行政院原子能委員會
委託研究計畫研究報告

具 TAU 蛋白結合潛力之前驅物合成方法開發

The development of TAU-specific radiolabel precursor

計畫編號： 1032001INER001

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目　錄

中文摘要

阿茲海默氏症(Alzheimer's disease, AD)及其他的失智症方面，Tau 纖維蛋白沉積或稱為神經纖維糾結(neurofibrillary tangles, NFTs)與認知功能異常及細胞死亡密切相關。先前的研究指出人類胚胎幹細胞分化形成的神經元提供良好的平台去研究人類 Tau 蛋白的分布、功能及機能異常，但人類胚胎幹細胞的使用有倫理上的限制。另一方面，因為脂肪組織可以由抽脂手術中大量取得，而且較不會對人體造成傷害，因此脂肪幹細胞有潛力發展為細胞再生療法或有潛力在體外研究 Tau 蛋白的病理表現情形。

本研究計畫將建立 TAU 專一性結合之輻射前驅物 ^{18}F-FEONM 之化學製程，以偵測 Aβ 斑塊。另一方面，幾丁聚醣(chitosan)能使間葉幹細胞聚集成球狀物(spheroid)，使其具更好的自我更新、分化能力以及更佳移植潛力。因此，實驗設計目的在測量並比較 ^{18}F-FET 及新合成 ^{18}F-FEONM 被貼盤脂肪幹細胞 (attached ADSC) 及成球脂肪幹細胞 (ADSC spheroid) 的細胞攝取量，結果顯示對 ^{18}F-FET 而言，細胞攝取量隨時間下降，貼盤脂肪幹細胞在 30 min 時細胞攝取量急遽下降，而成球脂肪幹細胞則在 60 min 時細胞攝取量急遽下降；對新合成 ^{18}F-FEONM 而言，貼盤脂肪幹細胞及成球脂肪幹細胞細胞攝取量在 30 min 達到高點，然後兩種型態脂肪幹細胞在 60min 及 120min 則逐步降低，另外，對 ^{18}F-FET 而言，成球脂肪幹細胞會比貼盤脂肪幹細胞增加 30 倍以上細胞攝取量，而對 ^{18}F-FEONM，成球脂肪幹細胞會比貼盤的脂肪幹細胞至少增加 15 倍以上的細胞攝取量，推測可能成球脂肪幹細胞與神經分化能力相關而導致對 ^{18}F-FEONM 及 ^{18}F-FET 的細胞攝取量增加。

中文關鍵字: 阿滋海默氏症、Tau 蛋白、神經纖維糾結、脂肪幹細胞、幾丁聚醣、球狀物

英文摘要

Tau filament deposition, or called neurofibrillary tangles (NFTs), in Alzheimer disease (AD), frontotemporal dementia (FTD), and other tauopathies correlates losely with cognitive dysfunction and cell death. Previous study indicated that human embryonic stem cell-derived neurons could be a good model to study human tau distribution, function and dysfunction. However, ESCs have the ethic problem. On the other hands, the waste adipose tissue obtained in the plastic surgery, such as liposuction, is readily accessible without causing damage to the donor. Therefore, adipose-derived stem cells (ADSCs) have the potential to be a convenient source for cell replacement therapy or have the potential to study the Tau pathology *in vitro*.

This project tried to synthesize a new Tau-specific radiolabel precursor, ^{18}F-FEONM that had potential to monitor the Aβ plaque. On the other hand, chitosan could promote the self-assembly of MSCs to form 3D spheroids that present better self-renewal property, differentiation capacity, and engrafting potential. The purpose of this experiment was designed to investigate the cell uptakes of ^{18}F-FET and new synthesized ^{18}F-FEONM for attached ADSCs and ADSC spheroids. For ^{18}F-FET, the cell uptakes of ADSC present a time dependent decrease with dramatically decrease at 30min, while ADSC spheroid showed time dependent decrease with delayed decrease at 60min. For ^{18}F-FEONM, the cell uptakes maintained a high level within 30min and then gradually declined at 60 and 120 min. Moreover, the cell uptake of ADSC spheroid was at least 15-fold higher than that of attached ADSC on TCPS for ^{18}F-FET. Meanwhile, for ^{18}F-FEONM, the cell uptake of ADSC spheroid was at least 30-fold higher than that of attached ADSC. We suggested that the sharp increase in cell uptake may be due to ADSC sheroids had the higher association with neuronal differentiation.

Keywords: Alzheimer’s disease (AD); Tau protein； neurofibrillary tangles (NFTs)；Adipose Derived Stem Cells (ADSCs); chitosan; spheroid

壹、計畫緣起與目的

Alzheimer's disease (AD) is one of the most common type of dementia and causes progressive loss of memory and other cognitive functions [1]. The two major histopathological results in brains of AD patients include extracellular senile plaques consisting of amyloid-β (Aβ) peptides and intracellular neurofibrillary tangles (NFTs), composed of abnormally hyperphosphorylated tau protein [2,3]. The tau pathology made up of the hyper-phosphorylated tau is also a common feature of several neurodegenerative disorders including frontolobardementias, the corticobasal degeneration, Progressive supranuclear palsy, Pick disease, Guam Parkinsonism dementia complex, and dementia pugilistica. The density of tau lesions exhibits directly correlation with dementia. Until now, there is no effective method available for AD and related tauopathies. Literature in animal models have demonstrated that tauopathy can present in the living brain, using either transgenic mice that express mutant human tau proteins specifically in the entorhinal cortex [4,5], or by injecting tau aggregates into specific brain regions [6,7]. However, these processes are not efficient, taking weeks to months to observe, and occur only in situations of high levels of exogenous or transgenic tau. Tau belongs to a kind of microtubule-associated protein localized in the axon of mature neurons [8,9], and a prerequisite for trans-synaptic propagation would be the localization of tau specifically at the synapse. There is increasing concern in mechanisms by which phospho-tau may propagate trans-synaptically via neuroanatomical pathways [10,11]. A better understanding of the mechanism of differential tau burden within the human dentate gyrus could increase our understanding of spread of neuropathology in AD.

Pharmacological treatment of AD currently includes cholinesterase

inhibitors and NMDA receptor antagonists. However, according to most studies therapeutics of both these groups provide mainly symptomatic benefits without counteracting the progression of the disease [12]. Drug research in the recent years has attempted to develop disease-modifying drugs hopefully able to delay the onset or counteract the progression of AD. Strategies targeting at Aβ pathology involve decreasing of Aβ production, preventing aggregation of Aβ into amyloid plaques, stimulating clearance of Aβ. Neither inhibitors of β-secretase or γ-secretase, nor stimulators of α–secretase have demonstrated satisfactory effecacy combined with low toxicity. Drugs targeting tau-protein are known to be divided into following groups: modulators of tau phosphorylation, inhibitors of tau-phosphorylating kinases (e.g. glycogen-synthase-kinase-3 β , cyclin-dependent kinase-5, p70-S6-kinase) and compounds that inhibit tau aggregation and misfolding [13]. AD is composed of complex multifactorial pathology, including multiple cycles and subcycles of self-amplifying neurode generative process [14,15]. Monotherapy targeting single process in this complicated cascade may explain disappointments in trials with agents affecting only one chain of this “circulus vituosus“ . So it would be advantageous to explore the possibilities of novel multi-target therapy, aimed to influence different disease-related mechanisms, resulting in additive or synergic therapeutic responses [16].

Stem cells differ from other kinds of cells in the body. All stem cells-regardless of their source - have three general properties: they are capable of renewing themselves for long periods; they are unspecialized; and they can give rise to specialized cell types. Stem cells can be roughly categorized into two types, embryonic and adult stem cells. As for the adult stem cells, it can be extracted from multiple matured animal tissues

that is fit the two purposes, self-renewal or multi-potential differentiation. In the recent years, there has been progressive advancement in various areas of stem cell therapy and restructure techniques. Resources of the stem cells have also increased along with the advancement [17-20]. The feasibility source of the adult stem cell has attracted scientific interest recently. Studies focusing on animal adipose tissues have the ability that adipose tissues can develop adult stem cells as a convenient source for cell replacement therapy. Besides, the waste adipose tissue obtained in the plastic surgery, such as liposuction, is readily accessible without causing damage to the donor. Several studies have focused on the identification of chondrogenic and osteogenic progenitors from adipose tissue, and it has been shown that the mesenchymal stem cells obtained from either normal adipose tissue or lipoma could have similar morphological and phenotypical features to the bone marrow mesenchymal cells [21-24]. ADSCs can differentiate into multiple lineages when cultivated under lineage-specific conditions, including osteogenic, adipogenic, and chondrogenic lineages [25]. Recently, ADSCs are found to be capable of transdifferentiating into mature cells not related with their original lineage, such as hepatocyte of endoderm origin and neuron of ectoderm origin [26,27]. This ability, together with their easy accessibility and low donor site morbidity, has made ADSCs good candidates for a broad range of cell-based therapeutics. The presence of pluripotency markers, including Oct-4, Sox-2 and Nanog, is important for the renewal and differentiation capabilities of ADSCs [28,29], but they are detected only in cells from early passages [30]. Therefore, maintaining the expression of stemness markers has become an important issue for in vitro culture of ADSCs.

ADSC and bone marrow-derived MSCs are recently proven to form 3D spheroids on chitosan membranes [31-33]. These cells attach and

spread on chitosan membranes before they retracted their pseudopodia to induce the formation of multicellular spheroids [31]. This process of spheroid formation was quite different from that present in suspension [34,35], on non-adherent polymer surfaces [36], or in hydrogels [37,38]. The Rho/Rho-associated kinase (ROCK) signaling pathway and the expression of cadherin molecules may be implicated in the process of spheroid formation [31,39]. However, the exact mechanism remains unclear.

In this project, it was demonstrated that ^{18}F-FDDNP has potential to monitor the Aβ plaque. Herein, we developed a reliable synthetic method for novel FDDNP analog, FEONM, in this study. Futhermore, we investigated the cell intakes of radiolabel precursors, ^{18}F-FEONM and ^{18}F-FET, between ADSCs and ADSC spheroids.

貳、研究方法與過程

一、Preparation of ^{18}F-FEONM

Chemical used in this project are as follows: 2-methylaminothanol, 2-bromoethanol, sodium metabisulfite, malononitrile, 2-Acetyl-6-methoxynaphthalene, p-toluenesulfonyl chloride, 1M TBAF. HCl was purchased from ACROS. K_2CO_3 was purchased from fish-scientific. DMF And pyridine were obtained from Sigma-Aldrich.

This project has successfully synthesized the new ^{18}F-FEONM analog and the diagram of preparation is present in Figure 1. First, 2-acetyl-6-methoxynaphthalene (compound **1)** is used as the starting material. The overbased hydrochloric acid (HCl concentration is 12N) is added to obtain compound **2** under reflux conditions with 66% yield. compound **2** is further reacted with 2-methylaminoethanol using Bucherer reaction to form compound **3.** In the literature, compound **1**, an aqueous solution of sodium hydrogen sulfate, and 2-methylaminoethanol placed in a high pressure reactor in a stringent condition can synthesize compound **3** with 68% yield. The purification is further performed by chromatography to obtain the compound **3** with 82% yield. Compoun **4** is synthesized by adding compound **3**, K2CO3, 2-Bromoethanol, and Anhydrous DMF for preparation. To compare with previous study that use compound **3**, malononitrile, anhydrous pyridine to prepare Compoun **4** with 84% yield, our method has 82% yield that allows for better convenience. Compound **5** is dissolved in anhydrous pyridine and then p-toluenesulfonyl chloride is added. Stirred for five hours at room temperature, then the production is purified by column chromatography

to obtain compound **6** with 60% yield. The fluorination reaction was performed by adding 1M TBAF at high temperatures to prepare final standard compound **7** with 80% yield.

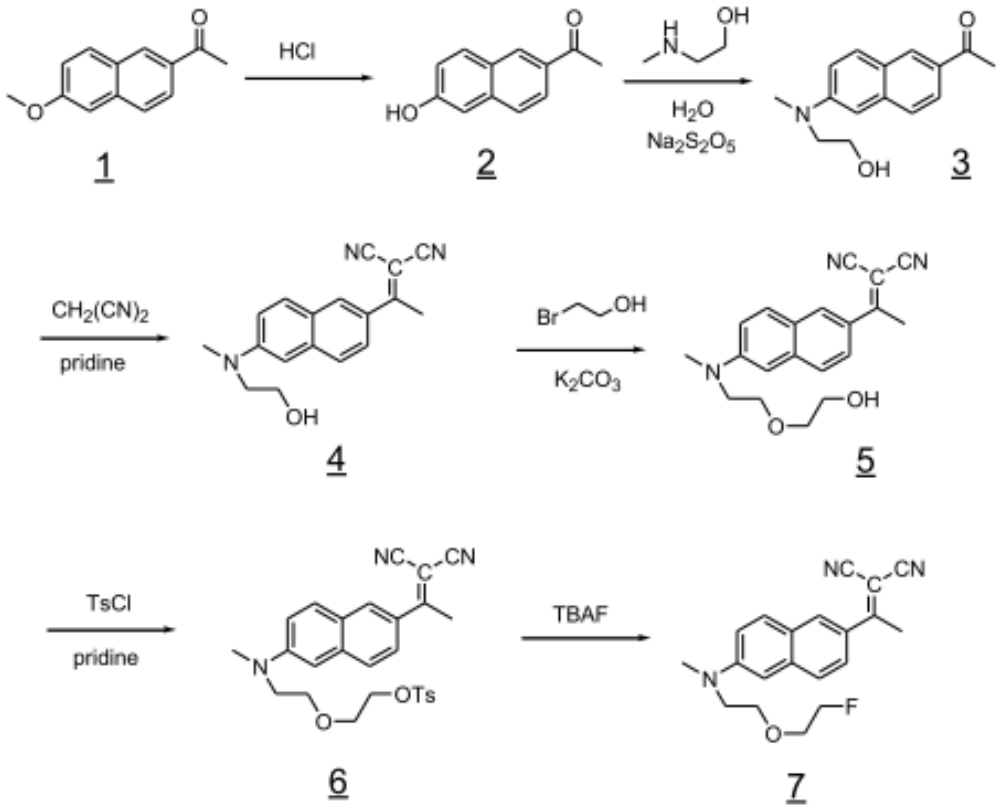


Figure 1. The synthesized flow chart of ^{18}F-FEONM

二、Isolation and culture of human adipose-derived stem cells

Human ADSC were obtained from the subcutaneous adipose tissue discarded during surgery following the procedures approved by the institutional review board. The human adipose tissue was minced and digested by 200 U/ml type I collagenase (Sigma, USA) in Hank's buffered salt solution (HBSS) for 1 h at 37℃. The cell suspension was centrifuged and then resuspended in the Dulbecco's modified Eagle medium (DMEM)/F12 medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and 1%penicillin-streptomycin (Invitrogen). Cells were placed in a flask and incubated at 37℃ with 5% CO2. The medium was refreshed once every 3 days. Cells of the third to the fifth passages were used in all the following experiments.

三、Analyses of surface markers for rat adipose-derived stem cells

Surface markers for each type of cells were quantified by flow cytometry using CD29, CD34, CD44, CD90, CD105 (1:1000, BD Biosciences, San Jose, CA) antibodies. 5×10^5 cells were washed twice with PBS, resuspended in 100 μl of PBS containing monoclonal antibodies and incubatedfor 30 min at 4°C. The cells were then washed twice and resuspended in 500 μl of PBS. Fluorescence analysis was performed with a flow cytometer (FACS Caliber, BD). The non-specific binding of the fluorescein isothiocyanate (FITC) and phosphatidyl ethanolamine (PE) conjugates were determined in control samples using a mouse IgG1-FITC and IgG1-PE negative control (Serotec).

四、Preparation of chitosan membranes

Chitosan powder (molecular weight 510 kDa and degree of

deacetylation 77%; Sigma) or sulfonated chitosan powder (molecular weight 140 kDa, degree of deacetylation 80%, and degree of sulfonation 50%; Hopax Chemicals, Taiwan) was dissolved in 1% aqueous acetic acid solution and stirred at room temperature for 12 h to obtain a 1% chitosan solution. The solution was filtered through a 100 μm mesh and then coated on coverslip glass (100 μl of solution on each 15 mm-diameter glass). The coated substrates were air-dried for one day, immersed in 0.5 N NaOH solution for 3 min, and washed extensively by distilled water. They were then air-dried for further experiments.

五、Cell seeding

ADSC (3×10^4 cells) were seeded on each membrane in 24-well tissue culture plates. Cell morphology was observed by an inverted microscope (Leica DMIRB). Cell grouping and spheroid formation were examined at 3 days.

六、Proliferation rate and viability of MSCs cultured on materials

The number of cells cultured on TCPS, chitosan membranes in basal medium was determined by Hoechst 33528 fluorescent dye assay at 1, 3, 5 days. Cells and cell spheroids were digested in papain (Sigma) solution and reacted with 0.1 mg/ml Hoechst 33528 dye (Sigma). The fluorescence was measured at room temperature by a fluorescence spectrophotometer (Hitachi F2500, Japan), with excitation at 365 nm and emission at 458 nm. The cell numbers were calculated against a standard curve obtained from known amount of cells. Cell viability was determined using propidium iodide (PI) (Sigma) staining and flow

cytometry for cells cultured in basal medium for 3 and 7 days. The solution of PI (concentration 2 mg/ml) was added to cell suspension before the analysis by the flow cytometer. The percentage of cells without being stained by PI was defined as the cell viability.

七、Cell uptake Experiments

^{18}F-FET and ^{18}F-FEONM were paepared. All experiments were performed in an rADSC cultured medium. Before the incubation with ^{18}F-FET and ^{18}F-FEONM, the cells were preincubated for 30 mm in 200 μL medium. Aliquots of 400 μL ^{18}F-FET or ^{18}F-FEONM (1.85 MBq/mL) were added and the cells were incubated at 37℃ for 5, 30, 60, 120 min. After stopping the tracer uptake with 1 mL ice-cold phosphate-buffered saline (PBS), the cells were washed three times with PBS at 4℃ and dissolved in 1.5 mL 0.1 N NaOH plus 2% Triton X. The radioactivity in the cells was measured with a gamma counter (Wallac, Thrku, Finland). In addition, the number of cells per well and the mean volume of the cells were determined electromechanically (CASI 1; Schaerfe System GmbH, Germany) for the calculation of the intracellular tracer concentration.The results were expressed as cpm/10^6 cells or as accumulation factor, which is calculated as the ratio between the intracellular tracer concentration and the concentration in the incubation medium.

八、 Statistical analysis

Numerical values were expressed as the mean ± standard

deviation. In all studies, three similar experiments were performed for each type of experiment. Reproducibility was confirmed for cells form at least three different donors. Statistical differences among the experimental groups were evaluated by analysis of variance followed by Student's t test. p-values <0.05 were considered statistically significant.

參、主要發現與結論

一、The synthesis of ^{18}F-FEONM

In the synthesized process of compound **3**, the use of atmospheric pressure, heating, and reflux condition was performed since there was no high pressure reactor in our laboratory. Therefore, we obtained the product with low yield and needed to purify by chromatography. Besides, compound **3** and **4** had the same Rf value from TLC analysis, we used Mass to the analysis of product before loading to chromatography. There were two steps in finishing the synthesized process. This process had the advantages of easily operation and good reproducibility. Compound **6** had a yellow bubble, while compound 7 present a yellow solid. Both compounds can be stored by filling nitrogen at 2 ~ 8°C for a long period. They were dissolvable in low polarity of dichloromethane.

The prominent pathological characteristics of AD include amyloid plaques and neurofibrillary tangles (NFTs), which are associated with the accumulation of β-amyloid and misfolded, hyperphosphorylated tau proteins. Literature has pointed out ^{18}F-FEONM can specifically bind to β-amyloid protein and was applied to the initial diagnosis of human Alzheimer's disease. This project further synthesizes the new ^{18}F-FEONM analogs and wishes to obtain the excellent diagnosis for AD. Following these process, the article provides a new method to prepare ^{18}F-FEONM analog with optimal adjustment.

二、The isolation and culture of adipose derived stem cells from human adipose tissue

As shown in Figure 1, the morphology of cultured adipose derived

stem cells showed a spindle-shaped pattern, similar to fibroblasts, Meanwhile, The growth of ADSC was very well.

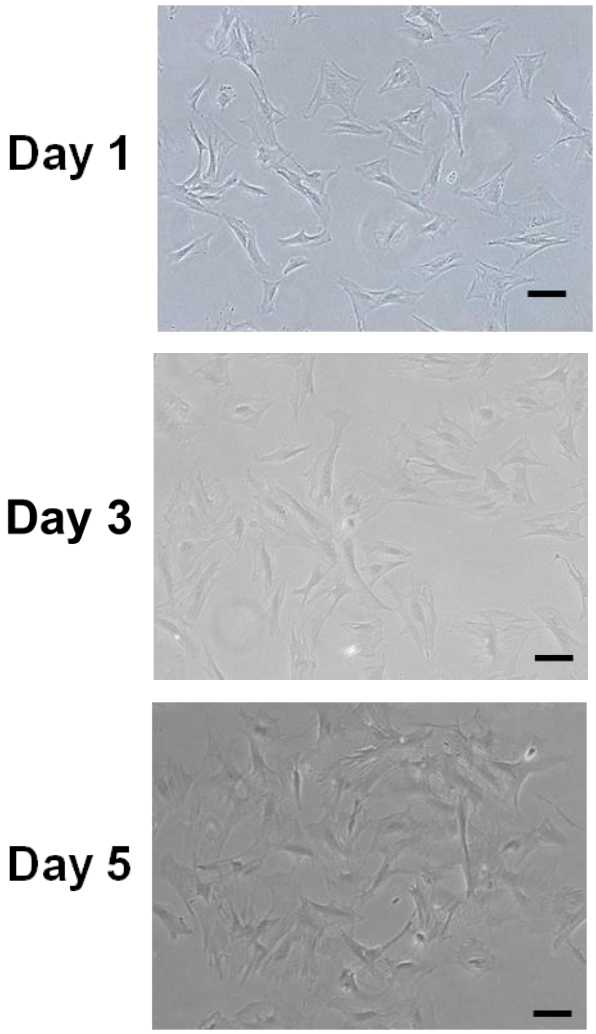


Figure 1. Human adipose-derived stem cells (hADSCs) isolated from human adipose tissue in ADSC cell culture medium at 1,3,5 days.

三、 The analysis of surface markers for ADSCs

Varieties of antibodies such as CD29 (+), CD34 (-), CD44 (+), CD90 (+), CD105 (+) were used to identify the surface markers of adipose derived stem cells. As shown in Figure 2, it showed negative for CD34 which was less than 5% of total amount of cells. However, ADSCs present positive for CD29, CD44, CD90, and CD105, which

occupied more than 95% of total amount of cells. These results were consistent to previous research and could be confirmed that most of the cells were human ADSC.

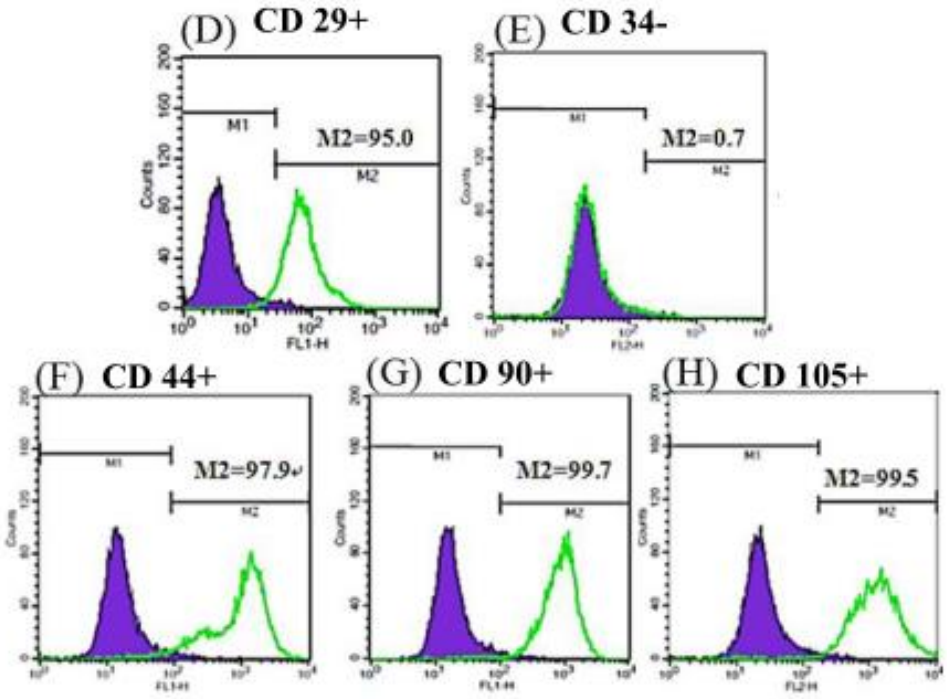


Figure 2. The characteristic surface markers of stem cells with $CD29^{+}$(A), $CD34^{-}$(B), $D44^{+}$(C), 90^{+}(D), $CD105^{+}$(E).

四、The morphology of ADSCs and ADSC spheroids

The ADSCs were detached by Trypsinase and then seeded onto 24-well tissue cultured plate with 5×10^{4} cells per well. After three days, we found that ADSCs could attach on the TCPS. But the ADSCs were gathered into a spheroid pattern on chitosan-coating TCPS.

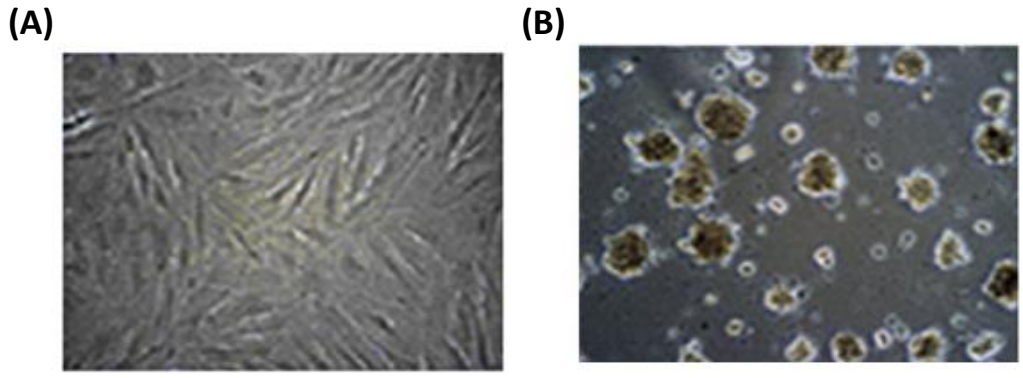
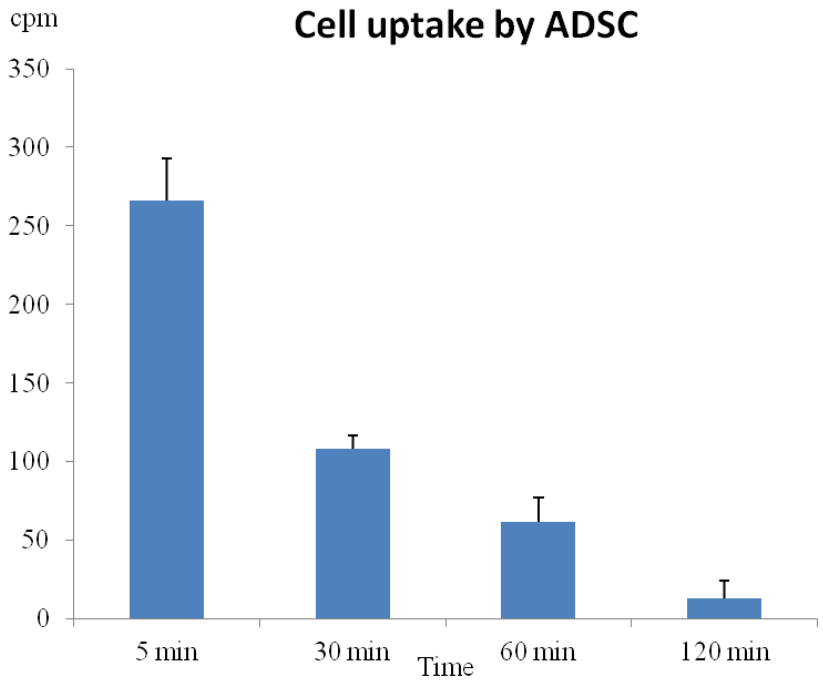


Figure. 3 The morphology of ADSCs cultured on (A)TCPS and (B) chitosan-coating TCPS.

五、The analysis of cell intake of ^{18}F-FEONM and ^{18}F-FET for ADSCs and ADSC spheroids

As shown in Figure 4, the difference of cell uptake of ^{18}F-FET was investigated between attached ADSCs and ADSC spheroid. For attached ADSCs, we observed that cell uptake of ^{18}F-FET was repidly decrease at 30 min and then was gradually declined at 60 and 120 mins (Figure 4). On the other hands, ADSC spheroid showed slightly decrease at 30 min and then was declined dramatically at 60 and 120 mins (Figure 4).

As shown in Figure 5, the difference of cell uptake of ^{18}F-FEONM was performed between attached ADSCs and ADSC spheroid. For attached ADSCs, we found that the peak of cell uptake of ^{18}F-FEONM was maintained within 30 min and then was gradually declined at 60 and 120 mins (Figure 5). For ADSC spheroid, the peak of cell uptake of ^{18}F-FEONM was continuous within 30 min and then was decreased at 60 and 120 mins (Figure 5).



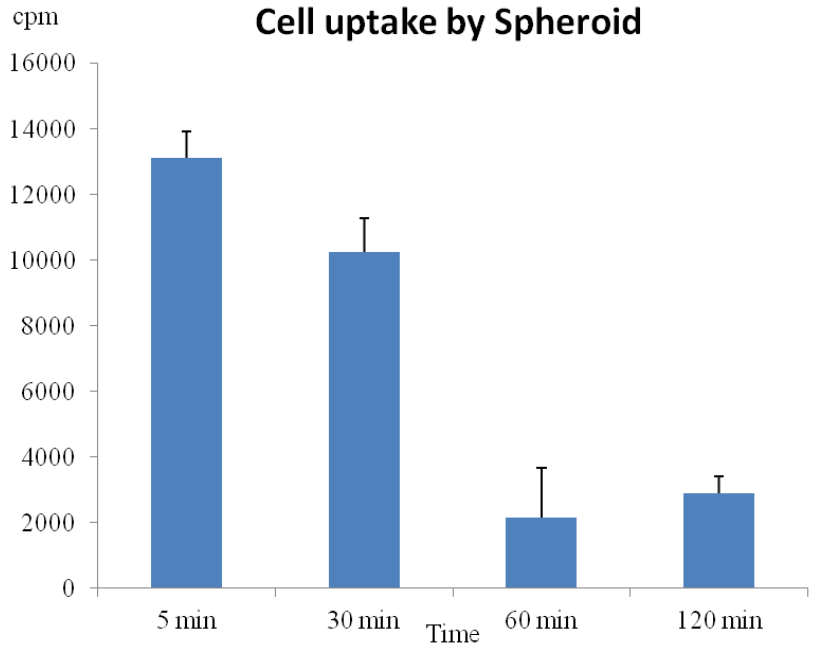
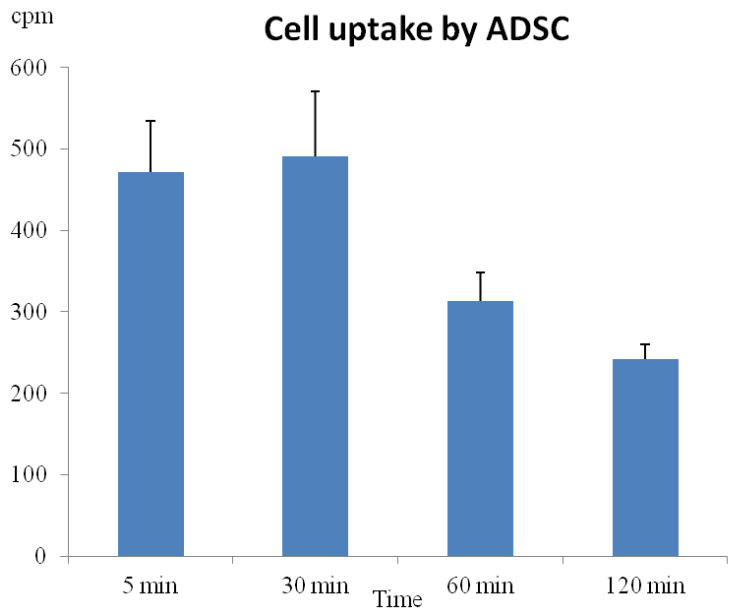


Figure 4. The cell uptake of ^{18}F-FET at 5 min, 30 min, 60 min, and 120 min for ADSCs and ADSC spheroids.



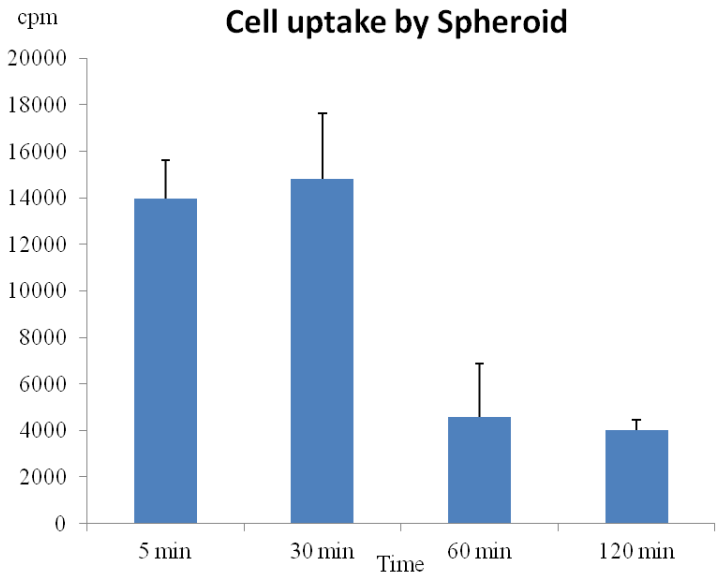


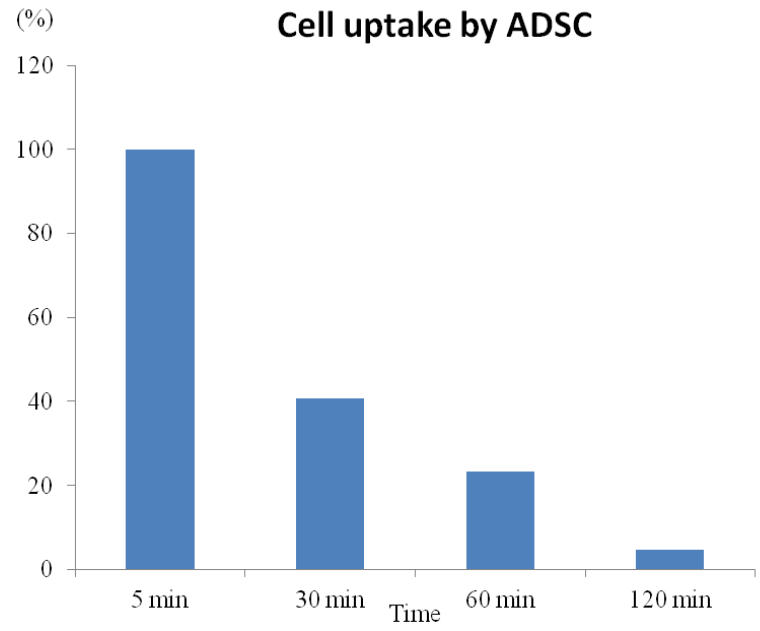
Figure 5. The cell uptake of ^{18}F-FEONM at 5 min, 30 min, 60 min, and 120 min for ADSCs and ADSC spheroids.

肆、討論

The value of cell uptake at 5min was selected as the baseline (100%) and the values of cell uptakes at 30, 60, 90 min were divided by baseline to obtain the relative percentages (compared to time point at 5 min), as shown in Figure 6 & 7. In ^{18}F-FET cell uptake analysis, attached ADAS showed gradually decrease with the percentages of 100%, 41%, 23%, 5% at 5, 30, 60, 120 min respectively, while ADAS spheroid exhibited sharply decrease with the percentages of 100%, 78%, 16%, 22% at 5, 30, 60, 120 min respectively. In ^{18}F-FEONM cell uptake analysis, attached ADAS showed decrease with the percentages of 100%, 104%, 66%, 52% at 5, 30, 60, 120 min respectively, whereas ADAS spheroid exhibited sharply decrease with the percentages of 100%, 106%, 33%, 29% at 5, 30, 60, 120 min respectively. The ^{18}F-FEONM cell uptakes were maintained within 30 min for attached ADAS and ADAS sphroid and then decreased at 60 and 120 min. In addition, the ratios of attached ADAS and ADAS spheroid were calculated at different time points, as shown in Figure 8. For ^{18}F-FET cell uptake experiment, the ADSC spheroids exhibited 49, 94, 35, 230-fold cell uptake than those of attached ADSCs at 5, 30, 60, 120 min respectively. Otherwise, the ADSC spheroids showed 30, 30, 15, 17-fold cell uptake than those of attached ADSCs at 5, 30, 60, 120 min respectively for ^{18}F-FEONM cell uptake experiment. This result showed that the cell uptake for spheroid form was higher than that of attached form no matter what ^{18}F-FET or ^{18}F-FEONM radiolabel probe.

From previous study, MSCs isolated from the adipose tissue of rats

were differentiated into neuron-like cells that express neuronal markers [40, 41]. Therefore, adipose tissue may apply as an alternative source of pluripotent stromal cells capable of neural differentiation and may have the potential for thetreatment of neurologic disorders. Besides, blocking Rho/ROCK by the Y compound significantly prevented the spheroid formation and decreased the stemness gene expression, indicating that the cytoskeletal rearrangement and spheroid formation may be crucial in keeping the stemness genes [42]. Rho/ROCK is one of the major regulators for cytoskeleton, cell migration and cell-cell adhesion. The Rho/ROCK-myosin signaling axis plays an essential role in the regulation of basic cell-cell communications in both mouse and human ESCs [43]. ROCK acts as one of the downstream molecules of Wnt/frizzled, which serves as the self-renewal signal for mouse mammary stem cells [44] and plays a variety of roles in stem cell activity. In addition, the 3D intermediate multicellular state has been described in many circumstances, such as embryoid bodies from ESCs and neurospheres from neuroprogenitors. This state may help further development of stem cells [45]. The mechanism need to further investigate for understanding the cell uptake of spheroid form was far higher than that of cell uptake of attached form.



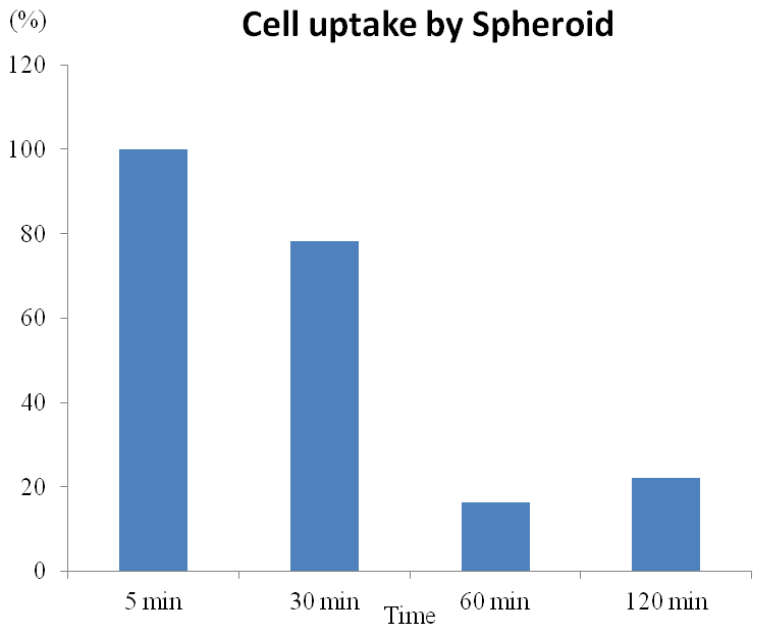
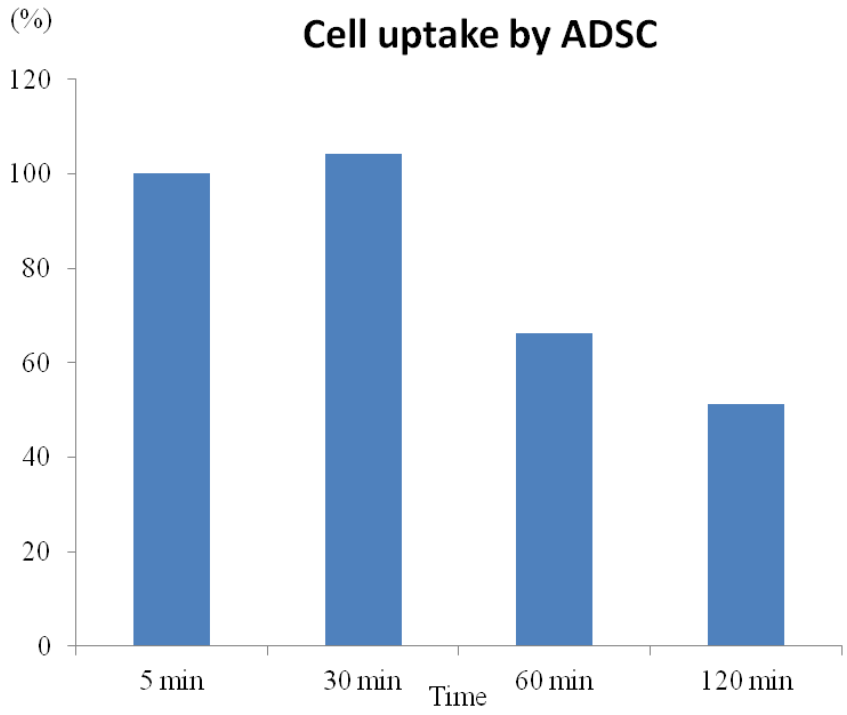


Figure 6. The percentage of cell uptake of ^{18}F-FET to compare with baseline setting at 5 min for different time points at 5 min, 30 min, 60 min, and 120 min for ADSCs and ADSC spheroids.



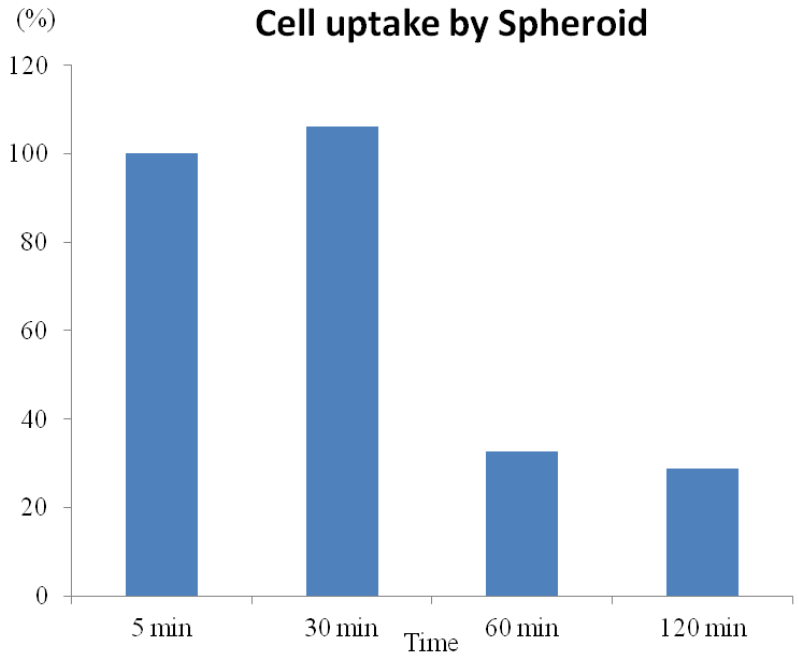
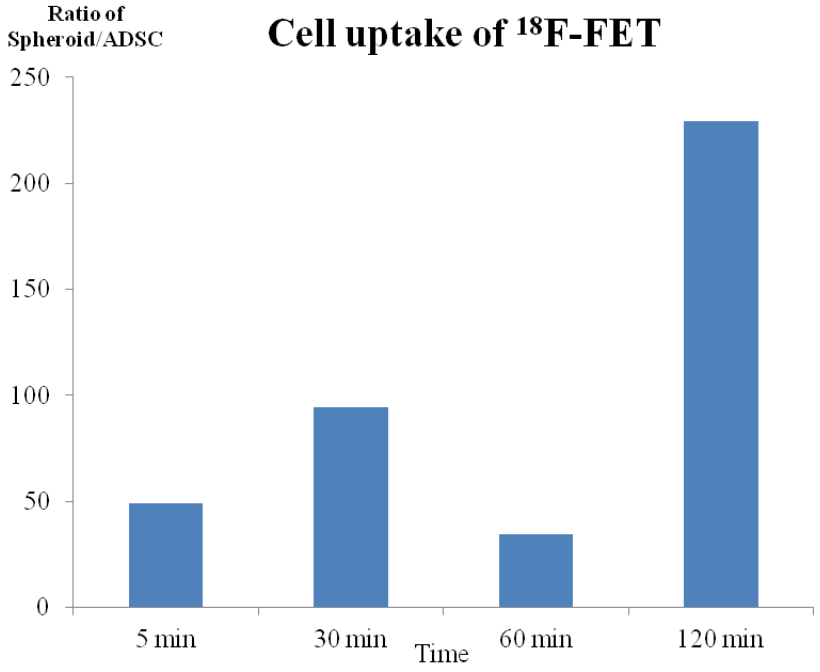


Figure 7. The percentage of cell uptake of ^{18}F-FEONM to compare with baseline setting at 5 min for different time points at 5 min, 30 min, 60 min, and 120 min for ADSCs and ADSC spheroids.



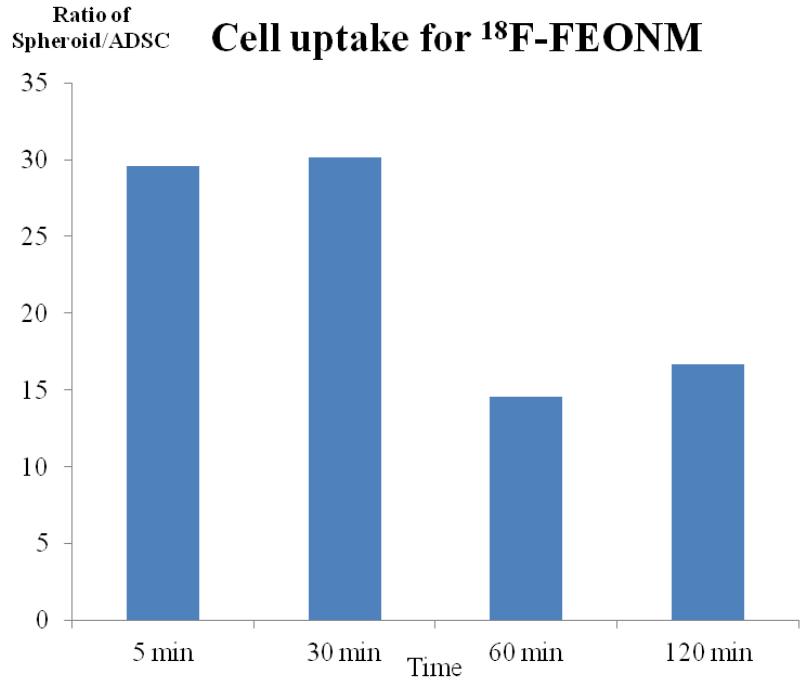


Figure 8. The ratio of cell uptake between ADSC spheroids and attached ADSCs at 5 min, 30 min, 60 min, and 120 min for ^{18}F-FET and ^{18}F-FEONM.

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