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Mechanism of Cancer Cell Death Induced by Hydrogen Discharged from Palladium Base Hydrogen Storage Alloy

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Abstract The mechanism of cancer cell death induced by hydrogen discharged from Pd-5at.% Ni hydrogen storage alloy has been investigated. Cancer cell (HeLa : cervical cancer cell) death was observed in the limited region within ~ 3 mm from the sample. The measurement of surviving fraction of cells revealed that almost all the cancer cells in the well of 96-well multi plate, 6.2 mm in diameter were extinct ($p < 0.01$), while no detectable influence was observed in the normal cells. From the fluorescent imaging experiment, it was indicated that the cell death induced by discharged hydrogen was due to the "Apoptosis" and hydrogen peroxide was detected in both intracellular and extracellular regions. Furthermore, the generation of hydrogen radical and hydroxyl radical was observed in the ESR measurement. From the results obtained, the mechanism of cancer cell death is proposed.

Introduction

Recently, the feasibility of the application of hydrogen to the treatment of diseases attracts attention in the medical field [1-5]. In an earlier work [6] on the application of hydrogen storage alloy (HSA) in the medical field, it has been found that hydrogen in a form of atomic hydrogen (hydrogen radical) causes the death of cancer cells, while no damage is detected in normal cells. In association with the mechanism of cancer cell death, it is reported that cytotoxic superoxide anion radical is generated in mitochondria [7] and superoxide dismutase converts it into hydrogen peroxide (H_2O_2) [8]. In normal cells, the enzyme (catalase) which is poor in the cancer cells detoxifies hydrogen peroxide into water : $2H_2O_2 \rightarrow 2H_2O + O_2$. Therefore, the selective influence of discharged hydrogen from HSA on cancer cells observed in the earlier work can be explained by a hypothesis that hydrogen radical emitted from the HSA induces the formation of superoxide anion and it is converted into hydrogen peroxide which brings a detrimental effect in the cancer cells, while in normal cells, hydrogen peroxide is decomposed by the enzyme, resulting in the selective death of cancer cells. However, the evidence for the hypothesis and the mechanism of cancer cell death is not well known in detail.

On the other hand, a biocompatibility is indispensable for the use of the HSA in human body. Pd-Ni solid solution alloys have superior characteristics, i.e., a good workability and an easy activation as well as an excellent biocompatibility. Moreover, since the plateau pressures can be controlled by adjusting alloy composition [9], a desired hydrogen discharging rate is easily obtained. The desorption plateau pressure of the Pd-Ni alloy increases with an increase in nickel content and the evolution of gaseous hydrogen is so violent in the alloy having a higher nickel content that the normal cells as well as the cancer cells may be suffered from a detrimental damage. It has been clarified [6] that Pd-5at.%Ni alloy has a mild evolution rate of hydrogen and hydrogen is continuously discharged over two days enough to induce the cancer cell death.

In the present work, Pd-5at.% Ni alloy is used as a hydrogen supplier and the mechanism of cancer cell death induced by hydrogen discharged from the HSA has been investigated.

Experimental

Sample Preparation

Pd-5at.%Ni alloy (denoted HSA hereafter) was prepared by arc-melting using pure materials (palladium > 99.9 wt% and nickel > 99.9 wt%) under argon gas atmosphere. After repeated rolling and annealing for stress relief at 923 K for 2 h, the alloy was rolled into a thin plate with 80 μm thickness and cut into a rectangular sheet of 2 mm wide and 4 mm long (ribbon sample). In the cell experiment, a cylindrical sample 3 mm in diameter and 80 μm thick was prepared for the observation of cell death region.

Cell experiment

In order to elucidate the range of cell death, HSA sample charged with hydrogen, denoted by H(+)HSA, and that free from hydrogen, denoted by H(-)HSA, were put in a laboratory dish (35 mm in diameter) containing cultured cancer cells (HeLa : cervical cancer cell) in medium solution. After incubating at 310 K for 24 h under 5 % CO_2 gas flow and 95 % humidity, the biological cells were observed using an optical microscope (OM). Then dead cells were stained by Trypan-blue reagent (dyeing for detection of dead cells) and the range of dead cells was measured using a stereomicroscope (SM).

In the measurement of surviving fraction of cells, normal cell (MDCK : renal epithelial cell) and cancer cell (HeLa) were employed. H(+)HSA and H(-)HSA ribbon samples were put in the wells of 96-well multi plate, 6.2 mm in diameter. The observation of cells was performed using OM and SM. The survival fraction of cells was measured using a cell counting kit (Cell Counting Kit-8).

Detection of Reactive Oxygen Species

Besides hydrogen radical (H・), Reactive Oxygen Species (ROS), such as superoxide anion ($\cdot O_2^-$), hydroxyl radical (・OH) and hydrogen peroxide (H_2O_2), are expected to be formed through the reaction with hydrogen discharged from the HSA. Electron spin resonance (ESR) measurement was carried out using a flat cell to detect H・ and ROS radicals. The HSA samples were put into ultra pure water for 24 h. Then radical trapping reagent CYPMPO (2-(5, 5-Dimethyl-2-oxo-2 λ 5-[1, 3, 2] dioxaphosphinan-2-yl) -2-methyl-3, 4-dihydro-2H- pyrrole 1-oxide) (10mM) was dissolved into the pure water and the solution was subjected to ESR measurement.

In order to detect H_2O_2 generated in the extracellular region (extracellular-H_2O_2), H_2O_2 Assay Kit (BIOXYTECH H_2O_2-560) was used. The HSA samples were put into ultra pure water for 6 h and 12 h. Then, the ultra pure water solution was poured in the reagent (mixture of R1 and R2, R1: Ammonium iron(II) sulfate + H_2SO_4 , R2: Sorbitol + Xylenol orange in water). After keeping the solution at room temperature for 30 min, absorbance spectrum near 560 nm was measured using a visible-ultraviolet spectrophotometer.

In association with the extracellular-H_2O_2, the generation of H_2O_2 in the cells (intracellular-H_2O_2) was also investigated using H_2O_2 detection reagent, DCDHF (2-(2, 7-dichloro-3, 6-diacetyloxy-9H-xanthen-9-yl)-benzoic acid). The alloys containing absorbed hydrogen H(+)HSA and free of hydrogen H(-)HSA were immersed in the wells containing cultured HeLa cells for 2 h, 4 h and 6 h. DCDHF reagent was simultaneously poured into the wells. Then, fluorescent image was observed using OM.

In order to detect ・OH formed in the intracellular region (intracellular-・OH) and the extracellular region (extracellular-・OH), Aminophenol Fluorescein (APF) reagent was used. For the detection of extracellular-・OH, APF reagent (5mM) was dissolved into PBS (Phosphate Buffered Saline). Thereafter, H(+)HSA ribbon samples were immersed into PBS. The intensity of fluoresce at 490 nm (fluorescence excitation wavelength) and 515 nm (fluorescence emission wavelength) was measured after 0.5h-, 2h-, 24h- and 48h-immersion of the HSA.

For the detection of intracellular-•OH, H(+)HSA was put in the laboratory dish where HeLa cells were cultured. After 6 h-immersion of the HSA, the PBS solution containing APF reagent was simultaneously poured into the dish and fluorescent imaging was performed.

Results and Discussion

Cell experiment

Cylindrical H(+)HSA sample was put in the 35 mm dish where cancer cells (HeLa) were cultured. Fig.1 shows the appearance of HeLa cancer cells near the HSA after 24h-immersion of the alloy. The dark blue region shows the dead cells. Cancer cell death is seen in a limited region, i.e., inside of the sample and ~3 mm outside of the sample. No influence is seen in the outer region beyond ~3 mm from the sample.

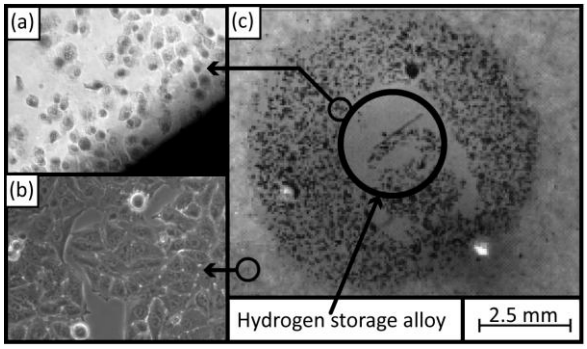

Fig. 1 The appearance of HeLa cancer cells near cylindrical Pd-5at.%Ni alloy (HSA) after 24h-immersion of the alloy. (a) dead cancer cells, (b) living cells and (c) the range of cell death, stained by Trypan-blue reagent.

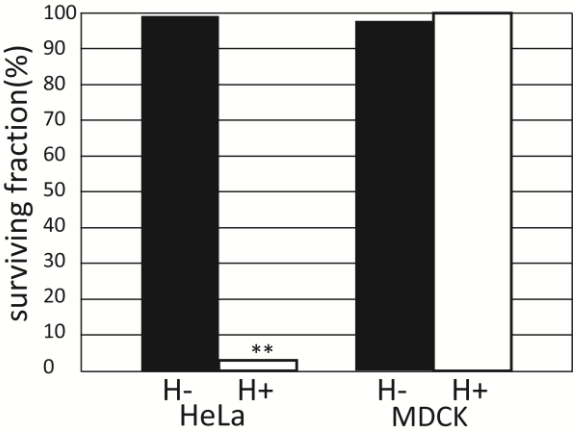

Fig. 2 The surviving fraction of HeLa cells after 48h-immersion of H(+)HSA and H(-)HSA. $^{**}P < 0.01$, by Student's t-test (n=4)

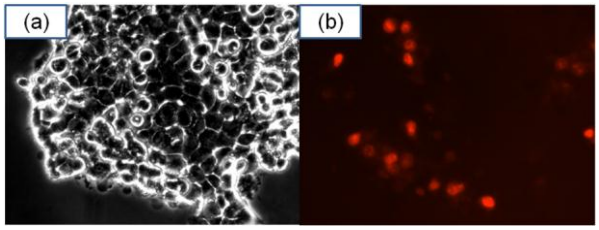

Fig. 3 Detection of dead HeLa cells using the reagent (Annexin V) for Apoptosis detection after 6 h-immersion of H(+) HSA.

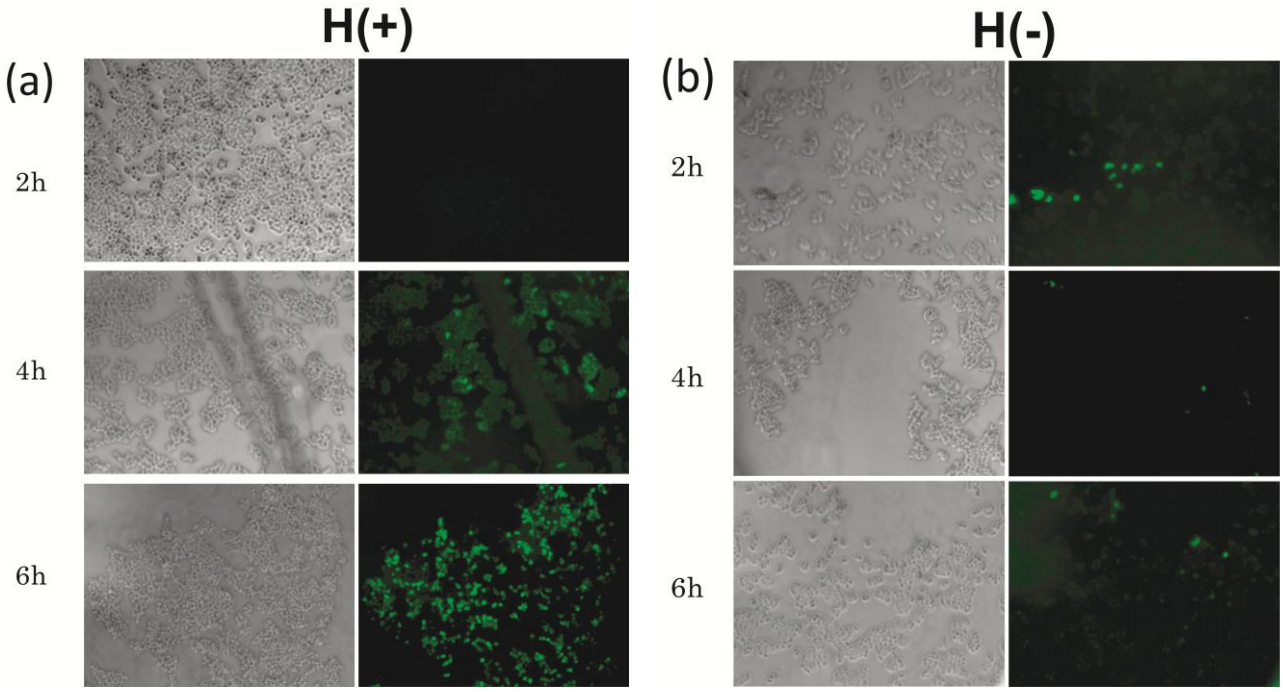

Fig. 4 Fluorescence images of H_2O_2 in cancer cells after 2~6h-immersion of (a) H(+)HSA and (b) H(-)HSA.

The surviving fraction of those cells after 48h-immersion of the H(+)HSA and H(-) HSA samples is shown in Fig.2. As compared with H(-)HSA sample, the susceptibility to discharged hydrogen are quite different. Cell death is observed only in the cancer cells, while no influence in the normal cells. Fig. 3 shows the fluorescent image of dead HeLa cells after 6h-immersion of H(+) HSA ribbon sample, using the reagent (Annexin V) for Apoptosis detection. A part of the cancer cells shows Apoptosis signals (in dead cells), indicating that the cell death induced by discharged hydrogen is possibly due to apoptosis, i.e., programmed cell-death (PCD).

Mechanism of Cancer Cell Death

It is well known that cytotoxic superoxide anion ($\cdot O_2^-$) is generated in mitochondria [7]. Then, superoxide dismutase (SOD) converts it into H_2O_2 [8] and an enzyme, such as catalase and glutathione peroxidase, detoxifies H_2O_2 into H_2O and O_2 in normal cells. Since the enzyme is poor in the cells, a very harmful hydroxyl radical ($\cdot OH$) that reacts with nucleic acid and proteins is possibly produced. Fig.4 shows the fluorescent image obtained using the H_2O_2 Detection Assay Kit, indicating the generation of intracellular-H_2O_2 in the dish containing HeLa cancer cells after various time from the immersion of the H(+)HSA and H(-)HSA samples. The generation of intracellular-H_2O_2 was also confirmed using anti-oxidant regeants, Ebselen and NAC, where the regeants scavenge oxygen, resulting in no fluorescence signal of H_2O_2. As seen in the stereomicrograph (left) and its fluorescent image (right), a significant cell death is detected from 6h and the fluorescent image is intensified at the same time. The reaction for the generation of H_2O_2 is supposed as : $H\cdot + O_2 \rightarrow H^+ + \cdot O_2^-$ in the solution and $2H^+ + 2\cdot O_2^- \rightarrow H_2O_2 + O_2$ in the cells (the latter reaction proceeds under the action of the enzyme SOD). The hydrogen radical possibly causes these reactions. Therefore, the existance of hydrogen radical discharged from the HSA as well as other radicals was examined by ESR measurement. Fig.5 shows ESR spectrum of medium solution after 24h-immersion of H(+)HSA sample. The generation of $H\cdot$ and $\cdot OH$ is detected in the solution, although no peaks of $\cdot O_2^-$ is seen in the spectrum. It is well known that H_2O_2 can easily pass the cell membrane. Hence, the detected

H_2O_2 (intracellular-H_2O_2) in Fig.4 possibly arises from the extracellular-H_2O_2 formed in the solution through the reaction and 2 H· + O_2 → H_2O_2. Fig.6 shows a change in H_2O_2 concentration in medium solution after 6h- and 12h-immersion of H(+)HSA sample. The intensity at the wave length of 560 nm increases with time, exhibiting the generation of extracellular-H_2O_2.

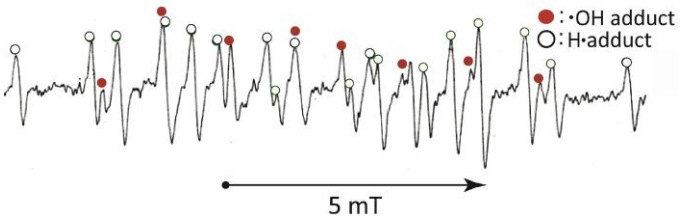

Fig. 5 ESR spectrum of medium solution after 24 h-immersion of H(+)HSA.

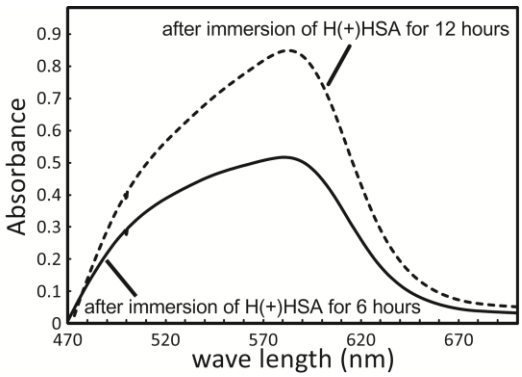

Fig. 6 Absorbance spectra of H_2O_2 in medium solution after 6h and 12h-immersion of H(+)HSA.

(a) (b) (c)

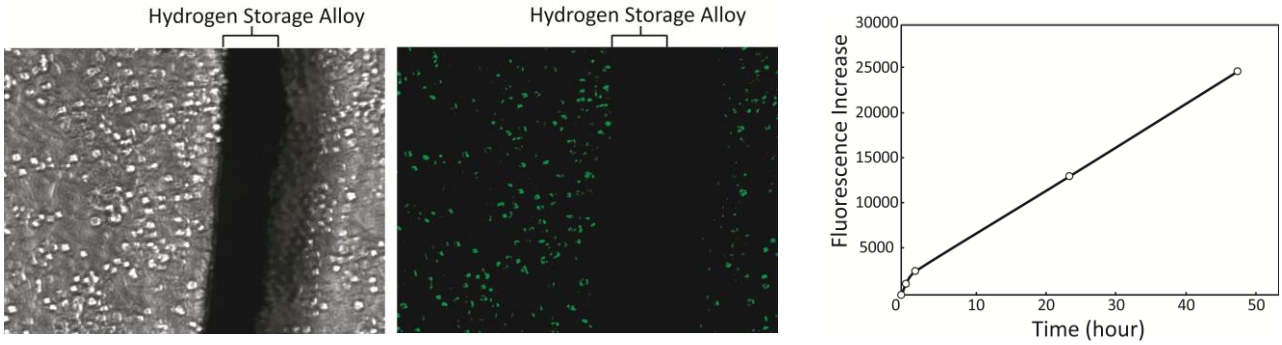

Fig.7 Microstructure of cancer cells. (a) under OM, (b) fluorescence image of ·OH radicals and (c) the change in fluorescence intensity of ·OH radical after immersion of H(+)HSA in PBS with time.

As seen in Fig.5, ·OH which is very harmful oxygen radical is detected in the solution. An inferred reaction for the generation of ·OH is : H· + H_2O_2 → H^+ + ·OH + OH^-. It is well known that ·OH can be formed through "Fenton reaction" (Fe^{2+} + H_2O_2 → Fe^{3+} + ·OH + OH^-) in the biological cells. Fig.7a shows the result of ·OH detection in HeLa cancer cells using the APF reagent. A distinct fluorescence image is seen in accord with the OM image of cancer cells and the amount of ·OH linearly increases with time (Fig.7c).

From the results obtained, the mechanism of cancer cell death is summarized in Fig.8.
(i) Hydrogen radical (H•) is discharged from the HSA : $H_{lat} \rightarrow H_{abs}$ (= H•) on the surface of HSA, (ii) The hydrogen radical reacts with oxygen in medium solution to form extracellular-H_2O_2 : $2\,H\bullet + O_2 \rightarrow H_2O_2$, (iii) The hydrogen peroxide formed in the solution transfers into the cell through the cell membrane : $H_2O_{2\ in\ sol.}$ (extracellular-H_2O_2) $\rightarrow$ $H_2O_{2\ in\ cell}$ (intracellular-H_2O_2) , (iv) In normal cells, intracellular-H_2O_2 is decomposed into oxygen and water under an action of the enzyme (catalase) : $H_2O_2 \rightarrow H_2O + O_2$, protecting the cells from the cytotoxic hydrogen peroxide. On the other hand, in cancer cells, intracellular-H_2O_2 is converted to hydroxyl radical by Fenton reaction : $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^{\cdot}$. This harmful hydroxyl radical attacks nucleic acid and proteins in cancer cells, leading to a selective death of cancer cells.

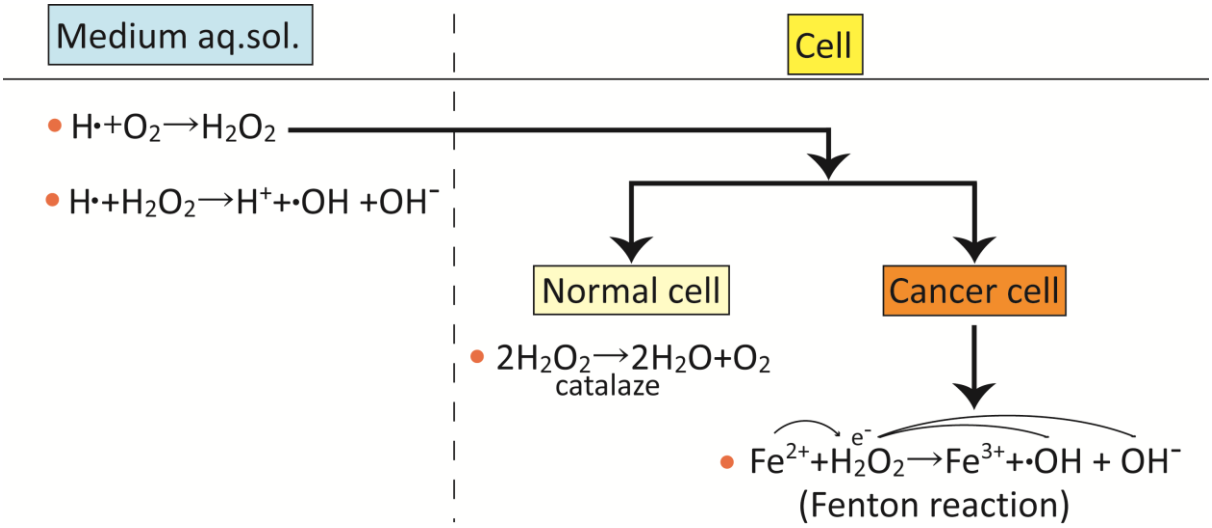

Fig. 8 Mechanism of cancer cell death.

Summary

The influence of hydrogen discharged from Pd-5at%Ni hydrogen storage alloy (HSA) on biological cells and the mechanism of cancer cell death has been investigated. Cancer cell (HeLa) death was observed in the limited region within ~ 3 mm from the sample. From the surviving fraction measurement, it was found that almost all the cancer cells in the well of 96-well multi plate were extinct ($P < 0.01$), while no detectable influence was seen in the normal cells. In the fluorescent imaging experiment, it was indicated that the cell death induced by discharged hydrogen was possibly due to the "Apoptosis" and hydrogen peroxide was generated in both intracellular and extracellular regions. Furthermore, the generation of hydrogen radical and hydroxyl radical was detected in the ESR measurement. The latter radical was also detected in the fluorescent imaging experiment.

From the results of cell experiment and fluorescent imaging, the mechanism of cancer cell death caused by discharged hydrogen from the HSA is proposed as follows;

1) Hydrogen radical (H•) is discharged from the HSA.

$H_{lat} \rightarrow H_{abs}$ (= H•) on the surface of the HSA (1)

2) The hydrogen radical reacts with oxygen in medium solution to form hydrogen peroxide.

$2\,H\bullet + O_2 \rightarrow H_2O_2$ in the solution (2)

3) The hydrogen peroxide formed in the solution transfers into the cell through the cell membrane.

$H_2O_{2\ in\ sol.} \rightarrow H_2O_{2\ in\ cell}$ (3)

4) In the normal cells, hydrogen peroxide is decomposed into oxygen and water under an action of the enzyme (catalase) and this reaction protects the cells from the cytotoxic hydrogen peroxide.

$H_2O_2 \rightarrow H_2O + O_2$ (4)

In the cancer cells, hydrogen peroxide is converted to hydroxyl radical.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^{\cdot} \quad \text{(Fenton reaction)} \qquad (5)$$

This harmful hydroxyl radical attacks nucleic acid and proteins in cancer cells, leading to a selective cancer cell death.

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