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Biodelivery of nerve growth factor and gold nanoparticles encapsulated in chitosan nanoparticles for schwann-like cells differentiation of human adipose-derived stem cells

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ABSTRACT

The constant release of neurotrophic factors through a nanomaterial-based delivery system can be an important strategy in medical and pharmaceutical fields for nerve tissue engineering. The present study was aimed at encapsulating NGF and AuNPs in chitosan nanoparticles (NGF-CNPs and AuNPs-CSNPs) and its evaluation on the differentiation potential of human adipose-derived stem cells (h-ADSCs) to Schwann-like cells. The NGF-CNPs were prepared by ionotropic gelation method with tripolyphosphate (TPP) as a crosslinker. After synthesis and characterization of nanoparticles, NGF encapsulation efficiency and release profile were observed by Bradford assay. Next, the effects of NGF-CSNPs and AuNPs-CSNPs on h-ADSCs survival were assessed through MTT assay. Also, the efficacy of Schwann-like cells differentiation was assessed by immunocytochemistry and real-time RT-PCR for S100β and MBP markers. NGF encapsulation efficiency was found about 85% and controlled and sustained release of NGF was observed during 7 days *in vitro* (74.63 ± 2.07%). The findings revealed that these nanoparticles are cytocompatible. The immunocytochemical analysis indicated that NGF-CSNPs and AuNPs-CSNPs could significantly increase the differentiated rate and myelinogenic potential of Schwann-like cells ($p < 0.05$). Besides, the expression level of GFAP, S100β, and MBP demonstrated significant upregulation in NGF-CSNPs and AuNPs-CSNPs groups compared to the control group ($p < 0.05$). Hence, it can be proposed that NGF-CNPs and AuNPs-CSNPs are capable of controlled release with improving the ability of h-ADSCs differentiation to Schwann-like cells. Also, the results show the potential future application of this differentiation in nerve tissue regeneration.

1. Introduction

The delivery system is useful in the controlled release of proteins, drugs, and genes. This system helps to more constant effect of drugs and materials on the target tissue and protects rapid degradation of drugs, lower administration of drugs, and minimized side effects of drugs [1]. Different polymers of natural or synthetic materials can be applied in a delivery system. These materials, however, should be biocompatible, easily fabricated and sterilized, and capable of high drug loading. Chitosan as a biomaterial with biodegradability, biocompatibility, and mucoadhesive properties is derived from the deacetylation of chitin and is used extensively in the delivery system [2,3]. Chitosan with amino groups has positive charges and can bind to molecules with negative charges. Accordingly, in the encapsulation of drugs using chitosan, tripolyphosphate (TPP) macromolecule are used as a cross linker with negative charges [4].

Neurotrophic factors are one of the important options in nerve tissue engineering. These proteins can stimulate cell proliferation and differentiation, as well as tissue growth. There are several kinds of neurotrophic factors such as nerve growth factor (NGF). This factor is found in different areas of the nervous system and cerebrospinal fluid (CSF) [5]. NGF is an essential protein in axonal regeneration, neurons, and neurotransmitter function. Also, it plays an important role in myelin sheath formation [6]. However, the level of NGF in the nervous system has an indirect relationship with age increase [5]. Thus, the decrease in NGF can establish many disorders such as Alzheimer's disease and Huntington's disease [7]. NGF has neuroprotective effects and alleviates age-related atrophy,

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injured nerves regeneration, and promote learning and memory functions [8,9]. Moreover, NGF has an effective role in the regulation of proteins, carbohydrates, and lipids metabolism in neurons [10]. These findings show that NGF can be beneficial for preventing or treating nerve injuries [11]. Nevertheless, due to the short half-life of proteins, their encapsulation and controlled delivery are serious problems in this regard. Recently, it has been shown that in addition to growth factors, AuNPs affect proliferation and differentiation of stem cells. Besides, AuNPs can be used as a carrier in drug delivery [12]. AuNPs have significant features such as easy synthesis in various sizes, high surface to volume ratio, and good biocompatibility and antimicrobial activity. Therefore, AuNPs have been investigated extensively in medicine field [13–15]. The size AuNPs is an important parameter in their administration and their effect on the treatment of diseases *in vivo*. Therefore, many studies have shown that 15–50 nm AuNPs can cross the blood-brain barrier and help to diagnosis and treatment of neurological disorders [16,17]. AuNPs has high mechanical strength so that when they are encapsulated with chitosan, they can be suitable for conduits fabrication in nerve tissue engineering [18].

The aim of the present study is to offer a controlled and slow release of NGF and AuNPs such that to maintain a high level of NGF and AuNPs during a long period of time. In this study, chitosan was used as a carrier for NGF and AuNPs encapsulation. After assessing the size, stability, and loading rate of encapsulated NGF and AuNPs, their release rate was evaluated during 7 days. Finally, the cytocompatibility of NGF-CSNPs or/and AuNPs-CSNPs and their effects on Schwann-like cells differentiation of h-ADSCs and myelinating potential were evaluated *in vitro*.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan (50–190 kDa) with a deacetylation degree more than 75%, tripolyphosphate (TPP), Coomassie blue G250, phosphate buffered saline (PBS), collagenase IA, 3–4,5-dimethyl thiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), Fetal Bovine Serum (FBS), and dimethyl sulfoxide (DMSO) were supplied by Sigma (USA). Phosphoric acid (85%), acetic acid, and absolute ethanol were purchased from Merck (Germany). NGF and AuNPs were obtained from R&D (USA) and US NANO (USA), respectively. Dulbecco s modified eagles medium (DMEM)/F12, trypsin/EDTA, and penicillin/streptomycin were purchased from Gibco (UK). B27, Human epidermal growth factor (H-EGF), and basic fibroblast growth factor (b-FGF) were purchased from Invitrogen. Anti-S100 and anti-myelin basic protein (anti-MBP), rabbit anti-mouse FITC, and goat anti-mouse Alexa flour were purchased from Abcam.

2.2. Preparation of NGF or AuNPs-loaded nanoparticles

Chitosan nanoparticles were synthesized according to the ionotropic gelation process [19]. Briefly, 4 mg/ml NGF or 25 ppm AuNPs was prepared in 0.1% chitosan solution under constant stirring. TPP was dissolved in Milli-Q and then 0.03% TPP solution was added to chitosan solution containing NGF or AuNPs slowly. The turbidity of the solution was considered as an indicator of the nanoparticles formation.

2.3. Characterization of NGF-CSNPs and AuNPs-CSNPs

The distribution of nanoparticles size and zeta potential were evaluated using Malvern Zetasizer Nano S (UK). Samples were diluted with water before measurement [20]. In order to determine of encapsulation efficiency (EE), the nanoparticles-containing solution was centrifuged at 10,000 rpm for 20 min and free NGF rate was determined by Bradford assay. In this method, 200 μl of supernatant was mixed with 800 μl of Bradford solution and was kept in a dark place for 15 min. Then, unbound NGF rate was measured at the wavelength of 595 nm by a spectrophotometer. EE was calculated as follows [21]:

$$EE(\%) = \frac{\text{Actual amount of drug loaded in nanoparticles}}{\text{Theory amout of drug loaded in nanoparticles}} \times 100$$

2.4. In vitro NGF release study of NGF-CSNPs

NGF loaded chitosan nanoparticles were added in PBS (pH 7.4) at room temperature (20–25 °C) under stirring. The release rate of NGF was measured by spectrophotometry method every day until 7 days. After NGF encapsulation, 1 ml of NGF-CSNPs was centrifuged at 10,000 rpm for 20 min. Then, 200 μl of supernatant was mixed with 800 μl of Bradford solution. The concentration of free NGF was analyzed by a spectrophotometer. Finally, the rate of NGF release was calculated using the following formula [22]:

$$\text{release rate}(\%) = \frac{E}{E0} \times 100$$

Where E is the concentration of releasing NGF and E0 is the loading concentration of NGF in the chitosan nanoparticles.

2.5. Isolation and morphology of human ADSCs

All procedures of this study are applied in accordance with Ethics Committee of the Medical Faculty in Isfahan University of Medical Sciences. Human adipose tissue was obtained from elective lipoaspirate samples of abdominal fat from female patients (age range: 20–45 years old). After receiving informed consent, isolation and culturing of cells were performed as previously described [23]. In this study, h-ADSCs were used at passages 3–4.

2.6. In vitro cytocompatibility of NGF-CNPs and AuNPs-CNPs

Cytotoxicity effect of NGF-CSNPs and AuNPs-CSNPs on h-ADSCs was evaluated by MTT assay on 1, 3, 5, and 7 days according to previous study [24].

2.7. Differentiation of h-ADSCs into Schwann-like cells phenotype

The cell differentiation procedure was carried out according to a previous study [24]. To determine the effect of NGF-CSNPs and AuNPs-CSNPs on Schwann cells differentiation, the cells were divided into 4 groups (i.e., 4 mg/ml of NGF-CSNPs in NGF group [25], 25 ppm of AuNPs-CSNPs in AuNPs group [26], 4 mg/ml of NGF-CSNPs, and 25 ppm of AuNPs-CSNPs in NGF/AuNPs) and added to differentiation medium. However, the control group had neither of the nanoparticles.

2.8. Immunocytochemistry technique

After fixation with 4% paraformaldehyde, the differentiated cells were permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Then, cells were incubated with primary antibody (anti-S-100 β; 1:500; and anti-major basic protein (anti-MBP); 1:500) in the dark at room temperature for 2 h and then, were placed in an incubator overnight. After washing with PBS, samples were exposed with secondary antibodies including rabbit anti-

Table 1
The list of primer sequences for RT-PCR analysis.

Gene	Primer sequences
GFAP	5′-CCGACAGCAGGTCCATGTG-3′ 5′-GTTGCTGGACGCCATTGC-3′
S100 β	5′-GGAGACGGCGAATGTGACTT-3′ 5′-ACTCGTGGCAGGCAGTAGTAA-3′
MBP	5′-GGCCCCGTGGATGGA-3′ 5′-GAGGCGCGAAAGGAGATG-3′
GAPDH	5′-GAAATCCCATCACCATCTTCCAGG-3′ 5′-GAGCCCCAGCCTTCTCCATG-3′

mouse FITC (1:500) and goat anti-mouse Alexa Flour (1:1000). Nuclear staining was performed with DAPI (1:1000). Finally, the cells were observed using a fluorescence microscope (Olympus BX51, Japan). Through a quantitative analysis, the number of positive cells was counted by Image J in captured photos.

2.9. Real-time reverse Transcription polymerase chain reaction

Real-time RT-PCR was used to evaluate the efficiency of NGF-CSNPs and AuNPs-CSNPs on specific Schwann cell and myelinization markers expression after h-ADSCs differentiation. Total RNA was extracted using High Pure RNA isolation kit (Roche, Germany) according to the manufacturer's protocol. Then, RNA was reverse transcribed to cDNA. Real-time polymerase chain reaction (PCR) was performed using steponeplus™ Real-time PCR detection system (Applied Biosystems) with Maxima SYBR Green/ROX qPCR Master Mix 2X (Thermo Scientific). The list of primers is depicted in Table 1. Relative gene expression level was analyzed with the comparative Ct method ($2^{-\Delta\Delta Ct}$). All samples were normalized to levels of glyceraldehyde 3- phosphate dehydrogenase (GAPDH).

2.10. Statistical analysis

In this study, all the data are presented as mean ± standard deviation (SD). Statistically significant differences were evaluated by one-way analysis of variance (one-way ANOVA) and the results were considered as statistically significant at $p < 0.05$.

3. Results

3.1. Encapsulation efficiency and release kinetics of NGF

Zetasizer analysis shows that the mean diameter of NGF-CSNPs is 147.04 ± 8.09 nm and the surface charge of nanoparticles has suitable stability (36.47 ± 1.88 mV). The encapsulation efficiency of NGF in chitosan nanoparticle is 83.93 ± 2.45%. Fig. 1A illustrates the *in vitro* release pattern of NGF determined at given time intervals. This encapsulation pattern in chitosan nanoparticle was provided a slow release while NGF release of CNPs was observed in a continuous and constant manner. As can be seen, there was not any burst release for 7 days. Finally, 74.63 ± 2.07% of NGF was released of NGF-CSNPs after 7 days.

3.2. Isolation and culture of h-ADSCs

Following h-ADSCs isolation, a spindle-like the shape of cells appeared on day 6 after culturing (Fig. 2A). When cells plated in

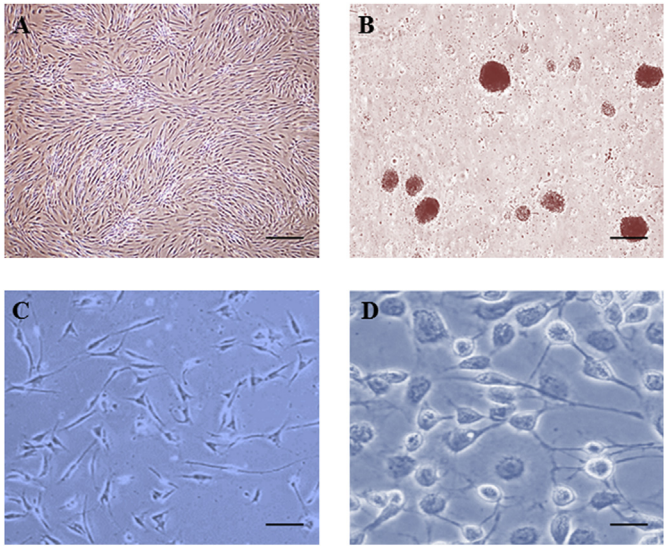

Fig. 2. Phase contrast image of h-ADSCs morphology of culturing (A), Neurospheres formation after h-ADSCs induction (B), Schwann like cells transdifferentiation from h-ADSCs (C,D). Scale bar is 200 μm in A & B, 150 μm in C and 100 μm in D.

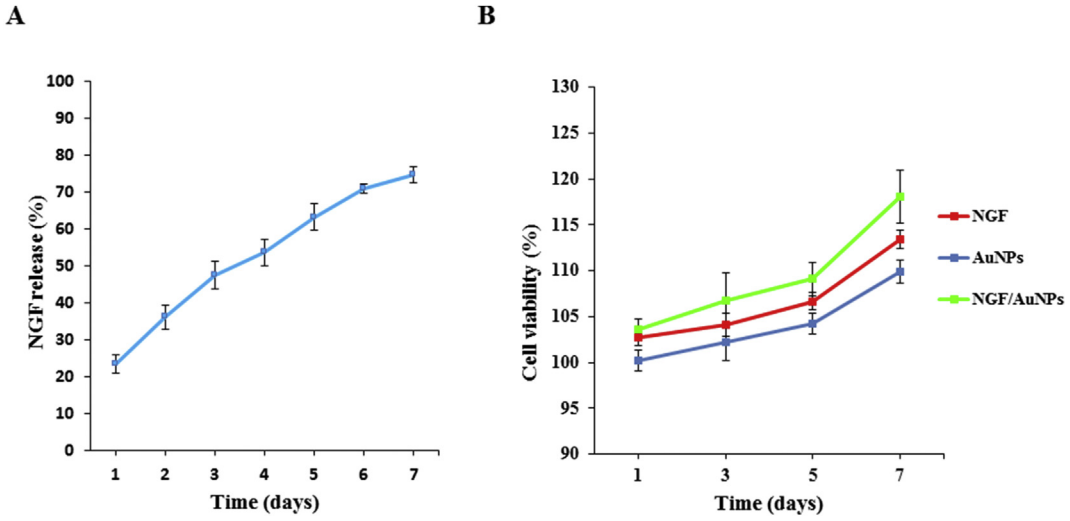

Fig. 1. Cumulative release profiles of NGF from NGF-CNPs in PBS, pH 7.4 at 37 °C (A). Comparison of cell viability in tissue culture plate (control), NGF loaded chitosan nanoparticle (NGF), AuNPs loaded chitosan nanoparticle (AuNPs) and NGF loaded chitosan nanoparticle and AuNPs loaded chitosan nanoparticle (NGF/AuNPs) groups by MTT assay on 1, 3, 5 and 7 days after seeding (mean ± SD)(B).

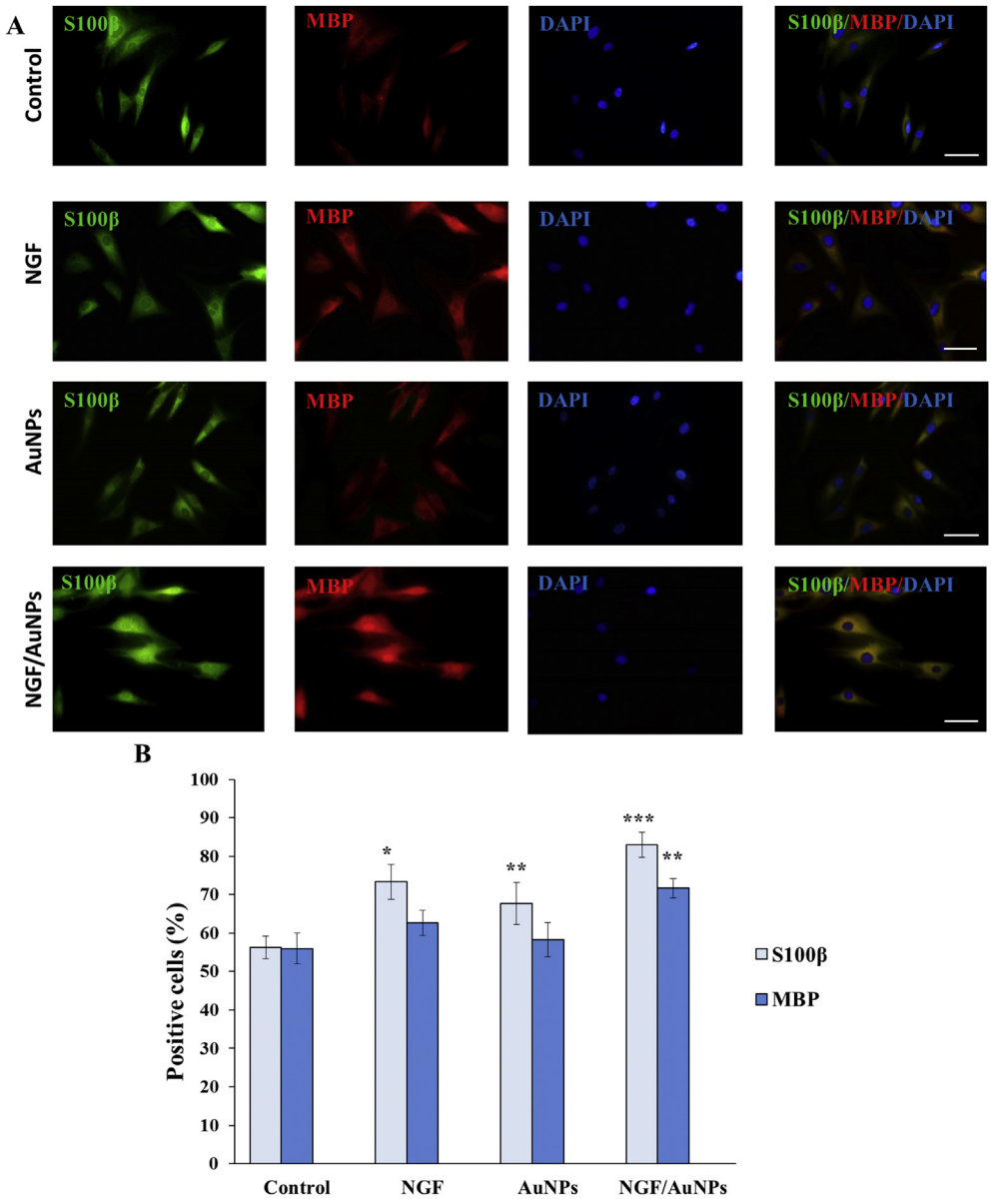

Fig. 3. Immunocytochemical staining for Schwann cells marker S100β (Green) and myelinating ability (MBP) (Red), merge (orange) for differentiated cells in different groups: Tissue culture plate (control), NGF loaded chitosan nanoparticle (NGF), AuNPs loaded chitosan nanoparticle (AuNPs), NGF loaded chitosan nanoparticle and AuNPs loaded chitosan nanoparticle (NGF/AuNPs). All nuclei were stained with DAPI. Scale bar is 100 μm (A). Comparative analysis of the mean percentages of S100β and MBP markers (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$) (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

DMEM/F12 medium were supplemented with EGF, b-FGF, and B27, neurospheres appeared after 7 days (Fig. 2B). Then, neurospheres were singled and exposed in differentiated medium and cells were differentiated into Schwann-like cells with bipolar and tripolar morphologies (Fig. 2C and D).

3.3. In vitro cytocompatibility of NGF-CNPs

The cytocompatibility effect of NGF-CNPs and AuNPs-CSNPs on h-ADSCs was evaluated by MTT assay on days 1, 3, 5, and 7 (Fig. 1B). Although it was noticed that NGF-CNPs and AuNPs-CSNPs have a positive effect on cell viability, there was no significant difference between treated and control groups on different days ($p > 0.05$).

3.4. Immunocytochemistry study

The Schwann-like cells differentiation efficiency was evaluated by immunocytochemistry analysis (Fig. 3A). To evaluate cell differentiation, the induced cells were stained with the markers against S100β (Schwann cell), MBP (myelinating potential) and cell nuclei were stained with DAPI. The mean percentage of S100β and MBP markers were determined after 7 days (Fig. 3B) and found that the mean percentage of positive cells for S100β was significantly increased in the presence nanoparticles containing NGF and AuNPs compared to control group ($83 \pm 3.26\%$) ($p < 0.001$) and the mean percentage of S100β positive cells treated with both of nanoparticles group was significantly more than NGF-CSNPs group and AuNPs-CSNPs group ($p < 0.05$). Also, the mean percentage of MBP in the differentiated cells in the presence of both of nanoparticles

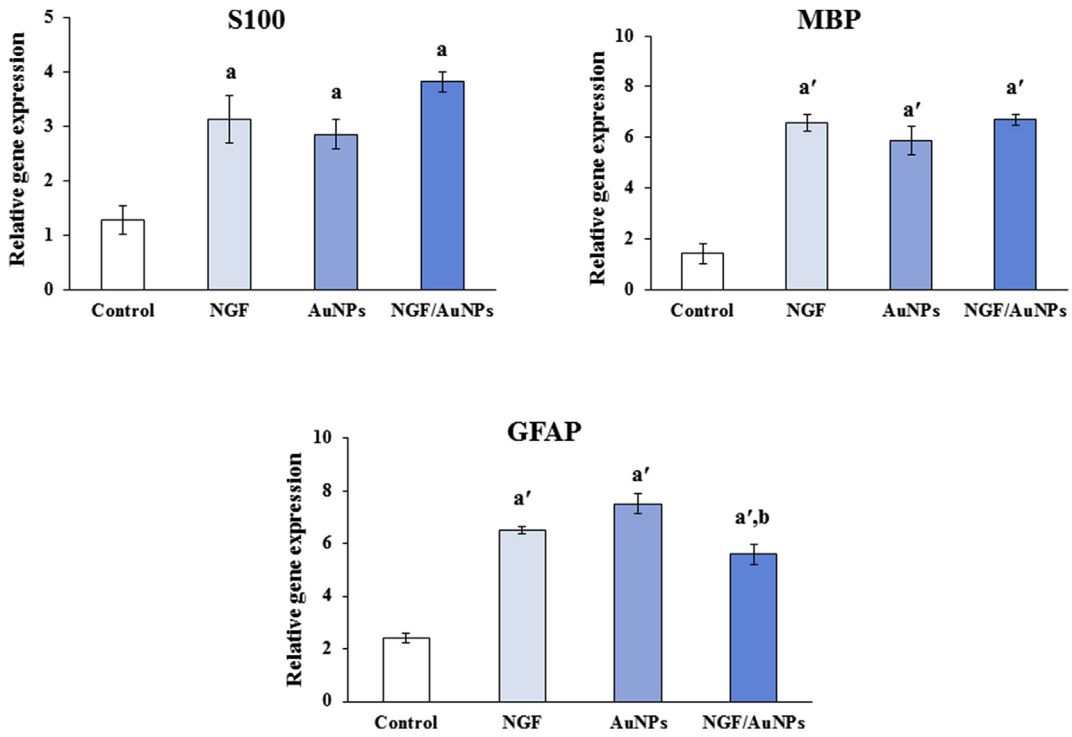

Fig. 4. Comparison of expression of S100β, MBP and GFAP markers in the differentiated cells by real time RT-PCR in groups treated with NGF, AuNPs and NGF/AuNPs groups versus control group (mean ± SEM, a: $p < 0.05$ compared to control groups, a′: $p < 0.01$ compared to control group and b: $p < 0.05$ compared to AuNPs group).

containing NGF and AuNPs was 71.66 ± 2.49% and there was a significant difference compared to other groups ($p < 0.05$). This significant difference in the mean percentage of S100β and MBP demonstrated that release of both NGF and AuNPs could provide a synergist effect on Schwann-like cells differentiation.

3.5. Real-time RT-PCR analysis

The effects of NGF-CSNPs and AuNPs-CSNPs were examined on Schwann-like cell differentiation and myelinating ability by real-time RT-PCR after 7 days (Fig. 4). The expression of S100β specific marker of Schwann cells was significantly upregulated in treated groups compared to control group ($p < 0.05$). Also, the level of MBP and GFAP (glial cell marker) expression in differentiated cells was significantly upregulated in the presence of nanoparticles compared to the control group ($p < 0.001$). The level of GFAP protein expression in a combination of NGF-CSNPs and AuNPs-CSNPs treated group was significantly downregulated compared to AuNPs-CSNPs ($p < 0.05$). However, there was no significant difference between NGF-CSNPs with AuNPs-CSNPs treated group and NGF-CSNPs group ($p > 0.05$).

4. Discussion

NGF is one of the prominent neurotrophic factors for central and peripheral nerve tissues preservation and development [27]. Therefore, this factor can be considered as a therapeutic agent in neurological disorders. In this regard, the short half-life of growth factors has forced researchers to improve this limitation with factors loaded in nanoparticles [25]. In the present study, the NGF and AuNPs loaded chitosan nanoparticles were prepared for the first time. Then, we evaluated the effect of NGF and AuNPs release on h-ADSCs differentiation into Schwann-like cells, as a source for treating various diseases such as peripheral nerve regeneration [28], multiple sclerosis (MS) [29], and diabetic neuropathy [30].

Despite some studies conducted on the effect of different neurotrophic factors on Schwann cells [31,32], the controlled release of neurotrophic factors on Schwann cells differentiation has not been assessed.

Recently, AuNPs have attracted much attention because of their physical and chemical properties in the biomedical field, especially neuroscience, including modulation of electrical activity and nerve regeneration in nerve injuries [33].

A study showed that the intraspinal administration of polyethylene glycol (PEG) functionalized gold nanoparticles after sciatic nerve injury in the mouse can promote neuron protection, remyelination, and hind limb motor recovery [34]. A previous study demonstrated the effect of gold nanoparticles on neurite extension of PC12 and determined that neurite outgrowth was enhanced using electrical stimulation. Finally, they reported that gold nanoparticles with nerve induction can be used for nerve regeneration [35]. In addition, gold nanocones as a supporting substrate influence the survival rate of rat cortical neurons and accelerated neurite outgrowth [36].

The size and stability of nanoparticles are important parameters in the nanoparticles preparation process. A study compared different AuNPs concentrations on cell proliferation and showed that 25 ppm AuNPs concentration has a better effect on cell proliferation and 50 ppm is a better choice for *in vivo* environment [26]. Therefore, in this study, 25 ppm AuNPs was chosen for AuNPs-CSNPs encapsulation.

The positive charge of nanoparticles reflected better stability. In addition, this positive charge was found to increase the interaction with a negative charge on cell membrane [37].

In this study, the surface charge of NGF-CSNPs was positive. The size of nanoparticles can affect cellular uptake and its easy penetration to blood vessels [38]. In this regard, it has been reported that spherical nanoparticles with a size smaller than 200 nm can

penetrate cells through clathrin-mediated mechanism [39,40]. Also, the surface to volume ratio in smaller nanoparticles is larger and may provide a higher drug loading capacity in encapsulation [41].

In vitro NGF release of NGF-CSNPs indicated that there is a controlled release of NGF during a period of 7 days without any burst release. Also, a study showed that NGF has released chitosan nanoparticles in a period of 12 days [25], which is consistent with the present study.

The toxicological properties of nanoparticles play an important role in the biology and medicine fields [42]. Owing to the biocompatible property of chitosan, it has received much attention as a delivery vehicle for growth factors and drugs encapsulation [43]. In the present study, the nanoparticles did not show any adverse effect on h-ADSCs viability. A study showed that 2 and 4 mg/ml of NGF-CSNPs significantly increased cell proliferation while the cell proliferation was inhibited by 6 mg/ml of NGF-CSNPs on days 4 and 6 [25]. So, the present study was performed using 4 mg/ml of NGF-CSNP.

By immunocytochemical and gene expression studies on Schwann cell markers and myelinating ability, it was evidenced that constant and controlled fashion of NGF and AuNPs release can lead to inducing Schwann-like cells from h-ADSCs. Moreover, it was revealed that expression levels of S100β and MBP in Schwann-like cells significantly were upregulated in NGF-CSNPs and AuNPs-CSNPs groups compared to control group.

The present study showed that NGF and AuNPs loaded chitosan nanoparticles are suitable for drug delivery regarding their practical size and stability. The sustained release of NGF was demonstrated during 7 days without any initial burst release. In addition, the NGF-CSNPs and AuNPs-CSNPs had no cytotoxic effect on cell viability and delivery of NGF and AuNPs through chitosan nanoparticles can increase the differentiation of h-ADSCs into Schwann-like cells and myelinating capacity *in vitro*. Therefore, we propose that NGF-CSNPs and AuNPs-CSNPs can be used in nerve tissue engineering *in vivo* and this strategy can help for therapeutic aspects in nerve injuries and neurodegenerative diseases.

Conflicts of interest

The authors declare no competing financial interests.

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