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Membrane Potential-dependent Uptake of ^{18}F-triphenylphosphonium - A New Voltage Sensor as an Imaging Agent for Detecting Burn-induced Apoptosis

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Abstract

Background—Mitochondrial dysfunction has been closely related to many pathological processes, such as cellular apoptosis. Alterations in organelle membrane potential are associated with mitochondrial dysfunction. A fluorine -18 labeled phosphonium compound: ^{18}F-triphenylphosphonium (^{18}F-TPP) was prepared to determine its potential use as a mitochondria-targeting radiopharmaceutical to evaluate cellular apoptosis.

Methods—Studies were conducted in both ex vivo cell lines and in vivo using a burned animal model. Uptake of ^{18}F-TPP was assessed in PC-3 cells by gamma counting under the following conditions: graded levels of extra-cellular potassium concentrations, incubation with carbonyl cyanide m-chlorophenylhydrazone (CCCP) and staurosporine. Apoptosis was studied in a burn animal model using TUNEL staining and simultaneous assessment of ^{18}F-TPP uptake by biodistribution.

Results—We found that stepwise membrane depolarization by potassium (K) resulted in a linear decrease in ^{18}F-TPP uptake, with a slope of 0.62+/−0.08 and a correlation coefficient of 0.936+/−0.11. Gradually increased concentrations of CCCP lead to decreased uptakes of ^{18}F-TPP. Staurosporine significantly decreased the uptake of ^{18}F-TPP in PC-3 cells from 14.2+/−3.8% to 5.6+/−1.3% (P<0.001). Burn induced significant apoptosis (sham: 4.4 +/−1.8% vs. burn: 24.6+/−6.7 %; p<0.005) and a reduced uptake of tracer in the spleens of burn injured animals as compared to sham burn controls (burn: 1.13+/−0.24% vs. sham: 3.28+/−0.67%; p<0.005). Biodistribution

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studies demonstrated that burn induced significant reduction in ^{18}F-TPP uptake in spleen, heart, lung, and liver, which were associated with significantly increased apoptosis.

Conclusions—^{18}F-TPP is a promising new voltage sensor for detecting mitochondrial dysfunction and apoptosis in various tissues.

Keywords

^{18}F-TPP; Mitochondrion; Membrane Potential; Apoptosis

INTRODUCTION

Mitochondrion plays a central role in cellular function and dysfunctional mitochondria are involved in many pathologic processes, such as inflammation, degenerative diseases, aging, cancer, and arthrosclerosis [1–4]. Membrane potential ($\Delta\psi_m$) is a reliable index of the status of mitochondrial function.

Cellular apoptosis is closely associated with the potential of the mitochondrial membrane [5, 6]. The depolarization of the inner mitochondrial membrane triggers the apoptosis pathway. Therefore, assessment of mitochondrial potential could be used to detect cellular apoptosis and the apoptosis related organ functional status. Monitoring the mitochondrial potential in vivo could be of clinical importance in monitoring disease process and assessing the efficiency of clinical treatment. Changes of mitochondrial membrane potential $\Delta\psi_m$ can be reflected by the uptake of lipophilic cations [7, 8].

A phosphonium cation, 18 Fluorine-triphenylphosphonium (^{18}F-TPP) has been developed as a new imaging agent tracer which might be used to non-invasively assess apoptosis-related mitochondrial dysfunction. This cation is strongly lipophilic, penetrates cellular membranes and accumulates in mitochondria, which is a most electro-negative organelle. Thus, the uptake of this tracer could serve as an indicator of the transmembrane voltage gradient. In the present study, we investigated the uptake of ^{18}F-TPP in an ex vivo cell culture system and in vivo distribution in burned and sham-burned mice, to evaluate its potential applications in monitoring disease status of the patients, using positron emission tomography (PET).

MATERIAL AND METHODS

Materials

^{18}F-TPP was synthesized as described by Shoup et al in our laboratory [9]. For ex vivo studies in cell culture system, the composition of the incubation medium is as follows (g/L): NaCl (150), KCl (5.3), $CaCl_2$ (1.16), $MgSO_4$ (0.67), NaH_2PO_4 (0.76), dextrose (5.8) and Hepes (5.2), with FBS 1.5% (v/v). The loading buffer solution is prepared as a standard solution except for the substitution of NaCl by equal molar K-asparate and lowering the Ca^{2+} concentration to 0.1mM. F-12K medium, fetal calf serum and Penicillin/streptomycin were obtained from ATCC (NY). Cell Death Detection kit (TUNEL) was obtained from Roche Molecular Biochemicals (Mannheim, Germany).

Animal

Wild-type C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) weighing 20 to 25 gm were randomly divided into groups (5 per group). The extent of apoptosis in spleen was evaluated by TUNEL staining. The study was approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital, Harvard University, and was in compliance with the “Guide for the Care and Use of Laboratory Animals” (Publication No. NIH 78-23, 1996).

PC-3 Cell Line Culture

The studies were conducted using prostate cancer cell line PC-3. The cells were cultured in 75-cm^2 tissue culture flasks in F-12K medium which was supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and streptomycin, at 37°C in a humidified 5% CO_2 incubator. Viability of the cells was checked by trypan blue exclusion staining and routinely was greater than 95%. Cells were harvested by treatment with 0.02% trypsin.

Time Course of ^{18}F-TPP Uptake by PC-3 cells

Cells were harvested by trypsinization, followed by washing three times with PBS and suspension in loading buffer at a concentration of 1×10^6 cells/ml. then, the cells were incubated in a water bath for one hour at 37°C, and then mixed with an equal volume of loading buffer containing 2.0 nM of ^{18}F-TPP. The cells were harvested at: 5 min, 30 min, 1.0 hour, 1.5 hours and 2.0 hours, and tracer accumulation in the cells were determined. At each time point, ^{18}F-TPP uptake was terminated by centrifugation at 1000rpm for 6 minutes. Radioactivity of the supernatant and the cell pellets were measured with a gamma counter (Beckman). Radiotracer uptake was expressed as the accumulation ratio (%) by dividing the radioactivity in the pellet by the total radioactivity in both supernatant and pellet per million cells.

The Effects of Proapoptotic Factors on ^{18}F-TPP Uptake by PC-3 cells

We explored the effect of TPP concentration on ^{18}F-TPP uptake by the PC-3 cells. Cells were incubated with ^{18}F-TPP tracer at graded concentrations of TPP: 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 nM. The most effective concentration of TPP was determined for subsequent studies.

The effects of potassium, carbonyl cyanide m-chlorophenylhydrazone (CCCP), staurosporine and valinomycin on ^{18}F-TPP uptake were further investigated by incubating the cells at different concentrations of individual compounds. PC-3 Cells were harvested and washed three times before being incubated with the compounds. Additive effects on uptake were further evaluated by studying different combinations of these factors.

Burn Injury Model

All animals received general anesthesia (Ketamine 40mg/kg body weight and Xylazine 5 mg/kg body weight, IP) prior to burn injury. The dorsal surface of the animals was shaved with animal hair clippers. A full-thickness thermal injury of 30% total body surface area (TBSA) was produced by placing the animals in a mold followed by exposure of the dorsal

area to a 90°C water bath for 9 seconds. The mice were immediately resuscitated by intraperitoneal injection of 1.5 ml saline. Sham burned controls received the same treatment except with the water bath set in room temperature. After the procedure, the mice were caged individually.

Twenty-four hours after the burn injury, euthanasia was performed by over dose of Phenobarbital. Organs were harvested for analyses of apoptosis and biodistribution.

Histopathological Examination

The spleen specimens were fixed in 10% formaldehyde and embedded in paraffin. Four μm tissue sections were stained with hematoxylin and eosin and examined with light microscopy by experienced pathologists that were blinded from the treatment conditions.

Evaluation of Cellular Apoptosis

Four μm tissue sections were deparaffinized in xylene and dehydrated in graded ethanol. Apoptotic cells were identified by using a Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) kit. Ten random fields from 5 slides per group were examined and the TUNEL-positive brown nuclei within the cells were scored as previously described [10]. Data were expressed as percentage of TUNEL-positive cells/100 cells. Digitized images were analyzed by two investigators.

Biodistribution Study

Pilot studies were conducted to determine the optimal time for assessing ^{18}F-TPP biodistribution in spleen. ^{18}F-TPP was injected to animals 24 hours after burn or sham burn injury, spleen was harvested at different time points after the injection and ^{18}F-TPP accumulation in the organ was measured. The radioactivity is expressed as the percentage of injected dose per gram tissue (%DPG).

Statistical Analysis

Data was presented as mean ± SEM. Statistical evaluations were performed using ANOVA and Student *t*-tests wherever indicated. *P* value less than 0.05 was considered significant and n represents the number of animals per group.

RESULTS

1. Time-dependent Accumulation of ^{18}F-TPP in PC-3 Cells and the Effects of Tracer Concentration on Uptake

Cells were incubated at normal potassium concentration (5.4 μM) and harvested at different time points. The results showed time-dependent uptake kinetics of ^{18}F-TPP. The cellular radioactivity increased gradually and reached a plateau at 60 minutes of incubation (Fig. 1, A). Therefore one hour was chosen as optimal incubation time to be used in the definitive studies.

Cells incubated with ^{18}F-TPP at graded concentrations demonstrated that the tracer uptake remained at a relatively stable level over the extracellular concentration range of 0.5 to 16

nM. The concentration of 2 nM demonstrated the peak uptake, which was chosen for further studies (Fig. 1, B).

2. Effects of Potassium Concentration on ^{18}F-TPP Uptake

Following the increased extracellular concentration of potassium, the uptake of ^{18}F-TPP showed a step-wise decrease. At the highest potassium concentration of 172.8 μM, the uptake of 18F-TPP reached its lowest level. The lowest potassium concentration of 10.8 μM corresponded with the highest level of ^{18}F-TPP uptake. Overall, there is an inverse linear correlation between the ^{18}F-TPP uptake and extracellular potassium concentrations (slope: 0.62+/− 0.78; correlation coefficient: r=0.936+/− 0.11. Fig. 2 A).

3. Effects of CCCP Concentration on ^{18}F-TPP Uptake

CCCP is a chemical inhibitor of oxidative phosphorylation which induces the uncoupling of protons in the electron transport chain[11]. As seen in figure 2B, increment of CCCP concentrations in the incubation medium from 2 μm to 5 μm resulted in a rapid reduction of ^{18}F-TPP uptake into the cells, although there was no further statistically significant decrease at higher concentrations. (Fig. 2 B).

4. Effects of Staurosporine, and the Combination of K, CCCP, Valionmycin and Staurosporine on ^{18}F-TPP Uptake

Significant reduction of ^{18}F-TPP uptake was found at staurosporine concentration of 8μM. (Fig. 3 A, control vs. staurosporine: $P<0.001$). Combination of the proapoptotic factors, such as high potassium/valionmycin and High potassium/valionmycin/CCCP, demonstrated similar effects on ^{18}F-TPP uptake as shown in Fig. 3 B.

5. Burn Induced Apoptosis in Spleen

We chose 24 hours after burn as the time point for evaluation of post burn apoptosis basing on our previous experience [9]. TUNEL stain on the sections of the spleens from the sham mice demonstrated diffusely scattered apoptotic cells at a rate of 4.4 +/−1.8% (Fig. 4 A). In contrast, burn induced significant cellular apoptosis in the spleen presenting an apoptotic index of as high as 24.6+/− 6.7 % (Fig. 4 B, C. sham vs. burn: $P<0.005$). The majority of the apoptotic cells were present in the white medulla of the spleen.

6. ^{18}F-TPP Uptake by Spleen and ^{18}F-TPP Biodistribution Changes after Burn Injury

The time course study on the uptake of ^{18}F-TPP by the spleen in mice showed the maximum accumulation at 20 minutes after a tail vein injection (Fig. 5. A). This time point was chosen for assessing the biodistribution in organs. ^{18}F-TPP uptake by spleens was reduced from 3.28 ±0.67% in sham burn to 1.13±0.24% in burned animals (Fig. 5 B, $p<0.005$).

The biodistribution studies also demonstrated a significant reduction of ^{18}F-TPP uptake in heart, lung, liver (sham v.s burn: $P<0.05$, Fig. 6). The results were consistent with the well-documented findings: burn induced a significant apoptosis status in multiple organs and tissues [9].

DISCUSSION

The change of mitochondria membrane potential is closely related to cell function and apoptosis. The present study aimed at evaluating the changes of mitochodrial potential in relation to ^{18}F-TPP uptake using an ex vivo cell culture system and an in vivo burn model. The ultimate goal of our study is to determine if this tracer can be used to evaluate the changes of mitochondrial membrane potential and the extent of apoptosis in multiple organ/tissues in vivo using PET imaging techniques. Thus, it may serve as a clinical index to monitor the organ function and disease status of patients, especially those suffering from critical illness.

Apoptosis, or programmed cell death, is involved in the etiology and pathogenesis following the progress of many disease conditions. The intrinsic pathway of apoptosis is mediated by permeabilization of the mitochondrial membrane [10], followed by release of proapoptotic signaling molecules, such as cytochrome c, and finally activation of the caspase family, such as caspase 6, 7 and 3. It has been proven that initiation of the complete post-mitochondrial cascade is accompanied with a decreased potential across the mitochondrial inner membrane. The extrinsic apoptotic pathway is triggered by interaction of death receptors, such as CD95, DR5, on the cellular membrane, which can activate caspase 8 as an initiator. Then caspase 8 cleaves the bcl-2 protein family and also increase the permeability of the mitochondrial membrane resulting in decreased membrane potential [11]. Furthermore, there are many interactions between the intrinsic and the extrinsic pathway which are closely associated with changes of the mitochondrial membrane potential. Thus the initiation of apoptosis can be monitored by alterations in membrane potential.

Membrane potential is maintained principally by the concentration gradient and membrane permeability to potassium and sodium. Increase of the extracellular potassium levels results in depolarization of the membrane potentials. The present study demonstrated that increase of extracellular potassium concentration resulted in significant decrease of ^{18}F-TPP uptake and a strong inverse linear correlation between potassium concentration in the incubation medium and ^{18}F-TPP uptake with a correlation coefficient of 0.936. The results indicated the sensitivity of this tracer uptake in relation to the alterations of mitodchondrial membrane potential. The sensitivity was further verified in conditions of altered membrane potential induced by multiple proapoptotic compounds. CCCP is a chemical inhibitor of oxidative phosphorylation which can cause an uncoupling of the proton gradient that is established during the activity of electron carriers in the electron transport chain [12]. CCCP causes the gradual destruction of living cells and as a result, leads to apoptosis [13]. Staurosporine is a broad-band protein kinase inhibitor, which can initiate cellular apoptosis through the intrinsic pathway. It has been well-documented that staurosporine leads to the loss of cross-membrane potential gradients. Valinomycin is a dodecadepsipeptide antibiotic. It functions as a potassium-specific transporter and facilitates the movement of potassium ions through lipid membranes “down” an electrochemical potential gradient [14]. Valinomycin triggers a rapid loss of the mitochondrial membrane potential and cytoplasmic acidification, leading to cysteine-active-site protease activation, DNA fragmentation and apoptosis [15]. The present study demonstrated that individual addition of these compounds into the incubation medium resulted in significant decrease of ^{18}F-TPP uptake by PC-3 cells (Figures 3, and 4).

Furthermore, combinations of these compounds resulted in similar effects in reducing ^{18}F-TPP uptake (Figure 5). These findings suggested a high sensitivity of ^{18}F-TPP as an indicator to detect the alterations of mitochondrial membrane potential of the cells.

The present biodistribution study demonstrated significant reductions of ^{18}F-TPP uptake in the spleen, as well as in the heart, liver and lung of the burned mice. It is documented that burn might enhance evident apoptosis in these organs [9, 16–18]. In our present study, we focused on investigating burn induced spleen apoptosis and ^{18}F-TPP uptake to verify the correlation between apoptosis and ^{18}F-TPP uptake. It also indicates the sensitivity of ^{18}F-TPP as a tracer to detect the changes of mitochondrial potential in both ex vivo system and living animals. Therefore ^{18}F-TPP is potentially a sensitive tracer which can be used as a PET imaging agent to non-invasively monitor apoptosis and functional status of multiple organs and tissues following various disease processes. It can also be used to evaluate the efficacy of treatment in clinical patients, especially those with critical illness with high risk of multiple organ failures.

Currently, the only available imaging agent that can be used to detect apoptosis is Annexin V [19], but it has inherent disadvantages. Apoptosis is not a one-time event but rather a continual process. Annexin V scan can only get a snapshot of the apoptosis status. The unique advantage of ^{18}F-TPP is to allow multiple “real-time” measurements. Also, ^{18}F-TPP targets the changes of membrane function, which provides a better understanding of the apoptosis, and apoptosis related functional status of the tissue.

In conclusion, the present study suggests the potential power and unique advantages of ^{18}F-TPP as a sensitive and accurate voltage sensor for detecting membrane potential alterations using PET. The findings established that ^{18}F-TPP is a promising imaging agent to detect apoptosis and apoptosis related organ function in clinical practice.

Acknowledgments

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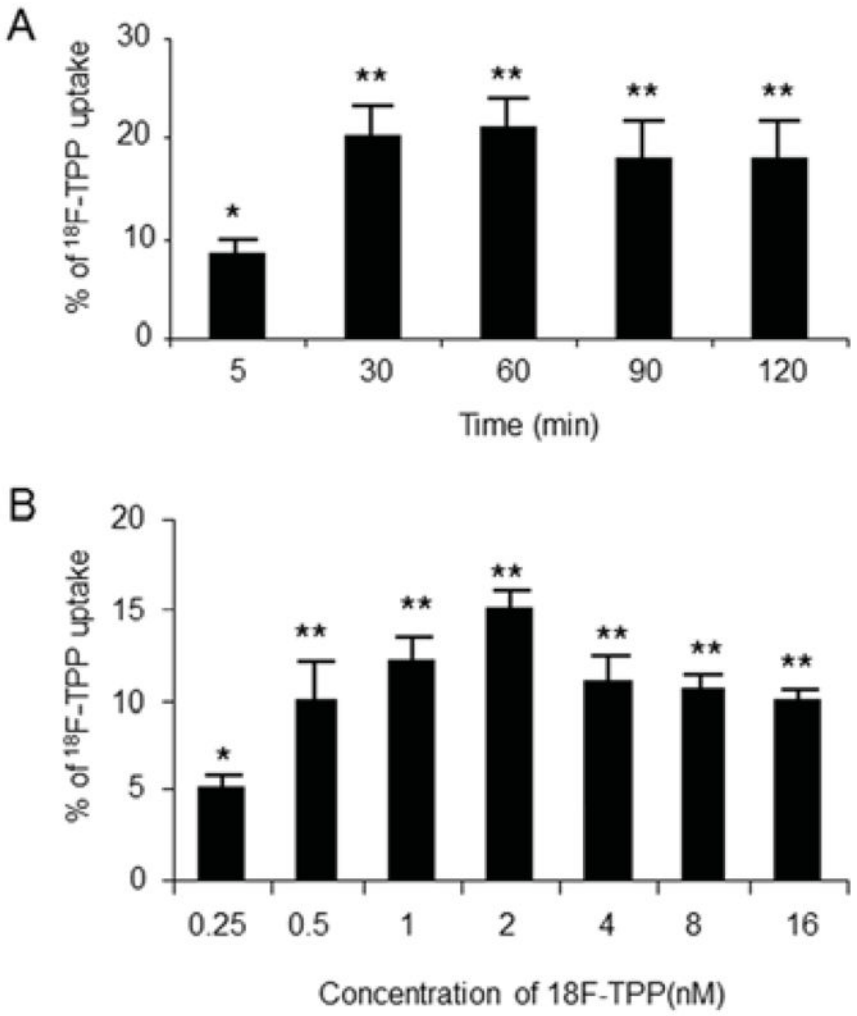

Fig. 1. Time course study and concentration study on ^{18}F-TPP uptake by PC-3 cells

PC-3 cells were incubated with normal K concentration (5.4 μM). Time course study demonstrated time-dependent uptake kinetics of ^{18}F-TPP by PC-3 cells. The cellular radioactivity increased gradually and reached the plateau at 60 minutes post incubation (Fig. 1 A: ANOVA, * vs. **: $P<0.05$; ** $P>0.05$). Cells incubated with ^{18}F-TPP at different levels of extracellular concentration. The maximum uptake was shown at the concentration of 2 nM (Fig. 1 B: ANOVA, * vs. **: $P<0.05$; ** $P>0.05$).

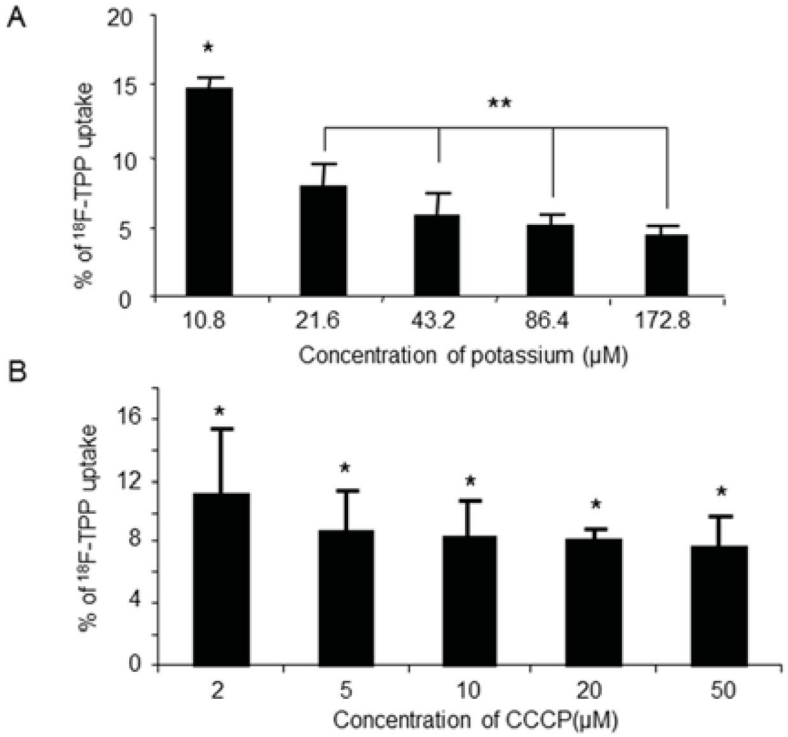

Fig. 2. Effects of potassium and CCCP concentrations on ^{18}F-TPP uptake by PC-3 cells

The uptake of ^{18}F-TPP was decreased as the extracellular potassium concentration increased. At the highest potassium concentration of 172.8 μM, the uptake was the lowest, while at the lowest potassium concentration of 10.8 μM, the uptake was at the highest. An inverse linear correlation between the uptake and extracellular potassium concentration (slope: 0.62+/− 0.78; correlation coefficient: r=0.936+/− 0.11) was found (Fig. 2 A: ANOVA, * vs. **: $P<0.05$; ** $P>0.05$). Gradually increased concentrations of CCCP lead to decreased uptakes of ^{18}F-TPP, which quickly reached a trough at CCCP concentration of 5μm (Fig. 2 B: ANOVA, * $P>0.05$).

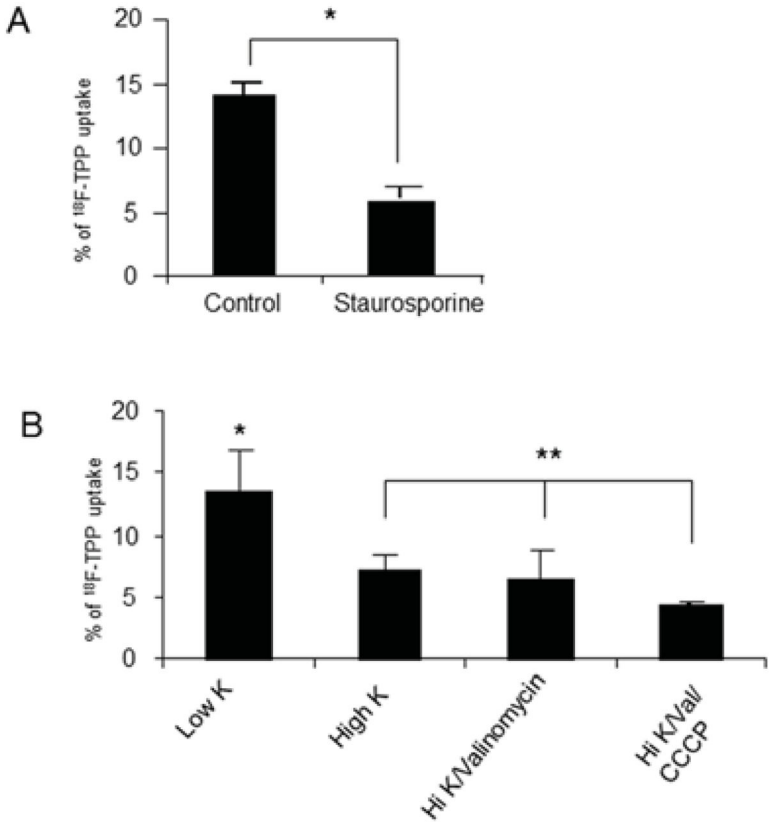

Fig. 3. Effects of staurosporine and combined proapoptotic factors on ^{18}F-TPP uptake

Staurosporine at 8μM lead to a significantly decreased uptake of ^{18}F-TPP in PC-3 cells compared with the sham treated cells (Fig. 3 A: *t* test: $P<0.001$). Combination of proapoptotic factors clearly demonstrated similar effects on the uptake (Fig. 3 B: ANOVA: * vs. ** $p<0.05$; ** $p>0.05$). Note: CCCP: 50 μM/L; Val: 1 μg/ml; Low K= 2 μM/L; high K= 172.8 μM/L.

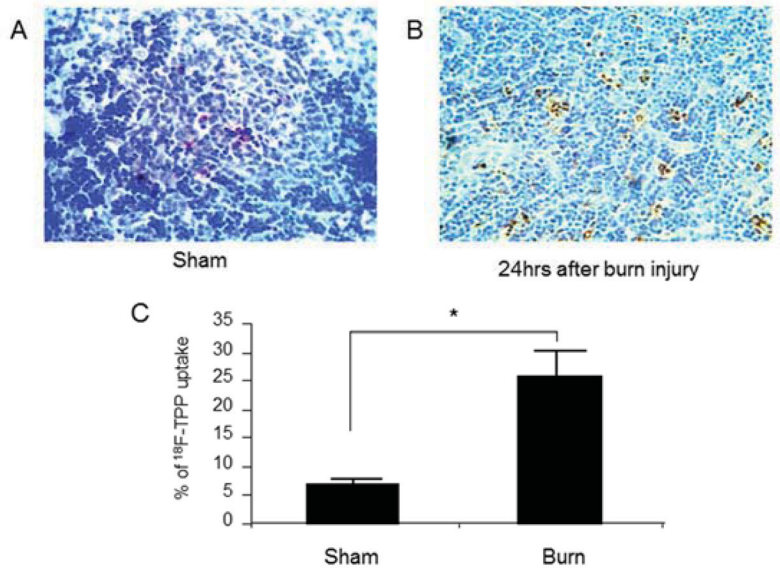

Fig. 4. Burn induced apoptosis in mouse spleen

TUNEL staining on the sections of the spleens from the sham mice demonstrated diffusely scattered apoptotic cells at a rate of 4.4 +/−1.8% in the white medulla (Fig. 4 A). By contrast, burn induced significant cellular apoptosis in the spleen presented an apoptotic index of as high as 24.6+/− 6.7 % (Fig. 4 B, C: sham vs. burn: *t* test, $p<0.005$). The majority of the apoptotic cells were present in the white medulla area.

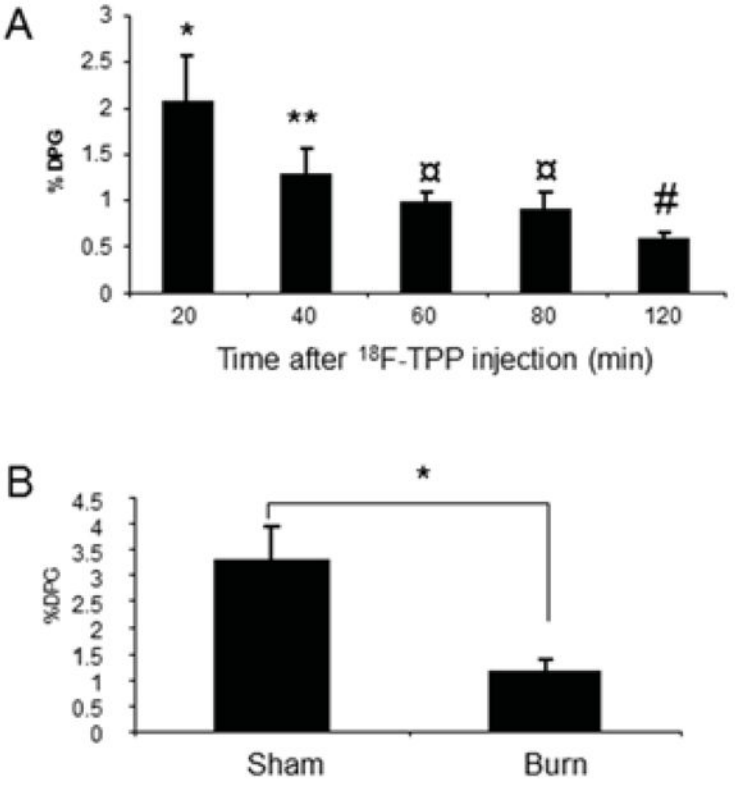

Fig. 5. Effects of apoptosis on ^{18}F-TPP uptake in spleen and ^{18}F-TPP biodistribution

Time course study on the uptake of ^{18}F-TPP by spleen in mice showed the maximum accumulation in 20 minutes after the injection of ^{18}F-TPP via tail vein. This time point was chosen for biodistribution studies (Fig. 5 A: ANOVA, * vs. the rest, ** vs. #: $P<0.05$; ¤, ** vs. ¤, and ¤ vs. #: $P>0.05$). Tracer uptake in burned mouse spleens was reduces to 1.13±0.24% compared with 3.28 ±0.67% (*t* test: $p<0.005$) in spleens of sham treated mice (Fig. 5 B).

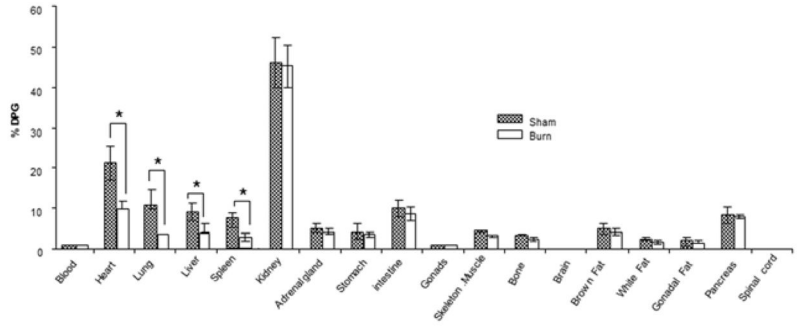

Fig. 6. ^{18}F-TPP biodistribution

The biodistribution study showed significant reduction of ^{18}F-TPP uptake in heart, lung, spleen, and liver in burned animals compared with sham treated ones(* burn vs. sham: *t* test, $P < 0.05$). No evident uptake differences were presented in other examined organs of burned animals by contrast with the sham treated ones (burn vs. sham: *t* test, $P > 0.05$).