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Influence of Carbon Points on Human Dermal Fibroblasts

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ABSTRACT
The data of an experimental study in order to determine the toxic dose of positively charged carbon dots (CD) on the culture of fibroblasts are presented. The study used fibroblasts isolated from the dermis of a healthy adult. The experimental part of the work consisted in assessing the morphology of fibroblasts depending on the concentration of the CD solution. For this purpose, 5 experimental and 1 control groups were formed. In the experimental groups, CD-solution was added in an increasing concentration – from 24.15 to 120.75 μg/ml. In the 5th and 6th groups, with a solution concentration of 96.6 and 120.75 μg/ml, respectively, signs of fibroblast destruction (cell fragments) appeared, with a characteristic devastation in the center of the visual field, with fragments of destroyed cells on the periphery. Fibroblasts with signs of destruction in the 5th group accounted for more than 20 %, in the 6th – more than 30 %. Thus, we have identified the minimum concentration of the CD solution, which has a toxic effect on human dermal fibroblasts, which was 96.6 μg/ml.

Keywords: *carbon points, carbon dots, dermal fibroblasts, toxic effect*

1. INTRODUCTION

According to the proliferative ability of organ-specific cellular elements, all organs and tissues can be classified into 3 groups:

1) organs and tissues, the cellular elements of which have an active or practically unlimited proliferation sufficient to completely replenish the structural defect in the damaged area (epithelium skin, mucous membranes of the respiratory tract, mucous membrane of the gastrointestinal tract, genitourinary system; hematopoietic tissue, etc.).

2) tissues with limited regenerative capacity (tendons, cartilage, ligaments, bone tissue, peripheral nerve fibers).

3) organs and tissues, where organ-specific cellular elements in natural conditions are not capable of proliferation (cardiac muscle, cells of the central nervous system).

In case of damage to tissues and organs of group 1, complete restoration of damaged tissues is possible, fabrics of the second and third groups due to limited the ability to regenerate is able to recover through the development of granulation connective tissue with the formation of a connective tissue scar at the site of damage.

At the same time, inadequate scar formation sometimes leads to the development of diseases. For example, excess growth of connective tissue is one of the main factors of pathogenesis. certain diseases (cardiosclerosis and associated him heart failure, joint contractures, adhesive disease, pneumosclerosis, cirrhosis of the liver, keloid scars), and insufficient formation of connective tissue leads to long-term healing wounds and is an urgent problem in surgery, accompanied by the failure of the sutures.

It is the wide proliferation of connective tissue in the body determines its universal properties and participation in regulatory mechanisms. The main components of connective tissue are:

1) fibrous structures of collagen and elastic types;

2) basic (amorphous) substance;

3) cellular elements (cells of fibroblastic row, macrophages, mast cells, adventitia cells, plasma cells, pericytes, adipocytes).

Cells of the fibroblastic series are of particular interest – they are the predominant population of cells of loose fibrous connective tissue and occupy one of the key places in its formation [1].

Fibroblasts are connective tissue cells that actively synthesizing proteins of the extracellular matrix, in particular, collagen.

In recent years, there has been an increased interest in the use of carbon nanomaterials as catalysts, electrodes, chromatographic, separating elements, as well as matrices for the cultivation and transplantation of cells.

Various types of carbon materials are used, including fullerenes, carbon nanotubes, and nanodiamonds.

The promise of using carbon nanomaterials in regenerative medicine is due to the wide range of their physical, mechanical, and chemical properties. Carbon dots (CDs) are luminescent carbon nanomaterials with good biocompatibility and low toxicity. CD have shown promising applications for in vitro and in vivo cell imaging. They enter cells without any further functionalization, and the fluorescence property of these particles can be used to visualize cells based on fluorescence [5, 6, 11].

The main factors determining their cytotoxicity and cellular/intracellular tracking include the chemical structure and the charge of surface functional groups. The toxicity of CD is highly dependent on the preparation protocol and appears only at higher concentrations [3, 9].

CDs-PEGs with a neutral charge are the most promising for biological applications, since they do not cause any disturbances in cell morphology, intracellular metabolism, and cell cycle up to concentrations of 300 μg ml-1. Negatively charged CDs-Pri delayed the G2/M phase of the cell cycle, stimulated proliferation, and led to higher oxidative stress; however, they did not penetrate into the cell nucleus. On the contrary, positively charged CDs-PEI are the most cytotoxic, entering the cell nucleus and causing the greatest changes in the G0/G1 phase of the cell cycle even at concentrations of about 100 μg ml [3].

Problem Statement

Carbon nanomaterials are increasingly used in regenerative medicine. However, their certain cytotoxicity is a limiting factor, which requires testing on various types of human cells.

Experimentally, in our study, we plan to evaluate the concentration of CD, from which the cytotoxic effect on dermal fibroblasts begins.

Research Questions

Since the toxicity of UT depends on the concentration, it is necessary to determine the threshold concentration above which the toxic effect on cells occurs.

Purpose of the Study

Purpose of the Study – in the experiment, by the method of counting and morphological assessment, to determine the toxic dose of carbon dots on cultured human skin fibroblasts.

2. RESEARCH METHODS

An experimental study with cultured fibroblasts was carried out in the research laboratory "Cell technologies and regenerative medicine" of the Medical Institute of NEFU.

The study used fibroblasts isolated from the dermis of a healthy adult, grown in DMEM/F-12 (1: 1) (1X), FBS 20 %, anti-anti (1x100), sodium pyruvate (1x100). They were incubated at 37 °C in an atmosphere enriched with 5 % CO_2 for 5 days.

Obtaining carbon dots was carried out in the educational scientific and technological laboratory "Graphene nanotechnologies" NEFU. CD were obtained by the hydrothermal synthesis method by mixing homogenized 10 g of dried bird cherry (Prúnus pádus) berries, 2 g of citric acid, 0.5 g of ethylenediamine and 0.5 g of polyethyleneimine (positively charged).

The resulting dry mixture was diluted with 20 ml of distilled water, placed in a polytetrafluoroethylene () autoclave at 180 °C for 5 hours. The resulting solution was centrifuged for 5 minutes at 14.5 thousand rpm. Then the solution was filtered through a track membrane with a pore size of 0.2 μm.

The concentration of CD was determined through the mass concentration. On a cover glass (weighed in advance), 400 μl of a solution of CD from bird cherry was dropped. After drying the glass with the solution was weighed again and then the mass of the cover glass itself was subtracted from the resulting number. Then this number was divided by 0.4 (since the volume solution was 400 μl). Then the CD solution was diluted from bird cherry with a known concentration in a ratio of 1: 5.25. The concentration of the working solution with YF was 24.15 μg/μL.

The presence of carbon dots in the solution was confirmed using optical spectroscopy methods: luminescence and UV-VIS absorption, infrared spectroscopy and Raman spectroscopy, as well as using a scanning electron microscope.

UV-VIS absorption spectra were measured on a Lambda 750 spectrophotometer, Perkin Elmer. Luminescence spectra were measured on a Perkin Elmer LS 50 B instrument. Fourier transform infrared spectra were obtained on a Varian 7000 FT-IR spectrometer.

Cattle measurements were carried out on an Integra Spectra, NT MDT. The experimental part of the work was to assess the morphology and viability of fibroblasts depending on the concentration of the CD solution.

The experimental part of the work consisted in testing the proliferative activity of fibroblasts depending on the concentration of the solution CD. We were divided into six groups, five Petri dishes 60x15mm in each group: 1 control group and 5 experimental groups.

Group I – control group without adding a solution with CD (independent growth was observed within 5 days). Group II – 10 μL of solution with CD was added only on day 1 (10 μL in total), Group III – on days 1 and 2 (20 μL); IV group – 1, 2 and 3 days (30 μl); Group V – days 1, 2, 3 and 4 (40 μl); Group VI – daily (5 days, total 50 μl).

Using a microscope, areas with fibroblast cells were found, which were photographed with a lens magnification of 4 x /0.13. The area of the photographed areas was calculated and the total was counted over an area of 1.8 cm^2.

Statistical analysis was carried out using the STATISTICA version 12.0 software (developed by StatSoft.Inc). The accumulation, correction, systematization of the initial information and visualization of the results obtained were carried out in Microsoft Office Excel 2016.

Aggregates of quantitative indicators, the distribution of which differed from normal, were described using the values of the median (Me) and the lower and upper quartiles (Q1–Q3). The Mann-Whitney U-test was used to compare independent populations due to the absence of signs of normal data distribution.

When comparing several samples of quantitative data, since the distribution was different from normal, the Kruskal-Wallis test was used, which is a nonparametric alternative to one-way analysis of variance. When comparing more than two dependent populations, the nonparametric Friedman test was used.

The critical value of the significance level (p) was taken equal to 0.05.

3. FINDINGS

One of the criteria for a possible toxic the effect on cells of CD is the morphology of fibroblasts. Changes in the shape of cells during their cultivation in a Petri dish in the presence of CD was observed under an inverted microscope. Morphological characteristics of dermal fibroblasts were assessed daily for 5 days:

On the first day (after 24 hours of cultivation), 100 % adhesion to plastic was observed in the control group and in all experimental groups. In almost half of the observed cells, processes begin to appear. The cells themselves are located at a sufficient distance from each other.

On the second day, in all the observed groups, an increase in size is characteristic, and swelling of fibroblasts is noted. The cells take the shape of a spindle. Almost all cells have elongated processes. The number of processes reaches 2–3 in some cells. The distance between the cells has decreased a lot.

The morphology of dermal fibroblasts on the third and fourth days is very similar. In all groups, pronounced signs of fibroblast proliferation are visible, the number of cells increased markedly.

Morphologically, fibroblasts are characterized by long processes, polygonal forms with several processes are found. The length of the processes is much longer. The arrangement of cells among themselves in all groups is dense, parallel. It should be noted that "vortex cords" appear.

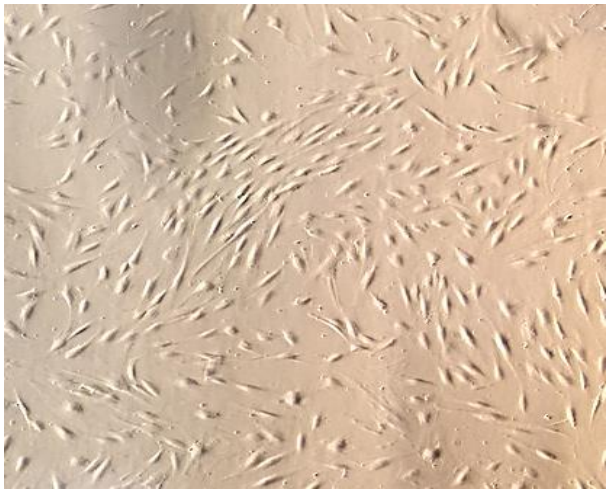
On the last fifth day of observation, imaging revealed significant differences depending on the concentration of CD. In groups, with the exception of groups 5 and 6, where the CD concentration was from 24.15 to 72.45 μg/ml, the morphology of fibroblasts was preserved and the number of cells increased.

In the 5th and 6th groups with a CD concentration of 96.6 and 120.75 μg/ml, respectively, cell destruction is noted. Remnants, fragments of fibroblasts are clearly visible in the visual fields.

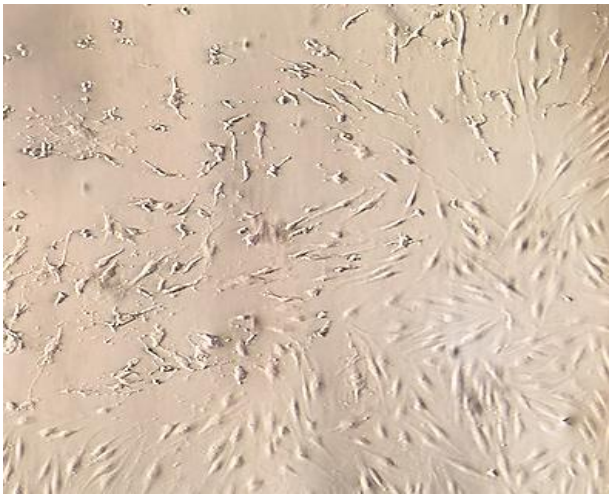
Visual fields with a high concentration of CD (groups 5 and 6) are characterized by the presence of local voids with fragments of destroyed cells along the periphery (Figure 01).

We found that the "destruction voids" of fibroblasts in the 5th experimental group occupy more than 20 % of the visual field, in the 6th group – more than 30 %.

It should be noted that the first five groups the dynamics of the growth of the median of the number of fibroblasts, the most pronounced in the control and the second experimental group (more than 15 once).



a



b

Figure 01. Human dermal fibroblasts after 5 days of cultivation in the presence of carbon dots: a – control (fibroblasts on culture plastic); b – in the presence of carbon dots at a concentration of 120.75 μg/ml.

In group 6, on day 3 of cultivation, the median number of cells has grown by almost 10 times, a similar sharp increase proliferative activity is also observed in group 5. However, from the 4th day in the 6th group, there was a sharp decrease in the number of intact fibroblasts. Their number on days 4 and 5 was almost equal to the initial data due to cell death (Figure 02).

The turning point for the two experimental groups with high concentration (groups 5 and 6) was the fourth to fifth days. It should be noted that from the third to the fourth day of observation, some degree of "inhibition" of fibroblast growth was revealed for all experimental groups, and in the fifth group, the number decreased almost to the initial level.

Friedman's test confirmed the statistically significant dynamics of the number of fibroblasts (16.48–19.36), the concordance coefficient was high (0.82–0.97), the level of statistical significance (p) varied from 0.001 to 0.002.

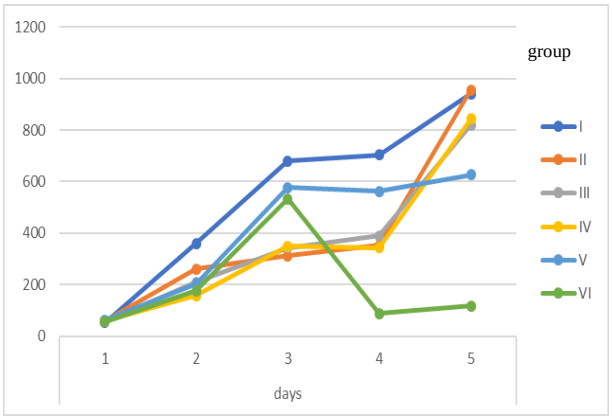


Figure 02. The number of fibroblasts on an area of 1.8 cm^2 in the comparison groups depending on the concentration of CD

We also compared the medians of the number of medians of six groups (Figure 02). As can be seen from the diagrams, on the first day of observation, the number of fibroblasts in the groups was approximately the same ($p > 0.05$). From the second to the fifth days, the largest number of fibroblasts was in the control group, the smallest – on the second day on the 4th ($p = 0.01$), on the third day 2 – 4th ($p = 0.0008$), on the fourth and fifth days – on the 6th ($p = 0.0031$ and 0.0002, respectively).

Next, we compared in pairs the median of each experimental group with the median of the control group for each day of the experiment using the Mann-Whitney test. On the first day, the median in the 5th and 6th groups was statistically significantly higher than in the control ($p = 0.026$ and 0.049, respectively), in the rest – did not differ significantly ($p > 0.05$). On the second day in the 3rd and 4th – it was lower than in the control ($p = 0.037$ and 0.003, respectively), on the third day – it was lower in groups 2-4 ($p = 0.001–0.000$), in the fourth day – in all groups ($p = 0.026–0.000$), and on the last (fifth) day – in the 5th and 6th groups ($p = 0.000$).

According to many studies, CD have very low toxicity [4, 10, 14, 15]. However, in our study, starting from a concentration of 96.6 μg/μL, signs of fibroblast destruction appeared, which were most pronounced in the 6th group, i.e. at the highest concentration (120.75 μg/μl). The results obtained are comparable with the data of researchers, where positively charged CDs with polyethyleneimine were the most cytotoxic, even at concentrations of about 100 μg/ml [3]. The revealed increase in the number of fibroblasts upon addition of CD is consistent with the results of other studies [2, 7, 8, 13].

According to [3], positively charged UTs with polyethyleneimine are the most cytotoxic, penetrating into the cell nucleus and causing large changes in the G0/G1 phase of the cell cycle, even at concentrations of about 100 μg/ml.

4. CONCLUSION

Thus, in our study, we experimentally revealed the minimum concentration of the UT solution, which has a toxic effect on the culture of human dermal fibroblasts, equal to 96.6 μg/ml.

In the 5th and 6th groups, at a solution concentration of 96.6 and 120.75 μg/ml, respectively, signs of destruction of fibroblasts (cell fragments) appeared, with a characteristic devastation in the center of the visual field, with fragments of destroyed cells on the periphery. Fibroblasts with signs of destruction in the 5th group accounted for more than 20 %, in the 6th – more than 30 %.

The results obtained once again prove that the toxicity of CD depends not only on its concentration in solution, but also on the manufacturing protocol.

The results we have obtained form new tasks for setting up experimental work on cell cultures. Diverse modeling using various cultures of human cells on the example of healthy fibroblast cell lines, will allow to give reliable results of the cytotoxic effect of carbon dots on cells.

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