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Oxygreen Process Applied on Nongerminated and Germinated Wheat: Role of Hydroxamic Acids

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Wheat samples were taken at different stages of germination characterized by their falling number (which is a relevant indicator of germination) from 400 to 60 s. Each batch was treated by the Oxygreen process, a treatment by ozone, in a closed sequential batch reactor. Leucotriene B4 (LTB_4) was induced by germination, but ozone treatment did not increase this effect. Extract obtained from these wheat batches was applied on human epithelial bronchial cells. Wheat extract from nongerminated wheat did not induce any DNA adduct. More the wheat germination gets underway, more DNA adducts are observed. In contrast, germination did not affect the cell viability. Ozonization of wheat exemplified genotoxic effects only if the wheat was germinated. The implication of hydroxamic acids is discussed. In conclusion, ozonization of wheat, of high milling quality, does not pose any problem.

KEYWORDS: Germination; wheat; ozone; oxygreen; DNA adduct; leucotriene B4; hydroxamic acid; ochratoxin A

INTRODUCTION

Over the world it is well-known that wheat is one of the main staple foods. The major use of wheat for human consumption is bread making. One criterion of wheat quality for bread making is the falling number which measures indirectly the level of α-amylase in grain. Indeed, an increase of α-amylase, which degrades starch into maltose, causes a decrease in bread-making quality due to modifications of flour properties (affects gas retention, dough handling, and bread texture). To estimate the degree of germination, an industrial and validated test, called the "falling number" method, is used to measure the effect of the α-amylase accurately [Quality Wheat CRC (*1*)]. The falling number is an indirect measure of starch quality through viscosity analysis of a water solution containing flour. The falling number is defined as the time in seconds required for stirring and allowing the stirrer to fall a measured distance through a hot aqueous flour or meal gel undergoing liquefaction due to α-amylase activity as determined by the AACC Hagberg Falling Number method. The lowest falling number accepted is 250 s; the highest falling number for wheat of best quality reaches 600 s [EC 1068/2005 amendment of EC 824/2000 (*2*)]. Nevertheless, it has been proven that germination is correlated with greater amounts of vitamins (vitamin E, vitamin C, β-carotene, riboflavin, thiamin, biotin, pantothenic acid) which exhibit antioxidant properties (*2–4*) offering interesting nutritional properties. More recently, Koehler et al. (*6*) observed an increase of folate and dietary fiber (*7*). In addition, several organic compounds are released following enzymatic hydrolysis of phytates, allowing availability of oligo elements such as magnesium, calcium, or iron (*8–11*). Another advantage of germination was pointed out by Hartmann et al. (*12*), who observed a rapid degradation of gliadin peptides toxic for patients suffering celiac disease. The Oxygreen process (*13, 14*) is a new wheat grain treatment by ozone, in specific and closed conditions, in a sequential batch reactor (described in international patent WO 01/43556 A1). The Oxygreen process operates on wheat grain with the aim to improve the quality of flour for many different applications, such as baking, Danish pastries, and English high ratio sponge cake production, and to avoid added ascorbic acid, gluten, and α-amylase for bread making. This process has been considered as safe, by the French agency for food safety (Afssa) in 2004, for these applications. Oxygreen takes place in the classical milling process.

In order to test the efficiency of the Oxygreen process (*13, 14*), we have selected batches of wheat. The first step was to evaluate if noncontaminated wheat exhibits any intrinsic toxicity. As ozone has been described as an inducer of germination [international patent WO 95/09523 (*15*)], in order to test whether germinated wheat could have a toxic effect, several extracts of wheat batches displaying various falling numbers at different stages of germination, contaminated or not by ochratoxin A, were tested on human epithelial bronchial cells.

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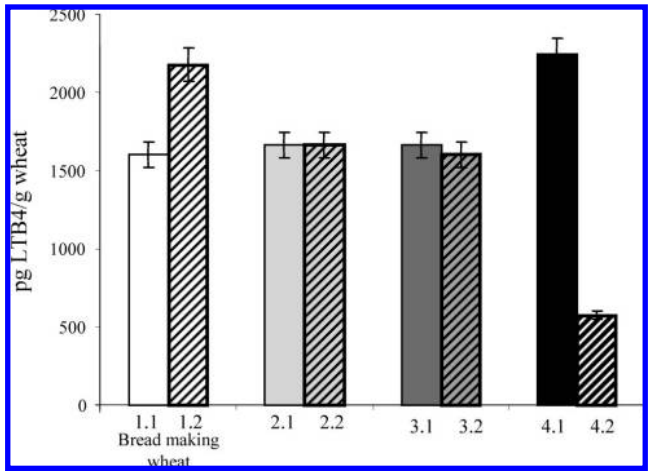

Figure 1. LTB_4 concentration, expressed in pg/g of wheat, in bread-making wheat (white, 1.1) or germinated wheat extract (gray, black) not treated [2.1 (falling number 143), 3.1 (falling number 132), 4.1 (falling number 62)] or treated (hatched) with the Oxygreen process (1.2, 2.2, 3.2, 4.2). For details of number, see Materials and Methods.

MATERIALS AND METHODS

Chemicals. Ochratoxin A (OTA) (benzene free; CAS Registry No. 303-47-9) was purchased from Sigma (Saint Quentin Fallavier, France). The following enzymes were purchased: proteinase K (used as received), RNase A, RNase T1 (boiled for 10 min at 100 °C to destroy DNases), and micrococcal nuclease (dialyzed against deionized water) from Sigma (Saint Quentin Fallavier, France); spleen phosphodiesterase (centrifuged before use) from Calbiochem (VWR, France); and nuclease P1 (NP1) and T4 polynucleotide kinase from Roche Diagnostics (Meylan, France). [γ-^{32}P]ATP (444 Tbq/mmol, 6000 Ci/mmol) was from Amersham (Les Ullis, France); Roswell Park Memorial Institute medium (RPMI) was prepared with Gibco products (Cergy Pontoise, France); phosphate saline buffer, trypsin, fetal calf serum, streptomycin, and penicillin were from Life Technologies (Cergy-Pontoise, France); rotiphenol (phenol saturated with Tris-HCl, pH 8) was from Rothsichel (Lauterbourg, France); salmon testis DNA was from Sigma and was purified before use; cellulose MN 301 was from Macherey Nagel (Düren, Germany); polyethylenimine (PEI) was from Corcat (Virginia Chemicals, Portsmouth, VA); Whatman No. 1 paper (ref 6130932) was from VWR (France), and PEI/cellulose TLC plates used for ^{32}P-postlabeling analyses were prepared in the laboratory at Toulouse, France. All reagents (potassium chloride, sodium hydrogen carbonate, sulfuric acid, phosphoric acid, hydrochloric acid, acetic acid, sodium dihydrogen phosphate) were of normal grade.

Germination Process. One wheat batch provided by a flour mill located in Brittany (France), with a starting falling number of 422, has been divided into four batches. One batch is used as control (called 1.1). The three other wheat batches were germinated by putting them for 24 h (called 2.1), 36 h (called 3.1), or 72 h (called 4.1) in water in the dark and at room temperature. The grains were collected by decantation and dried. The falling number of every batch was determined for evaluation of the germination process. Each batch has been divided in two parts. One-half has been treated by the Oxygreen process as described in Dubois et al. (*14*). They are respectively called 1.2, 2.2, 3.2, and 4.2.

Analysis of Leukotriene B_4. LTB_4 was quantified using a competitive immunoenzymatic method. LTB_4 enzyme immunoassay kit ACE-EIA was provided by Cayman, Spi Bio, France. This test is based on the competition between LTB_4 and the LTB_4−acetylcholinesterase (AChE) conjugate (LTB_4 tracer) for a limited amount of LTB_4 antiserum. The absorbance measured at 412 nm is inversely proportional to the free LTB_4.

Extraction of Wheat for Toxic Evaluation. Extraction of wheat has been conducted according to the protocol of mycotoxin extraction, described in detail by Molinié et al. (*16*). Briefly, 50 g of grind wheat was extracted with acetonitrile−water (9:1) containing 4% KCl and 0.8 mL of sulfuric acid. This first extract was defatted twice using *n*-hexane. Then the aqueous phase was submitted to extraction by $CHCl_3$. After evaporation of $CHCl_3$, the residue was dissolved in methanol and conserved at −20 °C.

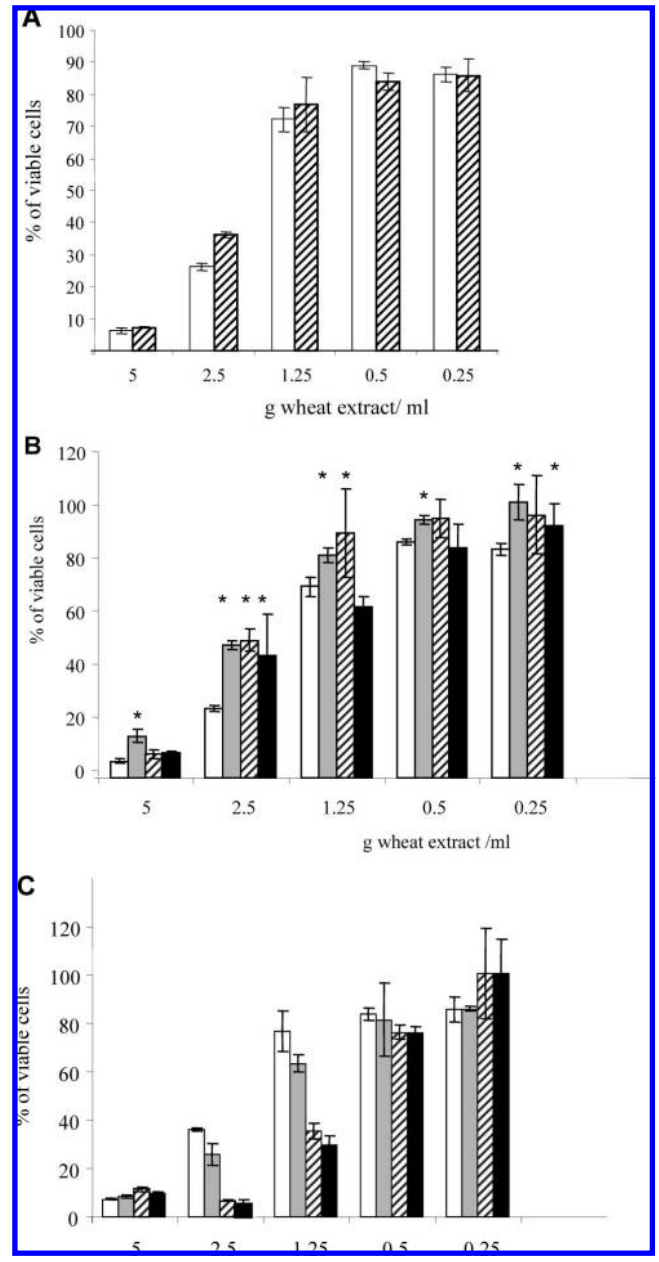

Figure 2. Cell viability according to the MMT test. (**A**) Human bronchial epithelial cells are exposed for 24 h to bread-making wheat extract, untreated (white) or treated by the Oxygreen process (hatched). (**B**) Human bronchial epithelial cells are exposed to bread-making wheat (white, 1.1) or germinated wheat [2.1 (gray), 3.1 (hatched), 4.1 (black)]. (**C**) Human bronchial epithelial cells are exposed to wheat treated by Oxygreen: bread-making (white, 1.2) or germinated [2.2 (gray), 3.2 (hatched), 4.2 (black)].

Cell Exposure. Human epithelial bronchial cells (WI26 provided by American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium, with Glutamax supplemented with penicillin/streptomycin (1%) and fetal bovine serum (10%). Cells were exposed during 24 h to increasing amounts of wheat extracts (0–5 g/mL of culture medium). The analyses were made in triplicate for DNA adducts detection and ten times for cytotoxicity analyses.

Cell Viability. Two tests were used to evaluate cytotoxicity: (i) MTT cell proliferation test allowing evaluation of metabolic activity of cells; (ii) LDH test quantifying cellular lysis. The data are expressed as the

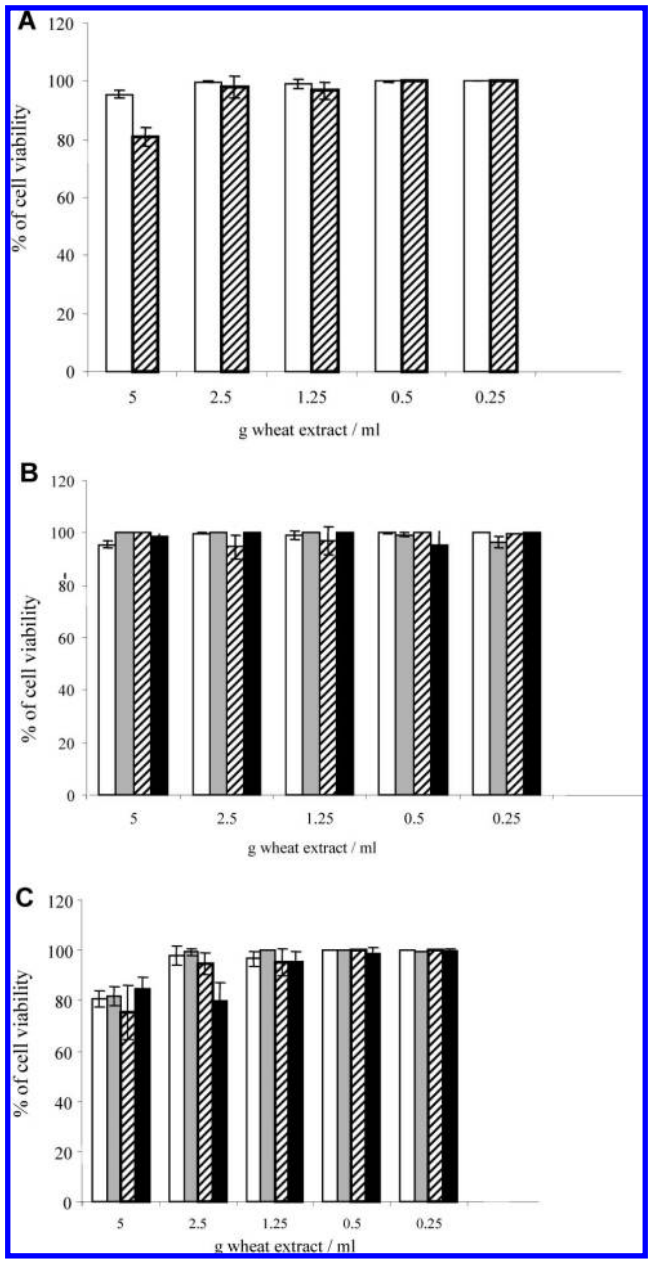

Figure 3. Cell viability according to the lactate dehydrogenase (LDH) test. (**A**) Human bronchial epithelial cells are exposed to various concentrations of bread-making wheat extract, untreated (white) or treated with the Oxygreen process (hatched). (**B**) Human bronchial epithelial cells are exposed to bread-making wheat (white; 1.1) or germinated wheat [2.1 (gray), 3.1 (hatched), 4.1 (black)]. (**C**) Human bronchial epithelial cells are exposed to wheat treated by Oxygreen: bread-making (white, 1.2) or germinated [2.2 (gray), 3.2 (hatched), 4.2 (black)].

average value of 10 independent analyses. Significant differences are mentioned on the histograms by an asterisk, determined by the Wilcoxon rank test.

MTT Test. The MTT test is a colorimetric assay system which measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. The viability of the cell was based on the succinate dehydrogenase activity of the viable cell. The cell titer 96 nonradioactive cell proliferation assay (Promega) is based on the cellular conversion of tetrazolium salt into yellow formazan absorbing at 570 nm. The viability of the cell is quantified by recording absorbance at 570 nm, using a 96 plate reader (Jobin Yvon, spectrofluo JY3D).

LDH Test. The test is based on measurement of activity of lactate dehydrogenase (LDH) which is a stable enzyme normally found in the cytosol of all cells but rapidly releases into the supernatant upon damage of membrane when cells die using the kit Cytotox 96 nonradioactive cytotoxicity assay, Promega, France. The cell death is quantified by recording absorbance at 490 nm, using a 96 plate reader (Jobin Yvon, spectrofluo JY3D).

DNA Adduct Detection. DNA was extracted from cells, purified, and postlabeled as described in detail by Pfohl-Leszkowicz and Castegnaro (*17*).

The separation of the DNA adduct is made using the contact transfer method. The solvent used in first dimension, D1, was 2.3 M NaH_2PO_4 buffer, pH 5.7, overnight; for D2, 4.8 M lithium formate and 7.7 M urea, pH 3.5, for 3 h; for D3, 0.6 M NaH_2PO_4 and 5.95 M urea, pH 6.4, for 3 h; for D4, 1.7 M NaH_2PO_4, pH 6, for 2 h.

The amounts of DNA adduct are expressed as relative adduct level (RAL) = number of adduct/10^9 nucleotides.

RESULTS AND DISCUSSION

Germination and LTB_4 Release. We have induced germination of wheat by putting wheat in water during variable times. We obtained four batches: 1.1 corresponds to the control batch, nongerminated wheat having a falling number of 422; 2.1, wheat put 72 h in water, falling number of 143; 3.1, wheat put 82 h in water, falling number of 132; 4.1, wheat put 96 h in water, falling number of 62. All batches have been split in two parts; one of them was treated by the Oxygreen process and is respectively numbered 1.2, 2.2, 3.2, and 4.2. LTB_4 is involved in cell growth regulation and differentiation. Following oxidative stress, production of LTB_4 could be stimulated. For this reason we first check the impact of the Oxygreen process on LTB_4 production. **Figure 1** shows the effect of the Oxygreen process on leukotriene B_4 released in wheat. The highest amount of LTB_4 was found in the most germinated wheat (batch 4.1). Interestingly, LTB_4 production was induced to the same level as in batch 4.1, when nongerminated wheat (batch 1.1) was treated by Oxygreen (batch 1.2). The Oxygreen process does not modify the LTB_4 concentration in batches 2.2 or 3.2.

The beginning of germination results in the induction of several physiological and biochemical activities, which confer particular characteristics to germinated grain. Notably, lipoxygenases and lipases are activated during the germination process (*18*); lipoxygenase plays a significant role during germination, allowing the specific oxidation of storage lipids and initializing their mobilization as a source of energy and carbon (*19*). Induction of these enzymes, in germinated wheat, results in the increase of the monounsaturated fatty acid content (oleic acid) or of the polyunsaturated fatty acid content (linoleic, linoleic, and arachidonic acids) released following the activity of lipase on triglycerides. Activation of phospholipase leads to the release of arachidonic acid and then leads to an increase in LTB_4 production. These explain why the highest amount of LTB_4 was found in batch 4.1, which corresponded to the most germinated wheat. Induction of LTB_4 production in the control batch treated by ozone (1.2) confirmed that ozone can activate phospholipases present in membrane of cells via the formation of lipid ozonation products (*20*). In addition, during the germination process, proteolytic enzymes are activated (*21*); it results in the release of amino acids from reserve proteins present in the aleurone layer, necessary to embryo development. In germinated wheat (4.1) proteolytic enzymes are released. When this later wheat was treated by ozone (4.2), the LTB_4 is no longer protected and thus is easily degraded by ozone, in our case reflected by the decrease of LTB_4 production.

Impact of Germination on Cell Viability. *MTT Test.* The viability of the cell has been measured by the MMT test, which detected the metabolic activity of the cell (**Figure 2**).

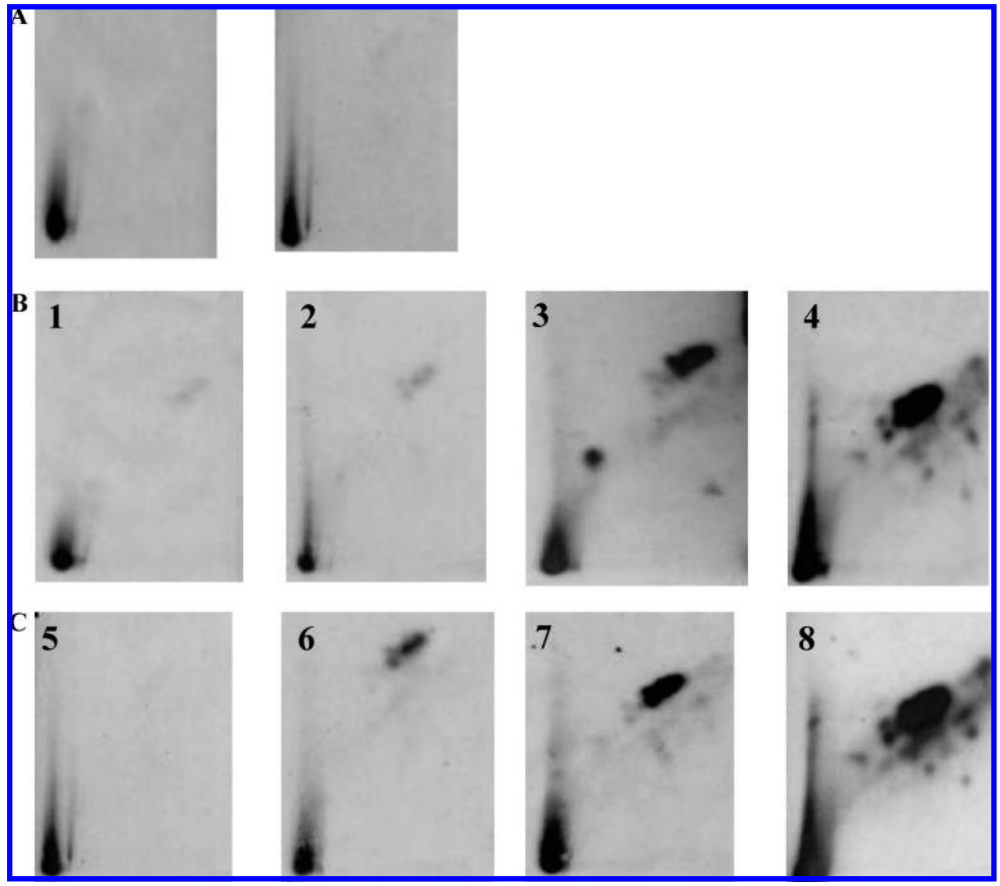

Figure 4. (**A**) DNA adduct pattern of human bronchial epithelial cells exposed to nontreated bread-making wheat extract (left panel, 1.1) or treated by Oxygreen (right panel, 1.2). (**B**) DNA adduct pattern from human bronchial epithelial cells exposed to control bread-making wheat (1) or germinated wheat [2.1 (2), 3.1 (3) 4.1 (4)]. (**C**) DNA adduct pattern from human bronchial epithelial cells exposed to wheat treated by the Oxygreen process: control bread-making wheat (5), 2.2 (6), 3.2 (7), and 4.2 (8).

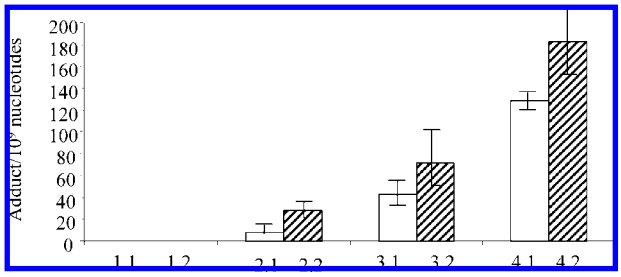

Figure 5. Amount of DNA adduct in human bronchial epithelial cells exposed to bread-making wheat (1) or germinated wheat (2–4) nontreated (white; 1.1, 2.1, 3.1, 4.1) or treated by Oxygreen (hatched; 1.2, 2.2, 3.2, 4.2).

Exposure to high concentration of nongerminated wheat extract (5 g/mL, 2.5 g/mL) decreased the cell viability (**Figure 2A**). This decrease was not observed when the extract was diluted. Treatment of nongerminated wheat (1.1) by the Oxygreen process did not modify the viability of human epithelial bronchial cells, whatever the concentration of extract applied on cells. **Figure 2B** compares the effect of germination state on cell viability. Whatever the wheat extract concentration, germination induced either proliferation (1.25, 0.5, and 0.25 g/mL) or reversed at least partially cytotoxicity, mainly with the concentration of 2.5 g/mL. Oxygreen applied on these germinated wheat modified the viability of cells (**Figure 2C**). The wheat extract obtained after ozonization of germinated wheat decreased the cell viability for the two intermediate concentrations (2.5 and 1.25 g/mL). The more the wheat was germinated, the more intense was the cytotoxic effect.

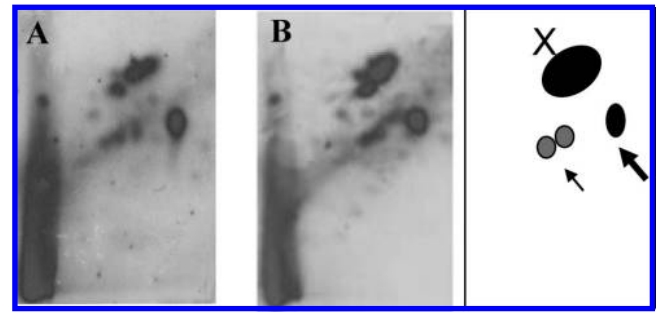

Figure 6. DNA adduct pattern of human epithelial bronchial cells exposed to naturally germinated wheat (left panel, **A**) and treated by Oxygreen (right panel, **B**). Scheme pointing OTHQ-related DNA adducts (faint arrow), C-C8dG OTA (bold arrow), and hydroxyamic acid related DNA adducts (X).

LDH Test. Cell mortality has been evaluated by the LDH test. When cells die, lactate dehydrogenase (LDH) contained in the cytosol is released in the extracellular medium. Thus, increased activity of this enzyme reflected membrane destruction due to death by necrosis. Treatment of nongerminated wheat by the Oxygreen process did not induce any cytotoxic effect whatever the concentration of wheat extract applied on cells (**Figure 3A**). In the same way, induced germination did not induce cell death (**Figure 3B**). Ozone treatment applied to wheat germinated or not induced only a slight cytotoxic effect (around 15%) with the highest concentration of wheat extract (5 g/mL) and with the most germinated (batch 4.2) one for 2.5 g/mL. Comparison of these data with those observed using the MTT

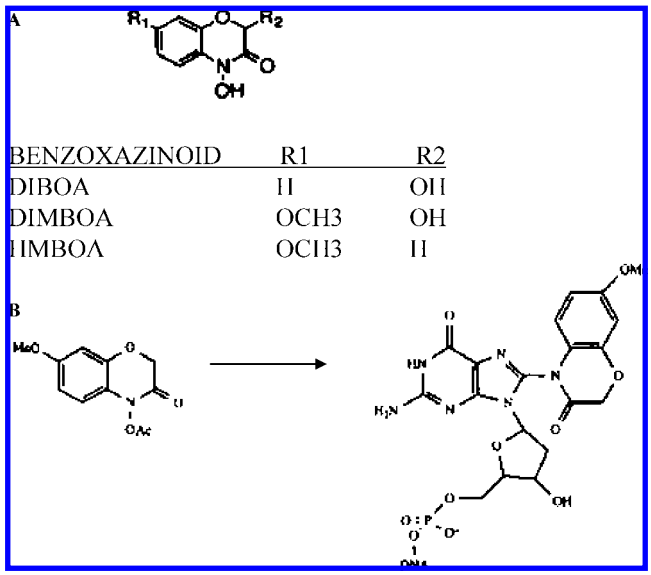

Figure 7. (**A**) Structure of benzoxazinoids. (**B**) AMBOA structure (HMBOA acetylated metabolite) and DNA adduct formation on the C8 of guanine.

test indicated that the decrease of cell viability was not due to necrosis of cells but to another mechanism such apoptosis, which could be modulated by LTB_4 (*22*).

Germination and DNA Adduct Detection. Genotoxicity of wheat extracts, reflected by DNA adduct detection, was tested on human epithelial bronchial cells. The cells were exposed during 24 h to the dose extract of 1.25 g/mL, which corresponded to the concentration for which differential responses with the different stage of germination have been observed on cell viability and on LTB_4 production. No DNA adducts are formed when cells were exposed to nongerminated wheat extract whether treated (1.2) or not (1.1) by the Oxygreen process (**Figure 4A**). In contrast, a smear of DNA adducts was observed when the cells were treated by germinated wheat extract (2.1, 3.1, and 4.1) (**Figure 4B**). The amount of total DNA adducts increased with the germination state of the wheat. Ozonation of germinated wheat (2.2, 3.2, and 4.2) emphasized the formation of these DNA adducts (**Figure 4C**). The amount of DNA adduct formed in cells ranged between 6.9 ± 2 to 183 ± 54.1 adducts/10^9 nucleotides. **Figure 5** compared the total DNA adducts. The increases were respectively 272% (18.8 adducts/10^9 nucleotides), 4.6% (3.1 adducts/10^9 nucleotides), and 42% (54.2 adducts/10^9 nucleotides) for germinated wheat 2.2, 3.2, and 4.2. It should be highlighted that the apparent low increase of DNA adduct between germinated wheat 3.1 and 3.2 is mainly due to a high standard deviation for 3.2. Indeed, the total DNA adduct level was 68 ± 7 for wheat extract 3.1, whereas it was 71.2 ± 45 for wheat extract 3.2, which corresponds to the same stage of germination but was treated by ozone. If we compared the average value including the positive variation, the increase of DNA adduct due to ozone treatment for this sample (3) corresponded to 54.8% (41 adducts/10^9 nucleotides).

Similar DNA adduct patterns (**Figure 6**) were observed when cells were treated by wheat extract from an industrial batch of wheat naturally germinated (falling number 120, collected in France in the year 2000). In addition, this wheat has been shown to contain a small amount of ochratoxin A (OTA) (0.5 μg/kg of wheat), which has induced the formation of three specific adducts of OTA: the C8-dG-OTA (bold arrow) and two specific adducts related to OTHQ (ochratoxin quinone) indicated by a faint arrow on the scheme in **Figure 6** (*23, 24*). Treatment of this wheat by Oxygreen leads to a dramatic increase of the DNA adducts related to germination, called X on the scheme in **Figure 6**. The increase was 100% (163 adducts/10^9 nucleotides versus 338 adducts/10^9 nucleotides). Although the amount of OTA was reduced by two by ozonation, OTA-related DNA adducts increased (75% for C8-dG-OTA, 2 adducts/10^9 nucleotides versus 3.5 adducts/10^9 nucleotides). This could be explained by the fact that adduction of OTA occurred after biotransformation mediated by lipoxygenase (*23, 24*).

It is now well-known that hydroxamic acids, natural antifungic and antimicrobial compounds, are produced by sprouts of Graminae such as wheat (*25, 26*). Indeed, cyclic hydroxamic acids, 2,4-dihydroxy-2*H*-1,4-benzoxazin(4*H*)-ones (DIMBOA) and its demethylated derivative DIBOA (**Figure 7**), are formed at the beginning of germination (*27–29*). DIMBOA can be reduced into the deoxy derivative (HMBOA). This latter derivative is acetylated into AMBOA (*30*), which reacts with DNA to form the DNA adduct on C8 of the guanine [**Figure 7** (*31, 32*)]. Moreover, recently Buchman et al. (*25*) demonstrated the genotoxic properties of these hydroxamic acids on human hepatic cell lines. Thus DNA adducts observed in cells exposed to germinated wheat extracts are due to these hydroxamic acids which have been generated during the germination of wheat. This biotransformation was activated by ozone treatment.

Concluding Remarks. This study shows that the Oxygreen process, applied on nongerminated wheat, does not induce any cytotoxic or genotoxic effect, whereas induced and natural germination of wheat generates toxic compounds. The action of ozone on germinated wheat exacerbates the cytotoxic and genotoxic effect related to the germination state of wheat. This study leads us to consider the importance of quality of the matrix, on which the Oxygreen process will be applied. In the milling industry, germinated wheat is not used, and as a consequence, the Oxygreen process has no reason to be used on them. Further experiments are needed to evaluate the risk of sprout-derived food, generally speaking, taking into account that during food processes these compounds are perhaps not stable.

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