

# Evaluation of the Cytotoxic Effect of Tissue Glue (Octyl 2-Cyanoacrylate) on H9C2 Cardiomyoblast Cells Using Extract Dilution Assay

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## Abstract

**Background:** Recently, Tissue Glue or Octyl 2-Cyanoacrylate (OCA) has been extensively used in cardiac surgeries as surgical adhesives to replace sutures. Since the binder is in contact with live tissues, its tissue compatibility and non-cytotoxicity have major effect on enhancing the healing process.

**Objectives:** The purpose of this study was to evaluate the cytotoxicity of OCA on rat cardiomyoblast cell (H9C2) using MTT assay.

**Materials and Methods:** Cells were cultured in the presence of various concentrations of the OCA extract. Then, morphological changes of the cultured cells were observed by light microscopy and their cytotoxicity by MTT assay. The morphology quality of the cells and their quantity were examined after 24-hour incubation up to 72 hours. Data were analyzed statistically by the ANOVA.

**Results:** The results showed that the percentage of survived cells amount of extract dilution ratio (1:4; 1:2; 1) were 80.4%; 79.6%, 77.5% respectively. Analysis of statistical test results does not show any significant differences between the optical density of the extract dilutions, and the control group indicating; consequently it has no significant cytotoxicity effect on the cells.

**Conclusions:** The outcome of this study is that the OCA has no toxic effect on H9C2 cells.

**Keywords:** Cytotoxicity, OCA, Tissue Glue

## 1. Background

Cyanoacrylates (CAs) with the appropriate human body compatibility have wide applications in medical sciences. They are used as an alternative to sutures in surgical treatments due to their strong adhesion property and rapid polymerization (1). They can be synthesized by reacting formaldehyde with alkyl cyanoacetate to obtain a prepolymer depolymerised into a liquid monomer by applying heat (2). In a cardiac surgery, the polymerization is necessary to stop bleeding quickly. Nowadays, tissue glue or Octyl 2-Cyanoacrylate (OCA) has been used vastly as a surgical adhesive. They are superseded suture and tie the end of vessels or other conduits to prevent bleeding (3). This technique is a simple and effective method for reconstruction and repair of sub-acute cardiac rupture. Also, this method can resolve the suture problem in ventricular infarction (4). Besides, using tissue glue in vascular connections does not cause limitation on the organ motion. Pericardial patch with an adhesive cyanoacrylate is easy to use and eliminates any needs for sutures

to control the bleeding heart (5). This adhesive tissue is also known as one of the materials to prevent bleeding from aorta. In general, cyanoacrylates produce free elements like formaldehyde when used in the body, which can cause systemic toxicity (6). There are no data available regarding toxicity studies of OCA on cardiac cell lines. While the possibility of OCA increases, neoplasia raises some concerns; further longitudinal studies are needed in this regard. The cytotoxicity effect of OCA on live cardiomyoblast cells was studied using MTT (3-(4-dimethyl thiazolyl)-2,5-diphenyl tetrazolium bromide) assay. The MTT assay is a cell culture method based on the reduction of yellow tetrazolium crystals and breaking them down by succinate dehydrogenase enzymes in to insoluble blue crystals (7).

## 2. Objectives

The cytotoxicity test was performed in order to evalu-

ate the presence and /or release of toxic leachable and degraded products.

### 3. Materials and Methods

#### 3.1. Preparation of Sample Extract

Cylindrical sample (*Neat OCA*) was made following ISO-10993:12 guideline (8). The ratio of surface area to volume of the sample and the mass in the extract volume was kept at 6 cm<sup>2</sup>/ml and 0.1 g/ml, respectively. Then, the sample was sterilized by ethylene oxide and placed in the extraction vehicle for 72-hour at 37°C while shaking constantly, and then the extract was filtrated.

An indirect contact test was performed as following process: negative control is the cells grown on polystyrene for cell cultures (without added OCA). The extract was separated by decantation up to achieve designated 100% extract. This extract was diluted with DMEM medium to gain various concentration percentages (1:2, 1:4) with culture medium and used immediately.

#### 3.2. Cell Line and Cell Culture Conditions

The cardiomyolast cell line, H9C2 (rat heart cardiomyoblasts), has been used for cytotoxicity evaluation. H9C2 cells have been obtained from National Cell Bank of Iran (NCBI). These cells were grown at 37°C in DMEM-medium supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 U/ml streptomycin (all from Life Technologies), at 37°C in a 5% CO<sub>2</sub>-humidified atmosphere. Viability of cells throughout the experiment were always > 95% as determined by trypan blue.

#### 3.3. Cell Viability Experiments

The effects of extract on viability of H9C2 cells were evaluated using MTT micro culture tetrazolium assay. Cells were seeded (Falcon, Strasbourg, France) and incubated in complete culture medium for 24 hours. Then, the medium has been removed and replaced with 2% FCS-medium containing extract dilutions (1:4, 1:2) and 1 undiluted OCA extract. After incubation for 24, 48 and 72 hours, the cells have been washed with phosphate-buffered-saline (PBS, Life echnologies) and incubated with MTT (2 mg/ml, Sigma-Aldrich) for an additional 4 hours at 37°C. Then, the insoluble product has been dissolved by addition of DMSO (Sigma-Aldrich). The absorbance of formazan dye was recorded at 570 nm (9). All tests were done three times and the medium was not replaced with fresh one until the end of the experiment. The formation of formazan as a product of MTT has been found to correlate with the number of living cells (10) and MTT assay has been performed before density limitation of cell growth occurred in control wells. The optical density read from the extract-treated wells was converted to a percentage of living cells against the control using the following formula.

$$(1) \frac{\text{Absorbance of treated cells in the each well}}{\text{Mean absorbance of control (untreated) cells}} \times 100$$

The dose- response curves were calculated at the above-mentioned dilutions and expressed as the mean percent fraction of control  $\pm$  SD of means (SDM). LC50 was determined by plotting the extract concentrations against the mean percentage of living cells at each dilution of extract.

#### 3.4. Morphological Alterations

Various dilutions of OCA extract (1:4, 1:2 and undiluted) were prepared and co-cultured with H9C2 cells. Cells have been observed under light inverted microscope for morphological alterations; for example, proliferation and distribution, granulation of cytoplasm and anchorage dependency from 24 to 72 hours.

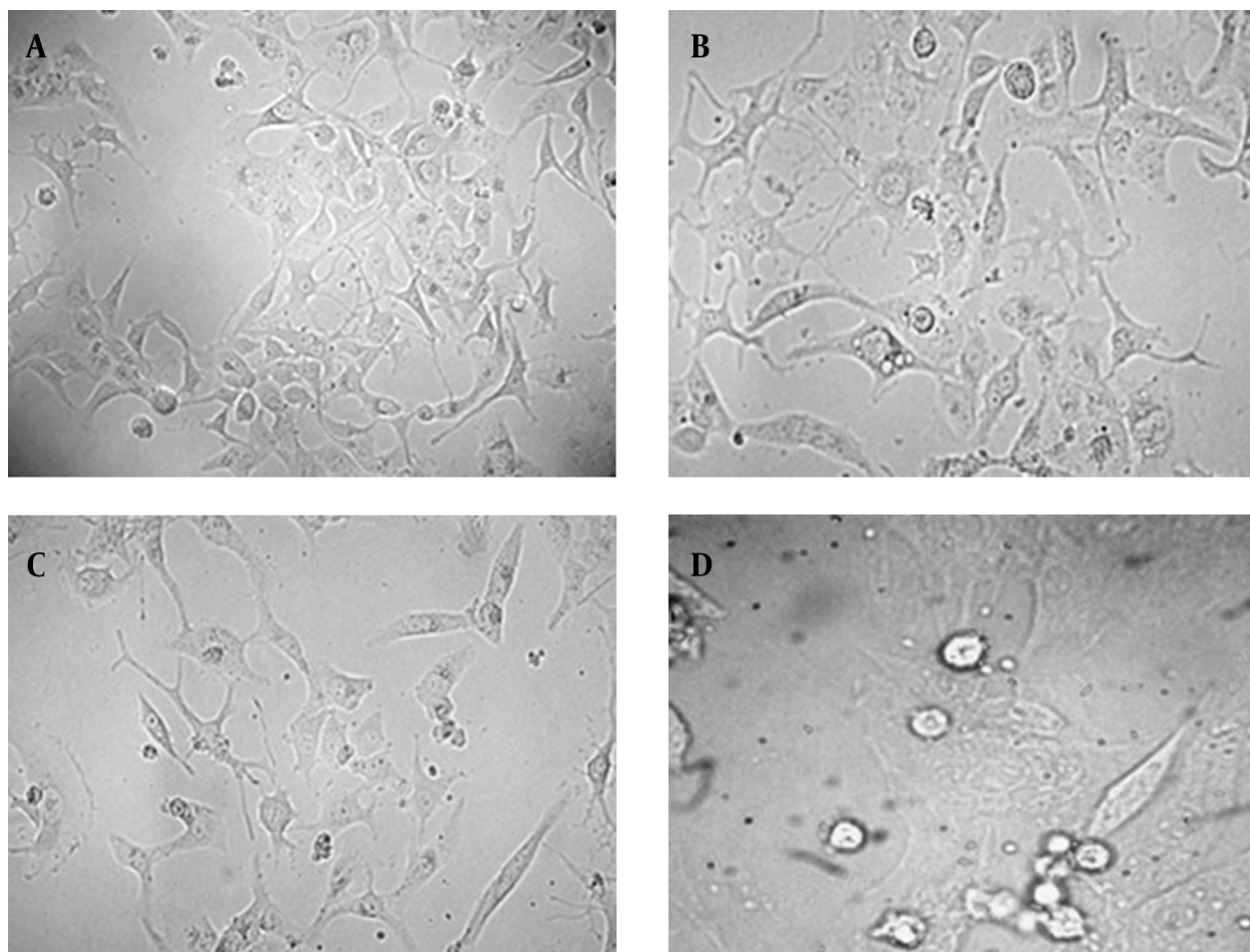
#### 3.5. Statistical Analysis

All data were presented as mean  $\pm$  SD. Statistical significance was determined by ANOVA and Tukey's multiple comparison tests. A P value < 0.05 was considered to be statistically significant.

### 4. Results

The effect of extracts has been assessed over three consequent days. To estimate the minimum time that is exposed to OCA extract, it is required to observe these effects at 72-hour. H9C2 cells were exposed to extracts with 1:4, 1:2 and 1 dilutions. The morphological assay of H9C2 cells was done two times; the first one was in our pilot study for determination of appropriate extract dilution and the second was done during the main study. After 72-hour of co-culture with undiluted extract, cell population and intercellular connections were decreased gently and significantly, respectively to moderate cytoplasmic granulations (Figure 1D). MTT test results and percentage of survival cells at different dilutions in comparison with the control were gathered in Table 1. There was no significant difference in percentage of surviving cells within consecutive days (1st, 2nd and 3rd day) ( $P > 0.05$ ). According to the results, incubation of cells for 24-hour with undiluted OCA extract resulted in 84%viability compared to 77.5% viability for 72-hour exposure. Treatment of cells for 72-hour with extract dilution (1, 1:2 and 1:4) resulted in 77%, 79% and 80% cell viability, respectively. A small change in inhibition was observed for the average of reduction in cell viability from 24-hour to 72-hour (around 7%) at all concentrations (Table 1). The estimated curves and their equations by regression analysis for viability data of H9C2 cells were shown in Figure 2. A good correlation between extract dilutions and cell viability for H9C2 cells was obtained from linear analysis ( $R = 0.873$ ,  $LC50\% = 84.77$ ).

**Figure 1.** Morphological Effect of Different Extract Dilutions on H9C2 Cells



Morphological observation by inverted phase-contrast microscope; (A), H9C2 cells were not treated with extract as a control. The morphological changes of H9C2 cells are shown at 1 concentration (undiluted extract) for 24-hour (B), 48-hour (C) and 72- hour (Orig. mag. X400) (D).

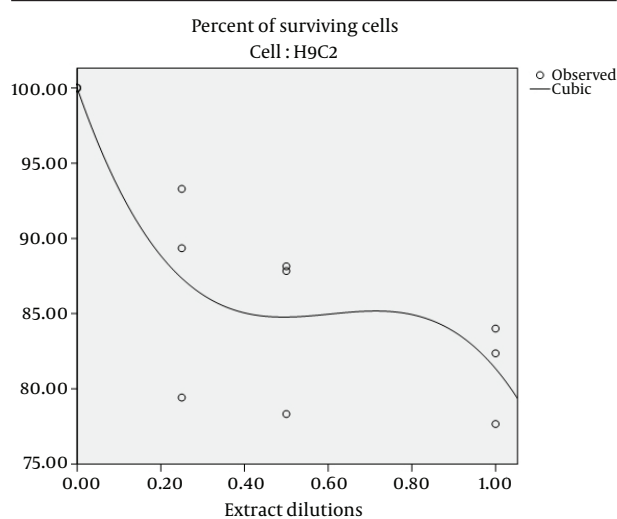
**Table 1.** The Percentage of Viability of H9C2 Cells (Mean  $\pm$  SD) Compared to Control in Different Extract Dilutions<sup>a</sup>

Cell Survival OCA Extract Dilutions	Percentage of Surviving Cells in Different				P Value <sup>b</sup>
	Control	1:4	1:2	1	
<b>First day and last day, 24 and 72</b>	100	86.4 $\pm$ 8.8	83.2 $\pm$ 12.1	80.8 $\pm$ 9.5	.509
<b>24 h</b>	100	93.3 $\pm$ 6.9	88.1 $\pm$ 16.3	84.0 $\pm$ 9.7	.311
<b>48 h</b>	100	89.3 $\pm$ 12.3	87.8 $\pm$ 2.3	82.4 $\pm$ 13.0	.190
<b>72 h</b>	100	80.4 $\pm$ 1.5	79.6 $\pm$ 5.3	77.5 $\pm$ 10.2	.081
<b>Total<sup>c</sup></b>		P value = .828			

<sup>a</sup>Data reported as mean  $\pm$  SD.

<sup>b</sup>analysis of variance.

<sup>c</sup>Repeated measurement analysis of variance.



**Figure 2.** Curve Estimation for Viability Data of H9C2 Cells by Regression Analysis

Cubic equation for H9C2:

$$\text{Viability} = 100.0 - 80.188 \times \text{Con} + 137.373 \times \text{Con}^2 - 75.84 \times \text{Con}^3$$

$$R = 0.873, \text{LC50\%} = 84.77$$

## 5. Discussion

The main concern about OCAs applications was the possible release of substances that may be harmful to the human body. Besides, the viscosity of the OCA glue was very high which made it impossible for performing the direct contact test. Therefore, cytotoxic effect of the released substances from the glue was evaluated using extract dilution test, and toxic extract of the cured sample on H9C2 cardiomyoblast cells was investigated.

One of the most severe problems in the cyanoacrylate surgical use is their toxicity. In this study, we demonstrate that polymerized OCA tissue adhesive is non-cytotoxic to H9C2 cells using a quantitative tetrazolium-based colorimetric MTT assay. The cells in contact with the polymerized OCA were 70% viable during the 72-hour incubation according to the MTT test results which are gathered in Table 1. Regarding the results, it was observed that in spite of decrease in the percentage of viable cells with an increase in the extracts concentration, none of the samples showed complete cytotoxicity for the H9C2 cells. Many studies have investigated the cytotoxicity of CA and its derivations. Various studies which have been conducted in-vivo have shown a good patient satisfaction and time taken to wound healing by applying cyanoacrylate. Use of cyanoacrylate shows more positive effect on the scar maturation in compare with topical silicone gel (11). Also, Cyanoacrylates have a positive effect on pathological scars at least comparable to that of silicone gel (12). Taravella showed a case in which 2-octyl-cyanoacrylate was used to treat a corneal perforation with excellent result (13). Besides, OCA can be a safe and cost-effective

alternative to topical sutures for total abdominal hysterectomy patients (14). In contrast, the OCA adhesives cause inflammation and tissue necrosis in vivo (15). Dissociation between vitro and vivo findings could be a reflection of the fact that difference in cell survival rates was overwhelmed by multiple factors that could play a part in vivo. For example, the monomers or glues may have different effects in terms of inducing cells to express pro-inflammatory or other molecules, as they have been described for other biomaterials (16). Laboratory-based researches show that CAs are toxic on cell cultures, using both direct and extract dilution tests (17). Nevertheless, few studies have been conducted on the toxicity of OCA on H9C2 heart cardiomyoblasts cells.

Therefore, conflicting results are reported about cyanoacrylate cytotoxicity. Several factors influence an extraction process, including experiment method, cell type and extraction medium volume as well as surface area of the test specimen, extraction period, temperature and mode. In this study, we tried to consider this case. Samples were extracted in accordance with ISO 10993-12 (8). OCA has a long monomer in its alkyl chain containing 8-carbons, and among other derivatives has longer alkyl chain and higher molecular weight which makes it less toxic. H9C2 (rat heart cardiomyoblasts) has been used for cytotoxicity evaluation. There are some contributions to use these cells. At first, they are important for mending of cardiac connective tissue cells and highly active metabolic ones (18). The second reason is that they are abundant in connective tissue and adjacent in tissue adhesive in surgical treatment of heart. The third one is in culture techniques, where they are reproducible and have the necessary characteristics.

In the present research, for the first time, the cytotoxicity effect of OCA on cardiomyoblast cells was studied in-vitro using MTT assay. MTT is an accurate, common and sensitive method based on health of cell membranes, sometimes considers the only practical method in these types of experiments. Most of the cytotoxicity determination techniques such as Agra/agar gel are derived in direct contact with culture area. By using material (OCA) in the experiment solidifies in moisture, it doesn't distribute properly and may fail to give accurate results contacting the cells. Only few studies have examined the effects of cyanoacrylate cytotoxicity on the cell. CA has been found to be cytotoxic to cells in vitro both in direct contact (19) and in extract dilution assays on L929 cell culture (20). Despite being toxic to L929 fibroblast cells after polymerization, our findings have shown that the OCA has no cytotoxicity effect on Cardiomyoblast cells. As a result, it can be concluded that CA glues cytotoxicity is totally cell type dependent.

OCA extracts exposing to the H9C2 cells produced low levels of cytotoxicity and there was no considerable difference in cytotoxicity of different concentrations of the extracts. Although the present research is a short-term in-vitro test which cannot be valid for in-vivo tests, it is



important to note that this result shows non-toxicity of OCA to H9C2 cardiomyoblast cells.

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