Protective and Sensitizing Effects of D₂O Treatment on Thermal Responses of Chinese Hamster Cells

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Modification of thermal sensitivity by deuterated water (D₂O) was examined in Chinese hamster V-79 cells. When cells were exposed to hyperthermia at 44°C in a medium containing 20 to 85% D₂O, cell survival was remarkably increased. Thus, D₂O applied during hyperthermia resulted in thermal protection. However, when cells were treated with D₂O (50%) medium for 4 to 24 hours and then exposed to hyperthermia in normal medium, thermal sensitization was observed. Increasing interval time at 37°C between D₂O and hyperthermic treatments, the thermal sensitization was rapidly reduced and reached to the normal level in about 4 hours of the interval. On the other hand, thermotolerance induced during step-up heating (42 → 44°C sequence) was reduced by pretreatment of cells with 50% D₂O medium for 4 or 24 hours. In split hyperthermia (44 → 44°C sequence) experiments, when cells were incubated at 37°C for 24 hour-interval in 50% D₂O medium, survival of cells was markedly reduced, and the shoulder of the survival curve almost disappeared. Thus, thermal sensitization also was observed with pretreatment of the cells with D₂O.

INTRODUCTION

Many chemical agents and altered culture conditions, which modify thermal sensitivity, have been utilized to analyze the mechanism(s) of thermal cell killing. Most of these agents and culture conditions reveal sensitizing effects on thermal responses of cells1. In recent works, however, protective effects on thermal cell killing have also been reported with several agents2–4, including deuterated water (D₂O)5–7. With D₂O, thermal cell killing at temperatures above 43°C is markedly reduced when the hyperthermic treatment of cells was done in a medium containing D₂O5–7. At temperatures below 42°C, however, heating in D₂O medium enhances cell killing7. Thus, the effects of D₂O in hyperthermic treatments of cells are rather complicated. In the present study, the modifying effects of D₂O on thermal sensitivity of

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Chinese hamster V-79 cells were examined in different treatment sequences such as simultaneous or separate treatments with D$_2$O and hyperthermia at 44°C.

MATERIALS AND METHODS

Cell Line, Media and Culture Conditions

Chinese hamster V-79 cells were maintained in growth medium (MEM-15), 1 liter of which contained 850 ml of Eagle's minimum essential medium (MEM), 150 ml of inactivated bovine serum and antibiotics. The cells were incubated at 37°C in a humidity-saturated condition with a mixture of 95% air and 5% CO$_2$. In these conditions, doubling time of cells was about 10.5 hours. The media containing D$_2$O (99.8% purity) were prepared by dissolving MEM powdered medium in graded concentrations of D$_2$O with the addition of bovine serum and antibiotics in the same manner as above. Hence, a final concentration of D$_2$O shown in the following experiments indicates the volume percentage. The pH (or calculated pD) did not fall below 7.2 during the experiments.

Heating Procedures

A water bath (Toyo Seisakusho, Osaka) was used for hyperthermic treatments, and the temperatures, measured by an electrothermometer, was maintained to a set temperature within ±0.05°C. Details of the heating procedures were already described$^{5,9}$. In brief, exponentially growing cells were placed in 0.03% trypsin solution for about 3 min, the trypsin solution was siphoned off, and a single-cell suspension in MEM-15 medium containing D$_2$O or not was obtained. Cell concentration was measured using a Coulter counter. The cells were serially diluted with MEM-15 with or without D$_2$O to attain appropriate cell concentrations and then a 5-ml aliquot of each suspension was transferred into plastic tubes. These tubes were immersed in a water bath for 10 min at 37°C prior to hyperthermic treatments. After the different periods of hyperthermic treatments, cells were rinsed twice with MEM solution by centrifugation (360 x g for 10 min) at 4°C, and then 1 ml from each of the 5 ml cell suspension was seeded in plastic plates filled with fresh MEM-15 medium.

In the experiments with D$_2$O exposure followed by hyperthermia in normal medium, cells exposed to D$_2$O medium for various periods were rinsed twice with MEM solution and then exposed to hyperthermia in normal medium in the same manner as above. With these methods, cell loss in the tubes was less than about 10% of the inoculated cell number. The trypsinization did not significantly influence heat sensitivity.

Survival Criterion

After treatments, cells were incubated 6 to 8 days to obtain macroscopic colonies composed of 50 cells or more. The colonies were stained with Giemsa solution, and the number of colonies per plate was counted. The cell survival fraction after each treatment was routinely estimated as the ratio of the number of colonies formed and the number of inoculated cells. The estimated fractions were normalized to the surviving fraction of the untreated (no heat and D$_2$O) control. Four replicate plates were used for each survival point, and the replicate experi-
ments were repeated two or three times. The data point represent the mean of at least eight plates, and the standard deviation is given for each survival point.

RESULTS

Thermal Protection by Simultaneous Treatments with D₂O and 44°C Hyperthermia

Cells in D₂O medium of graded concentrations (20—85%) were exposed to 44°C hyperthermia for 40 min. Cell survival, as shown in Fig. 1, sharply increased with increasing D₂O concentrations. An apparent plateau level of survival was observed in D₂O concentration range from 35 to 70%, and maximum survival was obtained at 85% D₂O. A concentration of 50% D₂O was chosen for the following experiments. Cells in 50% D₂O or normal medium were exposed to 44°C hyperthermia for various periods. The survival curves are shown in Fig. 2. Survival levels for heating in D₂O increased markedly as compared with those for heating in normal medium. The difference in these survival levels increased with increasing time of heating. The results show that thermal protection was obtained by the simultaneous treatments with D₂O and 44°C hyperthermia.

Thermal Sensitization by Pretreatment of D₂O

Cells were incubated at 37°C in 50% D₂O medium for 4, 8 or 24 hours, and then exposed to 44°C hyperthermia in normal medium. The survival curves for these treatments are shown in Fig. 3. The treatment with 50% D₂O at 37°C was less toxic. For example, survival value for

![Image of survival curve](https://academic.oup.com/jrr/article-abstract/26/2/238/1006412)

Fig. 1. Survival curve of cells exposed to 44°C hyperthermia in D₂O medium. Cells in medium containing D₂O with graded concentrations (0—85%) were exposed to 44°C hyperthermic treatment for 40 min.
cells treated with 50% D₂O for 24 hours was approximately 0.7, and the doubling time of cells in D₂O medium was 17.0 hours. Survival of cells treated with D₂O medium followed by heating decreased markedly as compared with the control of heating alone. The thermal cell killing was enhanced to a similar extent by pretreatment of D₂O from 4 to 24 hours.

Recovery kinetics of the thermal sensitization by pretreatment of D₂O was examined. After exposure to 50% D₂O for 4 hours, cells were incubated at 37°C in normal medium for up to 24 hours and then exposed to 44°C hyperthermia for 10, 20, 30 or 40 min. Recovery curves are shown in Fig. 4. The enhanced thermal cell killing was observed immediately after the removal of D₂O medium, and was rapidly reduced with increasing interval time at 37°C. Survival levels for cells appeared to reach the control levels of heat alone in about 4 hours of the interval. With 6 hour-interval, cell survival, after 44°C hyperthermia for 30 or 40 min, increased as compared to that for heat alone. The survival levels did not further increase in the treatments with 24 hour-interval.

Effects of D₂O Treatment on Step-up (42 → 44°C) and Split (44 → 44°C) Hyperthermia

In step-up heating of 42 → 44°C sequence, cells after treatment with 50% D₂O for 4 or 24
Fig. 3. Hyperthermic treatment time-survival curves of cells exposed to 44°C hyperthermia with or without D₂O pretreatment. Cells in 50% D₂O medium were incubated at 37°C for 4 hours (●), 8 hours (○) or 24 hours (▲), and then exposed to 44°C hyperthermia in normal medium. For the control, cells were exposed to 44°C alone (△).

hours were exposed to 42°C hyperthermia for 2 hours and then to 44°C hyperthermia for various periods in normal medium. Figure 5 shows the 44°C hyperthermic treatment time-survival curves of cells in step-up heating with or without D₂O pretreatment. Surviving cells after exposure to 42°C for 2 hours became resistant to the subsequent 44°C hyperthermia. Survival levels for cells exposed to 42°C for 2 hours decreased by pretreatment with D₂O for 4 or 24 hours. When cells were treated with D₂O medium for 4 hours immediately before step-up heating, cell survivals were reduced as compared with those for the step-up heating without D₂O treatment. In the case of D₂O treatment for 24 hours, cell survivals were reduced still more. The slope of this curve was almost similar to that for 44°C hyperthermia alone.

In split hyperthermia of 44 → 44°C sequence, cells were exposed to 44°C hyperthermia for 25 min, successively incubated at 37°C for 24 hours in 50% D₂O or normal medium, and then further exposed to 44°C for various periods. Survival curves are shown in Fig. 6. Survival of cells exposed to split hyperthermia in normal medium was markedly increased as compared with that for single hyperthermia at 44°C. Thermotolerance developed during a 37°C incuba-
Fig. 4. Recovery curves of cells exposed to the combined treatments of D$_2$O followed by 44°C hyperthermia as shown in Fig. 3. After incubation in 50% D$_2$O medium for 4 hours, cells in normal medium were further incubated at 37°C for various interval times and then exposed to 44°C hyperthermic treatments. Treatment at 44°C is 10 min (○), 20 min (●), 30 min (△) or 40 min (▲). The "H$_2$O" on the abscissa represents the control of 44°C hyperthermic treatment alone.

...ation after the initial hyperthermic treatment. When cells were incubated in D$_2$O medium during the interval of split hyperthermia, survival levels of cells with or without the second hyperthermic treatment were markedly reduced. The slope of the curve for the second hyperthermic treatment became steeper than that for split hyperthermia in normal medium and the shoulder almost disappeared.

DISCUSSION

In the present study, thermal protection was observed when cells were heated at 44°C in medium containing D$_2$O (Figs. 1 and 2). Similar phenomena with D$_2$O have been reported at temperatures above 43°C$^5$-$^7$. It is well-known that D$_2$O stabilizes cellular components such as membranes, microtubules and some enzymes against heat treatments$^{10-13}$, and increases protein-protein aggregation$^{14}$. Further, deuteration of molecules reportedly results in stronger...
intramolecular binding and/or increase in the strength of hydrophobic interactions\textsuperscript{11, 15, 16}. Therefore, the thermal protection may be due to stabilization of cellular components \textit{in situ} by hydrogen $\rightarrow$ deuterium exchange. However, the critical targets protected from thermal damage are remained to be determined.

On the other hand, thermal sensitization was observed when cells were heated in normal medium immediately after the removal of D\textsubscript{2}O medium (Fig. 3). The sensitizing effect was rapidly minimized with increasing the interval time at 37°C between D\textsubscript{2}O and hyperthermic treatments (Fig. 4). Almost all of deuterium $\rightarrow$ hydrogen exchanges occurs in short time\textsuperscript{17, 18}. Hence, cellular components including membranes and some enzymes may become unstable to heat in the course of deuterium $\rightarrow$ hydrogen exchange. In addition, doubling time of cells incubated in 50\% D\textsubscript{2}O medium became longer (i.e., 17 hours) than that for the control, and D\textsubscript{2}O arrests or delays cell division without serious or immediate effects upon growth\textsuperscript{19, 20}. 

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Fig. 5. Hyperthermic treatment time-survival curves of cells exposed to step-up heating with or without D\textsubscript{2}O pretreatment. After incubation in 50\% D\textsubscript{2}O medium for 4 hours ($\circ$) or 24 hours ($\bullet$), cells in normal medium were exposed to step-up heating, i.e., 42°C hyperthermia for 2 hours followed by 44°C hyperthermia for various periods. For the control, cells unpretreated with D\textsubscript{2}O were exposed to 44°C hyperthermic treatment alone ($\circ$) and step-up heating ($\bullet$).
Fig. 6. Hyperthermic treatment time-survival curves of cells exposed to split hyperthermia at 44°C with or without D₂O treatment. After exposure to 44°C hyperthermia for 25 min, cells were incubated at 37°C for 24 hours in 50% D₂O (△) or normal (○) medium and then further exposed to 44°C hyperthermia for various periods. For the control, cells were exposed to a single hyperthermic treatment at 44°C (○).

As an another possibility for thermal sensitization, elongation of heat-sensitive M and/or S phases and resulting increase in the proportion of cells in these phases may occur in 4 hours of incubation in D₂O medium. With more than 6 hours of the fractionation interval, however, cells became significantly resistant to hyperthermia (Fig. 4). A small fraction of intracellular deuterium, which may be unexchangeable and remain for long time, would act to protect cells against hyperthermic damage. In experiments of 44°C split hyperthermia, incubation of cells in 50% D₂O medium during a 24 hour-interval at 37°C decreased cell survival before the second 44°C hyperthermia. From this finding, it is suggested that cellular damage caused by hyperthermic treatment may be magnified through hydrogen → deuterium exchange.

Heat induces thermotolerance. Thermotolerance in both fractionation hyperthermia (42 → 44°C and 44 → 44°C) was partially inhibited by D₂O treatment (Figs. 5 and 6). Li et al. tried the kinetical analysis concerning the modification of thermotolerance by using D₂O. Fisher et al. also reported that induction of thermotolerance in split hyperthermia at 45°C
was impaired by the presence of D₂O. Thus, in the presence of D₂O, the development of
thermotolerance appeared to be specifically inhibited or at least substantially slowed. Further,
it is of interest that prior exposure to D₂O also affects the induction of thermotolerance at
42°C (Fig. 5). In recent reports, heat shock protein(s) has been suggested to play an important
role in the development of thermotolerance 26-28. It would be possible that the intracellular
deuterium = hydrogen exchange may inhibit the synthesis of heat shock proteins and result
in inhibition of the development of thermotolerance. However, unknown action(s) of D₂O
cannot be ruled out as possible mechanisms for the inhibition of thermotolerance.

In the present study, we clearly demonstrated the thermal sensitization and impairment
of thermotolerance by pretreatment of cells with D₂O. From the data, besides deuterated
cellular organells, hydrogen = deuterium exchange processes also appeared to affect thermal
cell killing. Since the mechanisms of hyperthermic cell killing is not clarified yet, D₂O may be
of value as a probe in the analysis of hyperthermic cell death.

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