ELECTROMAGNETIC RE-WARMING OF CRYOPRESERVED TISSUES

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ABSTRACT

Tissues can be preserved at very low temperatures by adding a cryoprotective agent (CPA), but these are toxic at high concentrations. Rapid electromagnetic heating allows lower concentrations of CPA to be used while still avoiding the danger of ice crystals forming during re-warming. We have investigated the suitability of four CPAs, measuring the rapidity and uniformity of heating in a 36mm tissue-equivalent sphere placed in a 430MHz applicator, and the toxicity. The uniformity of heating correlates with a stability factor that depends on the dielectric properties of the material. Uniform heating is also practicable in non-spherical samples provided they are approximately ellipsoidal.

INTRODUCTION

The ability to cryopreserve tissues, i.e. to store them at cryogenic temperatures, would have many benefits for transplantation surgery. Many individual types of cell can be successfully cryopreserved, but it has proved difficult to extend the techniques to larger volumes of tissue or even organs [1]. One reason for this is thought to be the formation of extra-cellular ice crystals, which damage the structure of tissues [2]. A possible solution has been proposed, which is to add a cryoprotective agent (CPA) in sufficient quantities that on cooling the aqueous component of the tissue becomes a glass rather than crystallising [3]. A problem with this approach is that the concentrations of presently used CPAs needed to achieve vitrification have toxic effects on the cells. At lower, less toxic concentrations, there is a greater likelihood of the formation of damaging ice crystals during the re-warming process, a process known as devitrification [4].

It is known that devitrification is less likely to happen at higher re-warming rates. The ability to heat tissues rapidly would therefore enable lower, less toxic concentrations of CPA to be used. However rapid heating by conduction (e.g. by placing the tissue in a water bath) is not feasible for tissue samples greater than a few millimetres in size because the thermal diffusivity is too low, and the inadequacy of such methods increases with the size of the sample. This means that electromagnetic heating methods are necessary. Initial attempts at this were made using conventional microwave ovens operating at 2450MHz, but early reports of success were not found to be repeatable in later studies [5]. Subsequent work showed the optimum frequency range for uniform electromagnetic absorption to be 300-1000MHz [6], and this lead Evans's group to design a resonant applicator operating at approximately 430MHz. This equipment was modified and refined by Robinson and Pegg [7] and was used for the experiments described here.

RF APPLICATOR

The electromagnetic applicator (Fig. 1) consists of a cylindrical cavity, designed to have three resonant modes all close to 430MHz but separated by a few megahertz to prevent cross-coupling. Each mode is excited independently by a synthesiser, a pulse-modulator and a 500W RF power amplifier to provide a strong electric field of rapidly changing polarisation at the centre of the cavity. Power is monitored by directional couplers and the input frequencies are continually adjusted to track the three cavity resonances. The average power is controlled by varying the duty cycle of the pulse-modulation.

We have previously demonstrated that rapid, uniform heating can be achieved in this cavity, when the sample is a 36mm diameter gelatin sphere incorporating a typical perfusate composition and various concentrations of the CPA dimethyl sulphoxide. We now present the results of a systematic comparison of the merits of four commonly used CPAs: dimethyl sulphoxide, ethanediol (ethylene glycol), propanediol (propylene glycol) and butane-1,2-diol. These were assessed for their physical properties – the rapidity and uniformity of heating – and for their toxicity to cells.

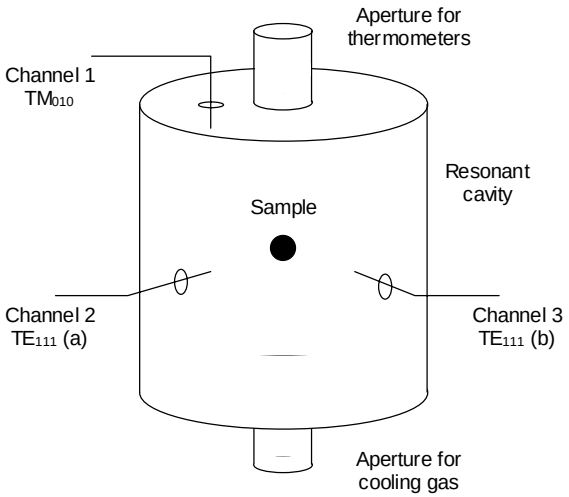

Fig. 1: RF applicator: the height and diameter of the cavity are both 560mm

HEATING RATES

Measurements were performed on each of the CPAs at various concentrations in Dulbecco's phosphate buffered saline. The perfusates were solidified with 6% gelatin and formed into 36mm diameter spheres, which were placed at the centre of the cavity in a holder made from low-loss plastic, and cooled to –60°C by passing cold gas up through the cavity. A fibre-optic thermometry system was used to monitor the temperature at up to eight points in the sphere without disturbing the electromagnetic fields.

Heating rates observed at 30% CPA concentration are given in Table 1, and show that all four CPAs enable suitably rapid re-warming. At 45% concentration of propanediol the heating rate was higher at 102.6±8.2 K/min, owing to the change in dielectric properties and the lower specific heat capacity. The RF power was modulated at a 20% duty cycle, implying that heating rates five times higher should be practicable with our system. Fig. 2 shows a typical result.

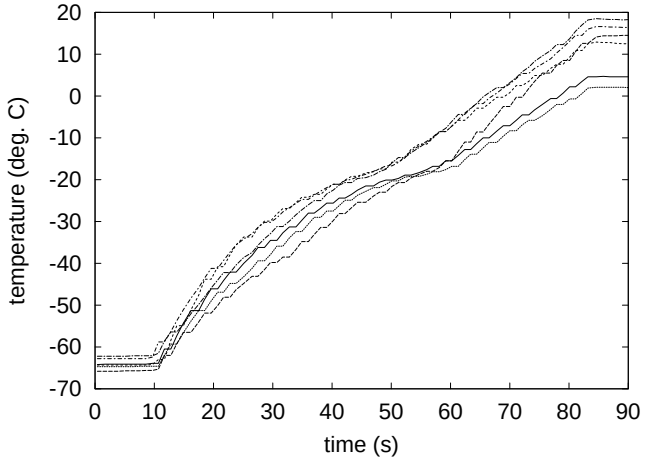

Fig. 2: typical variation of temperature during heating of propanediol (30% concentration): the lines represent the temperature at seven points within the sphere.

cryoprotectant	rate at -35°C (K/min)	final maximum difference at +5°C (K)
propanediol	64.7 ± 7.5 (42)	12.4 ± 4.1 (7)
butanediol	79.0 ± 32.6 (21)	33.1 ± 6.1 (5)
ethanediol	57.8 ± 3.4 (21)	16.1 ± 2.8 (5)
dimethyl sulphoxide	66.2 ± 6.0 (28)	16.8 ± 1.6 (5)

Table 1: rapidity and uniformity of heating in 36mm diameter spheres. Values are mean ± standard deviation (n).

UNIFORMITY OF HEATING

Table 1 shows that there were great differences in the uniformity of heating, defined as the final temperature difference between the hottest and coldest fibre probes when at least one of the probes had registered +5°C. As a difference of over 20K may lead to parts of the sample overheating before the rest has sufficiently re-warmed, this casts doubts on the suitability of butanediol. This is unfortunate as the CPAs that performed best in the toxicity tests (see below) were propanediol and butanediol. Increasing the concentration of CPA was found to improve the uniformity of heating in propanediol but make it worse in butanediol.

The possibility of uniform heating in the cavity is known to depend on the size and shape of the sample and on its dielectric properties: its permittivity ε and conductivity σ [8, 9]. For a spherical sample, one criterion is that the circumference should be less than the wavelength of the applied radiation in the sample: this was fulfilled in all the CPA solutions studied. A second criterion is that certain stability factors, which are functions of the permittivity and conductivity, should have a negative gradient with respect to temperature. A positive gradient will lead to electromagnetic absorption occurring preferentially in the warmer regions of the sample, an effect known as thermal runaway.

For an isolated region at higher temperature than its surroundings the relevant stability factor is K_s, defined at a frequency f by

$$K_s = \sigma \Big/ \left[\sigma^2 + (2\pi f \varepsilon')^2\right]^{1/3} \tag{1}$$

To assess the importance of this stability factor we measured the dielectric properties of perfusates at 430MHz using the resonant-cavity perturbation technique. We found that the temperature-gradient of K_s correlated with the observed increase or decrease in uniformity throughout the heating process. In Fig. 3 the standard deviation of the temperature measurements in propanediol (45% concentration) are plotted against their mean value, and on the same graph is plotted the temperature variation of K_s. For butanediol at 50%, the temperature-gradient of K_s is positive over most of the range –60 to 0°C. The poor uniformity observed in butanediol solutions is therefore a consequence of its dielectric properties.

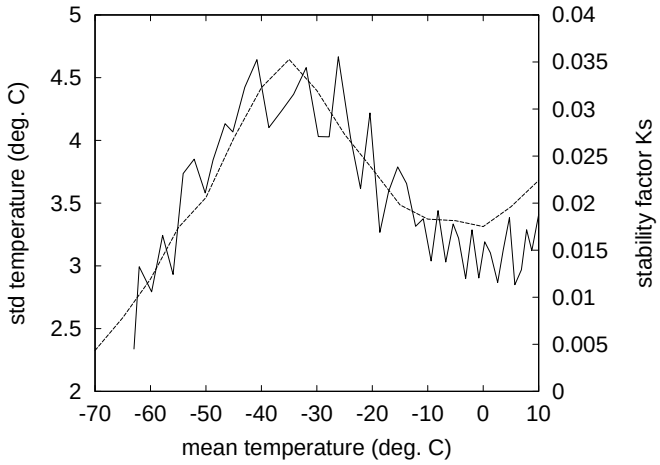

Fig. 3: Variation of standard deviation of temperature with mean temperature during heating of sphere (—), and variation of stability factor K_s with temperature (- - -)

TOXICITY

The toxicity studies were performed on ECV304 endothelial cells, and have been described fully elsewhere by Wusteman et al. [10]. Cells were exposed to each CPA at its minimal vitrifying concentration; then retrieved and counted. Exposed cells were also seeded into flasks and cultured for exactly 2 days. A cell survival index (CSI) was defined as the product of the retrieval and the cell multiplication factor observed after 2 days culture. Table 2 shows the CSI, expressed as a percentage of control, for the four CPAs. Butanediol was found to be the least toxic, although

propanediol was not significantly worse. Both butanediol and propanediol were significantly less toxic than the other two CPAs.

cryoprotectant	CSI (% control)
propanediol	63.6±17.1 (6)
butanediol	76.3±21.0 (9)
ethanediol	33.2±9.1 (8)
dimethyl sulphoxide	37.0±8.4 (4)

Table 2: toxicity of CPAs. Values are mean ± standard deviation (n).

SHAPE OF SAMPLE

Finally, we considered the effect of sample shape on the rapidity and uniformity of heating. Electromagnetic theory suggests that uniform absorption should be possible not only in spheres but also ellipsoids, provided that they are not too large. This is true even if all three principle axes of the ellipsoid are different [9]. To investigate this we made up gelatin solutions in various containers, using 30% propanediol as the CPA because it had been shown to have suitable dielectric properties. In a rod-shaped sample (height 64mm, diameter 14mm), which approximates to a prolate spheroid, the final temperature variation was 4.1 ± 1.7 K (n=3). In a disk-shaped container (height 5mm, diameter 32mm), approximating an oblate spheroid, the variation was 9.5 ± 2.4K (n=3). However in a cone-shaped container (height 20mm, diameter 24mm), which does not closely resemble any ellipsoid, the final temperature variation was 28.5 ± 2.3 K (n=4). These results confirm the importance of sample shape on uniformity of heating.

CONCLUSION

In conclusion, our work has demonstrated some of the conditions – CPA dielectric properties, CPA toxicity and sample size and shape – that are necessary for successful re-warming of vitrified tissues by electromagnetic absorption. The results show that uniform heating is possible in non-spherical samples, and indicate that propanediol is the most suitable CPA of those studied when both physical and biological properties are considered.

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