

Oxygen effect in radiation biology: Caffeine and serendipity

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The ‘hit theory’ developed in 1920s to explain the actions of ionizing radiation on cells and organisms was purely physical, and its limitation was its inadequacy to address the contemporary findings such as the oxygen enhancement of radiobiological damage, and the increased radiosensitivity of dividing compared to non-dividing cells. The textbooks written prior to 1970s did not either refer at all to oxygen as a radiosensitizer, or had mentioned it only in a passing manner; yet ‘oxygen effect’ was emerging as the central dogma in radiation biology! The oxygen effect in radiation biology is highly interdisciplinary encompassing atomic physics (i.e. interaction of photon with matter), radiation chemistry (formation of reactive oxygen species), molecular signalling, gene expression and genetic alterations in cells (mutation, cancer) or the cell death (apoptosis, necrosis, mitotic catastrophe, etc.). Cell death in higher organisms is now recognized as the precursor of possible error-free cell replacement repair.

Keywords: Oxygen effect, radiation biology, caffeine, serendipity.

THE early interpretation of the action of ionizing radiation on cells and organisms was based on the ‘hit’ or the ‘target’ theory, which regarded photons as bullets penetrating vital targets within the cells. This theory developed in 1920s could not, however, explain the contemporary observations such as oxygen enhancement of radiobiological damage in seeds¹ and *Ascaris* eggs² and the increased radiosensitivity of the dividing cells³. Thus, Lea⁴, a champion of the ‘target theory’ who wrote the first textbook on radiation biology, did not refer even once to oxygen as a radiosensitizer in his book of 416 pages. Another early textbook by S. M. Bacq and P. Alexander⁵ was also very inadequate in its treatment of whatever knowledge had been gathered by then on oxygen effect in radiobiology. Yet, M. S. Swaminathan’s two-credit lecture course on ‘Radiation Genetics’ during the early 1960s dealt with oxygen effect in such great detail, that it brought out elegantly the problem of radioresistant hypoxic tumour cells in cancer radiotherapy. At about the same time, research had begun at the Walter Reed Army Research Institute, USA, to develop chemical compounds that would afford greater radioprotection to normal cells than to cancer cells. Parallel research centred around development of compounds, which would preferentially potentiate the radio-

sensitivity of the hypoxic cells in solid tumours. It should thus be emphasized that the chemical modifiers of radiobiological damage were considered to fall into two distinct watertight compartments, viz. radioprotectors and radiosensitizers.

Caffeine, during the 1960s and early 1970s, was regarded as a radiosensitizer based on its potentiation of the UV-induced DNA damage in bacterial cells and mammalian cells *in vitro*. In those days, it was generally assumed that what is true for UV also held good for X- and γ -rays. Over one hundred papers by early 1970s had reported that caffeine potentiated the DNA damage induced by physical and chemical clastogens and mutagens by inhibiting the DNA repair enzymes and/or binding to sites of DNA lesions. These are catalogued by Kihlman⁶.

Serendipity and turning point

Having joined the newly formed Jawaharlal Nehru University (JNU), New Delhi in December 1970, I had the major responsibility of developing the laboratories and buildings, which involved frequent discussions with the architect and engineers and visits to the sites. The prospect for starting scientific research for the next couple of years seemed grim but for the generous offer of M. S. Swaminathan, then the Director, IARI to make use of his (as Director, IARI) laboratory space close to the IARI auditorium for initiating scientific work. The first thing that came to his mind was to initiate research centering around ‘oxygen effect’ which by then had become the central dogma both in basic and applied radiobiology for radiotherapy. Since the radiation-induced free radicals had been known to react with oxygen, their role in the development of ‘oxygen effect’ had become greatly recognized. One thing that needed careful consideration, however, was that the reactions involving free radicals and oxygen as well as their reaction products with vital target molecules and organelles in the cells are ultra-fast events and therefore, without fast kinetic research facilities, it would not be possible to pursue these in any meaningful manner. The determination of the lifetimes of potentially damaging oxygen-dependent free radicals (i.e. oxygen-sensitive sites) by various fast response techniques such as fast transfer, rapid mix, double-pulse depletion, gas explosion, liquid flow fast mixing and stopped flow mixing^{7–10} provided physical evidence of the ‘oxygen fixation’ hypothesis. The implication of this hypothesis is that

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in the absence of oxygen, several of the sequences of events initiated in the cells after the absorption of the low LET radiant energy are harmlessly recombined/quenched. An alternative to using fast kinetic facilities in cells with active metabolism is to use the common laboratory facilities in biological systems with highly reduced/retarded metabolic activity. In the USA, while E. L. Powers had standardized dry spores of *Bacillus megaterium* for such studies, R. S. Caldecott had chosen hull-less (naked) barley seeds (caryopses). Many advantages were indeed associated with barley seeds (IB65), obtained from K. B. L. Jain of IARI. Being pure-line inbred and self-pollinated, it was remarkably uniform in growth, so that seedling height (at 7 days after germination at constant temperature ($25 \pm 2^\circ\text{C}$) and illumination) could be used as a parameter. Having small number of somatic complement of chromosomes ($2n = 14$), the radiation-induced bridges and fragments at anaphases could be accurately scored in the early mitoses of the shoot-tip cells. The M_2 chlorophyll mutations, which could be readily scored, provided an excellent *genetic* parameter of radiation effect and its possible modification by any chemical or physical agent. It was, however, considered desirable to avoid the contact of the seeds *during* irradiation with air since passive diffusion of air (oxygen) into the seed could result in reaction of the radiation-induced free radicals with oxygen, and this would diminish the accuracy of assessing such reactions or their harmless decay (radical–radical recombinations) under controlled experimental conditions. This necessitated a fabrication of a glass manifold vacuum system to which seeds in glass ampoules could be fitted, evacuated to $\sim 10^{-3}$ Torr and then sealed off with a glass-working torch. With a design given by me, the late Hungarian glass technologist, I. Kiss at the National Physical Laboratories (NPL), New Delhi fabricated and got it tested successfully.

The experiments which had been planned were to assess *the times within which* the radiation-induced oxygen-sensitive sites (A_n sites) in seeds of varying moisture content would react with oxygen administered during post-irradiation oxic-hydration containing $\sim 1.8 \times 10^{-3}$ M of oxygen or would harmlessly decay following their post-irradiation anoxic hydration (containing $\leq 10^{-6}$ M O_2). The development of post-irradiation oxic damage would be assessed in terms of M_1 seedling injury, M_1 chromosomal aberrations in the early mitoses of shoot-tip cells, and M_2 chlorophyll mutations. Initial trials to check if everything was in perfect working condition had been started, when serendipity struck. This happened in the nature of stumbling upon the paper published by Ahnström and Natarajan¹¹. Their finding was that caffeine potentiated the gamma-ray-induced damage in germinating barley seeds probably by inhibiting the DNA repair, but it did not potentiate the damage induced by neutrons. They had explained the results on the basis of the differential spatial distribution of the DNA lesions induced by gamma-rays (sparsely ionizing) and neutrons (densely ionizing). Since the oxygen enhancement of radiobiological damage induced by neutron is far less than that induced by low LET X- and γ -rays (an aspect discussed in detail by M. S. Swaminathan in his research papers and lectures), I decided to test the effect of caffeine on the gamma-ray induced oxic and anoxic pathways of damage with their newly fabricated system. Thus, the experiments were initiated with caffeine. The very first experiment was rejected by the research scholar concerned, since caffeine protected the seeds against oxic pathway of damage. The second experiment also similarly yielded results contrary to the expectation based on the reports in the literature. That caffeine protected the irradiated seeds post-hydrated in oxic water was totally puzzling (Figure 1). The quality of caffeine even came under suspicion.

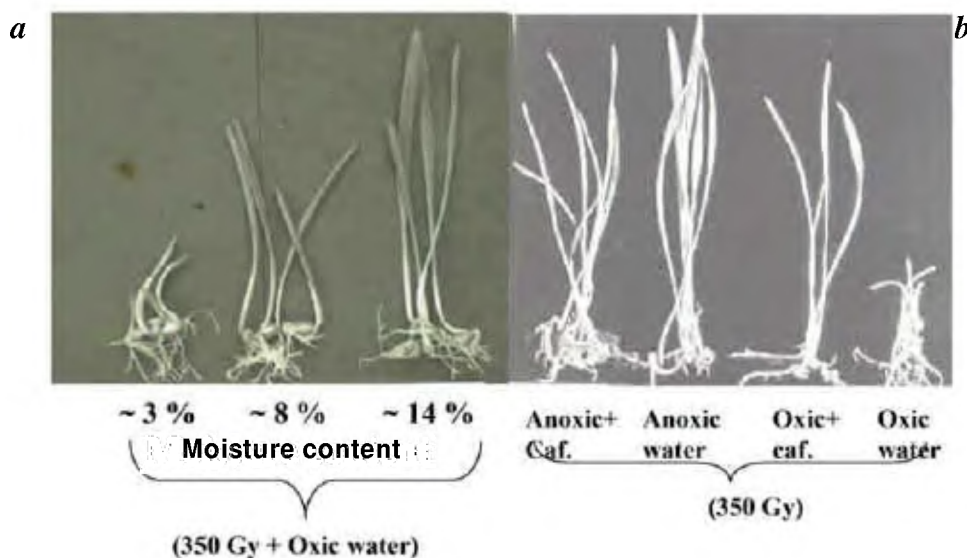


Figure 1. *a*, Development of O_2 -dependent seedling injury as a function of initial seed moisture content. There is no oxic damage in seeds of 14% moisture content; *b*, Caffeine protects against oxic damage but potentiates anoxic damage.

In each case, there was noticed remarkable potentiation of the seedling injury ascribable to the anoxic pathway of radiation damage. So, the experiment was repeated several times under the personal supervision of the author and all the experiments consistently showed that caffeine very significantly protected the barley seed system against post-irradiation oxygen-dependent (oxic) pathway of gamma-ray induced damage, but it potentiated the anoxic pathway of damage.

The results communicated to *International Journal of Radiation Biology (IJRB)* were rejected within a very short interval of time. All the three referees summarily rejected the paper. At that time the late A. R. Gopal-Ayengar, then the Director, Bio-Medical Group of Bhabha Atomic Research Centre, Bombay happened to visit New Delhi, and a fortuitous cancellation of his return flight to Bombay brought him to JNU. This was an opportunity for me to show him the caffeine experiments, discuss the results and the rejection of the manuscript by *IJRB*. The then editor-in-chief of *IJRB* (late M. Ebert) had great respect for Gopal-Ayengar, and had recognized him as an eminent radiation biologist. Gopal-Ayengar, once again communicated the paper with his own comments and recommendation to the editor-in-chief. The *IJRB* reconsidered its decision and agreed to publish the paper on a *condition* that a suitable postulate be put forward to explain the seemingly 'aberrant behaviour' of caffeine in the New Delhi barley system! Since over a hundred papers published by 1972 had already condemned caffeine as a harmful sensitizer of DNA damage induced by physical and chemical agents, I postulated that caffeine molecules and the radiation-induced oxygen-sensitive sites undergo 'mutually annihilatory reaction' to result in radioprotection against the oxic-pathway of damage. The paper was then accepted and thus the first paper was published in the *IJRB* in 1973. That this is indeed the mechanism of radioprotection was later demonstrated by me and my coworkers during the 1980s and 1990s using fast kinetic studies. These results briefly presented in this paper are dedicated to my great teacher M. S. Swaminathan, on the occasion of his 80th birthday. Coming to think of it, the fortuitous visit of late Gopal-Ayengar and his close scientific interaction and personal friendship with late Ebert, the then editor-in-chief of the *IJRB* also seem providential.

From observation to elucidation of mechanisms

The observations^{12–16} that caffeine affords significant radio-protection against oxic pathway of damage, but potentiates the anoxic pathway of radiation damage assessed in terms of M_1 seedling injury, M_1 chromosomal aberrations and M_2 chlorophyll mutations were followed by kinetic studies. These studies revealed that the rates of reaction of the radiation-induced O_2 -sensitive sites (A_n) with oxygen, as well as their decay in the absence of oxygen are dependent on both the initial seed moisture content and the post-hydration temperature. The method of deriving oxygen-sensitive sites from kinetic studies involving transfer of irradiated seeds from oxic to anoxic post-hydration and vice-versa is elaborated elsewhere^{17,18}. There is no significant radio-protection by caffeine when applied after 50 per cent of the radiation-induced oxygen-sensitive sites (A_n) have reacted with oxygen. Parallel experiments were performed to assess the influence of initial seed moisture content and post-hydration temperature on *two opposing processes*, viz. development of oxic damage due to reaction of A_n sites with oxygen, and their harmless decay during oxygen-free (anoxic) hydration. Several of these studies^{17,18} revealed that the radiation-induced oxygen-sensitive sites (A_n) induced by 350 Gy γ -rays in seeds of 3, 8 and 9% moisture contents react with oxygen 6 to 8 times faster than the rate of their decay in the *absence* of oxygen at 3 and 25°C; however, at 37°C, they react only 3 to 4 times faster with oxygen than their decay in the absence of oxygen in seeds of all the three, viz. 3, 8 and 9% moisture contents. Further, the initiation of decay of A_n sites in oxygen-free hydration starts much sooner in very dry (~3% moist) seeds than in those of 8 and 9% moisture contents (Table 1). This indicates the decay of a 'fast component' within the oxygen effect in dry seeds (~3% moisture content). Kinetics of development of chromosomal aberrations in shoot tip cells (seeds of 3% moisture) irradiated (350 Gy γ -rays) and then post-hydrated at $3 \pm 1^\circ\text{C}$ in oxygenated water *before* transfer to oxygen-free water are given in Table 2.

Actual physical evidence of the reaction of caffeine with the radiation-induced oxygen-sensitive species was obtained when I carried out part of the studies at the Centre for Fast Kinetic Research (CFKR), University of Texas at Austin (1984–85), and during my tenure at the BARC, Bombay

Table 1. Effect of initial seed moisture content and post-hydration temperature on the $t^{1/2}$ of decay of A_n sites in the absence of oxygen and time after which the decay of A_n sites is initiated

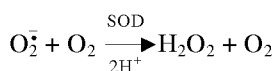
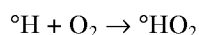
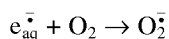
Post-hydration temperature	Initial seed moisture content			
	~3%		~8%	
	$t^{1/2}$ of decay of A_n sites (min)	Time after which decay of A_n sites is initiated (min)	$t^{1/2}$ of decay of A_n sites (min)	Time after which decay of A_n sites is initiated (min)
$3 \pm 1^\circ\text{C}$	194	< 5	234	120
$25 \pm 1^\circ\text{C}$	29	< 5	30	15

Source: Afzal and Kesavan¹⁸.

(1994–98). Several papers published^{19–22} established that caffeine reacts with the radiation-induced oxygen-sensitive species and reactive oxygen species (ROS) with reaction rate constant K ($M^{-1} s^{-1}$) given in Table 3.

Caffeine at a concentration of $5 \times 10^{-3} M$, and with reaction rate constant (K) of $1.5 \times 10^{10} M^{-1} s^{-1}$ for electrons¹⁹, would have a scavenging efficiency (KC) of $7.5 \times 10^7 s^{-1}$; in comparison, oxygen with its maximum intracellular concentration of about $1.8 \times 10^{-3} M$, and a reaction rate constant K of $1.5 \times 10^{10} M^{-1} s^{-1}$ for electrons²³ would have a scavenging efficiency (KC) of $2.7 \times 10^7 s^{-1}$, which is about 2.7 times less than that of caffeine. Thus, caffeine would outcompete oxygen for electrons. The consequence is the reduced level of formation of superoxide anion ($O_2^{\cdot-}$) and hydro-peroxy radicals ($^{\circ}HO_2$), and hydrogen peroxide (H_2O_2).

With the removal of electrons by caffeine the following harmful reactions would be reduced:



Furthermore, caffeine also scavenges hydroxyl radicals, singlet oxygen, hydroperoxides, hydrogen peroxide and superoxide. These studies established that caffeine reduces

(protects against) the oxic pathway of radiation damage by annihilating the radiation-induced precursor species which react with oxygen to form reactive oxygen species (ROS), besides scavenging the ROS also. Caffeine molecules are also mutually annihilated. It forms adducts with hydroxyl radicals¹⁹.

Since radiation-induced seedling injury shows correlation with peroxidase activity, the role of caffeine on the radiation-induced peroxidase activity under oxic and anoxic condition was studied in detail²⁴.

The data (Table 4) show that with increased seedling injury, there is concomitant (about 4-fold) increase in the peroxidase activity in the oxic pathway as compared to those of the anoxic pathway. Caffeine brings about a substantial reduction not only in the seedling injury, but also in the induced peroxidase activity. The authors, therefore, suggested that the removal of ROS by caffeine eliminates the need for initiating molecular signalling channel for activating the expression of peroxidase gene(s). In the context of ROS-induced molecular signalling, it should be mentioned that addition of ROS or depletion of cellular antioxidants results in apoptosis²⁵. Caffeine is known also to reduce or enhance the apoptosis induced by ionizing radiation under varying circumstances (reviewed by Devasagayam and Kesavan²⁶). The point is that caffeine possibly influences molecular signalling for gene expression through its primary action of scavenging the reactive oxygen species. The well-known removal by caffeine of the radiation-induced G_2 block in well-oxygenated systems does not necessarily result in enhanced frequency of chromosomal aberrations in the cells, presumably because of its induction of the p53-dependent apoptosis²⁶. The observed caffeine effect on the CHO cells irradiated under well-oxygenated and oxygen-depleted conditions using the premature chromosome condensation (PCC) technique probably falls in this category²⁷. The possibility that caffeine eliminates the error-prone DNA repair in the G_2 (by removing the G_2 block) in order to promote apoptosis as a prelude to an error-free cell replacement repair is likely, but it requires a more systematic investigation.

Table 2. Chromosomal aberrations in shoot-tip cells of barley seeds (~3 per cent moisture content) irradiated (350 Gy) *in vacuo* and then post-hydrated at $3 \pm 1^\circ C$ in oxygenated water before transfer to oxygen-free water

Time of transfer of seeds from oxygenated water to oxygen-free water (min)	Chromosomal bridges and fragments per anaphase cell
5	1.64 (102)
30	2.70 (104)
90	3.97(89)
150	4.90 (91)
210	4.85 (100)
480 (in oxygen-free water only)	0.80 (104)
480 (in oxygenated water only)	4.74 (101)

Values within parentheses represent the number of anaphase cells scored.

Note that the chromosomal aberrations shoot up from 0.80 to 1.64 per anaphase cell within 5 min of post-hydration in oxygenated water.

Table 3. The reaction rate constant K ($M^{-1} s^{-1}$) of caffeine

	Hydroxyl radicals ($^{\circ}OH$)	Singlet oxygen (1O_2)	Organic hydroperoxide ($^{\circ}RHO_2$)	Hydrogen peroxide (H_2O_2)	Superoxide ($O_2^{\cdot-}$)
Electrons ($e_{aq}^{\cdot-}$)					
	1.5×10^{10}	6.9×10^9	2.9×10^7	1.05×10^8	8.8×10^1
					7.5×10^1

Table 4. Differential modification of oxic and anoxic pathways of gamma ray-induced percentage seedling injury and peroxidase activity (units/mg protein) in 8-day old barley seedlings

Treatment	Nature of post-hydration			
	Oxic		Anoxic	
	Seedling injury	Peroxidase activity	Seedling injury	Peroxidase activity
+ Post-treatment				
350 Gy only	72.0	109.00	17.0	26.0
350 Gy + Caf.	42.0	62.0	35.0	44.0

Seed moisture at irradiation ~ 3.5% Radiation dose = 350 Gy *in vacuo*. Caffeine concentration = $3.8 \times 10^{-4} M$

Source: Singh and Kesavan²⁴.

Parallel studies on *Bacillus megaterium* spores exposed to 50 kVp X-rays at Austin, Texas¹⁹ and cobalt 60 gamma rays at the JNU, New Delhi²⁸ further confirmed that the observed radioprotection by caffeine is only against the oxic pathway of damage, and that greater the oxygen-enhancement ratio (OER), greater is the magnitude of caffeine-afforded protection. The OER values obtained for *B. megaterium* spores with 50 kVp X-rays and cobalt-60 gamma rays are 1.90 and 3.10 respectively. Consequently, caffeine exerts much greater radioprotection against the γ - than X-ray induced oxic damage. The 50 kVp X-rays have a much higher linear energy transfer (LET expressed as keV/ μ) than the cobalt 60 γ -rays. These results also possibly better explain the observations of Ahnström and Natarajan¹¹ that in germinating barley seeds, caffeine potentiated the damage induced by gamma rays, but not neutrons (which have much higher LET than gamma-rays).

Fallacy of caffeine being a harmful carcinogen and sensitizer of the DNA damage induced by physical and chemical agents

The review by Kesavan²⁹ deals with this question in detail. It is now settled that caffeine significantly reduces cancer risk caused by environmental and dietary carcinogens. Abraham has carried out several studies^{30,31}, which established the protective action of caffeine (he used coffee) against a variety of chemical carcinogens. The inhibition by caffeine of the carcinogenic action of cigarette smoke condensate (polynuclear aromatic hydrocarbons, PHA) in mouse skin is possibly due to scavenging of the reactive oxygen species by caffeine²⁹.

During the 1950s and 1960s, the reference radiation used for exposure of bacterial and mammalian cells *in vitro* for studying the caffeine effect was the UV of ~ 260 nm which largely induces cyclobutane type of pyrimidine dimers in the DNA. The enzyme photolyase induced by white light excises the dimers and allows the repair of the DNA, a process called *photoreactivation*. Caffeine effectively inhibits functioning of photolyase by binding to the damaged sites of the DNA^{32,33}. Over a period of time, most studies failed to make a distinction between the UV- and ionizing radiation-induced DNA lesions and it soon became generalized that caffeine is a sensitizer irrespective of the nature of interaction of a given radiation with matter²⁹. It should, however, be pointed out that in marked contrast to the damage induced by UV, the DNA damage induced by X-rays in mouse, *E. coli* or Ehrlich ascites tumour cells are not potentiated by post-treatment with caffeine²⁹. Weller *et al.*³⁴ used UV-B, which is not as efficient as UV-C in inducing pyrimidine dimers. The authors still observed significant differences in the effect of caffeine on the frequency of micronuclei induced by UV-B and gamma-rays. Caffeine significantly enhanced the frequency of micronuclei induced by UV-B, but not by gamma-rays.

Proof of caffeine's remarkable radioprotective action against lethal doses (7.50 Gy) of whole body irradiation of mice with gamma rays comes from the studies³⁵ conducted at BARC, Mumbai. In these studies, it was found that caffeine (80 mg/kg bw) administered 60 min before whole body exposure of Swiss albino mice to 7.50 Gy of gamma-rays affords substantial radioprotection. When 100 per cent of the irradiated mice were dead by day 25, more than 90 per cent of the animals survived even on day 90 (Figure 2), when the experiments had to be terminated for the reason that death as a parameter in the animal studies was opposed on ethical grounds.

Differential modification of the oxic and anoxic pathways of radiobiological damage: Caffeine is not unique

Kesavan and his co-workers¹²⁻¹⁵ were the first to demonstrate that *one and the same chemical compound* (caffeine) could bring about differential modification of the oxic and anoxic components of radiation damage to biological systems. This naturally raised questions on the text-book approach of classifying the chemical modifiers of radiation damage into two watertight compartments, viz. chemical radioprotectors and radiosensitizers. The paper on chemical radioprotection and radiosensitization of mammalian cells growing *in vitro*³⁶ is illustrative. It was, therefore, undertaken to investigate the influence of chemicals, which either donate – SH (protectors) or deplete – SH (sensitizers). The effects of several compounds on the oxic and anoxic pathway of radiation damage, which have already been published, are summarized in Table 5.

Powers³⁷ had postulated that removal of electrons by an agent would naturally diminish the extent of natural radioprotection in the cells by the occurrence of a protective reaction, viz. $^{\circ}\text{OH} + e_{\text{aq}}^- \rightarrow \text{OH}^-$. The implication is that $^{\circ}\text{OH}$ (hydroxyl radicals) are the most damaging species. Data from several studies in bacterial spore radiobiology using 50 kVp X-rays have supported the 'Electron Sequestration' hypothesis of Powers. Our data (Raghu and Kesavan, unpublished) in *B. megaterium* spores exposed to gamma rays reveal that predominantly hydroxyl radical scavengers (e.g. *t*-butanol) only protect against oxic damage, but do *not* potentiate the anoxic damage. On the other hand, the chemical agents, which have high reaction rate constants (K) for electrons and also the hydroxyl radicals modify the oxic and anoxic components of radiation damage differentially, viz. these protect against oxic damage, but potentiate the anoxic damage. These chemicals would be useful in cancer radiotherapy.

Serendipitous choice of caffeine has led to a better understanding of the major influence of electron and hydroxyl radical scavengers on the development of radiobiological damage in oxygenated (euoxic) and highly oxygen-depleted (hypoxic to anoxic) cell systems.

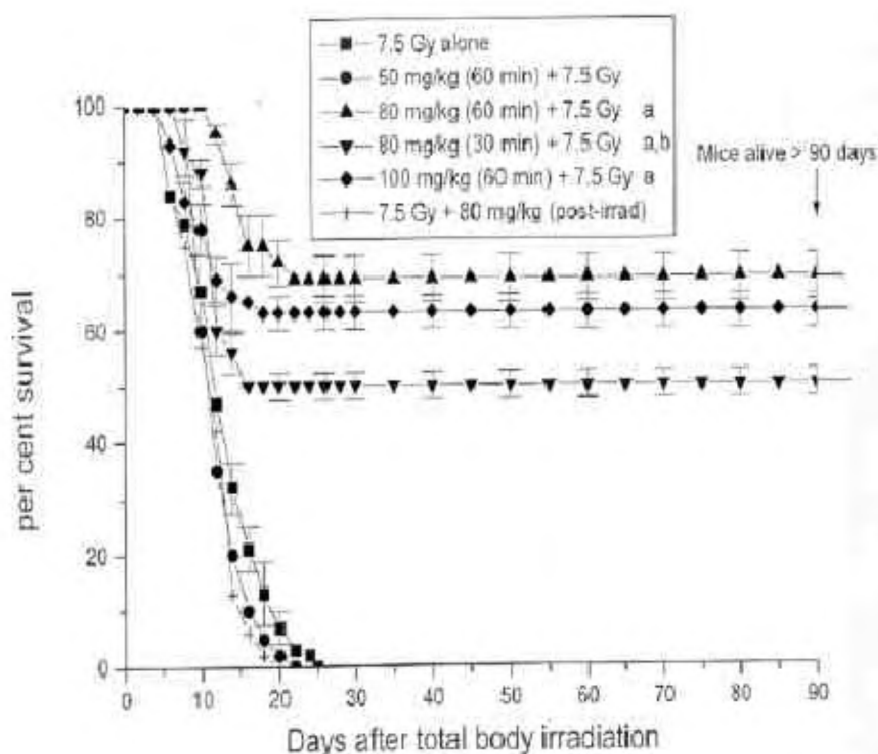


Figure 2. Radioprotection by caffeine of mice given whole body lethal dose of gamma rays.

Table 5. Influence of certain chemical compounds on the oxic and anoxic components of radiation damage in barley seed system

Chemical compound	Major attribute	Nature of modification of damage		Reference
		Oxic	Anoxic	
Caffeine	Scavenger of electrons, hydroxyl radicals, singlet oxygen, hydrogen peroxide and superoxide	Protection	Potentialiation	12, 13, 19–22
Cysteine	–SH donor; electron and hydroxyl radical scavenger	Protection	No effect	12, 49
Glutathione	–SH donor; electron and hydroxyl radical scavenger	Protection	Potentialiation	49, 51
Buthionine sulfoximine	Inhibitor of biosynthesis of glutathione; effective scavenger of hydroxyl radicals, singlet oxygen and moderate scavenger of hydrated electrons	Protection	Potentialiation	49, 51
WR 2721 (S-2 (3-aminopropylamino) ethylphosphorothioic acid)	SH- donor; electron and hydroxyl radical scavenger	Protection	Potentialiation	52, 53
Ascorbic acid	Vitamin C antioxidant; hydroxyl radical, organic peroxy radical and singlet oxygen	Protection	No effect	38
Potassium permanganate	Predominantly electron scavenger	Protection	Potentialiation	16, 38, 54
N-ethylmaleimide	–SH-depletor	Protection	Potentialiation at 4°C; but not at 37°C	55, 56
Potassium nitrate (KNO ₃)	Electron scavenger	Protection	Potentialiation	54
Potassium iodide (KI)	Hydroxyl radicals	Protection	Potentialiation	54
Potassium ferrocyanide (K ₄ Fe(CN) ₆)	Scavenger of hydroxyl radicals	Protection	Potentialiation	54
Catalase (4°C)	Electrons and hydroxyl radicals	Protection	Potentialiation	46
25°C	Electrons and hydroxyl radicals	Protection	No effect	46
Superoxide dismutase (4°C)	Dismutation of superoxide (O ₂ ^{•−})	Protection	Potentialiation	46
25°C	Dismutation of superoxide (O ₂ ^{•−})	Protection	Potentialiation	46

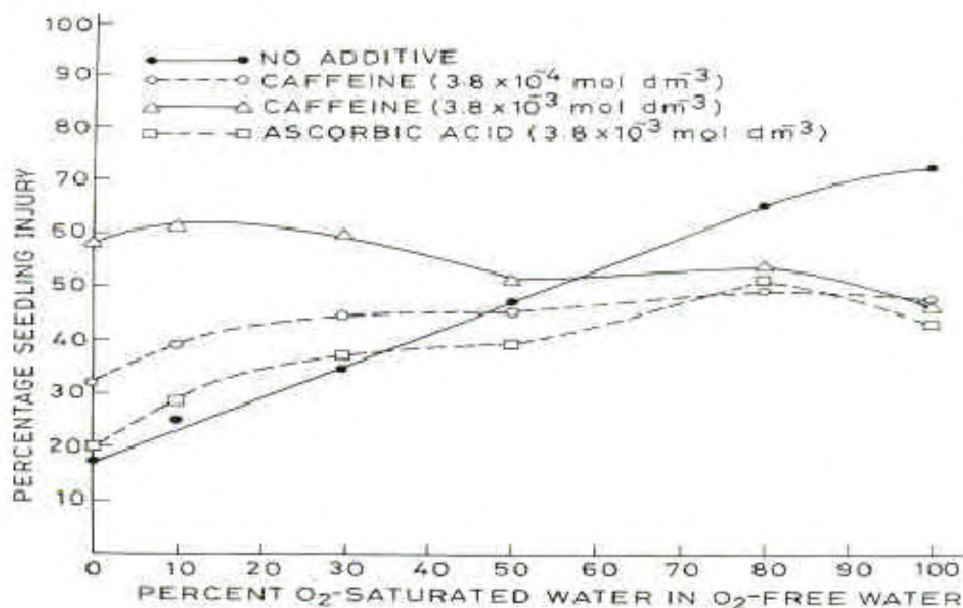


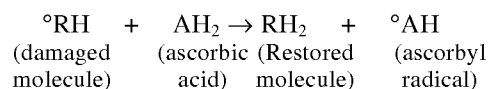
Figure 3. Radiobiological damage as a function of oxygen concentration and its modification by caffeine and ascorbic acid.

Role of oxygen concentration in determining the nature of chemical modification of radiobiological damage

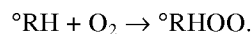
That the oxygen concentration indeed determines the mode and magnitude of modification of radiation damage by caffeine, ascorbic acid and potassium permanganate was demonstrated by Kesavan *et al.*³⁸. In their studies, the oxygen concentration in the post-hydration medium (OCHG) was adjusted at 0, 10, 30, 50, 80 and 100%, which correspond approximately to 1×10^{-6} , 1×10^{-4} , 5×10^{-4} , 9×10^{-4} and 1.8×10^{-3} M of oxygen respectively. While an O_2 -concentration of 1×10^{-6} M is fully anoxic, those in the range of 1×10^{-4} and 5×10^{-4} M are hypoxic. A linear response between seedling injury of barley seeds (exposed to 350 Gy ^{60}Co γ -rays) and O_2 concentration was observed (Figure 3). These chemicals potentiated, protected or exerted no effect, on γ -ray-induced seedling injury depending upon the concentration of OCHG. Thus, ascorbic acid did not exert any effect on seeds post-hydrated at OCHG of $\leq 30\%$, but afforded radioprotection at OCHG of $\geq 50\%$. Caffeine, on the other hand, potentiated the damage at OCHG of $\leq 30\%$, exerted no influence at OCHG of $\sim 50\%$ and afforded radioprotection at OCHG of $\geq 80\%$. Potassium permanganate enhanced the injury at OCHG of $\leq 10\%$, exerted no effect at OCHG of $\sim 30\%$ and afforded radioprotection at OCHG of $\geq 50\%$.

The differential modification of the post-irradiation seedling injury at different O_2 -concentrations by caffeine and ascorbic acid was noted in terms of peroxidase activity also (Table 6).

The ascorbic acid is known to be capable of a slow protective action³⁹ as follows:



The observation that ascorbic acid does not reduce injury at $\leq 30\%$ oxygen³⁸ suggests that it does not protect ${}^{\circ}RH$ against oxygen in the initial stages of post-hydration when a 'fast component' within oxidic damage possibly develops. There is evidence in the literature on barley seed radiobiology to implicate 'fast' and 'slow' components of reactivity of the radiation-induced oxygen-sensitive sites with oxygen or their decay in the absence of oxygen^{40,41}. Kesavan *et al.*³⁸ have deduced that ascorbic acid might donate electron to ${}^{\circ}RH$ to protect it against not the first fast component but rather the later 'slow' peroxidation as follows:



These are, however, not the only or even major pathways in the highly metabolizing cells or higher organisms. For instance, the *in vivo* mice experiments have shown that caffeine is far more radioprotective than ascorbic acid. Tests of radioprotective efficacy of equimolar concentrations of caffeine and ascorbic acid following whole body irradiation of mice have proved relevant that the former is far more protective than the latter^{42,43}. The magnitude of radioprotection (in terms of the frequency of formation of micronuclei following whole body irradiation of Swiss albino mice with 1 Gy of γ -rays) on equimolar basis of comparison was in the following sequence of increasing order for an acute dose of 10^{-2} M/kg bw:

Cysteamine > α -tocopherol > caffeine > buthionine sulfoximine > ascorbic acid

More studies are, however, required in the area related to the role of ROS in inducing molecular signalling, gene expression for repair of DNA and/or cell death for cell replacement repair, etc. These are all strongly indicated from several studies.

Mechanisms of anoxic radiation damage and its potentiation by caffeine

Many uncertainties still persist regarding the mechanisms of radiation-induced damage in the absence of oxygen, especially when cells and organisms are exposed to low LET (250 kVp X-rays and gamma-rays) ionizing radiations. From the point of radiation chemistry, the radiation-induced oxygen-reactive radicals harmlessly decay in the absence of oxygen.

Formation of radiation induced free radicals

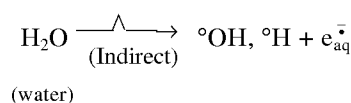
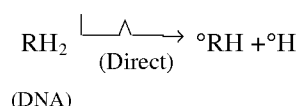


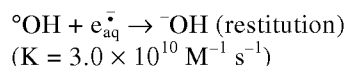
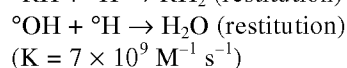
Table 6. Influence of caffeine (caf) and ascorbic acid (aa) on post-irradiation seedling injury and peroxidase activity depending upon O_2 -concentration in the post-hydration medium

Post-irradiation hydration/treatment	Percentage seedling injury	Peroxidase activity (units/mg protein)
None (no irradiation)	0	10.3
350 Gy + 0% O_2	17	23.8
350 Gy + 0% O_2 + caf.	58 (S)	66.9
350 Gy + 0% O_2 + aa	20 (NE)	25.0
350 Gy + 10% O_2	25	28.7
350 Gy + 10% O_2 + caf.	61 (S)	70.0
350 Gy + 10% O_2 + aa	29 (NE)	30.4
350 Gy + 30% O_2	34	39.5
350 Gy + 30% O_2 + caf.	59 (S)	65.2
350 Gy + 30% O_2 + aa	37 (NE)	40.9
350 Gy + 50% O_2	47	52.6
350 Gy + 50% O_2 + caf.	51 (NE)	57.0
350 Gy + 50% O_2 + aa	39 (P)	42.8
350 Gy + 80% O_2	65	79.9
350 Gy + 80% O_2 + caf.	53 (P)	58.2
350 Gy + 80% O_2 + aa	51 (P)	55.7
350 Gy + 100% O_2	72	99.3
350 Gy + 100% O_2 + caf.	46 (P)	50.2
350 Gy + 100% O_2 + aa	43 (P)	45.4

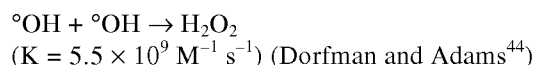
Concentration of caffeine and ascorbic acid: 3.8×10^{-3} M.
P, Protection; S, Sensitization; NE, no effect.

When oxygen is available, these radicals react with it and form superoxide (O_2^\bullet), hydroperoxyl radical ($^\bullet\text{HO}_2$), hydrogen peroxide (H_2O_2), organic hydroperoxide ($^\bullet\text{RHO}_2$), etc. When oxygen is absent, harmless recombinations/restitutions^{23,37} predominate as follows:

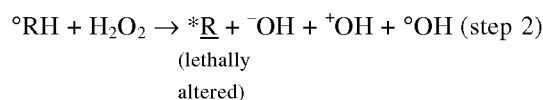
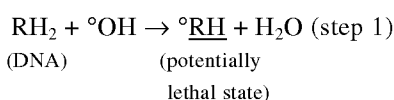
Radiation chemical events in the absence of O_2



As against these harmless recombinations, the following reactions could be potentially deleterious under anoxic conditions



Powers and his co-workers^{37,45} have postulated that under anoxic conditions the $^\bullet\text{OH}$ and H_2O_2 can promote *sequential double oxidation* to damage the vital molecule (say DNA) in the cell as follows:



The *R is the lethally damaged DNA molecule. It may be noted that $^\bullet\text{RH}$ represents potentially lethal configuration that can be restituted by donation of H-atom or be irreversibly altered by abstraction of H-atom.

The studies by Kesavan and co-workers^{47,48} in barley seed system, do not, however, lend support to the sequential double oxidation scheme of radiation damage in the *absence* of oxygen. In their studies, nitrous oxide-saturated (N_2O -saturated) post-hydration was even more radioprotective than nitrogen-saturated water for dry irradiated seeds. Part of the unpublished data (Table 7) illustrate this.

These and other already published data⁴⁷⁻⁴⁹ are quite intriguing. Since N_2O converts electrons into more damaging hydroxyl radicals^{37,50}, and also upsets an ideal equilibrium for harmless recombinations ($^\bullet\text{H} + \text{°OH} \rightarrow \text{H}_2\text{O}$ and $\text{e}_{\text{aq}}^- + \text{°OH} \rightarrow \text{°OH}^-$), the seedling injury following N_2O -saturated post-hydration is expected to be greater than that observed following N_2 -saturated post-hydration. On the contrary, in very dry seeds (~ 3.3 per cent moisture) the seedling injury is, however, significantly reduced following N_2O -saturated

Table 7. Influence of caffeine (3.8×10^{-4} M) on dry (~3.3 per cent) and wet (11.5 per cent) barley seeds exposed to 350 Gy γ -rays and post-hydrated at 4°C for 8 h in O₂-, N₂-, N₂O-saturated water

Dose of γ -rays + post-treatment at 4°C for 8 h	Initial seed moisture content			
	3.3%		11.5%	
	Percentage seedling injury	Peroxidase activity (units/mg protein)	Percentage seedling injury	Peroxidase activity (units/mg protein)
350 Gy + O ₂ -saturated	71.8	88.5	13.2	21.2
350 Gy + O ₂ -saturated + caf.	50.6 (P)	42.8	31.5 (S)	31.8
350 Gy + N ₂ -saturated water	14.0	25.9	13.4	20.7
350 Gy + N ₂ -saturated water + caf.	29.6 (S)	33.2	30.7 (S)	32.9
350 Gy + N ₂ O-saturated water	04.0	18.0	13.2	20.3
350 Gy + N ₂ O-saturated water + caf.	16.5 (S)	22.3	14.7 (no effect)	21.8

P = Protection; S = Sensitization.

post-hydration in comparison with that following N₂-saturated post-hydration. In moist (11.5 per cent) seeds, the level of seedling injury is just about the same (~13 to 14 per cent) irrespective of N₂- or N₂O-saturated post-hydration. Caffeine potentiates the radiation-induced seedling injury mediated by N₂- and N₂O in the dry (3.3 per cent moist) seeds, whereas in moist (11.5 per cent) seeds, it potentiates the N₂- but not the N₂O-mediated injury. These all reveal rather complex, yet very delicate physicochemical reactions in the radiation-induced sequence of events in the dry seed system, which has no active metabolism. Therefore, the nature of reactions leading to modification of radiation injury is largely physico-chemical.

There are, however, a few other considerations. One is that in very dry seeds, the free radicals are mostly the trapped electrons, H-atoms, $^{\circ}\text{RH}$ radicals; the hydrated electrons (e_{aq}^{\bullet}) and hydroxyl radicals ($^{\circ}\text{OH}$) are not likely to be significantly formed due to very little water content. That is why post-irradiation heat-shock leading to thermal annealing results in radioprotection against oxidative damage. The N₂O imbibed during post-hydration at 4°C for 8 h (the seeds have no/little metabolic activity at this temperature) could react with trapped electrons (\dot{c} trapped) and possibly convert them into $^{\circ}\text{OH}$ facilitating most likely *in situ* recombination (\dot{c} trapped + $^{\circ}\text{OH} \rightarrow ^{\circ}\text{OH}$). Hence, the N₂O-saturated post-hydration results in a level of seedling injury far greatly reduced than that observed following post-hydration in N₂-saturated water. In seeds of 11.5 per cent moisture content, however, hydrated electron (e_{aq}^{\bullet}) and hydroxyl radicals ($^{\circ}\text{OH}$) are possibly formed by radiolysis of water. Under these circumstances, N₂O might still disrupt the equilibrium between hydrated electrons and hydroxyl radicals by converting a fraction of the former into the latter. The result is a level of seedling injury that is comparable with that when the seeds are post-hydrated in N₂-saturated water. However, there is no doubt that these physicochemical reactions are quite important, and they vary with the nature of hydration and temperature.

The next question is about the potentiation by caffeine of the N₂O-mediated damage in dry (3.3% moist) but not in wet (11.5 per cent moisture) seeds. On the basis of the above

postulate that in dry (~3.3% moisture) seeds, there is no significant radiolytic formation of e_{aq}^{\bullet} and $^{\circ}\text{OH}$, and that the N₂O-saturated hydration could, however, convert trapped electrons into $^{\circ}\text{OH}$ which in turn could lead to *in situ* harmless recombination ($^{\circ}\text{OH}$ and $e_{\text{aq}}^{\bullet} = ^{\circ}\text{OH}$), the potentiating role of caffeine, under these circumstances, can be explained. The likely scenario is that caffeine with a high reaction rate constant ($K = 1.5 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) for electrons, could out-compete N₂O which has a lower $K = (5.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1})$ for electrons. The end result is interference by caffeine of the possible *in situ* recombination of electrons and hydroxyl radicals and the consequent increase in the level of injury above the level observed following N₂O-saturated post-hydration without caffeine. The lack of sensitizing effect of caffeine on the N₂O-mediated level of injury in seeds of 11.5 per cent moisture content is, as explained above, due to the formation of hydrated electrons and hydroxyl radicals in these wet seeds and the removal of both these species by caffeine notwithstanding the conversion of a fraction of the electrons into hydroxyl radicals by N₂O. In other words, caffeine does not significantly alter the equilibrium between the protective and damaging reactions involving electrons and hydroxyl radicals. This postulate needs to be verified with more experiments some of which would have to involve fast kinetic facilities.

Further, the studies to elucidate the specific expression and/or the suppression of expression of specific genes involved in the pathways of cell cycle, cell division, dsb repair, apoptosis, etc. in the O₂-free (hypoxic to anoxic) pathway of radiation (low LET) damage are urgently required. While a great deal of new knowledge has been gained regarding oxygen enhancement of radiobiological damage, there has been practically nothing known about radiation-induced pathways of biological effects in the absence of oxygen. Once these are elucidated, a better control over radiotherapy of cancer can be achieved.

In conclusion, it is fascinating that while the development of ROS is purely physicochemical, the ultimate radiobiological effects (mutations, cancer and cell death) result not directly from the ROS, but via their influence on molecular signalling and gene expression. With the absorption of

radiant energy, a chain of events gets triggered in the affected cell, and the final destiny of that cell is seemingly determined by itself in terms of fixation of an alteration in the DNA, or its repair, or its elimination altogether by cell death in one or another form (i.e. necrosis, apoptosis, etc.). The role of these events and the fate of individual cells in turn explain that carcinogenesis is not a single-step event, but multi-step sequential processes some of which are reversible.

In the end, it is reassuring that coffee and tea are better antioxidants than even vitamin C. But then as Paracelsus (1493–1541), a Renaissance physician, naturalist and philosopher-Father of Modern technology observed, only the dose determines that a thing is not poison in a world where all things are poison, and none without poison.

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