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Cytotoxicity of three-dimensional paper-based models from a three-dimensional paper-based printer

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23 # Cover letter

24
25 Dear Editor-in-Chief,
26
27 Please receive our article titled “Sterilization methods and cytotoxicity of three-
28 dimensional paper-based models from a three-dimensional paper-based printer” for
29 open evaluation in Nemesis journal.
30 1) Summarize the study’s contribution to the scientific literature: Our study aimed
31 to determine the possibility of using 3D models created with a low-cost, paper-based
32 3D printer in an operating room. Therefore, the influence of different methods of
33 sterilization on 3D models was tested, and the cytotoxicity of generated 3D models
34 was also determined.
35 2) Relate the study to previously published work: there was no previous works on
36 sterilization methods and cytotoxicity evaluation of three-dimensional paper-based
37 models generated using three-dimensional printer Mcor (Mcor technology, Eire).
38 3) Specify the type of article (for example, research article, systematic review, me-
39 ta-analysis, clinical trial): we provide with experimental research article.
40 4) Describe any prior interactions with Nemesis regarding the submitted manu-
41 script: we have no prior interactions with Nemesis journal.
42 5) Nemesis aim and scope relevance: There was no statistically significant differ-
43 ence for established statistical significance p=0.05 in cuboids dimensions before and
44 after sterilization regardless of sterilization method. For cytotoxicity, all 3D paper-
45 printed and sterilized samples showed higher cytotoxicity against normal, human,
46 adult dermal fibroblast culture when compared to positive control. The ANOVA sta-
47 tistical analysis confirmed that only 2-octyl cyanoacrylate coating of 3D paper
48 model improved the biological behaviour of the material.
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Abstract

Objective: Our study aimed to determine the possibility of using models created with a low-cost, paper based 3D printer in an operating room. Therefore influence of different methods of sterilization on models was tested and cytotoxicity of generated models was determined.

Material and methods: 30 cuboids divided into three groups were used for verification of shape stability after sterilization. Each group was sterilized either with: Ethylene oxide in temperature 55˚C, Hydrogen peroxide gas plasma in temperature 60˚C or Gamma irradiation at 21˚C, 25kGy. Each cuboid was measured using calliper three times before and three times after sterilization. Results were analysed statistically in Statgraphics Plus. Statistical significance was determined as $p< 0.05$. Sixty cylinders divided into six groups were used for cytotoxicity tests. Three of those groups were covered before sterilization with 2-octyl-cyanoacrylate. Each group was sterilized with one of the previously described methods. Cytotoxicity was tested by Nanostructural and Molecular Biophysics Laboratory in Technopark Lodz using normal adult human dermal fibroblasts. Survival of cells was tested using spectrophotometry with XTT and was defined as ratio of absorbency of tested probe to absorbency of control probe. Calcein/Ethidium dyeing test was performed according to LIVE/DEAD Viability/Cytotoxicity Kit protocol. Observation was done under Olympus GX71 fluorescence microscope. Results: There was no statistically significant difference for established statistical significance $p=0.05$ in cuboids dimensions before and after sterilization regardless of sterilization method. In XTT analysis all samples showed higher cytotoxicity against normal, human, adult dermal fibroblast culture when compared to positive control. ANOVA statistical analysis confirmed that 2-octyl cyanoacrylate coating of paper model improved biological behaviour of the material. It decreased cytotoxicity of the model independently of sterilization method. In calcein/ethidium dyeing test due to the high fluorescence of the background caused by cylinders of analysed substance it was impossible to perform the exact analysis of the number of marked cells.

Conclusions: Acquired results allow to conclude that Mcor Technology Matrix 300 3D paper-based models can be used in operating room only if covered with cyanoacrylate tissue adhesive.

Nemesis relevance: no statistically significant difference in cuboids dimensions before and after sterilization regardless of sterilization method. Presence of high cytotoxicity of 3D paper-based models without coating.

Keywords:

cytotoxicity; sterilization; three-dimensional printing, three-dimensional printer

Introduction

Within last few years patient-customized craniofacial reconstructions become new standard in maxillofacial surgery. Patient specific 3D printed models can be used as a template for pre-shaping titanium mesh [1-6] or bone grafts [1, 7, 8] before implantation, to simulate osteotomies [1, 9, 10], to improve cancer resection techniques [11, 12] and to better plan the surgery [7]. There are even possibilities to produce patient-specific implants using either subtractive [2, 13] or additive methods like selective laser sintering [6, 14-17]. All of these innovations lead to reduce operating time, minimize complications and produce better fitted implants [2], and improve functional and aesthetic postoperative results [2, 17].

The most commonly 3D printing technique in maxillofacial surgery is stereolithography using liquid resin which is photopolymerised with a laser beam [6]. Models created with this material have already proven their usefulness in medicine [1, 4, 14, 17-21]. Three-dimensional objects manufactured in such 3D printer are durable enough to successfully undergo sterilization and serve as a template during the surgery. Resins created especially for use in medical applications are guaranteed to be safely used in operating theatre. Unfortunately extremely high costs for hardware and material limits the routine use of this technique in maxillofacial surgery.

Mcor Technologies Matrix 300 printer, which was already validated for clinical applications [22, 23] allows to produce low-cost and durable paper-based 3D models. Contrary to other commercial solutions it was not designed to be used in medicine, but for design and architecture. Therefore, in order to safely use 3D paper-based models in operating theatre, we need to check if these models could be sterilized and if these 3D models present with any cell cytotoxicity.

Materials and methods

In this study Matrix 300 (MCor Technologies, Dunleer, Ireland) paper based 3D printer was used. It uses 80 gsm sheets of A4 paper and water soluble adhesive (MCor Technologies, Dunleer, Ireland) to produce detailed prints. The resolution of printing is 0,01mm. Printing process begins in PC running software controlling Matrix 300 printer called SliceIT (MCor Technologies, Dunleer, Ireland). This software allows creating or importing of earlier prepared .stl file models and transforms them into data necessary for the Matrix 300 printer. Every object is analysed and cut into 0,1mm layers equal to the thickness of the used A4 sheet. Such prepared 2D data are send to 3D printer where a cutting tungsten blade cut the object, layer by layer, from paper sheets. The layers are glued with Mcor Technologies Adhesive. When the

printing process is finished the printed objects are freed from the waste in a process called "weeding". In this stage the excess paper from around the printed 3D object is removed.

In order to check the possibility of safe intra-operative use of 3D models from MCor Technologies Matrix 300 printer we tested the influence of sterilization on 3D printed objects shape stability and we determined the 3D models cytotoxicity. To determine cytotoxicity XTT test and calcein/ethidium dyeing were performed by Nanostructural and Molecular Biophysics Laboratory in Technopark Lodz. Comparison of adhesive to a normal glue for wood and paper was also performed. For comparison, normal glue for paper/wood adhesion (joiner glue, Wytwornia Chemiczna Dragon, Krakow, Poland) was used for investigation. Lenticular shape samples were prepared in polyethylene mini dishes and later their macro and microstructure was compared i.e. scanning electron microscope (SEM) images were compared (Figure 1).

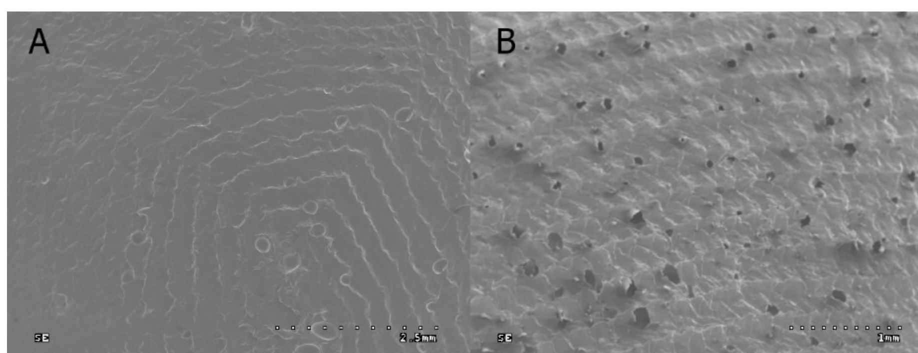

Fig. 1. Comparison of scanning electron microscope images of glue samples after drying. A. Surface of MCor adhesive; B. Surface of normal glue for paper/wood adhesion.

Also the spectral analysis (Table 1) of analyzed adhesives was performed with Thermo Noran system (Thermoscientific, Waltham, USA).

155 **Table 1.** Spectral analysis of Mcor Technologies adhesive and normal
156 wood/paper glue (Dragon). Comparison of percentage of number of atoms
157 creating adhesive molecules.

	Normal glue	Mcor Technologies adhesive
Carbon	62,86%	69,64%
Oxygen	36,64%	30,36%
Calcium	0,50%	-

158

159 In order to verify the influence of sterilization on the shape stability we printed 30
160 cuboids with dimensions 10x20x30mm. The cuboids were divided into three groups
161 by ten samples at random. For each group we tested one of current procedures of
162 low temperature sterilization used in hospitals: 1. Ethylene oxide in temperature of
163 55^0C, with a time of 4.5 hours and degazation period of 12 hours; Hydrogen perox-
164 ide gas plasma in temperature of 60^0C; and Gamma irradiation at 21^0C with 25kGy.
165 Each cuboid was measured by observer using a calliper three times before and three
166 times after sterilization (Table 2). Results were analysed statistically in Statgraphics
167 Plus (Summary Statistics, ANOVA, analysis of linear regression, t-test). Statistical
168 significance was determined as $p< 0.05$.

169 **Table 2.** Cuboids dimensions related to sterilization methods

Sterilization method	**Dimension**	**Before sterilization [mm]**	**After sterilization [mm]**	**Statistical significance (p)**
radiation	x	9.95	9.93	0.121
radiation	y	30.15	30.15	0.509
radiation	z	20.13	20.13	0.096
gas plasma	x	9.90	9.90	0.792
gas plasma	y	30.19	30.19	0.434
gas plasma	z	20.15	20.16	0.505
ethylene oxide	x	9.90	9.91	0.066
ethylene oxide	y	30.22	30.21	0.309
ethylene oxide	z	20.16	20.17	0.053

170

Before sterilization [mm] – mean dimension before sterilization [mm]

After sterilization [mm] – mean dimension after sterilization [mm]

Statistical significance (p) – statistical significance value calculated with t-test statistics. For each p value higher than 0,05 there is no statistically important change in dimension before and after sterilization.

In order to verify cytotoxicity of 3D prints from MCor Technologies sixty cylinders with a high of 3mm and diameter of 14mm were printed. They were divided into six groups with ten samples in each group. Three of those groups were covered before sterilization with 2-octyl-cyanoacrylate (Dermabond topical skin adhesive by Ethicon LLC, San Lorenzo, Puerto Rico) (Figure 2).

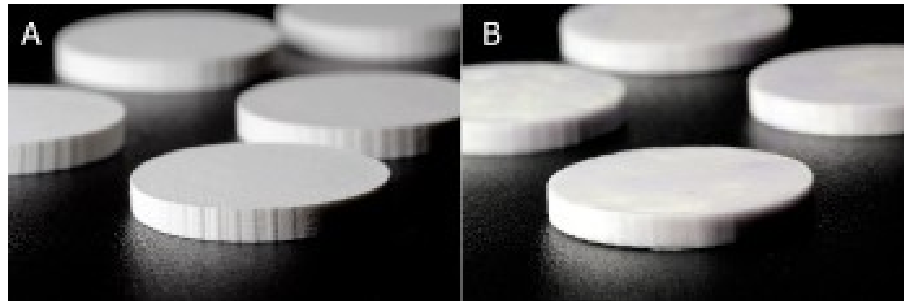

Fig. 2. 3D printed cylinders used for cytotoxicity determination tests. A. Before coating with 2-octyl-cyanoacrylate; B. After coating with 2-octyl-cyanoacrylate (Dermabond topical skin adhesive by Ethicon LLC, San Lorenzo, Puerto Rico).

The test cylinders were covered by 2-octyl-cyanoacrylate in the following pattern: the wall first, after five minutes the base and after further five minutes the second base. The same procedure was performed once again after ten minutes. Three different methods of sterilization (radiation, plasma, ethylene oxide) were used. Samples after sterilization were used for further cytotoxicity analysis:

Intact + ethylene oxide sterilization –10 pieces

Coated + ethylene oxide sterilization – 10 pieces

Intact + gas plasma sterilization - 10 pieces

Coated + gas plasma sterilization - 10 pieces

Intact + radiation sterilization - 10 pieces

Coated + radiation sterilization - 10 pieces

Additionally there were two control samples:

Control (+): intact medium and culture of XXT

Control (-): intact medium and culture washed with 50% ethanol.

202 The cytotoxicity of 3D printed paper blocks was tested by Nanostructural and Mo-
203 lecular Biophysics Laboratory in Technopark Lodz using normal adult human der-
204 mal fibroblasts (ATCC No. PCS-201-012, ATCC, Manassas, USA). Survival of
205 cells was tested using spectrophotometry with XTT and was defined as ratio of ab-
206 sorbency of tested probe to absorbency of control probe.

207 **Cell culture preparation**

208 Normal, human, adult dermal fibroblast (ATCC No. PCS-201-012, ATCC, Ma-
209 nassas, USA) culture was grown in 75 cm2 tissue culture dish (MIDSCI Company,
210 St. Louis, USA) using classical method of single layer cell culture until the phase of
211 late logarithmic growth. Cells after moving from temperature -150˚C and unfreezing
212 were put in a Fibroblast Basal Medium (ATCC No. PCS-201-030, ATCC, Manas-
213 sas, USA) enriched with Fibroblast Growth Kit – Low Serum (ATCC No. PCS-201-
214 041, ATCC, Manassas, USA) and antibiotics Penicillin and Streptomycin (Sigma
215 No. P0781, Sigma-Aldrich, St. Louis, USA). Cultures were grown in an incubator at
216 37˚C, 5% CO_2, 19% O_2 and humidity 100%. After reaching confluence of 80% cell
217 cultures were washed with Dulbecco`s Phosphate Buffered Saline (DPBS) (BI No.
218 02-023-1A, Biological Industries, Kibbutz Beit Haemek, Israel). In order to break
219 cells connections 0.6ml of trypsin/EDTA (Sigma No. T3924, Sigma-Aldrich, St.
220 Louis, USA) was used. Trypsinization was performed in incubator for five minutes.
221 The process was ended by diluting trypsin with growth medium five times. Cells af-
222 ter freeing them from single layer culture were suspended in growth medium and
223 were counted with Thoma chamber. Dead cells were marked with 0.4% trypan blue.

224 **Preparation of samples**

225 Discs of analysed substance were placed in an aseptic 6-well tissue culture plate
226 and were flooded with 3ml of complete Fibroblast Basal Medium. After 24 hours
227 medium containing substances unbound from analysed discs was added to cell cul-
228 tures. Negative control were cells treated with 50% ethanol. Method was taken from
229 norm PN-EN-ISO 10993-12 [24].

230 **Cytotoxicity analysis using spectrophotometric**
231 **method with XTT**

232 The XTT method uses the ability of mitochondrial dehydrogenases, especially
233 succinic to transform tetrazolic salt of XTT to formazan product. This reaction is
234 only possible in metabolically active cells. Cells in number $4x10^3$ cells/ml/well were
235 placed in an aseptic 96 well flat-bottomed spectrophotometric plate and were cul-
236 tured for 24 hours (minimal time required for cells attachment to the cell culture

237 plate). After this time conditioned medium was removed from the culture and was
238 replaced with 0.1ml medium containing substance unbound form analysed discs. Af-
239 ter another 24 hours of incubation medium containing unbound substances was re-
240 moved by aspiration, culture was washed with DPBS and 50 µl/well XTT mixture
241 (BI No. 20-300-1000, Biological Industries, Kibbutz Beit Haemek, Israel) was
242 added. Cultures with XTT were incubated for 4 hours in 37°C. Absorbance of for-
243 mozan solution was read on Multiskan GO (Thermoscientific, Waltham, USA) plate
244 reader for wave length 450nm were reference wave length was 630nm. Survival rate
245 of cells was established from the relation: Survival (%) = (A/Ak) x 100%, where A
246 is absorbance of sample treated with analysed substance, AK is absorbance of con-
247 trol sample (not treated with analysed substances).

248 Calcein/Ethidium dyeing

249 Calcein AM is commonly used to mark the living cells due to its ability to freely
250 transfer through the cell membrane. In the cell AM (acetometoxy) group is degraded
251 enzymatically which results in attaching calcium ions to the particle causing high
252 fluorescence of the probe. Ethidium homodimer enters cells with damaged mem-
253 branes and binds to nucleic acids, thereby dyeing dead cells.
254 Discs of analysed substance were placed in an aseptic 6-well tissue culture plate
255 and were seeded with cells in number $8x10^4$ cells/ml/well in 3ml growth medium.
256 After 24h incubation cultures were washed twice with DPBS and 3ml of fluorescent
257 dyes calcein/ethidium in DPBS were added according to LIVE/DEAD Viabil-
258 ity/Cytotoxicity Kit (Molecular Probes No. L3224, , Life Technologies, Waltham,
259 USA) protocol. After 30 minutes of incubation samples were washed with DPBS.
260 Observation was done under GX71 (Olympus, Tokyo, Japan) fluorescence micro-
261 scope.

262 Results

263 MCor Adhesive specification is not available from the producer (MCor Technolo-
264 gies, Dunleer, Ireland). The appearance of the MCor adhesive is very similar to a
265 normal glue for paper/wood adhesion i.e. cream, white opaque, and typical smell.
266 Lenticular shape samples were prepared in polyethylene mini dishes. The viscosity
267 of MCor adhesive is lighter than joiner glue. That is why the surface of MCor sam-
268 ple was just glossy meniscus, and in fresh joiner glue the surface was wrinkled. Af-
269 ter 24 hours of adhesives hardening, it was noticed significant surface deformation
270 in joiner glue. The surface formed concavity contrary to near flat surface of MCor
271 glue. Anyway the shrinkage was also observed in MCor adhesive. In Spectral analy-
272 sis occurred that both adhesives are comprised mostly of carbon and oxygen. How-
273 ever, in the normal glue small amount of calcium was also found (Table 1). SEM

274 analysis of samples showed smoother surface of MCor Adhesive compared to nor-
275 mal paper/wood glue (Figure 1).
276 There was no statistically significant difference for established statistical signifi-
277 cance p=0.05 in cuboids dimensions before and after sterilization regardless of ster-
278 ilization method (Table 2).
279 In XTT analysis samples showed higher cytotoxicity against normal, human, adult
280 dermal fibroblast culture when compared to positive control. However, statistical
281 analysis of gathered results showed that nearly all analysed samples showed statisti-
282 cally lower cytotoxicity than negative control. The only exception was group steril-
283 ised in ethylene oxide without Dermabond coating. In this group survival rate of
284 cells (average 17.24%) was similar to negative control group (average 16.65%). The
285 ANOVA statistical analysis confirmed that 2-octyl cyanoacrylate coating (Figure 2)
286 of paper model improved biological behaviour of the material. It decreased cytotox-
287 icity of the model independently of sterilization method (Table 3).

288 **Table 3.** Absorbance rates and cell survival rate depending on sterilisation
289 method. Survival (%) = (A/Ak)x100% where A is absorbance of sample
290 treated with analysed substance, and Ak is absorbance of control sample.

	Mean absorbance of sample	**Mean survival rate (%)**
Control (+)	0.224309091	100.00%
Control (-)	0.037354545	16.65%
EO INTACT	0.038663636	17.24%
EO COATED	0,046381818	20.68%
GP INTACT	0.042927273	19.14%
GP COATED	0.0838	37.36%
IR INTACT	0.053254545	23.74%
IR COATED	0.070872727	31.60%

291
292 Control (+) - intact medium and culture of XXT
293 Control (-) - intact medium and culture washed with 50% ethanol
294 EO INTACT – intact samples (not covered with dermabond cyanoacrylate) + eth-
295 ylene oxide sterilization
296 EO COATED – samples covered with dermabond cyanoacrylate + ethylene oxide
297 sterilization
298 GP INTACT - intact samples (not covered with dermabond cyanoacrylate) + gas
299 plasma sterilization
300 GP COATED - samples covered with dermabond cyanoacrylate +gas plasma ster-
301 ilization

IR INTACT - intact samples (not covered with dermabond cyanoacrylate) + radiation sterilization

IR COATED - samples covered with dermabond cyanoacrylate + radiation sterilization.

In calcein/ethidium dyeing test due to the high fluorescence of the background caused by cylinders of analysed substance it was impossible to perform exact analysis of number of marked cells. Samples not covered with 2-octyl cyanoacrylate strongly absorbed growth medium what caused increase in volume and stratification of samples.

Discussion

No dimensional change and no stratification after sterilization were the minimal requirements for 3D models that were planned to be used in the operating theatre. Three different, and current methods of sterilization were chosen for this study. None of those methods had any significant impact on size and structure of printed models. However, sterilization with gas plasma was delayed by the sorption of hydrogen superoxide by printed cuboids. This may result in failure of sterilization in case of bigger models.

In order to be safely used as a surgical template printed model should have as low cytotoxic effect as possible. Three-dimensional prints from Mcor Technology Matrix 300 paper-based printer had shown significant cytotoxic effect. Although cyanoacrylate adhesives are discussed as being cytotoxic themselves [25], coating the models with Dermabond cyanoacrylate tissue adhesive significantly reduced cytotoxicity. What is more, samples covered with Dermabond did not absorbed growth medium in calcein/ethidium dyeing test and showed no stratification effect at all in comparison to intact samples. Further studies regarding safe covering of 3D printed models are required.

330 331 332 333 334 335 336 337 338 339

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- **Ethical approval**: there was no need for ethical committee approval for this experimental study.
- **Informed consent**: there was no need for informed consent for this experimental study.

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Authors contribution:

Author	Contributor role
Kozakiewicz M	Conceptualization, Data curation, Investigation, Methodology, Validation, Resources, Writing original draft preparation, Writing-review and editing
Szymor P	Conceptualization, Data curation, Investigation, Methodology, Validation, Writing original draft preparation, Writing-review and editing
Olszewski R	Resources, Validation, Writing original draft preparation, Supervision, Writing original draft preparation, Writing-review and editing

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