The Development of a Novel *in vitro* Model Using Kidney Biopsy Specimens to Study the Effects of Warm and Cold Ischaemia on the Kidney

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Abstract: Traditional experimental methods of assessing renal function typically rely on whole kidneys and subsequent measures of glomerular filtration rate. In this study, we measured the viability of kidney biopsy samples of fixed length using formazan-based colorimetry in an attempt to evaluate the suitability of this assay in predicting whole organ viability.

A series of experiments were set up to study the response of the ischaemic rabbit kidney tissue of fixed length (5 mm) obtained using a 16-gauge needle to various preservative solutions at various temperatures. Samples were maintained at 37 °C in a Hereaus EK/O2 incubator in an atmosphere of 5% CO_2 in air, or at 1–4 °C in a Labheat incubator (Borolabs, U.K.) or at 20 °C in air for up to 96 hours.

The formation of formazan within renal biopsy cores was most rapid during the first hour and then levelled off for the rest of the assay period (4 hours). Formazan formation was marginally more from renal cortex than medulla, although the differences were not statistically significant.

The viability of kidney tissue was temperature dependent such that incubating a non-perfused kidney for 20 hours resulted in a 90% reduction in formazan formation and therefore viability at 37 °C compared to 1 °C.

Formazan formation from rabbit kidney biopsy samples taken on day 0 and placed singly wells of a 24 well flat-bottomed tissue culture plate containing 1.8 ml of preservative solution was assessed daily for 4 days. This was compared with the viability of tissue taken daily from whole kidneys perfused with the same preservative solution. Initial viability assessments were similar as were changes with time.

This assay was able to demonstrate the superiority of the currently available renal preservative solutions Soltran and Viaspan in maintaining the viability of renal tissue at low temperatures compared with other randomly selected solutions.

In conclusion, maintaining freshly obtained renal biopsy samples in a tissue culture system is a cheap, convenient and potentially useful model of renal ischaemia. Biochemical tests of cellular viability including formazan based colorimetry on isolated tissue may offer the opportunity to study the effects of ischaemia on the kidney, and may aid in the development of drugs for treating renal ischaemia.

Introduction

Research on the effects of ischaemia on the kidney and possible treatments for this, has been hampered by the lack of a suitable simple and inexpensive model. Traditionally such research has depended on the use of complicated surgical techniques on living animals followed by cumbersome assessments of glomerular function (Lieberthal et al. 1988; Cassie et al. 1959).

Warm renal ischaemic damage is a common clinical problem often occurring as a consequence of hypotension, surgery, sepsis, dehydration or haemorrhage and presenting as acute renal failure (ARF). Despite considerable experimental study, there are no drugs in routine clinical use with proven efficacy for abrogating the effects of this type of ischaemic damage on the kidney (Bates and Lin, 2005: Suzuki et al. 2005). Although most patients with ARF recover spontaneously, they often require a high level of expensive clinical care. Furthermore, ARF may be associated with high mortality rates (Metcalfe et al. 2002).

Prior to kidney transplantation, retrieved organs are ordinarily perfused with cold solutions to preserve them. These are fairly basic electrolyte solutions containing a few novel chemical agents.

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Kidneys are thus preserved for several hours. While they are preserved, kidneys still suffer cold ischaemic damage. Warm and/or cold ischaemic damage prior to transplantation has been shown to adversely affect long-term graft function and survival (Gjertson, 2002; Sienko et al. 2003; Salahudeen et al. 2004). The continued improvement in renal preservation fluids is needed, particularly with the ability to reverse ischaemic damage. This could potentially permit an increase in the number of marginal kidneys suitable for transplantation and help mitigate the tremendous suffering and mortality resulting from end stage kidney disease.

The main purpose of the present study was to develop and evaluate a simple and inexpensive model of warm and cold renal ischaemia using thin (biopsy) samples of renal tissue maintained in preservation solution in tissue culture conditions. Biochemical tests of cellular viability have been used on these samples instead of traditional means of assessing renal function. The results have been considered against predicted changes in renal function in similar clinical situations.

Materials and Methods

Kidneys

The kidneys of rabbits or rats were obtained shortly after necropsy of laboratory animals. These animals were used as control animals in other experimental research and the kidneys donated to this project after death. All kidneys used were removed from animals within 5 minutes of cardiac arrest.

All renal biopsies were taken with a 16 gauge (1.6 mm thick) biopsy gun (Single Action Biopsy Devise, K7/SABD-1615-T, Kimal plc, Arundel Road, Uxbridge, Middlesex, England, UB8 2SA.

These biopsy samples were taken up to 20 minutes after death. This time was chosen because of experimental work suggesting that in human kidney transplantation, warm ischaemia times of 10–20 minutes is not associated with a demonstrable difference in 1 year renal survival (see Yin and Teraski, 1988). Biopsy cores thus obtained were cut into 5 mm lengths and immediately placed in the relevant pre-cooled or prewarmed preservation solution in a Petri dish, thus sustaining less than 20 minutes warm ischaemia prior to incubation in the relevant preservative solution in wells of a 24 well tissue culture plate.

In some of our studies, kidneys were perfused with cold preservative within 2 minutes of death and incubated whole for a period prior to obtaining biopsies for viability assessments.

Statistical comparisons were carried out using the 2-sample t-test.

Culture system

5 mm long kidney biopsy samples were placed in each well of a 24 well flat-bottomed tissue culture plate (Becton Dickinson, U.K.) containing 1.8 ml of preservative solution. Samples were then maintained at 37 °C and 5% CO_2 in air in a Hereaus EK/O2 incubator, or at 1–4 °C in a Labheat incubator (Borolabs, U.K.) or at 20 °C in air for up to 96 hours.

Two commercially available kidney preservative solutions were used (Soltran, Baxter and Viaspan, Bristol-Myers Squibb).

MTT reduction assay

This technique is based on the formation of a blue water-soluble formazan compound from the pale yellow tetrazolium salt 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) after reduction by dehydrogenase enzymes principally NADH and NADPH in the mitochondria of living tissue. The intensity of colour formed gives a measure of tissue viability. Kidney samples were incubated in 1 ml of MTT solution (0.5 mg/ml) for up to 4 hours. Formazan formed within the kidney samples was solubilized in 400 or 800 µl of dimethylsulphoxide (DMSO) in a 48 well microtitre plate at room temperature for 1 hour. The plate was occasionally agitated to disperse the colour evenly. 200 µl of the resulting formazan solution was placed in a 96 well microtitre plate and the absorbance determined at 490 nm in a multi-well scanning spectrophotometer (MR 700, Dynatech laboratories) relative to a DMSO blank. 4–12 replicate biopsy samples were used for each assessment.

Experiments and Results

Time course of MTT production

Freshly obtained kidney biopsy samples were immediately incubated in MTT solution for up to 4 h (Fig. 1). Four replicate kidney biopsy samples were removed from the MTT solution at each time point. The formazan formed within the

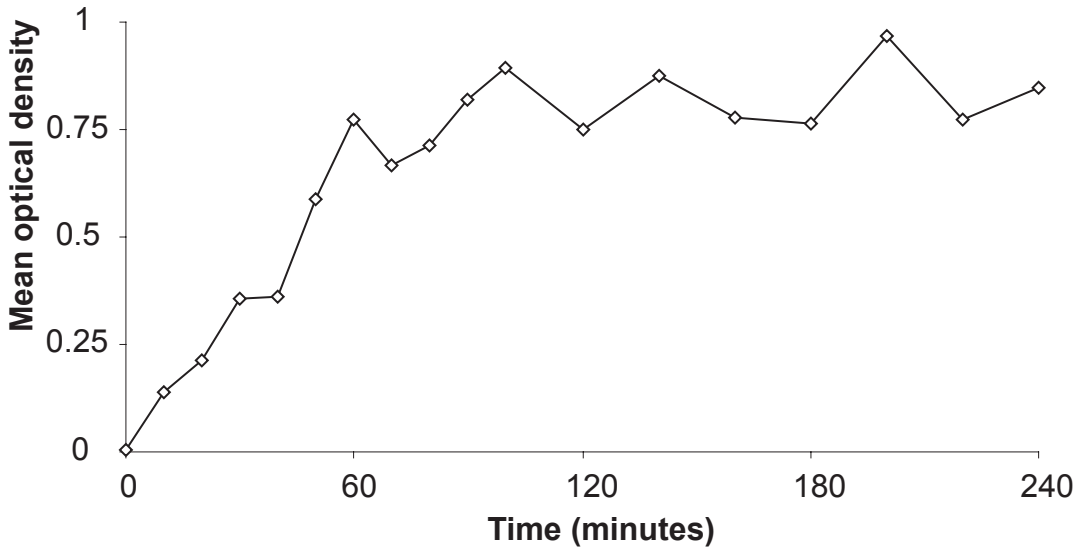

Figure 1. Time course of formazan formation by kidney biopsy cores after incubation for up to 4 hours in MTT.

kidney samples was solubilized in 400 µl of DMSO in a 48 well flat bottomed plate at room temperature for 1 hour. The plate was occasionally agitated to disperse the colour evenly. The absorbance of 200 µl of the resulting formazan solution was determined. Towards the end of the study, individual optical density (OD) readings were occasionally too high to be read by the spectrophotometer. A further 1:2 dilution was made in these cases and OD readings adjusted accordingly.

Formazan formation was most rapid in the first hour and then levelled off from 1–4 h. Background OD readings from kidney biopsy samples placed in DMSO without prior incubation in MTT (time 0) was low (0.005). OD readings were significantly higher at 60 min (mean, 0.771) than at 30 min (0.355) or at 10 min (0.139). Statistical comparisons were made between the 10 min and 60 min time points ($p = 0.029$). The difference between the optical density readings at 1 h (0.771) and 4 h (0.848) was not statistically significant ($p = 0.34$).

Variability in weight of kidney biopsy samples

We noted some variability in thickness of the biopsy cores obtained and therefore weighed a number of freshly obtained 5mm length cores.

The mean weight of 26 sample cores was 3.82 mg (range 1.7–5.8 mg, SD 1.026).

Comparison of formazan formation from renal cortex and medulla

MTT production from standard thickness and lengths of renal cortex and medulla were compared in two trials.

In this and subsequent studies, biopsy samples incubated in MTT for 30 minutes were solubilised in 400 µl of DMSO and samples incubated for 1 hour solubilised in 800 µl of DMSO at room temperature for 1 hour, and the absorbance of 200 µl the resulting formazan solution determined.

Kidney biopsy samples were taken from rabbits within 20 minutes of death and immediately placed in a Petri dish containing 30mls of pre-cooled Soltran (2 °C). These samples were briefly (up to 30 sec) removed from the preservative solution and cut to include 5 mm length renal cortex or renal medulla using a dissecting microscope. The samples were then immediately placed in 2 groups (cortex or medulla) in fresh pre-cooled soltran prior to use in the MTT assay (total pre-assay incubation period, 60 min).

In trial 1, six replicate samples were incubated in MTT for 30 min. Mean OD from renal cortex was 0.381 ± 0.089 and from medulla was 0.303 ± 0.06. Although MTT reduction by the renal cortex was 20.5% greater than from the medulla in this trial, this difference was not statistically significant ($p = 0.065$).

In trial 2, twelve replicate samples were incubated in MTT for 1 h. The absorbance of 200 µl of the resulting formazan solution was determined.

Mean OD from renal cortex was 0.391 ± 0.043 and from medulla was 0.371 ± 0.05. Again, MTT reduction by the renal cortex was 5.1% greater than from the medulla in this trial, but this difference was not statistically significant ($p = 0.35$).

Effect of temperature of incubation on formazan formation

In this and subsequent studies, the biopsy cores used contained variable amounts of cortex and medulla.

The left and right kidney of a rabbit were placed in an incubator approximately 5 min after necropsy for 20 hours and then biopsied. Two trials were carried out using whole kidneys that were not perfused with preservative solution.

In trial 1, formazan production was compared following incubation at 1 °C and 37 °C after 20 hours ($n = 12$).

In trial 2, formazan production was compared following incubation at 1 °C and 20 °C after 20 hours ($n = 4$).

Following incubation of the samples in MTT for 30 min, the formazan was solubilized as described and the absorbance of 200 µl of the resulting solution determined.

There was no significant difference in formazan formation from either kidney at time 0 h in either trial. In trial 1, mean OD from the kidney kept at 1 °C was 0.299 compared with 0.03 from the kidney kept at 37 °C (90% reduction in formazan formation; $p = 7.4 \times 10^{-7}$).

In trial 2, mean OD from the kidney kept at 1 °C was 0.448 compared with 0.347 from the kidney kept at 20 °C (12.5% reduction in formazan formation; $p = 0.34$).

Effect of preservation solution used to incubate kidney tissue on MTT production

In this study, the left and right kidney of a rabbit was obtained within 2 minutes of necropsy.

The renal artery of one of the kidneys was identified and cannulated with an 18 guage venflon and perfused with 50 mls of cold 1 °C Soltran. Both the perfused and non perfused kidneys were divided into two along the short axis using a scalpel blade. One half of each kidney was kept at 1 °C and the other half at 37 °C. After 24 hours, 12 biopsy cores were taken from each half kidney, cut into 5 mm lengths and assessed for tissue viability. Following incubation in MTT for

Table 1. A comparison of renal preservation following no treatment or perfusing kidneys with Soltran prior to incubation at 1 °C or 37 °C for 20 hours.

	Perfused with soltran	Perfused with soltran	Kidneys not perfused	Kidneys not perfused
Optical density	1 ºC	37 ºC	1 ºC	37 ºC
MTT 1	0.418	0.015	0.257	0.035
MTT 2	0.46	0.013	0.328	0.019
MTT 3	0.032	0.031	0.332	0.034
MTT 4	0.5	0.01	0.277	0.016
MTT 5	0.421	0.014	0.255	0.062
MTT 6	0.422	0.015	0.334	0.024
MTT 7	0.293	0.016	0.339	0.032
MTT 8	0.265	0.022	0.313	0.038
MTT 9	0.346	0.012	0.331	0.026
MTT 10	0.378	0.012	0.262	0.024
MTT 11	0.042	0.028	0.243	0.02
MTT 12	0.408	0.011	0.317	0.031
Mean OD	0.332	0.017	0.299	0.03
SD	0.15	0.01	0.04	0.01

1h, optical density readings were determined (Table 1).

Kidney tissue was significantly better preserved at 1 °C than at 37 °C, whether pre-perfused with preservative or not. The differences were highly significant ($P < 0.01$). The kidney perfused with soltran was better preserved than the non-perfused at 1 °C (MTT 0.332 and 0.299) although OD readings just failed to reach levels of significance ($P = 0.057$).

Comparison of the rate of decline in formazan formation from whole perfused kidneys and biopsy samples placed in preservative solution for up to 96 hours

Trial 1: In this trial, the renal arteries of rabbit kidneys were cannulated with a 20 guage venflon and perfused with 50 mls of cold 4 °C soltran or viaspan respectively within 2 minutes of cardiac arrest. 200,000 units of penicillin V, 16 mg dexamethasone, and 40 units of soluble human insulin were added to 2 litres of viaspan solution just prior to use as per the manufacturers recommendations. The kidneys were then placed in pots containing 100 mls of the same preservative solutions and maintained at 4 °C throughout the duration of the trial.

Following this, multiple kidney biopsy samples were taken from either kidney on day 0 as previously described. 6–12, 5 mm length replicate biopsies were placed singly in 24 well tissue culture plates containing pre-cooled (4 °C) soltran (SL) or viaspan (VL). MTT assays were carried out on the biopsy samples isolated on day 0 and maintained in culture for up to 4 days and compared with freshly removed biopsy samples taken from the perfused whole kidneys [soltran (SK) or viaspan (VK)] just prior to the assay (comparisons made at 24, 48 and 96 hours).

In this trial, samples were incubated in MTT for 1 h. Optical density readings from the VK and SK groups were 0.40 ± 0.09 and 0.469 ± 0.08 respectively at 0 h ($n = 6$). These results were not statistically different. ($P = 0.1495$). In all groups, there was a general tendency for a slow decline in formazan formation with time. By day 4, Optical density readings were as follows: SK, 0.35; VK, 0.25; SL, 0.28; VL, 0.24.

These results indicate that the decline in the viability of kidney tissue over time is similar (Fig. 2) whether loose kidney tissue is maintained singly in tissue culture or biospied from the whole organ. Results of studies with the renal preservative solutions Viaspan or Soltran showed similar trends.

Effect of renal tissue biomass on formazan formation

Fifteen, 5 mm length kidney biopsy samples were taken from rabbit kidneys as previously described and placed individually in a 24 well tissue culture plates containing viaspan for 24 hours.

These samples were individually removed, blotted on filter paper to remove excess preservative and weighed on a mettler balance.

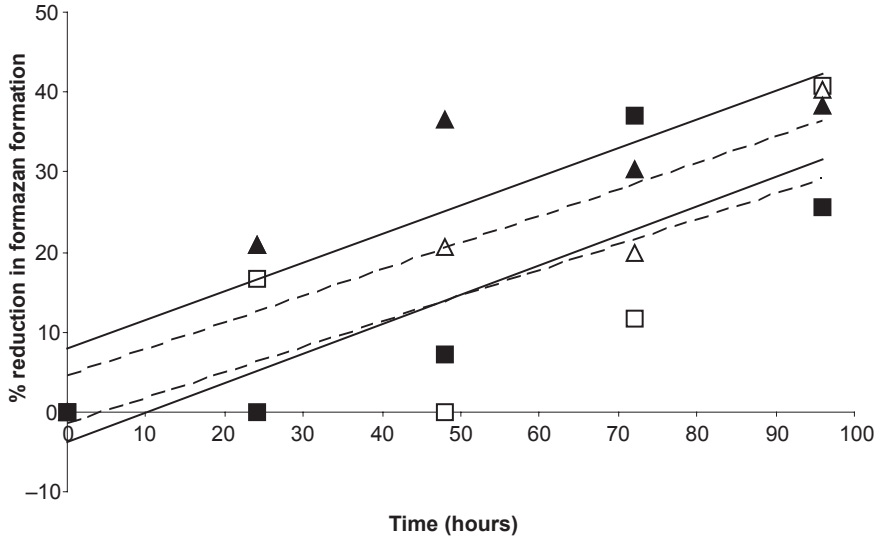

Figure 2. Percentage reduction in formazan formation within renal tissue over 96 hours compared to 0 hours. □ = soltran, Δ = viaspan, filled symbols and continuous linear regression lines = samples taken from whole kidneys, empty symbols and dashed linear regression lines = biopsy kidney tissue.

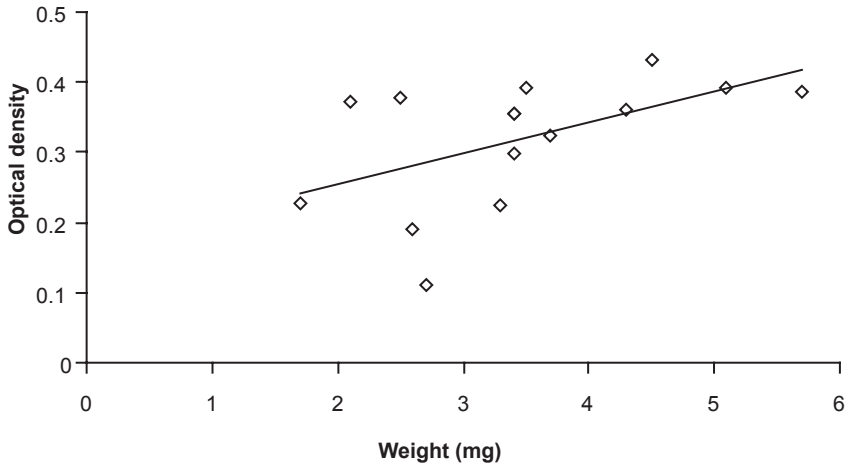

Figure 3. Formazan formation from kidney tissue samples of fixed length but different weight.

Following this, biopsy samples were incubated in MTT for 1h. There was a modest positive correlation between the amount of formazan formed and the weight of the tissue samples (Fig. 3). However, the increase in optical density was not directly proportionate to the increase in weight. The correlation coefficient was calculated as 0.53.

MTT formation from renal tissue from different animals

The MTT assay was applied in a similar fashion to renal tissue from rats in a series of different trials (data not shown). This showed a similar pattern of formazan formation with time. The quantity of formazan formed from the same biomass of tissue was similar as were changes following temperature dependent incubation and the preservation solution soltran suggesting that these results are not likely to be species specific.

Discussion

In these series of experiments, we have examined the viability of thin kidney tissue (1.6 mm × 5 mm) maintained under different conditions *in vitro*. We have used the MTT reduction assay based on the formation of a blue water-soluble formazan compound from the pale yellow tetrazolium salt 3-(4, 5 diethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) after reduction by dehydrogenase enzymes principally NADP and NADPH in the mitochondria of living tissue to assess viability. This assay has been used to assess the viability of microorganisms (Townson and Tagboto, 1991a). We have recently applied this technique to studying renal ischaemic damage (Tagboto and Townson, 2007), and have demonstrated good correlation between histological assessment of ischaemic damage and our biochemical test of viability (Tagboto and Griffiths, 2007).

Damage sustained by renal tissue following the interruption in blood flow is partially due to the direct effects of ischaemic injury but also in part due to the deleterious consequences of reperfusion. A limitation of this type of work is that it did not evaluate reperfusion injury, although the experimental design could be suitably modified to do this.

An attempt was made to standardise the biomass of kidney tissue used by taking samples of fixed thickness using a semi-automated biopsy devise and cut to a standard length. Previous work has shown that MTT production from living organisms is proportional to the biomass (Townson and Tagboto, 1991b). The relationship between biomass and optical density readings were demonstrated by this study, although the correlation was modest. It is possible that the use of thinner biopsy samples will permit better diffusion of preservative solution and MTT solution to cells within the centre of the core which may have implications for better preservation and formazan formation and may thus improve the correlation between biomass and formazan formation. The weight of samples showed considerable variability. Average weights of the biopsy samples were 3.82 mg (range 1.7–5.8 mg), representing a greater than 3 fold difference between the smallest and the largest samples. Examining biopsy samples visually, it is clear that the thickness of samples varied to some degree, explaining the difference in sample weights. Although the variability of the weight of our samples caused concern that weight adjusted

OD readings may be more appropriate, the impracticality of carrying this out within our resource constraints meant this was not done. This does not change the overall validity of this pilot study but should be addressed in further research. It will also be useful to experiment with biopsy needles of different gauges and from different manufacturers to see if this might decrease the variability between the samples. It is possible that the surface area of the kidney biopsy samples may correlate with formazan formation better than the weights of the samples. This needs to be examined in future work.

The MTT assay has previously been used to study ischaemic kidney tissue (Yin and Teraski, 1988). They examined the development of colour change in 1mm thick slices of kidney over 30 seconds by examining light reflected by this tissue placed on a clear area in a petri dish and covered with MTT solution. This method is more cumbersome than the technique described here and does not allow easy comparisons of kidneys tissue maintained under different conditions.

Formazan formation in renal tissue increased rapidly for the first hour and then more slowly over a 4 hour period. Although the amount of formazan produced at 4 h was greater than at 1h, this difference was not statistically significant. In two trials, MTT production from the renal cortex was marginally greater than from the medulla. However, the difference was not statistical significance.

It is well recognised that the viability of kidney after induced ischemia is temperature dependent (Lieberthal et al. 1988) and can be partially abrogated in the cold by commercially available preservation solutions. This assay was able to correctly predict this. Kidney tissue rapidly deteriorated at 37 °C. Even a small reduction in temperature to 20 °C offered significant protection against ischaemic damage. Compared with kidney tissue samples kept at 1 °C for 20 hours, formazan formation was 90% less if kidneys were kept at 37 °C but only 12.5% less at 20 °C. Ideally, kidney biopsy samples should have been incubated in the same gas phase irrespective of temperature of incubation. However, our limited resources meant that this could not be done.

Commercially available tissue culture medium (MEM) used with serum appeared to be superior to commercially available kidney preservation fluid at 37 °C but at 0–4 °C; commercially available kidney preservation fluid (Soltran or Viaspan) was superior (data not shown).

We conclude that the use of biopsy kidney tissue in culture followed by biochemical assessments of their viability may be a valuable substitute for whole organs in some types of research work.

Acknowledgements

This investigation received part financial support from the Iechyd Morgannwg Health Research and Development Consortium. I thank Dr. Simon Townson for providing laboratory space and advice about the conduct of this research.

Disclosure

The author reports no conflicts of interest.

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