† = Radionuclide within sperm head assumed to be homogeneously distributed.
‡ = Radionuclide within sperm head assumed to be localized within a narrow (0.1-μm-thick) outer shell.
Values show the dose to the spermatozoa head in mGy/MBq injected. Data are expressed as the mean of triplicate samples.

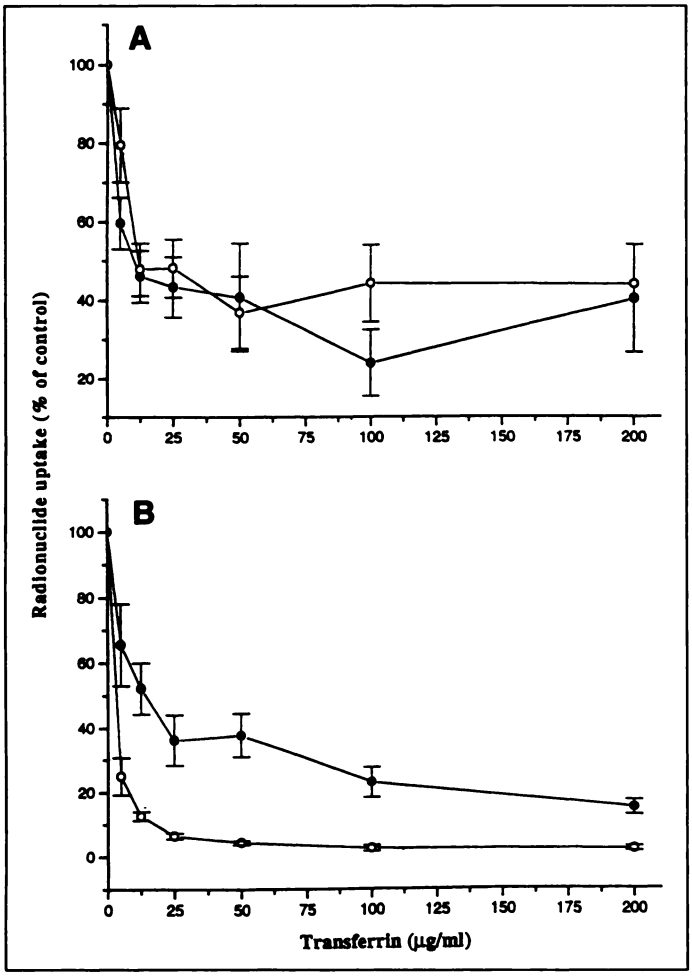

FIGURE 1. Effect of excess apotransferrin (○) or diferric human transferrin (●) on the uptake of ^{59}Fe (A) and ^{111}In (B) by isolated human spermatozoa after a 2-hr incubation at 34°C. Data represent mean ± s.e.m. for five independent experiments performed in quadruplicate.

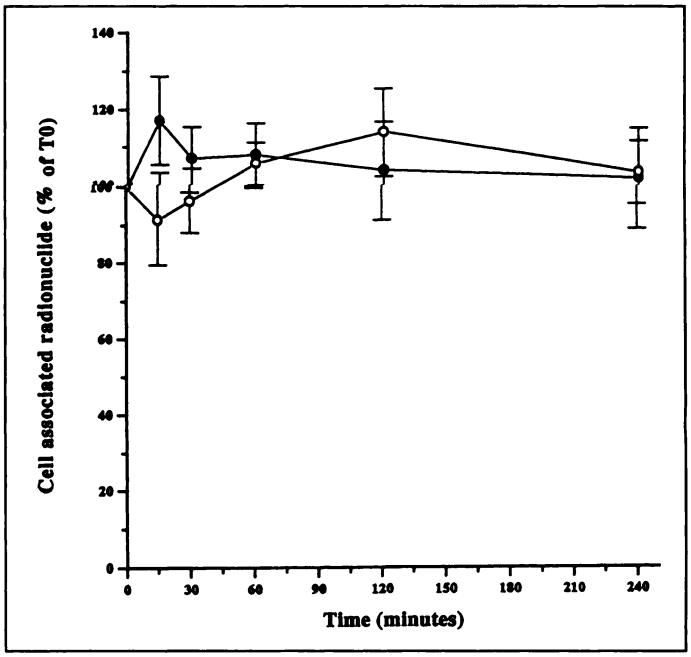

FIGURE 2. Retention of ^{59}Fe (●) or ^{111}In (○) by isolated human spermatozoa at 34°C. Data are expressed as a percentage of cell radionuclide content at T0, taken as the commencement of the reincubation period. Each point represents the mean ± s.e.m. for three independent experiments performed in triplicate.

study the role of transferrin in the accumulation of iron and indium radionuclides by human spermatozoa and the dosimetric implications of intracellular localization of these radionuclides. The assumption that spermatozoa express cell surface transferrin receptors is implicit to the interpretation of these studies. Although the interactions of iron and transferrin with Sertoli cells and earlier germ cell types are well-documented, comparatively little is known regarding iron accumulation by mature spermatozoa. Previous observations with antitransferrin receptor antibodies have indicated receptor expression on spermatocytes and round spermatids but not on elongated spermatids or spermatozoa (*20*) or confined to spermatocytes and early spermatids (*21*). In contrast, more recent data, showing the expression profile of transferrin receptor mRNA in rat adluminal germ cells, suggest that transferrin receptor mRNA is expressed throughout adluminal germ cell development, although this expression appears to decrease as cells differentiate from spermatocytes to elongated spermatids (*22*). This is probably attributable to a decreasing need to acquire and store iron as cells approach terminal differentiation and spermiation and is consistent with previous observations that rapidly proliferating cells express high levels of transferrin receptors (*6*). Because all cells have an obligate requirement for iron as a constituent of biological reduction-oxidation systems, however, it is conceivable that even mature cell types, such as spermatozoa in the reproductive tract, may express low levels of cell surface receptor to obtain sufficient iron for maintenance of intracellular systems. Our current data would support this contention; however, further studies are required to demonstrate conclusively that mature spermatozoa do express surface transferrin receptors.

TABLE 3
Effect of Temperature and Proteinase K Treatment on Uptake of Iron-59 and Indium-111 by Human Spermatozoa

Incubation conditions	Cell-associated ^{59}Fe	Cell-associated ^{111}In
34°C, no PK treatment	4,582 ± 242 (100)*	84,218 ± 9,168 (100)
34°C, PK treatment	3,569 ± 565	70,231 ± 15,641
	(77.9 ± 12.3)	(85.2 ± 18.6)
4°C, no PK treatment	659 ± 53	65,387 ± 3,584
	(14.4 ± 1.1)	(79.3 ± 4.2)
4°C, PK treatment	939 ± 123	40,169 ± 404
	(20.5 ± 2.6)	(48.7 ± 0.5)

*Values in parentheses show cell-associated activity expressed as a percentage of the control value (taken as uptake at 34°C without PK treatment).

Values represent counts per minute per million cells after a 2-hr incubation, followed by 45 min of treatment with 0.25 mg/ml PK pr PBS alone. Data are expressed as the mean ± s.e.m. for triplicate samples. PK = proteinase K.

Both ^{59}Fe-citrate and ^{111}In-chloride were readily accumulated by human spermatozoa in vitro, albeit to differing degrees. In contrast, no significant uptake of ^{99m}Tc-pertechnetate was observed. The inhibition of ^{59}Fe uptake by transferrin suggests competition for cell surface transferrin receptors between iron-transferrin and radionuclide-labeled transferrin, indicating that ^{59}Fe uptake by spermatozoa is likely to occur via a receptor-mediated route. Although the degree of inhibition of ^{59}Fe uptake was similar regardless of the degree of saturation of added transferrin, ^{111}In uptake was inhibited to a greater extent by nonsaturated-transferrin than by iron saturated transferrin. These data suggest that part of the apparent inhibition of ^{111}In accumulation by nonsaturated-transferrin is attributable to a lowering of the bioavailability of elemental indium rather than

to receptor blockade. This implies that spermatozoa may accumulate indium via both transferrin-dependent and -independent pathways.

The observation of marked inhibition of ^{59}Fe uptake at low temperature is consistent with uptake via an active transport route, presumably, receptor-mediated endocytosis. Conversely, spermatozoa accumulation of ^{111}In at 4°C was still 50% of that achieved at 34°C, even after proteinase K treatment to cleave cell surface radionuclide. The exact nature of the alternative uptake pathway for ^{111}In is not clear. These current data suggest that this process is unidirectional, relatively temperature-insensitive and not mediated via the transferrin receptor. Further studies are required to elucidate this pathway more thoroughly, however, because the mode of uptake of ^{111}In by spermatozoa is likely to affect the subsequent subcellular distribution, this has marked dosimetric implications for an Auger-emitting radionuclide.

The conditions used in these studies, in which spermatozoa have been incubated ex vivo in a physiological salt solution containing high activities of isotope, will clearly differ from the in vivo situation in which radionuclide access to the reproductive tract may be limited by the presence of the blood-testis barrier, the luminal wall of the epididymis and the rest of the male reproductive tract. However, seminal fluid contains relatively high concentrations of transferrin, and approximately 80% of this is secreted by testicular Sertoli cells (*23*). We have previously demonstrated transcellular transport of transferrin-binding radionuclides, including iron, indium and gallium, across isolated Sertoli cell monolayers and across intact models of the blood-testis barrier (*3–5*). It is likely, therefore, that such radionuclides will have access to the reproductive tract in vivo.

The radiation doses calculated within this work may be considered first approximations only. Several assumptions have been made regarding the amount of ^{111}In present in the fluid of the reproductive tract after administration of the radionuclide and the localization of ^{111}In within the sperm head. Furthermore, an in vitro experiment has been used to obtain a measure of in vivo cellular uptake of ^{111}In from the surrounding fluid, to mimic uptake by spermatozoa from reproductive tract fluid. Despite these limitations, these preliminary data indicate that the dose to mature spermatozoa from intravenous administration of diagnostic activities of ^{111}In, when localization and energy deposition at cellular level are considered, is likely to be substantially higher than that calculated using the conventional macroscopic approach. For example, diagnostic administration of 80 MBq of ^{111}In-chloride would result in a dose to spermatozoa of 4.3 mGy using conventional MIRD methodology. However, the dose could be as high as 670 mGy when energy deposition at the cellular level of the Auger spectrum and the specific cellular uptake indicated by our experiments are considered. In contrast, standard MIRD methodology is adequate for ^{59}Fe (*24*), which has no Auger component to its decay spectrum (*10*), and also for ^{99m}Tc-pertechnetate because, although it has low-energy electron emissions, this radiopharmaceutical does not appear to concentrate in spermatozoa.

CONCLUSION

This work demonstrates the need for further research to determine accurate values of in vivo uptake, localization and biological effects of ^{111}In within spermatozoa after administration for nuclear medicine procedures. It is possible that such investigations may indicate that the use of contraception after administration of ^{111}In and, possibly, other transferrin-binding radionuclides, such as ^{67}Ga, would be advisable.

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