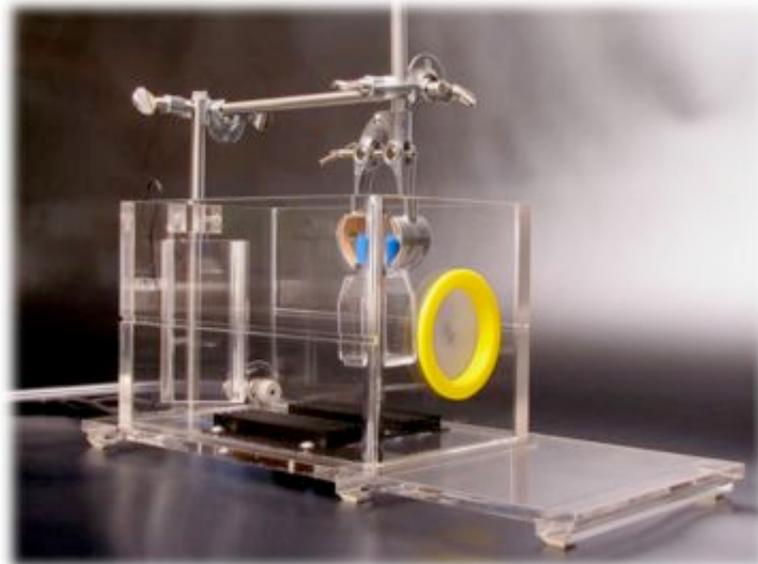

MANUAL

IVSWT Water Bath V2.0

Standardized Model for In-Vitro Shock Wave Treatment (IVSWT)



Introduction

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Innsbruck Medical University, Austria, October 2010

The number of in-vitro experiments in shock wave science increases continuously. This fact reflects how important basic research findings on the cellular and sub-cellular level are for the future progress of shock wave therapy. In some very emerging fields better mechanistic understandings may be prerequisite for translation into clinical use or will at least support the application of already well established indications.

Today's knowledge of shock wave effects on cell cultures includes the increase of proliferation, alteration of cell membrane receptors, increase and acceleration of cell differentiation, release of several kinds of growth factors and chemo-attractants as well as increased cell migration.

Besides reduction of animal experiments and cost-effectiveness, the biggest advantage of In-Vitro Shock Wave Treatment (IVSWT) may be the possibility of studying the specific behaviour of a certain cell type. In shock wave mediated tissue regeneration most likely all cells of the treated tissue are involved, even systemic effects are discussed. Nevertheless, each cell type plays a specific role and has its own intrinsic function. IVSWT enables us to detect this particular functions and may thereby give us better understanding of the complex underlying processes.

Some more applications of IVSWT are discussed. For example it may in future be used to enhance cell proliferation for (stem-) cell treatment purposes.

Distracting physical effects in most in-vitro models

Literature reveals various methods of applying shock waves onto cell cultures. In general all existing models are focused on how to apply shock waves onto cells. For example it is done by direct coupling to a sample with ultrasound transmission gel, coupling through water tanks or even through pork skin.

This fact leads to the problem that it is highly difficult to compare results as physical conditions of cell stimulation are quite different between the models. As no standardized model for in vitro trials exists all the different models have their respective advantage but are also associated with some distracting physical effects.

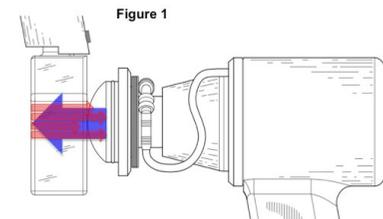


Figure 1

However, the question is not how to apply shock waves onto cells. This can easily be done by all the mentioned methods.

But: What happens to the waves after passing a cell culture? The main problem is that the difference of the acoustic impedance of the cell culture medium and the ambient air is that high that more than 99% of the shock waves get reflected (**Figure 1**). Due to the difference in acoustic impedance of the two media the waves are not only reflected but also a phase-shift of 180° occurs resulting in strong tensile forces to the cells (**Figure 2A-D**).

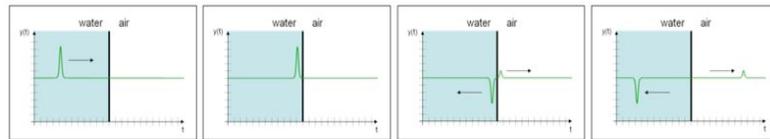


Figure 2A-D: Phase-shift of waves at water to air passage.

The acoustic impedance is defined as the product of the density of a material and its sound velocity $Z = \rho \times c$. For water the acoustic impedance is $Z_{\text{Water}} = 1'440'000 \text{ Ns/m}^3$, for air it's only 420 Ns/m^3 . The large difference of these two values results in reflection and phase shift of shock waves. The phase shift turns a positive pressure pulse into a tensile wave.

Even if this tensile force is not harmful to the cells it interferes with the idea of mimicking in vivo shock wave effects in vitro. In vivo these tensile forces do not occur due to the large body structures.

If the water to air transit is very close to the cells the back running waves can disturb the incoming ones. This may cause interference. Two types of interference are known. Constructive interference means that both waves are added thereby resulting in a doubled amplitude (**Figure 3A**). Destructive interference occurs if waves meet just oppositely. It causes abolishment of waves. (**Figure 3B**).

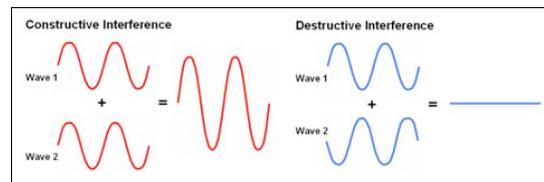


Figure 3A+3B: Interference between back running and incoming waves may occur.

Therefore IVSWT needs a model that enables shock waves to propagate after passing the cell culture. This can be done by putting cells into a water bath.

The prerequisites for in vitro experiments can be defined as:

- Probes e.g. cell cultures can be placed in the center of the shock wave path at the region of highest pressure of the shock waves as well as in front or behind this area.
- Vials or other cell culture containers have to consist of material with an acoustic impedance comparable to water. Most plastics fulfill these requirements while glass or metal do not.
- Shock waves have to be effectively coupled to the probes without any air gap or air bubbles in the shock wave path.
- No water to air transitions close to the probes shall occur to avoid reflections and large tensile forces.

Suitable materials for vials from an acoustic point of view are PE ($Z=1'760'000 \text{ Ns/m}^3$), soft-rubber ($Z=1'270'000 \text{ Ns/m}^3$), polyamid ($Z=1'960'000 \text{ Ns/m}^3$). Less ideal is PVC ($3'270'000 \text{ Ns/m}^3$) plexiglas ($Z=3'260'000 \text{ Ns/m}^3$) delrin ($3'450'000 \text{ Ns/m}^3$) polycarbonate ($2'770'000 \text{ Ns/m}^3$) or polypropylene ($2'400'000 \text{ Ns/m}^3$). Hard materials such as glass or metal shall not be used.

IVSWT water bath

Because of the above mentioned concerns we asked an engineer to build a water bath avoiding these problems. Basically this in vitro water bath consists of a plexiglas built container with a membrane to connect every kind of shock wave applicator. For coupling between this membrane and the applicator ultrasound transmission gel has to be used. The water bath is filled with degassed water to avoid cavitation that would occur if gas is soluted in the water. A heater at the bottom with a temperature sensor connected to a control unit enables to regulate temperature for imitation of in vivo conditions and to avoid cell cultures to cool down. Temperature can be held stable at 37 degrees centigrade as it is done in an incubator. A holder for the cell samples allows putting in any kind of culture flasks or tubes. Of course samples need to be completely filled with culture medium as air bubbles would block shock waves! A wedge shaped absorber at the back wall of the bath destructs waves in order not to get reflected and run back. This is to avoid reflection and its above mentioned distracting effects.

A further advantage compared to other IVSWT models is the possibility of varying the distance between the applicator and the culture flasks. Findings of our group and others who use this model clearly show that every cell type reacts very specifically to different treatment parameters. Moreover, defining the distance between the waves source and the sample is crucial as it enables us to control the cells to be at a specific position in relation to the focus of the applicator.

Besides our lab several other research groups already use this model and some of them presented their first results at the ISMST Basic Research Meeting in January 2010. The feedback was quite positive. Therefore this model seems to be a proper tool for IVSWT avoiding most of physical limitations in the experiments.

Conducting first experiments

According to our experience with cardiomyocytes, endothelial cells, fibroblasts and stem cells every cell type needs its specific treatment parameters. We therefore strongly recommend to perform a pilot trial for assessment of the adequate treatment parameters before conducting your planned experiments. This pilot trial needs to include different distances between the shock wave applicator and the sample as well as different energy flux densities. Also the most appropriate number of pulses should be established. The frequency of applying shock waves is still a matter of concern even in vivo. Hopefully, current investigation will give us more information about this topic.

Please note: To define the distance between your shock wave source and your sample can be very tricky. To work accurately you need to know the exact position of the source (e.g. in electro-hydraulic systems this means the tips of the electrode).

Moreover, you will need to find a position for your applicator that does not vary this distance every time by different indentation of the membrane!

References:

1. J. Holfeld: IVSWT - In Vitro Shock Wave Treatment. ISMST Newsletter 2010, No. 1 (available at www.ismst.com)
2. J. Holfeld J: Rationale for In Vitro Shock Wave Trails. 1st ISMST Basic Research Meeting, Vienna, Austria 2010

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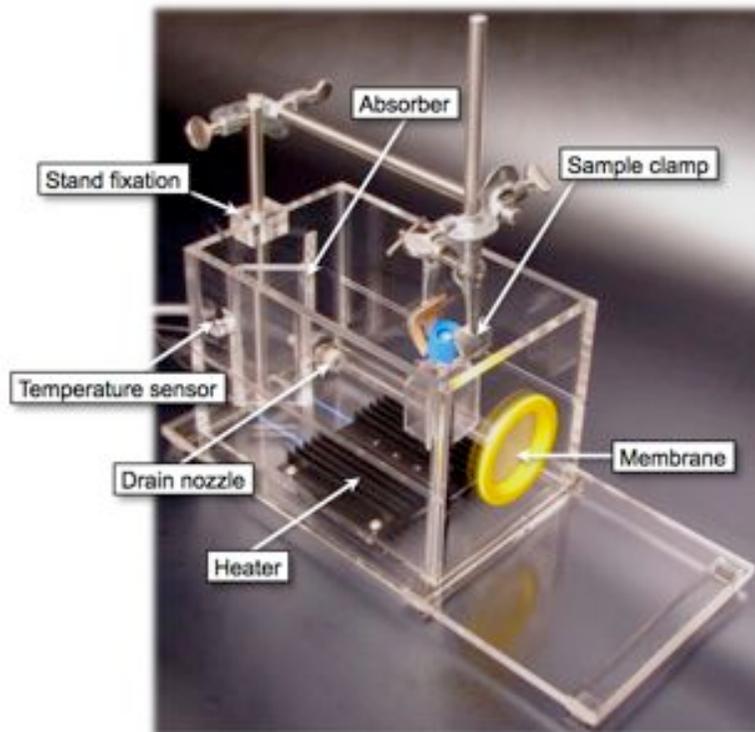
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Manual IVSWT Water Bath V2.0

The purpose of this model is to achieve standardization in IVSWT with respect to:

- direct sonic pulse and reflected parts of the shock wave,
- variable focal distance,
- temperature, and
- reproducibility.

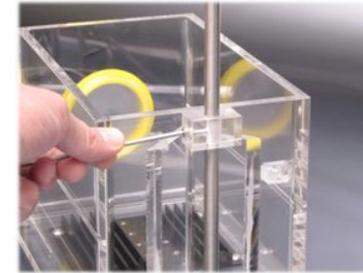
1.1. Overview:



Picture 1

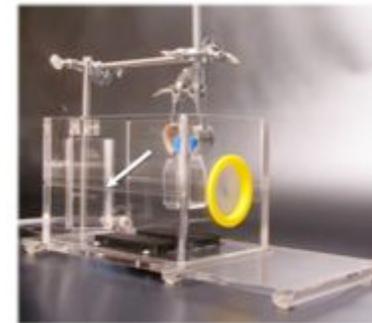
1.2. Sample holder

Insert the rod like shown in picture 1 into the fitting behind the absorber. Mount the stand by using the supplied screw and hex key for tightening.

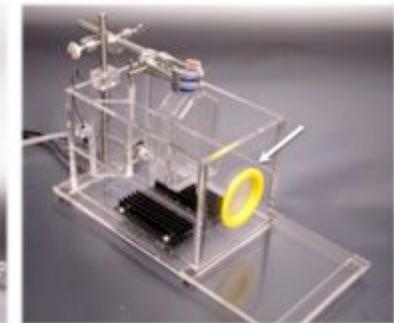


Picture 2

There are two ways of using the holder and talon clamps depending on the size of your sample container and whether it is necessary to adjust it vertically (see pictures 3A and 3B). The goal is to position the sample at the level of the leading line inset on both sides of the water bath (see white arrows in the pictures below!).



Picture 3A

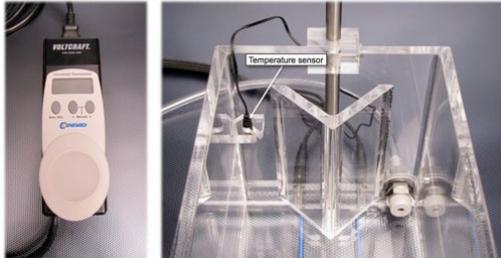


Picture 3B

The sample container needs to be completely filled with culture medium as air bubbles would block shock waves!

1.3. Temperature control

Picture 4A shows the UT100 temperature control unit. Position its temperature sensor into the holder at the left back corner of the water bath as shown in picture 4B.



Picture 4A

Picture 4B

In its original condition, the UT100 temperature control unit is programmed to establish and maintain a water temperature between 36.5 and 37° C. Please refer to the UT100 manual if any other specific temperature is required. The operating manual for the UT100 can be downloaded as a PDF form from the manufacturer's website:

<http://www.produktinfo.conrad.com/>

1.4. Water

Connect the heater as shown in picture 5; the electricity connectors may be arranged as desired.



Picture 5

Fill in approximately 3,200 ml of degassed tap water into the bath. Use degassed water only to avoid cavitation! The water should reach the neck of your cell culture container.

It is recommended to use pre-heated water as heating within the bath is very time consuming. If it is not possible to use pre-heated water, the heating time required to reach the preset temperature can be calculated as follows:

Example:

Desired temperature:	37°C
- filling temperature:	12°C
= difference:	25°C

heating time: 25 x 3 = 75 minutes

In other words, the heater raises the water temperature by about 1° C every 3 minutes until the preset temperature is reached.

1.5. Coupling the shock wave applicator



Picture 6

The leading lines inset on both sides of the water bath help you to see the level of your waves source. It will be necessary to position your cell culture container on the same level. When holding your shock wave applicator onto the membrane you need to use ultrasound transmission gel for coupling! Moreover, it is crucial to find a position that does not vary the distance between the shock waves source and your cell culture container by different indentation of the membrane.

1.6. Dismantling the test equipment

Emptying

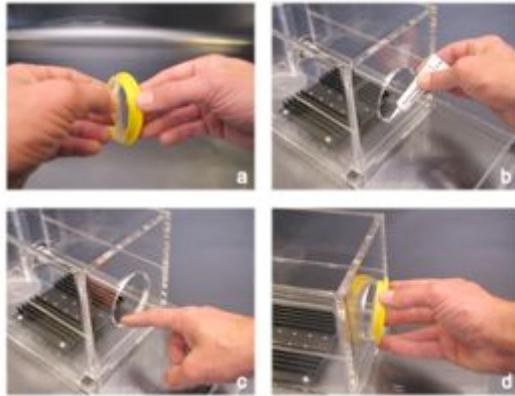
Use the 125 cm drain hose with the snap coupling to empty the water bath. Place the free end of the hose into a container placed on the floor underneath the test equipment.

WARNING: Draining starts immediately when the snap coupling is inserted into the nozzle on the back of the water bath.

Care & cleaning

Only use soft cloth to dry the water bath! After every use, wipe all surfaces using the cleaning and preserving liquid with the microfiber cloth provided.

1.7. Membrane change



Picture 7A-D

For changing the membrane take the yellow holding ring out of the water bath. Pull off the old membrane as shown in picture 7A and replace it by the new one. Then grease the circular opening of the water bath with any kind of commercial lubricant (e.g. Vaseline). Now insert the yellow holding ring with your new membrane.

1.8. Scope of delivery

- 1 IVSWT water bath V2.0
- 1 Temperature control unit UT100
- 1 Power cable
- 1 Membrane holding ring (yellow)
- 2 Coupling membranes
- 1 10mm rod and screw for fixation
- 1 3mm hex key
- 2 Talon clamps
- 1 4-Finger clamp
- 1 Niro-Achse 10 mm
- 1 Drain hose 125 cm
- 1 Manual IVSWT water bath V2.0
- 1 Manual temperature control unit UT100
- 1 Manual electricity transformer
- 1 Cleaning fluid
- 1 Microfiber cloth

1.10. Supplier

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