NEW CHITOSAN WOUND DRESSING – FIRST STEP - THE CYTOTOXICITY EVALUATION

Ewa Karuga[1], Adam Zając[2*], Zbigniew Rybak[1], Jerzy Hanuza[2,3]

[1] Department for Experimental Surgery and Biomaterials Research, Wroclaw Medical University, ul. Poniatowskiego 2, 50-326 Wrocław, Poland

[2] Faculty of Engineering and Economics, Wrocław University of Economics, ul. Komandorska 118/120, 53 - 345 Wrocław, Poland e-mail: adam.zajac@ue.wroc.pl

[3] Institute of Low Temperature and Structure Research, Polish Academy of Sciences, P.O. Box 1410, 50-950 Wrocław 2, Poland

Abstract

The cytotoxicity of chitosan with polyvinox (PCH) and crosslinked chitosan (CH) was studied and analyzed. Cell viability was determined by thiazolyl blue formazan (MTT) assay and cell morphology observations were carried out during cell culturing and MTT tests. Crosslinked chitosan was used as a protective foil (scaffold) for skin wounds. Studies in vitro and the other obtained in this work results prove that the studied materials CH and PCH do not cause cytotoxic activity to Balb 3T3 mouse fibroblasts. CH and PCH are promising biomaterials with prospective application as wound healing dressings.

Keywords: *wound dressing, chitosan, cytotoxicity*

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1. Introduction

Hard-to-heal-wounds are one of the major problems of modern medicine. There are no precise data on the incidence of chronic wounds, however, it is estimated that about 500 000 Polish citizens are consulted or hospitalized due to the damage to the skin of chronic nature [1].

The methods for treating such a kind of wounds include treatment of basic health problem causing the skin wounds incidence and applying an appropriate topical treatment [2]. Local treatment is mainly based on the selection of a suitable dressing material [3,4,5,6]. Despite significant progress in the field of biomaterials designed for a dressing material that meets the requirements of the patient as well as medical staff has still been sought. Scientists search for a material that will not only protect the wound against the external environment, but it also will stimulate tissue healing – being bioactive [7]. Chitosan may be such a material. Chitin and chitosan, apart from cellulose, are next two natural polymers of a great importance in nature. They are building constituents of plants, marine invertebrates, insects, cell walls and microorganisms [8,9]. Their properties have been characterized by various physico-chemical, biological and mechanical methods [10-19]. Chitosan has been successfully applied in biomaterials and drug-delivery systems [20,21], food additives and water purification [22] and supports for cells, enzymes [23] and catalysts [24].

2. Materials and methods

2.1. Scaffold preparation

Appropriately cross-linked chitosan was the basic material for the film used as a protective material for application to a skin wound. 0.65 g of chitosan (Sigma-Aldrich 419419) were placed in a Falcon-type tube and suspended in 18 ml buffered saline (Lublin Biomed 1108; PBS w/o Ca and Mg), then stirred for 20 min using a mechanical stirrer (Heidolph RZR 2021) with the rate 600 rev/min. Next, 1 ml of polivinylbuthyl ether (Avilin, in accordance with Ph.Eur) was added and the mixture was stirred with the rate 600 rev/min for 20 min. Subsequently, the mixture of 0.3ml of 90% L-lactic acid (AppliChem A3509, in accordance with PhEur) were added and stirred for 240min. Then, the pH was measured using an ion selective electrode (Shott Instruments) and the pH of the gel was adjusted to 6.5 with 0.1M NaOH (Sigma-Aldrich S5881). Water (Milli Q) was added to the gel to fill up the volume up to 20ml. 2.5 ml of the gel were placed in 5 ml volume syringes and sterilized by saturated steam under elevated pressure for 20min. The crosslinking mixture was obtained from the gel by adding an aqueous solution of calcium salts of polyphosphates sterilized by saturated steam under elevated pressure for 20 min. The chemical composition and the conditions for obtaining scaffolds for protective purposes are protected by a patent.

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2.2. Cytotocity evaluation of CH and PCH materials

The aim of this study was to determine the cytotoxicity of PCH and CH materials. The investigations were performed at the Department of Experimental Surgery and Biomaterials Research, Wroclaw Medical University. Cell viability was determined by MTT assay according to ISO 10993-5. Cell morphology observations were carried out during cell culturing and MTT tests.

The MTT assay was carried out using an indirect method. The study was conducted with the usage of the material extracts. This method is recommended by international standard-setting OECD organizations as a reference method for determining cytotoxicity.

The experiments were performed in accordance with PN-EN ISO 10993-5: 2009 'Biological evaluation of medical products. Cytotoxicity' [25] and PN-EN ISO 10993-12: 2009 'Biological evaluation of medical products - Part 12: Sample preparation and reference materials' [26].

2.3. Cell culture

The reference cell line - Balb/C 3T3 murine fibroblasts clone A31 was used in the test. The Balb 3T3 cell line was maintained as adherent cells in DMEM (Dulbecco's Modified Eagle's Medium, Lonza Sales Ltd), at 37°C in a humidified 5% CO_2 atmosphere using an incubator SteriCycle 381 (ThermoScentific). The medium was supplemented with 10% fetal bosine serum FBS (Lonza Sales Ltd), 25 mM Hepes and 4.5 g/l glucose (Lonza Sales Ltd).
Before the experiments the cytotoxicity test cells were passaged twice using 0.25% tripsin EDTA (Sigma Aldrich).

2.4. Extracts preparation

For the extract preparations the medium with the following composition: DMEM with 25 mM Hepes and 4.5 g of L glucose (Lonza Sales Ltd), 5% FBS (Lonza Sales Ltd) were used.

PCH, CH and control material extracts preparation was performed under sterile conditions in a laminar (Biohazard) MSC Advantage 1.2 (ThermoScentific).
The PCH and CH extracts were prepared as follows:
- CH samples with a mass of 2g/10cm^3 DMEM culture medium.
- PCH samples with a mass of 2g/10cm^3 DMEM culture medium.

As a positive control - 20 cm^3 of the medium with 5% FBS serum and phenol (MerckKGaA) (1.5 mg/cm^3 and 4 mg/cm^3) were used. For blank test 20 cm^3 of complete DMEM culture medium was applied.
Then the test and control extracts were incubated at 37 ± 1°C for 24 ± 2 h.
To evaluate the cytotoxic effect solutions of the tested and control extracts were used. The test extracts were diluted as follow: 100%, 50%, 25% and 12.5% using DMEM complete medium. In contrast, as the positive control solution phenol in DMEM culture medium at the following concentrations: 1.5 mg/cm^3; 4 mg/cm^3 was used.

2.5 Cytotoxicity test performance

The BALB 3T3 cells were placed in 96-well Nunc plates. In each well 100 µl of the cell suspension with a density of $1x10^5$ cells/cm^3 was placed, which is equivalent to $1x10^4$ cells/well.

After 24 hours, the cells stuck to the bottom of the well and divided, covering about 60% of the surface. After this time the culture medium was removed and tested and the control extracts in an amount of 100µl were added to each well. The cell plates were then incubated at 37 ± 1°C in 5% CO_2 for 24 and 48h.

2.6. Cell morphology evaluation

The cell morphology changes after exposure to the test and control extracts were evaluated in a contrast - phase inverted microscope CKX 41 (Olympus) after 24 and 48 h.

To evaluate the obtained results the criterion presented in Table 1. was used.

Table 1. Cell toxicity degrees of indirect – extract method of cytotoxicity assay (according to BS EN ISO 10993-5: 2009, Table 1)

Degree	Toxity	Cell morphology changes
0	**absence**	Single cytoplasmic granularity, no evidence of cell lysis, the lack of inhibition of cell growth
1	**poor**	Not more than 20% of the cells rounded, separating from the bottom, without cytoplasm densities, single cells ruptured
2	**moderate**	Not more than 50% of the cells rounded, without granularity, extensive cell lysis, empty areas between cells
3	**average**	Not more than 70% of the cells rounded, an extensive cell lysis
4	**strong**	Cell culture almost or completely destroyed

2.7. Cell Viability

The cell viability was determined with MTT assay according to PN-EN ISO 10993-5: 2009 [25].

The MTT test is based on mitochondrial dehydrogenase activity in viable cells to convert a yellow tetrazolium (MTT) into purple insoluble formazan. MTT results are directly related to the number of viable culture cells [33]. To perform the MTT assay MTT solution and a solution of bicarbonate isopropyl alcohol was prepared as described below:

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The MTT solution: 5 mg MTT (bromide 3 [4,5-Dimethyl-2-yl] -2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) were dissolved in 1cm^3 of PBS (phosphate-buffered saline) then 9 cm^3 of DMEM complete medium was added. Acidic solution of isopropyl alcohol: to 10 ml of isopropyl alcohol (PPH STANLAB) 38µl of hydrochloric acid (36% HCl) (PPH STANLAB) were added. After 24 and 48 h the cells were evaluated under the microscope. Then the test and control extracts were removed and the wells were rinsed with 100µl of DMEM. After rinsing, 100 ul of the MTT solution was added to each well and incubation lasted for 2 h at 37°C in 5% CO_2. After this time the MTT solution was decanted and 100 ul of isopropyl alcohol bicarbonate was added to each well. After 30 min the plate was placed in a spectrophotometer Epoch (Biotek) to read the absorbance at the wavelength of 570 nm. Cell viability was calculated by the following formula:

$$\mathbf{V=A_b / A_k x\ 100\ [\%]} \qquad (1)$$

where: V - cell viability expressed as a percentage; A_b – average absorbance of the test samples; A_k – mean absorbance of the control samples.

The evaluation of the cytotoxic effect of the material was achieved by comparing the results obtained from the morphological studies, and the results of cell viability. In accordance with the recommendations of the standard [8] a material is considered as cytotoxic when it causes morphological changes above 2 degrees and the cell viability is below 70%.

2.8. Statistical analysis

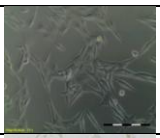
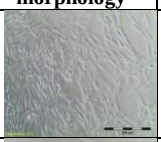
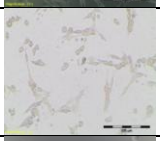
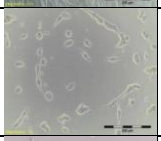
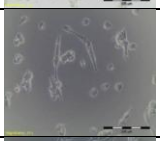
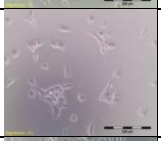
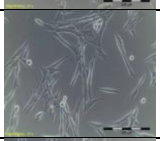
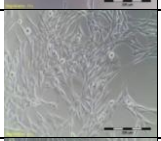
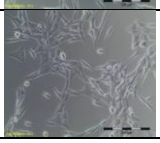
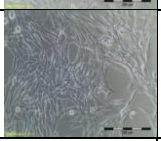
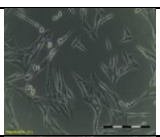
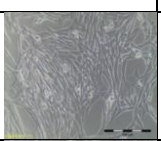
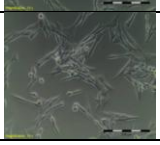
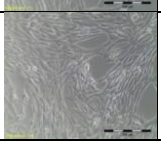
Statistical estimation of the results was carried out using Microsoft Excel spreadsheet. Then the data were implemented in the package StatSoft Statistica V.7.1 and MedCalc statistical program v. 9.02. Comparisons of the data involved univariate ANOVA, post-hoc test Newman-Keuls test Kruskal-Wallis ANOVA. The statistical evaluation adopted the level of statistical significance of p (α) <0.05.

3. Results and discussion

3.1. Cell morphology evaluation

Table 2. shows the average results of 18 repetitions of each experiment evaluating morphology of the cells after exposure to the test and control extracts for 24 and 48 hours. Fig. 1. shows examples of the images of cell morphology and toxicity degree after exposure to the test and control extracts after 24 and 48 hours.

Table 2. Cell morphology and toxicity degree after exposure to the test and control extracts after 24 and 48 hours.

Material	Extract	24 h		48 h	
		Cell morphology	Toxity degree	Cell morphology	Toxity degree
Blanc	DMEM cell medium		0		0
Positive control phenol	4 mg/cm^3		3		3/4
	1.5 mg/cm^3		2		3
CH	100%		0		0
	50%		0		0
	25%		0		0
	12.5%		0		0
PCH	100%		0		0
	50%		0		0
	25%		0		0
	12.5%		0		0

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3.2. Cell viability evaluation

18 iterations of MTT assay were performed for each sample (ie. for each material, for each extract solution and time - 24 and 48 h). Table 3. shows the mean values of cell viability.

Table. 3. The mean values of cell viability after exposure to the test and control extracts after 24 and 48 hours.

Material	extract	Cell viability % 24 h	Cell viability % 48 h
blanc	DMEM cell medium	100	100
Positive control phenol	4 mg/cm^3	57.76	36.02
	1.5 mg/cm^3	71.42	42.24
CH	100%	94.14	96.50
	50%	101.98	100.53
	25%	102.06	102.71
	12.5%	100.87	102.41
PCH	100%	94.35	97.33
	50%	97.33	103.08
	25%	95.21	99.38
	12.5%	95.56	100.21

Comparing the obtained from the morphological studies results, and the results of cell viability shows that the CH and PCH tested materials do not cause any cell morphology changes above 2 toxicity degree and do not cause the cell viability decrease below 70% which demonstrates that the CH and PCH materials do not exhibit cytotoxic effects on the Balb/C 3T3 cells.

3.3. Statistical analysis

3.3.1.Comparison of the cell viability

The results of the treated with the test CH and PCH materials extracts cell viability were compared with a constant reference value m=100 (the assumed value for the control - the culture medium) using the Student's t-test for the expected value. The results of this comparison are shown in Table 4.

Table. 4. Comparison of the cell viability

Time = 24 h										
parameter	**average**	**standard deviation**	**N**	**Standard error.**	**confidence - 95,0%**	**confidence +95,0%**	**constant reference**	**t**	**df**	**p**
CH100	94.14	10.08	18	2.376	89.13	99.15	100.00	-2.466	17	**0.0246**
CH50	101.98	6.88	18	1.623	98.55	105.40	100.00	1.219	17	0.2394
CH25	102.06	7.14	18	1.682	98.51	105.61	100.00	1.225	17	0.2373
CH12	100.87	11.35	18	2.675	95.23	106.52	100.00	0.325	17	0.7486
PCH100	94.35	9.61	18	2.265	89.57	99.13	100.00	-2.496	17	**0.0231**
PCH50	97.33	6.17	18	1.454	94.26	100.39	100.00	-1.839	17	0.0834
PCH25	95.21	8.31	18	1.959	91.07	99.34	100.00	-2.447	17	**0.0256**
PCH12	95.56	9.60	18	2.262	90.79	100.33	100.00	-1.962	17	0.0663
phenol15	71.42	7.48	18	1.763	67.70	75.14	100.00	-16.211	17	**0.0000**
phenol4	57.76	9.91	18	2.336	52.84	62.69	100.00	-18.080	17	**0.0000**

Time = 48 h										
parameter	**average**	**standard deviation**	**N**	**Standard error.**	**confidence - 95,0%**	**confidence +95,0%**	**constant reference**	**t**	**df**	**p**
CH100	96.50	7.50	18	1.767	92.77	100.22	100.00	-1.983	17	0.0637
CH50	100.53	8.46	18	1.994	96.32	104.73	100.00	0.265	17	0.7939
CH25	102.71	10.93	18	2.577	97.28	108.15	100.00	1.052	17	0.3072
CH12	102.41	9.99	18	2.355	97.45	107.38	100.00	1.025	17	0.3197
PCH100	97.33	10.25	18	2.416	92.23	102.42	100.00	-1.106	17	0.2839
PCH50	103.08	11.38	18	2.683	97.42	108.74	100.00	1.148	17	0.2669
PCH25	99.38	11.59	18	2.731	93.62	105.14	100.00	-0.228	17	0.8223
PCH12	100.21	17.02	18	4.013	91.75	108.68	100.00	0.053	17	0.9583
phenol1,5	42.24	7.55	18	1.779	38.49	45.99	100.00	-32.48	17	**0.0000**
phenol4	36.02	3.56	18	0.840	34.25	37.79	100.00	-76.15	17	**0.0000**

On the basis of the Student's t-test results for the expected value it was found:

24 h:

- a significantly lower cell viability ($p = 0.0246$) treated with the CH extract (100%) in relation to the assumed 100 value of the expected cell viability.
- a significantly lower cell viability ($p = 0.0231$) treated with the PCH extract (100%) of the PCH in relation to the assumed 100 value of the expected cell viability.
- a statistically significant lower cell viability ($p = 0.0256$) treated with the PCH extract in a dilution of 1:4 (25%) in relation to the 100 value assumed for the expected cell viability.
- a statistically significant lower cell viability ($p < 0.0001$) phenol treated at a dilution of 1.5 mg/cm^3 in relation to the 100 value assumed for the expected cell viability.
- a statistically significant lower cell viability ($p < 0.0001$) phenol treated at a dilution of 4 mg/cm^3 in relation to the 100 value assumed for the expected cell viability.

48 h:

- a statistically significant lower cell viability ($p < 0.0001$) treated with phenol at a dilution of 1.5 mg/cm^3 in relation to the assumed 100 value of the expected cell viability.

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- a statistically significant lower cell viability ($p < 0.0001$) treated with phenol at a dilution of 4 mg/cm^3 with respect to the 100 assumed value for the expected cell viability.

3.3.2. Comparison of the viability of the cells treated with the test material extracts for 24 h and 48 h exposure

Using the Student's t or the U Mann-Whitney tests a comparison of the cell viability of the CH and PCH extracts exposed for 24 h and 48 h. The results of the comparison are shown in Tables 5. and 6.

Table 5. The Student's t-test comparison of the cell viability for the cells treated with the test material extracts for 24 h and 48 h

parameter	average 24 h	average 48 h	t	df	p	No repetitions (24 h)	No repetitions (48 h)	standard deviation (24 h)	standard deviation (48 h)
CH100	94.14	96.50	-0.795	34	0.4321	18	18	10.079	7.497
CH50	101.98	100.53	0.564	34	0.5766	18	18	6.884	8.458
CH25	102.06	102.71	-0.212	34	0.8335	18	18	7.137	10.933
CH12	100.87	102.41	-0.432	34	0.6679	18	18	11.350	9.991
PCH100	94.35	97.33	-0.900	34	0.3745	18	18	9.611	10.251
PCH50	97.33	103.08	-1.885	34	0.0679	18	18	6.167	11.383
PCH25	95.21	99.38	-1.241	34	0.2230	18	18	8.312	11.586
PCH12	95.56	100.21	-1.010	34	0.3196	18	18	9.597	17.025
phenol15	71.42	42.24	11.652	34	**0.0000**	18	18	7.480	7.546
phenol4	57.76	36.02	8.757	34	**0.0000**	18	18	9.911	3.565

Tab. 6. Comparison of the U Mann-Whitney test of the cell viability of the cells treated with the test material extracts for 24 h and 48 h

Parametr	Rang sum 24 h	Rang sum 48 h	U	Z	p level	No repetitions 24 h	No repetitions 48 h
CH100	314.0	352.0	143.0	-0.6011	0.5478	18	18
CH50	360.0	306.0	135.0	0.8542	0.3930	18	18
CH25	336.0	330.0	159.0	0.0949	0.9244	18	18
CH12	328.0	338.0	157.0	-0.1582	0.8743	18	18
PCH100	309.0	357.0	138.0	-0.7593	0.4477	18	18
PCH50	276.0	390.0	105.0	-1.8034	0.0713	18	18
PCH25	299.0	367.0	128.0	-1.0757	0.2821	18	18
PCH12	320.0	346.0	149.0	-0.4113	0.6809	18	18
phenol15	492.0	174.0	3.0	5.0305	**0.0000**	18	18
phenol4	484.0	182.0	11.0	4.7774	**0.0000**	18	18

Comparing the viability of the cells treated with the test material extracts for 24 h and 48 h it was found:

- a statistically significant lower cell viability ($p < 0.0001$) treated with phenol at a dilution of 1.5 mg/cm^3 exposed for 48 h and 24 h.
- a statistically significant lower cell viability ($p < 0.0001$) treated with phenol at a dilution of 4 mg/cm^3 exposed for 48 h and 24 h.

3.3.3. Simultaneous evaluation of the impact of the time of exposure, the concentration of the extract and the type of the studied CH or PCH material on the cell viability

Simultanoeus statistical evaluation was made to control: the time of exposure, the concentration of the extract and the type of the studied materials on the cell viability using the ANOVA test for factorial designs. The results of this evaluation are shown in Table 7.

Table 7. The results of the concomitant impact of the exposure time, the concentration and the material on the cell viability

Parameter	SS	degrees of freedom	MS	F	p	impact assessment
free term	2821396	1	2821396	27759.5	0.0000	
time	480	1	480	4.72	**0.0306**	indirect effects
concentration	1151	3	384	3.78	**0.0111**	the greatest impact
Material	396	1	396	3.90	**0.0493**	the smallest impact
Time*Concentr.	9	3	3	0.03	0.9934	
Time*Material	235	1	235	2.31	0.1293	
Concentration *Material	350	3	117	1.15	0.3306	
Time*Concentr. *Material	99	3	33	0.33	0.8067	
error	27645	272	102			

The results show that the variability of the studied factors range such as the exposure time, the concentration of the tested material extracts and the type of the CH or PCH tested material on the cell viability, the concentration of the CH and PCH test material extracts has the largest influence. The exposure time and the type of the material is also important, but much less significant. However, there was no significant effect of the other studied interaction factors.

4. Conclusion

An *in vitro* examination is the first step in the process of complex biological evaluation of biomaterials. Cytotoxitciy tests allow to perform a basic selection of potential biomaterials that may cause potential systemic toxicity in the future *in vivo* tests [27,28]. Our studies of cytotoxic effects of CH and PCH materials was carried out using an indirect method - extracts using the MTT colorimetric assay, and it was found that these materials do not exhibit toxicity to Balb 3T3 cells and do not affect the morphology changes in the cells. Ribeiro et

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al. [29] also observed absence of any cytotoxic effect of the tested chitosan hydrogels. The authors reported no increased mortality of the cultured cells exposed to chitosan. Chang et al. [30] and Yang et al. [31] conducted a study of cytotoxicity of chitosan materials for wound treatment, including burn wounds [30]. The results confirm absence of cytotoxicity of chitosan materials addressed to wound treatment. Radhakumary et al. [32], performed cytotoxicity assay by direct and indirect contact, they also found no negative effect of chitosan material on cell cultures. *In vitro* studies and the obtained results allow us to conclude that the CH and PCH test materials do not cause any cytotoxic activity to Balb 3T3 mouse fibroblasts. CH and PCH are promising biomaterials with potential usage as a medical product for wound treatment. At present comprehensive biological studies of their biocompatibility by *in vitro* methods are being carried out.

5. References

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