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Effects of high-energy-pulse-electron beam radiation on biomacromolecules

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To study the molecular mechanism of high mutation frequency induced by high-energy-pulse-electron (HEPE) beam radiation, the effects of HEPE radiation on yeast cells, plasma membrane, plasmid DNA, and protein activity were investigated by means of cell counting, gel electrophoresis, AO/EB double fluorescent staining, etc. The results showed that the viability of yeast cells declined statistically with increase of absorbed doses. The half lethal dose (LD_{50}) was 134 Gy. HEPE beam radiation had little influence on the function of plasma membrane and protein, while it could induce much DNA damage of single strand breaks (SSB) and double strand breaks (DSB) that were required for gene mutation. The *G*-value for DSB formation of HEPE beam radiation in aqueous solution was 5.7 times higher than that caused by ^{60}Co gamma rays. HEPE can be a new effective method for induced mutation breeding and deserves further research in the future.

high-energy-pulse-electron beam, biomacromolecule, radiation damage

Radiation, including γ-rays, low energy ion beam, fast neutrons, laser beam, UV radiation, etc. has been widely applied to induce mutation breeding and made great progress in plant and microorganism breeding. Many well-adapted and high-yielding varieties, such as wheat, cotton, rice, and industrial yeast and bacteria strains, have been selected and cultivated through radiation, bringing huge economic and social benefits to our society. However, since the efficiency of mutation induction and selection of current methods is relatively low and ineffective, induced mutation requires screening of large populations which is laborious and costly[1]. New effective methods with higher mutation creation ability are highly desired.

HEPE has mainly been used in pulse radiolysis[2] and material surface modification[3] to date. There have been few reports about the application of HEPE in mutation creation and selection. Some previous studies have revealed that HEPE radiation holds the virtue of small physiological damage in M1 generation and wide mutation frequency in M2 generation[4,5]. According to typical radiation biology theories, absorbed dose-rate exhibits its strong influence on relative biological effectiveness (RBE), which was called dose-rate effect relationship[6]. The higher the dose-rate is, the greater the RBE. The absorbed dose-rate of HEPE on biomaterials may reach 10^{10} Gy·s^{-1}, which is much higher than that of γ-rays (usually under 60 Gy·s^{-1}) and those of other radiation methods. With such high dose-rate, HEPE can produce high density radicals in a very short time, resulting in a large number of DNA double strand breaks, bringing mutation effects with high mutation efficiency and wide variety.

For the purpose of investigating the possible molecular mechanism of high mutation creation efficiency in HEPE breeding, the typical biomaterials of yeast cells, plasmid DNA, and cellulase protein were selected in this

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research, and the damage of HEPE on yeast cells, DNA, protein activity, and plasma membrane were minutely observed..

1 Experimental

1.1 Materials and apparatus

The original yeast strain YE1 (*saccharomyces cerevisiae*) was isolated from industrial alcohol mold culture. Plasmid PBR322 was provided by Protein Research Institute of Tongji University; acridine orange (AO) was purchased from Sigma-Aldrich Corporation.

High-energy pulsed electron accelerator was equipped in Shanghai Institute of Applied Physics, Chinese Academy of Sciences, with electron beam energy 10 MeV, electric current 1.7 A, pulse time 2 ns, and absorbed dose rate 10^9 Gys^{-1}.

1.2 Radiation and death rate measurement of yeast cells

Yeast YE1 was cultured in YPD medium (2% peptone, 1% Yeast extract, 2% glucose) at 37℃ for 48 h, and 1 mL cell suspension was centrifuged at 5000 r/min for 10 minutes, then air-dried in Clean Bench, sealed, and stored at 4℃. Samples were exposed to different absorbed doses at room temperature from 0 to 200 Gy. With 2 nanoseconds (ns) every pulse and 2 Gy each pulse, the dose rate of HEPE was calculated as 10^9 Gys^{-1}. After radiation, Yeast samples were diluted with sterile distilled water to 10^6 fold, and 200 μL volume of each dilution was inoculated to Petri dishes of YPD solid medium (2% peptone, 1% Yeast extract, 2% glucose, 2% agar). Total yeast number was counted after 48 h of incubation at 36℃. Count the total yeast flora of radiated and non-radiated samples, and calculate the death rates.

1.3 AO/EB double fluorescent staining of yeast cells[7]

Yeast (*saccharomyces cerevisia*) was cultured at 36℃ for 48 h. 1 mL cell suspension was taken and centrifuged at 10000 r/min for 30 seconds, and then washed twice in phosphate buffered saline (PBS, pH 7.2). Adjust cell concentration to 1×10^7 cells/mL, take 50 μL of this suspension to a new centrifugal tube, and then add 2 μL of fluorescence dye acridine orange/ethidium bromide (AO/EB, 100 mg/L), followed with 15 minutes of incubation at 37℃ in dark. Cells were then washed with PBS three times and observed under fluorescence microscope, with excitement wavelength 490 nm. Count 500 cells immediately.

1.4 DNA radiation and lesion analysis

Plasmid PBR322 was purified as described by Sambrook et al.[8]. After precipitation with 75% ethanol, plasmid DNA was resuspended in TE buffer (Tris-Cl, EDTA, pH = 7.6), $OD_{260/280}$=1.86, and DNA concentration was 3.2 g/L. 10 μL plasmid DNA was taken for each sample. Two groups of samples were prepared. One was DNA solution, and the other was dried in freeze-drying system for 4 hours, sealed and irradiated by different absorbed doses from 0 to 900 Gy. After radiation, DNA samples were diluted and loaded onto 1% agarose gel (TAE buffer, 5 V/cm, 50 min). Gel images were then recorded by a Gel Doc equipment of Bio-Rad Laboratories. DNA bands were quantitated using the image analysis software of Quantity one 4.5.2. The percentage of each type of plasmid DNA was calculated as described by Werner Friedland et al.[9].

1.5 Protein radiation and analysis of biological activity

Two groups of protein samples of cellulase were prepared, one was dried cellulase powder with 7.36% water percentage and the other was cellulase solution (100 g/L, TE buffer, pH=7.2). The samples were sealed and irradiated with different absorbed doses. After radiation, dried samples were also diluted to 100 g/L. Determine the enzyme activity of each sample and evaluate the damages caused by different doses of HEPE radiation.

Cellulase activity was measured using sodium carboxymethyl cellulose (CMC) as a substrate. The reaction mixture contained 1.5 mL of 1% CMC, 0.5 mL of cellulase samples. 3 mL DNS solution was added to the blank to stop enzymatic reaction, and then incubated at 50℃ for 30 min. After the above reaction, 3 mL of DNS was added to each sample (except the blank), and then 42 mL of distilled water was added to make a final volume of 50 mL. The absorbency was read at 530 nm.

1.6 Statistical analysis and equation fitting

Calculate the standard deviation of the data in repetitive experiments, fitting to different equation models by least square method according to the four dose-response equations provided by WHO and logistic model. Test the significance and Simulation Degree[10].

1) Linear equation: $Y = a + bD$; 2) Quadratic equation: $Y = a + cD^2$; 3) linear and quadratic equation: $Y = a + bD + cD^2$; 4) exponential equation: $Y = a + KD^n$; 5) logistic equa-

equation: $Y = a/[1 + \exp(b+cD)]$. Y: dose response value, D: absorbed dose.

1.7 Measurement of absorbed dose

Absorbed dose was measured by pulse radiolysis system, with Potassium thiocyanate dosimeter. Water radiolysis products convert the thiocyanato into anion free radical. According to the transient absorption of this anion free radical at 480 nm[11] (ε = 7600 $mol^{-1} \cdot cm^{-1}$), the absorbed dose of water can be calculated. Faraday pail and digital oscilloscope are used as measure instruments for real-time beam current, and then fit the data of absorbed dose and beam current into a linear equation. Absorbed doses of other samples received were then calculated by the data shown in an oscilloscope. Doses were corrected by the density ratio of samples to water.

2 Results and discussion

2.1 Dose response curve of yeast cells to HEPE

The dose response curve of yeast cells to HEPE is shown in Figure 1. Survived yeast counts decrease significantly ($P < 0.05$) with increasing radiation doses, fitting linear and quadratic model: $Y = 99.682 - 0.035D - 0.0025D^2$ ($P < 0.01$, $R^2 = 0.9585$). Y: survival rate of yeast cells (%). D: absorbed dose (Gy). The dose response curve fits the quadratic model, which indicates that HEPE cause minor physiological damage to yeast cells. The half lethal dose of HEPE for the yeast cells calculated is 134 Gy.

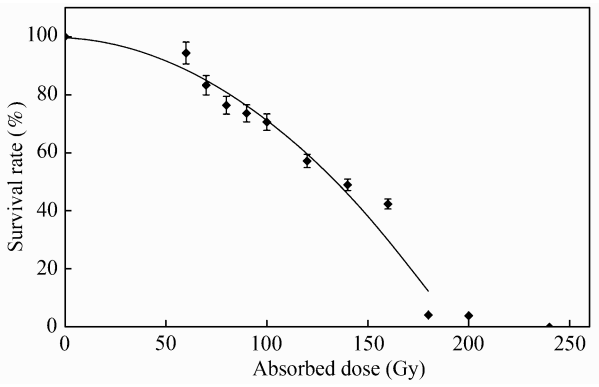

Figure 1 Dose-response curve of yeast cells to High-energy-pulse-electron beam radiation.

2.2 Cell membrane damage by HEPE

Cell membrane damage by HEPE was studied using AO/EB double fluorescent staining. Acridine orange (AO) permeates all cells and makes the nucleus appear green. Ethidium bromide (EB) is only taken up by cells when cell membrane integrity is lost, and stains the nucleus red. Thus live cells with intact plasma membrane appear a normal green nucleus; while cells that have lost membrane integrity present an overall orange even red appearance under a fluorescence microscope.

As shown in Figures 2 and 3, cells of blank control without radiation were almost all dyed green and the green-stained rate decreased tardily, fitting the linear model of $Y = 94.2748 - 0.0189D$ ($P < 0.01$, $R^2 = 0.9369$), Y: cell rate with intact membrane (%), D: absorbed dose (Gy).

The percentage of cells with intact membrane decreased tardily, from maximum 93.3% to minimum 81.2%, with the increase of radiation doses. At half the lethal dose of yeast cells (134 Gy), there is only 2.5% of damaged cell membrane. This indicates that HEPE causes very low direct damage to cell membrane.

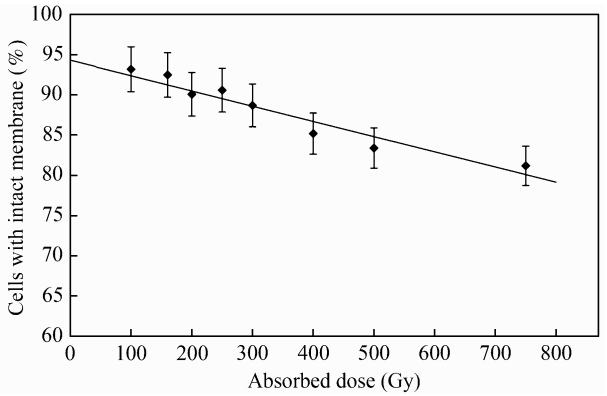

Figure 2 Membrane damage of yeast cells by high-energy-pulse-electron beam radiation.

2.3 Plasmid DNA damage by HEPE

Nature supercoiled plasmid DNA (SC) can be transformed into open circle forms (OC) and linear forms (LI) for single-strand-breaks and double-strand-breaks by ionizing radiation. Plasmid pBR322 was irradiated by different doses and the agarose gel electrophoresis results are shown in Figure 4. The percentage of each type of plasmid DNA was calculated and the results are shown in Figure 5. The value of supercoiled DNA obtained was corrected by a factor of 1.7 for the reduced binding of ethidium bromide to this form of DNA[12].

With the increase of absorbed doses, double strand breaks of plasmid DNA in aqueous solution increased more rapidly than dried plasmid DNA. This tendency fits the radiation-chemistry theory of aqueous solution.

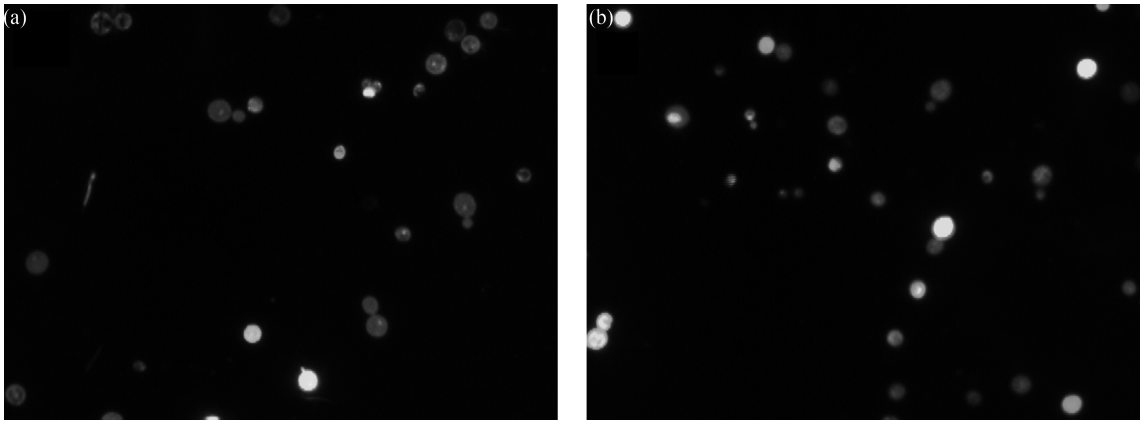

Figure 3 Acridine orange and ethidium bromide fluorescent staining of yeast cells. (a) Unirradiated cells; (b) irradiated cells of 750 Gy.

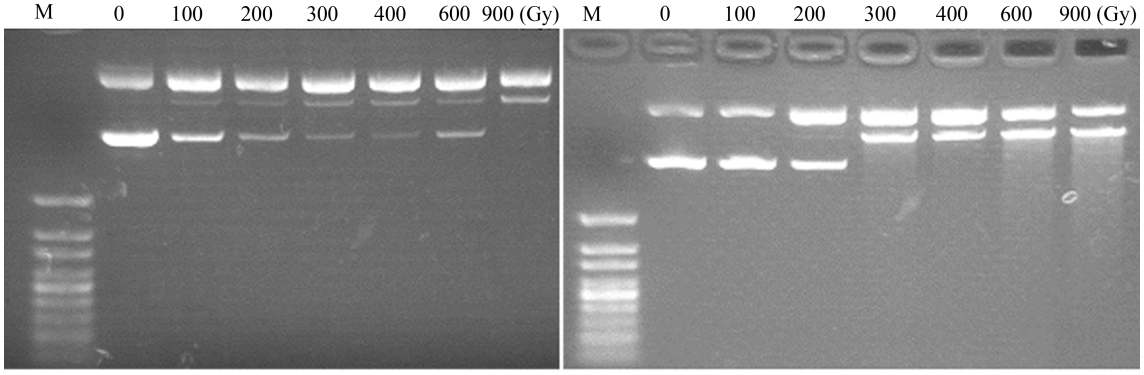

Figure 4 Plasmid DNA lesion analysis by agarose gel electrophoresis. The left is the dried DNA, and the right is aqueous DNA.

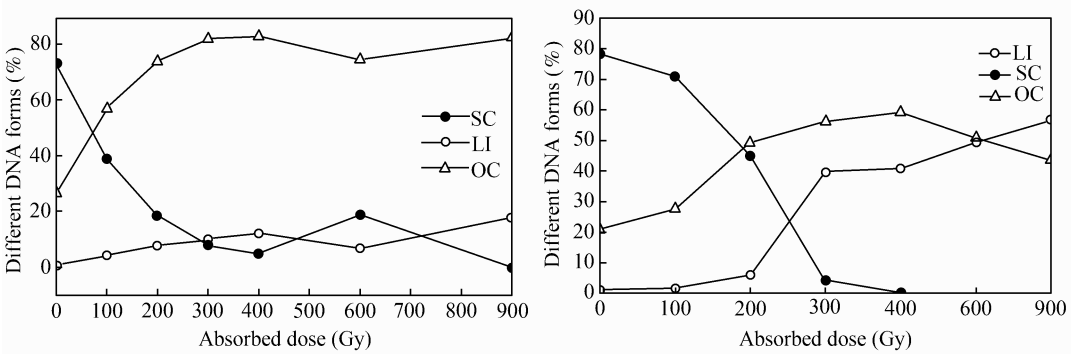

Figure 5 Quantitated plasmid DNA lesion analysis by High-energy-pulse-electron beam radiation. The left is the dried DNA, and the right is aqueous DNA.

Radiolysis Products of water contained plentiful HO·, which caused more DNA damage than in dried state. There was a special change at the dose of 600 Gy for dried plasmid DNA: the nature supercoiled forms increased while the linear and open circle firms decreased. The possible reason is that there are two processes proceeding in dried DNA during the process of radiation: strand breaking and cross linking, for the interaction of free-radicals during radiation process. At the dose of 600 Gy, the cross linked DNA produced more than the strand-broken DNA, thus causing the total increase of supercoiled plasmid DNA.

Furthermore, according to the percentage of LI and SC, The G-values for the formation of SSBs and DSBs [G(SSB) and G(DSB), respectively] can be calculated from the slopes of the fitted lines in plots of yield as described by Spotheim-Maurizot[13]. The dried plasmid DNA: $G(\mathrm{SSB}) = 1.01\times10^{-9}\ \mathrm{Gy^{-1}\cdot u^{-1}}$, $G(\mathrm{DSB}) = 5.25$

$\times 10^{-11}$ $Gy^{-1} \cdot u^{-1}$ and the plasmid DNA in aqueous solution $G(SSB) = 3.68 \times 10^{-10}$ $Gy^{-1} \cdot u^{-1}$, $G(DSB) = 2.43 \times 10^{-10}$ $Gy^{-1} \cdot u^{-1}$. The $G(DSB)$ value of DNA in water solution was greater than that in dried state. This result indicated that appropriate intracellular water ratio can improve the DNA lesion and thus improve the mutation efficiency.

2.4 Protein damage analysis of HEPE

With the increase of absorbed doses, cellulase activity increased first and then decreased as shown in Figures 6 and 7. Enzyme activity can be improved by low dose radiation, with the improvement of 6.82% for cellulase solution at 100 Gy and 3.37% for dried cellulase at 200 Gy. This phenomenon was also reported by other researchers[14]. High dose reduces the enzyme activity, and the maximum percentage loss tested is 13.48% (aqueous solution, 900 Gy). The possible mechanism is that at low dose, the radiolysis products of water react with cellulase protein, changing the molecular conformation, making the active center exposed more forwardly, thus improving the enzyme activity. While at high absorbed dose, numbers of amino acids are subjected to oxidative damage, destroying the molecular structure of the active center and thus depressing the enzyme activity.

In aqueous cellulase, water radiolysis products, including hydrated electron, hydrogen, and hydroxyl radicals, act with enzyme protein by addition or oxidation reaction, especially oxidizing the disulfide linkage, so

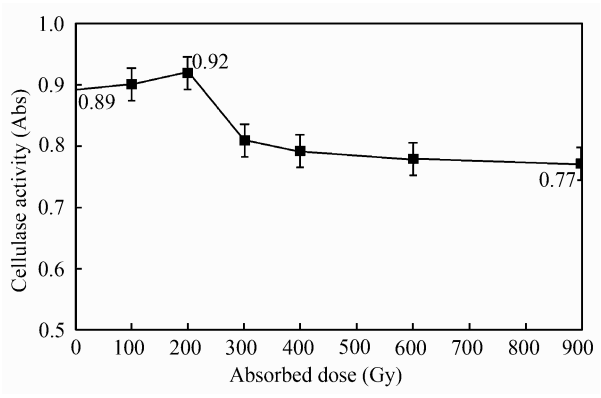

Figure 6 Relationship of doses and dried cellulase.

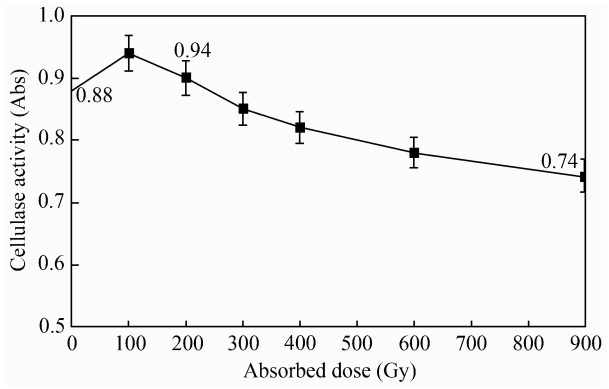

Figure 7 Relationship of doses and aqueous cellulase.

aqueous cellulase can be damaged stronger by HEPE radiation than dried cellulase. Besides, it should be taken into account that there is 7.36% water ratio in dried cellulose, and this partly compensated the difference. It can be concluded that comparing with the yeast cell death, HEPE radiation causes less damage to cellulase activity, with the maximum damage 13.5% at 900 Gy.

3 Conclusion

(1) In our result, the values of $G(SSB)$ and $G(DSB)$ of HEPE for plasmid DNA in aqueous solution are 3.68×10^{-10} $Gy^{-1} \cdot u^{-1}$ and 2.43×10^{-10} $Gy^{-1} \cdot u^{-1}$. However, by gamma radiation, they are 3.15×10^{-9} $Gy^{-1} \cdot u^{-1}$ and 4.23×10^{-11} $Gy^{-1} \cdot u^{-1}$ in Shao's research[15], and 6.53×10^{-9} $Gy^{-1} \cdot u^{-1}$ and 6.9823×10^{-11} $Gy^{-1} \cdot u^{-1}$ in Klimezak's report[16]. The G(DSB) of HEPE radiation in aqueous solution is 5.7 times higher than that of gamma radiation.

(2) Efficient mutation induction requires more DNA damage and less cell death rate. HEPE beam radiation causes low cell death rate, micro-lesion to plasma membrane, and protein activity, while producing enough DNA double strand breaks required for DNA mutation. The advantage of inducing high DNA lesion and low physiological damage to other vital biomacromoleculars is the main mechanism for HEPE to create high efficiency mutation, and HEPE can be used as a new effective breeding method to bring into application.

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