IN VITRO, DONE ON STEM CELL CULTURES BIOCOMPATILITY TESTING OF NERVOUS CONDUITS

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Abstract: *Extensive nerve injuries, often leading to nerve defects, represent a very challenging pathology with a complex therapeutic approach: trophic molecules (1 and 2 insulin-like growing factors, NGF, BDNF, 3, and 4/5neurotrophins), vascularized nerves, metabolic manipulation of pulsating electric fields. Nowadays, the researches are axed on peripheral nerve tissue engineering, consisting of viable Schwann or Schwann-like cells implants in nerve conduits manufactured from biocompatible natural and synthetic polymers. In our study we evaluated polymer biocompatibility, both by qualitative (Giemsa cell cytochemical staining) and quantitative methods (MTT test). According to the cytotoxicity scale from the SR EN ISO 10993-5:2003 European standard, it can be concluded that, although the synthetical polymers are slightly cytotoxic, their mixing with biopolymers led to a significant increase in viability, thus achieving a good biocompatibility, with the consequent selection of the optimal variants of polymer shuffles in order to achieve nerve conduits.*

Cuvinte cheie: *celule stem, biocompatibilitate, conducte nervoase*

Rezumat: *Leziunile nervoase extensive, soldate adesea cu defecte nervoase, reprezintă o patologie extrem de provocatoare cu o abordare terapeutică complexă: molecule trofice (factorii de creştere insulin-like 1 şi 2, NGF, BDNF, neurotrofinele 3 şi 4/5), nervi vascularizaţi, manipularea metabolică a câmpurilor electrice pulsatile. În prezent, cercetările sunt axate pe ingineria tisulară a nervilor periferici, constând în implantarea de celule Schwann sau Schwann-like viabile în conducte nervoase obţinute din polimeri naturali şi sintetici biocompatibili. În cadrul studiului nostru am evaluat biocompatibilitatea polimerilor atât prin metode calitative (colorarea citochimică a celulelor Giemsa) cât şi prin metode cantitative (testul MTT). Conform scalei de citotoxicitate din standardul european SR EN ISO 10993-5:2003, s-a constatat că, deşi polimerii sintetici sunt uşor citotoxici, amestecarea lor cu biopolimeri conduce la o creştere sensibilă a viabilităţii, deci o bună biocompatibilizare a acestora, cu selecţia în consecinţă a variantelor optime de amestecuri de polimeri în scopul obţinerii de conducte nervoase.*

INTRODUCTION

Composite biomaterials and particularly biodegradable/bioerodable materials present a special interest for nanotechnology, both for medicine potting, including gene therapy and for tissue engineering. Composite biomaterial biocompatibility evaluation represents a fundamental stage in the establishment of product safety and application. This fundamental process must be considered when the material has medical applications. The biological evaluation of medical devices is controlled by both European ISO 10993 and American Food and Drug Administration (FDA) standards.

Biomaterials were used in the medical domain as implantable devices, artificial organs, prostheses, stomatology, bone repair, medicine delivery systems and recently in tissue regenerative engineering (Wang et al.,2004) [1]. For medical applications, polymer biocompatibility is a key factor, refering both to minimal cytotoxicity and to biofunctionality.

For the past 15 years, the researches were concentrated on the development of an *in vitro* testing methodology, the currently used systems going from simple subcell fractions to primary cultures, cell lines to organotypical cultures (Bhogal et al.,2005) [2].

Cell cultures represent an important mean for the preliminary evaluation of the biomaterialul cytotoxicity. A great variety of cell types from stem cells, non-differentiated fybrolast-like cells or epithelial-like cells and also highly, tissue specific, differentiated cells can be isolated from various tissues and species. They are grown for different periods of time or/and cryopreserved for future use (Brendler-Schwaab,1994) [3]. Furthermore, mammalian cell cultures are the most indicated for cytotoxicity evaluation because the vast majority of chemical substances that can cause diseases or death in humans and animals are cell excreted (Katti et al.,2002) [4]. Cell tests can give essential information of the potential effects of a compound on a cell and represent a basis for future molecular studies.

Generally it is accepted that cytotoxicity involves the characterization of different aspects of cell functions, such as viability and proliferation, loss of plasmatic membrane integrity, decrease of cell adhesion, biosynthetical activity and altered cell morphology (Silva et al.,2005) [5]. According to the structural characteristics and biomedical applications, the tested materials can be evaluated by specific methods, either by direct contact methods, the cells being seeded directly on the material

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(Ferruti et al.,2005) [6] or by indirect contact methods (Kirkpatrick and Dekker,1992) [7].

The qualitative evaluations of the morphological changes induced by cytotoxicity, such as substrate cell detachments, cell rounding, granule formation, nucleus condensation and cytoplasm fragmentation are achieved by microscopic examination of the either alive cell or fixed and coloured cells. More information on the effect of the biomaterial on cell morphology are delivered by electronic (SEM) or transmission (TEM) microscopy scanning.

Because toxicity can be determined by a series of cell events, such as cell morphology changes, proliferation, differentiation, excitability and/or cell communication, these systems can need improvement before being accepted to evaluate the risks of a certain biomaterial (Bhogal et al, 2005) [2] by adding known metabolites (Coecke et al.), (1999) [8]. Cell metabolic systems can be divided in three categories: (**1**) metabolic competent indicator cells, such as hepatocytes; (**2**) co-cultures systems, that include a metabolic non-competent indicator cell type, such as fibroblasts and a metabolic competent cell type, such as hepatocytes; (**3**) genetic changed cell lines, that can act either as indicators of some metabolic pathways or as toxicity indicators. Co-cultures are very valuable in cytotoxicity tests due to their capacity of *in vitro* cell network formation (Ward et al.,1998) [9]. Furthermore, tissue engineering recent researches were facilitated by the development of *in vitro* complex models (Lavik and Langer,2004) [10].

MATERIAL AND METHODS

CN were achieved by mixing synthetic polymers [polyvinylic alcohol (PVA), polyvinyl chloride (PVC) and polyethyleneglycol (PEG) with natural polymers (non-denatured type I collagen, hidrolyzed collagen and chitosan). It was established the optimal mix report of these polymers. The biopolymer NC achieved by us represented adequate supports for MSC, in order to test them for peripheral nerve regeneration. Nervous conduits (NC) biocompatibility screening was done on a mesenchymal stem culture. These were achieved from adipose tissue harvested by liposuction. In the first table we listed the composite materials that we had tested.

Table no 1. Types of tested composite materials

Crt. No.	NC composition	COMPONENT REPORT
1	Polyvinylic alcohol (PVA_M) - Collagen	1:1
2	Polyvinylic alcohol (PVA_M) – Hidrolyzed collagen (HC)	9:1
3	Polyvinylic alcohol (PVA_M) - Chitosan	4:1
4	Polyethyleneglycol (PEG) – Collagen	1:1

Biocompatibility evaluation was achieved by the morphological analysis of the cells grown for 6 days.

Morphological analysis

NC biocompatibility evaluation was achieved both *in toto* and on sections.

For the *in toto* cell morphological evaluation, the cells grown on the NC were fixed in 4% paraformaldehyde in PBS and stained with DAPI and hematoxylin eosin (H&E). The examination of the cells stained with DAPI was done at an Axiostar Pluss fluorescence microscope (Zeiss).

For the morphological evaluation of the sections, the cells grown in the NC were fixed in 4% paraformaldehyde in PBS and included in paraffin. 6 µm sections were stained with hematoxylin eosin (H&E).

RESULTS

NC manufactured from **1:1 PVA_M - Collagen** presented a good compatibility. On their surface were identified mesenchymal stem cells (MSC) both in fluorescence microscopy by DAPI staining(Fig. 1a) and in optic microscopy by H&E staining (Fig. 1b). MSC adhered firmly on the PVA_M – Collagen 1:1 nerve conduit (NC) (Fig. 1c).

Figure no. 1a-b. Human MSC on a 1:1 PVA_M – Collagen nerve conduit (NC). DAPI (a) and H&E (b) *in toto* staining.

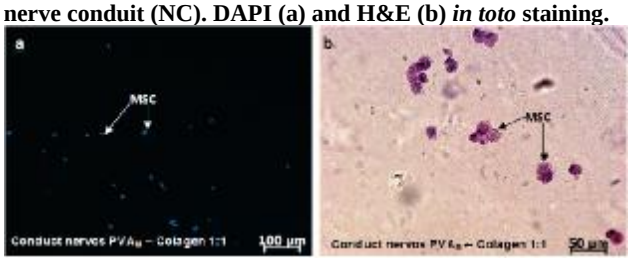
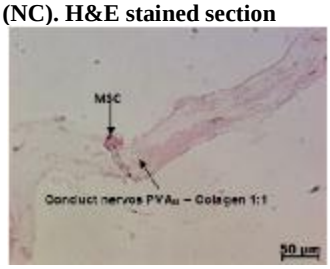
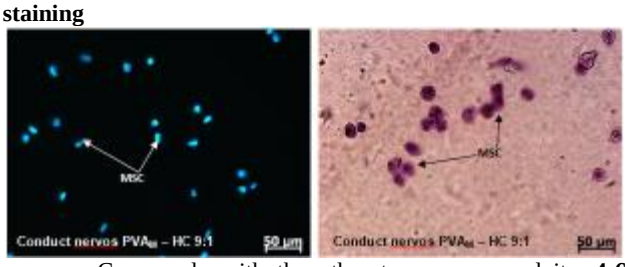


Figure no. 1c. Human MSC on a PVA_M – Collagen 1:1 nerve conduit (NC). H&E stained section



NC manufactured from **9:1 PVA_M – Hydrolysed collagen** presented also a good compatibility. On their surface were identified MSC, both in fluorescence microscopy by DAPI staining(Fig. 2a) and in optic microscopy by H&E staining (Fig. 2b). The sections showed that MSC adhered firmly to the 9:1 PVA_M – Hydrolyzed collagen NC (Fig. 2 c). The number of cells that adhered to the NC was bigger than in the case of 1:1 PVA_M – Collagen conduit.

Figure no. 2a-b. Human MSC on a 9:1 PVA_M – Hydrolyzed collagen nerve conduit (NC). In toto DAPI (a) si H&E (b) staining



Compared with the other two nerve conduits, **4:6 PVA_M - Chitosan NC** presented the best biocompatibility, on their surface being identified more mesenchymal stem cells both in fluorescence microscopy by DAPI staining (Fig. 3a) and in optic microscopy by H&E staining (Fig. 3b). The same aspect was observed on sections, too (Fig. 3c).

1:1 PEG – Collagen nerve conduits presented a good compatibility. On their surface were identified mesenchymal stem cells, both in fluorescence microscopy by DAPI staining (Fig. 4a) and in optic microscopy by H&E staining (Fig. 4b). The sections showed that MSC adhered firmly to the 1:1 PEG – Colagen (Fig. 4 c).

In all the cases, mesenchymal stem cells that adhered to the NC had a normal morphology.

Figure no. 2c. Human MSC on nerve conduit 9:1PVA$_M$ – hydrolyzed collagen. H&E stain section

Figure no. 3 a-b. Human MSC on a 4:6 PVA$_M$ – Chitosan nerve conduit. DAPI and H&E in toto staining (a) and (b)

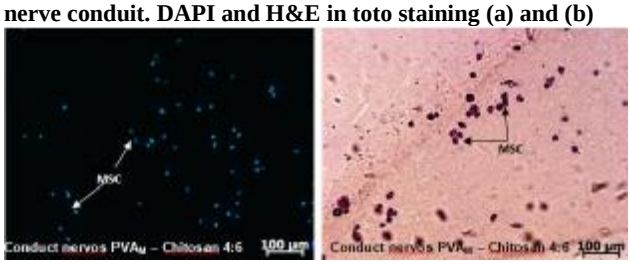


Figure no. 3 c. Human MSC on a 4:6 PVA$_M$ – Chitosan nerve conduit. Section stained with H&E

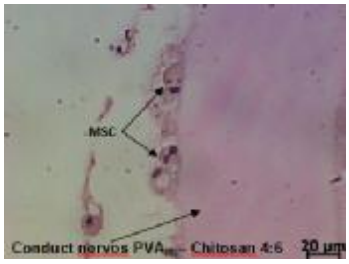


Figure no. 4 a-b. Human MSC on a 1:1 PEG - Collagen nerve conduit. DAPI and H&E in toto staining (a) and (b)

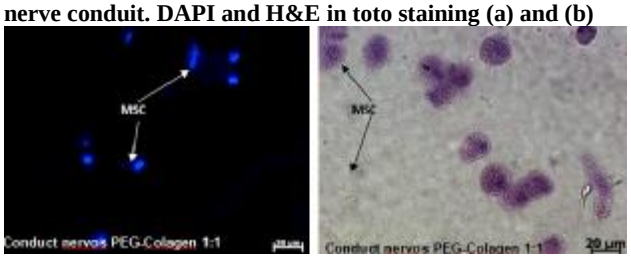
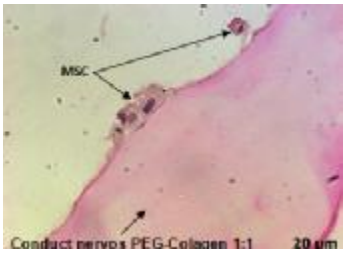


Figure no. 4 c. Human MSC on a 1:1 PEG - Collagen nerve conduit. Section stained with H&E.



CONCLUSIONS

- Nerve conduits (NC) achieved by the shuffle of synthetic polymers [polyvinylic alcohol (PVA), polyvinyl chloride (PVC) and polyethyleneglycol (PEG)] with natural polymers (non-denatured type I collagen, hidrolyzed collagen and chitosan), having an optimal mixture report, were tested for their biocompatibility and there were selected the best biocompatible polymer shuffles in order to achieve nerve conduits, namely:
  - the PVA: hydrolyzed collagen mixture in a combination report of 9:1
  - the PVA: non-denatured collagen mixture in a combination report of 1:1
  - the PEG: non-denatured collagen mixture in a combination report of 1:1
  - the PVA: chitosan mixture in a combination report of 2:3
  - the PVC: hydrolyzed collagen mixture in a combination report of 2,33:1
- The achieved biopolymer nerve conduits represented adequate supports for MSC embedding in order to test them for peripheral nerve regeneration.
- 1:1 PVA$_M$ – Collagen, 9:1 PVA$_M$ – Hydrolyzed collagen, 4:6 PVA$_M$ – Chitosan and 1:1 PEG – Collagen presented a good biocompatibility demonstrated by the adherence on their surface of mesenchymal stem cells that kept a normal morphology after 6 days of culture.
- From the four nerve conduits, the best biocompatibility had the 4:6 PVA$_M$ – Chitosan conduit, followed by 9:1 PVA$_M$ – Hydrolyzed collagen, 1:1 PVA$_M$ – Collagen and 1:1 PEG – Collagen.

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