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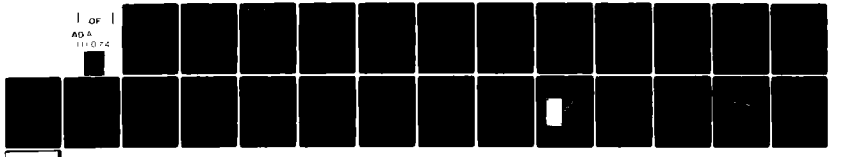

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The study of freeze-thaw damage to endothelial cells necessitated the development of a suitable in vitro system. After investigating various substrates, Leighton tubes containing a plastic coverslip proved to be most versatile for studying alterations to endothelial cells following thaw. The polymethylpentene coverslip was the key to its versatility. It may be easily cut to allow portions of the same monolayer to be processed for examination

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by phase contrast, brightfield and both scanning and transmission electron microscopy. At the same time, the attached handle allows for easy manipulation without disturbing the monolayer. Also, endothelial cells remain better attached to the plastic substrate than to glass coverslips following a freeze-thaw insult. The Leighton tube itself is ideal for temperature studies because it is water tight allowing for submersion in refrigerated water baths, and the media covering the cells may be assayed for cellular enzymes released into the media. In this manner, biochemical markers may be correlated with the same cells used for all forms of microscopy.

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I. Development of a Suitable in Vitro System

Author(s) Lynn R. Trusal, C.J. Baker, A.W. Guzman

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Title: Freeze-Thaw Induced Damage to Endothelial Cells in Vitro

I. Development of a Suitable In Vitro System

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Running Head: In Vitro Freeze-Thaw System

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SUMMARY

The study of freeze-thaw damage to endothelial cells necessitated the development of a suitable in vitro system. After investigating various substrates, Leighton tubes containing a plastic coverslip proved to be the most versatile method for studying alterations to endothelial cells following thaw. The polymethylpentene coverslip was the key to its versatility. It was easily cut which allowed portions of the same monolayer to be processed for examination by phase contrast, brightfield, and both scanning and transmission electron microscopy. At the same time, the attached handle allowed easy manipulation without disturbing the monolayer. Also, following a freeze-thaw insult, endothelial cells remained better attached to the plastic substrate than to glass coverslips. The Leighton tube itself was ideal for temperature studies because it was water tight which allowed submersion in refrigerated water baths. The media covering the cells can be assayed for cellular enzymes released into the media. In this manner, biochemical markers from the same cells may be correlated with cellular structrure.

KEY WORDS: endothelial cells, Leighton tubes, freeze-thaw, polymethylpentene coverslip.

INTRODUCTION

Endothelial cells are exposed to the temperature of the circulating blood because of their strategic location along the vessel wall. In frostbite, endothelial cells suffer a freeze-thaw insult when an extremity freezes. In order to study freeze-thaw damage to endothelial cells, it was necessary to develop a suitable in vitro system for demonstrating cellular alterations caused by freezing.

Towards this aim, we describe the use of Leighton tubes containing a plastic (polymethylpentene) coverslip for growth and examination of bovine endothelial cells following a freeze-thaw injury. Such a system permits cellular examination of the monolayer by phase contrast, brightfield, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In addition, release of cellular enzymes also indicate cellular damage and may be correlated with the ultrastructure of these same cells.

This procedure provides an easy but comprehensive method for processing and studying monolayer cultures following temperature exposure. Ultrastructural evaluations of freeze-thaw damaged cells are not discussed since they are dealt with in great detail in the following paper.

MATERIALS & METHODS

Cell Preparation

Aortas from newly slaughtered calves served as the source of endothelial cells. Aortas were transported from the slaughter house on ice with both ends clamped to maintain sterile conditions. Each aorta was rinsed twice with Earl's balanced salt solution (EBSS)(Ca^{++} and Mg^{++} free, pH 7.4) to remove blood residue. As previously described, endothelial cells were obtained by collagenase perfusion (1 mg/ml, Type 1, Worthington Bioch. Corp., Freehold, NJ) (1). Following incubation for 15 min at 37°C, the collagenase solution containing freed cells was collected and pooled with a rinse solution consisting of EBSS which contained remaining loose endothelial cells released from the luminal surface. After centrifugation at 750 rpm for 5 min, the cells were resuspended in media, counted and seeded at a density of $5\text{-}6 \times 10^5$ cells/ml as follows: in Leighton tubes (Costar, Cambridge, MA) containing a plastic coverslip (9x55 mm), on glass coverslips (11x22 mm), teflon coated glass coverslips (2) or collagen (0.25%) coated glass coverslips (3). Growth media consisted of Medium 199 (M.A. Bioproducts, Walkersville, MD) containing 25 mM HEPES buffer and supplemented with 20% fetal bovine serum, 2mM L-glutamine, 50µg/ml gentamicin and 2.5µg/ml fungizone (310 m Osm/ℓ , pH 7.4). After 2-3 days in a 37°C, 3% CO_2 incubator endothelial cell growth on the coverslips reached confluency.

Only primary cultures were studied to decrease the chance of mycoplasma contamination and improve in vivo comparisons. A direct fluorescent microscopic technique (4) was used to determine the presence of mycoplasma contamination (Bio Assay Systems, Inc., Cambridge, MA).

Identification of Endothelial Cells

Endothelial cells were positively identified by both electron microscopy and immunofluorescence techniques. TEM was used to determine the presence of Weibel-Palade (W-P) bodies which have been shown to be specific for endothelial cells of several species and vessels (8,9).

Primary cultures of bovine endothelial cells were subjected to a modified procedure of Jaffe et al. (5) for the demonstration of Factor VIII antigen. This blood factor has been demonstrated to be produced by and specific for endothelial cells (5,6,11) and is not found in smooth muscle or fibroblast cell cultures (5). Briefly, cultures of bovine endothelial cells were fixed in 100% acetone, rinsed in phosphate buffered saline (PBS), and incubated for 45 min with rabbit antibody to human Factor VIII antigen (Behring Diagnostics, Somerville, N.J.) (6) diluted 1:10 in PBS. This was followed by four PBS rinses for 15 min each and 45 min staining by flourescein-isothiocyanate conjugated IgG goat anti-rabbit globulin (FITC-GARG) (Cappel Laboratories, Cochranville, PA) diluted 1:10 with PBS. After four rinses in PBS for 15 min each, the coversiips were mounted on glass slides and examined by reflected halogen fluorescence. Photographs were taken using Kodak Tri-X film. Control cultures were treated identically except PBS or normal bovine sera was substituted for rabbit antisera during the initial incubation, prior to FITC-GARG staining.

Freeze-Thaw Procedure

For freeze-thaw studies, experimental tubes were submerged in a 95% ethanol refrigerated bath (Neslab, Portsmouth, NH). The tubes were water tight and plastic coverslips did not require transfer to new containers for freezing. All types of glass substrates required transfer to 1 oz glass prescription bottles for freezing. The rate of freezing was approximately $1^{o}C$/min and was monitored with a thermocouple permanently mounted in a sample tube contain-

ing the same amount of media (1 ml) that covered the cells. The media froze between −10 to −13°C, and was lowered to -15 or -20°C, before thaw in a 37°C water bath. Control tubes were maintained at 37°C until processed with experimentals. The rate of freezing did not exceed 1°C/min since clinical frostbite usually occurs at slow cooling rates. Likewise, although the thawing rate of 20°C/min is not considered rapid for optimal cell survival in vitro, it is faster than the rate obtained by the recommended method for thawing of clinical frostbite cases (7).

Processing for Light Microscopy

Following thaw, the glass coverslips or portions of the plastic coverslip used for light microscopy were rinsed briefly in PBS (Ca^{++} and Mg^{++} free, pH 7.4) at 22°C to remove excess media and fixed in Carnoy's (1 part glacial acetic acid:3 parts absolute ETOH:6 parts chloroform) prior to staining with 1% Toluidine Blue O staining. Pieces of coverslip used for phase contrast or brightfield examination were mounted cell side down on glass coverslips (25x 60mm) for viewing and photography.

SEM & TEM Processing

Those portions of plastic coverslip used for SEM and TEM were processed as previously described (10). Briefly, coverslips were rinsed, fixed in 2.5% buffered glutaraldehyde, postfixed (1% buffered osmium tetroxide) and dehydrated in ethanol. Pieces of coverslips for SEM examination were critical point dried, mounted on stubs, and sputter coated with gold-palladium. Those pieces of coverslips for TEM were embedded in Epon-Araldite. After polymerization for 3 days (60°C), ultrathin sections were cut and stained with uranyl acetate and lead citrate for viewing.

Enzyme Determinations

Immediately after thaw, media (1ml) was removed from the Leighton tube by syringe, filtered through a Millipore filter (Type GS) to remove cellular debris and refrigerated. Lactic dehydrogenase (LDH) was chosen as a general cytoplasmic enzyme marker and determinations were performed as soon as all samples were collected. Measurements were made using the Worthington/Gilford LDH (P–L) kit (Worthington Bioch. Corp., Freehold, NJ) according to package insert SM 527020 and the Gilford Automated System, Model 3402 (Gilford Instru. Lab., Inc., Oberlin, OH). The six printed values were averaged for a reportable LDH activity in U/ℓ. Tubes with 1 ml of media served as background controls for each temperature.

RESULTS

Endothelial Cell Testing and Identification

Direct fluorescent examination of stained cells revealed no pinpoint fluorescence characteristic of mycoplasma contamination. Use of only primary cultures probably decreased the chance of such contamination.

Figure 1 illustrates positive immunofluorescence of Factor VIII antigen in bovine endothelial cell cultures. Control cultures exhibited only background fluorescence.

Figure 2 demonstrates the presence of W-P bodies in a bovine endothelial cell. Therfore, both methods have positively identified the cultured cells as endothelial in origin.

Choice of Substrate

Since endothelial cell cultures were subjected to freeze-thaw conditions, the ability of the substrate to permit normal monolayer growth and retain the cells following thaw was very important. All substrates except the teflon coated coverslips yielded good monolayer growth, with polygonal endothelial cells growing in close apposition. Teflon was found unsuitable because cells would not grow on the teflon droplets thereby interrupting the monolayer. All glass substrates proved unsuitable for the freeze-thaw conditions since 90 to 95% of the cells detached after thaw, with a few cells remaining around the periphery of the coverslip. Plastic coverslips retained 50-90% of the monolayer following thaw. Occasionally the monolayer had a tendency to peel off in certain areas, but gentle handling minimized this occurrance. Figure 3 illustrates the subjective evaluation of the various substrates with regard to growth characteristics, integrity of the monolayer following thaw, differential staining and ease of processing for microscopy.

Light Microscopy

Bovine endothelial cells grown on plastic polymethylpentene substrates may be stained with a variety of histological stains or examined by phase contrast. Figure 4 illustrates a phase contrast photomicrograph of control cultures (37°C) while Figure 5 is a photomicrograph of control cells (37°C) stained with Toluidine Blue O. Dead cells remaining after seeding are visible as refractive bodies (arrow) in phase contrast or pyknotic bodies (arrows) in brightfield.

All glass coverslips presented no problems for viewing by either phase contrast or brightfield. Plastic coverslips presented no problems for viewing by either phase contrast or brightfield when mounted on large glass coverslips using PBS and inverted for viewing. Typical permanent mounting on glass slides hindered examination at higher magnifications unless the plastic coverslips were mounted on slides cell side up and, in turn, covered with a glass coverslip. This may be sealed with clear fingernail polish allowing the cells to be viewed through only the thickness of the glass coverslip.

SEM

Scanning electron microscopy was possible with cells on either glass or plastic substrates, but plastic ones were more versatile because they could easily be cut into appropriate sizes for mounting on aluminum stubs and they retained the monolayer better following thaw. Figure 6 illustrates a typical scanning electron micrograph of bovine endothelial cells maintained at 37° (control). Nuclei rise slightly from the centers of the cells.

TEM

The important test for the substrates was their ability to be processed for TEM. Glass coverslips proved very poor in this regard because of difficulty in separating Epon-Araldite from the glass following polymerization. Teflon coated

coverslips separated well but were unsuitable in cell growth characteristics. Glass coverslips and glass coated with collagen tended to shatter, splinter or cleave into layers upon separation using liquid nitrogen immersion. Plastic coverslips embedded for either parallel or perpendicular sectioning always allowed quick and easy separation of the coverslip from the embedding resin (10).

Figure 7 is a transmission electron micrograph of a bovine endothelial cell maintained at 37°C. Cellular integrity was intact including nuclear and mitochondrial membranes. The cytoplasm contained thin filaments (F), mitochondria (M) and numerous pinocytotic vessicles (V) near the plasma membrane.

Enzyme Data

LDH was assayed for its presence in the media following thaw in experimental cultures and after incubation at 37°C for controls. Release of LDH did not occur appreciably until after freezing at -10 or below. The small number of cells did not present quantitation problems and as few as 5×10^5 cells released 100-250 U/ml following a freeze-thaw injury. Data from a sample experiment is shown in Fig. 8.

DISCUSSION

Any in vitro cell culture system designed to study cellular injury must be versatile enough to allow processing and subsequent examination by the major forms of microscopy. It was also desired to use other parameters, such as the release of enzymes, to evaluate cellular damage. We have found the culture tube containing a polymethylpentene plastic coverslip to be a system suitable for studying freeze-thaw damage to bovine endothelial cells.

The Leighton tube was particularly suited for temperature studies which required submersion of the cell culture in a refrigerated water bath. It was self-contained, water tight and did not require transfer of the coverslip to another container for freezing. It was our experience that individual glass coverslips (11x22mm) proved difficult to manage in this regard because they required transferring to glass prescription bottles for freezing. The coverslips were often broken and the monolayer damaged because of excessive handling. This problem was alleviated in the Leighton tube by the presence of a coverslip handle.

Temperature equilibration between the water bath and the media did not prove a problem. By means of a thermocouple, it was possible to demonstrate the temperature of the media lagged only $1^{\circ}C$ behind that of the external water bath. Therefore, there was a short latent period for temperature equilibration between the water bath and the media covering the cells.

It was also found that endothelial cells remained better attached to plastic than to glass following a freeze-thaw insult. Prior use of glass coverslips demonstrated that most of the cells detached following thaw, while plastic coverslips retained the majority of the monolayer following thaw.

Another advantage of the large (9x55mm) plastic coverslips was that they could be cut prior to processing for microscopy. The first portion of coverslip was cut off and mounted cell side down on a large glass coverslip for phase

contrast examination. Other pieces were excised and fixed for staining by different histological stains such as Toluidine Blue O, Hemotoxylin and Eosin, and Acridine Orange. The remaining piece of coverslip with attached handle was then fixed with glutaraldehyde and post fixed in osmium tetroxide in preparation for SEM and TEM examination. Following dehydration, the portion for SEM examination was excised and critical point dried; while the last portion of the coverslip was embedded in resin prior to TEM examination, in either cross section or "en face" (9). Thus, portions of the same monolayer were processed separately and examined by four forms of microscopy.

In addition to using the in vitro system for examining freeze-thaw alterations in endothelial cells on both the light and electron microscopy level, we were interested in evaluating the versatility of the culture tube as a means of studying cellular damage via release of a cellular enzyme. The enzyme chosen was the cytoplasmic enzyme LDH. Since a freeze-thaw insult was known to cause membrane damage, release of LDH helped in assessing membrane integrity following thaw. In spite of the small area ($5cm^2$) for monolayer growth, 1.0 ml of media needed to cover the cells probably facilitated detection of enzymes released into the media.

In order to later improve in vivo comparisons and decrease the chance of overgrowth by smooth muscle and bipolar cells, we chose to use only primary cultures of endothelial cells. The use of such primaries sometimes presented the problem of obtaining enough cells for processing by several procedures. Many of the techniques using plastic substrates utilized large flasks for cell culturing. This method, although satisfactory, required a greater number of cells and a longer growth period to achieve confluency. Cultures containing as few as 5×10^5 cells yielded a confluent monolayer in two days with the coverslip large enough for SEM, TEM and light microscopic examination.

In conclusion, the Leighton tube with a polymethylpentene coverslip was found very versatile for studying the effects of various temperatures on bovine endothelial cells. Its advantages are summarized as follows. The tube is self-contained and water tight allowing submersion for temperature studies. Release of cellular enzymes may be correlated with the same cells used for microscopy examination, and the large plastic coverslip allows all forms of microscopy to be done on the same monolayer. The plastic coverslip is easily cut while the coverslip handle allows processing without disturbing the monolayer. Finally, the cell monolayer remains better attached to the plastic coverslip following a freeze-thaw injury, and plastic coverslips permit easy resin embedding and separation for electron microscopy.

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DISCLAIMER STATEMENT

The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other official documentation.

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Figure 1. Photomicrograph of immunofluorescence to Factor VIII antigen demonstrated in bovine endothelial cells. Positive fluorescence to this factor identifies the cells as endothelial. x 8,215.

Figure 2. Transmission electron micrograph of bovine endothelial cell containing Weibel-Palade bodies (arrows), which also positively identify the cells as endothelial in origin. x 45,730.

Figure 3. Chart illustrating a subjective evaluation of four different substrates used for growth and processing of bovine endothelial cells.

Figure 4. Phase contrast photomicrograph of a confluent monolayer of bovine endothelial cells growing on a plastic substrate. Note integrity of intact monolayer. x 150.

Figure 5. Light micrograph of a control (37°C) culture of bovine endothelial cells stained with Toluidine Blue O. Round pyknotic cells (arrows) are dead cells not removed with rinses following seeding. x 205.

Figure 6. Scanning electron micrograph of control (37°C) bovine endothelial cells growing on a plastic substrate. Nuclei can be seen to bulge from the center of the cells. x 2,965.

Figure 7. Transmission electron micrograph of a control (37°C) bovine endothelial cell. Nuclear and mitochondrial membranes appear intact. Pinocytotic vesicles (V), 70-100 Å filaments (F) and mitochondria (M) are evident in the cytoplasm. x 19,000.

Figure 8. Graph illustrating release of LDH from bovine endothelial cells following exposure to different temperatures (^{o}C). Enzyme is measured in U/ℓ.


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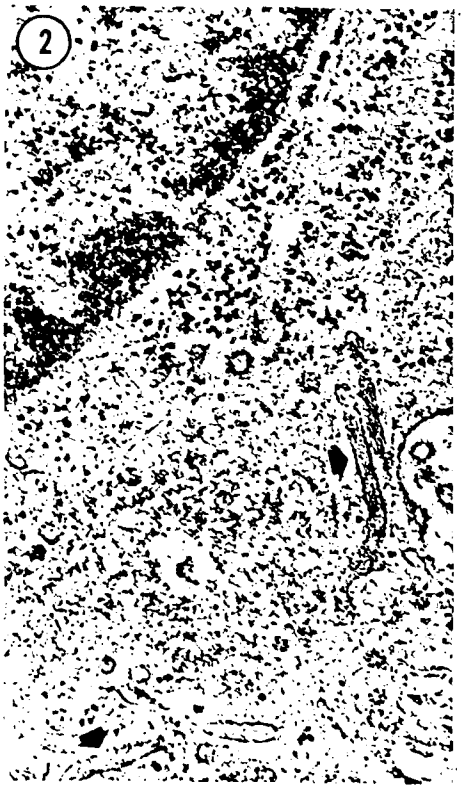
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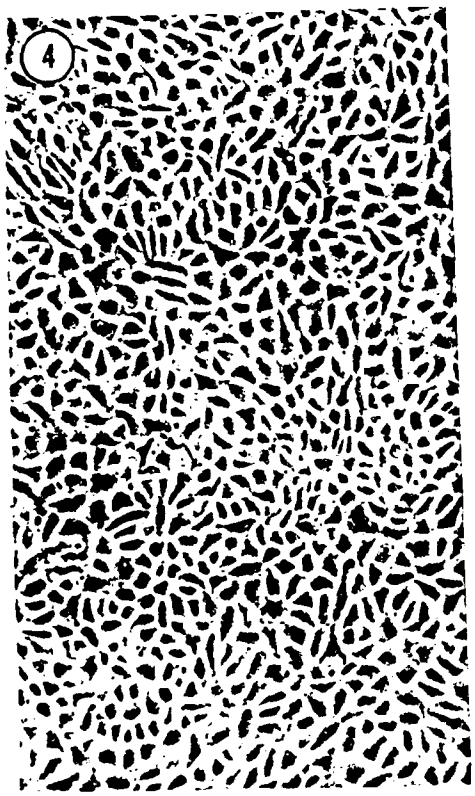
EVALUATION OF CELL SUBSTRATES

SUBSTRATE	CELL MONOLAYER			MICROSCOPY		
	GROWTH	INTEGRITY	STAINING	LIGHT	SEM	TEM
GLASS COVERSLIPS	EXCELLENT	POOR	EXCELLENT	EXCELLENT	EXCELLENT	POOR
GLASS COVERSLIPS (TEFLON COATED)	POOR	POOR	EXCELLENT	EXCELLENT	—	GOOD
GLASS COVERSLIPS (COLLAGEN COATED)	EXCELLENT	POOR	EXCELLENT	EXCELLENT	—	POOR
PLASTIC COVERLSIPS (COSTAR LEIGHTON TUBE)	EXCELLENT	GOOD	EXCELLENT	GOOD	EXCELLENT	EXCELLENT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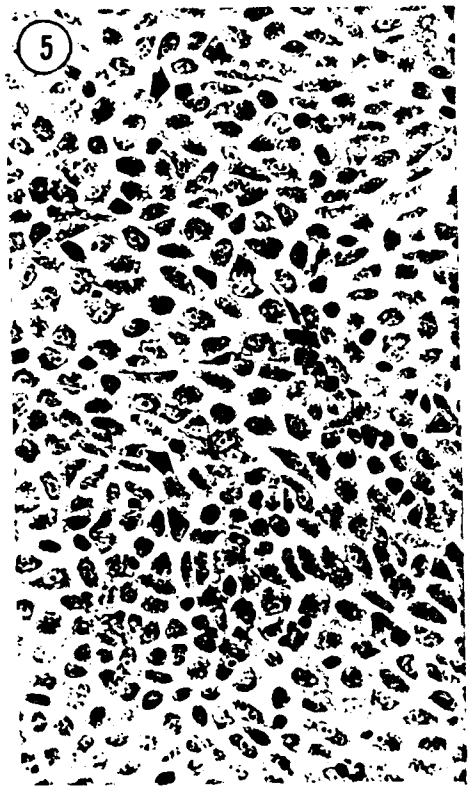
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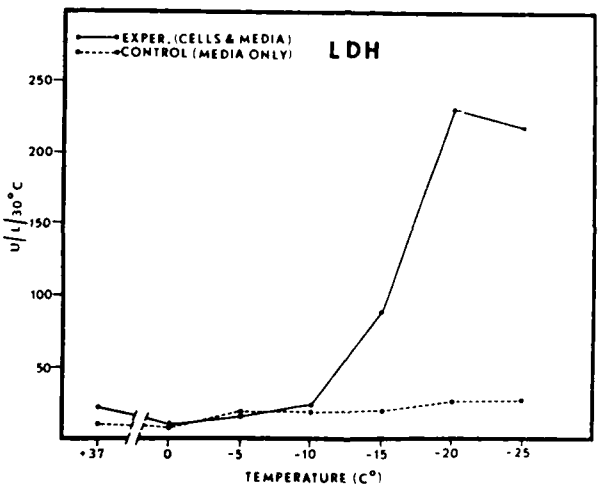
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