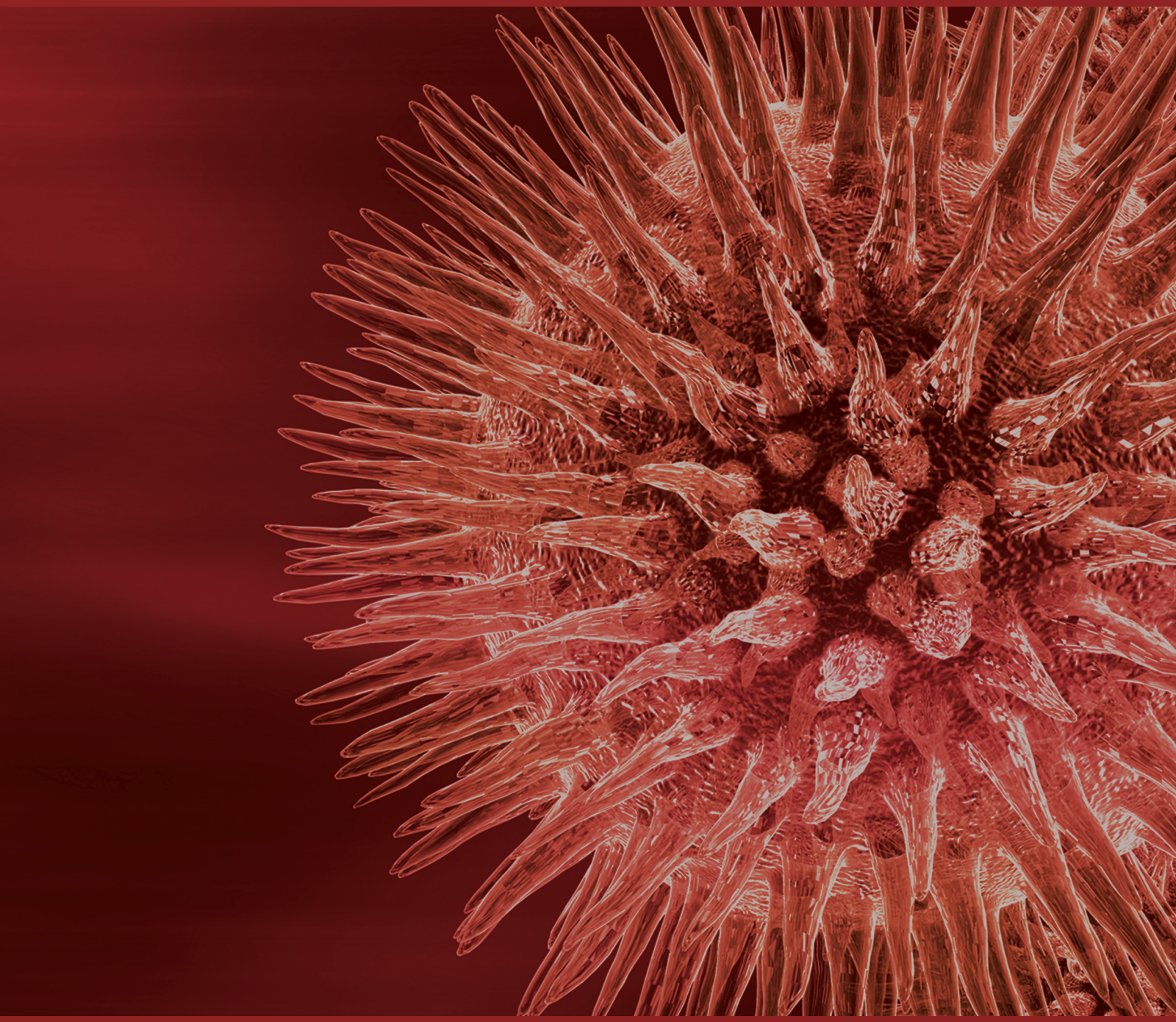
BioMed Research International

DNA Damage and Oxidative Stress in Human Disease

Guest Editors: Sharbel Weidner Maluf, Norma Possa Marroni, Vanina D. Heuser, and Daniel Prá



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Editorial

DNA Damage and Oxidative Stress in Human Disease

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The impact of DNA damage in human diseases is gaining attention since the mid-1990s. DNA damage and oxidative stress are known factors for the origin and progression of cancer. DNA damage and oxidative stress have also been implicated in so diverse diseases such as brain injury, pulmonary diseases, and other chronic inflammation-related disorders.

This special issue contains eight papers, covering several aspects of the implication of DNA damage and oxidative stress. Three papers focus on brain, colorectal, and skin cancers and one paper focuses on the genomic stability and oxidative stress in the cancer-predisposing genetic syndrome ataxia telangiectasia. One paper discusses the implication of nutrients in genomic stability in cell cultures. Two papers discuss the effect of a vitamin and a neuroprotectant on diabetes and traumatic brain injury, respectively. Another paper focused on the impact of autophagy on idiopathic pulmonary fibrosis.

"*The vitamin D receptor (VDR) gene polymorphisms in Turkish brain cancer patients*" by B. Toptas et al. provides evidence for the first time that the risk of meningiomas might be related to polymorphisms in the nuclear receptor of vitamin D, an important factor for the regulation of cell division and proliferation. In "*Oxidative stress in the pathogenesis of colorectal cancer: cause or consequence?*" M. Perše reviews the interplay between the several risk factors that have been implicated in colorectal cancer, a very common type of cancer in Western countries, which has a complex etiology. N. C. Jenkins and D. Grossman show, in "*Role of melanin in melanocyte dysregulation of reactive oxygen species,*" that the presence of melanin in the skin appears to be a double-edged sword: it protects melanocytes as well as neighboring keratinocytes in the skin through its capacity to absorb UV radiation, but its synthesis in melanocytes results in higher levels of intracellular ROS that may increase melanoma susceptibility.

L. B. Ludwig et al. provide that ionizing radiation is more efficient than bleomycin to induce chromosomal instability in ataxia telangiectasia patients and that this instability is not related to a systemic increase in oxidative stress. In "*The influence of micronutrients in cell culture: a reflection on viability and genomic stability,*" A. L. V. Arigony et al. addresse the effect of several vitamins and minerals by reviewing their role in metabolic routes related to DNA homeostasis. The paper presents lines of evidence whether while in deficiency or excess in cell culture the micronutrients reviewed can reduce or increase the level of DNA damage and influence cell proliferation and viability. Finally, the authors advocate which

nutrients should deserve more attention in future studies focusing on the increase of genomic stability and cell fitness under culture conditions.

"*Vitamin C intake reduces the cytotoxicity associated with hyperglycemia in prediabetes and type 2 diabetes*" by S. I. R. Franke et al. compares the levels of vitamin C intake, which is among the most abundant antioxidants obtained from diet, with the levels of markers of hyperglycemia, DNA damage, and cytotoxicity in subjects with type 2 diabetic or with risk of developing the disease. The authors observe that vitamin C intake slightly higher than the dietary recommendation for healthy individuals can be beneficial to the subjects by preventing the cell death of white blood cells that have been reported in the literature to be associated with diabetes complications.

In "*Therapeutic time window for edaravone treatment of traumatic brain injury in mice,*" K. Miyamoto et al. deal with the edaravone administration postcontrolled cortical impact (CCT) resulting in a significant reduction in the injury volume and oxidative stress. These findings suggest that edaravone could prove clinically useful to ameliorate the devastating effects of traumatic brain injury (TBI). "*Self-eating: friend or foe? The emerging role of autophagy in idiopathic pulmonary fibrosis,*" by G. A. Margaritopoulos et al. highlights some key issues regarding the process of autophagy and its possible association with the pathogenesis of idiopathic pulmonary fibrosis.

Advances in molecular biology and bioinformatics are allowing researchers to gain an increased understanding of the function and regulation of genes and to identify pathways that are affected. Currently, the search for biomarkers related to disease is gaining increasing attention and especially biomarkers for oxidative stress and DNA damage became more and more valuable instruments for unraveling disease pathogenesis and facilitating prediction, prevention, and treatment of diseases.

Acknowledgments

We would like to express appreciation to the authors for their excellent contribution and patience in assisting us. Finally, the fundamental work of the reviewers of these papers is also greatly acknowledged.

Sharbel Weidner Maluf
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Vanina D. Heuser
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Research Article

Vitamin C Intake Reduces the Cytotoxicity Associated with Hyperglycemia in Prediabetes and Type 2 Diabetes

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Hyperglycemia leads to the formation of free radicals and advanced glycation end-products (AGEs). Antioxidants can reduce the level of protein glycation and DNA damage. In this study, we compared the levels of vitamin C intake, which is among the most abundant antioxidants obtained from diet, with the levels of fasting plasma glucose (FPG), glycated hemoglobin (A1C), DNA damage, and cytotoxicity in prediabetic subjects and type 2 diabetic subjects. Our results indicated that there was no significant correlation between FPG or A1C and DNA damage parameters (micronuclei, nucleoplasmic bridges, and nuclear buds). FPG and A1C correlated with necrosis ($r = 0.294$; $P = 0.013$ and $r = 0.401$; $P = 0.001$, resp.). Vitamin C intake correlated negatively with necrosis and apoptosis ($r = -0.246$; $P = 0.040$, and $r = -0.276$; $P = 0.021$, resp.). The lack of a correlation between the FPG and A1C and DNA damage could be explained, at least in part, by the elimination of cells with DNA damage by either necrosis or apoptosis (cytotoxicity). Vitamin C appeared to improve cell survival by reducing cytotoxicity. Therefore, the present results indicate the need for clinical studies to evaluate the effect of low-dose vitamin C supplementation in type 2 diabetes.

1. Introduction

Diabetes mellitus (DM) includes a group of diseases that are characterized mainly by high levels of serum glucose (hyperglycemia) and a deficiency in or resistance to the action of the hormone insulin. There are approximately 200 million diabetics worldwide, and type 2 DM (DM2) accounts for 90–95% of all DM cases. DM2 is a very relevant pathology because of its high prevalence and related complications, including macrovascular (cardiovascular disease and ischemic encephalopathy), microvascular (coagulation dysfunction, nephropathy, and neuropathy), and biochemical (e.g., dyslipidemia) disturbances [1].

Clinical and experimental evidence suggests that DM complications are associated with metabolic disturbances that result mainly from hyperglycemia. Advanced glycation end-products (AGEs) are formed by a nonenzymatic reaction between glucose and basic amino acids, and their level is directly correlated with serum glucose levels [2]. The serum AGE level is a marker of late DM complications [3]. Glucose self-oxidation also leads to free radicals and oxidative stress formation, with the latter occurring when the concentration of free radicals is higher than the antioxidant capacity [4]. Oxidative stress is among the main causes of DM progression due to cell and tissue injury [1]. Glycated hemoglobin (A1C) is an altered form of hemoglobin that is produced by the action

of AGEs, and it is considered to be a good marker of the average level of serum glucose over the previous weeks; A1C was shown to correlate with an increased risk of DM complications [2].

Antioxidant defenses comprise endogenous and exogenous enzymatic and nonenzymatic mechanisms. Vitamin C is a key exogenous nonenzymatic antioxidant that is found in high concentrations in serum and within cells. Vitamin C is an enzymatic cofactor and antioxidant that is capable of shifting between its oxidized and reduced forms by electron donors and that protects, at the intracellular level, DNA, proteins, and lipids against oxidative stress [5].

This study aimed to evaluate the relationship between the dietary intake of vitamin C, which is the main antioxidant in human blood, and the levels of A1C and primary DNA damage, which are two markers of oxidative stress, in prediabetic and DM2 subjects.

2. Materials and Methods

We evaluated the extent of DNA damage in whole blood samples collected from 70 prediabetic and DM2 subjects. All of the subjects were enrolled in the Ambulatory "Serviço Integrado de Saúde" of the University of Santa Cruz do Sul or were attending the Brazilian "Family Health Strategy," both in Santa Cruz do Sul, RS, Brazil. The study protocol was approved (CAAE: 03981212.8.0000.5343) by the internal human experimentation ethics committee of the University of Santa Cruz do Sul, and all of the subjects gave written informed consent for their participation. The code of ethics of the World Medical Association (Declaration of Helsinki) was followed throughout the study.

Peripheral blood samples from all of the subjects were collected during the morning. Subjects were asked to fast and rest for an 8 h period before blood sampling, and only those who followed this recommendation were included in the study. Blood samples were immediately processed for fasting plasma glucose (FPG), A1C, DNA damage, and cytotoxicity levels. First, a blood subsample was centrifuged to obtain serum to measure the fasting glucose level. Second, another blood subsample was mixed with ethylenediaminetetraacetic acid for A1C determination. Third, a blood subsample was mixed with heparin for the cytokinesis-blocked micronucleus cytome assay (CBMN Cyt) analysis. The biochemical evaluations were conducted and the slides for the comet assay were prepared immediately after collection. FPG was measured using an enzymatic-spectrophotometric method, and A1C was measured using an HPLC method with the Bio-Rad Variant II Turbo Hemoglobin Testing System (Bio-Rad Laboratories, Hercules, CA, USA). Equipment, reagents, standards, and protocols for evaluating fasting plasma glucose (FPG) and A1C were, respectively, supplied by Biosystems S.A. (Barcelona, Spain) and Bio-Rad Laboratories, Hercules, CA, USA.

For the CBMN Cyt analysis, samples were sent to the Cytogenetics Laboratory of the Porto Alegre Clinics Hospital. The CBMN Cyt measurement was performed according to the method described by Fenech and Morley [6] and as adapted by Maluf [7]. Blood samples (0.5 mL) were placed in 5 mL of culture medium containing RPMI 1640 supplemented with 20% fetal bovine serum and 2% phytohemagglutinin. The culture flasks were incubated at 37°C for 44 h, then cytochalasin B (final concentration 6 μg/mL) was added, and the resulting suspensions were incubated for another 28 h. Thereafter, cell suspensions were treated with a hypotonic agent (KCl) and fixed in a 3 : 1 solution of acetic acid and methanol. Drops of the cell suspension were then placed on microscope slides (at least 2 per individual) and stained with Giemsa. The analysis was completed according to the standard criteria for CBMN Cyt [8]. Two thousand binucleated lymphocytes were analyzed per individual to assess the frequency of micronuclei (MN), nuclear buds (NBUD), nucleoplasmic bridges (NPB), apoptotic cells, and necrotic cells. The results are expressed as per 1000 cells.

The intake of vitamin C was determined using the Virtual Nutri 1.0 (São Paulo, SP, Brazil) software following the procedures described by Prá et al. [9]. In short, the habitual diet was evaluated on 3 nonconsecutive days (2 weekdays and 1 weekend day) using a validated questionnaire that contains open questions about typical food intake at each meal. Home measures were presented during the interview to aid in the interpretation of the amount of food ingested.

Pearson's and Spearman's correlations and nonlinear curve-fitting tests were used to evaluate correlations between fasting glucose, A1C, DNA damage, and vitamin C. Statistical evaluations were performed and graphs were plotted using GraphPad Prism 4.0 (San Diego, CA, USA). The level of statistical significance was set at $P < 0.05$.

3. Results

Table 1 presents the characteristics of the study subjects. Most of the study subjects were female and most were more than 40 years old. Regarding the intake of vitamin C (Table 2), the prevalence of deficiency was 54.2% for men and 34.8% for women. The minimum and maximum mean intakes were 5 and 290 mg/day, respectively. Age correlated with FPG ($r = 0.307$; $P = 0.030$) and A1C ($r = 0.428$; $P = 0.002$). FPG correlated with A1C ($r = 0.631$; $P < 0.001$).

No significant correlations between FPG or A1C and DNA damage parameters (micronuclei, nucleoplasmic bridges, and nuclear buds) were observed. FPG and A1C correlated with necrosis (Figure 1; $r = 0.294$; $P = 0.013$ and $r = 0.401$; $P = 0.001$, resp.). Vitamin C intake correlated negatively with necrosis and apoptosis (Figure 2; $r = -0.246$; $P = 0.040$, and $r = -0.276$; $P = 0.021$, resp.). No significant correlation was observed between vitamin C intake and the DNA damage parameters (micronuclei, nucleoplasmic bridges, and nuclear buds). Necrosis and apoptosis were lower in subjects with an adequate intake of vitamin C in relation to those with an inadequate intake, but only for men (Figure 3). Vitamin C intake did not correlate significantly with A1C ($r = -0.175$; $P = 0.148$).

The nucleoplasmic bridges correlated with nuclear buds ($r = 0.278$; $P = 0.020$). Necrosis correlated with apoptosis ($r = 0.624$; $P < 0.001$).

Table 1: Characteristics of the studied cohort of prediabetic and type 2 diabetic adult individuals ($n = 70$).

Sex (male/female)			24/46			
	Average ± SD	Minimum	Maximum	P25	P50	P75
Age (years)	51.9 ± 10.9	28	76	46	53	60
Fasting serum glucose (mg/dL)	109.3 ± 39.5	73	315	89	97	119
Glycated hemoglobin (%)	6.5 ± 0.70	5.7	8.7	6.0	6.4	6.9
Micronuclei (‰ cells)	0.58 ± 0.95	0.0	7.0	0.0	0.5	1.0
Nucleoplasmic bridges (‰ cells)	1.55 ± 1.47	0.0	11.0	0.5	1.0	2.0
Nuclear buds (‰ cells)	1.02 ± 1.03	0.0	7.0	0.5	1.0	1.0
Necrosis (‰ cells)	3.54 ± 2.30	0.0	12.0	2.0	3.0	4.5
Apoptosis (‰ cells)	4.66 ± 2.85	1.0	13.0	2.5	4.0	6.5

P: percentile.

Table 2: Assessment of the intake of vitamin C according to sex in the studied cohort of prediabetic and type 2 diabetic adult individuals ($n = 70$).

			Vitamin C intake (mg/day)									
	EAR	Mean ± SD	Percentile of usual intake distribution									Assessment comments
			10th	20th	30th	40th	50th	60th	70th	80th	90th	
Men ($n = 24$)	75	80.5 ± 65.7	9.6	18.5	43.9	53.5	61.9	89.7	103.5	116.9	174.3	Prevalence of inadequacy is >50% but <60%
Women ($n = 46$)	60	88.5 ± 57.3	18.6	34.0	53.7	63.1	73.2	87.2	113.6	145.4	181.5	Prevalence of inadequacy is >30% but <40%

SD: standard deviation; EAR: estimated average requirement [10].

4. Discussion

There is growing evidence that DM is linked to oxidative stress. Oxidative stress has been shown to be involved in many of the micro- and macrovascular complications that are associated with DM. DNA damage is among the well-known molecular effects of oxidative stress, and DNA damage increase has been observed in many chronic diseases with increased oxidative stress. The molecular evidence of increased DNA damage in white blood cells of diabetics is somewhat controversial, despite that changes in the DNA repair capacity of such cells are well documented [11–13].

Several studies have shown that vitamin C can improve metabolic dysfunctions associated with DM through, among other mechanisms, its antioxidant potential [14, 15]. It is well documented that vitamins are also capable of affecting DNA damage at different levels, including inhibiting damage formation, facilitating damage removal by DNA repair, and/or promoting cell death of the damaged cells through necrosis or apoptosis [16]. On the other hand, there are few studies in the literature (e.g., [17]) that link vitamins to DNA damage and cytotoxicity.

The mean vitamin C intake of the subjects in the present study (Table 2) was approximately the current estimated average requirement (EAR), which represents the adequate intake level and is used to evaluate nutrient intake for groups and individuals, according to dietary reference intake (DRI) [10]. A large portion of the cohort had inadequate intake of vitamin C, which was higher for men (>50% and <60% for men versus >30% and <40% for women). The vitamin C intakes of most of the individuals were lower than the 90–100 mg per day level recommended by Carr and Frei [18] for chronic disease risk reduction in nonsmoking men and women. The maximum intake observed among the studied subjects was approximately 300 mg/day, which is substantially lower than the 2000 mg/day level set by the Institute of Medicine (IOM) [10] as the maximum intake for healthy men and women. Therefore, the intake of all of the subjects was much lower than the level at which health risks start to occur.

FPG and A1C were correlated with age, as has been observed in previous studies [19, 20]. No correlations between DNA damage endpoints and age, FPG, or A1C were observed. Several studies have shown that there is no increase in DNA damage in DM2 [21, 22]. Other studies have indicated that an increase in micronuclei will not likely occur in early DM2 [23], but only in uncompensated DM2 (i.e., A1C > 8%) [24]. In our study, only 4 (approximately 6%) out of the 70 subjects had uncompensated DM2 (A1C > 8.0%); therefore, it was not likely that the sample would present an increase in chromosomal DNA damage. Until recently, it was not clear why individuals with early DM2 do not exhibit increased chromosomal DNA damage even though large levels of oxidative stress have been reported by several authors. It is likely that this effect might occur as a result of the increase in the frequency of necrosis and apoptosis (cytotoxicity) observed in early DM2. Human studies and those of rodent models of DM2 have indicated an increased

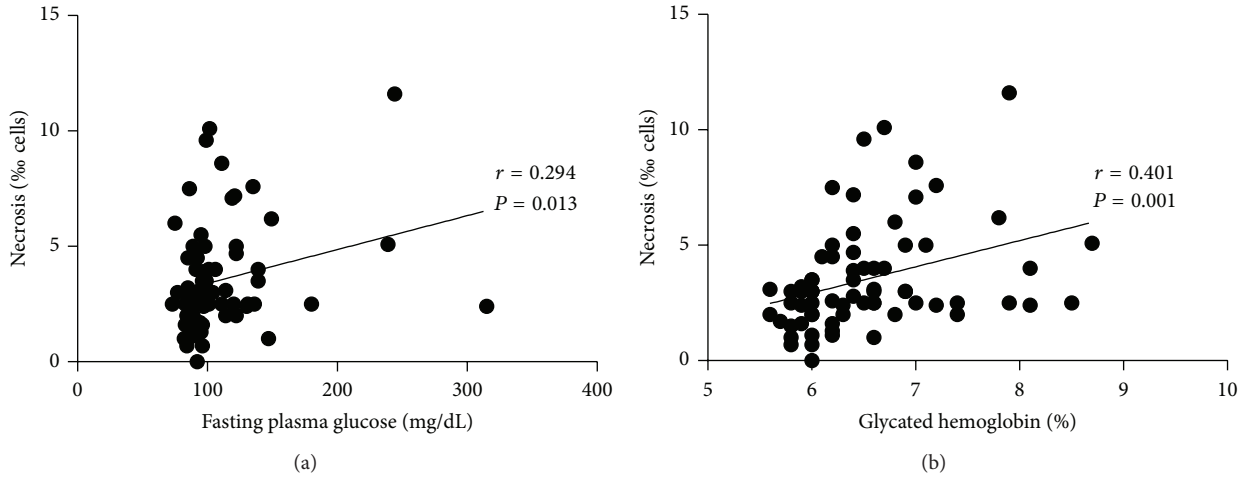

Figure 1: Correlations between fasting plasma glucose (a), glycated hemoglobin (b), and necrosis in prediabetic and type 2 diabetic adult subjects ($n = 70$). r and P: correlation coefficient and level of significance, respectively, according to the Spearman correlation test.

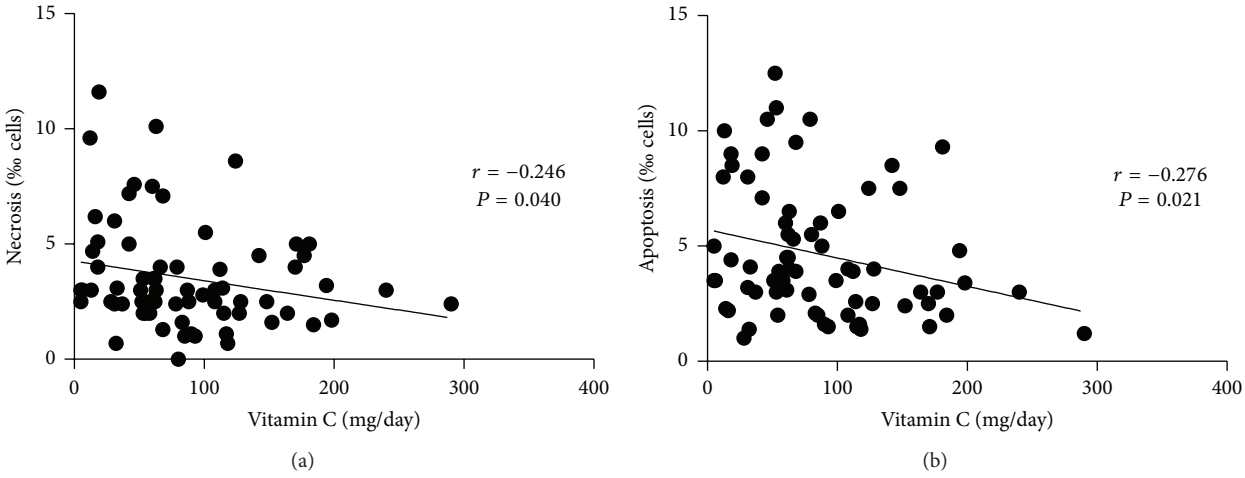

Figure 2: Correlations between vitamin C intake, necrosis (a), and apoptosis (b) in prediabetic and type 2 diabetic adult subjects ($n = 70$). r and P: correlation coefficient and level of significance, respectively, according to the Spearman correlation test.

frequency of necrosis and apoptosis [25]. We also observed an increase in apoptosis and necrosis in prediabetes [23]. There are two possible explanations for the cytotoxicity observed in DM2: (i) glucose self-oxidizes and generates oxidative stress that damages different cell compartments, possibly leading to cell death [26], and (ii) iron participates in several redox reactions and can generate a large amount of reactive oxygen species that could also mediate cell death. There are several studies that show a link between iron and DM2 risk, for example, due to the high cycling of hemoglobin arising from the short half-life of red blood cells [23]. However, the exact link between cytotoxicity and DNA damage in DM2 remains to be elucidated.

Vitamin C reduces the levels of oxidative stress. Additionally, vitamin C might have roles in DNA repair, reducing the extent of DNA damage [27]. There is evidence that diabetics have depleted serum levels of vitamin C and, therefore, require intake levels that are slightly higher than those recommended for non-diabetic individuals. Choi et al. [15] evaluated the relationship between fasting plasma vitamin C, lymphocyte primary DNA damage (comet assay), and A1C in 427 DM2 individuals and observed a similar negative correlation between DNA damage and serum vitamin C. Because dietary intake was not evaluated by Choi et al. [15], the potential use of their data for dietary recommendations is limited. In contrast, in our study, we showed that cytotoxicity decreases when the EAR is met, but only among men. The EAR for men is higher (75 mg vitamin C per day) than for women (60 mg vitamin C per day). Therefore, the present results reflect a low intake of vitamin C in our study population, and the results indicate that intake levels that are slightly higher than the DRI for vitamin C are linked to a reduced risk of DM2-associated complications, at least in normal dietary conditions. The present results also indicate the need for clinical studies that evaluate the effect of low-dose vitamin C supplementation in DM2. It is important to

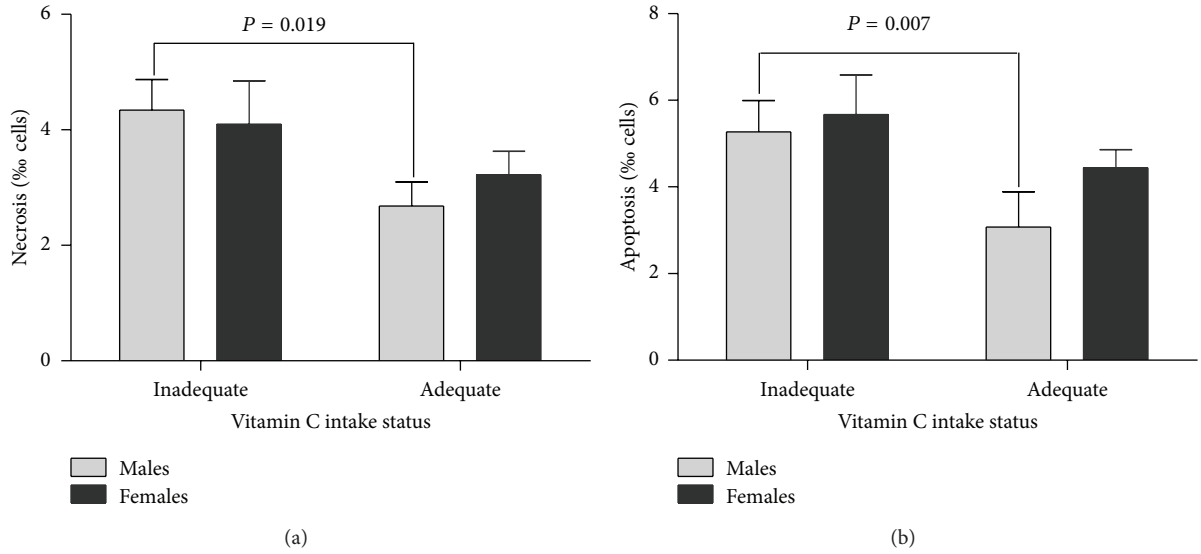

Figure 3: Necrosis (a) and apoptosis (b) according to adequate vitamin C intake status and sex in prediabetic and type 2 diabetic adult subjects ($n = 70$). Values are presented as the mean ± standard error of the mean. P: level of significance according to the Mann-Whitney U test. Adequate vitamin C intake was based on the estimated average requirement [10]. For clarification, see Table 2.

highlight that many previous studies have used large vitamin C dosages; under these conditions, vitamin C was shown to act as a prooxidant.

The present study has several limitations as follows: (i) we did not include control subjects (individuals without prediabetes or DM2), and we had only a few individuals with uncompensated DM2, which is different than similar studies and could represent a limitation in comparing the results; (ii) the sample size was small, which did not allow for the generation of dietary recommendations; (iii) we did not measure the plasma level of vitamin C but instead measured the dietary level, which could be subject to recall errors; (iv) we did not consider the different doses and types of hypoglycemiant drugs that were taken by the patients who were under medical treatment; and (v) we did not evaluate the mechanism by which DM2 induces cytotoxicity and how vitamin C modulates that cytotoxicity. Despite these limitations, the correlation observed between vitamin C intake and cytotoxicity is important when designing interventions to reduce the complications that are associated with hyperglycemia in DM2.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Chromosome Instability and Oxidative Stress Markers in Patients with Ataxia Telangiectasia and Their Parents

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Ataxia telangiectasia (AT) is a rare neurodegenerative disorder, inherited in an autosomal recessive manner. Total blood samples were collected from 20 patients with AT, 13 parents of patients, and 17 healthy volunteers. This study aimed at evaluating the frequency of chromosomal breaks in spontaneous cultures, induced by bleomycin and ionizing radiation, and further evaluated the rates of oxidative stress in AT patients and in their parents, compared to a control group. Three cell cultures were performed to each individual: the first culture did not receive induction to chromosomal instability, the second was exposed to bleomycin, and the last culture was exposed to ionizing radiation. To evaluate the rates of oxidative stress, the markers superoxide dismutase (SOD), catalase (CAT), and thiobarbituric acid (TBARS) were utilized. Significant differences were observed between the three kinds of culture treatments (spontaneous, bleomycin, and radiation induced) and the breaks and chromosomal aberrations in the different groups. The oxidative stress showed no significant differences between the markers. This study showed that techniques of chromosomal instability after the induction of ionizing radiation and bleomycin are efficient in the identification of syndrome patients, with the ionizing radiation being the most effective.

1. Introduction

Ataxia telangiectasia (AT) is a rare neurodegenerative disorder, autosomal recessive inherited [1, 2]. The carriers are apparently born normal; however, by 2-3 years old, clinical manifestations appear. The frequency of occurrence of the syndrome in the United States is approximately one in 40,000 live births [3].

Ataxic movements and ocular telangiectasia are among the pathological manifestations [4–7]. AT syndrome is caused by a mutation in the gene located on chromosome 11q22-23 encoding the protein ATM kinase [2, 8]. ATM recognizes double-stranded breaks in DNA and signals the cell-cycle checkpoints [2].

AT patients are sensitive to ionizing radiation [9, 10] and have chromosomal instability, defects in cell-cycle checkpoints [2, 11], and, therefore, increased risk of developing cardiovascular disease and cancer [6, 12]. Hypersensitivity and chromosomal instability characteristic of AT patients can be evaluated through the high frequency of chromosomal breaks and gaps, rearrangements, aneuploidy, and translocations [13–15] observed in cells spontaneous carriers [16] or

in cells exposed to mutagens [13–15]. Furthermore, patients with AT exhibit high rates of chromosomal rearrangements involving autosomal chromosomes 7 and 14. Changes are often found as translocations and inversions involving these chromosomes [17].

Chronic oxidative stress is a common feature of AT [14, 18], but the involvement of ATM protein or the degree of oxidative stress is not elucidated. Bleomycin is already widely used in the evaluation of chromosomal instability in patients with clinical suspicion of AT, but is rarely used as a supplementary examination or routine for the parents of these patients. Based on this, the present work is to provide important information to incorporate new routine tests in identifying patients with AT. Early identification of these is of paramount importance for prenatal counseling, diagnosis, improvement or insertion and treatments for the various manifestations of the disease. The early diagnosis of AT would help in the prevention of cancer, where there is an increased frequency of this disease among patients and their parents.

In this sense, the present study evaluated the frequency of spontaneous, radiation-induced and bleomycin-induced chromosome breakage, as well as the level of oxidative stress in AT patients and parents of patients with the syndrome by comparing the results with the control group.

2. Materials and Methods

2.1. Patients and Blood Sampling. This study was developed at the Cytogenetics Laboratory from the Medical Genetics Service of the Hospital de Clínicas de Porto Alegre, Brazil, between August 2011 and June 2012. We evaluated the chromosomal instability and oxidative stress in blood cells sampled from 20 AT patients, aged 15.3 ± 10.2 years and 13 parents aged 41.0 ± 10.6 years of the AT patients. The control group was composed of 17 healthy individuals aged 22.4 ± 13.4 years.

Subjects were enrolled after signing an informed consent term in accordance with the guidelines of the Ethics Committee at Hospital de Clínicas de Porto Alegre (HCPA), RS, Brazil. Ten-milliliter samples of heparinized peripheral blood were collected, immediately protected from light, and stored at 4°C for evaluation. All subjects answered the personal health questionnaire of the *Commission for Protection Against Environmental Mutagens and Carcinogens* (ICPEMC).

2.2. Cell Cultures, Treatments, and Chromosome Preparations. For the chromosomal instability analysis, three cell cultures were performed to each individual included in this study. A protocol adapted from 1960 [19] was used. Slides were stained with GTG-banding technique [20].

The first culture did not receive induction to chromosomal instability, thereby representing the spontaneous cultures of each individual. The second culture was exposed to bleomycin (6 μg/mL of culture). The third culture was exposed to 3 Gy (ionizing radiation from a ^{137}Cs γ-ray source). The irradiation was performed at the Gamma Cell 1000 Elite irradiator.

The breaks and chromosomal aberrations observed in the three treatments of all individuals groups were reported. A minimum of 4 and a maximum of 50 metaphases made by culture were analysed, depending on the cell growth of each culture. Chromosomal aberrations were classified as chromatid breaks (chtb), chromosome breaks (chrb), chromosome fragments, translocations (t), deletions (del), interstitial deletions, dicentric chromosomes, triradial and quadriradial figures, Rings chromosome (r), Additional materials (add), isochromosomes (iso), chromatid failures (chtg), chromosomal failures (chrg), and fragile sites.

To calculate the frequency of chromosomal instability flaws and fragile sites were excluded, because they are not considered chromosome breakage. The other changes were included in the count, and the number of breaks for each occurrence of each chromosome abnormality observed was recorded.

2.3. Definition of Chromosomal Changes. The chromosome breaks are defined as all discontinuities in the chromosome that are experiencing a greater distance than the width of a chromatid, which may involve one (chtb) or both chromatids (chrb) breaks giving rise to acentric chromosome fragments [21]. The translocations are formed when there is an exchange of portions of two or more chromosomes [22]. Deletions are characterized by the loss of a portion of a chromosome. These deletions can be terminal (del) or interstitial (interstitial deletion). Interstitial deletions are defined when there is loss of a middle segment of the chromosome [22]. The chromosomes are dicentric chromosomes with two centromeres. They are formed from two chromosomes break and union portions of centromere [22]. The figures are chromosomal rearrangements involving more than one chromosome and more than one break point, which can be classified as either triradial or quadriradial [23]. The chromosomal rings are formed when there is loss of telomeric portions of chromosomes resulting from the union of edges forming a circular chromosome [22]. Additional materials of unknown origin were classified as add. These additional materials can be formed by insertion, duplication, or translocation of chromosome segments [22]. Isochromosome is formed when a loss occurs in a portion of the chromosome and the other portion is doubled [22]. Failures chromatid and chromosomal are defined as all discontinuities in one (chtg) or both chromatids (chrg) of chromosomes that have a distance less than one chromatid [21]. The fragile sites are regions where there is abnormal chromatin compaction [24]. They are viewed as a glitch in the chromosome.

2.4. Oxidative Stress. For preparation of the samples, 5 mL of whole blood was collected from each patient. The samples were centrifuged for 5 minutes at a speed of 3000 RPM. After centrifugation, plasma was separated from red blood cells using a disposable Pasteur pipette. The plasma was stored in an Eppendorf.

Red blood cells were centrifuged for 5 minutes at a speed of 3000 RPM and washed three times with saline solution. After washing, the red blood was collected and stored in a 1.5 mL Eppendorf with a buffer prepared with magnesium sulfate ($MgSO_4$) and acetic acid. The Eppendorf's plasma

Table 1: Frequency of chromosomal breaks observed in the different groups and treatments.

	Frequency of chromosomal breaks		
	Spontaneous	Bleomycin (6 μg/mL)	Radiation (3 Gy)
Patients			
Frequency (min × max)	0.00–1.30	0.43–5.65	0.47–7.32
χ frequencies/cell	0.18	1.63	2.10
N° cells (min × max)	30 × 50	30 × 50	30 × 50
Parents			
Frequency (min × max)	0.00–0.13	0.00–1.27	0.23–0.93
χ frequencies/cell	0.02	0.56	0.52
N° cells (min × max)	30 × 50	5 × 50	21 × 50
Control group			
Frequency (min × max)	0.00–0.03	0.00–0.90	0.10–0.93
χ frequencies/cell	0.01	0.47	0.56
N° cells (min × max)	30	4 × 30	13 × 30

χ: average frequency of chromosomal breaks per cell.

and red blood cells were stored in a freezer at −80°C until processing.

To assess oxidative stress, markers TBARS (thiobarbituric acid), SOD (superoxide dismutase), and CAT (catalase) were used.

2.5. Measure of Substances That React in TBARS. The technique of TBARS is the sample heating with thiobarbituric acid and the consequent formation of a colored product, measured in a spectrophotometer at 535 nm. The occurrence of staining is due to the presence of Malondialdehyde (MDA: an indicator of oxidative stress) and other substances from lipid peroxidation in biological material. Plasma samples were placed in test tubes with 0.75 mL of trichloroacetic acid (TCA) 10%, 0.25 mL of homogenate, 0.5 mL of thiobarbituric acid (TBARS) 0.67%, and 25 mL of distilled water. The tubes were stirred and heated to a temperature of 100°C. After the tubes were cooled, 1.5 mL of n-butyl alcohol was added, to extract the pigment formed. After that the samples were placed in a shaker (Biomatic) for 45 seconds and centrifuged for 10 minutes at 3000 RPM (1110 ×g). Finally, the colored product was removed and read in spectrophotometer (CARY 3E-UV-Visible Spectrophotometer Varian) with a wavelength of 535 nm. The concentration of TBARS obtained was expressed in ng per mg total protein [25, 26].

2.6. Evaluation of the Activity of CAT. The rate of decomposition of hydrogen peroxide (H_2O_2) is measured spectrophotometrically at 240 nm, and this wavelength H_2O_2 has maximum absorbance. An incubation mixture containing a final volume of 1000 μL with reagents was prepared: 50 mM phosphate buffer, pH 7.4 and 0.3 M H_2O_2. In quartz cuvette, 955 μL phosphate buffer and 25 μL plasma were added, which were placed in the apparatus and minus the blank. 20 μL of hydrogen peroxide was added and readings were performed at 240 nm. The results were expressed as pmoles/g tissue per mg total protein [26, 27].

Table 2: Statistical analysis comparing the frequency of breaks of different treatments for each group.

Groups	Treatments			P value of the Friedman test
	Spontaneous	Bleomycin (6 μg/mL)	Radiation (3 Gy)	
Patients	a	a, b	b	0.006
Parents	a	b	b	0.001
Control group	a	b	b	0.000

Treatments with at least one letter in common exhibit similar frequency of breaks ($\alpha = 0.05$).

2.7. Evaluation of the Activity of SOD. The technique for determination of SOD, second Misra and Fridovich [28], is based on inhibition of the formation of superoxide dismutase in adrenochrome autoxidation of epinephrine. Whereas epinephrine remains stable in acidic solutions and spontaneously oxidizes in basic solutions, favoring the formation of adrenochrome, SOD can be measured spectrophotometrically by following the change in absorbance at 480 nm of epinephrine which has a peak absorbance. To perform the reaction, a mixture was prepared with a final volume of 1 mL with bicarbonate buffer (0.05 M, pH 10.2) and plasma epinephrine (4 mM). The absorbance used was 480 nm, at 30°C. The line pattern was developed with increasing concentrations of SOD (20–100 nM) to determine the concentration that produces the same whether the inhibition of autoxidation of epinephrine by 50%, and the results were expressed as USOD/mg of total protein. An enzyme activity is defined as the amount of enzyme which is able to inhibit 50% of autoxidation of epinephrine [26].

2.8. Quantification of Total Protein. For the quantification of total protein, the method of Bradford [29] was used. The Bradford method is a technique for determining total protein using the Coomassie Brilliant Blue dye BG-250. This

Table 3: Statistical analysis comparing the chromosomal aberrations (CAs) between the different groups within each treatment.

Treatments	Chromosomal aberrations	*P* value of the Kruskal-Wallis test
Spontaneous	Chromatid breaks (chtb)	0.910
Bleomycin (6 μg/mL)		0.007*
Radiation (3 Gy)		0.000*
Spontaneous	Chromosome breaks (chrb)	0.289
Bleomycin (6 μg/mL)		0.036*
Radiation (3 Gy)		0.197
Spontaneous	Chromosome fragments	0.022*
Bleomycin (6 μg/mL)		0.009*
Radiation (3 Gy)		0.270
Spontaneous	Translocations (t)	0.097
Bleomycin (6 μg/mL)		0.100
Radiation (3 Gy)		0.004*
Spontaneous	Deletions (del)	0.002*
Bleomycin (6 μg/mL)		0.073
Radiation (3 Gy)		0.004*
Spontaneous	Interstitial deletions	1.000
Bleomycin (6 μg/mL)		0.293
Radiation (3 Gy)		1.000
Spontaneous	Dicentric chromosomes	1.000
Bleomycin (6 μg/mL)		0.439
Radiation (3 Gy)		1.000
Spontaneous	Triradial figures	1.000
Bleomycin (6 μg/mL)		0.005*
Radiation (3 Gy)		0.001*
Spontaneous	Quadriradial figures	1.000
Bleomycin (6 μg/mL)		0.228
Radiation (3 Gy)		0.222
Spontaneous	Ring chromosomes (r)	1.000
Bleomycin (6 μg/mL)		0.587
Radiation (3 Gy)		0.943
Spontaneous	Additional materials (add)	1.000
Bleomycin (6 μg/mL)		1.000
Radiation (3 Gy)		0.287
Spontaneous	Isochromosomes (iso)	1.00
Bleomycin (6 μg/mL)		0.293
Radiation (3 Gy)		1.00
Spontaneous	Chromatid failures (chtg)	0.027*
Bleomycin (6 μg/mL)		0.461
Radiation (3 Gy)		0.014*
Spontaneous	Chromosomal failures (chrg)	0.557
Bleomycin (6 μg/mL)		0.021*
Radiation (3 Gy)		0.461
Spontaneous	Fragile sites	0.591
Bleomycin (6 μg/mL)		0.314
Radiation (3 Gy)		0.004*

*demonstrates the chromosomal aberrations with statistically significant differences.

method is based on the interaction between the dye BG-250 proteins and macromolecules containing amino acids basic side chains or aromatic amino acids. In the reaction pH, the interaction between the protein and the high molecular weight dye BG-250 causes the displacement of the equilibrium to form anionic dye, which absorbs strongly at 595 nm in a spectrophotometer [30].

2.9. Statistical Analysis. To evaluate the difference of the frequencies of chromosomal breaks between the different treatments (Spontaneous, bleomycin, and radiation) in each group (patients, parents, or controls) the Friedman test was used.

Statistical analysis of chromosomal abnormalities and total number of breaks between different groups and treatments were performed using the Kruskal-Wallis test. The correlation between total chromosome breakage and different oxidative stress markers was performed using the Spearman test. When statistical analysis showed significant results, post hoc Bonferroni correction was concucted. Multiple comparisons were performed with SPSS, version 15.0, with a significance level of 0.05.

3. Results and Discussion

The sample number and analyzed cells varied depending on the difficulty of each individual cell growth. The rates of cell growth were variable among individuals sampled and from culture to culture. Overall, we observed a sharp decline in growth in the cultures of the group of patients among the three treatments, as described next. Cell growth failure was observed: in patients: 45% samples included in spontaneous and radiation treatments, and 50% samples on treatment with Bleomycin; in parents: 7.7% samples in spontaneous and radiation treatments, and 23.08% in the sample on treatment with Bleomycin and in controls: 23.53% samples in treating spontaneous and 11.76% in the treatment with radiation and Bleomycin.

Regarding the frequency of chromosome breakage, the average of those in the spontaneous treatment of patients with AT demonstrated to be increased compared to the control group, as shown in Table 1. In relation to treatment with induction of DNA damage, patients with AT demonstrated hypersensitivity to the effects of bleomycin and ionizing radiation compared to the control group, confirming what has been described by Huo et al. [9], Sun et al. [10], Abraham [31], and Barzilai et al. [32] (Table 1). Hypersensitivity for bleomycin was demonstrated to be about three times higher than that recorded in the parent groups and controls. Already, hypersensitivity to ionizing radiation proved to be about four times higher in the group of patients than that observed in the groups of parents and controls.

The average frequency of chromosome breakage in parents was almost similar to that obtained by the control group, showing no significant differences as observed in the study by Sun et al. [10] (Table 1).

Statistically, the frequency of chromosomal breaks observed in the three treatments showed significant differences ($\alpha = 0.05$) in patients, parents, and controls, as shown in Table 2, and in the breaks and chromosomal aberrations in the different groups (Table 3). Increased rearrangements were not observed involving the chromosomes 7 and 14.

Table 4: Statistical analysis comparing the total number of breaks in each treatment between the different groups.

Treatments	Groups			*P* value of the Kruskal-Wallis test
	Patients	Parents	Control group	
Spontaneous	a	a, b	b	0.007
Bleomycin (6 μg/mL)	a	b	b	0.001
Radiation (3 Gy)	a	b	b	0.001

Treatments with at least one letter in common exhibit similar number of breaks ($\alpha = 0.05$).

Statistically, the total number of breaks in each of the different treatment groups showed significant differences (Table 4). In spontaneous treatment, differences were observed between patients and controls, and for treatment with bleomycin and radiation, such differences were observed between patients and parents and between patients and controls (Table 4).

No significant differences were observed between different markers of oxidative stress, as shown in Table 5.

The correlation analysis between the total number of breaks of the spontaneous treatment in each group relative to each index marker of oxidative stress showed no significant differences, as shown in Table 6.

A factor that may have influenced the growth of these cultures was the amount of DNA damage generated after induction with bleomycin and radiation. The damage caused to the DNA interferes with their replication, triggering mutations, various types of chromosomal abnormalities, or cell death by apoptosis [33]. For microscopic analysis, cells are stationed in metaphase by the use of colchicine—in this stage, the cells have been through the process of repairing damage (G_2 phase), where cells that have undergone higher levels of DNA damage are eliminated via apoptosis. Gilad et al. [34] showed that ionizing radiation inhibited cell growth in the 6 patients who were included in the study, proving the difficulty of cell growth in patients with the syndrome.

The variation in the amount of cells analyzed for each patient may have influenced the low rates of chromosomal rearrangements involving chromosomes 7 and 14 in patients with AT observed. It was then possible to observe that the evaluation techniques of chromosomal instability after exposure to ionizing radiation or bleomycin are effective in identifying patients with the syndrome, and ionizing radiation was considered the most effective.

Prospects are expected to increase the sample size to conduct further sampling of patients and standardizing the number of cells analyzed. A much more detailed analysis of each patient was developed in order to make more accurate assessments by chromosomal abnormalities and indices of oxidative stress. The inclusion of the analysis of the marker of oxidative stress glutathione peroxidase (GPx) is also

Table 5: Average indices of oxidative stress observed.

	TBARS (ng/mg prot.)	CAT (pg/mg prot.)	SOD (USOD/mg prot.)
χ Patients	3.36	1.11	21.16
χ Parents	3.03	0.95	27.04
χ Control group	3.42	0.73	20.79

χ: average indices of oxidative stress observed.

Table 6: Correlation of the total number of spontaneous breaks of treatment of each group in relation to indexes of different markers of oxidative stress.

Groups	Markers of oxidative stress			
	TBARS	CAT	SOD	
Patients	0.774	0.518	0.200	P value of the Spearman correlation
Parents	1.000	0.809	0.499	
Control group	0.222	0.223	0.631	

($\alpha = 0.05$).

expected. The GPx develops its role in situations where there are low levels of hydrogen peroxide, while CAT operates in situations of high concentrations [35, 36]. Therefore, with the inclusion of this marker and with the addition of a larger number of patients, it will be possible to measure reliably whether or not this happening oxidative stress is strong evidence in patients with AT.

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Review Article

The Influence of Micronutrients in Cell Culture: A Reflection on Viability and Genomic Stability

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Micronutrients, including minerals and vitamins, are indispensable to DNA metabolic pathways and thus are as important for life as macronutrients. Without the proper nutrients, genomic instability compromises homeostasis, leading to chronic diseases and certain types of cancer. Cell-culture media try to mimic the *in vivo* environment, providing *in vitro* models used to infer cells' responses to different stimuli. This review summarizes and discusses studies of cell-culture supplementation with micronutrients that can increase cell viability and genomic stability, with a particular focus on previous *in vitro* experiments. In these studies, the cell-culture media include certain vitamins and minerals at concentrations not equal to the physiological levels. In many common culture media, the sole source of micronutrients is fetal bovine serum (FBS), which contributes to only 5–10% of the media composition. Minimal attention has been dedicated to FBS composition, micronutrients in cell cultures as a whole, or the influence of micronutrients on the viability and genetics of cultured cells. Further studies better evaluating micronutrients' roles at a molecular level and influence on the genomic stability of cells are still needed.

1. Introduction

Micronutrients, essential nutrients that are needed in small amounts, are as important for life as macronutrients. Micronutrients comprise all of the vitamins, such as A, D, and E, as well as the minerals, such as calcium, zinc, and iron. The *in vivo* role of micronutrients is well established, and several studies have examined the effects of micronutrients on genomic stability [1–21]. Approximately 40 micronutrients are required in the human diet, and for each micronutrient, proper metabolism demands an optimal level of intake. A micronutrient deficiency distorts the metabolism in numerous and complicated ways, many of which may lead to DNA damage.

Micronutrients are required for optimal macronutrient metabolism because of micronutrients' critical role in intermediate metabolism. Invariably, metabolism requires the concomitant involvement of one or more vitamins and minerals. Chronic degenerative disease etiology and the rate of pathogenesis are thus intimately associated with micronutrient imbalances. Nutrition research has recently highlighted the role of several nutrients in regulating the genomic

machinery [22]. More specifically, a number of vitamins and micronutrients are substrates and/or cofactors in the metabolic pathways regulating DNA synthesis and/or repair and gene expression [23]. A deficiency in such nutrients may result in the disruption of genomic integrity and alteration of DNA methylation, thus linking nutrition with the modulation of gene expression. In many cases, the response to a nutrient deficiency also seems to be genotype specific. Gene-nutrient interactions are thus a fascinating example of physiological responses to the environment/diet at the molecular level [22].

Minerals and vitamins are indispensable to DNA metabolic pathways [24, 25]. Although there is still no clear evidence for a diet that optimally protects against DNA damage, in terms of either proportions or combinations of specific micronutrients, many studies that conducted *in vitro* and in animal models have demonstrated the roles of micronutrients in maintaining genomic stability. For example, vitamins C and E deficiencies are known to cause DNA oxidation and chromosomal damage [26, 27]. Vitamin D exhibits antioxidant activity, stabilizes chromosomal structure, and prevents DNA double-strand breaks [28]. Similarly, magnesium is an essential cofactor in DNA metabolism that plays a role in maintaining the high fidelity of DNA transcription [29]. Whereas either an excess of or a deficiency in iron may cause DNA breaks [30], a carotenoid-rich diet reduces DNA damage [31], but excess retinol may be carcinogenic in certain individuals [32]. In a final example, vitamin B-12 deficiency is associated with the formation of micronuclei [5, 24], and reduced transcobalamin II in the serum is associated with chromosomal abnormalities [33].

Given the importance of micronutrients *in vitro*, the optimization of cell viability and genomic stability warrants further studies. Cell-culture media mimicking the *in vivo* environment may help to generate *in vitro* models of a cell's response to different stimuli. The composition of these media includes certain vitamins and minerals, but unfortunately, in many common culture media, the only source of micronutrients is fetal bovine serum (FBS), which contributes to only 5–10% of the media composition. Moreover, the appropriate proportion of micronutrients is not always provided because the precise composition of each batch of FBS is in fact extremely variable [34].

Certain micronutrients, such as calcium, folate, magnesium, and iron, have been reported as key elements in cellular processes, including the proliferation, survival, and even differentiation of cell cultures [35–38]. However, the particular concentration of micronutrients in a culture as well as the cell type may trigger different responses. Further studies of micronutrients' roles at a molecular level and influence on genomic stability are still required.

2. Aims and Scope

This review summarizes and discusses studies showing the influence of some micronutrients on cell viability and genomic stability, with a particular focus on *in vitro* models. *In vivo* evidences are presented to illustrate the relevance of the nutrients to genomic stability. Papers were retrieved from PubMed using the following search terms: micronutrients, vitamins, minerals, cell culture, proliferation, viability, and genomic stability. Additional publications were collected by cross-referencing the primary articles retrieved. The review does not aim to include all nutrients that could influence genomic stability; then, only the following nutrients were included vitamins A, B7, B9, B12, C, and E and minerals Cu, Fe, Mg, Se, and Zn. According to Friso and Choi [39], an imbalance of such dietary nutrients as folate, zinc, vitamin C, and selenium can alter genomic and/or gene-specific DNA methylation, resulting in many different molecular effects on gene expression and integrity, in turn affecting cell growth, tissue differentiation, cancer incidence, and aging. To better address the selected micronutrients' effects in cell viability and genomic stability, we considered the information available regarding either their deficiency or excess.

3. Micronutrients and Their Influence on Genomic Stability

DNA damage is one of the most important factors that can compromise homeostasis, resulting in chronic (e.g., atherosclerosis) and even degenerative diseases, including Alzheimer's disease (AD) and certain types of cancer [40]. A deficiency in or imbalance of certain micronutrients has been described as mimicking radiation or chemicals, causing single- and double-strand breaks (SB) or lesions in DNA, or even both [20].

In Table 1, micronutrients whose imbalances cause DNA damage are listed, as well as the nutrients' food sources and possible health effects. In general, micronutrients can either act directly on the genome to prevent mutations or protect the genome indirectly by serving as enzyme cofactors in the cellular processes that modulate transformation [41, 42]. Therefore, any imbalance may result in a degree of DNA damage.

The role of diet in determining genomic stability is more important than previously imagined. It has been found that diet affects all pathways relevant to genomic stability, including exposure to dietary carcinogens, activation and detoxification of carcinogens, DNA repair, DNA synthesis, and cell apoptosis [23, 43]. All of these critical pathways are dependent not only on enzymes but also on substrates and cofactors, a few of which are only available at the right concentration when the dietary intake of key minerals and vitamins is adequate [44]. As a result, a dietary deficiency in certain micronutrients required for DNA maintenance may exert effects similar to inherited genetic disorders that impair the activity of enzymes required for genomic stability [23, 45–47]. Additionally, such a deficiency may damage DNA to a similar extent as significant exposure to known carcinogens, such as ionizing radiation [43].

3.1. Vitamin A. Vitamin A is also referred to as retinoic acid, retinol, retinal, α- and β-carotene, lycopene, lutein, zeaxanthin, β-cryptoxanthin, or astaxanthin. The role of vitamin A and provitamin A (carotenoids) in DNA damage has recently been reviewed by Azqueta and Collins [65]. The well-established antioxidant properties of vitamin A have

Table 1: Micronutrients linked to genomic stability, dietary requirements, and effects of deficiency and excess.

Micronutrient	EAR for adults (not pregnant or lactating)	General health effects of deficiency	Effects of deficiency related to genome instability	UL for Adults	Effects of excess related to genome instability	References
			Vitamin			
Vitamin A	500–625 RAE	Blindness, impaired immunity, and dermal alterations	Increased sensitivity to DNA-damaging agents	3000 RAE	Congenital malformations while in pregnancy. Cancer risk increase for smokers	[48–50]
Vitamin B7 (biotin)	30 μg*	Dermal alterations, immune dysfunction, neurological symptoms, and congenital malformations during pregnancy	Chromatin structural alterations	NA (safe up to 20,000 μg)	Congenital malformations. Increase in DNA damage	[51–54]
Vitamin B9	320 DFE	Anemia and other hematological alterations, pregnancy complication (e.g., neural tube defect)	Uracil misincorporation in DNA; DNA strand breaks	1000 DFE	Increased cancer risk (promotion effect)	[43, 51, 55, 56]
Vitamin B12	2 μg	From lack of energy to irreversible severe damage to nervous system	DNA strand breaks	1000 μg	Unknown	[43, 51]
Vitamin C	60–75 mg (95–110 if smoker)	Dermatological alterations associated to collagen synthesis and immune impairment	DNA strand breaks	2000 mg	DNA damage related to oxidative stress	[43, 49, 55]
Vitamin E	12 mg	Increase in chronic disease risk	DNA strand breaks	1000 mg	DNA damage related to oxidative stress	[43, 49, 57, 58]
			Mineral			
Copper	700 μg	Anemia and other blood dysfunctions, impaired growth, and neurological alterations	Oxidative DNA damage increase	10000 μg (under review)	DNA damage associated to oxidative stress, particularly to liver	[59, 60]
Iron	6–8.1 mg	Anemia and other blood dysfunctions, impaired growth, and neurological alterations	DNA damage increase	45 mg	DNA damage associated to oxidative stress, particularly to liver	[21, 43, 60]
Magnesium	255–350 mg	Rare because Mg deficiency is unusual	DNA repair deficiency	NA	Unknown	[61, 62]
Selenium	45 μg	Decreased activity of glutathione peroxidase leading to increased risk of degenerative diseases and impairment in immunity	DNA strand breaks	400 μg	Tumor incidence seems to be reduced in high doses supplementation	[49, 63]
Zinc	6.8–9.4 mg	Dermal alterations, growth retardation, immune dysfunction, neurological symptoms, night blindness, and adverse outcomes during pregnancy	DNA strand breaks	40 mg	DNA damage increase	[43, 60, 64]

*Adequate intake not EAR.

EAR: estimated adequate requirement; DFE: dietary folate equivalents; RAE: retinol activity equivalents; UL: upper level; NA: not available.

facilitated studies measuring oxidative damage both *in vivo*, in animal studies and human clinical trials, and *in vitro*. Whereas high concentrations of provitamin A carotenoids can cause DNA damage, perhaps by acting as prooxidants, nonvitamin A carotenoids that can significantly reduce such damage [66].

The functions of vitamin A are related to night, day, and color vision; epithelial-cell integrity against infections; the immune response; hemopoiesis; skeletal growth; male and female fertility; embryogenesis. Paradoxically, either an excess of or a deficiency in retinoic acid results in similar malformations in certain organs, including the mammalian kidney [67]. Many eye pathologies are due to vitamin A deficiency, including night blindness, conjunctival xerosis and corneal injuries. Similarly, hypervitaminosis A, resulting from the storage of excess vitamin A in the body, can damage various systems. Very large doses of vitamin A, especially in young children, can increase the intracranial pressure, leading to headache, nausea, and vomiting [68]. It has also been established that adequate vitamin A intake is required for normal organogenesis, immune function, tissue differentiation, and vision. Given these requirements, vitamin A deficiency, which is widespread in the developing world, is responsible for at least one million instances of unnecessary death and blindness each year [69].

3.2. Vitamin B7. Vitamin B7, also known as biotin, acts as a cofactor for the biotin-dependent enzymes pyruvate carboxylase, propionyl-CoA carboxylase, crotonyl-CoA carboxylase, and two isozymes of acetyl-CoA carboxylase [70]. These enzymes catalyze key steps in important metabolic pathways, including fatty acid biosynthesis, gluconeogenesis, and amino acid metabolism [71]. Vitamin B7 deficiency due to inadequate dietary intake or congenital defects in biotin absorption or metabolism results in the inactivation of all five biotin-dependent enzymes. This condition is known as multiple carboxylase deficiency (MCD) [72, 73], whose symptoms include ketoacidosis, lactic acidosis, feeding difficulties, skin rashes, and neurological abnormalities, such as subependymal cysts, hypotonia, seizures, and ataxia. In severe cases, or if MCD is left untreated, the condition can lead to coma or death [74].

It has been demonstrated that biotin plays a role in DNA-strand breaks and the cellular response to strand breaks (SB). More specifically, biotin supplementation increased DNA breaks in cell cultures, although it is unknown whether this finding is relevant to whole organisms [75]. In contrast, *in vivo*, a high biotin intake in combination with a low intake of multiple other nutrients has been associated with increased genomic stability [53]. Biotin deficiency rarely occurs spontaneously in animals, including humans [76], but can be induced by consuming large amounts of raw egg white, which contains avidin, known to inhibit biotin absorption from the intestinal tract, or by taking anticonvulsants [77].

3.3. Vitamin B9. A deficiency in vitamin B9, also known as folic acid or folate, is common in people who consume few fruits and vegetables. Vitamin B9, as well as other vitamins from the B complex, plays an important role in genomic stability, and a deficiency can cause chromosomal breaks in human genes [78]. Vitamin B9 deficiency can also lead to (a) an elevated rate of DNA damage and altered DNA methylation, both of which are risk factors for cancer [78–80], possibly including colon cancer [81] or (b) an increased homocysteine concentration, an important risk factor for cardiovascular disease [82]. These defects may also play a significant role in developmental and neurological abnormalities [78, 79]. However, in animals with existing preneoplastic or neoplastic lesions, folicacid supplementation increases the tumor burden [83]. In contrast, the adequate intake of vitamin B9 can increase genomic stability and possibly reduce cancer risk [84–87] because vitamin B9 is a key carbon donor during nucleotide biosynthesis [88].

3.4. Vitamin B12. Vitamin B12, or cyanocobalamin, deficiency is associated with pernicious anemia and neurological pathologies varying from a minor decrease in cognitive function to neurodegenerative disorders, although the role of vitamin B12 in these conditions requires further investigation [89, 90]. The lack of understanding of the underlying molecular mechanisms may be due to the experimental limitations of the available classical cell-culture models [89]. Nevertheless, vitamin B12 is known to play an important role in genomic stability, and a deficiency in vitamin B12 can lead to DNA damage [81]. Vitamin B12 is also required for the synthesis of methionine and *S*-adenosyl methionine, the common methyl donor required for the maintenance of the DNA methylation patterns that determine gene expression and DNA conformation [91].

Despite controversies in the literature regarding the prevalence of vitamin B12 deficiency, this deficiency seems to be more common among people aged 65–76 years [92]. However, the symptoms of vitamin B12 deficiency caused by poor diet, digestive problems, and/or inadequate absorption in elderly people can be nonspecific, rendering a diagnosis more difficult. Furthermore, neurological symptoms may appear before anemia; in fact, only approximately 60% of elderly people with vitamin B12 deficiency are anemic [92, 93]. In cell-culture models, sufficient vitamin B12 can be provided to the cells by the FBS [89].

3.5. Vitamin C. Vitamin C, also known as ascorbate or ascorbic acid, is a micronutrient required for innumerable biological functions, specifically serving as a cofactor for certain important enzymes [94]. One type enzyme is the prolyl hydroxylases, which play a role in collagen biosynthesis and the downregulation of hypoxia-inducible factor- (HIF-) 1, a transcription factor that regulates many genes responsible for tumor growth, energy metabolism, and neutrophil function and apoptosis. Vitamin C-dependent inhibition of the HIF pathway may provide alternative or additional approaches to controlling tumor progression, infection, and inflammation [94].

As vitamin C exhibits antioxidant properties that provide protection against oxidative stress-induced cell damage by scavenging reactive oxygen species (ROS), the effects of this vitamin on cancer chemoprevention [95, 96] and cancer treatment [97] as well as sepsis [98] and neurodegenerative

diseases (e.g., Alzheimer's disease) [99] have been studied. In fact, ingesting inadequate levels of vitamin C can mimic radiation exposure. In the literature, numerous human supplementation studies have used biomarkers of oxidative damage to DNA, lipids (lipid oxidation releases mutagenic aldehydes), and protein. Although these studies have yielded both positive and negative results, if the fact that blood-cell saturation occurs at approximately 100 mg/day is taken into consideration, the evidence suggests that this level of vitamin C intake minimizes DNA damage [20]. Unfortunately, vitamin C deficiency is common in poor communities, so measures to improve the consumption of vitamin C-rich foods should be considered [100].

3.6. Vitamin E. Vitamin E, which comprises compounds from the tocopherol and tocotrienol families, is required to prevent peripheral neuropathy and hemolytic anemia in humans, which arise due to vitamin E deficiency. Vitamin E functions as a vital lipid-soluble antioxidant, scavenging hydroperoxyl radicals in the lipid milieu. The human symptoms of vitamin E deficiency suggest that this vitamin's antioxidant properties play a major role in protecting erythrocyte membranes and nervous tissues [94]. Additionally, these antioxidant properties play a role in genomic stability, particularly because vitamin E is a potent peroxyl radical scavenger. Vitamin E is also a chain-breaking antioxidant that prevents the propagation of free radicals in membranes and plasma lipoproteins [101].

Recently, Ni and Eng [102] demonstrated that α-tocopherol can selectively protect SDH (var^+) cells from oxidative damage and apoptosis and rebalance the redox metabolites nicotinamide adenine dinucleotide (NAD^+ and NADH). Another interesting recent study [103] evaluated the amount of the oxidation product 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formed from the DNA nucleoside deoxyguanosine (dG) after vitamin exposure. In the case of vitamin E, no DNA damage was induced in cultured cells. Taken together, these results reinforce the role of this vitamin in maintaining DNA integrity and stability. Although the direct comparison of the study outcomes is complicated by varying definitions of vitamin E deficiency, the available data suggest that children and the elderly are most vulnerable to this deficiency and that men may be at higher risk than women [104].

3.7. Copper. Copper is an essential trace element, serving as a cofactor for many enzymes in different biological processes. In contrast to iron, the copper concentration not only in the blood but also in individual organs is maintained at constant levels beginning in early childhood, indicating the presence of robust homeostatic mechanisms [105]. Adequate copper intake permits the normal utilization of dietary iron, as intestinal iron absorption, iron release from stores (e.g., in the macrophages of the liver and spleen), and iron incorporation into hemoglobin are copper-dependent processes. In addition to preventing anemia, copper assists in blood coagulation and blood-pressure control; the crosslinking of connective tissue in the arteries, bones, and heart; defense against oxidative damage; energy transformation; the myelination of the brain and spinal cord; reproduction; hormone synthesis. In contrast, inadequate copper intake has adverse effects on the metabolism of cholesterol and glucose, blood pressure control and heart function, bone mineralization, and immunity [106].

The excessive accumulation of copper in the body can contribute to the development of cancer due to copper's role in causing DNA damage [107]. Curiously, in addition to the robust mechanisms maintaining copper homeostasis and copper's rapid excretion, mammals express copper-dependent enzymes that are central players in antioxidant defense. Thus, whereas copper can induce ROS formation when involved in Fenton-like or Haber-Weiss reactions, copper-dependent processes can also help to clear ROS [105]. For further information on the relationship between copper and DNA damage, please refer to the recent review published by Linder [105].

3.8. Iron. Iron is a crucial nutritional element for all life forms that plays a critical role in the cell, including electron transport and cellular respiration, proliferation and differentiation, and the regulation of gene expression [3]. Iron can undergo univalent redox reactions, resulting in oxidized and reduced forms known as ferric (Fe^{3+}) and ferrous (Fe^{2+}) iron, respectively. Due to iron's oxireduction, which can contribute to ROS generation, as well as iron's role in Fenton and Haber-Weiss reactions, this nutrient is also potentially deleterious. These reactions occur when an inorganic nutrient, such as Fe^{2+} or Cu^+, is in excess and donates an electron to H_2O_2, leading to OH production. The ROS generated by Fenton chemistry can contribute to major pathologies, such as cancer, atherosclerosis, and neurodegenerative diseases [38].

Free radicals can cause serious damage to the genome. Depending on the dose and type, inorganic nutrients can protect against or contribute to oxidative stress [108]. Peroxidases and especially catalase, which use heme iron as a cofactor, decompose H_2O_2. If the resultant reactive species are not efficiently removed, these species can induce the formation of the more active OH or peroxynitrite, which may result in DNA oxidation. Therefore, deficiencies in such nutrient-dependent antioxidant enzymes can increase oxidative stress and favor the genomic instability [109].

In addition, iron is a cofactor of many important enzymes related to DNA repair mainly as clusters of iron sulphur. For example, the glycosylases MutyH and NTHL1 involved in base excision repair (BER) and mismatch repair (MMR) and the helicases ERCC2 and BACH1 acting in the nucleotide excision repair (NER) possess iron-sulphur clusters in their structure [110, 111]. The increased DNA damage sensitivity in cells with impaired Fe/S protein biogenesis may include the loss of nucleotide excision repair because maturation of XPD is defective. Since the Fe/S cluster of XPD is required for its DNA helicase activity *in vitro* [110].

Although excess iron can cause oxidative DNA damage in rats and has been associated with an increased risk of cancer and heart disease in humans [20], iron deficiency also appears

to lead to oxidative DNA damage and is associated with cognitive dysfunction in children. The importance of iron in normal neurological function has been well established, as neurons require iron for many physiological processes, including electron transport and axonal myelination, and as a cofactor for many enzymes involved in neurotransmitter synthesis [112, 113]. In contrast, inadequate iron intake results in anemia, immune dysfunction, and adverse pregnancy outcomes, such as premature birth. Maintaining physiological iron levels via dietary intake is thus mandatory for health. However, iron deficiency is still very common in the human population, particularly among children and pregnant women [114].

3.9. Magnesium. Magnesium is indispensable to life, as this micronutrient is involved in many important biological processes. Magnesium has multiple functions in all cellular processes, including DNA replication and protein synthesis, and also serves as a cofactor for DNA-repair proteins and in the maintenance of a cell's redox status, cell-cycle regulation, and apoptosis [29]. Magnesium deficiency or the displacement of Mg^{2+} by other toxic, divalent metal ions leads to increased genomic instability, which has been implicated in many diseases [115] and may result in inhibited DNA repair, oxidative stress, accelerated aging, and increased cancer risk [29, 116]. Studies have indicated that higher magnesium consumption may protect against certain inflammatory disorders, such as insulin resistance [117], hypertension [118], diabetes mellitus [119], and cardiovascular disease [118].

Magnesium is not genotoxic at physiologically relevant concentrations and in fact maintains low mutation frequencies by facilitating high-fidelity replication and by supporting all DNA-repair processes and chromosomal segregation during mitosis [29]. In fact, it is an essential cofactor in NER, BER, and MMR processes, where magnesium is required for the removal of DNA damage [120]. All downstream activities of major base excision repair proteins, such as apurinic/apyrimidinic endonuclease, DNA polymerase beta, and ligases, require magnesium. Thus, this element may act as a regulator for the base excision repair pathway for efficient and balanced repair of damaged bases, which are often less toxic and/or mutagenic than their subsequent repair product intermediates [121]. Magnesium is also important for the fidelity of DNA replication, impacting cell cycle and apoptosis [61].

Animal and human epidemiological studies have demonstrated inverse correlations between magnesium levels and cardiovascular disease [29] or the incidence of certain types of cancer, including colorectal cancer [122, 123]. Additionally, magnesium deficiency is one risk factor for premature aging [29]. The relationship between magnesium levels and tumorigenesis is more complex, with magnesium deficiency increasing tumor incidence in animals and humans, whereas magnesium promotes the growth of preexisting tumors due to profound changes in magnesium homeostasis in tumor cells. Thus, the protective effects of magnesium are restricted to the early stages of tumor development [29]. According to Ford and Mokdad [124], despite the role of magnesium in maintaining good health, historically, much of the population of the United States has not consumed adequate amounts of this nutrient. Additionally, there are significant racial and ethnic disparities in magnesium intake.

3.10. Selenium. The trace element selenium is another well-established micronutrient essential for mammalian health [125]. Selenium is a constituent of the small group of selenocysteine-containing selenoproteins [126], including glutathione peroxidase, thioredoxin reductase, selenoprotein P, and selenoprotein R, which are primarily involved in antioxidant activity and the maintenance of a cell's redox state [127–130]. Due to selenium's key role in redox regulation and antioxidant function, this nutrient is critical for membrane integrity, energy metabolism, and protection against DNA damage [126]. However, in certain cases, selenium can also lead to oxidative DNA damage [20], increased infection risk, and altered mood [131]. Whether selenium exerts positive or negative effects *in vivo* or *in vitro* is related to dose. Interest in organoselenide chemistry and biochemistry has increased over the last two decades, mainly because a variety of organoselenium compounds can be used as antioxidants, enzyme inhibitors, neuroprotective, antitumoral, or anti-infectious agents, as well as cytokine inducers and immunomodulators [125, 132–135]. In fact, an interaction with the zinc finger structures of DNA repair proteins may occur by essential trace elements such as certain selenium compounds, which appear to exert anticarcinogenic properties at low concentrations but may compromise genetic stability at higher concentrations [136].

Selenium deficiency alone is not common in developed countries, but an inadequate intake of this mineral has been associated with the development of cancer, asthma, and coronary disease, among other chronic conditions [137]. When required, dietary supplementation must be performed carefully, given the intrinsic toxicity of high selenium levels [138].

3.11. Zinc. Zinc is one of the most important micronutrients due to the prevalence of zinc-dependent enzymes in metabolic processes; zinc's vital role in several bodily functions, such as vision, taste perception, cognition, cell reproduction, growth, and immunity; the beneficial effect of zinc supplementation on many disease states [139]. In fact, zinc is a component of over 300 proteins, including over 100 DNA-binding proteins with zinc fingers, Cu/Zn superoxide dismutase, the estrogen receptor, and the synaptic transmission protein [20]. Zinc also has a crucial role in the biology of p53, in that p53 binds to DNA through a structurally complex domain stabilized by zinc atom, possibly increasing the response to anticancer drugs [140].

Zinc deficiency is a health problem in many communities, especially among adolescents, due to the pubertal growth spurt [139]. At the molecular level, there is evidence of a relationship between zinc deficiency and increased chromosomal breaks, possibly due to increased oxidative damage stemming from a loss in the activity of Cu/Zn superoxide dismutase or the zinc-containing DNA-repair enzyme Fapy glycosylase, which repairs oxidized guanine [20]. Unfortunately, nearly half of the world's population is at risk of inadequate zinc

intake, so public health programs are urgently needed to reduce zinc deficiency [139].

3.12. Summary of the Effects of the Selected Micronutrients on Genomic Stability. Taking the preceding discussion and other evidence from the literature into account, the adequate intake of micronutrients seems to have an important role in genomic stability. In contrast, an imbalance of the same micronutrients may also negatively impact the DNA, possibly via oxidative stress, consequently causing or contributing to different human diseases. It is thus highly relevant to elucidate the mechanism underlying the response to and repair of oxidative stress and this mechanism's relationship to the DNA damage response pathways, all of the inorganic nutrients (vitamins and minerals) and disease, including carcinogenesis. An understanding of the possible influences on genomic stability, even in cell culture, is also in current demand.

4. Cell-Culture Medium and Micronutrients That Increase Genomic Stability: Is the Concentration Relevant?

According to Ferguson and Fenech [141], the last decade of studies on micronutrients and genomic stability have improved dietary recommendations based on the prevention of DNA damage or the maintenance of genomic integrity. In light of this, the development of *in vivo* and especially *in vitro* models to more robustly evaluate DNA damage is necessary.

Table 2 presents interesting data regarding the micronutrients that may interfere with genomic stability and the micronutrient concentration values found in typical cell-culture media, FBS, and human serum. Unfortunately, data are not available for all of the micronutrients in the media, and even the proportions of micronutrients in FBS, as an organic product, are not all well characterized. Additionally, as demonstrated by Bryan et al. [34], the concentration of many micronutrients in FBS can vary significantly between batches.

Although cell-culture media attempt to provide an environment similar to the *in vivo* milieu of cell development, there is an evident imbalance of micronutrients between the media and human serum. Certain micronutrients are present in these media at concentrations higher than those found in human serum (e.g., vitamins B7 and B12), whereas other nutrients are present at significantly lower concentrations than in human serum (e.g., iron and zinc). A recent study [103] called attention to the composition of multivitamin supplements, which may trigger unwanted health outcomes due to the synergistic oxidative effects of the component vitamins and metals. In this research, the vitamins' chemical oxidation potencies were studied by measuring the amount of the oxidation product 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formed from the DNA nucleoside deoxyguanosine (dG) after vitamin exposure. The micronutrients evaluated by the authors were the vitamins A, B1, B2, B3, B6, B12, and C; β-carotene; folic acid; α-, δ-, and γ-tocopherol. The minerals copper, iron and zinc were also examined. All of these micronutrients were tested in cell culture, alone or in combination, taking the human serum levels of each micronutrient into account. The main conclusion reported was that certain vitamins, alone or in combination with metals (e.g., vitamin C and copper), can induce DNA damage. However, cells in culture and *in vivo* have distinct needs for nutrients and growth factors, as the cells' activity in each environment may differ due to interactions with other cells or parts of the larger organism. Thus, examining physiological concentrations of micronutrients *in vitro* may not be the most appropriate approach.

As mentioned above, each cell type may have a distinct requirement for micronutrients. Depending of the origin of the cell and its role *in vivo*, the cell may specifically have a higher affinity for one micronutrient over another. In the case of iron, for example, which is stored in specific tissues, including the spleen, liver, and bone marrow [142], the primary cells or immortal cell lines derived from these tissues may have a greater need for this specific micronutrient. In the case of certain neuronal cells, which require iron for cell development [143], the demand for iron may also be higher than in other cell types. Although the evaluation of micronutrients' influence on DNA damage and integrity as well as on cell development, including the related enzymes and proteins, should be continued, the micronutrient concentrations relevant not only to human but also to cell-culture genomic stability must be considered.

5. Could Changes in a Culture's Micronutrient Composition Influence the Viability and Genetics of the Cultured Cells?

Cells are typically maintained at an appropriate temperature and CO_2 concentration (usually 37°C and 5% CO_2 for mammalian cells) in an incubator. Beyond these parameters, the most commonly varied factor in culture systems is the growth medium. The recipes for growth medium can vary in pH, glucose concentration, growth factors and the presence of other nutrients and micronutrients. The development of synthetic basal formulations for mammalian cell-culture applications has been facilitated by the contributions of many investigators. In particular, the definition of the minimally required nutrients by Harry Eagle in the 1950s spawned an iterative process of continuous modification and refinement of the exogenous environment to cultivate new cell types and support the emerging applications of cultured mammalian cells. This process led to the development of highly potent, basal nutrient formulations capable of sustaining serum-free cell proliferation and biological production [152]. However, the growth factors most often used to supplement cell-culture media are still derived from animal blood, such as FBS. FBS has become the supplement of choice for cell culture-based research, containing an array of proteins, growth factors, and ions necessary for cell viability and proliferation *in vitro*, including certain vitamins and minerals [153]. Currently, the use of these ingredients is minimized or eliminated wherever possible in favor of chemically defined media, but this substitution is not always possible.

Bryan et al. [34] stated that one of the major obstacles to obtaining human cells of a defined and reproducible

Table 2: Concentrations (in μmol/L) of micronutrients that can increase genomic stability in traditional cell-culture media and FBS versus human serum.

Micronutrients	Cell culture medium*								10% FBS**	Mean human serum concentration***	Status cell-culture medium versus human serum
	MEM	DMEM	L-15	M-199	HAM F-10	HAM F-12	RPMI-1640	DMEM/HAM F12			
Vitamins											
Vitamin A	NA	NA	NA	3.1×10^{-1}	NA	NA	NA	NA	3.0×10^{-2}	2.0	Lower
Vitamin B7 (Biotin)	NA	NA	NA	4.1×10^{-2}	1.0×10^{-1}	3.0×10^{-2}	8.2×10^{-1}	1.0×10^{-2}	Trace	4.0×10^{-4}	Higher
Vitamin B9	2.3	9.1	2.3	2.3×10^{-2}	3.0	3.0	2.3	6.0	Trace	5.0×10^{-3}	Higher
Vitamin B12	NA	NA	NA	2.8×10^{-1}	1.0	1.0	4.0×10^{-3}	5.0×10^{-1}	Trace	3.0×10^{-4}	Higher
Vitamin C	NA	NA	NA	1.4×10^{-2}	NA	NA	NA	NA	Trace	50.0	Lower
Vitamin E	NA	NA	NA	NA	NA	NA	NA	NA	0.0003	30.0	Unknown
Minerals											
Copper	NA	NA	NA	NA	1.0×10^{-2}	1.0×10^{-2}	NA	5.0×10^{-3}	Trace	14.0	Lower
Iron	NA	2.5×10^{-1}	NA	1.7	3.0	3.0	NA	1.6	3.0	23.0	Lower
Magnesium	8.0×10^{2}	8.0×10^{2}	1.8×10^{2}	NA	6.2×10^{2}	6.1×10^{2}	4.1×10^{2}	1.1×10^{3}	Trace	8.0×10^{2}	Lower/similar
Selenium	NA	NA	NA	NA	NA	NA	NA	3.0×10^{-2}	3.0×10^{-2}	11.0	Lower
Zinc	NA	NA	NA	NA	1.0×10^{-1}	3.0	NA	1.5	Trace	17.0	Lower

NA: not available.

*MEM: minimum essential medium; DMEM: Dulbecco's modified eagle medium; L-15: Leibovitzs medium 15; M-199: medium-199; HAM F-10 and F-12: Ham's nutrient mixture F-10 and F-12; RPMI-1640: Roswell Park Memorial Institute medium; DMEM/HAM F-12: Dulbecco's modified eagle medium/Ham's nutrient mixture F-12. The vitamin and mineral concentrations described were obtained from the webpages of the key suppliers.

**The values for the vitamin A, vitamin E, and selenium concentration in FBS were found in [144], and the iron concentration in FBS was determined analytically.

***The references citing the micronutrient concentrations in human serum are as follows: vitamins A [145], B7 [146], B9 [147], B12 [148], C, and E [149]; Mg [150]; Cu, Fe, Se, and Zn [151]. The concentration of the vitamins and minerals in the media were obtained from the manufacturers.

standard, and thus suitable for use in medical therapies, is the routine necessity of supplementing cell-culture media with FBS. In this study, FBS variants were evaluated, in terms of both elemental (micronutrient) composition and the variants' effects on the expression of a group of proteins associated with the antigenicity of primary human umbilical vein endothelial cells (HUVECs). A combination of inductively coupled plasma mass spectrometry (ICPMS) and flow cytometry was used to achieve these experimental objectives. Statistically significant differences in antigenic expression during cell culture were demonstrated for a set of trace elements in FBS (e.g., lithium, boron, magnesium, phosphorus, sulfur, potassium, titanium, vanadium, chromium, manganese, iron, copper, zinc, gallium, and selenium). The lack of reproducibility and the variation in protein expression in the primary human cells was attributed to the FBS supplementation.

Culture conditions for cell lines are known to affect gene expression [154–156], while stem cells grown in different types of serum exhibit variable differentiation and proliferation characteristics [157, 158] the same cell line, if cultivated in different conditions, can present different phenotypes. Nevertheless, the cellular requirement for a specific micronutrient is directly correlated with the cell type, the rate of cell grow, and the stage of cell differentiation. In light of this, it is important to observe that minimal attention has been dedicated to the composition of FBS and the micronutrient supplementation of media in cell cultures or the fact that micronutrients can influence the viability and genomic stability of cultured cells.

In Tables 3 and 4, a few examples of the effects of vitamins and minerals in cell culture and on genomic stability, drawn from the literature, are highlighted.

5.1. Vitamin A. For vitamin A, but possibly applicable to many other micronutrients, the studies presented in Table 3, conducted at low concentrations, which tend to show protective effects, whereas higher concentrations are associated with increased DNA damage [65]. This finding is consistent with the known ability of β-carotene to act as a prooxidant, rather than as an antioxidant, at high concentrations and under high oxygen tension [178]. The physiological concentrations of micronutrients should always be evaluated and, if possible, at least used as a maximum in studies evaluating the viability and genomic stability of cell cultures. However, as can be verified in Table 2, there is a lack of data regarding the presence of vitamin A in cell-culture media.

5.2. Vitamin B7 (Biotin). Biotin plays an important role in regulating gene expression, thus mediating certain aspects of cell biology and fetal development [179]. The effects of biotin deficiency are detailed in Table 3 and are related to decreased rates of cell proliferation, impaired immune function, and abnormal fetal development. An excess of biotin is also mentioned and can exert reproductive and teratogenic effects. However, as can be verified in Table 2, cell-culture media containing higher levels of biotin than human serum are common. More studies evaluating the effects of the high biotin levels in cell cultures are necessary.

5.3. Vitamin B9. Folate depletion appears to enhance carcinogenesis, whereas folate supplementation above what is presently considered to be the basal requirement confers a protective effect [180]. A few examples of folate deficiency and supplementation are described in Table 3, and the relationship between this vitamin and cell proliferation and apoptosis has been demonstrated. Furthermore, as can be verified in Table 2, the folate levels in the cell-culture media evaluated are typically higher than those levels found in human serum. It is well established that folate deficiency can influence the genomic stability of cultured cells [81, 181], yet there is still a lack of data evaluating whether folate levels above the physiological range can impair cell growth. Elevated levels of folic acid should be examined, as in tumor-prone animals, both folate deficiency and supplementation promote the progression of established neoplasms [83, 182]. As a folate overload is more common than a deficiency in *in vitro* studies, the former should be most thoroughly evaluated.

5.4. Vitamin B12. Vitamin B12 deficiency has been described as similar to chemicals that damage DNA by causing single- and double-strand breaks [20]. As demonstrated in Table 3, in a cellular model designed to better understand vitamin B12 deficiency in the brain, the growth and differentiation of neuronal cells were affected [89]. Additionally, supplementation with certain cobalamin compounds protected the cells from neurotoxicity and increased cell growth [170, 171]. Unfortunately, *in vitro* research demonstrating a direct link between vitamin B12 deficiency or overload and genomic stability in human cells has not yet been published. Based on Table 2, however, high concentrations of vitamin B12 are more common in cell-culture media than in human serum.

5.5. Vitamin C. In Table 3, a few examples of the influence of vitamin C in cell cultures are provided. Different concentrations of this vitamin result in distinct responses, ranging from DNA damage (at higher concentrations) to the protection of DNA (at lower concentrations). Importantly, the concentration of vitamin C in current cell cultures is not available in Table 2, as possibly only trace levels are present in media. As the cellular response to vitamin C may be dose-dependent, a similar concentration of this vitamin in culture media to that in human serum should be evaluated.

5.6. Vitamin E. *In vivo* vitamin E supplementation is still being discussed [183], and more *in vitro* studies will be required to better understand the protective effects of vitamin E on cell viability and genomic stability. Nevertheless, certain results (Table 3) have been consistent with the concept that α-tocopherol, combined with ascorbic acid or alone, can protect against oxidative DNA damage [175] and reduce apoptosis and autophagy [177] under certain conditions. Unfortunately, the current *in vitro* concentration of vitamin E is also not available in Table 2, as possibly only trace levels are present in media. Given this observation, it is interesting to observe that the *in vitro* studies of vitamin E described in Table 3 adopted concentration values similar to that of human serum (approximately 30 μmol/L) and that the results were positive for the cell cultures.

Table 3: Examples from the literature of vitamins' effects in cell culture and on genomic stability.

Micronutrient	Main effects on cell viability and genomic stability	Cell type	Additional information regarding the form and concentration of the micronutrient evaluated	Status in relation to physiological concentration	References
Vitamin A	Enhanced the levels of 8-oxo-dG DNA damage but significantly inhibited M1dG formation especially after induction of M1dG by H_2O_2 or B[a]P; increased production of reactive oxygen species and formation of promutagenic DNA lesions	Lung epithelial cells	Beta-carotene (5 μmol/L)	Similar	[159, 160]
	Caused oxidation of dG and cytotoxicity, giving rise to an almost complete cell death	Leukemia cells (HL-60)	Retinol (2 μmol/L) and ascorbic acid (50 μmol/L)	Similar	[161]
	Induced apoptosis by increasing apoptotic protein p53 and decreasing antiapoptotic Bcl-2 as well as nuclear ATM; also induced DNA fragmentation	Gastric cancer cells (AGS)	Beta-carotene (100 μmol/L)	Higher	[162]
	DNA damage on HepG2 which was also concordant to increased apoptosis and necrosis of cells	Hepatocarcinoma cells (HepG2)	Beta-carotene (4 μmol/L) and 8 μmol/L)	Similar	[163, 164]
	Reduced levels of total DNA adducts and increased apoptosis levels in cells coexposed to benzo(a)pyrene and retinoic acid		Retinoic acid (1 μmol/L)	Lower	
Vitamin B7 (biotin)	Increased strand breaks and cellular response to strand breaks	T-lymphocyte cell line (Jurkat)	25×10^{-6} μmol/L and 0.01 μmol/L	Lower and higher	[75]
	Affects biotinylation of proteins, gene expression, and metabolism of interleukin-2; rates of proliferation and apoptosis were not affected by biotin status		25×10^{-6} μmol/L, 25×10^{-5} μmol/L and 0.01 μmol/L	Lower and higher	[165]
Vitamin B9	Increased levels of excision repair and apoptosis	Lymphocytes	Folate ($< 2.3 \times 10^{-3}$ μmol/L)	Lower	[166, 167]
	Decreased apoptosis and increased cell proliferation	Neural stem cells (NSCs)	Folic acid (8.4×10^{3} μmol/L)	Higher	[168, 169]
	High concentration accelerated growth; increased metabolic activity, proliferation, and apoptosis; decreased differentiation	Human colon cancer cells (HT29)	Folic acid (0.021 μmol/L and 0.21 μmol/L) with other micronutrients involved in folate-methionine cycle	Similar and higher	[56]

Table 3: Continued.

Micronutrient	Main effects on cell viability and genomic stability	Cell type	Additional information regarding the form and concentration of the micronutrient evaluated	Status in relation to physiological concentration	References
Vitamin B12	Reduced cell proliferation and increased differentiation	Neuroblastoma cells (NIE115)	Vitamin B12 (total absence)	Lower	[89]
	Chronic exposure inhibited neurotoxicity	Retina cells (primary cultures from fetal rats)	Methylcobalamin (1 μmol/L)	Higher	[170]
	Absence is likely to result both in reduced cell proliferation and in cell death, as inhibition of DNA synthesis generally results in apoptosis	Human erythroleukemic (K562) and murine lymphoma (BW5147) cell lines	Cobalamin (total absence and 3.7×10^{-3} μmol/L)	Lower and higher	[171]
Vitamin C	Physiological concentrations of AA were not toxic, while high concentrations of AA induced DNA strand breakage in a dose-dependent manner, whereas AA2P were not genotoxic	Human dermal fibroblasts (HDFs)	Ascorbic acid (AA) and ascorbic acid 2-Phosphate (AA2P) (total absence or 20, 100, and 500 μmol/L)	Lower, similar, and higher	[172]
	Enhanced DNA-protein crosslinks and cytotoxicity	Chinese hamster cells (V79)	Ascorbic acid (1000 μmol/L)	Higher	[173]
	Decreased number of 8-hydroxydeoxyguanosine adducts	Mouse keratinocyte cell line	Ascorbic acid (2,27 μmol/L and 4,54 μmol/L)	Lower	[174]
	Protective effect against DNA damage induced by X-ray treatment	Human lymphoblastoid cells (Raji)	Ascorbic acid (60 μmol/L)	Similar	[175]
Vitamin E	Protective effect against DNA damage induced by H_2O_2 treatment	Raji cells	α-Tocopherol (30 μmol/L)	Similar	[175]
	Reduced DNA fragmentation and apoptotic body formation, possibly favoring DNA repair	African green monkey kidney (Vero), human colon carcinoma (Caco-2), and dysplastic oral keratinocyte (DOK) cells	Vitamin E (25 μmol/L)	Similar	[176]
	Reduced apoptosis and autophagy	Cultured trophoblasts and villous explants obtained from human placentas at term	Vitamin E (50 μmol/L) with vitamin C (50 μmol/L)	Higher	[177]

5.7. Copper. As can be verified in Table 2, there is a marked lack of copper in common cell-culture media, even when supplemented with FBS. Thus, cells in culture are typically exposed to an environment deficient in a micronutrient critical for the formation of detoxifying enzymes, which may impact cell development and possibly genomic stability and survival rates. It is important to note that the copper concentrations evaluated in cell culture (Table 4) are generally above the human physiological range, so toxic effects in cultures should be expected. Thus, the optimization of the copper concentration in cell cultures is necessary to maintain cell viability and genomic stability and to avoid the deleterious effects of this metal.

5.8. Iron. In Table 4, it is important to note that the results of Lima et al. [187] may be expected in a cell culture in which the requirements for micronutrients are quite different from those *in vivo*. In this study, the concentrations evaluated were generally higher than the values measured in human serum (Table 2), and even the lowest concentration of iron applied for the authors (22.38 μmol/L) would be considered high for cells in culture. For HL-60 leukemia cells, as demonstrated in [201], the iron concentration range for optimal cell proliferation is very narrow (2-3 μmol/L). In contrast, in the studies in which the iron levels were between 5 and 10 μmol/L, these levels generally benefitted the cultures analyzed, or at least no damage was observed [187–189].

5.9. Magnesium. As presented in Table 4, several studies on the effects of magnesium deficiency on cultured cells have demonstrated reduced oxidative stress, cell-cycle progression, cell growth, and cell viability [190, 191, 202–207]. Killilea and Ames [192] specifically investigated the consequences of long-term and moderate magnesium deficiency in normal human cells in comparison with more typical magnesium levels, using a concentration observed in normal human serum (0.8 mmol/L). No alterations were observed in the cells cultured in the medium containing normal magnesium levels. Additionally, based on studies conducted either in bacteria or in mammalian cells in culture, there is no evidence for the genotoxic effects of magnesium salts at physiologically relevant doses [29], indicating that adequate micronutrient levels in cell-culture media may improve cell viability and genomic stability. As shown in Table 2, the levels of magnesium currently found in cell-culture media are very similar to those levels in human serum, which is very unusual for micronutrients in general.

5.10. Selenium. The differential toxicities elicited by selenocompounds need to be taken into account in *in vivo* and *in vitro* supplementation studies [194]. The references in Table 4 evaluated different forms of selenium and certain salts that may be more toxic to the cellular environment than others. Due to the importance of selenium as well as many other micronutrients discussed in this review, the micronutrient concentration in the media, as well as the FBS, intended for cell culture should be controlled and adjusted to the physiological range, if applicable. By comparing the human serum concentration of selenium in Table 2 with those concentrations described in the experiments cited in Table 4, it is apparent that the concentrations below the physiological range benefitted the cell culture, although high concentrations of selenium compounds potentially negatively affected tumor cells.

5.11. Zinc. The role of zinc in genomic stability was recently reviewed by Sharif et al. [208]. Additionally, a few brief examples of zinc's influence on cell viability and genomic stability are provided in Table 4. A possible conclusion from the *in vitro* assays is that when the zinc concentration used is below the human serum value (Table 2), the results tend to be beneficial for the cultured cells. In contrast, zinc concentrations above the physiological level can damage cultured cells. Again, it is interesting to observe that certain cell-culture media (e.g., HAM F-10 and F-12), even when supplemented with FBS, cannot provide enough of this micronutrient for appropriate cell development and genomic stability once the concentration falls below the physiological range.

6. What Must Be Done: Limitations of the Available Evidence and Conclusions

Micronutrients are clearly important for cell development and genomic stability, and many of the micronutrients mentioned are necessary for the DNA synthesis and repair mechanisms. Table 5 provides an overview of the current data regarding the effects of deficiencies or excesses of the micronutrients addressed in this review on genomic stability. The micronutrient levels found in the discussed cell-culture media and the status of research on each micronutrient are also highlighted. Evidently, much research has been performed, but more specific studies focusing on cell cultures are still required.

Even though there are some highly enriched media available as basal media for serum-free cell culture, like Medium 199 or Ham F-12 nutrient mixture, the most common source of micronutrients currently used in cell cultures is still FBS. The limitations of FBS in providing adequate micronutrient concentrations have been analyzed and described in the literature [34]. Given that cell- and tissue-culture models are generally important in scientific research, the development of standards *in vitro* methods is mandatory. These new standards will decrease dependence on animal serum, a supplement with an undefined, variable composition that can considerably influence experimental results [209]. Furthermore, according to van der Valk et al. [209], an improved exchange of information regarding newly developed serum-free media may be beneficial. It has also become clear that nearly every cell type has distinct requirements for media supplementation, and especially, as discussed in this review, for micronutrient supplementation. A universal cell- and tissue-culture medium may not be feasible, as different cell types have different receptors involved in cell survival, growth and differentiation, and release different factors into the surrounding environment.

Besides this, it is important to highlight that although the formulations of the classical cell culture media are unchanged for a long time, since their development, the quality and

Table 4: Examples from the literature of minerals' effects in cell culture and on genomic stability.

Micronutrient	Main effects on cell viability and genomic stability	Cell type	Additional information regarding the form and concentration of the micronutrient evaluated	Status in relation to physiological concentration	References
Copper	Increased cytotoxicity and ROS formation	HepG2	50, 100, 150, and 200 μmol/L	Higher	[184]
	Reduced mitochondrial activity and cell viability and increased DNA damage	Chinese hamster ovary cells (CHO-K1)	24.55, 35.40, 48.31, 89.23, 116.77, 170.75, 339.45, and 450.35 μmol/L	Higher	[185]
	Increased the DNA damage in a dose-dependent manner and also reduced rates of DNA synthesis and histone acetylation	Leukemia cells (HL-60)	Total absence, 10, 20, 50, 100 and 200 μmol/L	Lower, Similar, and Higher	[186]
Iron	Inhibited DNA synthesis in proliferative cells	Human lymphocytes	Iron suiphate (22.38, 44.76, and 89.52 μmol/L)	Similar and Higher	[187]
	Possibly accelerated aging process and death at concentrations >10 μmol/L, whereas 5 μmol/L increased protein content	Cerebellar granule cells	Ferric nitrilotriacetate (5, 10, 15, 20, and 40 μmol/L are shown	Lower, Similar, and Higher	[188]
	Genotoxic effects	Primary nontransformed colon cells and preneoplastic colon adenoma cell line (LT97)	Ferric nitrilotriacetate (10, 100, 250, 500, and 1000 μmol/L)	Lower and Higher	[189]
Magnesium	Inhibited cell proliferation and promoted endothelial dysfunction by generating proinflammatory, prothrombotic, and proatherogenic environment	Human endothelial cells	Magnesium sulphate (100, 500, and 1000 μmol/L)	Lower and Higher	[190]
	Inhibited growth more drastically in normal than in transformed cells and altered cell-cycle progression	Normal (HC11) and transformed (MCF-7) breast epithelial cell lines	Total absence, 10, 30, 50, 100, 300, and 500 μmol/L	Lower	[191]
	Inadequate concentration accelerated cell senescence	Normal human fibroblasts (IMR-90)	100, 400, and 800 μmol/L	Lower and Similar	[192]
	Incision repair completely inhibited in absence of Mg^{2+} as well as at very high concentrations, whereas optimal concentrations essential in all steps of NER	Human lymphoblastoid (AHH1) and clonal human epithelial adenocarcinoma (HeLa S3) cell lines	400 and 800 μmol/L	Lower and Similar	[193]

Table 4: Continued.

Micronutrient	Main effects on cell viability and genomic stability	Cell type	Additional information regarding the form and concentration of the micronutrient evaluated	Status in relation to physiological concentration	References
Selenium	Methylseleninic acid, L-selenocysteine, selenodiglutathione, or selenite-induced cell death in micromolar concentrations, whereas selenomethionine or ebselen was not toxic within the concentration range tested	HepG2, human hepatoma cell line (Huh-7), and mouse hepatoma (Hepa 1-6)	Sodium selenite, L- or DL-selenocysteine, selenodiglutathione, selenomethyl-selenocysteine, sodium selenate, L- or DL-selenomethionine, methylseleninic acid, ebselen, selenomethionine, and selenodiglutathione (0.1×10^{-3} to 1000 μmol/L)	Lower, Similar and Higher	[194]
	Induces G1-cell cycle arrest and apoptosis via multiple signaling pathways, which may play a key role in methylselenol-induced inhibition of cancer cell proliferation and tumor cell invasion	Human sarcoma cell line (HT1080)	Seleno-L-methionine (SeMet) (total absence, 1.25, 2.5, and 5 μmol/L)	Lower	[195]
	Decrease in cell damage and protection against oxidative stress	HepG2 cells	Selenium methylselenocysteine (0.01, 0.1, 1, and 10 μmol/L)	Lower and Similar	[196]
			Selenium methylselenocysteine (1 μmol/L)	Lower	[197]
Zinc	Increased oxidative DNA damage; disrupted p53, NFκB, and AP1 DNA binding; decreased DNA repair	Rat glioma cell line (C-6)	Zn sulfate and Zn carnosine (4.0 μmol/L)	Lower	[198]
	Decreased cell growth and viability, increased DNA SB and cytotoxicity in Zn-depleted cultures as well as at concentrations of 32 and 100 μM; reduced genomic damage in cultures supplemented with 4 or 16 μM	Human lymphoblastoid cell line (WIL2-NS)	Zn sulfate and Zn carnosine (total absence, 0.4, 4.0, 16.0, 32.0, and 100.0 μmol/L)	Lower, Similar, and Higher	[199]
	Decreased cell viability in Zn-depleted cultures (0 μM) as well as at concentrations of 32 and 100 μM for both Zn compounds and increased DNA SB, apoptotic, and necrotic cells in Zn-depleted cultures	Primary human oral keratinocyte cell line (HOK)			[200]

Table 5: Overview of the data addressed in this review.

Micronutrient	Evidence of genomic instability induction		Concentration in common cell-culture media versus physiological concentration	Optimal concentration proposed for cell culture
	Deficiency	Excess		
Vitamin A	+	+	Lower	Studied
Vitamin B7	+	+	Higher	Requires more studies
Vitamin B9	+	+	Higher	Studied
Vitamin B12	+	NA	Higher	Studied
Vitamin C	+	+	Unknown	Studied
Vitamin E	−	+	Unknown	Studied
Copper	+	+	Lower	Studied
Iron	+	+	Lower	Studied
Magnesium	NA	+	Similar	Studied
Selenium	+	−	Lower	Studied
Zinc	+	+	Lower	Studied

NA: Not available.
(−) Negative: the available data indicate no effect.
(+) Positive: the available data indicate an effect.

purity of single components used as supplements, are likely to have increased considerably. However, some losses of important substances could have occurred, including trace elements, vitamins, growth factors, and lipids and this should be better addressed before defined a serum-free media. In fact, the threshold for developing and using a new well-defined medium, given that the current FBS-supplemented culture media work well, is high [209]. At the very least, an evaluation of FBS composition, in terms of micronutrients and possibly other factors, should be strongly considered in the laboratories that focus on *in vitro* studies. Knowledge of the micronutrient composition of FBS may help to minimize the bias in experimental results. However, maintaining both successful and consistent cell cultures can be difficult, as FBS is a complex natural product and may vary between batches, even if obtained from a single manufacturer. More specifically, the quality and concentration of both bulk and specific proteins in cell cultures can affect cell growth [210]. Adjusting the *in vitro* micronutrient levels to physiological values will guarantee a better environment for cell development, mimicking the *in vivo* milieu.

Further studies on the effects of micronutrients on cell viability, proliferation, and stability, as well as gene expression and integrity are still required, but the information already available is a sufficient call to action. As mentioned by Ferguson and Fenech [141], most investigations have been limited to studying the effects of single micronutrients and have not considered genetic consequences. Thus, there is an important need for studies that also examine nutrient-nutrient and nutrient-gene interactions. Determining the physiological range of such significant micronutrients as iron and then adjusting the concentrations currently found in cell-culture media may be beneficial for *in vitro* assays. More specifically, the viability and genomic stability of cell lines and primary cultures may be improved. Depending on the cell type (primary, immortalized, tumor, or normal) and origin (lung, hepatic, neural, or other), the requirement for a micronutrient may vary widely, so this subject should be carefully evaluated. Finally, the form of the micronutrient used in supplementation media may also influence experimental results. For example, according to Jacobs et al. [211], whether iron has toxic effects is directly related to the presence of a chelating agent, which reduces the concentration of free ferric ion and promotes the formation of ferritin.

Once the relationship between an *in vivo* imbalance of micronutrients and genomic stability, which may cause many diseases, including cancer, is established, it will be mandatory to better understand *in vitro* micronutrient supplementation. In fact, certain simple questions, such as *"is the concentration of this micronutrient sufficient for the development of this cell?"* or "*are the levels of this micronutrient similar to the levels observed in human serum?",* may aid the proper design of *in vitro* studies.

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Review Article

Oxidative Stress in the Pathogenesis of Colorectal Cancer: Cause or Consequence?

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There is a growing support for the concept that reactive oxygen species, which are known to be implicated in a range of diseases, may be important progenitors in carcinogenesis, including colorectal cancer (CRC). CRC is one of the most common cancers worldwide, with the highest incidence rates in western countries. Sporadic human CRC may be attributable to various environmental and lifestyle factors, such as dietary habits, obesity, and physical inactivity. In the last decades, association between oxidative stress and CRC has been intensively studied. Recently, numerous genetic and lifestyle factors that can affect an individual's ability to respond to oxidative stress have been identified. The aim of this paper is to review evidence linking oxidative stress to CRC and to provide essential background information for accurate interpretation of future research on oxidative stress and CRC risk. Brief introduction of different endogenous and exogenous factors that may influence oxidative status and modulate the ability of gut epithelial cells to cope with damaging metabolic challenges is also provided.

1. Introduction

There is growing support for the concept that reactive oxygen species (ROS), which are known to be implicated in a range of diseases, may be important progenitors in carcinogenesis [1]. In the last decade, growing number of reports investigating association between ROS and carcinogenesis have been published. Reports have proposed various consequences of oxidative stress that may be linked to carcinogenesis [2–4].

The aim of this review is to briefly summarize proposed mechanisms of oxidative stress that are implicated in carcinogenesis, to review evidence linking oxidative stress with colorectal cancer (CRC), and to provide essential background information for accurate interpretation of future research on oxidative stress and CRC risk. For this reason, we provide brief introduction of different endogenous and exogenous factors that may influence oxidative status and modulate the ability of gut epithelial cells to cope with damaging metabolic challenges.

2. Proposed Mechanisms of Oxidative Stress in the Pathogenesis of Cancer

When free radicals are produced in excessive and uncontrollable amounts, they and their derivative products may react with various cellular macromolecules, such as lipids, proteins, and DNA and may modulate gene expression.

2.1. Lipid Oxidation. ROS have the ability to oxidize polyunsaturated fatty acids (PUFAs), which take part in cell membrane constitution. This reaction initiates lipid peroxidation, a chain reaction that produces other free radicals and substances such as malondialdehyde (MDA), conjugated dienes, hydroperoxides, lipoperoxides, and toxic aldehydes [2, 5]. Lipid peroxidation changes the fluidity of cell membranes, reduces the capacity to maintain an equilibrated gradient of concentration, and increases membrane permeability and inflammation [6]. Namely, leakage of normal intracellular enzymes into extracellular fluids results in chemotaxis of neutrophils and other inflammatory cells to the site of injury

[7]. In addition, products of lipid peroxidation (particularly MDA and 4-hydroxy-2-nonenal (HNE)) may act as signaling transducers and at low levels modulate several cell functions including gene expression and cell proliferation [2, 8–10]. They also have high reactivity with DNA bases. MDA, which is one of the best known breakdown products of lipid peroxides, was found to react with DNA dG, dA, and dC forming M_1G, M_1A, and M_1C DNA adducts, respectively. These etheno-DNA adducts are mutagenic, and evidence indicates that they may contribute to cancer, including CRC [11–13].

2.2. Protein Oxidation. Proteins are also susceptible to ROS and are frequent target of increased production of free radicals. ROS oxidize structural proteins and inhibit proteolytic system. Such reactions lead to alteration of structure of proteins or alteration of enzyme functions. The latter can have a wide range of downstream functional consequences, such as inhibition of enzymatic and binding activities, increased or decreased uptake by cells, inactivation of DNA repair enzymes, and loss of fidelity of damaged DNA polymerases in replicating DNA [14]. Oxidized proteins are catabolised in order to reform amino acids. Moderately oxidized soluble cell proteins are selectively and rapidly degraded by the proteasome, while severely oxidized proteins (carbonyl byproducts) are poor substrates for degradation and accumulate in cells [15]. It is assumed that accumulation of such damaged material over time contributes to various age-related pathologies in man [14, 16]. Namely, accumulation of damaged proteins in cell acts as an inhibitor of the proteasome, which decreases the capacity for removal of oxidized proteins, accelerates the accumulation of misfolded and damaged proteins, and affects cellular lysosomal system. This in turn hampers protein turnover and gradually leads to further structural and functional alterations of cell organelles [17].

2.3. DNA Oxidation. ROS are also known to cause oxidative nucleobase modifications in DNA (i.e., oxidized thymines, oxidized cytosines, oxidized adenines, oxidized guanines), which may lead to carcinogenesis via mispair/mutagenic potential of the modified base. For example, formation of 8-oxodG in DNA leads to G $\rightarrow$ T transversions during replication unless the damage is repaired by base excision repair (reviewed in [18]). Recent evidence demonstrates that 8-oxodG in the nucleotide pool can be metabolized to form 8-oxodGTP, which can then incorporates into DNA during cellular replication or during DNA repair leading to A $\rightarrow$ C transversions [19]. The dGTP nucleotide pool is mainly located in the cytoplasm and is thus more available for attack by ROS in comparison to dG incorporated in DNA, which is located in the nucleous and protected by histones [19]. Oxidation of DNA may affect DNA methylation due to oxidation of DNA at either the methylated cytosines or guanines in CpG sequences. DNA adduct formation at the guanine of CpG sequences inhibits binding of the DNA methyltransferase to the adjacent cytosine residue and thus results in hypomethylation of DNA. DNA methylation plays an important role in gene regulation (overexpression or silencing) [18]. DNA hypomethylation occurs in many cancers, including colorectal cancer.

2.4. Modulation of Cellular Signaling. Redox environment is critical factor in cellular signaling. ROS play important roles as intracellular signaling molecules. They are involved in various physiological cellular processes. Under homeostatic conditions, ROS are critical to multiple signal transduction pathways by acting as second messengers. ROS regulate key cellular functions such as proliferation, differentiation, growth, and apoptosis through cellular signaling. Among the most known pathways are NF-κB, the phosphatidyl inositol-3 kinase (PI3K)/Akt pathway, heat shock proteins, and the mitogen-activated protein kinase (MAPK) pathway. However, beneficial or harmful role of ROS depends on their concentrations. Under conditions of oxidative stress where levels of ROS are imbalanced with antioxidants, ROS can be detrimental for the cell itself, leading to uncontrolled proliferation, inflammation, or apoptosis [1, 20–24].

3. Oxidative Stress and Colorectal Cancer

Colorectal cancer (CRC) is one of the most common cancers worldwide, with the highest incidence rates in western countries [25]. It is estimated that most of the cases of CRC occur sporadically (70–80%), while approximately 15% of CRC cases develop as a result of inherited factors, such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal carcinoma (HNPCC) [26]. Changes in worldwide variations in the incidence rates, together with the results of migrant studies, show that sporadic human CRC may be attributable to various environmental and lifestyle factors, such as dietary habits, obesity, and physical inactivity [27].

Colon cancer originates from the epithelial cells that line the bowel. These cells divide rapidly and have a high metabolic rate, which has been found as a potential factor that may be responsible for increased oxidation of DNA [28]. Study on primary rat colonocytes has shown that cells from lower crypt sections are more sensitive towards hydrogen peroxide damage than differentiated cells at the surface of the crypt [29]. Since proliferating cells (stem cells and their dividing daughter cells) in the colon are based in the lower part of the crypt, this may show that proliferating cells are putative target cells of colon carcinogenesis. Stem or progenitor cells have been shown to be very sensitive to the redox environment. Their self-renewal and differentiation depend largely on redox environment in the gut mucosa. Proliferating cells are also exceptionally sensitive against DNA damage because the DNA is present as single strand in the S-phase of the cell cycle and serves as template for the complement strand in daughter cells. DNA damage in single strand could lead to varying mutations in the DNA of daughter cells, which could not be repaired [29]. DNA damage can result in either cell cycle arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, all of which are associated with colon carcinogenesis [4]. However, recent evidence has suggested that the generation of ROS may play important role in all

phases of carcinogenesis, that is, the initiation, promotion, and progression stages [30].

3.1. Markers of Oxidative Stress in CRC. In the last decade association between oxidative stress and CRC has been intensively studied. Excellent review of some more commonly used biomarkers of oxidative stress (such as MDA, HNE, acrolein, isoprostanes, glutathione status, tyrosine oxidation, nitration) in human disease and discussion about shortcomings related to validation criteria and other confounding factors has been published by Dalle-Donne et al. [31].

It was found that the human colorectal tumors (adenomas and carcinomas) have increased levels of different markers of oxidative stress, such as increased levels of ROS (measured by chemiluminescence), nitric oxide (NO) [32], 8-oxodG in DNA [33], lipid peroxides, glutathione peroxidase (GPx), catalase (CAT) [34], and decreased methylation of cytosine in DNA [33]. Besides lipid modifications also increased leukocyte activation in carcinogenic tissue was found [34], which indicates possible contribution of inflammatory cells to a further oxidative stress [32]. It was found that the level of DNA lesions varied between colon and rectum tissues, being lower in the former than in the latter [35].

In addition to colon tumors, significantly increased levels of 8-oxodG in DNA in leukocytes [33] and serum [36] of cancer patients were also detected. Guz and coworkers [33] found significantly decreased methylation of cytosine residues in DNA of leukocytes of patients with colorectal tumors in comparison to the levels found in leukocytes of healthy subjects.

It was found that whole-blood levels of ROS (measured by chemiluminescence) were higher in patients with history of sporadic CRC in comparison with age- and gender-matched healthy controls. No difference in the whole-blood levels of ROS was found between patients gene carriers for HNPCC or patients with FAP and their corresponding healthy controls. These results suggest that ROS may play a role in the etiology of sporadic cancer. However, since they lack important information about patient's lifestyle habits (smoking, drinking, use of antioxidants, exercise, etc.), additional investigations are needed before any conclusions can be made [37].

4. The Protective Mechanisms against Oxidative Stress

All these previously mentioned findings support the hypothesis that oxidative stress may be implicated in colorectal carcinogenesis. However, living organisms are constantly exposed to numerous challenges (in the outer or inner environment) that can significantly affect redox potential of their cells. Therefore, they have developed various protective mechanisms that provide cells with enormous capacity of redox homeostasis. These antioxidative protective mechanisms can be divided into three levels of defence (Figure 1) [38].

The first level of antioxidative defence is represented by the organization of oxygen transport or by the proteins, which bind iron and in this way prevent the Fenton's reaction. *The second level* includes two primary defense systems, that is, detoxification enzymes that may be controlled by the level of the xenobiotics and antioxidant system that reduce free radical species and maintain the redox state of the cell. *The third level* of defence includes enzymes that repair the oxidative damage of lipids, proteins, carbohydrates and nucleid acids [38]. Some of these enzymes are different proteolytic enzymes, glycosylases, endo- and exonucleases, DNA ligases, DNA polymerases, and so forth. For example, repair and removal of DNA containing oxidized bases in vivo are regulated by DNA glycosylases [19], mainly through the base excision repair (BER) although certain types of oxidative lesions also appear to be repaired by nucleotide excision repair (NER) and mismatch repair (MMR) [18]. Cells that possess complex DNA repair system are composed of BER, NER, global genome repair (GGR), and the transcription-coupled repair (TCR) [39]. Failure in these protective mechanisms may represent one of the risk factors in the etiology of CRC.

4.1. The Role of Protective Mechanisms in Oxidative Stress Induced CRC. The antioxidant defence system is known to be composed of numerous antioxidants which work collectively. Antioxidants are divided into primary (superoxide dismutase (SOD), CAT, GPx, glutathione reductase (GR)), secondary (vitamin E, vitamin C, beta-carotene, uric acid, bilirubin, and albumin) and tertiary (biomolecules damaged by free radicals) defence elements in the cell [40]. Thus, micronutrient antioxidants may have by virtue of their free radical scavenging properties important role in the redox homeostasis. Patients with adenomatous polyps had significantly lower levels of all measured micronutrient antioxidants (α- and γ-tocopherol, lutein, β-cryptoxanthin, lycopene, and α- and β-carotene) in their colon mucosa than their healthy control subjects. However, their serum levels of these antioxidants were similar in both groups [41]. In contrast, another study reported that colorectal cancer patients had significantly decreased levels of antioxidant enzymes, vitamins C and E in the serum than corresponding healthy control group [36]. Low intake of the micronutrient selenium (Se) has been implicated as a risk factor in CRC. Epidemiological studies linking Se intake to CRC risk have found strong evidence for a link to adenoma risk [42].

Much of research has recently been focused on the investigation of genetic factors that may affect susceptibility to CRC. Several single-nucleotide polymorphisms (SNPs) in genes implicated in antioxidative protective system, such as eosinophil peroxidase, myeloperoxidase [43], SOD2 (MnSOD), and selenoprotein, have been found [42, 44]. Selenoproteins are group of ~25 proteins with incorporated selenocysteine. They are implicated in various protective mechanisms against oxidative stress. For example, the GPx are antioxidative enzymes, thioredoxin reductases (TR) function in redox control, selenoprotein P (SePP) transports selenium to tissues, and selenoprotein S (SelS) is involved in removing unfolded protein response [42].

It was found that genetic variation in various selenoprotein genes may influence susceptibility to CRC. For example, study on 832 CRC patients and 705 healthy controls showed

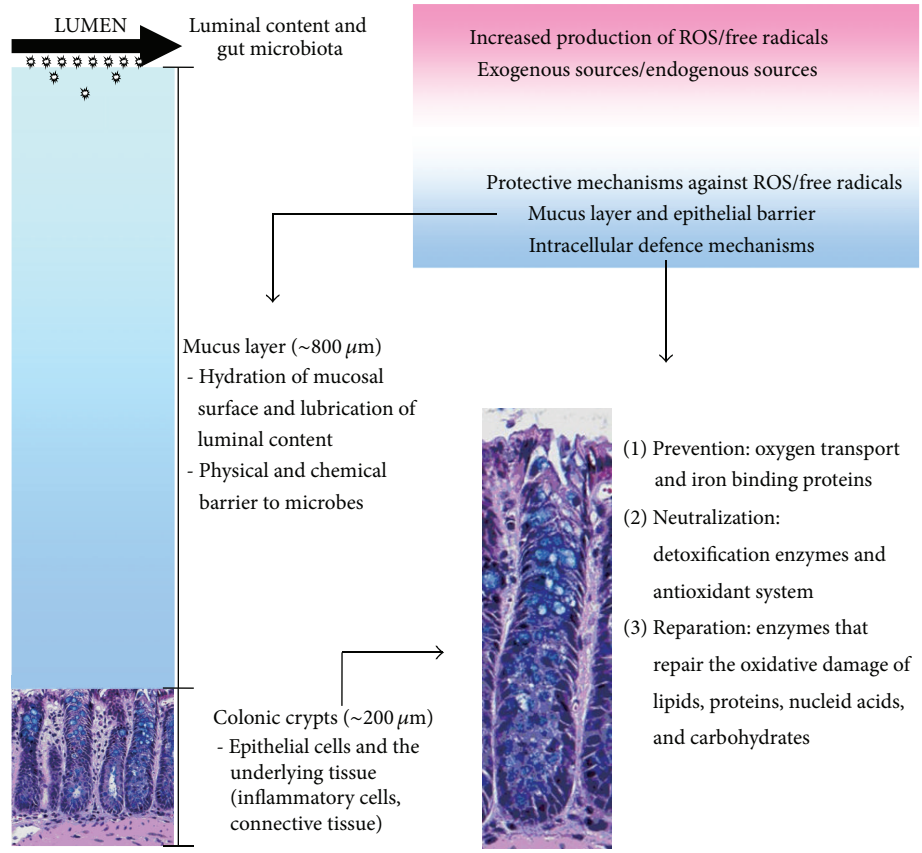

Figure 1: Shematic representation of the colonic barrier and intracellular protective mechanisms against oxidative stress.

significant association between SNPs in *SEPP1*, *GPX4*, and *SELS* genes and risk of CRC [42]. Another study on 827 patients with CRC and 733 healthy controls found association between SNPs in *SEP15* and *SELS* genes and altered risk of CRC [44]. These SNPs have been shown to have functional consequences. It was suggested that these variants play a role in cancer development and may thus represent potential biomarkers for CRC risk. Furthermore, findings from two population-based case-control studies of colon (n = 1555 cases, 1956 controls) and rectal (n = 754 cases, 959 controls) cancer support an association between selenoprotein genes and CRC development and even survival after diagnosis. Results also suggested that the impact of cancer susceptibility from genotype may be modified by lifestyle [45]. Importance of antioxidative protective system has been recently demonstrated on animal model. Induction of inflammatory colon carcinogenesis in GPx-3 deficient mice resulted in an increased tumor number along with a higher degree of dysplasia, increased inflammation, increased proliferation, hyperactive Wnt signaling, and increased DNA damage [46]. In addition, genetic variation in the MAPK signaling pathway, downstream target for ROS, has been shown to be associated with CRC risk and survival after diagnosis [47]. There are also studies investigating diet-gene interactions and the mechanisms by which food components regulate gene expression to modify CRC susceptibility [48].

5. Sources of Free Radicals in Colon: Beneficial, Harmful, or Confounding

Human colonic contents are diverse mixture of bile, mucus, desquamated epithelial cells, various microorganisms and their fermentation products, undigested or unabsorbed food and its metabolic products, such as metals, salts, toxins, mutagens, carcinogens, and dissolved gases (like nitrogen, hydrogen, carbon dioxide, methane, oxygen). It is believed that intestinal mucosa is constantly challenged with diet- and bacterial-derived oxidants and carcinogens. Chronic exposure of such challenging conditions may then lead to uncontrolled generation of free radicals, redox imbalance, and DNA damage, which can affect intestinal metabolic homeostasis with cancer as an endpoint [33].

It is noteworthy to recognize that epithelial cells in the gut mucosa of a healthy individual are not in direct contact with the luminal content or gut microbiota. The secreted mucus layer is very thick (~800 μm in the rodent colon) and represents both a physical and chemical barrier to microbes. Its function is also to keep the mucosal surface well hydrated and to lubricate luminal content [49]. Gut mucosa is composed of a thick secreted mucus layer, a layer of epithelial cells and the underlying nonepithelial tissue, composed of inflammatory cells, connective tissue, and so forth (Figure 1) [49]. All these components are intrinsically linked in a complex physiology.

In addition, the gut mucosa is not constantly exposed to harmful challenges. The gut mucosa is exposed to various beneficial and modifying factors (e.g., healthy food, exercise) that can counteract deleterious effects of harmful challenges. To get an insight into this comprehensive and complex field, brief introduction of harmful, beneficial, and modifying effects of some endogenous and exogenous factors on antioxidant status are represented in the following section.

5.1. Gut Microbiota. The gut microbiota (termed microflora) contains a broad spectrum of microorganisms, which resides in the gastrointestinal tract and play important role in human health and disease (reviewed in [50]). They are essential for the host's wellbeing in terms of nutrition and mucosal immunity. Certain members of the gut microbiota have been shown to promote the host's health. However, there are also numerous studies that have implicated some members of the gut microbiota in the development of CRC due to different mechanisms including generation of reactive metabolites (*E. faecalis* produces hydroxyl radical-potent source of oxidative stress on the intestinal epithelium) [51] and carcinogens, alterations in host carbohydrate expression and induction of chronic mucosal inflammation [52]. The human intestinal habitat contains 300–500 different species of bacteria, varying significantly in content between individuals, which may potentially represent huge variability in the formation of free radicals among men.

5.2. Inflammation. Rapidly growing body of evidence indicates that chronic inflammation is important factor in development of carcinogenesis [7, 53]. It is widely known that CRC is a complication of a chronic inflammatory state in the bowel. Patients with inflammatory bowel disease (ulcerative colitis or Crohn's disease) have 6-fold increased risk to develop CRC compared with the general population [46]. Excessive and uncontrollable production of ROS for a longer period of time results in persistent injury of cells in the tissue and consequently persistent inflammation. Besides damaged cells also inflammatory cells produce soluble mediators, which act by further recruiting inflammatory cells to the site of injury and producing more reactive species [53, 54]. This sustained inflammatory/oxidative environment leads to an enhanced production of hydroperoxides in a vicious circle, which can damage healthy epithelial and stromal cells in the vicinity of injury and over a long time may lead to carcinogenesis. The role of chronic inflammation and oxidative stress in carcinogenesis is excellently explained elsewhere [7, 53].

5.3. Food. It has been demonstrated that dietary fatty acids affect the lipid content of tissue and result in differential susceptibility to peroxidation [55–58]. Lipids and fatty acids obtained from dietary fats are metabolized and incorporated into the phospholipids of the cell membranes of many cell types and serve as precursors for many biologically active molecules, as well as being important for cell signalling or different intensity of inflammation response [59, 60]. A substantial increase in the PUFA content may overcome the protective action of the antioxidant system and increase susceptibility to lipid peroxidation [61]. We have recently demonstrated that long-term consumption of an high-fat mixed-lipid (HFML) diet significantly increased the production of lipid peroxides in the liver [62] and skeletal muscle [63] and increased development of CRC [62]. On the other hand, fish oil has been found to reduce oxidative DNA damage [58, 64]. It was recently shown that a high-fat, low-calcium, and vitamin D diet induces oxidative stress in the colon [65]. Hemoglobin from either red meat or bowell bleeding may act as an enhancer of oxidative damage in the bowel [66].

On the other hand, micronutrient antioxidants may have by virtue of their free radical scavenging properties important role in the redox homeostasis. Nutritionally derived antioxidants such as vitamin E and C, beta-carotene [67], flavonoids and polyphenoles may provide second line of defence against the production of ROS. Epidemiological studies evaluating the occurrence of polyps after supplementation with vitamin E and β-carotene have yielded mixed results [41], while experimental studies demonstrated protective effects. It has been already shown that vitamin E has antiproliferative properties in cancer cell lines, while different natural antioxidants such as gallic acid [68], polyphenols [69], vitamin D [70], vitamin A [71] pharmacological compounds like bis-1,7-(2-hydroxyphenyl)-hepta-1,6-diene-3,5-dione (BDMCA) [72] have potential to inhibit CRC development in dimethylhydrazine (DMH) model, which is well-established CRC animal model and possesses many characteristics found in human sporadic CRC [73].

5.4. Obesity. Obesity, particularly abdominal obesity, was associated with increased risk of CRC and was found to affect oxidative status in obese people [74–77]. It is known that adipose tissue produces various adipocytokines (e.g., adiponectin, leptin, and numerous cytokines such as TNFα, IL-6, IL-8, and IL-10) that are implicated in normal functioning of the body. Evidence has shown that increasing obesity alters levels of adipocytokines, increases circulating oestrogens, decreases insulin sensitivity, and raises the inflammatory response. It causes so-called metabolic syndrome or low-grade chronic inflammation, which may be responsible for constant increase in production of free radicals. It is assumed that over time such conditions (oxidative stress) create environment favorable to the CRC development [74–77].

5.5. Aging. Accumulated evidence suggests that aging is associated with increased production of free radicals, resulting in increased oxidation of lipids, proteins, and genetic material [78]. Oxidative conditions cause progressive structural and functional alterations of cellular organelles and changes in redox-sensitive signalling processes [40, 79, 80]. Such cellular conditions contribute to increased susceptibility to a variety of diseases, including inflammation and cancer [81]. Oxidative stress as a consequence of increased production of nitrogen or oxygen reactive species has been demonstrated in inflammatory bowel disease and CRC.

5.6. Physical Activity. Regular exercise may help to prevent colon cancer due to an improvement in the cell's

antioxidant defence system. It has already been demonstrated that exercise improves the antioxidant defence system in various tissues. Exercise stimulates various signaling pathways in cells, such as MAPK and NFκB, which results in increased expression of important enzymes associated with cell defence (MnSOD and GPx) and adaptation to exercise (eNOS and iNOS) [21, 22]. Many of the biological effects of antioxidants appear to be related to their ability not only to scavenge deleterious free radicals but also to modulate cell-signalling pathways. The modulation of signalling pathways by antioxidants could thus help prevent cancer by preserving normal cell cycle regulation, inhibiting proliferation, inducing apoptosis, inhibiting tumor invasion and angiogenesis, suppressing inflammation, and stimulating detoxification enzyme activity [30, 82, 83]. Exercise has been found to decrease the expression of inducible nitric oxide synthase (iNOS), as well as TNF-α, in the colon of azoxymethane-(AOM-) treated mice [84].

6. Concluding Remarks

As demonstrated previously, gut mucosa possesses various protective mechanisms to neutralize effects of increased production of free radicals, that is, thick secreted mucosal layer, which represents important physical and chemical protective defence to luminal content and strong antioxidative protective mechanism. We provided data demonstrating that oxidative status and ability of antioxidant system to respond to various conditions are influenced heavily by a number of physiological and environmental factors. Various redox-dependent mechanisms in an organism could be affected by different endogenous or exogenous factors with protective (scavenging), harmful (accelerating), or modifying effects on production of free radicals. Recent studies have found an association between a genetic variant in some genes of antioxidative protective mechanisms and CRC risk. On the basis of the known and suggested role of previously mentioned proteins (selenoproteins, SOD, etc.) in cell protection mechanism, it is possible that factors that affect their pattern of colonic expression may modulate the ability of gut epithelial cells to cope with damaging metabolic challenges. For instance, low dietary Se intake or genetic variation in the selenoprotein genes may influence expression or function of selenoproteins, respectively. As demonstrated, these studies represent ground for various speculations. Thus, before we can answer the question whether oxidative stress is a cause or a consequence of cancer development, further studies elucidating the role of antioxidative protective defence and other confounding factors in the pathogenesis of CRC are needed.

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Research Article

The Vitamin D Receptor (VDR) Gene Polymorphisms in Turkish Brain Cancer Patients

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Objective. It has been stated that brain cancers are an increasingly serious issue in many parts of the world. The aim of our study was to determine a possible relationship between Vitamin D receptor (VDR) gene polymorphisms and the risk of glioma and meningioma. *Methods*. We investigated the VDR Taq-I and VDR Fok-I gene polymorphisms in 100 brain cancer patients (including 44 meningioma cases and 56 glioma cases) and 122 age-matched healthy control subjects. This study was performed by polymerase chain reaction-based restriction fragment length polymorphism (RF LP). *Results*. VDR Fok-I ff genotype was significantly increased in meningioma patients (15.9%) compared with controls (2.5%), and carriers of Fok-I ff genotype had a 6.47-fold increased risk for meningioma cases. There was no significant difference between patients and controls for VDR Taq-I genotypes and alleles. *Conclusions*. We suggest that VDR Fok-I genotypes might affect the development of meningioma.

1. Introduction

The term of brain cancer is a heterogeneous group of neoplasms which occurs in intracranial tissue and the meninges. The incidence of malignant brain tumors is rare and approximately 2% of all cancers in adults [1, 2]. According to the result of several studies, the frequencies of brain tumors are of different rates according to countries worldwide. In addition, these differences can be observed among different ethnic groups in the same country [1]. Primary brain tumors are classified according to histopathological features like various other cancer types into the following major histologic groups: tumors of neuroepithelial tissue including astrocytoma, glioblastoma, oligodendroglioma, and ependymoma, tumors of meninges including meningioma and hemangioblastoma, and tumors of cellular region including pituitary tumors and craniopharyngioma [3]. Glioblastoma and meningioma are two of the most common and most malignant primary brain tumors. Gliomas are responsible for approximately 80% and meningioma is responsible for nearly 13–26% of all primary malignant brain tumors [4–6]. Primary brain tumors are a very heterogeneous group of diseases that are still not fully understood in their pathological mechanism. Despite all these developments, the contribution of genetic factors in the development of brain tumors is still not fully understood. Understanding of the genetic risk factors is very important for choosing the most suitable cancer treatment [6, 7].

Vitamin D is a steroid hormone that regulates several endocrine functions and cell functions such as, proliferation and differentiation. [8, 9]. The $1,25(OH)_2D$ is one of potent regulators of cell growth and differentiation, which is an effect on cell death, tumor invasion, and angiogenesis, which makes it a candidate compound for cancer regulation [9]. It is known that vitamin D inhibits malignant cell proliferation and differentiation in several tissues such as breast, colon, skin, and brain [10]. The nuclear functions of vitamin D require binding to the vitamin D receptor (VDR) [11]. The vitamin D receptor (VDR) is involved in multiple pathways such as insulin-like growth factor (IGF) signaling; it also has a role in the inflammation and estrogen-related pathways that may be related to the prognosis of cancer [12, 13]. The gene encoding the VDR is located on the chromosome

12q12-14 [14]. Various studies suggested the effect of VDR gene polymorphisms in the development of several types of carcinoma such as breast, prostate, and colon carcinoma [9, 15–17]. One of the VDR gene polymorphisms is the Fok-I (rs2228570) polymorphism, which is located in exon 2 and results in an alternative transcription initiation site, leading to alter the activity of VDR protein. The polymorphic Fok-I site in exon 2 results in different translation initiation region due to thymine (T) to cytosine (C) substitution. The one of the most well known of these polymorphisms such as Taq-I (rs731236), which are located in exons 9, and a T/C nucleotide substitution (ATT to ATC) leading to a synonymous change at codon 352 (isoleucine) [18]. It has been reported that VDRs localized in neuronal and glial cells affect the metabolism of brain cells and change the expression of VDR [18, 19]. It has been reported that the potential effect of vitamin D on the treatment of cancer was first identified in myeloid leukemic cells [20]. Synthetic vitamin D analogues are among the preferred options in the treatment of central nervous system tumors. In addition, phase II clinical studies have correlated with positive effects of vitamin D therapy on glioblastoma cells [21–26]. The increased vitamin D synthesis in glioma cells after treatment can regulate cell proliferation [19]. However, the consist of this effect is required in VDR gene expression [20, 25]. Several studies have determined the contribution of VDR polymorphisms in various types of cancer. The aim of this study was to evaluate the association between polymorphisms in the VDR Fok-I and Taq-I and the risk of brain cancer in Turkish patients.

2. Materials and Methods

Subject Selection. We investigated the VDR Taq-I and VDR Fok-I gene polymorphisms in 100 brain cancer patients (including 44 meningioma cases and 56 glioma cases) and 122 age-matched healthy control subjects who were in the follow-up Cerrahpaşa Faculty of Medicine: Department of Neurosurgery in Istanbul University. The mean ages of glioma and meningioma patients and control group were 44.75 ± 15.63, 50.26 ± 12.68, and 47.22 ± 10.63 years, respectively.

The specimens were taken after obtaining informed consent, and the study was conducted prospectively. The Medical Ethics Committee of Istanbul Medical Faculty approval was obtained for the study. The protocol followed was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects).

2.1. Polymorphism Analysis. Blood specimens were collected in tubes containing EDTA, and DNA samples were extracted from the whole blood by a salting out procedure (Miller et al., 1988) [27]. Genotyping was performed by the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RF LP). For Taq-I polymorphism, the following primers were used to amplify the VDR gene: 5′-CAG AGC ATG GAC AGG GAG CAA G-3′; 5′-GCA ACT CCT CAT GGG CTG AGG TCT CA-3′. For detection of the Taq-I RFLP, 50–100 ng genomic DNA was amplified with 1x polymerase chain reaction (PCR) buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM of each primer, and Taq polymerase in a 50 μL reaction volume. The PCR conditions were as follows: Initial denaturation step of 94°C for 4 min followed by 5 cycles of 94°C for 45 sec, 64°C for 60 sec, and 72°C for 2 min, and a further 25 cycles of 94°C for 30 sec, 64°C for 30 sec, and 72°C for 45 sec. PCR products were digested with *TaqI* restriction enzyme at 65°C, electrophoresed on 2% agarose gels, and stained with ethidium bromide. Genotypes were determined as TT (490, 245 bp), Tt (490, 290, 245, and 205 bp), or tt (290, 245, and 205 bp) for Taq-I polymorphism [28]. The primers (MBI Fermentas, Lithuania) for Fok-I polymorphism were 5′-GAT GCC AGC TGG CCC TGG CAC TG-3′; 5′-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3′. The DNA template was amplified by PCR using 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 mM of each primer, and Taq polymerase (MBI Fermentas, Lithuania) in a 50 μL final volume. The PCR conditions involved an initial denaturation of 4 min at 94°C and followed by 30 cycles of 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. A final extension step at 72°C for 4 min was also studied. PCR products were digested with *FokI* restriction enzyme (MBI Fermentas, Lithuania) at 37°C for 3 h followed by electrophoresis in a 2% agarose gel. The FF genotype (homozygote of common allele) shows only one band of 272 bp in agarose gel. The ff genotype (homozygote of infrequent allele) generates two fragments of 198 and 74 bp. The heterozygote displays three fragments (272, 198, and 74 bp) [29].

2.2. Statistical Analysis. Statistical analyses were performed using the SPSS software package (revision 16 SPSS Inc., Chicago, IL, USA). Data are expressed as means + SD. Differences in the distribution of genotypes or alleles between cases and controls were tested using the chi-square statistic. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated to estimate the risk of glioma and meningioma. Values of $P < 0.05$ were considered statistically significant. Haplotype frequencies D' and r^2 were calculated using Haploview 4.0 programme.

3. Results

The analysis included 100 brain cancer (including 44 meningioma cases, 56 glioma cases, and 122 healthy controls. Table 1 shows the clinical characteristics of the study groups. Genotype and allele frequencies of meningioma cases, glioma cases, and controls are shown in Table 2. The risks of meningioma and glioma associated with VDR genotypes are shown in Table 3. There was a significant difference in the distribution of VDR Fok-I genotypes in meningioma patients but no significant difference, was detected in glioma patients ($P > 0.05$) (Table 2). VDR Fok-I ff genotype was significantly increased in meningioma patients (15.9%) compared with controls (2.5%), and carriers of Fok-I ff genotype had an increased risk for meningioma cases ($P = 0.004$) (χ^2: 10.33 OR: 6.470, %95 CI: 1.749–23.926). The VDR Taq-I genotype frequencies for meningioma, glioma, and control cases were not significantly different ($P > 0.05$). In addition, vitamin D

Table 1: General demographic informations and parameters of patients and control groups.

Parameters	Meningioma cases ($n = 44$)	Glioma ($n = 56$)	Controls ($n = 122$)
Age (years) (mean ± SD)	50.26 ± 12.68	44.75 ± 15.63	47.22 ± 10.63
Gender *n* (%)			
Male	18 (40.9%)	32 (57.1%)	51 (41.8%)
Female	26 (59.1%)	24 (42.9%)	71 (58.2%)
Histological characteristic of tumors			
Astrocytoma *n* (%)		11 (19.6%)	
Glioblastoma multiforme *n* (%)		22 (39.3%)	
Oligodendroglioma *n* (%)		8 (14.3%)	
Oligoastrocytomas *n* (%)		6 (10.7%)	
*Others *n* (%)		9 (16.1%)	

Values as average ± standard deviation. *Ependymoma, hemangioblastoma, paraganglioma, and so forth.

Table 2: Genotype and allele frequencies of meningioma cases, glioma cases, and controls.

SNP	Controls *n* (%) ($n = 122$)	Meningioma *n* (%) ($n = 44$)	χ^2	*P* value	Glioma *n* (%) ($n = 56$)	χ^2	*P* value
*Fok-I genotype							
FF	56 (45.9%)	19 (43.2%)			28 (50.0%)		
Ff	63 (51.6%)	18 (40.9%)	10.527	0.005	23 (41.1%)	4.598	0.1
ff	3 (2.5%)	7 (15.9%)			5 (8.9%)		
*Fok-1 alleles							
F	175 (71.7%)	56 (63.6%)			79 (70.5%)		
f	69 (28.3%)	32 (36.4%)	1.997	0.157	33 (29.5%)	0.052	0.818
#Taq-I genotype							
TT	65 (53.3%)	23 (52.3%)			32 (57.1%)		
Tt	44 (36.0%)	18 (40.9%)	0.703	0.704	18 (32.2%)	0.275	0.872
tt	13 (10.7%)	3 (6.8%)			6 (10.7%)		
#Taq-I alleles							
T	174 (71.3%)	64 (72.7%)			82 (73.2%)		
t	70 (28.7%)	24 (27.3%)	0.064	0.800	30 (26.8%)	0.137	0.710

Chi-square test was used to compare alleles and clinic pathological characteristics in the study group. *n*: number of individuals.
*For Fok 1 polymorphism (rs2228570): F is referred to as T allele, and f is referred to as C allele.
#For Taq 1 polymorphism (rs731236): T is referred to as T allele, and t is referred to as C allele.
Due to the same base changes T-C or C-T for both polymorphisms, it should be shown as the initial letter of the polymorphism.

Table 3: The risk of meningioma and glioma associated with VDR genotypes.

SNP	Controls	Meningioma	OR (95% CI)	*P* value	Glioma	OR (95% CI)	*P* value
Fok-1							
FF	56 (45.9%)	19 (43.2%)	1.050 (0.774–1.425)	0.756	28 (50%)	1.089 (0.787–1.508)	0.611
Ff + ff	66 (54.1%)	25 (56.8%)			28 (50)		
FF + Ff	119 (97.5%)	37 (84.1%)	6.470 (1.749–23.926)	0.001	51 (91.1%)	3.631 (0.899–14.665)	0.053
ff	3 (2.5%)	7 (15.9%)			5 (8.9%)		
Taq-I							
TT	65 (53.3%)	23 (52.3%)	1.022 (0.711–1.468)	0.909	32 (57.1%)	1.073 (0.810–1.421)	0.631
Tt + tt	57 (46.7%)	2 (47.7%)			24 (42.9%)		
TT + Tt	109 (89.3%)	41 (93.2%)	1.043 (0.943–1.153)	0.460	50 (89.3%)	1.005 (0.403–2.508)	0.991
tt	13 (10.7%)	3 (6.8%)			6 (10.7%)		

haplotypes were evaluated for association with brain cancers. Haplotype analysis revealed that there was no relationship between VDR polymorphisms and brain cancers (for glioma cases: D': 0.17, LOD: 1.08, and r-squared: 0.029 and for meningioma cases D': 0.199, LOD: 1.06, and r-squared 0.034) (Tables 4 and 5).

4. Discussion

For the first time, we demonstrated the positive association of VDR Fok-I gene variants with meningioma cases. We also determined that VDR Fok-I ff genotype might affect development of meningioma, but we found that there was no statistically significant difference between VDR polymorphisms with glioma.

Vitamin D is an important factor in the regulation of cell division and differentiation. The VDR gene is a member of nuclear receptor superfamily. The VDR gene located on chromosome 12. For a long time, several polymorphisms in VDR gene have been investigated for functional significance and potential effects on disease susceptibility [17]. Many studies reported that vitamin D has an antiproliferative effect on many cancer types, which is promoting apoptosis in a variety of malignant cells, such as glioma, neuroblastoma, leukemia, lymphoma cells, breast cancer, and colon cancer [28, 30, 31], and numerous vitamin D analogs have been produced for the treatment of several cancer types. Alternations of vitamin D levels may be related to the changing in the expression of several transcription factors, cell cycle arrested proteins, growth factor, and other genes [32]. Several studies implicated that the ff and Ff genotypes of the VDR gene are associated with a decreased transcriptional activity. VDR Fok-I polymorphism changes the size of the VDR protein [33–36]. The shorter VDR variant could be less active; therefore, this variation may lead to more aggressive disease prognosis [28, 37]. There are some studies showing that vitamin D deficiency affected the brain morphology and cellular proliferation and growth factor signaling in other tissue [19, 38–40]. Brain tumors are relatively rare than other cancer types, but some of the brain cancers are fatal cancer types [41]. Meningiomas are one of the most frequent neoplasms of the brain tumors which account for nearly 13–26% and originate from the arachnoid cells or meningothelial cells [5, 42, 43]. Most of the meningioma cases are sporadic tumors. Today, we have a very little knowledge about genetic risk factors of meningioma. The pathophysiology of meningioma may be associated with a few genes, such as NF2, ATM, GST, CYP450, TP53, KRAS, and MNI. Especially, these genes are related to DNA repair, cell cycle regulation, tumor suppressor, and hormone metabolic pathways [44]. Malmer et al. reported that there may be a relationship between the ATM gene variants which regulate for cellular response against DNA damage and meningioma risk, [45]. Ting et al. reported that the activation of the ATM gene is mediated with VDR phosphorylation when genotoxic stress; furthermore, VDR gene mutation associated with inhibition of ATM gene expression [46].

Sadetzki et al. reported that the genotype of KRAS was related to the increased the risk of (nearly 2 fold) meningioma [47]. VDR expression rates were associated with KRAS mutation in several cancer types. Several studies suggest that cellular effects of VDR may be associated with MAPK signaling pathways, especially KRAS mutation in several cancer types such as breast and colorectal cancers [48, 49]. Some meningiomas might originate from arachnoids cells within the cranium, and other meningioma types may associated with increased bone density [50]. Furthermore, vitamin D also acts directly on osteoblasts, which is modulate differentiation, and regulates mineralization of the extracellular matrix in osteoblastic cells [33]. Previous studies reported that MN1 gene was a candidate genetic risk factor for sporadic meningioma cases [51]. Sutton et al. demonstrated that protein levels of MN1 was significantly in a relationship with vitamin D mediated transcription mechanism in osteoblastic cells [32]. All of these findings gave us an idea that vitamin D gene variations may be a genetic risk factor for the development of meningioma cases. Finally, we found that there was statistically significant difference between the control and meningioma patients for Fok-I ff genotypes. The individuals who had VDR Fok-I ff genotype had an increased risk for meningioma.

Despite an extensive literature study, there was no study that reported the relationship between VDR polymorphisms and brain cancer susceptibility. In conclusion, the present results suggest that the Fok-I polymorphism in the VDR gene

Table 4: Haplotype frequencies of glioma cases and controls.

Block	Haplotype	Case, control ratio counts	χ^2	P
Block 1				
TT	0.547	0.534, 0.553	0.114	0.7359
TC	0.170	0.198, 0.157	0.892	0.3448
CT	0.168	0.171, 0.166	0.017	0.8968
CC	0.115	0.097, 0.124	0.546	0.46

*For Fok-1 polymorphism (rs2228570): F is referred to as T allele, and f is referred to as C allele.
#For Taq-1 polymorphism (rs731236): T is referred to as T allele, and t is referred to as C allele.
Due to the same base changes T-C or C-T for both polymorphisms, it should be shown as the initial letter of the polymorphism.

Table 5: Haplotype frequencies of meningioma cases and controls.

Block	Haplotype	Case, control ratio counts	χ^2	P
Block 1				
CT	0.543	0.512, 0.554	0.476	0.4904
CC	0.183	0.250, 0.159	3.581	0.0584
TT	0.153	0.125, 0.163	0.726	0.394
TC	0.121	0.114, 0.124	0.062	0.8032

*For Fok-1 polymorphism (rs2228570): F is referred to as T allele, and f is referred to as C allele.
#For Taq-1 polymorphism (rs731236): T is referred to as T allele, and t is referred to as C allele.
Due to the same base changes T-C or C-T for both polymorphisms, it should be shown as the initial letter of the polymorphism.

may be related to the risk of meningioma. However, further studies with large number of subjects are needed to explain this kind of relationship between VDR-Fok-I genetic variants and meningioma risk.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Self-Eating: Friend or Foe? The Emerging Role of Autophagy in Idiopathic Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis is the most common and severe form of idiopathic interstitial pneumonias. Despite an exponential increase in our understanding of potentially important mediators and mechanisms, the pathogenesis remains elusive, and little therapeutic progress has been made in the last few years. Mortality in 3–5 years is still 50%. Autophagy, a highly conserved homeostatic mechanism necessary for cell survival, has been recently implicated in the pathogenesis of pulmonary disorders. In this paper we aim to highlight some key issues regarding the process of autophagy and its possible association with the pathogenesis of idiopathic pulmonary fibrosis.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive fibrosing interstitial pneumonia of unknown cause whose pathogenesis, despite recent advances, is still not fully understood [1]. It is currently believed that IPF is the result of repeated injuries in different sites of the alveolar epithelium with dysregulation of cellular homeostasis followed by aberrant wound healing and inadequate repair of the epithelial damage. Median survival from the time of diagnosis is approximately 3 years, and surprisingly the mortality rate exceeds that of many cancers [2]. Lung transplantation is the only therapeutic approach which can affect survival, but it is limited to highly selected patients. However, recent clinical trials have yielded encouraging results regarding the use of pirfenidone and N-acetyl-cysteine in mild to moderate disease [3, 4].

In view of the limited current knowledge and poor survival of the disease, the need for an elucidation of the pathogenesis becomes imperative. Recently, the process of autophagy, a term derived from the Greek and meaning "self-eating," has been implicated in the pathogenetic pathway of IPF [5, 6]. Autophagy is a highly conserved homeostatic mechanism by which cells transport damaged proteins and organelles such as mitochondria to lysosomes for degradation in both health and disease conditions. It contributes to cellular homeostasis by (a) providing an alternative source of metabolic fuel, (b) removing damaged cellular components which are toxic to the cell such as dysfunctional mitochondria or aggregated proteins, and (c) promoting cell death [7].

In previous studies, the term of selective autophagy has been introduced in order to underline the selective targeting of cargoes for degradation. Mitophagy refers to selective digestion of mitochondria, and xenophagy refers to selective degradation of invading pathogens and bacteria [8, 9]. The selective degradation of protein aggregates is regulated by p62, a cytosolic chaperone protein.

Most of the current knowledge regarding the role of autophagy in cell homeostasis was obtained by studies in yeast and mice, whereas its role in human diseases has only been slightly investigated and appears to be highly pleiotropic as it may either represent an adoptive prosurvival response or, if deregulated, promote cell death and morbidity. Autophagic cell death differs from apoptotic cell-death; the former does

not involve caspase activation. Nonetheless, the two processes can coexist [10].

In this paper, we aim to highlight the current knowledge regarding the process of autophagy and its role in various pulmonary disorders with a particular focus on IPF.

2. Classes and Regulation of Autophagy

There are three mechanisms by which autophagy can occur. Microautophagy involves a nonselective surrounding of cytoplasmic components directly by lysosomal membranes. The chaperone-mediated autophagy involves a selective transport to lysosomes of cargoes that contain a specific pentapeptide motif (KFERQ). Macroautophagy is characterized by the presence of autophagosome, a double-membraned vesicle that surrounds the damaged component to be degraded following fusion with endosomes and lysosomes. Chaperone mediated autophagy and microautophagy are less studied whereas macroautophagy (referred as autophagy hereafter) is the best studied mechanism since autophagosomes are easy to detect with fluorescence and electron microscopy [11]. The process consists of in four distinct steps: (a) the formation of an isolation membrane, (b) the formation of an autophagosome with engulfment of the cargo, (c) the fusion of the autophagosome with a lysosome, and (d) the degradation of the cargo by lysosomal enzymes with regeneration of metabolic precursor molecules to be used for anabolic pathways [7].

A series of autophagy-related genes (Atg) are involved in the regulation of the process. Beclin-1, an interacting protein, in complex with class III phosphatidylinositol 3-kinase (PI3 K) and Atg14 acts as a major positive regulator of autophagy [12]. The rapamycin-sensitive mammalian target of rapamycin (mTOR)/class I PI3 K pathway acts as a major negative regulator of autophagy [13, 14]. Autophagosome formation requires two ubiquitin-like conjucation systems: the Atg5–Atg12 conjucation system and the Atg8 (microtubule-associated protein-1 light chain [LC] (3) conjugation system by which LC3 is converted from LC3-I (free form) to LC3 II (conjugated to phosphaditylethanolamine form) a step which is considered critical for autophagosome formation [15–17]. The fusion of autophagosome with lysosome requires the involvement of a GTPase termed Rab-7 and lysosome-mediated membrane protein (LAMP) −1 and −2.

Autophagy can be measured with various methods, and each one has its advantages and limitations. Electron microscopy can visualize early-stage autophagosomes but is less sensitive for the visualization of late-stage autophagosomes [18]. Fluorescence-based methods such as the use of green fluorescent protein (GFP)-LC3 are also used. They are based on the fact that when autophagy is induced and LC3b becomes part of the newly formatted autophagosome, the GFP-LC3 changes its cellular localization from a diffuse cytosolic pattern to a punctuate pattern [19]. Western blot analysis has demonstrated that LC3b-II correlates well with the number of autophagosomes and, thus, with autophagic activity [20]. Western blot analysis of p62, a cytosolic chaperone protein that has an LC3b binding domain [21], can also be used as an increase of p62 levels is associated with the reduced autophagic activity [22]. These methods have the disadvantage of evaluating autophagy in a certain time point (i.e., static measures) and may not reflect the autophagic activity properly as an increase of either the number of autophagosomes or the levels of LC3b-II may be due to the enhanced induction of autophagosome formation or to inhibited fusion with lysosomes which in reality means low autophagic flux. In order to distinguish between these two options, autophagic flux assays are used (i.e., dynamic measures). In cell cultures, LC3b-II levels are measured with Western blot in presence and absence of inhibitors of autophagic degradation such as chloroquine, leupeptin, and bafilomycin-A [23]. Another pitfall regarding the measurement of autophagy in lung diseases is that the process is cell-dependent as it was shown in COPD [24, 25] where it is enhanced in the epithelium and impaired in alveolar macrophages. Thus, the study of autophagy in samples such as whole lung homogenates may not represent what happens in a specific subset of cells. A combination of static and dynamic methods is currently recommended with a careful definition of the type of cell in which autophagy is being measured [7].

3. Autophagy in Pulmonary Diseases

Autophagy has been implicated in the pathogenesis of several pulmonary diseases (Table 1) and represents a potential therapeutic target in current and future clinical trials (Table 2) [26]. Chronic obstructive pulmonary disease (COPD) is the best studied lung disorder regarding the role of autophagy and represents an example of the cell-specific role of autophagy in the context of the same disease. In lung biopsy samples, it was observed that the number of autophagosomes as well as the levels of LC3b-II and other autophagy-related proteins was increased and moreover was correlated with disease severity [27]. Similar results were obtained in lung epithelial cell lines and fibroblasts exposed to cigarette smoking extract (CSE) which is the primary causative agent of COPD [27, 28]. Genetic depletion of Beclin-1 and LC3b decreased cell death in exposed cells, and mice deficient in LC3b did not develop emphysema after exposure to CSE [24, 27, 29]. On the other hand, in alveolar macrophages, autophagic flux was impaired, and the effect of smoking was further supported by the fact that similar changes were observed in alveolar macrophages of non smokers exposed to cigarette smoking [25]. Therefore, in conclusion, enhanced autophagy in epithelial cells has a deleterious effect by promoting cell death and developing of emphysema, and impaired autophagy in alveolar macrophages contributes to the deficient transport of bacteria to lysosome, namely, xenophagy, and to the higher propensity of COPD patients to bacterial acute respiratory infections which lead to acute exacerbations.

Cystic fibrosis (CF) is a disease characterized by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene which is associated to intracellular accumulation of misfolded proteins that are supposed to be cleared by autophagy. It has been observed that in CF human epithelial cell, mutant CFTR proteins impair autophagosome formation via depletion of Beclin-1 [30] and increase

Table 1: Autophagy and pulmonary diseases.

Study	Model	Disease	Stimulus/effect
Chen et al. 2008 [24]	*in vivo/in vitro*	COPD	CS induces autophagy in epithelial cells.
Monick et al. 2010 [25]	*in vitro*	COPD	CS-induced cell death and emphysema are regulated by LC3β activity.
Chen et al. 2010 [29]	*in vivo/in vitro*	COPD	CS decreases autophagy in alveolar macrophages.
Luciani et al. 2010 [30]	*in vivo/in vitro*	CF	Defective autophagy in CF
Abdulrahman et al. 2011 [31]	*in vivo/in vitro*	CF	Treatment with rapamycin induces autophagy and reduces the burden of *B. cenocepacia*.
Singh et al. 2006 [33]	*in vitro*	MTB infection	Induction of autophagy by IFNγ or rapamycin enhances mycobacteria killing.
Gutierez et al. 2004 [34]	*in vitro*	MTB infection	Induction of autophagy by IFNγ eliminates mycobacteria through an IRGM mechanism.
Lee et al. 2011 [35]	*in vivo/in vitro*	PH	Autophagy protects against hypoxia-induced PH.
Parhitko et al. 2011 [36]	*in vivo/in vitro*	TSC	Genetic-pharmacologic inhibition of autophagy blocks autophagy in TSC.
Gills et al. 2007 [39]	*in vivo/in vitro*	Lung cancer	Nelfinavir activates autophagy and exhibits antiproliferative activity in lung cancer.
Mi et al. 2011 [80]	*in vivo/in vitro*	PF	Antagonism of IL-17A induces autophagy and protects against fibrosis.
Patel et al. 2012 [5]	*in vivo/in vitro*	IPF	TGF-β1 inhibits autophagy in lung fibrobasts. Rapamycin induces autophagy in the BLM model and reduces the degree of fibrosis.
Araya et al. 2013 [6]	*in vitro*	IPF	Insufficient autophagy promotes myofibroblast differentiation and collagen deposition.

COPD: chronic obstructive pulmonary disease, CS: cigarette smoking, CF: cystic fibrosis, MTB: mycobacterium tuberculosis, IFNγ: interferon γ, PH: pulmonary hypertension, TSC: tuberous sclerosis complex, PF: pulmonary fibrosis, IPF: idiopathic pulmonary fibrosis, TGF-β1: transforming growth factor-β1, BLM: bleomycin.

Table 2: Clinical trials targeting autophagy in pulmonary diseases.

Identifier	Condition	Intervention	Phase	Status
NCT00969306	SCLC	Chloroquine, A-CQ100	Phase 1	Not yet recruiting
NCT00933803	NSCLC	Paclitaxel Carboplatin Hydroxychloroquine Bevacizumab	Phase 1 Phase 2	Active Not recruiting
NCT01649947	NSCLC Recurrent NSCLC	Paclitaxel Carboplatin Hydroxychloroquine Bevacizumab	Phase 2	Recruiting
NCT00728845	Recurrent Advanced NSCLC	Bevacizumab Carboplatin Hydroxychloroquine Paclitaxel	Phase 1 Phase 2	Terminated
NCT01687179	LAM	Sirolimus and hydroxychloroquine	Phase 1	Recruiting

SCLC: small-cell lung cancer, NSCLC: non-small cell lung cancer, LAM: lymphangioleiomyomatosis.

of reactive oxygen species (ROS) and transglutaminase-2 production which contributes to the excessive inflammation seen in CF. Restoring Beclin-1 restores autophagy measured by LCEb-II and p62 levels and GFP-LC3 puncta and leads to increased clearance of protein aggregates and marked reduction of inflammation in a mouse model of CF [30]. Interestingly, treatment with N-acetylcysteine (NAC) also has beneficial effects suggesting a possible implication of antioxidants in the therapy of CF. Infection by *Burkholderia cenocepacia* is potentially lethal in patients with CF, and it was observed that it is associated with downregulation of several autophagic genes in alveolar macrophages [31]. Treatment

with rapamycin induced autophagy and reduced lung inflammation.

Alveolar macrophages provide the first line of defense against invading microbes. Infection by *Mycobacterium tuberculosis* is also associated with inhibition of the formation of autophagosomes which inhibits mycobacterial killing by alveolar macrophages [32]. Stimulation of alveolar macrophages with interferon-γ (IFN-γ) upregulates a GTPase, namely, IRGM-1, and activates autophagy [33]. Induction of autophagy by rapamycin also enhances mycobacterial killing [34].

Autophagy seems to be implicated in the development of pulmonary hypertension (PH). In lung samples of human PH and lung vasculature, there was an increased expression of LC3b and GFP-LC3 puncta formation, a marker of autophagosome formation in GFP-LC3-transfected endothelial cells. When mice genetically deficient in LC3b were exposed to chronic hypoxia, they demonstrated an evidence of increased pulmonary hypertension compared to wild-type mice suggesting a protective role of autophagy by limiting hypoxia-dependent vascular cell proliferation [35].

Tuberous sclerosis complex (TSC) is an autosomal dominant tumor suppressor gene syndrome caused by germline mutations in the TSC1 or TSC2 genes. Patients with TSC have multisystem manifestations such as neurologic disease, benign tumors in multiple organs, and pulmonary lymphangioleiomyomatosis (LAM). Genetic and pharmacologic autophagy inhibition blocks tumorigenesis in xenograft and spontaneous models of TSC [36] and hence may represent a potential therapeutic target for TSC.

Autophagy has functional implications in the pathogenesis of cancer, but only few studies have been performed specifically in lung cancer, and the role of autophagy in response to chemotherapeutic agents using cultured human lung A549 adenocarcinoma cells [37, 38] has been evaluated. Interestingly, Nelfinavir, an HIV protease inhibitor, exerted pleiotropic biochemical and cellular effects that included induction of endoplasmic reticulum (ER) stress, autophagy, and apoptosis *in vitro* and *in vivo* and exhibited antiproliferative activity in lung cancer cells [39].

4. Autophagy and IPF: Indirect Pathogenetic Links

A growing body of evidence suggests that there may be a pathogenetic link between IPF and autophagy. Oxidative stress, endoplasmic reticulum (ER) stress, and hypoxia, all mechanisms that participate in the pathogenesis of IPF [40–42], are well-known inducers of autophagy [43–45]. On the other hand, viral infections, which have also been hypothesized to favor the development of fibrosis, seem to have an inhibitory effect on autophagy.

ER is an organelle that serves general functions such as facilitation of protein folding and transport of newly synthesized proteins. Oxidative stress, disturbances in calcium regulation, glucose deprivation, and viral infection can cause ER stress which leads to increase of the unfolded proteins [46]. In neuroblastoma cells, ER stress induces autophagy and is associated with cell survival [47]. However, ER-induced autophagy may be cell specific because it was seen that in colon and prostate cancer cell lines activation of autophagy by chemical inducers reduces cell death, whereas in normal human colon cells and in nontransformed embryonic fibroblasts, it contributes to cell-death [48]. In lung epithelial fibroblast cell lines, autophagy induced by chemicals resulted in increased accumulation of LC3-II and activation of unfolded protein response, a compensatory mechanism to ER stress [49] suggesting a possible protective role of autophagy.

It is now accepted that IPF is the result of multiple injuries in the epithelium which leads to early death of type II alveolar epithelial cells (AECs II) and aberrant wound healing. In cases of familial IPF, it was shown that mutations of the surfactant protein-C gene (SFTRC) lead to accumulation of misfolded proteins, induction of ER stress, and apoptosis of AEC II [50–53]. In sporadic IPF and regardless the absence of mutations, an increase of ER stress and apoptotic markers in AEC II was also observed [54]. Recently, it was also observed that ER stress is implicated in the differentiation of fibroblast into myofibroblast which is considered a key event in the pathogenesis of IPF [40].

Recent evidence suggests that oxidative stress, defined as an imbalance of the generation of ROS in excess of the capacity to neutralize them, promotes autophagy. According to a hypothesis [55], mild levels of oxidative stress activate autophagy in order to eliminate the damaged organelles and thus to promote cell survival. On the other hand, acute or persistent oxidative stress, which has been hypothesized in the pathogenesis of IPF, leads to an increase of intracellular ROS, damage of the lysosomal membrane with intracellular release of potent hydrolases, dysregulation of autophagy with perpetuation of the oxidative injury, and initiation of a vicious circle that leads to apoptotic cell death. In transformed and cancer cell lines treatment with hydrogen peroxide (H_2O_2) induced autophagy and promoted caspase-independent cell death, whereas knockdown of specific autophagic genes with small interfering RNAs (siRNAs) prevented H_2O_2-induced autophagic cell death [56]. Starvation, which is a known inducer of autophagy, is associated with an increase of intracellular ROS and leads to autophagosome formation and autophagic degradation in CHO and HeLa cells [57]. In the former scenario [56], ROS-induced autophagy led to cell death, whereas in the latter [57] it represented a mechanism which is essential for cell survival. In the model of human bronchial epithelial cells treated with cigarette smoke extract, an increase of intracellular ROS was observed, and the activation of autophagy had a deleterious effect promoting the death of epithelial cells [26].

ROS and markers of oxidative stress are evident in IPF, and levels of ROS are negatively correlated with lung function [58–60]. The overproduction of ROS may cause lung injury and promote a tissue microenvironment which favors fibrosis over regeneration. Glutathione, an antioxidant agent, has been found to be decreased in IPF [61]. NAC is capable of stimulating glutathione synthesis, increasing the intra- and extracellular levels, and thereby partially restoring glutathione levels [62, 63]. NAC has been found to have favourable effects on the lung function of patients with IPF

and mainly in those with less progressed disease [4, 64]. However, results of a recent trial showed some conflicting results which need further careful investigation [65].

Autophagy is sensitive to oxygen tension, and hypoxia inducible factor 1-α (HIF 1-α) has been implicated as a regulator of autophagy and of turnover of damaged mitochondria under hypoxic condition. HIF 1-α target gene, namely, Bcl-2/adenovirus E1B 19 kDa—interacting protein-3 (BNIP3), also regulates hypoxia-induced autophagy [45, 66]. However, even in the case of hypoxia, the dual role of autophagy has been emerged as in another study, it was observed that prolonged hypoxia induces autophagic cell death through a BNIP3 dependent mechanism [67].

It is now believed that hypoxia can lead to alveolar epithelial cell apoptosis initiating the cascade of fibrogenesis. In fact, it was observed in both animal models of bleomycin-induced fibrosis and in lung tissues of IPF patients that HIF 1-α is overexpressed and may exert its role in early stages of fibrogenesis as it was localized in areas of active fibrosis and in normal areas of IPF lung but not within the fibroblastic foci which represent areas of established fibrosis [42].

Autophagy, as part of the host defence system, has been targeted by viral proteins through the evolution of mechanisms of virus escape. The alpha-herpesvirus HSV-1 inhibits autophagy (i.e., xenophagy) through the actions of ICP34.5 and US11 proteins. ICP34.5 protein directly binds Beclin1 leading to the inhibition of the autophagosome formation [68]. As part of the intrinsic antiviral response, infected cells block protein synthesis through the PKR-mediated phosphorylation of eIF2a translation initiation factor, a process that also leads to the upregulation of autophagy [69]. The ICP34.5 and US11 proteins inhibit the phosphorylation of eIF2a at temporally distinct phases of HSV-1 infection, thereby releasing the block to protein synthesis and subsequently inhibiting the induction of autophagy [70, 71]. Similar to alpha-herpesviruses, beta-herpesviruses like hCMV are extremely efficient in blocking autophagosome formation through the TRS1 viral protein which directly interacts and inhibits Beclin1 [72]. Gamma-herpesviruses seem to employ a different mechanism for the inhibition of autophagy which relies on the acquisition of cellular homologues of Bcl-2 protein including BHRF1 and BALF-1 of EBV, Orf16 of Kaposi's sarcoma-associated herpesvirus (KSHV), and M11 of murine γ-herpesvirus 68 (γ-HV68) [73]. Bcl-2 protein apart from its antiapoptotic role acts as a potent inhibitor of autophagy through Beclin binding. Bcl-2 is regulated through JNK phosphorylation, upon which Bcl-2 is released from Beclin-1 allowing for the activation of autophagy. The viral Bcl-2 analogues expressed by several gamma-herpesviruses lack the JNK phosphorylation domain, thereby escaping JNK regulation and acting as dominant inhibitors of autophagy.

Several studies have suggested a link between IPF and occult viral infections in the lung, including herpesviruses, adenovirus, hepatitis C, and Torque teno virus. It has been hypothesized that these viruses may represent injurious agents in the context of the "multiple hits" hypothesis. The Epstein-Barr virus (EBV) has been detected in both familial and sporadic IPF [74], and EBV protein and DNA expression have been found in IPF lung tissues [75, 76]. EBV replication has been demonstrated in type II alveolar epithelial cells, and EBV latent membrane protein 1 (LMP-1) expression was detected in the alveolar epithelium in IPF patients, findings that were associated with poor prognosis [77]. Moreover, from the clinical point of view, antiviral treatment has been reported to stabilise the course of IPF [74]. Recently, our group has detected the presence of HSV-1 in patients with fibrotic idiopathic interstitial pneumonias, since the virus presented similar incidence in two different biological samples, tissue, and bronchoalveolar lavage fluid. We have also found that the presence of HSV-1 can enhance fibrosis by inducing the transcription of molecular pathways which promote fibrotic, angiogenetic, wound healing, and innate immunity processes, suggesting a probable role of infectious factors in the pathogenesis of lung fibrosis [78]. Proof of concept experiments of the involvement of herpesviruses in lung fibrosis come from experimental models of pulmonary fibrosis with the MHV-68 murine gamma-herpesvirus. Importantly, experimentally established pulmonary latent infection of mice with MHV-68 could confer higher susceptibility to bleomycin or FITC-induced fibrosis [79] in comparison to the uninfected control mice, thereby supporting a multiple/recurrent hit hypothesis where the herpesvirus presence alters the lung microenvironment and acts as a cofactor in experimentally induced models of pulmonary fibrosis.

5. Autophagy in IPF

Recently, Mi et al. have shown that IL17A, a cytokine that induces production of collagen and promotes epithelial-mesenchymal transition (EMT) through a transforming-growth-factor-β1- (TGF-β1-) dependent mechanism, inhibits autophagy in mouse epithelial cells [80]. Moreover, they observed that in the murine model of BLM-induced fibrosis, antagonism of IL17A activated autophagy, decreased the production of collagen, attenuated fibrosis and increased survival. This protective effect was abolished after blocking autophagy with 3-methyladenine (3-MA).

Patel et al. studied markers of autophagic activity and concluded that autophagy is not induced in human IPF lungs [5]. More in detail, they observed that the levels of LC3-II were lower and the levels of p62 were higher in IPF lungs compared to controls. Moreover, they observed a decrease in the number of autophagosomes with electron microscope in IPF lungs. In order to provide a plausible answer, they used fibroblast cell lines and showed that TGF-β1, a profibrotic cytokine which is overexpressed in IPF, inhibits autophagy. Silencing of LC3 and beclin-1 genes and, hence, inhibiting autophagy enhanced the expression of fibronectin and α-smooth cell actin, a marker of myofibroblast which is a key cell in the process of fibrogenesis. In the BLM model, they observed that treatment with rapamycin enhanced autophagy and protected from fibrosis. Based on these observations, the authors concluded that autophagy protects against the development of fibrosis.

A growing body of evidence at both clinical and biological level suggests that IPF is a disease of aging characterised by premature age-related changes in alveolar epithelial cells [81]. It is also accepted that autophagy functions less as tissue

ages due to insufficient formation of autophagosomes or to deficient elimination after fusion with lysosomes [82]. Recently, Araya et al. also attempted to clarify the role of autophagy in IPF [6]. They suggested that insufficient autophagy leads to epithelial cell senescence as they observed an increased expression of p62 and ubiquitinated proteins, both markers of decreased autophagy as well as an increased expression of p21 which is a marker of cellular senescence. On the other hand and in agreement with previous observations of cell-specific effect of autophagy, they showed that insufficient autophagy in lung fibroblasts leads to the differentiation to myofibroblasts without any effect on their senescence and to increased production of extracellular matrix which are critical steps in the fibrogenetic process.

6. Conclusion

Over the last decade, there has been an explosion in the research field regarding the possible mechanisms involved in the pathogenesis of IPF and clinical trials in order to find a therapeutic agent able, at least, to stabilise the course of the disease. Despite all these efforts, pathogenesis of IPF is still not fully understood. A universally accepted therapeutic agent has not yet been found. Recently, it has been suggested that there should be an "oncologic" approach in the pathogenesis of IPF. According to this hypothesis, multiple pathways may be involved simultaneously in the pathogenesis of IPF, and future therapeutic approaches should target these pathways simultaneously [83]. Moreover, this hypothesis has been strengthened by the observation that there are certain similarities between IPF and lung cancer biology [2]. Autophagy has been recently implicated in the pathogenesis of lung diseases, and only few studies exist in the field of IPF, and thus its role is rather obscure. Therefore, more studies are needed in order to clarify the role of autophagy in IPF and in order to develop novel therapeutic approaches.

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Research Article

Therapeutic Time Window for Edaravone Treatment of Traumatic Brain Injury in Mice

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Traumatic brain injury (TBI) is a major cause of death and disability in young people. No effective therapy is available to ameliorate its damaging effects. Our aim was to investigate the optimal therapeutic time window of edaravone, a free radical scavenger which is currently used in Japan. We also determined the temporal profile of reactive oxygen species (ROS) production, oxidative stress, and neuronal death. Male C57Bl/6 mice were subjected to a controlled cortical impact (CCI). Edaravone (3.0 mg/kg), or vehicle, was administered intravenously at 0, 3, or 6 hours following CCI. The production of superoxide radicals ($O_2^{\bullet-}$) as a marker of ROS, of nitrotyrosine (NT) as an indicator of oxidative stress, and neuronal death were measured for 24 hours following CCI. Superoxide radical production was clearly evident 3 hours after CCI, with oxidative stress and neuronal cell death becoming apparent after 6 hours. Edaravone administration after CCI resulted in a significant reduction in the injury volume and oxidative stress, particularly at the 3-hour time point. Moreover, the greatest decrease in $O_2^{\bullet-}$ levels was observed when edaravone was administered 3 hours following CCI. These findings suggest that edaravone could prove clinically useful to ameliorate the devastating effects of TBI.

1. Introduction

In spite of the fact that traumatic brain injury (TBI) is a major cause of death and disability, particularly in young people, and given the huge socioeconomic costs of caring for affected persons, there is still no adequate treatment available to ameliorate its damaging effects [1, 2]. The overall incidence of TBI in the United States is estimated to be 540 cases per 100,000 persons and the prevalence of long-term disability is estimated to be between 3.2 and 5.3 million. In 2000, the economic impact of TBI in the United States was estimated to be $9.2 billion in lifetime medical costs and $51.2 billion in lost productivity. Falls and motor vehicle accidents are the leading causes of TBI, with most cases transferred immediately to an emergency department [3]. Given that moderate and severe TBIs are associated with neurologic and functional impairments [4], further intensive care following initial treatment and diagnostic assessment are also usually required.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a derivative of antipyrin and was approved as free radical scavenger for the treatment of acute cerebral infarction in Japan [5]. Edaravone was first reported to strongly scavenge hydroxyl radicals (OH^-) produced by the Fenton reaction *in vitro* and to decrease lipid and L-tyrosine oxidation [6]. The effects of edaravone have been studied in relation to brain ischemia in animals and humans, and decreased brain edema, infarction, endothelial damage, and oxidative damage have been reported [6–11]. Edaravone has also been used in other neural injury models such as spinal cord injury [12], TBI [8, 13, 14], and brain hemorrhage [15] and was found to reduce lesion size and oxidative stress levels. We have previously

reported that intravenous edaravone (3.0 mg/kg) treatment immediately after cortical impact suppressed traumatic neural damage in rodents [13] and decreased hydroperoxide ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radical formation in both rodents and patients with TBI [13, 16]. Edaravone treatment at 2 hours and again at 12 hours also decreased neuronal loss in a dose-dependent fashion (0.75, 1.5, or 3 mg/kg) in the CA3 layer of the hippocampus after TBI [8], while at 3 mg/kg i.v it suppressed apoptotic neuronal cell death and oxidative damage after TBI [14]. In spite of these positive outcomes, the therapeutic time window of edaravone on TBI has not been examined in detail.

It has been suggested that reactive oxygen species (ROS) generation is activated in the lesion area after TBI, leading to the initial production of superoxide ($O_2^{\bullet-}$) and nitric oxide ($^{\bullet}NO$) radicals. These ROS then react and metabolize to form stronger oxidants in the form of peroxynitrite ($ONOO^-$), hydroxyl ($^{\bullet}OH$), carbonate ($CO_3^{\bullet-}$), and nitrogen dioxide ($^{\bullet}NO_2$) radicals [17, 18], which in turn react with proteins, lipids, sugars, and nucleotides and impair the normal physiological function of cells. Although it is considered that $O_2^{\bullet-}$ does not have strong oxidative potential and that edaravone does not scavenge $O_2^{\bullet-}$ *in vitro* [6], we previously reported that mice deficient in $Gp91^{phox}$ (NOX2), a subunit of NADPH oxidase and a generator of $O_2^{\bullet-}$, exhibited reduced lesion size and oxidative stress following TBI [19]. Moreover, knockout mice lacking interleukin-1, a proinflammatory cytokine, were less susceptible to neuronal cell death than their wild-type littermates and displayed less inducible nitric oxide synthase gene expression and reduced $O_2^{\bullet-}$ and $ONOO^-$ production during ischemia [20, 21].

In the present study, we investigated the therapeutic time window of edaravone on TBI and oxidative metabolite generation in mice following a controlled cortical impact (CCI). We also evaluated the temporal profile of ROS production, oxidative stress, and neuronal cell death in order to estimate the relationship between the effect of edaravone on brain damage and the sequence of events leading to ROS generation following CCI.

2. Materials and Methods

2.1. Animals and CCI Model. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Showa University (#00158). Young adult male C57/BL6 mice (8–12 weeks of age, 20–26 g per body weight) were anesthetized with 2% sevoflurane in N_2O/O_2 (70%/30%) and positioned in a computer-guided stereotaxic system (Leica Angle Two, Leica Microsystems, Wetzlar, Germany) incorporating an electromagnetic CCI device (Benchmarked Stereotaxic Impactor, Leica Microsystems). Following a midline scalp incision, a 4 mm^2 opening was made in the skull 3 mm lateral and 2 mm caudal of bregma, thereby exposing the right parietotemporal cortex. A CCI was carried out at a depth of 1.0 mm from the dura mater at a stroke velocity of 3.7 m/second, using an impact device with a rounded tip of approximately 1.2 mm in diameter (Figures 1(a) and 1(b)) [22].

After removing hemorrhaged blood resulting from the impact, the skull was covered with 4 mm diameter artificial dura (GORE Preclude, W. L. Gore & Associates, Newark, NY, USA) and a 5 mm diameter artificial bone plate made from dental cement (GC Fuji I, GC Corporation, Tokyo, Japan). The core body temperature of the mice was maintained at 37°C during the surgery.

2.2. Experimental Design. We performed 3 experiments as given in the following.

Experiment 1. The animals were divided into 4 experimental groups to determine the possible therapeutic time window of edaravone (3.0 mg/kg, $n = 10$ for each group). Group 1 (Ed 0 h): edaravone was administered immediately (0 hour) after the CCI. Group 2 (Ed 3 h): edaravone was administered 3 hours following CCI. Group 3 (Ed 6 h): edaravone was administered 6 hours following CCI. Group 4, vehicle (Vh): this served as the control group in which normal saline was given immediately (0 hour) following CCI ($n = 9$). All animals were sacrificed 24 hours following CCI (Figure 1(d)).

Experiment 2. Mice ($n = 3$ in each group) were divided into 4 groups. They were sacrificed group 1: immediately (0), group 2: 3 hours, group 3: 6 hours, and group 4: 24 hours after CCI to investigate the temporal profiles of ROS, oxidative stress, and neuronal death in the brain (Figure 1(e)).

Experiment 3. Three groups were set ($n = 3$ in each group) to investigate the effect of edaravone treatment to ROS (Figure 1(f)). Group 1 (Ed 0 h): edaravone was administered immediately (0 hour) after the CCI and *in situ detection* of $O_2^{\bullet-}$ was performed 4 hours after CCI. Group 2 (Ed 3 h): edaravone was administered 3 hours following CCI and *in situ detection of* $O_2^{\bullet-}$ was performed 4 hours following CCI. Group 3 (Vh 3 h): vehicle was administered 3 hours after CCI and *in situ detection of* $O_2^{\bullet-}$ was also performed 4 hours following CCI as the control group.

2.3. Administration of Edaravone. Edaravone, a free radical scavenger, was a gift from Mitsubishi Tanabe Pharma (Osaka, Japan). Animals were placed in the supine position and anesthetized with sevoflurane administered by inhalation through a face mask. The skin over the left clavicle was incised to expose the left jugular vein. Edaravone dissolved in saline was slowly administered at a dosage of 3.0 mg/kg body weight (100–150 μL volume) into this vein.

2.4. Tissue Preparation. Under sodium pentobarbital (50 mg/kg, i.p.) anesthesia, the animals were transcardially perfused with 0.9% NaCl followed by 2% paraformaldehyde (PFA) in 50 mM phosphate buffer (pH 7.2). The brain and skull were then removed intact and postfixed in 2% PFA overnight, after which the skull was carefully removed, and the brain was immersed in 20% sucrose for 2 days for cytoprotection. The brain was next frozen in liquid nitrogen-chilled 2-methylbutane and coronally cryosectioned at a thickness of 50 μm from bregma to approximately 3.9 mm caudal

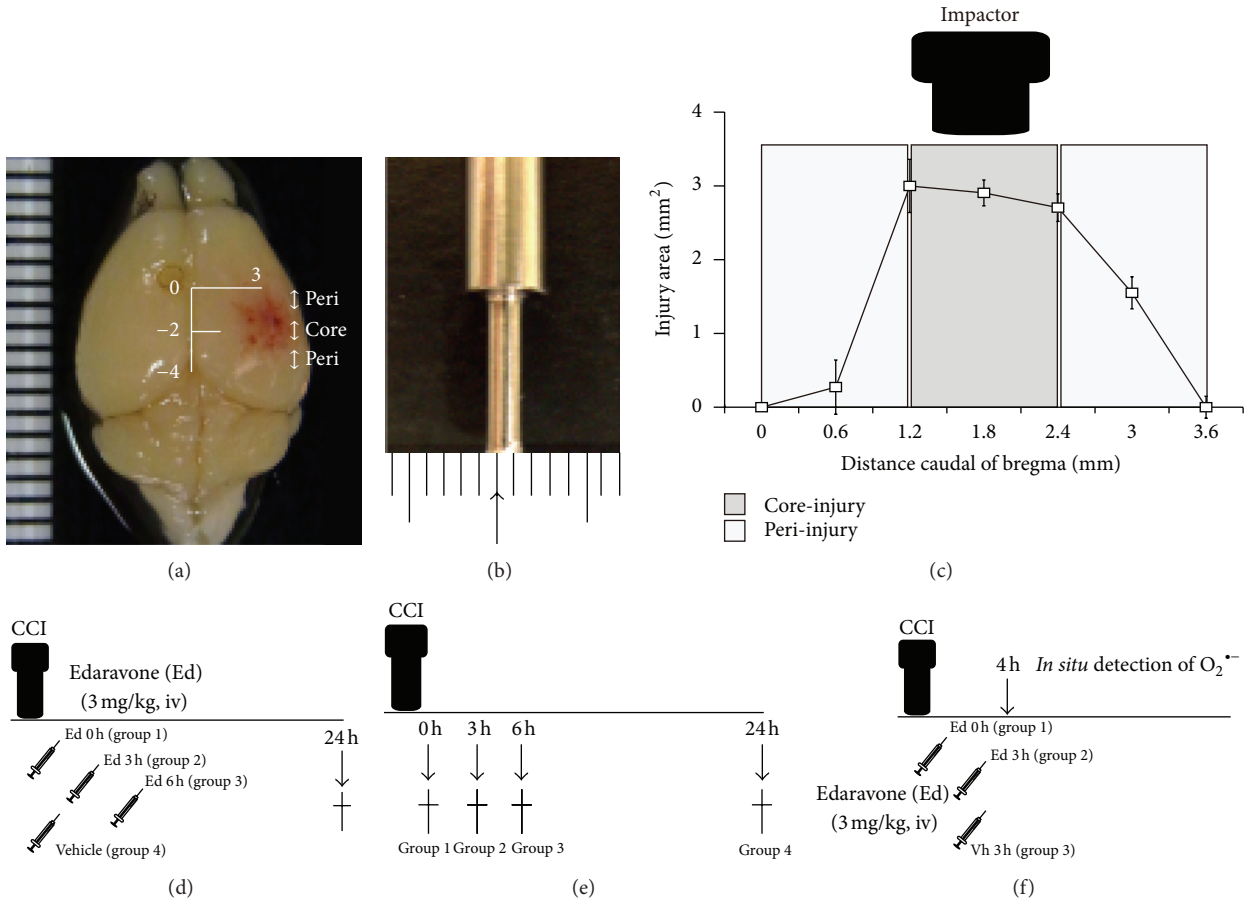

Figure 1: Establishment of TBI model and experimental protocol. (a) Brain images following TBI. The contusion was conducted over the right parietotemporal cortex 2.0 mm caudal of bregma and 3.0 mm lateral of the midline. (b) High power images of the CCI device showing a rounded tip of approximately 1.2 mm in diameter. (c) Coronal cryosections (thickness 50 μm) from bregma to approximately 3.9 mm caudal of bregma encompassing the injury region were used to determine the injury area. The core-injury area was defined as the direct impact region 1.2 mm to 2.4 mm caudal of bregma. The peri-injury area was defined as being <1.2 mm and >2.4 mm caudal of bregma. (d) The free radical scavenger edaravone was injected intravenously into the jugular vein following CCI. To determine the possible therapeutic time window, edaravone (3.0 mg/kg, $n = 10$ for each time point) was administered either immediately (0) or 3 or 6 hours following CCI. As a vehicle-treated control group, saline was administered immediately (0 hour) following CCI ($n = 9$). (e) Temporal profiles of ROS, oxidative stress, and neuronal death in the brain following CCI were determined immediately (0) and at 3, 6 and 24 hours after CCI ($n = 3$). (f) Edaravone treatment to ROS was investigated. Edaravone was administered to group 1: immediately (0 hour) or group 2: 3 hours after the CCI and *in situ detection of* $O_2^{\bullet-}$ were performed 4 hours post-CCI. As the control, Group 3: vehicle was administered 3 hours post-CCI and *in situ detection of* $O_2^{\bullet-}$ was also performed 4 hours following CCI.

of bregma, thus ensuring coverage of the injured region (Figure 1(c)). The sections were then immediately immersed in PBS containing 0.1% Tween 20 (PBST) for subsequent histological assessment.

2.5. Fluoro-Jade B and Toluidine Blue Staining. Fluoro-Jade B (FJB) staining was used to detect degenerating neurons as previously reported, with only minor modifications [23, 24]. A series of sections was collected at 600 μm intervals from 0 to 3.6 mm caudal of bregma (7 sections per mouse) and mounted on poly-L-lysine-coated glass slides. After air drying, the slides were incubated with freshly prepared 0.06% potassium permanganate for 15 min and rinsed with distilled water. The sections were then immersed in 0.0005% FJB (Millipore, Billerica, MA, USA) in a dark room for 30 min, after which they were completely air-dried before being immersed in xylene and enclosed in malinol (Muto Pure Chemicals, Tokyo Japan). A second series of adjacent sections was stained with toluidine blue (TB) [25]. The sections were observed with a fluorescence microscope (Biozero 8100, Keyence, Osaka, Japan).

2.6. Measurement of Lesion Volume. FJB- and TB-stained sections were used to semiquantify the injury area based on sections that displayed FJB labeling or little or no TB staining. Some sections, particularly those near the injury core, lacked part of the neocortex due to severe tissue damage; this area was also included in our calculations of lesion volume. The outlines of the affected regions were traced, and the areas were calculated using NIH Image software. The

injury volume was then determined by summing these areas. This was performed by an investigator who was blinded to the experimental groups.

We further defined core- and peri-injury areas. Given that the diameter of the impact tip was around 1.2 mm, we defined the core-injury area as encompassing the region 1.2 mm to 2.4 mm caudal of bregma, with the peri-injury area surrounding this (Figure 1(c)).

2.7. Immunostaining of Nitrotyrosine (NT). Another series of sections at 600 μm intervals (6 sections per animal) was used to label for nitrotyrosine (NT), a peroxynitrite ($ONOO^-$) oxidative metabolite, by free-floating immunohistochemistry. After immersion in 0.3% H_2O_2, the sections were incubated in 5% normal horse serum and immersed overnight with a polyclonal affinity-purified rabbit anti-NT antibody (1 : 1000, Upstate Biotechnology, Lake Placid, NY, USA). The sections were then incubated with biotinylated goat anti-rabbit IgG (1 : 200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by an avidin-biotin complex solution (Vector, Burlingame, CA, USA) using diaminobenzidine (Vector) as a chromogen. The area of dark brown NT-immunopositive staining as well as that of the severely damaged core-injury region as determined using NIH Image software and the injury volume calculated by summing these areas.

To determine the identity of the NT-positive cells, we colabeled for various cell markers. After blocking, the sections were coincubated with anti-NT antibody and either monoclonal mouse anti-NeuN antibody (1 : 2000, a neuronal marker, Millipore) or monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody (1 : 2000, an astroglial marker, Sigma, St. Louis, MO, USA), followed by the secondary antibodies Alexa 488-conjugated goat anti-rabbit IgG antibody (1 : 400) and Alexa 546-conjugated goat anti-mouse IgG antibody (1 : 800, Molecular Probes, Eugene, OR). Nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 : 10,000; Roche, Mannheim, Germany).

2.8. In Situ Detection of $O_2^{\bullet-}$. Hydroethidium (HEt) rapidly penetrates into the brain parenchyma, reacts with $O_2^{\bullet-}$, and generates ethidium (Et) which can be detected at an emission wavelength of 510–550 nm [26, 27]. Mice were anesthetized with 2% sevoflurane in N_2O/O_2 (70%/30%) and were administered 1.0 mg/mL HEt solution (in 0.9% NaCl containing 1% DMSO) into the left jugular vein. Fifteen minutes after HEt infusion, the animals were perfuse-fixed and their brains frozen and sectioned. To identify the Et-positive cells, some sections were also stained with primary antibody for NeuN or GFAP. All sections were nuclear-stained with DAPI. Fluorescence was detected using an Axio Imager optical sectioning microscope with an ApoTome slider module.

2.9. Statistical Analysis. Data were expressed as mean ± SEM. Statistical comparisons were made by Student's *t*-test for two groups and by one-way ANOVA followed by *Tukey-Kramer* tests for multiple group comparisons. A value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Edaravone Has a Therapeutic Time Window of 6 Hours for the Treatment of TBI. We commenced by investigating the effect of edaravone and the optimal therapeutic time window for its administration in our experimental TBI model. Animals were administered either vehicle (0 hours; $n = 9$) or 3.0 mg/kg edaravone (0, 3, or 6 hours after CCI) ($n = 10$) (Figure 1(d)) and the efficacy of this treatment was evaluated at 24 hours based on FJB (Figure 2) and TB (Figure 3) staining.

One vehicle-treated mouse died during the experiment. The total injury volume calculated from FJB staining was significantly reduced in edaravone-treated animals (0 h: 4.83 ± 0.32 mm^3, $P < 0.01$; 3 h: 3.13 ± 0.43 mm^3, $P < 0.001$; 6 h: 4.31 ± 0.50 mm^3, $P < 0.001$) compared with that in the vehicle-treated cohort (0 h: 6.38 ± 0.34 mm^3). Interestingly, the total injury volume in mice treated with edaravone at 3 hours was significantly less than that which occurred in those treated at 0 hours ($P < 0.05$), with the former animals also displaying the strongest neuroprotection observed across all groups. No significant differences in injury volumes were recorded between the 0- and 6-hour or the 3- and 6-hour treatment groups (Figure 2).

To confirm these results, we determined the injury volume based on TB staining and obtained similar results (Figure 3). The total injury volume detected by TB staining was significantly reduced in the edaravone-treated groups (0 h: 4.78 ± 0.40 mm^3, $P < 0.05$; 3 h: 3.09 ± 0.39 mm^3, $P < 0.001$; 6 h: 3.75 ± 0.51 mm^3, $P < 0.01$) compared with the vehicle-treated controls (0 h: 6.14 ± 0.42 mm^3), with the best result again being obtained for the 3-hour treatment group compared with the 0-hour group ($P < 0.05$).

To estimate which region of the affected tissue edaravone rescued from cell death, we also analyzed the volume of the core- and peri-injury areas separately using FJB staining (Figure 2(d)). The animals treated with edaravone at 0 hours had a significantly lower peri-injury volume compared with controls treated with vehicle at the same time point (2.08 ± 0.17 mm^3 versus 3.09 ± 0.21 mm^3, $P < 0.01$), but no significant difference was observed for the core-injury volume (2.75 ± 0.18 mm^3 versus 3.29 ± 0.19 mm^3). In contrast, edaravone treatment at both 3 and 6 hours significantly reduced the TBI-induced volumes in both the core- (3 h: 1.64 ± 0.31 mm^3, $P < 0.001$; 6 h: 2.06 ± 0.16 mm^3, $P < 0.05$) and peri- (3 h: 1.49 ± 0.21 mm^3, $P < 0.001$; 6 h: 2.11 ± 0.36 mm^3, $P < 0.001$) injury sites. To be more specific concerning the core injury area, edaravone treatment at both 3 and 6 hours rescued the frontal region to a greater extent than that seen for the 0-hour treatment (Figure 2(b)). Notably, the core-injury volume following edaravone administration at 3 hours was significantly less than that measured in response to edaravone treatment at 0 hours. The results for TB staining were similar to those for FJB staining (Figure 3).

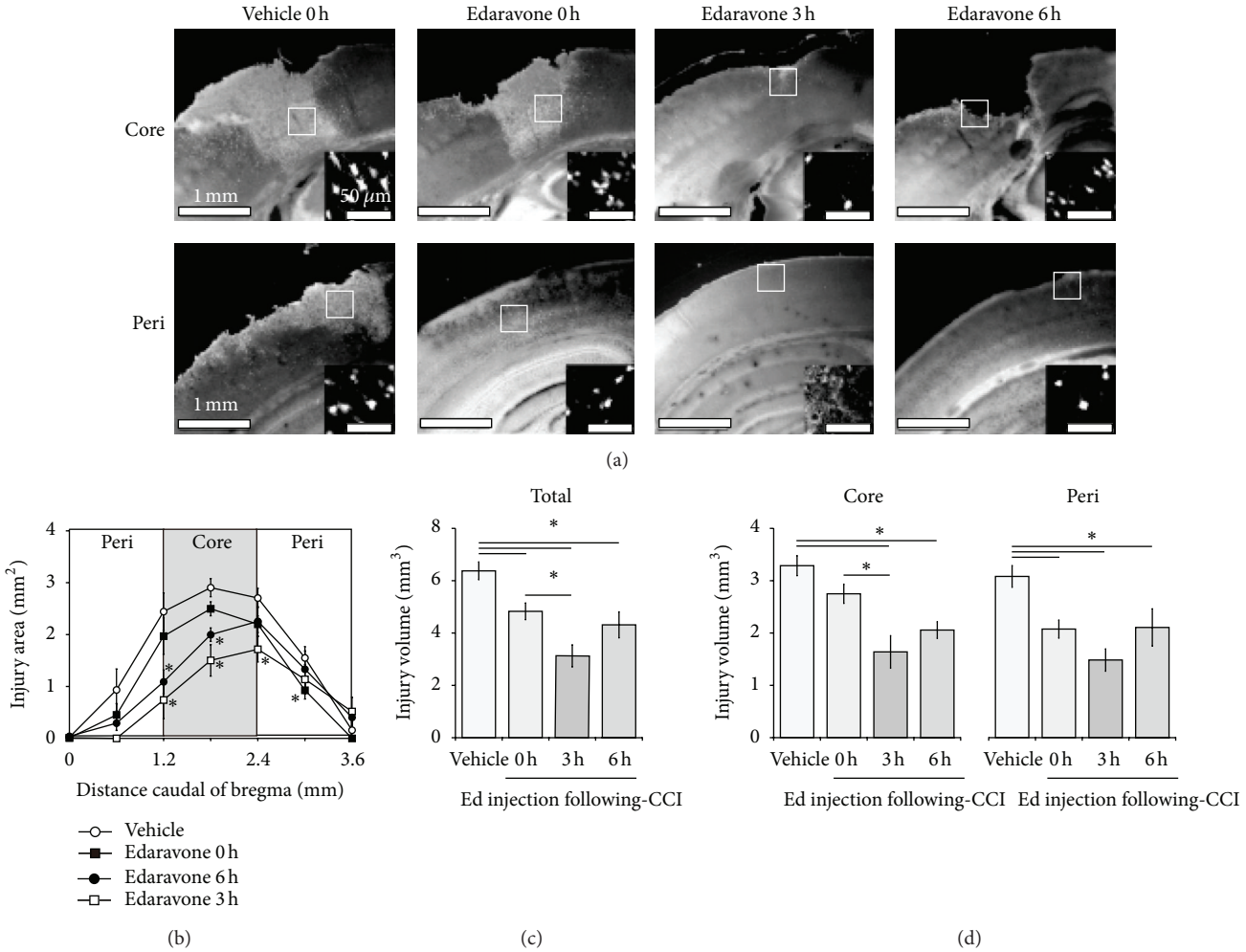

Figure 2: Effect of edaravone on TBI following CCI. (a) Representative core- (*upper*) and peri-injury (*lower*) images of FJB staining at 24 hours following CCI. Edaravone was injected 0, 3, or 6 hours following CCI; vehicle treatment occurred at 0 hours. The inset shows a higher power view of the boxed region in the main image. (b) The TBI area was semiquantified for each experimental group. Edaravone ($n = 10$) treatment at 3 and 6 hours led to a significant decrease in the core-injury area compared with the vehicle-treated control ($n = 9$). The injury volume was calculated by integration of the TBI areas and expressed as total (c) or core- and peri-injury (d) volumes. Data are expressed as mean ± SEM. Asterisk (∗) indicates a significant difference between groups based on *Tukey-Kramer* tests (P values are described in the text).

3.2. Edaravone Treatment Suppresses Oxidative Stress following TBI. The previous results revealed that edaravone reduces the area affected by TBI and that it can exert a therapeutic effect when administered up to 6 hours following injury. As previous studies have reported that edaravone acts as a radical scavenger and reduces oxidative stress in a number of diseases [28–30], we therefore next investigated whether the effect of edaravone in our model was due to the suppression of oxidative stress. To assess this, we determined the NT-positive volume 24 hours after CCI in animals treated with either vehicle ($n = 9$) at 0 hours or edaravone ($n = 10$) at 0, 3, or 6 hours (Figure 1(d)). The total NT-positive volumes for the edaravone-treated groups at 0 hours ($3.91 \pm 0.15\,\text{mm}^3$, $P < 0.001$), 3 hours ($2.28 \pm 0.30\,\text{mm}^3$, $P < 0.001$), and 6 hours ($3.15 \pm 0.45\,\text{mm}^3$, $P < 0.001$) were significantly less than those of the vehicle-treated control mice ($6.14 \pm 0.38\,\text{mm}^3$) (Figure 4). There was also a marked difference between the 0- and 3-hour edaravone treatment groups ($3.91 \pm 0.15\,\text{mm}^3$ versus $2.28 \pm 0.30\,\text{mm}^3$, $P < 0.001$). Similar results were obtained for the core- and peri-injury volumes of the animals treated with edaravone at 3 hours (core: $1.33 \pm 0.16\,\text{mm}^3$, $P < 0.001$; peri: $0.95 \pm 0.38\,\text{mm}^3$, $P < 0.001$) and 6 hours (core: $1.83 \pm 0.23\,\text{mm}^3$, $P < 0.01$; peri: $1.32 \pm 0.22\,\text{mm}^3$, $P < 0.01$) compared with the vehicle-treated controls (core: $3.29 \pm 0.28\,\text{mm}^3$; peri: $2.85 \pm 0.15\,\text{mm}^3$). However, 0-hour edaravone treatment only produced a significant ($P < 0.05$) decrease in the NT-positive peri-injury volume (core: $2.81 \pm 0.28\,\text{mm}^3$; peri: $1.94 \pm 0.22\,\text{mm}^3$). Moreover, the animals treated with edaravone at 3 hours showed significantly greater neuroprotection at both the core- and peri-injury sites versus the 0-hour treatment group (Figure 4).

3.3. Neurodegeneration in the Core Injury Area Occurs at 6 Hours following CCI and Spreads to Peri-Injury Area with Time. We next determined the time course (Figure 1(e)) of

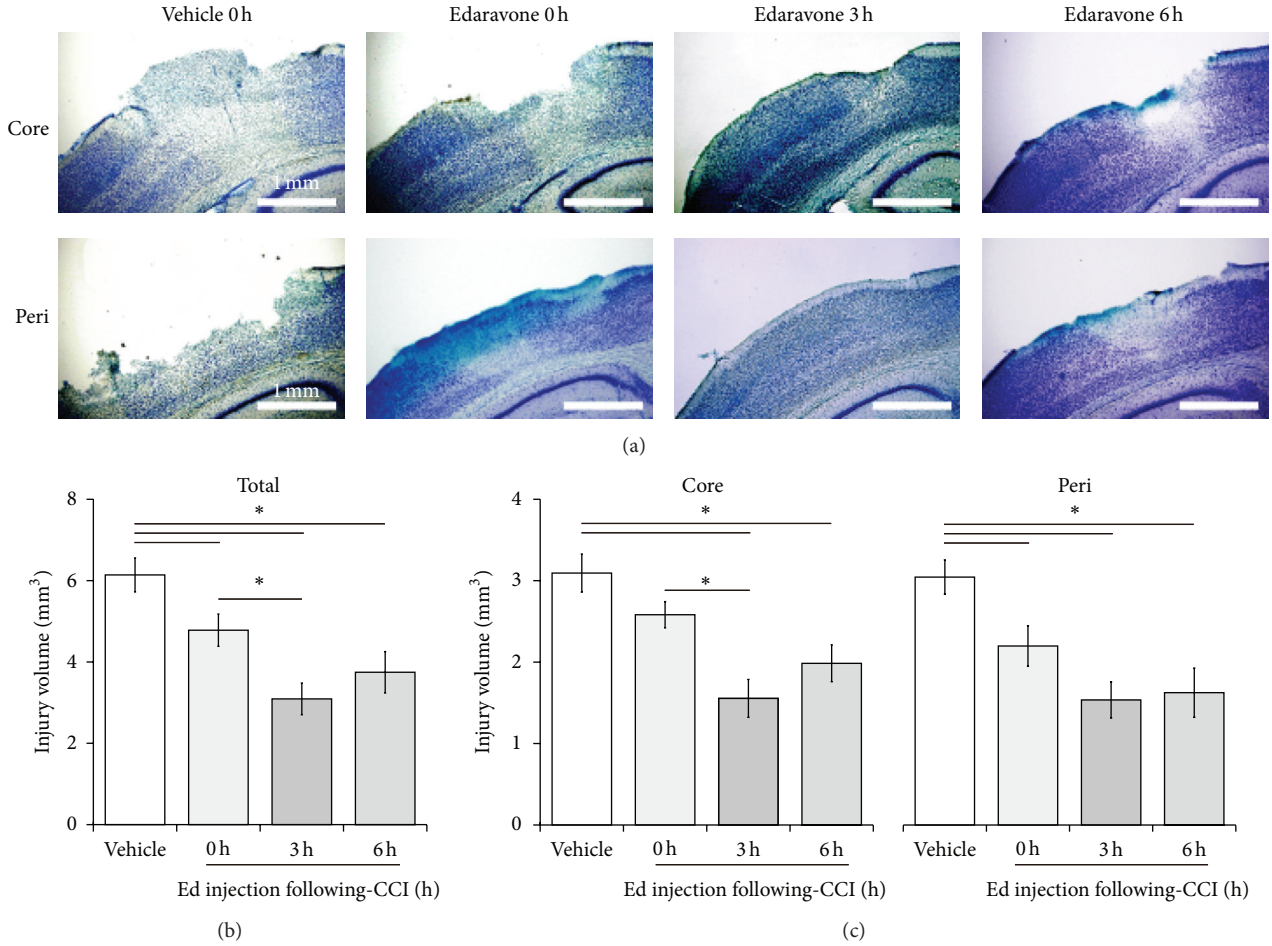

Figure 3: Effect of edaravone on TBI after CCI based on TB staining. (a) Representative core- (*upper*) and peri-injury (*lower*) images of TB staining 24 hours after CCI. The region with little or no staining was defined as the injury area. Edaravone ($n = 10$) was injected at 0, 3, or 6 hours and vehicle ($n = 9$) at 0 hours after CCI. The total (b) or core- and peri-injury (c) areas were semiquantified in each experimental group. Data are expressed as mean ± SEM. Asterisk (∗) indicates a significant difference between the groups based on *Tukey-Kramer* tests.

neurodegeneration following CCI ($n = 3$ for each time point). Only a few FJB-positive cells were present in the core-injury area 3 hours after CCI. By 6 hours, however, diffusely scattered FJB-positive cells were observed not only in the core injury area but also in the peri-injury area. By 24 hours, both the number of FJB-positive cells and the size of the affected area had increased even further (Figure 5). The injury volume was significantly greater at 6 ($2.49 \pm 0.30\ mm^3$, $P < 0.01$) and 24 hours ($6.38 \pm 0.34\ mm^3$, $P < 0.01$) compared with 0 ($n = 3$, $0.10 \pm 0.03\ mm^3$) and 3 ($0.19 \pm 0.05\ mm^3$) hours (Figure 5(c)).

3.4. Oxidative Metabolites Increase 6 Hours after CCI in Neurons. We subsequently investigated the temporal profile (Figure 1(e)) of oxidative stress by using NT immunostaining after CCI ($n = 3$ for each time point). Minimal immunoreactivity was observed immediately after CCI (0 h). However, dark brown NT staining began to appear from 6 hours following CCI in the neocortex around the epicenter of the impact site. By 24 hours, the NT-positive area had expanded and some of the tissue at the impact site had been lost (Figure 6(a)). The volume of the NT-positive region increased significantly in a time-dependent manner after CCI, accounting for $0.18 \pm 0.03\ mm^3$, $0.38 \pm 0.10\ mm^3$, and $2.07 \pm 0.63\ mm^3$ ($P < 0.05$ versus 0 hour) and $6.14 \pm 0.38\ mm^3$ ($P < 0.01$ versus 0 hour) at 0, 3, 6, and 24 hours after CCI, respectively (Figure 6(b)).

Colabeling with cell markers at the core-injury site indicated that the oxidative stress was occurring mainly in neurons (Figure 6(c)).

3.5. ROS Increases in Neurons 3 Hours after CCI. We next determined the time course (Figure 1(e)) of ROS generation based on the *in situ* detection of $O_2^{\bullet -}$ using HEt at 0, 3, 6, and 24 hours following CCI ($n = 3$ for each time point). A low Et signal was initially observed in the core-impact region. However, by 3 hours following CCI, this signal had increased markedly and continued to rise slightly up until the 24-hour time point (Figure 7(a)). Colabeling for Et and various cell markers revealed that the affected cells were mainly NeuN-positive neurons (Figure 7(b)).

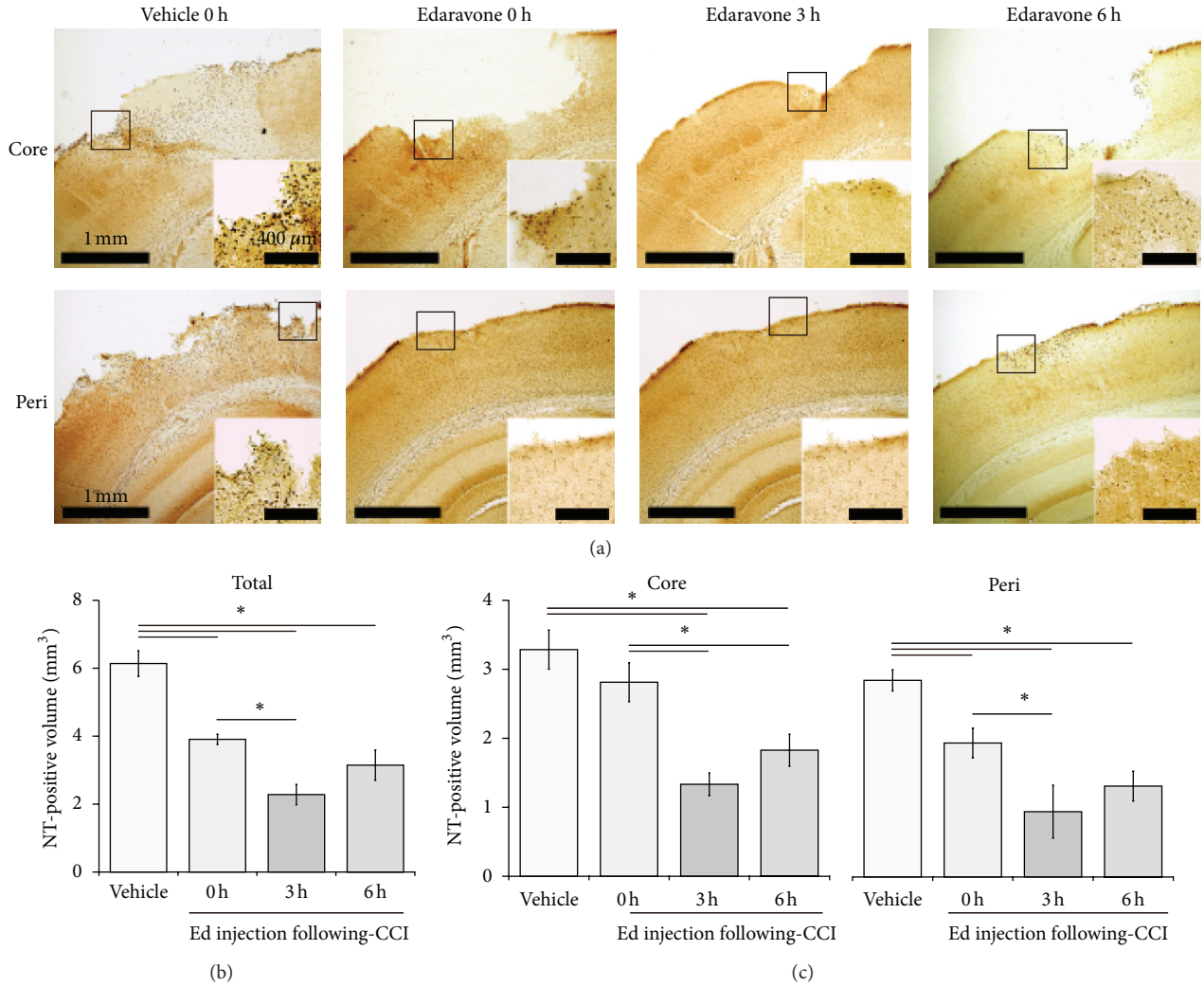

FIGURE 4: Effect of edaravone on oxidative stress following CCI. (a) Representative core- (*upper*) and peri-injury (*lower*) images of NT staining at 24 hours following CCI. Edaravone (n = 10) was injected at 0, 3, or 6 hours following CCI; vehicle (n = 9) treatment occurred at 0 hours. The inset shows a higher power view of the boxed region in the main image. The injury volume was calculated by integration of the TBI areas (based on seven 50 μm coronal sections at 500 μm intervals) and expressed as total (b) or core- and peri-injury (c) volumes. Data are expressed as mean ± SEM. Asterisk (∗) indicates a significant difference between groups based on *Tukey-Kramer* tests (P values are described in the text).

3.6. Edaravone Suppresses the ROS Production Cycle following CCI. Finally, we also investigated the effect of edaravone treatment 3 hours following CCI on $O_2^{\bullet-}$ generation at the core-injury site using HEt injection. Mice (n = 3 in each group) were administered edaravone 0 or 3 hours following CCI or vehicle 3 hours following CCI, with the Et signal evaluated 4 hours after injury as a measure of $O_2^{\bullet-}$ generation. As illustrated in Figure 7(c), vehicle-treated brains showed a large number of affected cells and a strong Et signal intensity. Fewer cells were affected following edaravone treatment at 0 hours, but the intensity of the Et signal became stronger thereafter. However, the 3-hour treatment resulted in only a few affected cells and a very weak Et signal, suggesting that edaravone administered at this time point suppressed the ROS and oxidative stress cycle and provided a greater neuroprotective effect.

4. Discussion

Edaravone is a free radical scavenger approved in Japan for the treatment of stroke. It could be a suitable candidate for treating TBI as well, given the results of several rodent studies showing that this drug is able to decrease neuronal cell death. However, the therapeutic time window of edaravone on TBI has not been examined in detail. In the present study, we have demonstrated that the intravenous injection of edaravone (3 mg/kg) decreased TBI and reduced oxidative stress when administered after a delay of up to 6 hours following CCI. We also determined the temporal profiles of ROS production, oxidative stress, and neuronal cell death in order to understand the relationship between brain damage and the sequence of events underlying ROS generation following CCI.

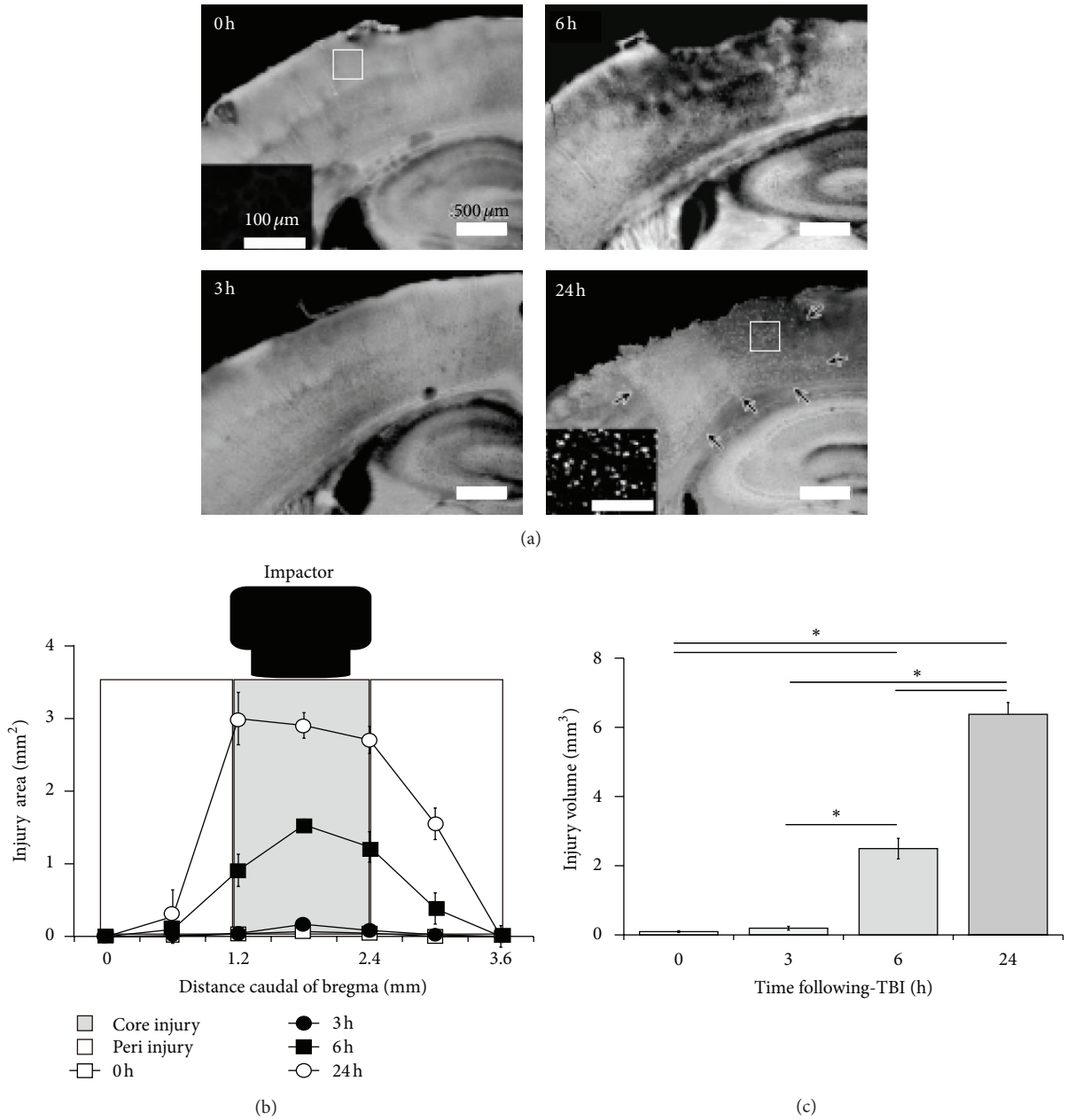

Figure 5: Detection of neuronal cell death by FJB labeling after CCI. (a) Neuronal cell death, as indicated by FJB labeling, increases in a time-dependent manner after CCI ($n = 3$ at each time point). No FJB-positive cells were observed at 0 (also see inset) and 3 hours after CCI; however, by 6 and 24 hours (also see *inset*), FJB labeling and an increase in the area of cortical disruption produced by the contusion were observed. (b) Seven 50 μm coronal sections at 500 μm intervals were used to semiquantify the area of FJB immunoreactivity, together with the area of cortical disruption produced by the TBI. This analysis revealed a marked increase in the affected area at 6 and 24 hours. Data are expressed as mean ± SEM ($n = 3$). (c) A significant increase in the TBI volume was observed 6 and 24 hours following CCI. Data are expressed as mean ± SEM ($n = 3$). Asterisk ($*$) indicates a significant difference between groups based on *Tukey-Kramer* tests (P values are described in the text).

We previously reported that the intravenous administration of edaravone (3 mg/kg) immediately after TBI suppressed cortical damage [13]. Another study also demonstrated that edaravone injected 2 and 12 hours after TBI decreased neuronal cell loss in the CA3 layer of the hippocampus in a dose-dependent fashion (0.75, 1.5, or 3 mg/kg) [8]. In the present study, we examined the therapeutic time window of intravenously injected edaravone (3 mg/kg) on TBI and showed that edaravone administered for up to 6 hours at least after CCI suppressed the lesion size. Comparisons of lesion sizes following edaravone treatment at 0, 3, and 6 hours after CCI demonstrated that edaravone treatment at 3 hours provided the greatest neuroprotective effect compared with the other treatment groups and that

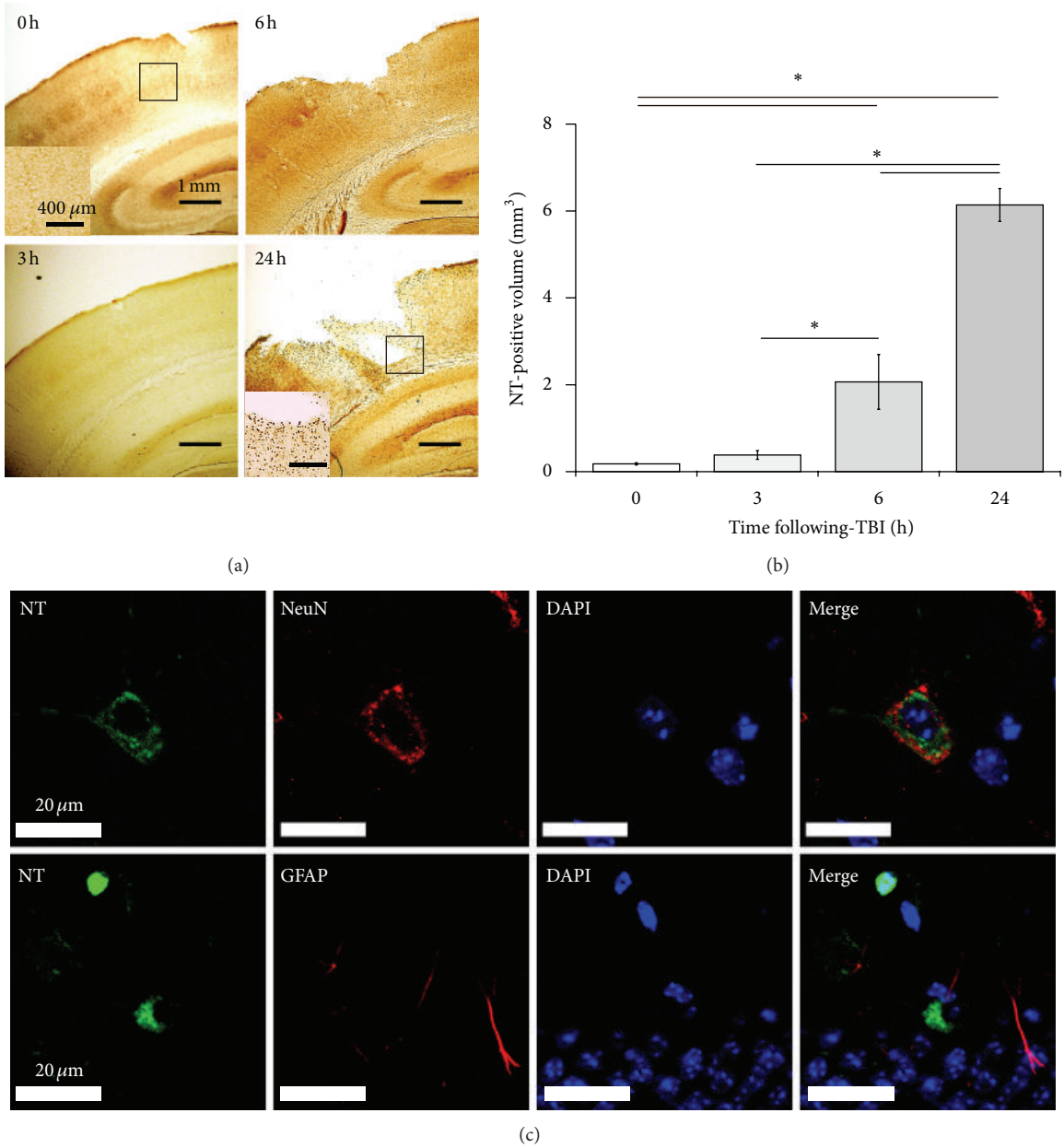

Figure 6: Detection of oxidative stress by NT labeling following CCI. (a) Very few NT-immunopositive cells were observed at 0 (also see *inset*) and 3 hours following CCI; however by 6 and 24 hours (also see inset) this number, and the area of cortical disruption produced by the contusion, had increased markedly. (b) Semiquantification of the NT-positive volume revealed a significant increase at 6 and 24 hours after CCI. Data are expressed as mean ± SD ($n = 3$). Asterisk (∗) indicates a significant difference between groups based on *Tukey-Kramer* tests (P values are described in the text). (c) Multiple immunofluorescence staining of NT and cell markers. The NT-positive staining overlapped with that of the neuronal marker, NeuN (*green, upper panel*), but not with the astroglial marker, GFAP (*green, lower panel*). The sections were also counterstained with the nuclear dye DAPI (*blue*).

a significant difference was observed compared with treatment at the 0-hour time point. To estimate the extent of the neuroprotective effect, we further compared the lesion size by measuring both core- and peri-injury areas. Edaravone treatment 6 hours after CCI decreased the lesion size significantly both in the peri- and core-injury sites compared with control, but the area was slightly larger than that seen in mice treated with edaravone 3 hours after CCI. Although mice treated with edaravone at the 0-hour time point showed a significantly decreased lesion size in the peri-injury site compared with vehicle-treated animals, no statistically significant differences with respect to the core-injury site size were seen. Furthermore, the size of the core-injury site in the 0 hour edaravone treatment group was significantly greater than that measured in mice treated 3 hours after CCI. In particular, the lesion size in the frontal

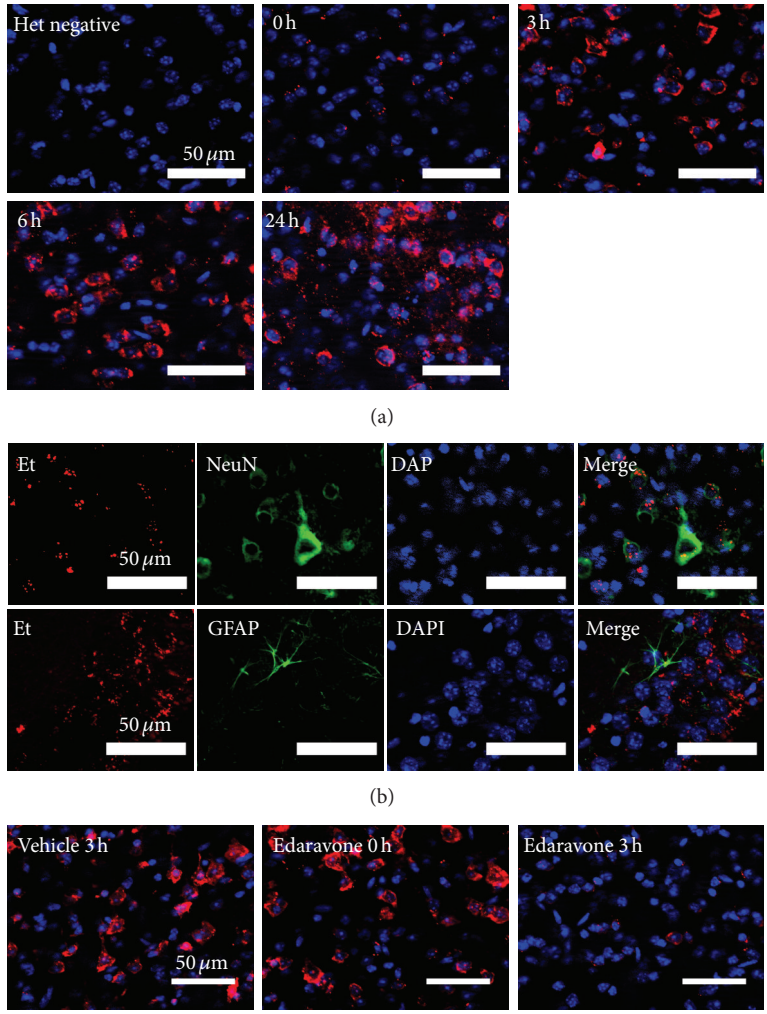

FIGURE 7: *In situ* detection of ROS as $O_2^{\bullet-}$ using Het. (a) No $O_2^{\bullet-}$ (Et) signals (red) were detected in control animals. In mice subjected to TBI, a low level of $O_2^{\bullet-}$ was observed immediately after CCI. By 3 hours this level had increased markedly, remaining high 6 and 24 hours after CCI. (b) Multiple immunofluorescence staining of $O_2^{\bullet-}$ and cell markers. $O_2^{\bullet-}$ (Et) signals (*red*) strongly colocalized with the neuronal marker NeuN (*green, upper panel*) and to lesser extent with the astroglial marker GFAP (*green, lower panel*), with nuclei labelled by DAPI (*blue*). (c) The effect of edaravone treatment on $O_2^{\bullet-}$ production after CCI ($n = 3$ in each group) is shown. As a control, vehicle was administered 3 hours post-CCI (vehicle 3 h). Edaravone was administered at 0 hours (edaravone 0 h) or 3 hours (edaravone 3 h) after CCI. The production of $O_2^{\bullet-}$ was evaluated based on the *in situ* detection of the Et signal (*red*) in the core-injury area 4 hours after CCI. Nuclei (*blue*) were labeled with DAPI.

area was significantly different between these two groups. Observations made with respect to NT immunostaining as an indicator of oxidative stress also demonstrated a similar tendency to that seen with lesion size.

We subsequently examined the temporal profiles of ROS production, oxidative stress, and neuronal cell death in order to understand the relationship between brain damage and the sequence of events giving rise to ROS generation following CCI. Superoxide detected by HEt was used for the determination of ROS levels because $O_2^{\bullet-}$ is initially increased after injury [26], while the oxidative metabolites $ONOO^-$ and $^{\bullet}OH$ also contribute to increasing oxidative stress and damage in tissue [11]. The $O_2^{\bullet-}$ signal was initially observed 3 hours following CCI and increased with time up to 24 hours. The oxidative stress detected by NT, which is a metabolite of L-tyrosine oxidation by $ONOO^-$ [31], was observed at 6 hours following CCI in the core-injury region and increased for up to 24 hours with extension to the peri-injury area. This was reflected in the rise in $O_2^{\bullet-}$ production and the concomitant increase in neuronal cell death detected by FJB staining, suggesting that excessive $O_2^{\bullet-}$ production after CCI might result in the induction of oxidative stress and neurodegeneration in the brain.

From the results of the temporal profiles of ROS production, oxidative stress, and neuronal cell death, the effects of treatment with edaravone were consistent with the time points which fall before, during, and after the production of ROS, respectively. While a precise explanation for this cannot

be given, we suggest that it could have something to do with the half-life of edaravone, which is reported to be approximately one hour [29]. Therefore, the animals treated with edaravone immediately after (0 hour) CCI might actually have had a decreased effective concentration of this drug at the time of maximum ROS production. By 6 hours following CCI, a weaker neuroprotective effect was observed due to the fact that significant oxidative damage and neuronal cell death had already occurred. Nonetheless, treatment at this time point still had a therapeutic effect given that the level of ROS continued to increase for 24 hours. These results suggest that there might be an optimal time for treatment with edaravone to suppress the degree of injury. An experimental study in which mice were exposed to hypobaric conditions for 3 hours after TBI supports our present results; these animals showed exacerbated secondary traumatic injury severity because of a greatly heightened inflammatory response due to the hypobaric condition [32]. We recently found that edaravone improved cerebral blood flow (CBF) after TBI [33]. In this way, CBF in control animals in that study was significantly reduced, probably as a consequence of vasospasm, in the ipsilateral hemisphere of the brain 3 to 6 hours after CCI [33]. Treatment with edaravone significantly ameliorated the CBF. Other studies have reported that edaravone improves blood circulation in the heart [34] and lung [27], suggesting that edaravone treatment may suppress traumatic ischemic injury, as observed in the present study, by ameliorating circulation.

This finding is important given that most TBIs are associated with falls and motor vehicle accidents. Therefore, in most cases the onset of TBI is clear and patients are transferred to an emergency department within a few hours. From a clinical perspective, it is noteworthy that delayed treatment with edaravone in our study was more efficacious than administration immediately after the CCI; the time delay between the onset of ROS production and neuronal cell death means probably that these cases would fall within a therapeutic time window for the treatment of TBI. At the present time, edaravone (60 mg/day injected intravenously in 2 divided doses) is used in Japan for the treatment of stroke. Therefore, the dosage of edaravone in our study was approximately triple compared with clinical use. Further study is required to determine the minimum dosage of edaravone necessary to suppress ROS production and lesion size in this animal model and for this finding to be translated to the clinical setting whereby edaravone is used to suppress the lesion size in clinical cases of TBI.

Although many studies have reported that oxidative stress contributes to TBI, very few have linked this directly to ROS production in the brain. We have previously reported that both patients suffering a neurological emergency and animals subjected to TBI display increased blood alkoxy-radical levels as detected by an electron spin resonance (ESR) spin trapping method [13, 35]. Based on the rapid elevation of intracellular Ca^{2+} and the impairment of CBF, the source of the $O_2^{\bullet-}$ is considered to be primarily the mitochondria [36]. The intracellular $O_2^{\bullet-}$ impairs mitochondrial function and induces neuronal cell death. Extracellular $O_2^{\bullet-}$ might be produced by NADPH oxidase in microglia/macrophages following CCI [19]. Based on our previous demonstration that mice deficient in one of the subunits of NADPH oxidase, $Gp91^{phox}$, display decreased $O_2^{\bullet-}$ levels and that inhibition of $Gp91^{phox}$ by apocynin reduces the severity of TBI *in vivo* [19, 36, 37], it appears that this extracellular $O_2^{\bullet-}$ also contributes to the induction of neuronal cell death in response to injury. Therefore, both intra- and extracellularly produced $O_2^{\bullet-}$, together with their associated metabolites, may play important roles in the generation of oxidative stress following CCI.

Many clinical trials of antioxidant agents or radical scavengers have been performed in cases of cerebral infarction, subarachnoid hemorrhage, and TBI [15, 38, 39]. However, the only agent which has been granted approval to date is edaravone [9, 40], even though patients are treated without concomitant monitoring of ROS or oxidative metabolite levels. Human TBI presents as a more complex and diverse condition than animal experimental models. Therefore, care needs to be exercised when determining the likely therapeutic time window of edaravone. We suggest that edaravone and possibly other radical scavengers or antioxidants should be administered in response to ROS generation, meaning that the bedside monitoring of ROS could shed more light on the potential benefit of these agents. Recently, some groups have reported that Overhauser enhanced MRI, which is a double resonance technique, creates images of free radical distribution in small animals by enhancing the water proton signal intensity by means of the Overhauser effect [41]. Although not available for bedside monitoring, this technique could be adapted to directly monitor ROS levels in the brain. We have also previously reported that one particular ROS, the alkoxy radical, is increased both in patients suffering a neuroemergency and in animals subjected to TBI and can be monitored by ESR [13, 35]. These new techniques could represent powerful tools for the diagnosis of ROS production prior to oxidative stress, thereby facilitating more effective treatment.

5. Conclusion

In the present study, we have demonstrated that edaravone suppresses neuronal damage in mice subjected to a CCI, with the greatest effect observed when the drug is given 3 hours after TBI. This time window is consistent with the increase in ROS produced in the cortex after CCI. We therefore suggest that edaravone could prove clinically useful to ameliorate the devastating effects of TBI. To ensure optimal efficacy, however, it is critical that ROS levels are measured concomitantly.

Conflict of Interests

No competing financial interests exist.

Acknowledgments

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Research Article

Role of Melanin in Melanocyte Dysregulation of Reactive Oxygen Species

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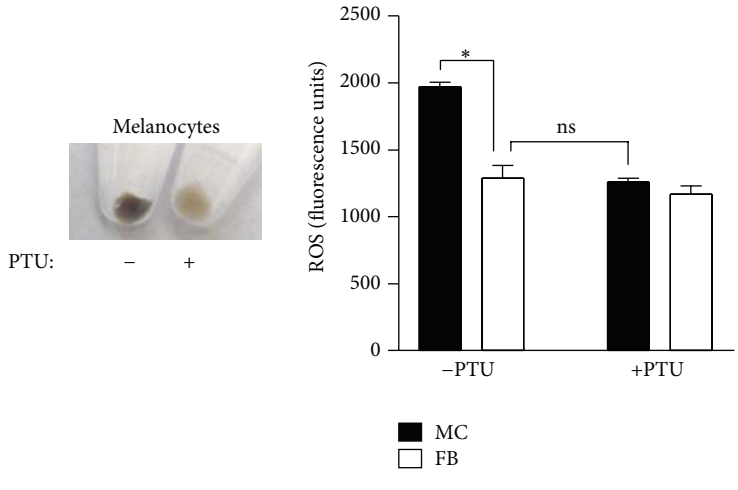
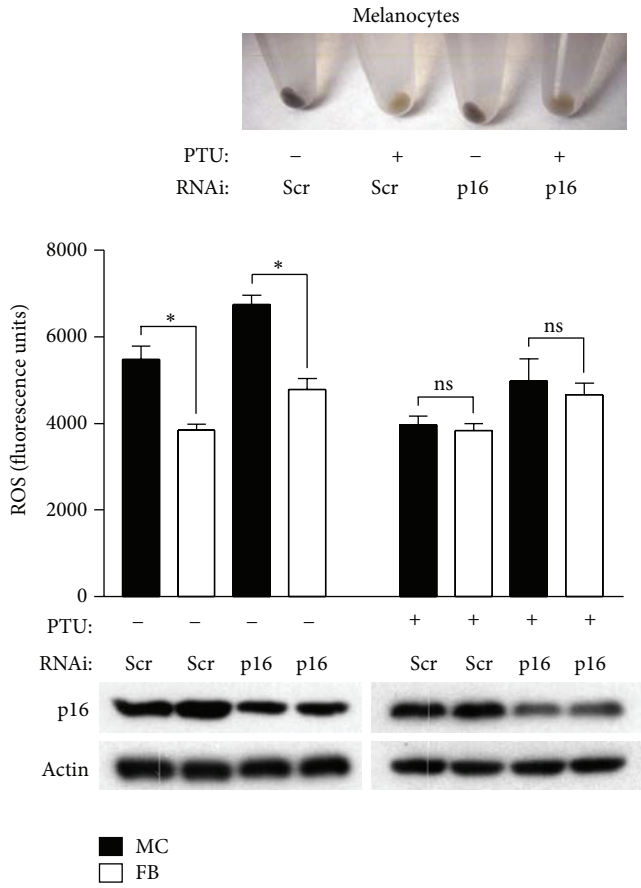
We have recently reported a potential alternative tumor suppressor function for p16 relating to its capacity to regulate oxidative stress and observed that oxidative dysregulation in p16-depleted cells was most profound in melanocytes, compared to keratinocytes or fibroblasts. Moreover, in the absence of p16 depletion or exogenous oxidative insult, melanocytes exhibited significantly higher basal levels of reactive oxygen species (ROS) than these other epidermal cell types. Given the role of oxidative stress in melanoma development, we speculated that this increased susceptibility of melanocytes to oxidative stress (and greater reliance on p16 for suppression of ROS) may explain why genetic compromise of p16 is more commonly associated with predisposition to melanoma rather than other cancers. Here we show that the presence of melanin accounts for this differential oxidative stress in normal and p16-depleted melanocytes. Thus the presence of melanin in the skin appears to be a double-edged sword: it protects melanocytes as well as neighboring keratinocytes in the skin through its capacity to absorb UV radiation, but its synthesis in melanocytes results in higher levels of intracellular ROS that may increase melanoma susceptibility.

Inactivation or loss of $p16^{INK4A}$ (p16) is a common event in many tumor types although germ-line mutations in *p16* are disproportionately associated with melanoma predisposition [1]. The p16 protein inhibits the kinase activity of cyclin-dependent kinases 4 and 6, preventing the hyperphosphorylation of retinoblastoma-related pocket proteins that are required to release E2F transcription factors necessary for cell-cycle progression. Thus the canonical tumor suppressor function of p16 is to prevent division of stressed or damaged cells by holding them in the late G1-S transition to allow adequate time for DNA repair, or promoting their irreversible exit from the cell-cycle into a senescent state [2]. We have recently reported a potential alternative tumor suppressor function for p16 relating to its capacity to regulate oxidative stress, demonstrating that the depletion of p16 by RNAi in human cells led to increased levels of intracellular reactive oxygen species (ROS) and the oxidative DNA lesion 8-oxoguanine that was independent of cell-cycle phase [3]. We observed that oxidative dysregulation in p16-depleted cells was most profound in melanocytes, compared to keratinocytes or fibroblasts. Moreover, in the absence of p16 depletion or exogenous oxidative insult, melanocytes exhibited significantly higher basal levels of ROS than these other epidermal cell types. Given the role of oxidative stress in melanoma development [4], we speculated that this increased susceptibility of melanocytes to oxidative stress (and greater reliance on p16 for suppression of ROS) may explain why genetic compromise of p16 is more commonly associated with predisposition to melanoma rather than other cancers.

It is not known why melanocytes maintain higher levels of ROS than other cell types, but we hypothesized a role for melanin since its presence is a distinguishing feature of melanocytes and melanin synthesis is known to generate ROS [5]. A previous study found a correlation between levels of melanin and ROS, showing that both were elevated in melanocytes from dysplastic nevi compared to those from normal skin of the same individual [6]. Melanogenesis is pro-oxidative, commencing with the oxidation of L tyrosine to



(a)



(b)

Figure 1: Inhibition of melanin synthesis reduces intracellular ROS in melanocytes. (a) Human melanocytes (MC) were either untreated (–) or treated (+) with 200 μM PTU (Sigma) for 14 days (left panel). Fibroblasts (FB) were isolated from the same donors. Endogenous ROS were detected by the addition of 20 μM DCFDA (Invitrogen) and measured as previously described [3]. Error bars represent S.E.M. of triplicate determinations, and results are representative of two experiments performed. $^{*}P = .003$ (two-sided t test). ns, not significant. (b) PTU treatment of melanocytes transfected with either a control scrambled (Scr) siRNA sequence, or siRNA specific for p16, decreases melanin content (upper panel). Error bars represent S.E.M. of ROS determinations made from three separate donors (middle panel). $^{*}P = .04$, $^{**}P = .03$ (paired two-sided t test). ns, not significant. Representative Western blot showing p16 levels in siRNAi-transfected cells (lower panel).

dopaquinone, an enzymatic process that can be inhibited by N phenylthiourea (PTU). To evaluate the role of melanin in melanocyte oxidative dysregulation, we derived melanocytes and fibroblasts from three separate individuals, and cells were cultured in the absence or presence of PTU for 14 days. This was sufficient to deplete most of the melanin in melanocytes (Figure 1(a), left). Intracellular ROS levels were then quantitated by fluorometric analysis following treatment with the cell-permeable fluorophore DCFDA. As previously reported [3], melanocytes exhibited significantly higher ROS levels compared to donor-matched fibroblasts (Figure 1(a), right). By contrast, treatment with PTU resulted in a reduction of basal intracellular ROS levels in melanocytes comparable to those of fibroblasts (Figure 1(a), right). PTU-treated fibroblasts, on the other hand, showed no significant difference in intracellular ROS from their untreated counterparts.

Next we evaluated the pro-oxidative role of melanin in the context of p16 depletion. Donor-matched fibroblasts and melanocytes were transfected with either control or siRNA specific for p16 [3] to deplete endogenous p16 protein (Figure 1(b), lower). Depletion of p16 in both cell types led to increases in intracellular ROS, with ROS levels consistently higher in melanocytes compared to fibroblasts under both control conditions and following p16 knockdown (Figure 1(b), middle). The removal of melanin by PTU (Figure 1(b), upper) was associated with reduction of ROS levels in melanocytes comparable to fibroblasts, even under conditions of p16 depletion (Figure 1(b), middle). These results implicate melanin as the cause of increased oxidative stress in normal and p16-depleted melanocytes.

It is established that chronic oxidative stress and resulting oxidative damage promote carcinogenesis. Melanocytes are more susceptible to oxidative damage due to maintenance of higher levels of ROS [3]. Loss of p16 function through methylation-mediated gene silencing, mutation, or gene deletion, as is commonly found in melanoma [1], would be predicted to further increase ROS levels and correspondingly increase oxidative damage. Elevated levels of ROS in melanocytes are likely compounded by the relative deficiency of this cell type in the repair of oxidative DNA lesions [7]. Both acute and chronic UV radiations induce ROS in the skin, and we have previously shown that the administration of the antioxidant N acetylcysteine prior to and following acute UV exposure delays melanoma onset in a mouse melanoma model [4]. In this same model system, loss of p16 accelerates UV-induced melanoma development [8]. Although melanocytes may be protected by endogenous melanin which can directly absorb UV-generated photons and oxygen radicals [9], at higher UV doses melanin can be oxidized leading to the generation of ROS [10]. However, we have found in the absence of UV exposure that the pro-oxidative nature of melanin production is directly associated with higher melanocyte basal levels of intracellular ROS, which increase significantly following p16 depletion. Thus the presence of melanin in the skin appears to be a double-edged sword: it protects melanocytes as well as neighboring keratinocytes in the skin through its capacity to absorb UV radiation, but its synthesis in melanocytes results in higher levels of intracellular ROS that may increase melanoma susceptibility. Further studies may elucidate whether the pro-oxidative nature of melanin biosynthesis is indeed the basis for predisposition of individuals with inherited p16 mutations that are more likely to develop melanoma over other cancers.

Conflict of Interests

The authors have no conflict of interests to declare.

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