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Albumin coated copper-cysteine nanozyme for reducing oxidative stress induced during sperm cryopreservation

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ABSTRACT

In the present work, SOD mimetic nanozyme (NACu-Cys) consisting of Cu-Cys complex and nano-albumin (NA) were synthesized. After characterizing the nanozyme, its superoxide dismutase (SOD) behavior was evaluated by inhibition of the pyrogallol autoxidation method. The results revealed that NACu-Cys exhibited SOD mimetic activity with a half inhibition concentration (IC_{50}) value of 7.0×10^{-3} μM and a turnover number (k_{cat}) of $5.4 \times 10^{7}\ s^{-1}$. In the next step, this nanozyme was applied as a protective agent against oxidative stress induced by sperm cryopreservation. Increasing the motility, raising the viability and reducing the apoptosis occurred as a result of NACu-Cys additions to human sperm freezing medium. Comparison between the natural SOD and SOD mimic behavior of NACu-Cys revealed that this nanoparticle has the ability to be used as oxidative stress decrescent during cryopreservation process.

1. Introduction

Reactive oxygen species (ROS) including superoxide radical anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) could usually be generated by either a metabolic process or the sources such as environmental stress, ionizing radiation, redox and heavy metals, smoking and pollution [1]. At high level of ROS, the antioxidant defense system in the body is suppressed and consequently the oxidative stress occurrs. Oxidative stress damages biological tissues, and leads serious diseases including ischemia-reperfusion disorders, cardiovascular conditions, inflammatory processes, neurodegenerative disorders, cancer, and diabetes mellitus [2]. Furthermore, it effects fertility status and thus, it has been studied extensively in recent years. Researches have revealed that ROS negatively effects sperm function, both in vivo and in vitro. On the other hand, sperm preparation for assisted reproductive technologies such as sperm cryopreservation is a clinical process that leads to ROS generation [3].

Antioxidants play a vital role in cell protection from oxidative stress caused by ROS. One of the natural antioxidant defense systems is based on SOD (superoxide dismutase) function. SOD removes ROS by dismutation of $O_2^{\cdot -}$ into O_2 and H_2O_2 [4]. However, applications of natural SOD are limited due to their high cost, short plasma half-life, and also the disadvantages such as chemical stability, cell permeability and immunogenicity. To overcome these limitations, researchers focused on designing artificial enzyme having SOD mimetic activity [5]. Most of the SOD mimetics have been designed based on a redox active metal center, similar to the metals exists in active site of the natural SOD, and a chelating agent [6]. Copper-zinc SOD is a natural SOD species which is most common in eukaryotic cells. In this type of SOD, copper ion plays the main role in the catalysis of $O_2^{\cdot -}$ dismutation but zinc ion participates in the enzyme structure forming [7]. Hence, many copper complexes of amino acid residues [8], peptides [9], macrocyclic compounds and Schiff-bases [4] have been reported to exhibit SOD like activity. Consequently, our research team also developed a copper complex containing cysteine residue (Cu-Cys complex) which exhibited SOD mimetic activity [10,11].

Recently, researchers have emphasized on constructing biocompatible and highly active artificial enzymes. Therefore, they considered the protein-based nanoparticles as artificial enzymes due to their biocompatibility, non-antigenicity, metabolization and easy modification. One of these protein-based nanoparticles is nano-albumin (NA) [12]. The remarkable properties of albumin such as antioxidant activity [13], high solubility, long circulatory half-life, and many binding sites have attracted researchers' attention to use nanoalbumin carrier for a large number of drugs. For example human serum albumin nanoparticles have been used in some FDA (food and drug administration) approved drugs such as Abraxane™ [14].

In the present report, a novel SOD mimetic nanoparticle consisting

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of Cu-Cys complex and albumin were synthesized using nab-technology (nano-albumin bound technology) [15]. The structure of NACu-Cys was analyzed using different spectroscopic methods, dynamin light scattering (DLS), energy dispersive X-ray spectroscopy (EDS), field emission scanning electron microscope (FESEM) and atomic force microscope (AFM) images. The SOD mimetic activity and enzyme kinetic parameters of NACu-Cys was evaluated by pyrogallol autoxidation method [16]. To confirm the SOD mimetic activity of NACu-Cys in vitro, its cryoprotective activity against cryodamage of human sperm was investigated and the results were compared with those obtained by natural SOD.

2. Experimental section

2.1. Materials and apparatus

Pyrogallol, Tris buffer, EDTA, hydrochloric acid, and copper acetate was purchased from Merck. L-cysteine, bovine serum albumin (BSA), bovine erythrocyte Cu-Zn superoxide dismutase (SOD, EC.1.15.1.1) and eosin-nigrosin were purchased from Sigma-Aldrich. Ham's F10 medium was obtained from Life Technology, Carlsbad, CA and human serum albumin were supplied by Life Global, Guelph, Canada. YO-PRO-1 and propidium iodide (PI) with the Apoptosis Assay Kit was got from Invitrogen, Carlsbad, CA and used for staining and flow cytometry assays of apoptosis and necrosis in sperm cells.

NACu-Cys was prepared by ultra-sonication homogenizer, UHP-400 (Development of ultrasonic technology, Iran). UV–Vis spectra were recorded by Carry 100Bio spectrophotometer (Varian, Australia). Fluorescence spectra were obtained with spectrofluorimeter model MPF-4 (Hitachi, Japan). Fourier transform infrared (FT-IR) spectrum was measured with a Perkin-Elmer 343 spectrometer (USA), using KBr disc. Dynamic light scattering (DLS) (Brookhaven, USA) were used to measure size of the nanoparticles. The chemical composition was considered by energy dispersive X-ray spectroscopy (EDS). And the images of NACu-Cys were obtained by field emission scanning electron microscope (FESEM), using Mira 3-XMU FESEM (Tescan Co, Brno, Czech Republic) and atomic force microscopy (AFM) by using a Park Scientific model CP-Research (VEECO). Far-UV circular dichroism (CD) of NACu-Cys was measured in the wavelength range from 190 to 260 using a spectropolarimeter model 215 (Aviv, USA) and quartz cuvette (one cm path length, 300 µl). All fluorescence signals of labeled spermatozoa were analyzed by flow cytometer FACScan (Becton Dickinson, San Jose, CA) equipped with Flomax software. Inductively coupled plasma–optical emission spectroscopy (ICP-OES), Optima 72,000 model (Perkin–Elmer, USA) was used for determination of copper concentration in Cu-Cys complex. The motility was assayed by the computer-assisted sperm analyzer (CASA) system (SCA^{TM} motility module; Microptic, Barcelona, Spain).

2.2. Preparation and characterization of NA and NACu-Cys

Cu-Cys complex was synthesized by a solvent free method [17]. Briefly, L-cysteine (0.10 g) and copper acetate (0.08 g) were mixed and ground by an agate mortar till acetic acid smell was released. After 5 h, the reaction was completed and the sky blue microcrystals were obtained. In order to remove the unreacted cysteine or copper acetate the microcrystals were washed with methanol three times (Scheme 1A).

NACu-Cys was prepared by mixing two following components: at first, 1 ml BSA solution (5% w/v) which was pre-saturated with chloroform 1% was prepared and then, 1 mg Cu-Cys complex was dispersed in a mixture of chloroform (230 µl) and ethanol (20 µl). These two solutions were then mixed and homogenized at 20 kHz with amplitude 28%. The resulting product was rotary evaporated at 25 °C for 15 min under reduced pressure then lyophilized (Scheme 1B). NACu-Cys showed markedly greater water solubility than Cu-Cys complex. NA was prepared similar to NACu-Cys preparation except the addition of Cu-Cys complex.

The protein content of NACu-Cys was determined by Bradford method [18]. Briefly, into a 96-well titer plate 5 µl of NA or NACu-Cys and 245 µl of Bradford reagent were added and mixed. The mixture was incubated at 22 °C for 10 min and then the absorbance of the solution measured at 595 nm. The concentration of unknown sample determined using BSA standard curve. The copper content of NACu-Cys was determined using ICP-OES. For sample preparation, 2.5 mg L^{-1} of NACu-Cys was dissolved in a mixture of aqua regia (300 µl, 1:3, nitric to hydrochloric acid) and hydrogen peroxide (150 µl). EDS was used for elemental analysis of NACu-Cys. DLS, FESEM and AFM were used for size determination and morphology images of NACu-Cys. And also, the UV–Vis, fluorescence and FT-IR spectra of NACu-Cys and starting materials were recorded. The secondary structure of BSA, NA and NACu-Cys were compared by CD spectroscopy.

2.3. SOD mimetic activity and kinetic assay of nanoparticles

The autoxidation of pyrogallol at alkaline pH yields $O_2^{\cdot -}$ followed by o-hydroxy-o-benzoquinone and other polymer products. When SOD reacts with $O_2^{\cdot -}$, the formation rate of the o-hydroxy-o-benzoquinone and other polymer products are decreased. One unit of SOD is defined as the amount of enzyme that inhibits the rate of pyrogallol autoxidation by 50%. 0.05 M Tris/HCl buffer at pH 8.2 containing 10^{-4} M EDTA were used for enzyme assay at 27 °C [16]. The standard rate of pyrogallol autoxidation typically contained a certain concentration of pyrogallol, which produces an autoxidation rate of 0.07 (absorbance per minute) at 325 nm. Based on this condition, the amount of SOD or its mimetic compounds that inhibits the autoxidation rate of pyrogallol by half, is defined as one unit of activity and called half-maximal inhibitory concentration (IC_{50}).

The steady-state kinetics of Cu-Cys complex, NACu-Cys and natural Cu-Zn SOD in the presence of pyrogallol (as substrate) was obtained at 325 nm in Tris/HCl, while the concentration of SOD and its mimetics were 3.08×10^{-8} M. Progressive curves of the reactions were obtained at various pyrogallol concentrations, and the initial rates were used to plot Michaelis-Menten curves. The kinetic parameters of the Cu-Cys complex, NACu-Cys and natural SOD were calculated based on the data obtained by Lineweaver-Burk plot.

2.4. Sample collection and preparation

This study was approved by the ethical committee of Tehran University of Medical Sciences. Informed written consent was obtained from all patients. Semen samples after 72 h of sexual abstinence were collected from 11 fertile men who have normal classical sperm parameters according to the World Health Organization (WHO, 2010) criteria (volume ≥ 1.5 ml, cell concentrations $\geq 15 \times 10^6$ cells/ml, total motility ≥ 40%, and normal sperm cell morphology ≥ 4%) were in sterile plastic containers by masturbation in the privacy room adjacent to the laboratory. After liquefaction, one portion of the neat semen samples was separated for performing routine semen analysis based on WHO 2010 by CASA system. To isolate sperm cells, liquefied semen were washed two times (400*g*, 5 min) in Ham's F10 medium containing 10% HSA; subsequently, sperm were selected using swim up technique in the same medium. After 1 h, the sperm suspension containing motile sperms was collected and subdivided into four equal aliquots: first aliquot was examined without being exposed to Cu-Cys, NACu-Cys or SOD (as control group). But, to the three remaining aliquots 100 units ml^{-1} of either Cu-Cys, or NACu-Cys or natural SOD were added. For each group, the sperm suspensions were adjusted to equal concentrations (5×10^6 sperm cells/ml). Then the cryopreservation solutions were mixed with each parts of sperm suspension drop by drop. Subsequently, after equilibration at room temperature for 10 min, straws were kept at 3 cm above the level of liquid nitrogen for 30 min and finally transferred in to liquid nitrogen tank. After 14 days, samples