Osmotically inactive volume, hydraulic conductivity, and permeability to dimethyl sulphoxide of human mature oocytes

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Controlled ovarian stimulation during an *in vitro* fertilization cycle usually produces large numbers of oocytes and, consequently, it is likely that more embryos will be generated than can be transferred in a given cycle. It is desirable to freeze-bank surplus oocytes before insemination to avoid the ethical and legal complications of disposing of or storing embryos. Although many attempts have been made to cryopreserve human oocytes, to date, post-thaw survival has been poor, and viable pregnancies after *in vitro* fertilization have been rare. A possible explanation for the lack of success is that the freezing methods have been adapted from animal studies but have not been optimized for the human oocyte. In this study, video microscopy was used to determine the volumetric responses of mature human oocytes to changes in osmolarity during preparation for freezing. A Boyle van't Hoff plot of data collected in static experiments with fresh human oocytes gave a value of 0.19 ± 0.01 (mean $\pm$ SEM) for the osmotically inactive volume. Dynamic measurements during exposure to dimethyl sulphoxide at room temperature (22°C) were analysed by a two-parameter transport model and produced values of 1.30×10^{-6} cm atm⁻¹ s⁻¹ for the hydraulic conductivity of the plasma membrane and 3.15×10^{-5} cm s⁻¹ for dimethyl sulphoxide permeability (chi-squared = 0.43, df = 20) of fresh human oocytes. Oocytes that had failed to fertilize had a slightly lower hydraulic conductivity and dimethyl sulphoxide permeability and, after exposure to 1.5 mol dimethyl sulphoxide l⁻¹, these cells appeared to become permeable to normally impermeable solutes. These permeability properties have been used to design a protocol for the addition and removal of dimethyl sulphoxide to control the magnitude of volumetric changes.

Introduction

During routine *in vitro* fertilization treatment (IVF) the ovaries undergo controlled ovarian stimulation to induce the growth of many antral follicles, which can be aspirated to retrieve mature oocytes. After fertilization, more than one embryo is normally replaced in the uterus to increase the chance of conceiving. Any surplus embryos of good quality are usually cryopreserved so that, if the transfer fails, they can be thawed for a further pregnancy attempt without the need for another round of ovarian stimulation. This strategy is successful (Marcus *et al.*, 1997) but the creation of embryo banks raises many complex ethical and legal considerations, including the fate of orphaned embryos and the time limit set by regulatory authorities for storage (Trownson and Dawson, 1996). Successful oocyte cryopreservation would avoid these problems and make economical use of oocytes obtained in each cycle. The technique could also facilitate egg donation cycles and might be used to preserve oocytes in patients

before they undergo chemo- or radiotherapy (Apperley and Reddy, 1995), although the emergency nature of cancer treatment may preclude this strategy.

Numerous attempts have been made to freeze-thaw human oocytes but success rates have been disappointing because frozen-thawed oocytes are susceptible to zona hardening, cytoskeletal injury and abnormal meiotic divisions (Oktay *et al.*, 1998). Evidently, the protocols that have been used in cryopreservation are still not optimal for this cell type and the biophysical properties determining the response of human oocytes to osmotic stress during freezing and thawing require investigation. Before cryopreservation, cells are equilibrated with a cryoprotective agent (CPA) to limit the extent of ice formation, but the solutions are hyperosmotic and CPAs penetrate cells more slowly than water. As a consequence, shrinkage occurs initially, as water is withdrawn down the osmotic gradient, and then there is a return to normal volume as the CPA penetrates the cell. During the subsequent process of freezing, ice forms preferentially outside the cell, concentrating extracellular solutes and again exposing the cell to hypertonic conditions (Mazur, 1963). While rapid cooling puts the cell at risk of

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lethal intracellular freezing, an excessively slow rate of cooling overexposes the cell to the dangers of hypertonic conditions. Finally, when the cell is returned to isotonic solution after thawing, there is an inward osmotic flux and the possibility of excessive swelling and lysis of the oocyte. Thus, the permeabilities of the cell membrane to both water and CPA are important factors in determining optimum cooling rates.

The volume changes that occur during freezing and thawing can be calculated if the total surface area, volume and osmotically inactive volume of the cell are known, together with the permeability parameters of the oocyte plasma membrane to water and the CPA. In the present study, the osmotic responses of human oocytes to a range of non-isotonic solutions of impermeant solutes was measured by microscopy. These measurements were used to construct a Boyle van't Hoff plot, from which the non-osmotic volume (V_o) was derived. Video microscopy was used to record the time course of the volumetric responses during exposure of oocytes to the CPA, dimethyl sulphoxide (DMSO) at 1.5 mol l^{-1} , which is a concentration widely used in cryopreservation. The dynamic data were analysed by a two-parameter model to yield values for the hydraulic conductivity (L_p) and solute permeability (P_s). These parameters have been used to propose a method for the controlled addition and removal of CPA to and from oocytes that will reduce the risk of harmful swelling.

Materials and Methods

Mature oocyte collection

All oocytes were donated by patients who had given consent to the use of their oocytes for research which had been approved by the local ethics committee of the Leeds Teaching Hospitals NHS Trust. Fresh human oocytes at metaphase II were analysed within 2 h of follicular aspiration after downregulation with a GnRH analogue and stimulation using exogenous gonadotrophins (MacNamee and Brinsden, 1992). Oocytes that had failed to fertilize (FTF) were collected after 24–48 h in culture and those that were morphologically normal, spherical and with a polar body were selected for study. Before exposure to non-isotonic solutions, each oocyte was incubated in Leibovitz L-15 medium containing 1 mmol ethylenediaminetetra acetic acid l^{-1} (EDTA) for 10 min at 37°C to ensure uniform spherical shrinkage (Younis *et al.*, 1996).

Determination of the osmotically inactive volume (V_o) of human oocytes

Hypertonic and hypotonic solutions were prepared by the addition of sucrose or pure water to Leibovitz-L15 medium (320 mosmol kg^{-1}). The osmolality of each solution was adjusted using a freezing point osmometer (Advanced Instruments Ltd) to 230, 600, 1000 and 1400 mosmol kg^{-1} , respectively. Individual oocytes were transferred to isotonic medium at room temperature (22°C) with siliconized mouth

pipettes, and the diameters were measured under an inverted microscope with a calibrated micrometer ($n = 8$). The individual oocytes were then pipetted into 70 μl droplets of hypertonic or hypotonic solution in a humidified vessel to prevent evaporation. The cells were left to equilibrate for 5 min at room temperature before the new diameters were measured. Cell volume was calculated before and after exposure to non-isotonic solution, assuming spherical shrinkage. The volumes relative to the initial volume in isotonic medium were plotted against the reciprocal of osmolality. The data were fitted to a straight line by the least-squares method and extrapolated to determine the cell volume in an infinitely concentrated solution (Toner *et al.*, 1990).

Calculation of the permeability parameters of human oocytes

Solutions of 1.5 mol dimethyl sulphoxide l^{-1} (DMSO) and 1.5 mol DMSO l^{-1} plus 0.1 mol sucrose l^{-1} were prepared in Leibovitz-L15 medium and sterilized by filtration. A flow chamber was assembled on the stage of an inverted microscope with Hoffman optics (Nikon, Kingston-on-Thames) to visualize the dynamic response of oocytes to these non-isotonic solutions. The chamber consisted of a channel 0.5 cm wide and 4 cm long with a slight elevation at one end to aid unidirectional flow. A peristaltic pump delivered bathing solution to the elevated end of the chamber at a rate of 0.4 $ml\ min^{-1}$. Video recording apparatus attached to the inverted microscope continuously recorded, at a magnification of $\times 400$, the volumetric response of the oocytes. Individual oocytes were transferred to the flow chamber and maintained in a static position with a holding pipette controlled by a micromanipulator (Narishige). The chamber was then perfused sequentially with (a) isotonic medium (Leibovitz-L15), (b) 1.5 mol DMSO l^{-1} , (c) 1.5 mol DMSO l^{-1} plus 0.1 mol sucrose l^{-1} and (d) isotonic medium again. The oocytes were exposed to each treatment for 1, 10, 10 and 5 min, respectively, at room temperature. Before repeating the experiment on a new oocyte, the chamber was flushed with fresh medium to remove any residual DMSO or sucrose. The experiment was performed on eight fresh oocytes and 12 FTF oocytes.

The video recording was analysed on a PC using frame grabber apparatus (DT55 vision EZ frame grabber card). Frames were paused every 30 s and the cross-sectional area of the oocyte was measured using a computer graphics package (Global Lab Image Software). Cell volume was calculated from these values and converted into a volume relative to that in isotonic medium. The data were pooled to calculate the mean relative volume, at each 30 s time point, for both fresh and FTF oocytes. The volumes measured during the first 10 min were fitted to a two-parameter model defined by the following equations (Kleinhans, 1998):

$$Jv_w = -L_p (\pi^e - \pi^i)$$

$$Jv_s = V_o \times P_s (C^e - C^i)$$

where Jv_w = volumetric water flux; L_p = hydraulic conductivity; π^e and π^i = external and internal osmolality,

respectively, assuming non-ideal behaviour of the intracellular solutes as described by Pegg *et al.* (1987) and Arnaud and Pegg (1990); Jv_s = volumetric flux of DMSO; P_s = DMSO permeability; C^e and C^i = external and internal concentrations of DMSO, respectively; and V_s = partial molar volume of DMSO. In accordance with Kedem and Katchalsky (1958), solute concentration was expressed in mol l⁻¹. V_s was calculated by dividing the molecular weight of DMSO (78.13) by the density of DMSO at 20°C (1.1014 g cm⁻³) giving the result: 71 cm³. No correction was made for change in density with temperature. Integration was performed using a simple forward integration scheme with time-step control implemented in a purpose-written PASCAL program running on a standard PC. A weighted least squares best fit was obtained by iteration to minimize the chi-squared statistic described below.

Statistical analysis

Means $\pm$ SD were calculated for each volume measurement. The Boyle van't Hoff regression was calculated by the method of least squares. The goodness of fit to the DMSO permeation data was quantified by calculating a chi-squared (χ^2) statistic using the following equation:

$$\chi^2 = \sum \{ (V_{\text{obs}} - V_{\text{pred}}) / \sigma \}^2$$

where V_{obs} and V_{pred} are the observed mean and predicted volume, respectively, at each data point and σ^2 is the variance associated with the experimental determination of each mean volume. The degrees of freedom are $n - 2$. This approach follows that described by Walcerz *et al.* (1995).

Results

Osmotically inactive volume of human oocytes

Mature oocytes closely follow a Boyle van't Hoff relationship and thus exhibit an ideal osmometric response over the range 230–1400 mosmol kg⁻¹ (Fig. 1). Extrapolation of the data to infinite osmolality gave an osmotically inactive volume of 0.19 ± 0.01 (mean $\pm$ SEM).

Permeability parameters for human oocytes

The exposure of oocytes to EDTA before exposure to non-isotonic solutions resulted in uniform spherical shrinkage which permitted the calculation of cell volume from measurements of cross-sectional area (Fig. 2a–d). The mean volumetric change of the oocytes during exposure to the cryoprotective media at each 30 s time point was calculated by pooling the data from each of the eight fresh oocytes. These data were plotted together with the standard error (Fig. 3).

A number of assumptions concerning the basic cell parameters were made to analyse the data. The measured diameters in the study ranged from 100 to 150 μ m. The mean

Fig. 1. Boyle van't Hoff plot of the osmotically inactive volume of mature human oocytes ($n = 8$).

radius of the vitellus in isotonic solution (63 μ m) gave a normal oocyte volume of 1.0×10^{-6} cm³. The physiological water content was assumed to be 800 pl (8×10^{-7} cm³) (B. J. Fuller, personal communication). Since the Boyle van't Hoff plot indicated that 81% of the cell volume was osmotically active, it was assumed that the cells contained insignificant amounts of osmotically inactive water. The measured osmolality of the Leibovitz medium was 320 mosmol kg⁻¹ and the inclusion of 0.1 mol sucrose l⁻¹ raised it to 436 mosmol kg⁻¹. These concentrations of impermeant solute were assumed for the relevant stages of the experiment.

The data obtained from the oocytes during the first 10 min of exposure to 1.5 mol DMSO l⁻¹, using the assumed parameters, were modelled to give a best fit curve (Fig. 4), which shows that the oocytes shrink to approximately 60% of their isotonic volume within the first 50 s of exposure to cryoprotective media, as cellular water is withdrawn by the osmotic gradient. The cell then starts to re-expand as DMSO diffuses across the membrane. After 10 min of exposure to 1.5 mol DMSO l⁻¹, the oocyte volume is approximately 90% of that in isotonic solution. FTF oocytes were slightly less permeable to DMSO (Fig. 5). The permeability properties of human oocytes were calculated from the model (Table 1).

The response to the complete sequence of solution changes using these values is shown (Fig. 6) with the line of best fit to the fresh oocyte data. The fit was good in stages 1 and 2 (when the oocytes were incubated in 1.5 mol DMSO l⁻¹ in Leibovitz medium and then transferred to a medium containing 1.5 mol DMSO l⁻¹ plus 0.1 mol sucrose l⁻¹ in Leibovitz medium). The observed pattern was modelled less well in stage 3 (when the oocytes were finally transferred to isotonic Leibovitz medium). The overall value of chi-squared was 2.60. The volume of FTF oocytes consistently exceeded that predicted during the second and third stages. The fit to the data collected from these oocytes could be obtained in stage 2 if a reduced transmembrane osmotic differential of impermeant solutes to 90% of the theoretical value was assumed. These findings indicate that these oocytes had become leaky to the Leibovitz salts.

In the present study, the one step addition of 1.5 mol

Fig. 2. The morphological appearance of mature human oocytes during exposure to isotonic and non-isotonic solution at room temperature. (a) A morphologically normal oocyte in isotonic Leibovitz medium. (b) A shrunken oocyte after exposure for 30 s to 1.5 mol DMSO l⁻¹. (c) A swollen oocyte after sequential exposure to 1.5 mol DMSO l⁻¹, 1.5 mol DMSO l⁻¹ supplemented with 0.1 mol sucrose l⁻¹ and reintroduction of isotonic Leibovitz solution. (d) An oocyte exposed to 1.5 mol DMSO l⁻¹ at room temperature without prior incubation in 1 mmol EDTA l⁻¹. Scale bar represents 28 μ m.

DMSO l⁻¹ caused a 40% decrease in cell volume, and the subsequent direct transfer to isotonic solution caused the oocytes to swell to 140–150% of the physiological volume. Values calculated for the biophysical properties of the oocytes have been used to produce a model that uses a two-step procedure for the addition and removal of cryoprotectant that limits volumetric changes to approximately 30% (Fig. 7).

Discussion

Many attempts have been made to cryopreserve mature human oocytes but, in general, post-thaw viability rates have been disappointing and the fertilization rates correspondingly poor (Mandelbaum *et al.*, 1988). However, a few pregnancies and live births have been reported in humans (Chen, 1986; Van Uem *et al.*, 1987; Porcu *et al.*, 1997), cows (Fuku *et al.*, 1992), rabbits (Al-Hasani *et al.*, 1989) and mice

Fig. 3. Mean relative cell volumes at room temperature of fresh human mature oocytes. The oocytes were exposed during stage 1 (0–10 min) to 1.5 mol DMSO l⁻¹, during stage 2 (10–20 min) to 1.5 mol DMSO l⁻¹ plus 0.1 mol sucrose l⁻¹ and, during stage 3 (20–25 min), to isotonic Leibovitz solution ($n = 8$).

Fig. 4. The curve of best fit, using the two-parameter model, for data on the dynamic responses of the volume of freshly isolated human oocytes immersed in 1.5 mol DMSO l⁻¹ in Leibovitz solution. The derived values of the permeability parameters are: hydraulic conductivity: 1.30×10^{-6} cm atm⁻¹ s⁻¹; solute permeability for dimethyl sulphoxide: 3.15×10^{-5} cm s⁻¹. Chi-squared = 0.43.

Fig. 5. The curve of best fit, using the two-parameter model, for data on the dynamic responses of the volume of failed to fertilize (FTF) human oocytes immersed in 1.5 mol DMSO l⁻¹ in Leibovitz solution. The derived values of the permeability parameters are: hydraulic conductivity: 1.00×10^{-6} cm atm⁻¹ s⁻¹; solute permeability for dimethyl sulphoxide: 2.50×10^{-5} cm s⁻¹. Chi-squared = 0.83.

(Whittingham, 1977; Glenister *et al.*, 1987), demonstrating that the technique is feasible. Mature oocytes are arrested at metaphase of the second meiotic division when the cell has a highly organized cytoskeletal system, numerous cytoplasmic organelles and a zona pellucida. Damage to any of these structures during freezing can reduce viability after thawing. The decrease in the rate of fertilization could be due to hardening of the zona pellucida (Wood *et al.*, 1992; Carroll *et al.*, 1993), and this hypothesis is supported by the better results obtained with intracytoplasmic sperm injection (ICSI) than with IVF (Gook *et al.*, 1995; Kazem *et al.*, 1995) and the creation of viable pregnancies (Porcu *et al.*, 1997; Polak de Fried *et al.*, 1998). Reports that both DMSO and cooling at temperatures well above 0°C may irreversibly damage the cytoskeleton in human oocytes (Johnson and Pickering, 1987; Pickering and Johnson, 1987; Van der Elst *et al.*, 1988;

Table 1. Permeability properties of human oocytes calculated from a two-parameter transport model

	Fresh oocytes	Oocytes that had failed to fertilize
Hydraulic conductivity (cm atm ⁻¹ s ⁻¹)	1.30×10^{-6}	1.0×10^{-6}
Solute permeability for dimethyl sulphoxide (cm s ⁻¹)	3.15×10^{-5}	2.5×10^{-5}
Chi-squared (df = 20)	0.43	0.83

Fig. 6. Curve plotted for the entire experiment using the best-fit values from stage 1 fresh human oocyte data (●) and failed to fertilize (FTF) oocyte data (□).

Fig. 7. Calculated osmotically induced excursions in cell volume when mature human oocytes are exposed in sequence to 0.8, 1.5, 0.64 and 0 mol DMSO l⁻¹ in isotonic media. This scheme would restrict volume changes to $\pm 30\%$.

Pickering *et al.*, 1990) are of greater concern.

Freezing protocols were derived originally from animal studies, but the size and structure of mature oocytes differ significantly among species. Therefore, extrapolation to the human oocyte is unsafe and studies *inter alia* of osmotic stress in these cells during the freezing procedure are required to design cryopreservation protocols.

The present results show that mature oocytes behave as

ideal osmometers between 230 and 1400 mosmol kg⁻¹ and indicate that the osmotically inactive volume is about 0.19. Non-aqueous cellular material contributes a large component to this volume but it is not unusual for a proportion of the cellular water to be osmotically inactive too. However, in human oocytes, it seems that there is little non-osmotic water since approximately 80% of the cell volume is osmotically active. Reported measurements of the non-osmotic volume of mature oocytes in other species indicate that this may be a general phenomenon: 0.18–0.20 for murine oocytes (Leibo, 1980; Toner *et al.*, 1990; Hunter *et al.*, 1992a); 0.1 for non-human primate oocytes (Younis *et al.*, 1996); and 0.23–0.29 for human oocytes (Hunter *et al.*, 1992a,b; McWilliams *et al.*, 1995). This conclusion has important implications for the calculation of changes in cell volume during the addition and removal of cryoprotectants (Arnaud and Pegg, 1990).

Membrane permeability modelling is commonly used to derive parameters describing water and solute permeability from experimental measurements of changes in cell volume. The Kedem and Katchalsky (1958) formalism is most commonly used in such analyses. However, Kleinhans (1998) has emphasized that the underlying assumption that solute moves exclusively through the same channels as water is rarely secure. When this assumption is not justified, the derived parameters have no precise physical meaning, but merely describe the experimental curve in a more or less empirical way. Since it is highly improbable that this assumption is met by oocytes, the earlier and simpler two-parameter (L_p and P_s) formalism has been used in the present study. The resulting values of L_p and P_s describe the experimental data adequately and can be used to predict the effect of changing protocols for the addition and removal of cryoprotectants.

The curve of best fit was determined by minimizing the squares of the differences between the mean observations and the values predicted by the model, weighted by the variance associated with each data point. The value of the chi-squared statistic indicates the goodness-of-fit; in this case, with 20 degrees of freedom, the transitional value for $P < 0.05$ is 31.4 whereas, in the present study, chi-squared = 0.43 (an extremely good fit). This approach cannot assign individual tolerances to the derived parameters and, indeed, Walcerz *et al.* (1995) have argued that the nonlinearity and asymmetry of the data preclude the use of standard methods to estimate the error associated with L_p and P_s . In fact, it is unclear what value individual error measures would have, since L_p and P_s are used together to predict the complete curve, and it is the correspondence between that curve and the data that is important.

In the present study, a value of 1.30×10^{-6} cm atm⁻¹ s⁻¹ was obtained for the hydraulic conductivity (L_p) of mature oocytes at room temperature in the presence of 1.5 mol DMSO l⁻¹. There is no general agreement over the units used for expressing L_p but, in SI units, the above value equates to 1.31×10^{-13} m³ N⁻¹ s⁻¹. In another commonly used unit, the above result corresponds to 0.78 µm atm⁻¹ min⁻¹, which compares well with reported measurements in the same species: 0.40–0.70 µm atm⁻¹ min⁻¹ and 0.64–0.84 µm atm⁻¹ min⁻¹ for human fresh and FTF oocytes, respectively

(Bernard *et al.*, 1988; Hunter *et al.*, 1992a,b; Paynter *et al.*, 1997). Values are also in a similar range in other species: 0.36–0.77 µm atm⁻¹ min⁻¹ for murine oocytes (Leibo, 1980; Bernard *et al.*, 1988; Hunter *et al.*, 1992a; Paynter *et al.*, 1997; Pfaff *et al.*, 1998) and 0.47 µm atm⁻¹ min⁻¹ for bovine oocytes (Ruffing *et al.*, 1993).

The solute permeability of the membrane to DMSO was estimated to be 3.15×10^{-5} cm s⁻¹ (0.315 µm s⁻¹), which is comparable with values obtained in previous studies of human and murine oocytes of 0.26 µm s⁻¹ (Paynter *et al.*, 1997) and 0.16–0.24 µm s⁻¹, respectively (Paynter *et al.*, 1997). Studies that investigated the membrane permeability of oocytes to different CPAs reported values for propylene glycol of 0.82 µm s⁻¹ and 0.31–0.48 µm s⁻¹ in human and murine studies, respectively (Fuller *et al.*, 1992). In contrast, the permeability of murine oocytes to glycerol is two orders of magnitude lower, at 0.002 µm s⁻¹ (Jackowski *et al.*, 1980).

A slightly lower water permeability (1.0×10^{-6} cm atm⁻¹ s⁻¹) and solute permeability for DMSO (2.5×10^{-5} cm s⁻¹) (chi-squared = 0.83) was observed when FTF oocytes were compared with fresh oocytes. This result is in contrast to the measurements of hydraulic conductivity reported by Hunter *et al.* (1992a), although these authors commented upon the extreme variation in their results. The most notable difference in the present study between fresh and FTF oocytes was in the apparent leakiness to normally impermeant salts after exposure to DMSO. It is unclear whether these permeability changes are connected with the inability of oocytes to be fertilized or are secondary to exposure to the conditions for IVF or to ageing *in vitro*. However, changes in the zona are unlikely to have been responsible since this is a highly permeable membrane.

The one-step addition of 1.5 mol DMSO l⁻¹ to oocytes produced an approximately 40% decrease in cell volume, and the subsequent direct transfer to isotonic solution caused the oocyte to swell to 140–150% of the original volume. It is not known whether such changes are harmful in themselves, but the permeability data determined in this study can be used to design methods that reduce osmotic stress to the oocyte. Indeed, a two-step procedure for the addition and removal of DMSO that limits volumetric changes to $\pm 30\%$ has been established. This limit was based on the tolerance of other cell types and, if future research indicates that the margin is too generous, this model can be adapted easily for stricter limits. For a tolerance limit of $\pm 30\%$, oocytes should at first be equilibrated with 0.8 mol DMSO l⁻¹ for about 10 min and then transferred to 1.5 mol DMSO l⁻¹ for about 20 min before freezing. After thawing, oocytes should be transferred to 0.64 mol DMSO l⁻¹ for about 20 min before being returned to isotonic conditions. Stepwise changes in CPA exposure are already in common practice, but the present study has produced a theoretical basis and a quantitative indication of the expected impact on cell volume. Unfortunately, testing the practical benefits of this new protocol is likely to be slow because of the scarcity of fresh oocytes for research.

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