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Age-associated features of mitochondrial potential ($\Delta\Psi_m$) changes induced by rare-earth based nanoparticles

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Age-specific effects of redox-active nanoparticles (NPs) (rare-earth orthovanadates and CeO_2) of various geometrical parameters on mitochondrial potential ($\Delta\Psi_m$) have been studied in isolated rat hepatocytes. It was shown that extra small (1–2 nm) CeO_2 NPs at high concentrations cause a slight decrease of $\Delta\Psi_m$ only in the hepatocytes of 3-month-old rats. The orthovanadate NPs suppress $\Delta\Psi_m$ in concentration-dependent manner, whereas the sensitivity of mitochondria to the lower NPs concentration is higher for 20 month-old rats. Thiol protector glutathione was shown to prevent completely NPs-induced $\Delta\Psi_m$ decrease. For the old animals, thiol protection against the NPs as well as exogenic pro-oxidants (H_2O_2 and *t*-BHP) action was more expressed. $\Delta\Psi_m$ stabilization in the presence of the NPs was observed at pro-oxidant conditions only for the young rats. It was suggested that the reduction of mitochondrial functions under effect of the orthovanadate NPs is due to increase of reactive oxygen species (ROS) production, and it had age-related character and depended on the thiol buffer system.

Keywords: Rare-earth based nanoparticles, oxidative stress, mitochondrion, hepatocytes, prooxidant, antiradical, age.

Исследованы возрастные особенности влияния редокс-активных наночастиц (НЧ) (ортованадатов и CeO_2) с различными геометрическими параметрами на митохондриальный потенциал ($\Delta\Psi_m$) в изолированных гепатоцитах крыс. Показано, что экстрамалые НЧ (1–2 нм) CeO_2 в высокой концентрации вызывают незначительное снижение $\Delta\Psi_m$ только в гепатоцитах 3-х месячных крыс. Ортованадатные НЧ подавляют $\Delta\Psi_m$ в зависимости от концентрации и чувствительность митохондрий к меньшим концентрациям НЧ выше у 20-и месячных животных. Тиольный протектор глутатион предотвращает вызванное НЧ снижение $\Delta\Psi_m$ полностью. У старых животных защитное действие тиолов против воздействия как НЧ, так и экзогенных прооксидантов (H_2O_2 and *t*-BHP) более выражено. Стабилизация $\Delta\Psi_m$ в присутствии НЧ под воздействием прооксидантов наблюдалась только у молодых животных. Можно предположить, что снижение митохондриальной функции, вызванное НЧ, зависит от возрастания генерации активных форм кислорода, имеет возрастные особенности и зависит от тиольной буферной системы.

Вікові особливості зміни мітохондріального потенціалу ($\Delta\Psi_m$), викликані наночастинками на основі рідкоземельних елементів. *К.А.Аверченко, Н.С.Кавок, В.К.Клочков, С.Л.Єфімова, М.Ю.Малюкіна, О.О.Сєдих, С.А.Клімов.*

Досліджено вікові особливості впливу редокс-активних наночастинок (НЧ) (ортованадатів та CeO_2) з різними геометричними параметрами на мітохондріальний потенціал

($\Delta\Psi_m$) в ізольованих гепатоцитах щурів. Показано, що екстрамалі НЧ (1–2 нм) CeO_2 у високій концентрації викликають незначне падіння $\Delta\Psi_m$ тільки у гепатоцитах 3-місячних щурів. Ортованадатні НЧ пригнічують $\Delta\Psi_m$ залежно від концентрації і чутливість мітохондрій до менших концентрацій НЧ вище у 20-місячних тварин. Тіольний протектор глутатіон (GSH) повністю запобігає викликаному НЧ зниженню $\Delta\Psi_m$. У старих тварин захисна дія тіолів проти впливу як НЧ, так і екзогенних прооксидантів (H_2O_2 and *t*-BHP) більш виражена. Стабілізація $\Delta\Psi_m$ у присутності НЧ під впливом прооксидантів спостерігалася тільки у молодих тварин. Можна припустити, що зниження мітохондріальної функції, викликане НЧ, залежить від зростання генерації активних форм кисню, має вікові особливості і залежить від тіольної буферної системи.

1. Introduction

Nanotechnologies have opened numerous perspectives in biomedical area. Nanomaterials allow multiple functionalization, optical and magnetic properties, redox activity to be combined that lead to development of the novel imaging and therapy approaches. Biomedical applications of rare-earth based nanoparticles (NPs) were also reported [1, 2]. Recently it was shown that the rare-earth based NPs can be used for *in vitro* and *in vivo* bio-object imaging [3, 4]. Such NPs exhibit stable light emission, narrow luminescence bands and size-independent emission wavelength. Gd^{3+} ions being the most commonly used ions for T1-based MRI contrast enhancement [5] or antioxidant like sensors. It was shown that the antioxidant sensor properties of the NPs can be explained by the smaller size rendering the role of the surface more important or by differences of the surface properties due to the citrate complexes or of crystallinity [6].

Oxygen nonstoichiometry is responsible for the activity of cerium oxide NPs in biochemical redox processes when they react with reactive oxygen species (ROS) and free radicals. Use of CeO_2 NPs as free radical scavengers has great prospects because the oxidative stress plays a critical role in damaging of vital functions (e. g. at irradiation) [7, 8]. The reason for the antioxidant activity of CeO_2 is interconversion of the redox processes associated with the interconversion Ce^{4+} Ce^{3+} on the nanoparticles surface. We suppose that in orthovanadate Eu^{3+} doped NPs such mechanism is also applicable as a result of the conversion Eu^{3+} Eu^{2+}. However, it is known that NPs not containing variable-valency ions can also have antioxidant activity, for example, fullerenes. Data about properties of the nanoceria is contradictory: some researches report about ability of CeO_2 NPs in *in vitro* system demonstrate the activity like superoxide dismutase (SOD) [9–11] and have neuroprotective [12] and anti-inflamatory properties. But data about cytotoxic and proapoptic effects of CeO_2 are also reported [13, 14]. Prooxidant\antioxidant properties of all NPs depends on their sizes, shapes, hydrophilicity/hydrophobicity, heterogeneity and porosity of the surface and this is may determine the end result of interaction between the particles and biological microenvironment. The nanocrystals growth mechanisms also have particular relevance for the NPs that may undergo chemical transformations in environmental or biological milieu. So, demonstrated by the NPs prooxidant or antioxidant action, namely, the change of oxidative balance in living systems has to be investigated in different levels and in dynamics as well. Obviously, that in the cells toxic effects of NPs implemented through the imbalance of the ROS generation. Cells mitochondria are primary sources and main targets of the ROS, and they are very sensitive to the oxidative damage. The mitochondrial transmembrane potential ($\Delta\Psi_m$) allows assessing the mitochondrial activity and cell viability, as well as apoptosis activation in the cell [15, 16]. Quantitative microfluorimetry with JC-1 allow monitoring of the $\Delta\Psi_m$ in single living cells [17–19]. In the present research we report ability of rare-earth based NPs (luminescent orthovanadates $n ReVO_4:Eu^{3+}$ (Re = Gd, Y, La) and CeO_2 with different form-factors) to alter the mitochondrial potential of isolated hepatocytes of different aged rats — 3 month-old and 20 month-old.

2. Experimental

2.1. Synthesis of the NPs

Synthesis of $n ReVO_4:Eu^{3+}$ (Re = Gd, Y, La) and CeO_2 water colloidal solutions was carried out according to the method reported earlier [4, 20]. NPs with different form-factor, namely — spherical (with average size of 1–2 nm), spindle form (25×8 nm), rod-like (57×6–8 nm), CeO_2 (with the average size of 1–2 nm and 8–10 nm) were ob-

tained. The NPs were characterized using Transmission electron microscopy (TEM-125K electron microscope, Selmi, Ukraine). Standard deviation does not exceed ±10 % from the average size of the particle.

Dynamic Light Scattering and Phase Analysis Light Scattering of the NPs were performed to find out the question about influence of the cell culture medium (the Eagle's medium with 10 % fetal serum pH = 7.4) on the particle size distributions and ζ-potential was measured using Zeta-PALS/BI-MAS (Brookhaven Instruments Corporation, USA) kit like ascribed in [21]. Changes in distribution of the fractions and coagulation of the NPs in the Eagle's medium were not observed.

2.2. Fluorescence analysis of the cells

Fluorescence analysis of the cells was performed with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide) as described in [21, 22]. Hepatocytes were isolated from 3 month-old and 20 month-old male the Wistar normal rats by the method described earlier [23] in accordance with the International Rules of "The European Convention for the protection of vertebrate animals used for experimental and other scientific purposes" (Strasbourg, 1986) approved by the III-rd National congress on bioethics of Ukraine (Kyiv, 2007). The cells number was counted and their viability was evaluated by trypan blue exclusion. In the experiments the samples with viability higher than 90 % were used. The cells (5×10^5 cell/ml) were stained with JC-1 (10^{-6} M) in the Eagle's medium with 10 % fetal serum pH 7.4 at the room temperature for 60 min. After the cell staining, 25 or 50 μL of the stock NPs solution (1 g/L) was added to each well, then the cells were treated by the NPs for 24 h and assayed for mitochondrial membrane potential. The final concentrations of the NPs in the samples were 0.025 or 0.05 g/L, respectively.

In separate experiments inorganic hydroperoxide H_2O_2 (1 mM) and organic hydroperoxide *t*-butylhydroperoxide (*t*-BHP) (50 μM or 5 μM) were used as model oxidants. 2 mM glutatione (GSH) or 5 mM *n*-acetyl cysteine (NAC) were applied as protectors against development of oxidative stress: after staining of the cells with JC-1, the thiols were added to the wells 1 h before adding of the oxidants or the NPs.

Microfluorimetry was described elsewhere [24, 25]. Further modifications of the quantitative image method were made to improving the accuracy of the measurements permitted to analyze the mitochondrial function *in situ* [26–28, 21]. The fluorescence microscopy was performed using inverted epifluorescence microscope (IX-71; Olympus, Tokyo, Japan). Images were taken using digital camera (C-5060 Olympus, Tokyo, Japan) connected to the microscope. DP-soft 5.0 software (Olympus, Tokyo, Japan) was used for the images processing. Ratio imaging was done using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA) to measure the mean green and red intensities of the objects. The relative $\Delta\Psi_m$ changes occurring in the cellular area at the time point was evaluated by using $F_{exp}/F_{control}$. The data was averaged from ~25–30 cells per cover slip for every analyzed point. Results were expressed as the mean ±SEM of five repetitions.

The results were statistically processed by means of the software Statistika v. 5.0 (StatSoft, USA) and Origin 6.1 (Origin Lab Corporation, USA) using the Student's *t*-criterion. The results differed statistically and significantly at $p < 0.05$.

3. Results and discussion

In our previous research we have shown that in biotic and abiotic systems the NPs influence on free-radical processes differs significantly. In dependence of the microenvironment and experimental conditions the NPs demonstrate protective properties or enhanced induction of oxidative stress [29]. Also we have shown that the main target of orthovanadate NPs with different form-factors is mitochondrion, and interaction of mitochondrion with the NPs depends on time of interaction and shape of the NP [21]. In present research we investigate age-associated features of the NPs influence on the mitochondrion of isolated hepatocytes.

(1–2 nm) CeO_2 NPs at concentration of 0.05 g/l caused slight decrease of $\Delta\Psi_m$ only in the hepatocytes of young rats that can correlate with ability of the extrasmall NPs to penetrate intracellular organelles including mitochondria. According to literature penetration of the extrasmall NPs into cells occur by the mechanism of adsorption-diffusion without endocytosis. It allows the NPs to penetrate directly into cytosol and intracellular substructures such as nucleus and mitochondrion [30]. $\Delta\Psi_m$ changes are associated with high reactivity of the extrasmall NPs and their tendency to aggregate and ability to damage the cellular structures. This data correlated with data about high

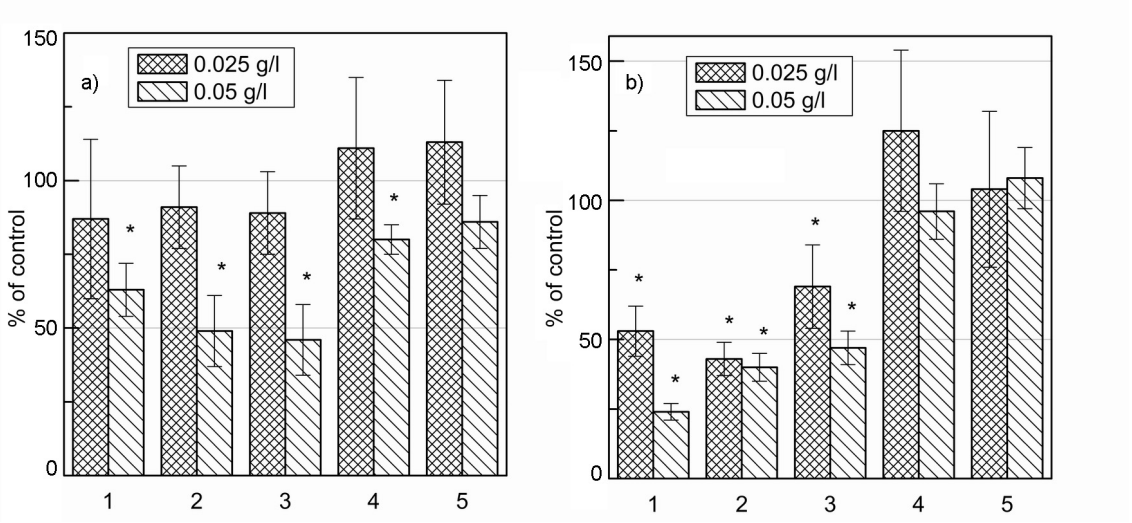

Fig. 1. Influence of exposure with NPs (1 — spherical, 2 — spindle, 3 — rod-like, 4 — CeO_2 1–2 nm, 5 — CeO_2 8–10 nm) in different concentrations (0.025 and 0.05 g/l) for 24 h on $\Delta\Psi_m$ of single hepatocytes of different aged rats (a — 90 day; b — 20 month). ($^* p < 0.05$ compared to control).

prooxidant activity of these particles in biosystems of different complexities [29]. Suppression of the mitochondrial activity occurs in dependence of concentration of the orthovanadate NPs, and less concentration (0.025 g/l) had more expressed effect for 20 month-old rats as compared to the 3 month-old rats (Fig. 1). In addition at higher concentration (0.05 g/l) suppressive effect for the extrasmall orthovanadate NPs was more expressed. The most age differences in response of $\Delta\Psi_m$ to the NPs was observed for the smallest orthovanadate NPs: in hepatocytes of the old rats these particles inhibited mitochondrion more significantly. This fact can be explained by different initial state and reactivity of the antioxidant defense-system. Confirmation of the age-associated features of influence of the NPs on redox-balance of the cells was obtained with using NAC and GSH as protectors against harmful influence of the NPs on $\Delta\Psi_m$. GSH unlike to NAC neutralizes negative effects of the particles on mitochondrion completely. GSH is one of the main components of the thiol redox buffer in the cell. Depletion of the pool of reduced GSH and change in the GSH/GSSG ratio may be mediated by enzymatic processes of detoxification of the nanoparticles [31]. Exogenous GSH is able to penetrate the hepatocytes unchanged and protect as extracellular, so the intracellular structures, and the most importantly, mitochondrion and nuclei. NAC is able to protect cells and mitochondrion only partly because it is a progenitor of energy-consuming process of synthesis of GSH and this effect may not be enough for neutralization of the NPs in conditions of decrease of the mitochondrial function and synthesis of ATP. We found that in the hepatocytes of 20 month rats GSH expressed significant the protective effect against influence of the orthovanadate NPs as well as oxidative stress developed by prooxidants (H_2O_2 and *t*-BHP) (Fig. 2). With the aging the activity of enzymes of antioxidant defense system increases, but the level of GSH exogenous substrate decreases and this fact explains the observed differences of exogenic GSH influence in the cells of 3 month-old and 20 month-old rats (Fig. 2). NAC also demonstrates more expressed protective effect against toxic concentration of *t*-BHP in the cells of the old rats.

Previously it was shown that in biotic system the spherical orthovanadate NPs enhanced effect of oxidative stress simulated by H_2O_2 and *t*-BHP and pre-incubation of samples with 8–10 nm CeO_2 prevented development of oxidative stress induced by H_2O_2 [29]. Paradoxical stabilization of the mitochondrial potential in hepatocytes of the young rats in prooxidant conditions modeled by H_2O_2 and *t*-BHP was shown (Fig. 3a, b). The effect was not observed in hepatocytes of 20 month old rats. Conceivably, the adaptive resources of cells are mobilized under influence of NPs and prooxidants, and the final effect is the preservation of $\Delta\Psi_m$ level. In contrast to the young ones, in the old animals independently on the presence of prooxidants the mitochon-

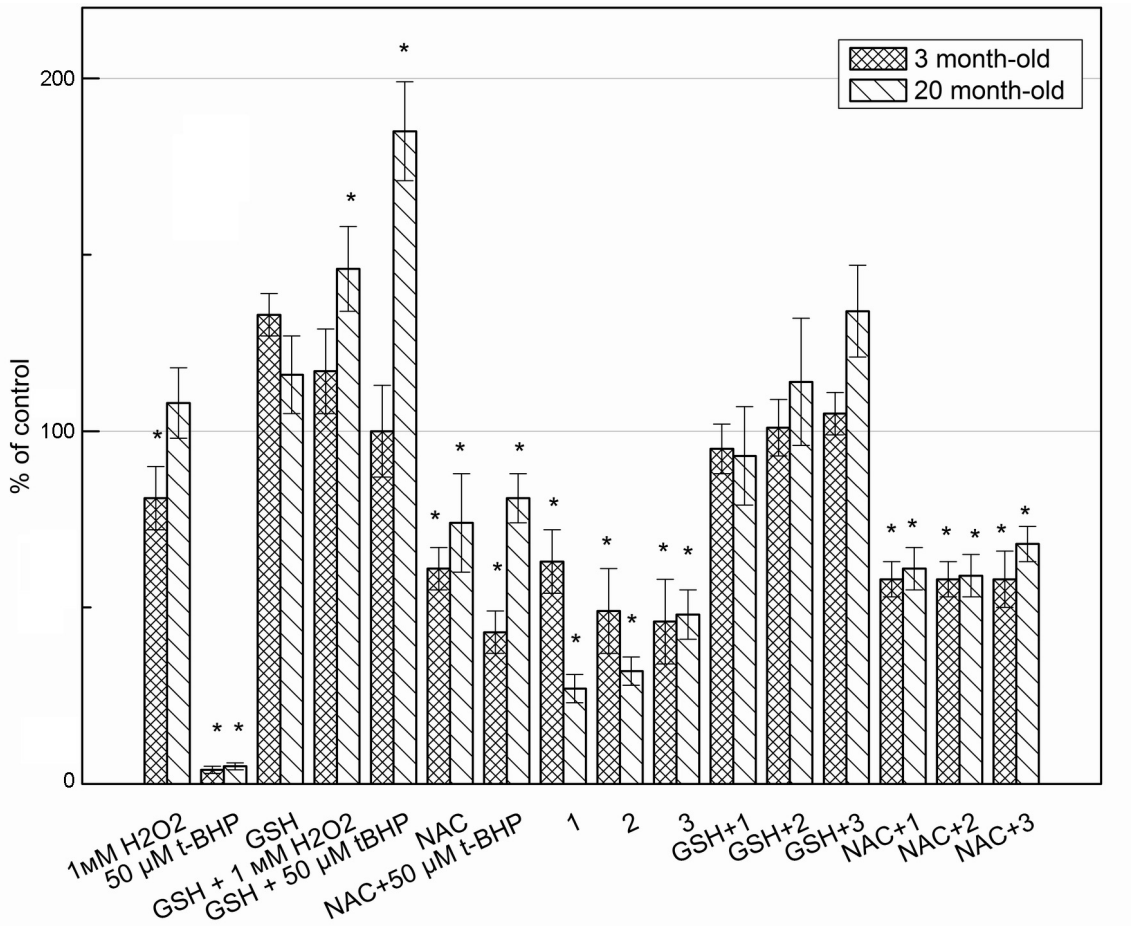

Fig. 2. Influence of exposure with NPs (1 — spherical, 2 — spindle, 3 — rod-like; c = 0.05 g/l) and model oxidants (H_2O_2 (1 mM); TBHP (50 μM)) for 24 h on $\Delta\Psi_m$ of single hepatocytes after or without pretreatment (for 1 h) with 2 mM GSH and 5 mM NAC. (* $p < 0.05$ compared to control).

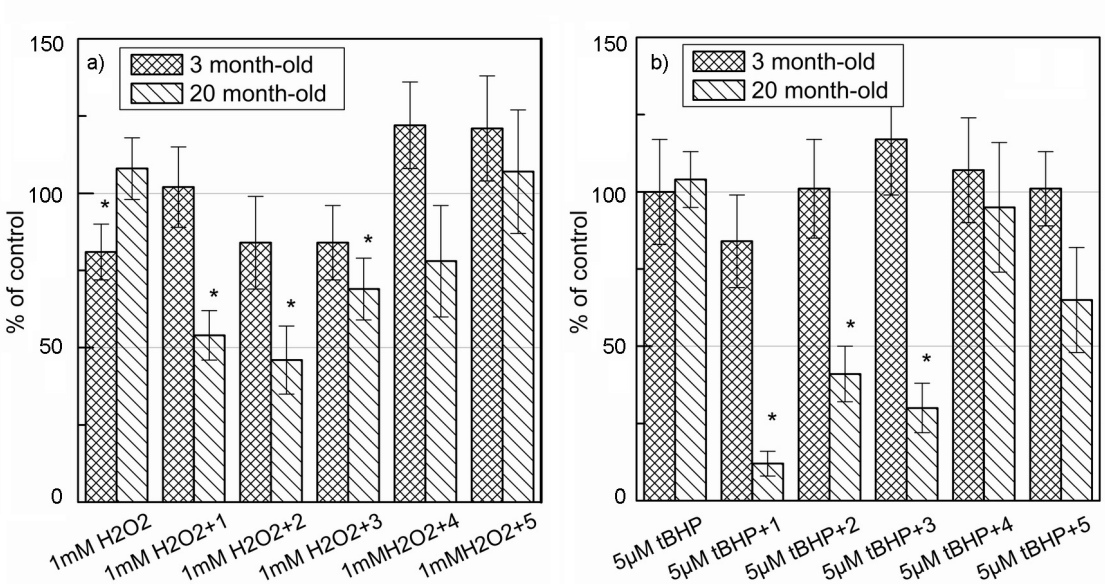

Fig. 3. Effect of all types of nanoparticles (1 — spherical, 2 — spindle, 3 — rod-like, 4 — CeO_2 1–2 nm, 5 — CeO_2 8–10 nm) (c = 0.05 g/l) on $\Delta\Psi_m$ of single hepatocytes of different aged rats (3 month and 20 month) with induction of oxidative stress using H_2O_2 (1 mM) — (a) and t-BHP (5 μM) — (b) (* $p < 0.05$ compared to control).

drial potential decreased under influence of the NPs: in presence of *t*-BHP (Fig. 3b) the mitochondrial potential decreased more significantly compared to H_2O_2 (Fig. 3a). Such differences can be explained by the higher mobility of defense systems of the young organism and by the fast adaptive rebuilding induced by ROS. This fact provides the evidence that the NPs induced mitochondrial function failure is ROS-dependent, has the age-related character and depends on the thiol buffer system.

4. Conclusions

Thus, the data obtained show that extrasmall NPs of the both investigated types can have suppressive action on mitochondria due to high penetration activity and prooxidant properties. Such effects were more expressed for the orthovanadate NPs because of the higher prooxidant activity. In addition the orthovanadate NPs action revealed significant age specificity: in the cells of 20 month animals the decrease of $\Delta\Psi_m$ was more expressed. This fact is explained by the aging changes in antioxidant defense system and functional states of the cells membranes, by synthesis and level of GSH decreased with the aging on the ground of increased activity of the GSH-using enzymes. Protective effect of exogenous GSH in hepatocytes of the old rats confirms this assumption. The adaptive resources of the cells mobilized under simultaneous influence of the NPs and prooxidants in the cells of 3 month-old rats and paradoxical stabilization of the mitochondrial potential observed. The effect was absent in hepatocytes of the old rats due to the lower mobility of the defense system and presence of antioxidants (GSH). Age-specific changes of biosystems should be considered in evaluation of NPs bioactivity that is the main point in development of nanotherapeutics.

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