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# Fructose and related phosphate derivatives impose DNA damage and apoptosis in L5178Y mouse lymphoma cells

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**Abstract**

Glycation between reducing sugars and amino groups of long-lived macromolecules results in an array of chemical modifications that may account for several physiological complications. The consequences of the reaction are directly related to the reactivity of the sugars involved, whether aldoses or ketoses, phosphorylated or non-phosphorylated. So far, most studies have been focused on glucose, while fructose, a faster glycating agent, attracted minor attention. We have recently demonstrated that under *in vitro* conditions fructose and its phosphate derivatives can modify plasmid DNA faster than glucose and its phosphate metabolites. In the present study we provide further evidences suggesting that fructose and its phosphate metabolites, at the tested conditions, are cytotoxic and inflict deleterious DNA modifications to L5178Y cells in culture. Damage was verified by viable cell counts, MTT assay, colony forming ability, induction of mutation in the thymidine kinase gene, internucleosomal DNA cleavage, and single strand breaks. The intensity of the tested sugars to impose damage increased significantly in the following order: sucrose = glucose 1-phosphate < glucose < glucose 6-phosphate < fructose 1-phosphate = fructose < fructose 6-phosphate. Aminoguanidine, an inhibitor of the glycation reaction, inhibited internucleosomal DNA cleavage. Taken together, these results suggest that fructose triggers deleterious modification in cultured cells through the glycation process, and thus should deserve more attention as an agent that may induce physiological complications. 

*Keywords:* Fructose; DNA; Glycation; Cytotoxicity; Mutation; Apoptosis

## 1. Introduction

The free carbonyl groups of reducing sugars can react with free amino residues of biological macromolecules in a series of complex chemical processes known as the Maillard reaction (non-enzymatic glycosylation) or glycation [1]. Glycation is initiated by the generation of acid labile Schiff base adducts [2,3] that can undergo subsequent rearrangements into more stable Amadori or Heyns products [4–7]. These early glycation products are considered to be intermediates in the reaction and undergo slow and complex transformations [8] to form irreversible advanced glycation end products (AGEs) [9,10]. AGEs, which include heterogeneous structures of complex modifications, tend to accumulate on long-live biological molecules, and are able to cause a variety of cross-links with high fluorescence characteristics [11,12]. In general, all molecules that have free amino groups, whether these are proteins (both intracellular and extracellular), nucleic acids, low molecular weight amines and certain lipoproteins and lipids are subjected to glycation in vivo. Logically, short-lived molecules, such as proteins in plasma and blood, are expected to be influenced primarily by the early glycation products, while long-lasting molecules like collagen, lens crystalline, myelin and DNA are expected to be altered as they irreversibly accumulate AGEs. Thus, glycation is a type of chemical modification that takes place slowly and continuously throughout the life span [13]. The accumulation of modifications in long-lived macromolecules has been implicated in the pathophysiology of aging [10,14], diabetes [9–12,15,16], and Alzheimer's disease [17,18]. Hitherto, the Amadori products have been detected on many proteins, and AGEs have been found to accumulate over time on a number of long-lived extracellular proteins in vivo [11,19–21]. Beside long-lived proteins

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as targets for glycation, in vitro studies with nucleic acids have demonstrated that the amino groups of DNA bases can also react non-enzymatically with reducing sugars. Incubation of DNA with reducing sugars raised chromophores and fluorophores with similar spectral properties when compared with advanced glycated protein products [22,23]. Several investigations have shown that DNA structure and function are affected after incubation with the intracellular occurring reducing sugars, glucose and glucose 6-phosphate [22,24], and the reactive glycated products formed from glyceraldehyde 3-P and lysine [25]. Lee and Cerami [26] showed that increased intracellular levels of glucose 6-phosphate increased plasmid DNA mutation in *Escherichia coli.*

In principle, all reducing sugars whether aldoses or ketoses [5] and even molecules related to sugars, such as ascorbic acid [27], can initiate glycation in vivo. However, most studies so far were mainly focused on glucose as the reducing sugar. Glucose is assumed to be the major source of glycation based on its abundance and association with diabetic complications [10,15,16]. Glucose however, is the least reactive among sugars [28], and in vitro studies suggested that fructose, as compared with glucose, is a more potent initiator of glycation [28–30]. The non-enzymatic reaction between fructose and amino groups (fructation) has not yet attracted special attention, although fructose has been mentioned in comparative studies including various sugars [28]. The evaluation of a possible role of fructose in modifying DNA in vivo is interesting for the following reasons: a) In some organs, such as ocular lens, kidney, and peripheral nerves, fructose is synthesized from sorbitol through the polyol pathway [31]; b) Although in healthy subjects extracellular concentrations of fructose are lower than that of glucose, its higher reactivity suggest fructose as a strong candidate for fructation in vivo. In diabetic subjects however, fructose may play a greater role since concentration of fructose approach and even exceed that of glucose in ocular lenses [32] and in peripheral nerves [33]; c) 10-20% of the hexose bound to human ocular lens proteins was found to be attached via carbon 2, indicating that the proteins had reacted with endogenous fructose [29]; d) While dietary fructose has some adverse side effects, it is still advocated as a preferred sweetener for diabetic subjects [34]; e) The recent increase in the dietary consumption of fructose [35] might effect the concentration of fructose, and fructose metabolites in blood and tissues; f) The distinct metabolism of fructose in the liver stimulates the formation of several unique metabolites namely fructose 1-phosphate (F1P), dihydroxyacetone phosphate, glyceraldehyde and glyceraldehyde 3-phosphate [36], and it should be mentioned that several sugar phosphates display highly accelerated glycation compared to their non-phosphate analogues [28].

In a previous study [37] we have demonstrated that long-term fructose consumption compared to glucose, elevates plasma fructosamine and glycated hemoglobin levels in blood, and accelerates aging as assessed by several age-related variables in collagen from skin and cortical bones of male rats. Recently, we also showed that fructose and its metabolic phosphate derivatives triggered DNA damage in pBR322, verified by gel electrophoresis and transformation capacity into an *Escherichia coli* host [38].

The aim of the present investigation was to further examine the cytotoxicity and DNA damage induced in L5178Y cells after exposure to fructose and related phosphate derivatives. We demonstrate here that fructose and especially fructose 6-phosphate inflicted cell damage, DNA modifications, apoptosis, and mutation in mouse lymphoma cells.

## 2. Materials and methods

### *2.1. Cell line and growing conditions*

The mouse lymphoma cell line (L5178Y), heterozygous for a null mutation in thymidine kinase (TK-/+), was purchased from the American Type Culture Collection (USA). L5178Y cells are non-adhesive and were cultured at 37 C in humidified 5% $CO_2$.

### *2.2. Culture mediums*

a) Minimal Essential medium, pH 7.7 (MEM)-composed of Fischer's Medium (Sigma Chemical Company, St. Louis, MO) containing 1.0 mmol/L sodium pyruvate (Sigma), 1 g/L fluoronic F-68 (Sigma), 2.5 mg/L amphotericin B (Sigma), 50 mg/L gentamicin sulfate (Sigma), and 0.5% (v/v) heat-inactivated horse serum (Sigma).
b) Growing medium, pH 7.7-based on the MEM, but contains a higher amount [10 vs. 0.5% (v/v)] of heat-inactivated horse serum to promote growths.
c) HAT medium, pH 7.7-based on the growing medium, and in addition contains 5 mmol/L hypoxanthine, 2 mmol/L aminopterin, 0.8 mmol/L thymidine, and 3 $\mu$mol/L glycine.
d) HT medium, pH 7.7-similar to the HAT medium, but lacking aminopterin.
e) Non-selective cloning (NSC) medium, pH 7.7-composed of 40% (v/v) soft agar (Sigma), 26% (v/v) growing medium, 14% (v/v) used sterile growing medium, 10% (v/v) tryptose broth (Sigma), and 10% (v/v) heat-inactivated horse serum.
f) Selective cloning (SC) medium, pH 7.7-based of the NSC medium and contains 0.1 mmol/L of the toxin bromodeoxyuridine (Sigma). Bromodeoxyuridine (BDUr) is a nucleotide analog that can be incorporated into the genomic DNA following phosphorylation by TK. Thus, cells with functional TK will include BDUr in their DNA and perish, whereas the toxin does not affect TK-/- cells, and they will survive and produce countable colonies.

### 2.3. Assessment of viable cells

L5178Y cells were cultured in 10 ml MEM ($10^5$ cells/ml) for 72 h in the presence of various concentrations of the reducing sugars (fructose and glucose) or their related phosphate derivatives namely: fructose 1-phosphate (F1P), fructose 6-phosphate (F6P), glucose 1-phosphate (G1P) and glucose 6-phosphate (G6P), added directly to the medium. To evaluate the influence of osmotic pressure, cells were cultured with sucrose, a non-reducing sugar, while the negative control received no sugar in the medium. Sugars and their phosphate derivatives were purchased from Sigma Chemical Company, St. Louis, MO. Two assays were used to assess viable cells:

a) Trypan blue dye exclusion for direct cell counts. Aliquots (20-100 $\mu$l) of cell suspensions were mixed with equal volume of 0.05% (w/v) Trypan Blue. Viable cells that excluded the dye were counted in a hemocytometer.
b) MTT dye reduction assay. The reduction of the MTT salt (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) to colored formazan was assayed by a method adapted from that of Romije et al. [39]. An aliquot (1000 $\mu$l) of cell suspensions was centrifuged (120 x g) and supernatants were removed. Cells were washed twice with 500 $\mu$l of 0.05 mol/L phosphate buffered saline (PBS) pH 7.7, resuspended in 150 $\mu$l of PBS containing 15 $\mu$g MTT and incubated at 37 C for 4 h. Cells were centrifuged, the supernatant removed, and cells were resuspended in 150 $\mu$l dimethyl sulfoxide. The absorbance of the blue colored formazan was measured within 30 min at 540 nm in a 96-wells plate, using a microplate reader. A standard curve was prepared from untreated (control) cells to confirm linearity between absorbance at 540 nm and cell number.

### 2.4. Selection for TK+/- cells

L5178Y cells demonstrate two independent pathways for utilizing thymidine in DNA synthesis. One operates via the dihydrofolate reductase (normal pathway), and the other uses thymidine kinase (emergency route). When TK+/- cells are cultured in suspension, some tend to spontaneously mutate to give thymidine kinase-deficient cells TK-/-. Therefore, in order to guarantee before each experiment a cell population with low counts of TK-/- spontaneous mutants, cells were cultured, prior to each experiment, in HAT medium with aminopterin that inhibits dihydrofolate reductase, thus killing TK-/- cells. Following 48 hr in the HAT medium, the surviving TK+/- cells were rinsed with PBS to eliminate the residual influence of aminopterin, and allowed to recover for 72 h in HT medium.

### 2.5. Selection for TK-/- mutant cells

TK+/- cells were cultured for 48-72 h in 10 ml of MEM ($1x10^5$ cells/ml) in the presence of fructose or glucose and their related phosphate derivatives added directly to the culture medium. To evaluate the influence of osmotic pressure, cells were cultured with sucrose, a non-reducing sugar, under identical conditions. A positive control was cultured for 2 h in the presence of a known mutagen, ethyl methanesulfonate (EMS), at 0.25 and 0.50 mmol/L. The negative control received no sugar in the medium. Cells were then harvested by centrifugation (120 x g), washed twice with 10 ml of PBS, resuspended in 10 ml of growing medium, and allowed to recover for 24 h and express their genetic lesions (expression time). Following the expression period, cells were collected by centrifugation and resuspended in 1 ml NSC medium. An aliquot (100 $\mu$l) was serially diluted up to 10000 and cells were cultured in suspension for 14 days in NSC medium, to estimate their ability to form viable colonies. The remaining cells (2x450 $\mu$l) were cultured in suspension for 14 days in SC medium (containing BDUr) to determine the number of TK-/- mutant cells. Induced mutation frequencies were calculated as described by Clive & Spector [40].

### 2.6. Determination of internucleosomal DNA cleavage

L5178Y cells were placed in 10 ml of MEM ($8x10^5$ cells/ml) and exposed for up to 96 h to various concentrations of reducing sugars or related phosphate derivatives added directly to the medium. Incubation for 12-24 h with anisomycin (5 mmol/L) was used as a positive apoptosis inducer [41], while in the negative controls no reducing sugars were added to the medium. Internucleosomal DNA cleavage was visualized by the characteristic DNA apoptotic laddering in gel electrophoresis [42]. Genomic DNA from $10^5$ cells was prepared according to Zhu and Wang [43], without the use of phenol/chloroform extraction to avoid partial DNA degradation, and involves cell lysis with sarkosyl (Sigma), proteinase K (Sigma) digestion, and RNAase A (Sigma) treatment. The amount of DNA in each preparation was quantified as previously described [38] following a short run (25 min) on an agarose (2%) mini gel. Quantified DNA preparations were subjected to electrophoresis in a 2% agarose gel and the characteristic apoptotic ladder was visualized following staining with ethidium bromide (1.0 mg/L) and photographing under UV light. In order to confirm the participation of the glycation reaction in DNA cleavage, 2 mmol/L aminoguanidine (Sigma), an inhibitor of the glycation reaction, was added to the incubation mediums containing 110 mmol/L fructose or F1P.

### 2.7. Determination of single strand breaks of nuclear DNA

The principle of the single cell gel electrophoresis assay (SCGE), or the comet assay, is based upon the ability of

denatured cleaved DNA fragments to migrate out of the cell under the influence of an electric potential, whereas undamaged super-coiled DNA remains with the confines of the cell membrane when a current is applied. Cells were immobilized in a low melting point agarose followed by gentle cell lysis, electrophoresis, staining with a fluorescent dye (SYBR green), and visualizing under a fluorescence microscope. The SCGE assay was conducted following the instructions of a commercial kit (R&D Systems, kit TA-800).

## 3. Results

### *3.1. Cell viability*

The influence of the tested sugars and their related phosphate derivatives on L5178Y cells viability was estimated by direct cell counts (Figure 1), and the MTT assay (Figure 2), respectively. The most severe effects on cell survival were observed in L5178Y cells cultured in the presence of F6P. Increasing amounts of F6P resulted in a logarithmic decline of viable cell counts (Figure 1F). At levels of 33, 50, 66, and 132 mmol/L, F6P suppressed cell counts by a factor of 3, 5.5, 12.5 and 200, respectively compare to the control. Fructose and F1P decreased viable counts by a factor 2 and 10 compare to the control at levels of 110 and 165 mmol/L, respectively (Figure 1B and 1D). Glucose (Figure 1A) and G6P (Figure 1E), were less potent than fructose and G1P, and at 165 mmol/L, decreased cell counts to 50% of the control. G1P (Figure 1C) and sucrose (Figure 1G), both non-reducing sugars, showed no harmful signs in L5178Y cells at levels up to 165 mmol/L. However, at higher concentrations they strongly decreased cell counts probably as a result of the increased osmotic pressure.

The ability of L5178Y cells to reduce MTT to blue formazan was used for the spectrophotometric determination of viable cells, and is presented in Figure 2. Figure 2A shows a linear relationship between cell number and absorbance (540 nm) over the range $1x10^4$ to $10x10^4$ cells per assay. Among the tested carbohydrates, F6P showed a pronounced negative effect on the ability of L5178Y cell to reduce MTT. MTT reduction was completely diminished as F6P levels reached 100 mmol/L (Figure 2G). At 165 mmol/L fructose and F1P (Figure 2C and 2E) lowered dye reduction to 80% of the control, while glucose and G6P (Figure 2B and 2F), at the same level, caused a decrease of 50%. G1P (Figure 2D) at 165 mmol/L showed only a minor effect on dye reduction. Increasing the concentration of the tested sugars to 280 mmol/L completely diminished dye reduction probably due to the influence of the increased osmotic pressure.

### *3.2. Colony-forming ability*

The ability of L5178Y cells to recover, following treatment with reducing sugars for 72 h, was estimated by counting the number of colonies formed when treated cells were allowed to re-culture in suspension in the NSC medium containing agar (Figure 3). L5178Y cells treated either with glucose, sucrose or G1P at levels up to 165 mmol/L (Figure 3A, 3C and 3G) demonstrated only a minor decrease in their ability to form colonies. In contrast, cells recovery was considerably reduced following the treatment with fructose or F1P at 55, 110 and 165 mmol/L (Figure 3B and 3D). Treatment with G6P up to 130 mmol/L resulted in a minor decrease in cell recovery, while increasing the level to 165 mmol/L caused a drastically reduction in the colony-forming ability. F6P caused a severe damage to L5178Y cells demonstrating only a residual ability to form colonies (Figure 3F).

### *3.3. Mutant frequency in the TK gene*

The mutant frequency induced in the TK gene of L5178Y cells following the treatment with reducing sugars, their related phosphate derivatives, and a known mutagen (EMS) are presented in Figure 4. It is apparent that glucose, G1P and G6P are less potent in causing mutation to L5178Y cells compared to fructose and its related phosphate derivatives. Glucose doubled mutation frequency only when L5178Y cells were treated at the highest tested level (165 mmol/L). No signs of induced mutation were observed following the treatment with G1P (110 and 165 mmol/L) and G6P (65 and 100 mmol/L). In contrast, treatment with fructose and F1P (110 and 165 mmol/L) increased mutation frequency by 2.2-4 times. Furthermore, among the tested carbohydrates, F6P is clearly the most potent sugar. Treatment with F6P at levels of 65 and 110 mmol/L for 48 h increased the mutant frequency of TK in L5178Y cells by 6 and 11 times, respectively. At this point it should be mentioned that these results are far below those obtained in the positive control following treatment with EMS for 2 hr. EMS at 0.25 mmol/L inflicted a 20-fold increase in TK mutant frequency, and 50-fold at level of 0.50 mmol/L (data not shown).

### *3.4. Assessment of internucleosomal DNA cleavage in L5178Y cells*

The ability of reducing sugars and their related phosphate derivatives to induce internucleosomal DNA cleavage in L5178Y cells was visualized by the characteristic DNA apoptotic laddering (Figure 5). DNA from the control cells did not show any signs of laddering (lane 1). To negate the possibility that sugar-induced osmotic pressure caused the observed apoptotic signs, we tested the influence of sucrose, a non-reducing sugar, on the DNA of L5178Y cells. Exposure to sucrose (165 mmol/L, 96 h) caused no signs of laddering (lane 2). Anisomycin, serving as an agent known to induce apoptosis, caused the characteristic DNA laddering pattern following a 12 h incubation period (lane 12). Glucose (110 mmol/L) showed only minute signs of DNA

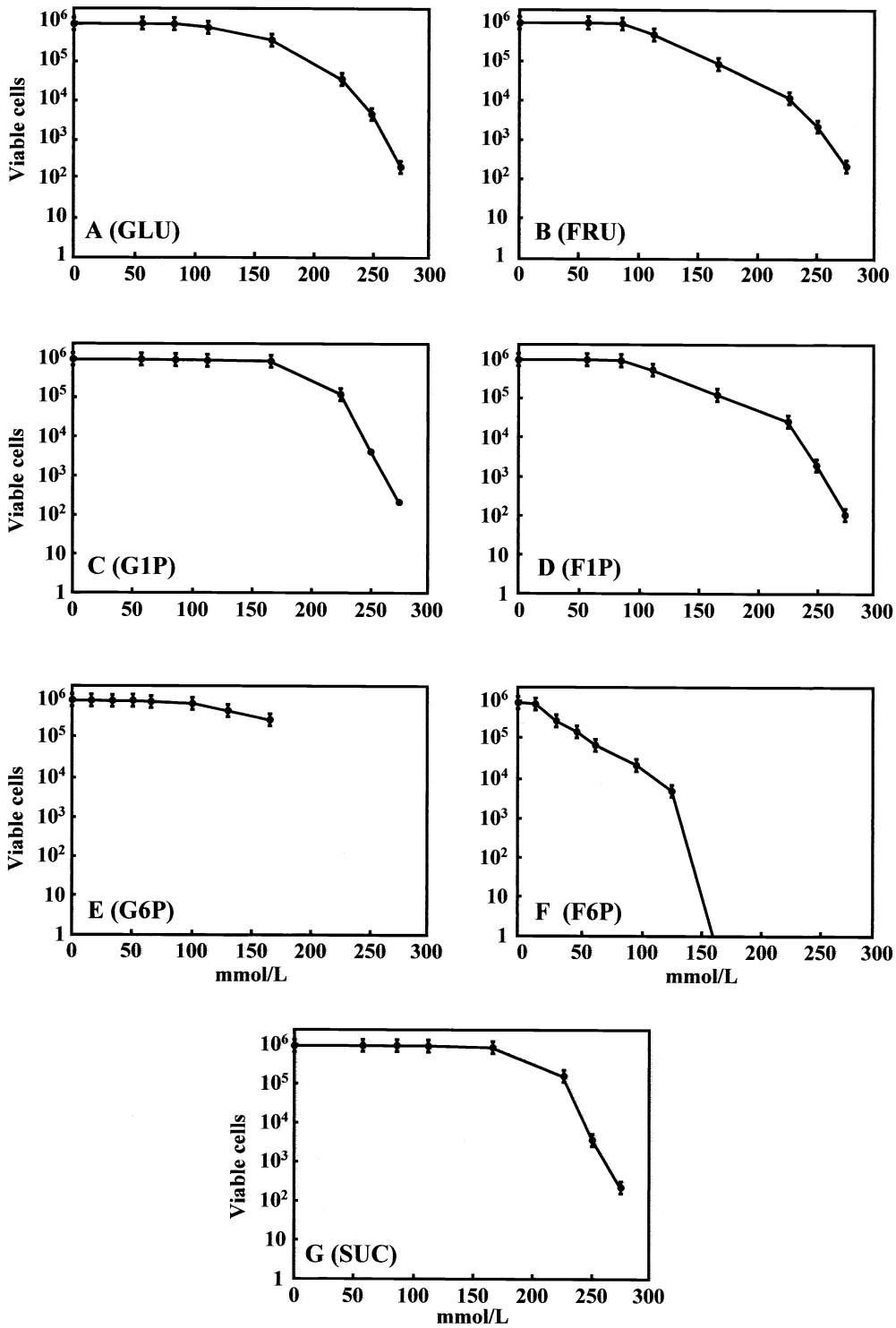


Fig. 1. The influence of glucose (GLU), glucose 1-phosphate (G1P), glucose 6-phosphate (G6P), fructose (FRU), fructose 1-phosphate (F1P), fructose 6-phosphate (F6P) and sucrose (SUC) on the survival of L5178Y cells following incubation in minimal growing medium (0.5% horse serum) for 72 h. Viable cells were counted using a hemocytometer following staining with Trypan Blue. Values are means + SD of three experiments for each carbohydrate, and are plotted on a semi- logarithmic scale.

laddering after treatment for 96 h, (lane 3), while exposure to fructose or F1P (110 mmol/L, 96 h) resulted in eminent DNA laddering (lanes 4 and 5, respectively). Adding aminoguanidine (2 mmol/L) to the incubation medium suppressed internucleosomal DNA cleavage (lanes 6 and 7). G1P and G6P at 110 mmol/L had no effect on DNA pattern (data not shown). Among the tested sugars, F6P was the most effective in inducing internucleosomal DNA cleavage.

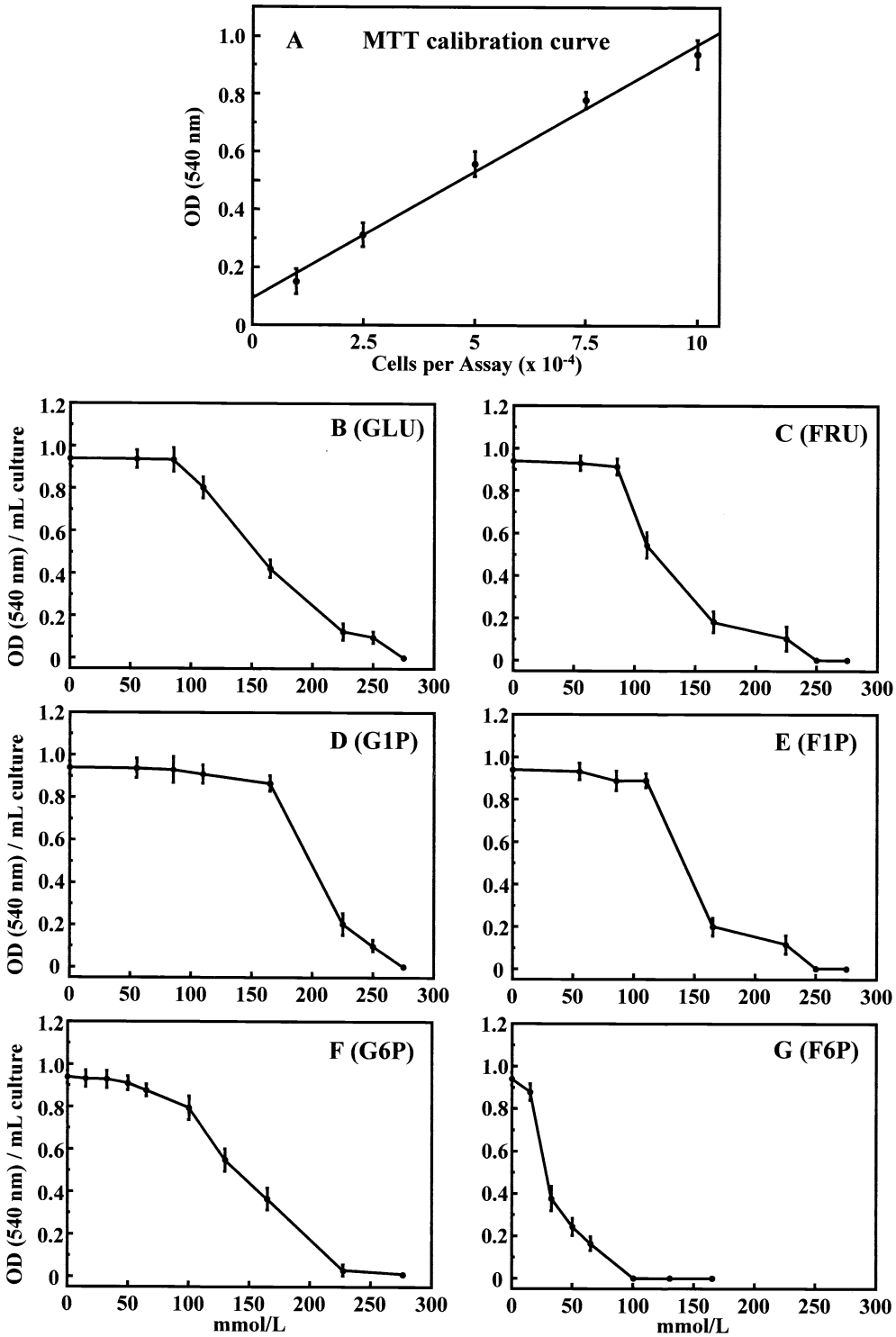


Fig. 2. MTT assay of L5178Y cells following incubation in minimal growing medium (0.5% horse serum) for 72 h with various amounts of either glucose (GLU), glucose 1-phosphate (G1P), glucose 6-phosphate (G6P), fructose (FRU), fructose 1-phosphate (F1P) or fructose 6-phosphate (F6P). Values are means + SD of three experiments for each carbohydrate.

F6P treatment for 36 h resulted in a ladder pattern in a dose-dependent manner (lanes 8-11). Furthermore, treating cells with F6P at 66 mmol/L for 72 h completely degraded the high-molecular DNA (data not shown).

### *3.5. DNA single strand breaks*

Single strand brakes (SSB) of nuclear DNA was also evaluated by SCGE (Figure 6), and is indicated by the

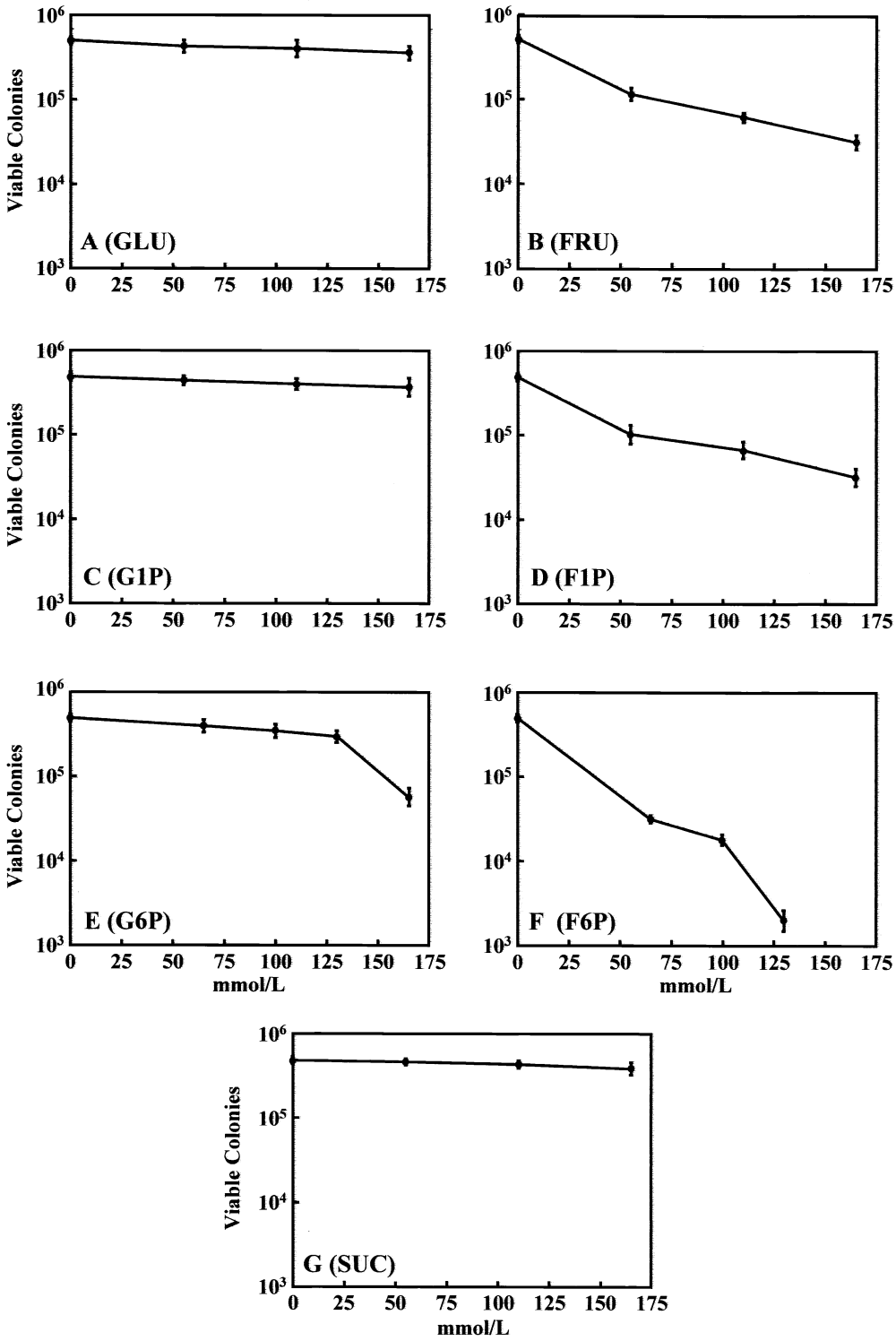


Fig. 3. Colony-forming ability of L5178Y cells treated with glucose (GLU), glucose 1-phosphate (G1P), glucose 6-phosphate (G6P), fructose (FRU), fructose 1-phosphate (F1P), fructose 6-phosphate (F6P), or sucrose (SUC). Cells were cultured in minimal essential medium (0.5% heat-inactivated horse serum) containing the respected carbohydrates for 72 h (48 h for F6P), recovered and re-cultured in non-specific cloning medium (10% heat-inactivated horse serum) for 14 days. Values are means + SD of three experiments for each carbohydrate.

shape, decreased staining density, and increase total migration length and area of the fluorescent-stained DNA. SYBR-stained DNA appeared as a bright mass in the fluorescent microscope system. DNA from the control and glucose-treated cells was primarily visualized as a round area that did not migrate in the gel to any great extant (Figure 6a and

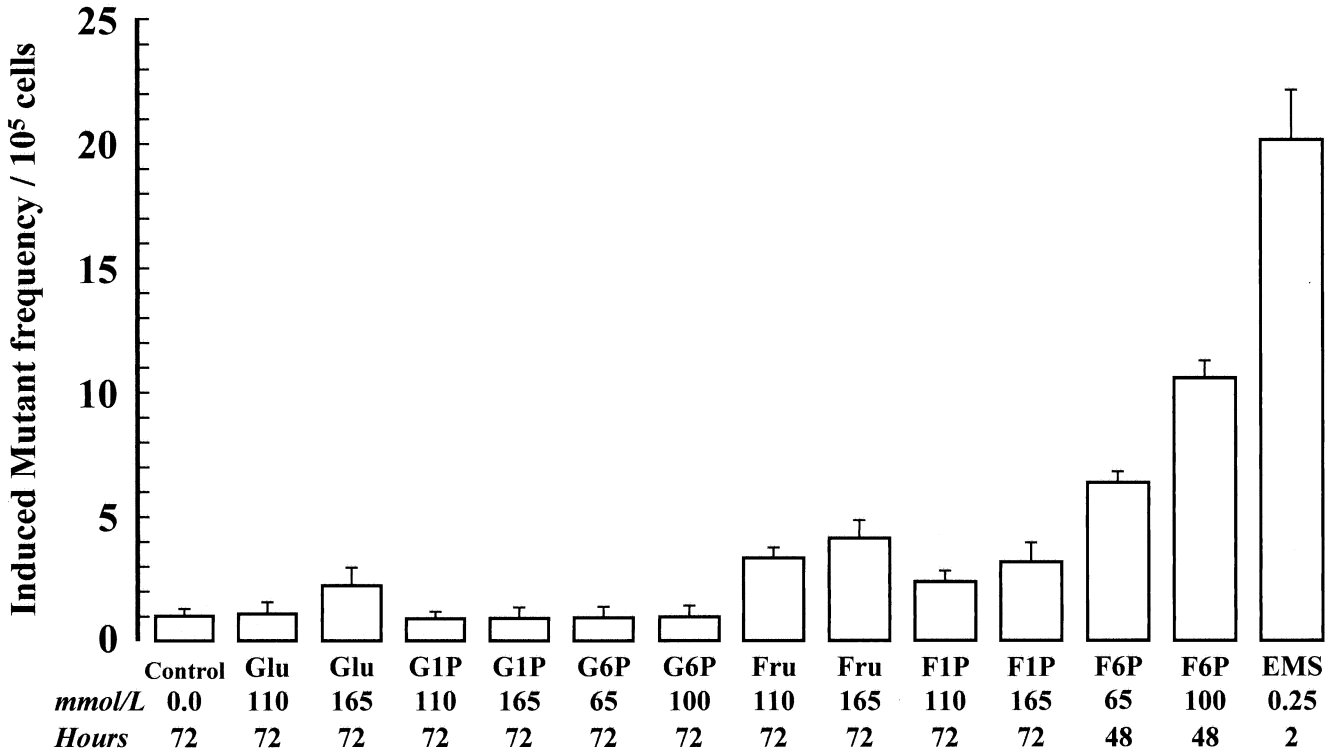


Fig. 4. Induced mutation frequencies in the thymidine kinase gene of L5178Y cells due to the treatment with either glucose (Glu), glucose 1-phosphate (G1P), glucose 6-phosphate (G6P), fructose (Fru), fructose 1-phosphate (F1P), fructose 6-phosphate (F6P), or the mutagen ethyl methanesulfonate (EMS) in minimal growing medium (0.5% horse serum). Results for each carbohydrate and EMS were related to the control that received an arbitrary value of one unit. Values are means + SD of three experiments for each carbohydrate.

6b). In contrast to the control and glucose-treated DNA, DNA treated with fructose (110 mmol/L, 96 h) presented a moderate tail in most cells (Figure 6c). Most striking was the treatment with 100 mmol/L F6P for 36 h. DNA damage was seen in most cells as a bright area accompanied by a comet-like tails that migrated extensively in the gel (Figure 6d). The comet tail was due to the increased migration of DNA fragments to the anode.

## 4. Discussion

We have previously demonstrated that fructose consumption accelerates glycation and age-related parameters in rats [37], and when incubated with PBR322, *in vitro*, fructose induced DNA modifications and damage [38]. The purpose of the present study was to further explore the damage occurred to mammalian cells, in culture, following their exposure to fructose and its phosphate derivatives. Cell damage was determined by cell viability, colony forming ability, induced mutagenesis, apoptosis, and the occurrence of SSB. We observed that the magnitude of the damage is directly correlated with the reactivity and concentration of the tested reducing sugars. In the present study we have shown that fructose and its phosphate derivatives, F1P and F6P, are more potent agents than glucose, G1P and G6P in inflicting damage to cultured L5178Y cells. The extent of damage observed with F6P compared to fructose, is in accordance with observations demonstrating that several sugar phosphates react more readily than their non-phosphate analogs [25,28,38,44]. The fact that G1P showed a low reactivity is probably due to the loss of its reducing capacity after substitution the active aldehyde residue on carbon 1 with a non-reducing phosphate group. In contrast, F1P inflicted approximately the same damage as fructose, since the reactive carbonyl group is present on carbon 2, and F1P retains its reducing power even after phosphorylation on carbon 1.

The degree of damage observed with fructose compared to glucose is consistent with the results of previous in vitro studies suggesting that fructose is a more potent initiator of glycation [28–30,38]. The differences between the reactivity of these two sugars are due to the extent at which they exist in their open chain free aldehyde form. Although the bulk of both sugars in culture are present as the cyclic (pyranose) structure, the open ring aldehyde form represents 0.7% of the total fructose, but only 0.002% of the total glucose [28]. Because of this 350-fold difference in the level of the free aldehyde form, fructose can initiate glycation in a higher rate than glucose. When in vitro glycation of proteins is involved, fructose is 7.5 to 10-fold faster then glucose [29,30]. This is probably due to the highly reactive

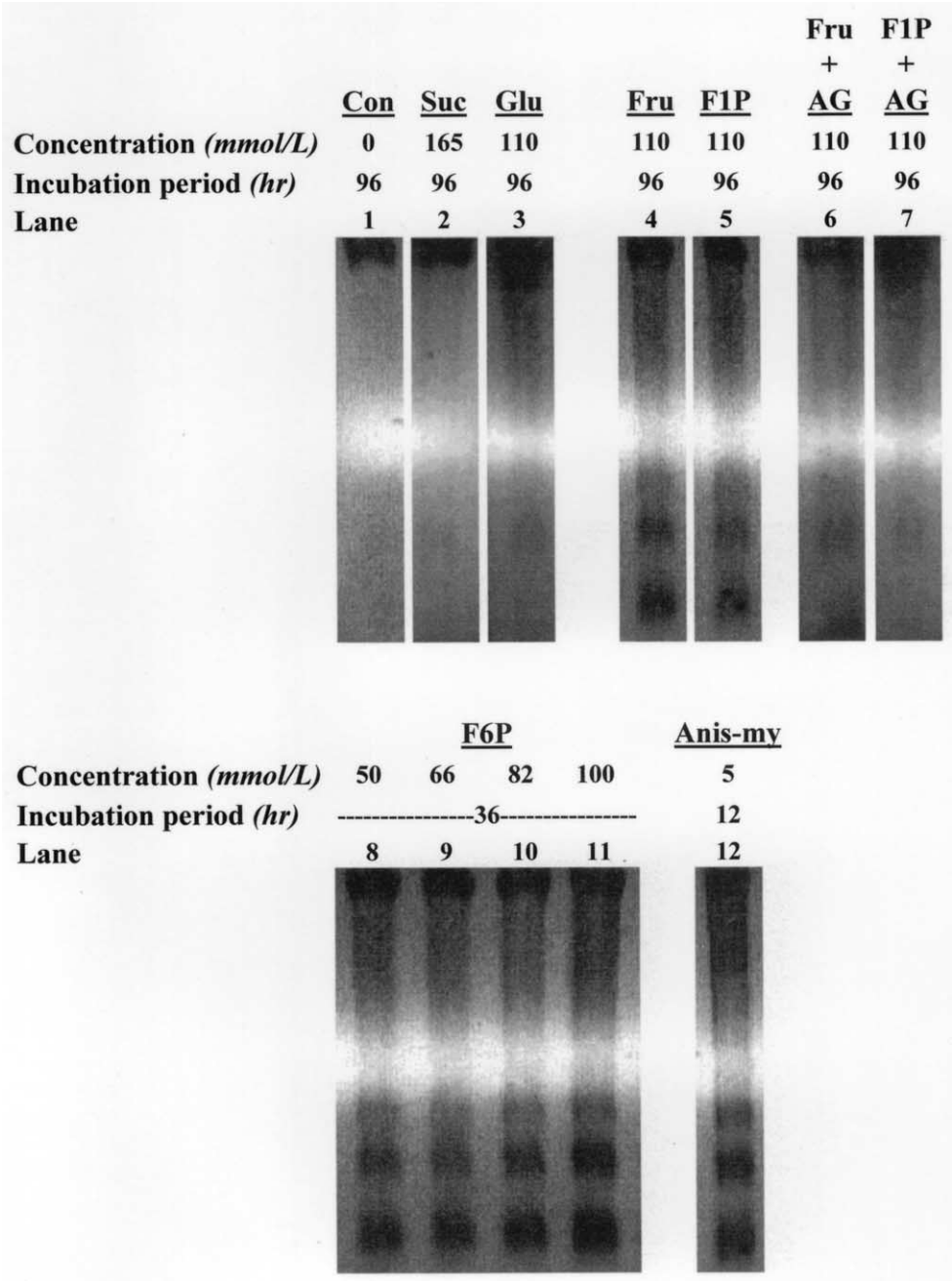


Fig. 5. Internucleosomal DNA fragmentation in L5178Y cells exposed to glucose (Glu), sucrose (Suc), fructose (Fru), fructose 1-phosphate (F1P), and fructose 6-phosphate (F6P) in minimal Fischer medium (0.5% horse serum). Fructose and F1P (110 mmol/L) were also incubated in the presence of 2 mmol/L aminoguanidine (AG). No carbohydrates were added to the negative control (con), while anisomycin (Anis-my) served as a known apoptotic inducer. Cells were lysed, and DNA extracted as described in "Material and Methods". The DNA underwent electrophoresis in a 2% agarose gel containing 1.0 mg/L ethidium bromide.

Heyns compounds obtained from ketones which are converted faster to advanced glycated products compared to Amadori products of aldoses [30]. In addition, it should be pointed out that beside glycation, oxidation processes could not be excluded as responsible for the observed cytotoxicity and DNA damage following the treatment with fructose and its related metabolites. The term glycoxidation, or autoxidative glycation, is frequently used to emphasize the intimately involvement of oxidation in glycation processes. It is well recognized that apart from glycation, reducing monosaccarides are also prone to autoxidation [2,23]. The initial step of sugar autoxidation involves enolization of $\alpha$ hydroxy aldehyde, and this process should also proceed more rapidly with fructose than with glucose [45]. The generated ketoaldehydes are highly reactive [46] and may further attack free amino groups of macromolecules to yield additional Amadori or Heyns products. In addition, Amadori or Heyns products can also enolize and thereby reduce

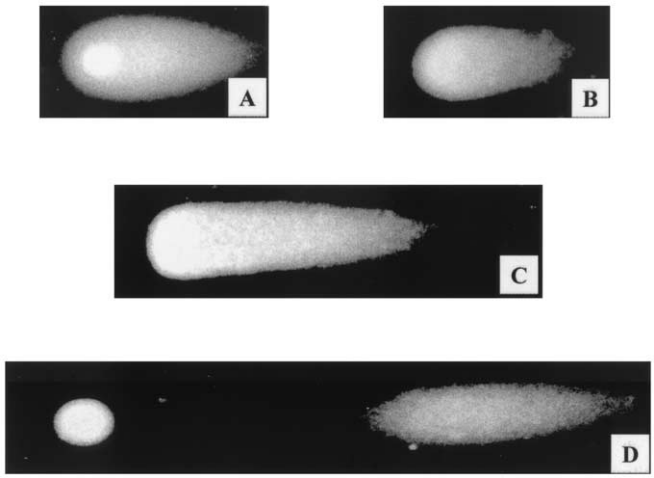


Fig. 6. Photographic images (200X) of fluorescent-stained DNA in an agarose gel. L5178Y cells were treated with or without reducing sugars in minimal Fischer medium (0.5% horse serum). Cells were sandwiched between layers of agarose, lysed and subjected to electrophoresis. Cells were then stained with a fluorescent dye (SYBR green) and examined by fluorescence microscopy. (A) Control; (B) 110 mmol/L glucose, 96 h; (C) 110 mmol/L fructose, 96 h; (D) 100 mmol/L fructose 6-phosphate, 36 h.

molecular oxygen, yielding oxygen radicals and dicarbonyl compounds. Autoxidation of free sugars, Amadori or Heyns products, and dicarbonyl compounds is an oxygen-dependent process catalyzed by transition metals such as iron and copper. Several studies showed that superoxide free radicals are generated during autoxidation of reducing sugars and Amadori products [46–48]. However, there are supporting evidences to suspect hydroxyl radical-mediated attack [49–51]. Thus far, it is not obvious which steps of oxygen radical generation are more important, but several studies showed that the induced-damage was prevented when antioxidants and glycation inhibitors were used [52,53]. Although hydroxyl radicals are powerful oxidants, their short life span suggests an immediate site-specific attack [54], following their generation by the Fenton reaction in the proximity of a transition metal-bound target macromolecule, to generate a profound damage. An involvement of glycation in sugar-induced apoptosis is supported by the inhibitory effect of aminoguanidine, an inhibitor of the glycation reaction [55]. However, it has been reported that aminoguanidine has antioxidant properties in addition to its preventing role in glycation [56,57]. Thus, we could not rule out the possibility that aminoguanidine had effects other than as an inhibitor of glycation in the present study.

Considering the inflicted damage, the present study, as well as our previous one [38], clearly demonstrate that the intensity of the tested reducing sugars to cause injure is in the following order: sucrose= G1P < glucose < G6P < F1P = fructose < F6P. Sucrose and G1P are non-reducing sugars and can be use to estimate the ability of the tested cells to tolerate osmotic pressure.

At this point it should be emphasized that reflecting our observations in a mammalian cell line into physiological conditions of the mammalian organism must be done with great caution. Cell damage, mutation, apoptosis, and SSB were hardly detected in L5178Y cells following treatment with glucose (up to 110 mmol/L). However, under diabetic conditions, various body cells exposed to elevated levels of glucose for prolong periods, may exhibit functional deterioration. Glucose is converted into fructose by the polyol pathway, and the fructose level increases in several organs under diabetic conditions [32,33] and may reach 15 mmol/L in lens [32]. Fructose, produced in tissues demonstrating reactive polyol pathway, may be circulated and transported into other organs via the glucose transporter GLUT2 [58], and inflicts its deleterious effects. In healthy subjects however, the actual concentration of fructose in vivo is low when no fructose is being consumed, and levels of 2.2 mmol/L have been recorded soon after a fructose meal [59]. These levels are not as high as those used in our experiment, but we assume that the functionality of cells exposed to low levels of fructose may deteriorate after a long duration. Yet, no attempt was made to investigate the influence of increasing dietary fructose consumption on the concentration of its reactive metabolites in tissues, which could in turn potentiate glycation. Glyceraldehyde and glyceraldehyde 3-phosphate, among the major metabolites of fructose, are most reactive glycation agents acting several times faster than equimolar amounts of glucose or fructose [28,60]. Glycation products of glyceraldehyde 3-phosphate and lysine, in vitro, produced DNA modification and apurinic/apyrimidinic sites in double-stranded DNA in addition to SSB [25]. Lal et al. [61] showed that fructose-3-phosphate, a novel phosphomonoester, is produced in the diabetic lens. This metabolite is a precursor of 3-deoxyglucosone, a potent glycating agent. Thus, the rational of studying the influence of sugars in levels beyond their cellular physiological concentration was to minimize the time necessary to achieve a measurable effect. It is therefore suggested that in vivo glycation (fructation) in mammalian tissues is a possible event that may negatively affect cell viability and DNA integrity.

In conclusion, we have demonstrated that fructose, F1P, and F6P inflicted damage to mammalian cells in culture probably by inducing glycoxidation. The increase usage of fructose by the food industry and still being recommended to diabetic subjects raise serious dietary as well as health controversies. Studies aimed to further elucidate the negative role of fructose both in health and under diabetic conditions are under investigation.

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