Digest Journal of Nanomaterials and Biostructures Vol. 8, No. 2, April - June 2013, p. 551 - 560

EVALUATION OF CYTOTOXICITY OF NATURAL NANO-ATTAPULGITE AND ITS ENHANCEMENT OF VERO CELL PRODUCTIVITY

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Natural Nano-attapulgite (NATP) is widely used industrially due to its high specific surface area. To determine its potential in the large-scale mammalian cell culture and tissue engineering, the cytotoxicity of NATP is evaluated using African green monkey kidney (VERO) cells on both the cellular and molecular levels. Our researches show that NATP not only does not exhibit any cytotoxicity, but also substantially enhances proliferation of VERO cells which is useful to pare the production cost of biomedical products. These results suggest that nano-attapulgite can serve as potential in large-scale mammalian cell cultures and tissue engineering, respectively.

(Received February 8, 2013; Accepted March 20, 2013)

Keywords: Attapulgite, Cytotoxicity, Cell Cultivation, Cell viability

1. Introduction

Mammalian cell cultivation is widely adopted in fundamental research and biomedical applications such as production of hormones, interferon, enzyme growth factors, and vaccine monoclonal antibody [1, 2]. However, owing to the different culturing conditions, complexity, and accumulated metabolism products [2], mammalian-cell cultures cannot meet the requirement demanded by large-scale production. Reducing the production cost and improving the cell cultivation efficiency and production yield are thus imperative. Optimization of the cell culture conditions and using inexpensive and biologically friendly materials are feasible options [3, 4]. However, the task is very challenging since the materials must satisfy several requirements such as removal of toxic metabolites, nontoxicity, biocompatibility, and low cost.

Natural attapulgite (NATP), a natural clay mineral composed of hydrous magnesium silicate and having a unique three-dimensional nanoscale fibrous structure, has the chemical formula of $Si_8O_{20}Mg_5(Al)(OH)_2(H_2O)_4 \cdot 4H_2O$ [5, 6]. Owing to the absence of the alternating locations for epipelagic oxygen in the tetrahedral layer, the crystalline structure of attapulgite includes two bands of silica tetrahedral linked by magnesium ions in octahedral coordination and capillary channels [6-8]. NATP has unique properties such as large specific surface area (about 146 ~ 210 m^2/g), surface activity, porous and adsorption ability, ion exchange, and salt resistance [9]. NATP is used industrially in thermoplastic polymers, catalyst supports, silicate/epoxy nanocomposites, rubber, and adhesives. Recent research has focused on extending the applications of NATP to environmental applications, biosensors, fluorescent probes, and biocarriers [10-15]. NATP and modified-NATP have been used to evaluate the flocculation efficiency and mechanism in freshwater containing harmful algal blooms. The materials are potentially useful in the large-scale treatment of harmful algal blooms [10]. By depositing hemoglobin/attapulgite and horseradish peroxidase/attapulgite nanohybrids on glassy carbon (GC) electrodes, new biosensors have been developed to measure H_2O_2 [11, 12]. NATP is a good matrix for protein immobilization

and preparation of third-generation biosensors without using mediators. The biosensors exhibit rapid response, wide linear range, high sensitivity, low detection limit, as well as good stability and repeatability [11, 12]. NATP is also a potential candidate in cell imaging. Compared to SiO_2-based composites, attapulgite-based lanthanide composites offers longer luminescence lifetime, higher quantum yields, lower cytotoxicity, and better biocompatibility [13]. In addition, NATP can be fabricated into biocarriers [14, 15]. For instance, pH-sensitive CTS-g-PAA/APT/SA composite hydrogel beads for controlled drug delivery of diclofenac sodium and modified attapulgite/polyurethane bioactive macromolecular carriers have been prepared. Introduction of clay into the polymeric network may offer simple and unique approaches to prepare new controlled drug delivery systems and improve the biologically active groups on the carrier and its ability to fix microbes. However, there have been few reports on the application of attapulgite to mammalian cell culture. In this work, NATP is incubated with Vero cells and the cytotoxicity and other biological properties are evaluated comprehensively.

2. Materais and methods

2.1 Dispersion and Purification of ATP

The crude attapulgite (CATP) supplied by Jiangsu Junda AT Materials Co., Ltd, was a 200 mesh with purity higher than 90%. It was further dispersed and purified prior to use in the mammalian culture. 10 g of the CATP power were dispersed into 100 mL of 5 mol L^{-1} HCl solution and stirred for 10 min to create the CATP suspension. To purify the CATP, gravitational precipitation was employed and the precipitate was discarded several times. The CATP-refined suspension was further crushed and dispersed overnight by ultrasonication. Afterwards, the suspension was washed repeatedly with the $NaHCO_3$ solution and millipore water until it became neutral. It was then dried at 80 $^{\circ}C$ in vacuum to obtain NATP powders with purity reaching 99.9%. With regard to the mammalian cell culture, NATP suspensions with different concentrations were prepared. The refined NATP power was dispersed in Millipore water, stirred overnight, and sterilized before introducing to the culture medium.

2.2 Cell Culture

The VERO cell line, isolated and extracted from kidney epithelial cells of an African green monkey (IBCB, CAS, Shanghai), had been extensively manipulated genetically to produce influenza vaccine. The basic medium used was Dulbecco's modified eagles medium (DMEM, Sigma-Aldrich) supplemented with 1% L-glutamine (Stem Cell Technologies, Vancouver British Columbia), 10% of fetal bovine serum (FBS, Gibco), 1% of penicillin streptomycin (Gibco, Grand Island), and various concentration of NATP. The stock solution of NATP was inoculated to the cell culture medium and diluted freshly to reasonable concentrations using the complete culture medium. The VERO cell cultures were processed in a humidified incubator under 5% CO_2 at 96% relative humidity and 37 ^{o}C. The experiments were performed under a clean atmosphere to minimize endotoxin contamination that may interfere with the toxicity profile of the NATP.

2.3 Cell Viability

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide) assay was used to measure the Vero cell viability [16]. The MTT assay was a colorimetric assay to measure the activity of enzymes that reduced MTT to formazan dyes to yield a purple color, thereby enabling the assessment of the viability and proliferation of cells. In addition, this assay could be used to determine the cytotoxicity of materials, since those materials would stimulate or inhibit cell viability and growth. In our experiments, Vero cells were inoculated into 96-well microplate at a density of 3 x 10^4 cells per well and incubated overnight under 5% CO_2 at 96% relative humidity and 37 ^{o}C. The culture media were replaced with fresh ones mixed with the NATP suspension at concentrations of 20 μg/mL and 150 μg/mL and co-cultured for 1, 2, and 3 days, respectively. The MTT experiments were conducted according to the MTT protocol.

2.4 Flow cytometry/Cell apoptosis and necrosis

Annexin V and PI assay kit (KenGEN Biotech, China) and flow cytometry were used to determine whether NATP caused cell apotosis and necrosis. The Vero cells incubated in the NATP suspension at concentrations of 20 μg/mL and 150 μg/mL were selected as target cells in the experiments. A positive control composed of Vero cells was treated with proapototic compound staurosporine (phosphate buffer solution, PBS). In the logarithmic growth phase of the cells, Annexin V-FITC and PI staining was performed and the materials were subjected to flow cytometry to discern apoptotic and necrotic cell death phenotypes resulting from NATP.

2.5 Apoptotic DNA fragmentation

The DNA fragmentation kits and agarose gel electrophoresis were employed to further elucidate the effects of NATP on cell apotosis. The positive control and Vero cell incubated in 20 μg/mL NATP were precipitated in the presence of the indicated amount of PEG/NaCl. Agarose gel electrophoresis was conducted on the Vero cells treated with camptothecin for 3 h to generate DNA fragments. Direct analysis of the precipitated supernatants from the positive control and incubated with NATP by gel electrophoresis by 1.5% agarose gel revealed an oligonucleosomal ladder of fragmented DNA.

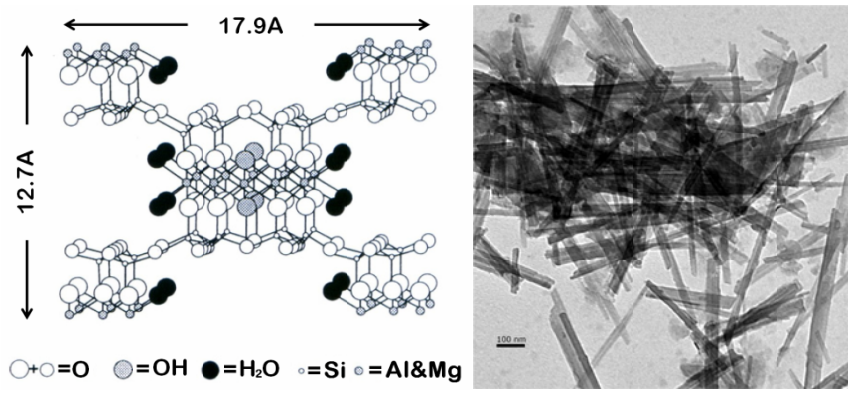
2.6 LDH production

LDH assay kits (Jiancheng Bioengin. China) were employed to measure endogenous lactate dehydrogenase release from the Vero cells. Here, the positive control and Vero cell incubated in the NATP suspension at concentration of 20 μg/mL and 62.5 μg/mL were cultured for 24 h, and then the LDH value was measured according to the instruction of the LDH assay kits.

3. Results and discussion

3.1 Dispersion and purification of ATP

As shown in Fig. 1A, attapulgite belongs to the 2:1 phyllosilicate classification where the sheets of silica tetrahedral are periodically inverted with respect to the tetrahedral bases [5, 17]. Owing to this inversion, the octahedral sheets are periodically interrupted and terminal cations must complete their coordination spheres with water molecules. This structure exhibits a fibrous morphology. The typical NATP TEM morphology in Fig. 1B shows that NATP is a highly dispersed individual rod-like fibrous structure without aggregation. Because of ultrasonication, compared to the commercial attapulgite powder, the length of each NATP fiber in this study decreases to hundreds of nanometers while the diameter remains constant at approximately 10 nanometers. One important objective is to improve the purity of the NATP power. The TEM images also reveal no contamination in the CATP powders and it eliminates the possibility that contamination interferes with the toxicity profile of the NATP.



(a) (b)

Fig. 1: (a) Crystal structure of attapulgite and (b) NATP TEM morphology.

3.2 Vero cell morphology

The cytotoxicity of NATP against Vero cells is evaluated systematically by monitoring the cell morphology, cell viability, apoptosis and necrosis, apoptotic DNA fragmentation, as well as LDH production. The cell morphology change is a sensitive and reliable method to determine cytotoxicity [18]. In this work, the overall appearance of the Vero cells is examined by inverted microscopy (TE2000-S, Nikon, Japan) after culturing for 24 h, and the Vero cell images are acquired by white light microscopy (×40), as shown in Fig. 2. The images of the Vero cell microtome section (Fig. 2D) is obtained by transmission electron microscopy (JEOL, Japan). No NATP particles are observed from the inside of the Vero cells, illustrating the NATP particles are not easily endocytosed or permeated into the Vero cells. In comparison with the untreated cell lines (Fig.2A), no obvious morphological changes in the Vero cells are observed in the presence of 20 μg/mL NATP (Fig. 2B) and 150 μg/mL NATP (Fig. 2C), and the densities of the Vero cells incubated with NATP are much higher than those of the positive control. In other words, NATP at the appropriate concentration can improve the growth of Vero cells and it is further testified by the cell viability.

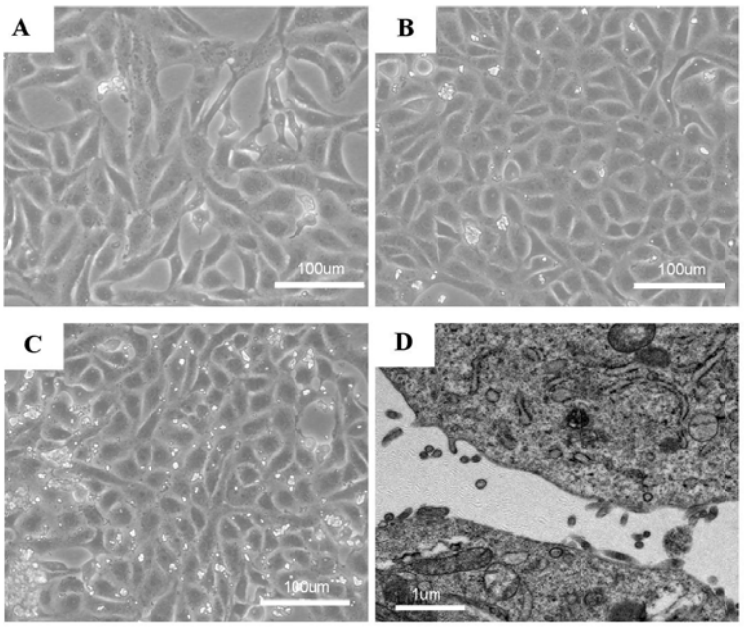


Fig. 2: Morphology of Vero cells: (A) Positive control, (B) incubated with 20 μg/mL NATP for 24 h, (C) 150 μg/mL NATP for 24 h, and (D) TEM image of Vero cell incubated with NATP.

3.3 Cell Viability

Fig. 3 shows the viability of Vero cells incubated with NATP at a concentration of 20 µg/mL and 150 µg/mL after 24 h, 48 h, and 72 h. In comparison with the control group, apparent increase in the absorbance value of the MTT is observed from the Vero cells incubated using either low or high NATP concentration, suggesting that NATP has negligible cytotoxicity even at a relatively high concentration. The cell viability is an indication of living or dead cells on a cell sample. In this perspective, the cell viability is a hallmark of cell productivity. Fig. 3 also shows that the NATP suspension improves the yield of the Vero cells. At concentrations of 20 µg/mL and 150 µg/mL, the yield improves to 27.4% and 118% on the third day and 35.6% and 115.2% on the fourth day, respectively. The results suggest that NATP is a commercially practical in improving the cell productivity and reduce biological products cost.

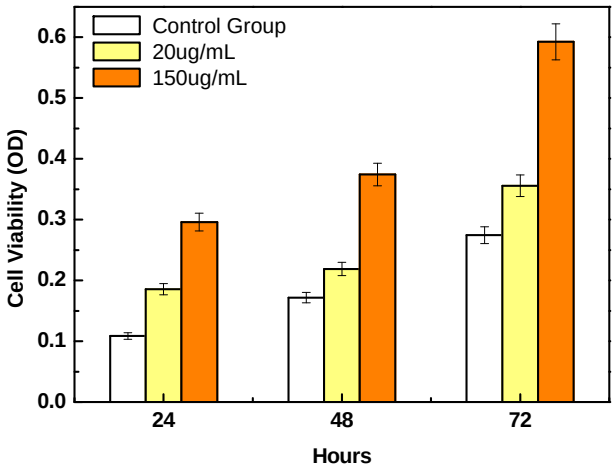


Fig. 3: Viability of Vero cells detected by the MTT assay. The Vero cells are incubated in NATP suspensions (20 µg/mL, and 150 µg/mL) for 24, 48, and 72 h. To improve statistics, all the tests are conducted in triplicates to obtain the mean values.

3.4 Cell apoptosis and necrosis

Apoptosis describes programmed cell death. Although numerous flow cytometric assays have been developed to evaluate cell apoptosis and necrosis, the most popular approaches are based on detection of Annexin V binding to apoptotic cells and uptake of propidium iodine (PI) by dead or dying target cells [19]. One of the early events that occur in the apoptotic pathway is translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Annexin V, one phospholipid-binding protein, has a high affinity for PS. Conjugated with fluorescent tag FITC, Annexin V can be used with flow cytometry to measure PS translocation. That is, Annexin V-FITC staining (green fluorescence) can identify apoptosis at an early stage. However, Annexin V has its limitation since it precedes the loss of membrane integrity which accompanies the later stages of cell death resulting from either apoptotic or necrotic processes. Due to the permeability difference of propidium iodide (PI) through the cell membranes of damaged and live cells, PI staining (red fluorescence) can identify early apoptotic cells (Annexin V-FITC+/PI-) from dead cells (AnnexinV-FITC+/PI+,). It is thus important to evaluate the effects of NATP on cell apotosis and necrosis. Figs. 4A, 4B, and 4C show the results obtained from the 20 µg/mL NATP, 150 µg/mL NATP, and positive control, respectively. The gated signal is divided into four groups: intact live cells (Annexin V-FITC-/PI-, left-down), apoptotic cells (Annexin V-FITC+/PI-, right-down), necrotic cells (Annexin V-FITC+/PI+, right-up), and large nuclear fragments (Annexin V-FITC+/PI+, left-up). Fig. 4D shows that a simple compilation of apoptosis and necrosis experimental results detailing the relative amount of live, apoptotic, and necrotic cells. The percentage of necrotic cells incubated with NATP goes up slightly, from positive control (0.5%) to 20 µg/mL NATP (0.9%) and 150

μg/mL NATP (1.5%). The main reason is believed to be that the physical stress, especially shearing, ruptures the Vero cells membrane and causes quick cell necrosis at a relatively high concentration of NATP. With regard to apoptosis, there are no apparently differences among the positive control, Vero cell incubated with 20 μg/mL NATP, and Vero cells incubated with 150 μg/mL. Hence, cell apoptosis is not induced by NATP and this conclusion is vital to commercial applications.

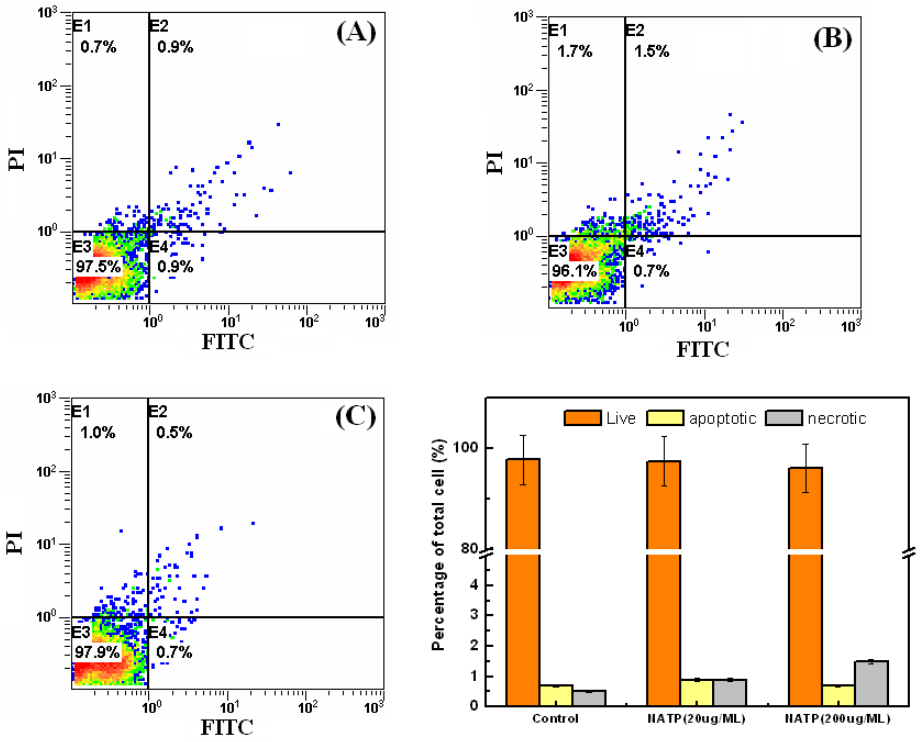


Fig. 4. Annexin V-FITC and PI staining of Vero cells incubated with NATP suspensions at concentrations of 20 μg/mL and 150 μg/mL for 24 h, respectively. (A) 20 μg/mL NATP suspension, (B) 150 μg/mL NATP suspension, (C) Control with PBS, (D) simple compilation of experimental data detailing the relative proportion of live, apoptotic, and necrotic cells. The Vero cells stained with FITC represent early apoptosis, whereas those stained with PI represent necrosis. The data are shown as mean values obtained from three separate experiments. Statistical analysis is performed according to the Student's t-test and a mean $P < 0.05$ denotes a lack of significant difference.

3.5 Apoptotic DNA fragmentation

DNA fragmentation, that is, nuclear DNA degraded into nucleosomal units, is another symbolization of apoptotic cell death and occurs in response to various apoptotic stimuli in a wide variety of cell types. In general, this degrades genomic DNA in the internucleosomal linker regions and produces 180- to 185- base-pair DNA fragments which give the characteristic "ladder" appearance in Agarose gel electrophoresis [20]. This laddering is a sensitive means to distinguish between apoptosis and toxic cell death [21]. Fig. 5 shows that the whole precipitation of DNA from the positive control and culture incubated with NATP. No ladder pattern can be observed from the Vero cells incubated with NATP, thus indicating that apoptotic DNA fragmentation is not induced by NATP.

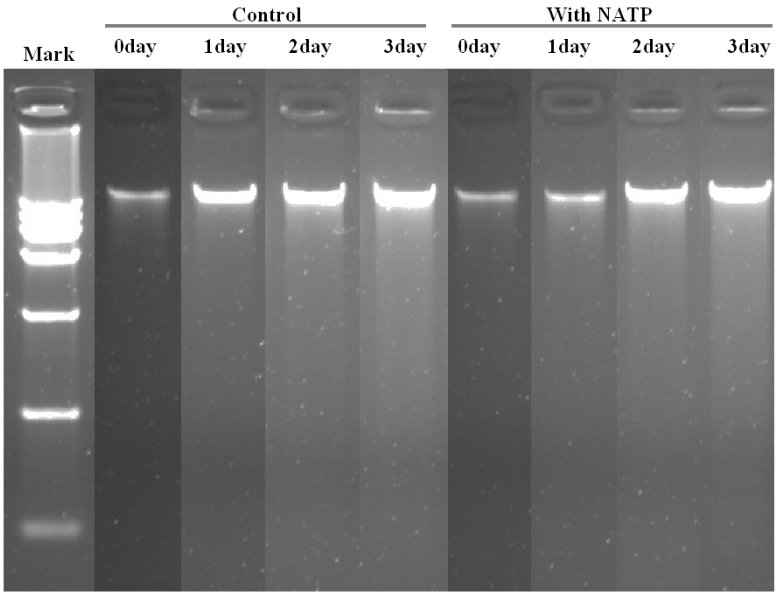


Fig. 5: DNA fragmentation assay. Group 1: Positive control; Group 2: Vero cell incubated with NATP. MW, 15 kb DNA ladder. This image is recomposed from the original Agarose gel electrophoresis image.

3.6 LDH production

In addition, lactate dehydrogenase (LDH), a soluble cytosolic enzyme present in most eukaryotic cells, is released into cell culture when the cell death is caused by breakage of the plasma membrane integrity. An increase in the LDH activity in the culture supernatant is proportional to the number of lysed cells. As an indicator of cell membrane integrity, the LDH activity can be used as a general means to evaluate the cytotoxicity resulting from environmental substances [22]. Fig. 6 shows that the LDH value increases from 0.22 of the positive control to 0.25 and 0.32 for the NATP (20 µg/mL) and NATP (62.5 µg/ml), respectively. The results are consistent with Vero cell necrosis resulting from NATP. In the beginning, adding NATP to the cell medium can break the integrity of the membrane in a small section of Vero cells due to physical stress which leads to the cell necrosis and LDH released from the necrotic cells. In addition, the LDH values of the Vero cells incubated with NATP are smaller than those of the positive control, demonstrating that the release of LDH is independent of NATP-induced cell apoptosis and necrosis.

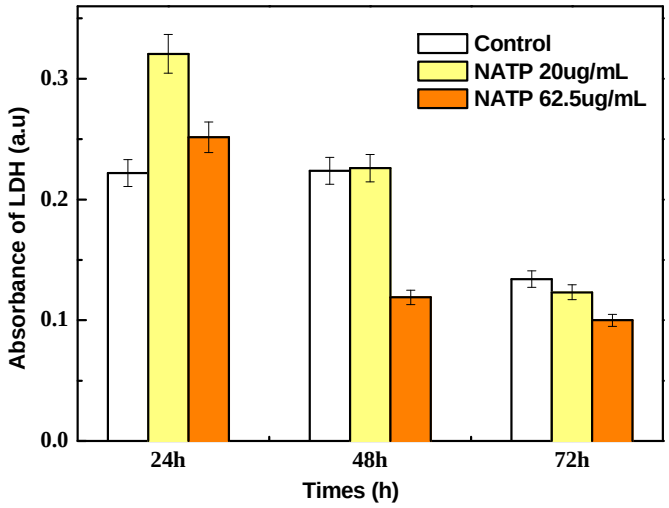


Fig.6. Production of LDH when Vero cells are incubated with NATP suspension at the concentration of 20ug/mL and 62.5ug/mL, respectively. The tests are repeated three times to get the mean values and standard deviations.

4. Conclusion

Highly discrete and purified natural attapulgite has been prepared for African green monkey kidney (VERO) cell culture. The cytotoxicity is assessed systematically by monitoring the cell morphology, cell viability, apoptosis and necrosis, apoptotic DNA fragmentation, and LDH analysis. The materials do not exhibit cytotoxicity in the Vero cell culture and small necrotic induction is observed from the *in vitro* study. NATP enhances the productivity of Vero cells when the NATP concentration is proper. The results suggest that NATP can improve cell productivity and pare the production cost of biomedical products.

Acknoledgements

This work was jointly supported by National Natural Science Foundation of China (No. 11079010), the National Key Technology R&D Program of China (No. 2008BAK41B01, 2007AA10Z170, 2012IASCAAS-BJTU-01), Fundamental Research Funds for the Central Universities (No. 2011JBM133) and Hongguoyuan Person Project of Beijing Jiaotong University of China, Hong Kong Research Grants Council (RGC) General Research Funds (GRF) No. CityU 112212, and City University of Hong Kong Applied Research Grant (ARG) No. 9667066.

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