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Gerontomodulatory and Youth-Preserving Effects of Zeatin on Human Skin Fibroblasts Undergoing Aging *In Vitro*

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ABSTRACT

Our studies have shown that zeatin, (6-[4-hydroxy-3-methyl-but-2-enylamino]adenine), a cytokinin plant growth factor, has gerontomodulatory, youth preserving and anti-aging effects on serially passaged human adult skin fibroblasts undergoing aging *in vitro*. There were no immediate negative or toxic effects in terms of cell attachment, cell proliferation, cell survival, cytoskeletal organization, and cellular growth by treatment with zeatin concentrations between 1 and 200 μM. During long-term treatment, cells could be maintained throughout their replicative lifespan in the presence of 40, 80, and 200 μM zeatin, but the optimal concentration of zeatin's anti-aging and youth preserving effects was found to be 80 μM. Life-long serial passaging of human skin fibroblasts in the presence of zeatin resulted in the prevention of cell enlargement, reduction of intracellular debris, prevention of actin polymerization, and enhancement of cellular ability to decompose hydrogen peroxide and to cope with ethanol and oxidative stresses. Most importantly, anti-aging and beneficial effects of zeatin were observed without any induction of additional cell proliferation or an increase in the maximum proliferative capacity, thus ruling out any potentially harmful and carcinogenic effects.

INTRODUCTION

THE HAYFLICK SYSTEM of serially passaged normal diploid cells undergoing cellular aging *in vitro* is a well established experimental model system used extensively in biogerontological and anti-aging rejuvenation research.[1] Some recent examples of screening of such compounds and their successful development into various cosmeceutical and neutriceutical products include β-alanyl-L-histidine dipeptide (carnosine),[2,3] and N^6-furfuryladenine or kinetin.[4] Of these, the anti-aging and beneficial effects of kinetin on human skin fibroblasts[4] and on fruitflies were originally reported by us.[5,6] These studies were followed by our detailed analysis of the molecular pathways through which kinetin appears to bring about its biological effects. For example, we had shown that kinetin prevents and reduces the Fenton-reaction mediated oxidative damage to DNA[7] and inhibits the glycoxidation-mediated damage to proteins.[8] There is some evidence that kinetin makes a complex with Cu,Zn-superoxide dismutase (SOD) and can indirectly enhance catalase and glutathione peroxidase activities in order to counteract hydrogen peroxide (H_2O_2) stress.[9,10]

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Since kinetin is a member of the cytokinin group of plant growth factors,[10,11] it is important to find out whether the anti-aging and antioxidative effects of kinetin can be observed for other cytokinins also. Out of several natural and synthetic cytokinins, 6-[4-hydroxy-3-methyl-but-2-enylamino] adenine, commonly known as zeatin, is one of the most widely studied cytokinins with respect to its growth modulatory and anti-senescence effects in plants.[11] Although kinetin was the first cytokinin to be isolated in 1955 from the autoclaved samples of Sperm Whale DNA,[12] it was considered to be a thermally rearranged product, until our demonstration of the natural occurrence of kinetin in DNA and cell extracts.[13] Zeatin, on the other hand, was purified from immature kernels of the corn, *Zea mays*,[14] and was later identified to be present in the tRNA of a wide variety of organisms.[11,15] Figure 1 shows the chemical structure of zeatin, which occurs in two forms, *trans* and *cis*, but the cytokinin activity of zeatin is mainly attributed to its more stable *trans* form.[11,16] There is a large body of information available regarding the biosynthesis, activity, and degradation of zeatin and zeatin riboside in bacteria, fungi and higher plants.[17–21] However, almost nothing is known about the effects of zeatin on animal and human systems.

Here we report the results of our studies on the short-term and long-term treatment of human skin fibroblasts with zeatin, which show that, as in the case of kinetin, zeatin also has several beneficial and youth-preserving effects on human cells undergoing aging *in vitro.*

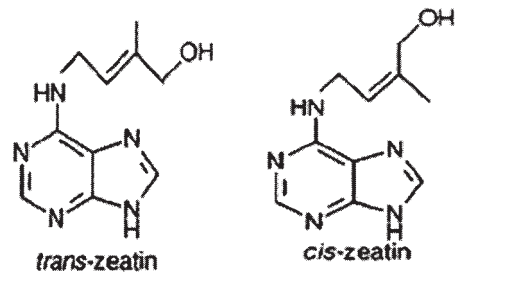

FIG. 1. Chemical structures of *trans* and *cis* zeatin.

MATERIALS AND METHODS

Cell culture and serial passaging

Primary cultures of normal diploid adult skin fibroblasts, designated ASF-2, were established from mammary skin biopsies obtained from a healthy young female donor as described before.[22–24] Cells were grown in T_{25} plastic flasks (growth area 25 cm^2, Cellstar, Germany, and Sarstedt Inc, USA) in an incubator at 37°C, 5% CO_2, atmospheric oxygen conditions, and 95% humidity, in Dulbecco's minimum essential medium (DMEM; Bio Whittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (Biological Industries, Beit Haemek, Israel), 400 μM glutamine and 100 U/mL penicillin/streptomycin (BioWhittaker). This final medium is referred to as the "complete medium." In the near-confluent state, cells were trypsinized and subcultured at a 1:4 or 1:2 split ratio repeatedly until the end of their proliferative capacity *in vitro*. Full records of the input and output of number of cells was kept by counting the cell numbers using a Coulter counter. Cumulative population doubling level (CPDL) achieved after serial passaging was determined in accordance with the well-established protocols for the Hayflick system of replicative senescence.[22–24] Frequent checks for mycoplasma (tested by fluorescent DNA staining by Hoechst 33258) showed that the cultures were free from mycoplasma infection throughout their replicative lifespan.

Zeatin treatment

Trans-zeatin (6-[hydroxy-3-methyl-but-2-enylamino] adenine), molecular weight 219.25, 99.5% purity, was purchased from OleChemim Ltd (Czech Republic). Since zeatin is only slightly soluble in water, a stock solution (8 mM) was prepared by dissolving zeatin in a minimum amount of 1N HCl (at about 30 mg zeatin per mL 1N HCl), followed by its appropriate dilution in Hanks' buffer, and filter sterlization through 0.2-μm sterile filters. The stock solution was stored in fridge at 4°C, and was used for experiments by diluting it in the complete medium. Similarly, 8 mM stock solution of zeatin riboside ([9-(β-D-Ribofuranosyl]-trans-zeatin; molecular weight 351.4, purchased from

Sigma Aldrich) was prepared. Pilot tests were performed to rule out any effects of the solvent HCl by testing its maximum equivalent concentrations (0.4% in 8 mM stock solution) on the attachment, survival and growth of ASF-2 cells. No biological effects of HCl could be detected, and therefore, no further controls with HCl were included in experiments on zeatin and zeatin riboside within the test concentrations (between 1 and 500 μM).

Cell attachment, survival, and short-term growth

In order to rule out any immediate negative effects of zeatin treatment, the number of cells attached to the growth surface of cell culture flasks was determined. An equal number of cells (about 10,000 cells per cm^2 of growth area) were seeded in multiple wells in 24-well tissue culture plates (growth area 1.9 cm^2) with or without different concentrations of the test compound. After 6 h, the number of attached cells were counted in treated and untreated cells after trypsinisation and cell counting as described above.

Cell survival was measured by the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. About 16,000 cells were seeded per well in a 96-well plate 24 h before the experiment. Cells were then treated with different concentrations of zeatin or zeatin riboside for 3 days, after which MTT (Sigma, M2128) was added at 0.5 mg/mL in medium. After 4 h, MTT was removed and isopropanol and HCl were added to dissolve the MTT crystals for 12–16 h. The absorbance at 595 nm was measured using a spectrophotometer. Data for "3-day cell survival" was statistically analyzed by Student's *t*-test.

Short-term growth experiments were performed using freshly prepared cell suspensions from mass cultures of early passage ASF-2 cells. About 10,000 cells were seeded into six sets of 24-well plates and treated with various concentrations of zeatin. The number of cells were counted after different days of treatment in two wells by following the normal method of cell trypsinization and counting using a Coulter counter. The third well in each test category was fixed in cold methanol and stained with Giemsa stain for permanent record and for photography. The experiment was carried on until the cultures became fully confluent and no further growth of cells was possible.

Long-term serial passaging of ASF-2 cells was performed in at least three parallel cultures in T_{25} flasks with or without the addition of 40, 80, and 200 μM *trans*-zeatin until the end of the replicative lifespan. Cultures were considered to be irreversibly senescent after complete cessation of cell growth during a period of more than 1 month after last subculturing and showing more than 95% cells with senescence-specific β-galactosidase staining.[26] During serial passaging, fresh complete medium with or without zeatin was replaced on twice a week basis throughout the replicative lifespan of ASF-2 cells.

Other methods

Apoptosis assay. The extent of apoptosis in treated and untreated cell cultures was determined by DAPI-staining of DNA, using fluorescence microscope. Staurosporine (0.2 μM) was used as an inducer of apotosis for standardising the method.

DNA synthesis assay. Proportion of cells undergoing DNA duplication, and thus entering the next round of cell division, was determined by labelling the cells with bromodeoxyuridine, using a commercially available kit from Roche, Germany.

Actin staining. The pattern of cytoskeletal actin staining was studied by staining the cells with fluorescent ligand FITC-labelled phalloidin, using a fluorescence microscope.[25]

β-gal staining. Senescence-specific β-galactosidase staining at pH 6.0 was performed following Dimri's method.[26]

Hydrogen peroxide (H_2O_2) decomposition assay. H_2O_2 decomposition assay *in vitro*, which is a measure of the general antioxidant ability of cells combining catalase and glutathione peroxidase activities, was determined by spectrophotometeric method.[27]

RESULTS

Immediate and short-term effects of zeatin

In order to rule out any immediate harmful effects of zeatin on human skin fibroblasts, a battery of tests were performed on young cells

Table 1. Effects of Short-Term Treatment of Early Passage Young Human Skin Fibroblasts with Zeatin

Characteristic	*Untreated cells*	*Zeatin-treated cells*
Attachment frequencies	80–90%	80–90%
S-phase–positive cells	75–80%	75–80%
3-day cell survival	90–100%	90–105%
β-gal–positive cells	<5%	<5%
Apoptotic cells	<5%	<5%

(early passage, less than 30% lifespan completed) and on senescent cells (late passage, more than 80% lifespan completed). Table 1 summarizes these results which showed that zeatin concentrations up to 200 μM had no negative effects on the cell attachment frequency, on 3-day cell survival, on BrdU-labeling index, on the number of senescence-specific β-galactosidase positive cells and on the number of spontaneously apoptotic cells. However, higher concentrations of zeatin (250 μM, 500 μM, 1 mM, and 2 mM) affected the 3-day cell survival significantly with less than 15% cells surviving at the highest concentrations.

These observations were further confirmed by one step growth analysis during a period of up to 11 days. Figure 2 shows that ASF-2 cells could grow and proliferate at almost similar rates in the absence or presence of zeatin at concentrations up to 80 μM. However, there was a slight slowing down of growth in 200 μM zeatin-treated cultures, but a significant slowing down of growth in 500 μM zeatin-treated. Therefore, for all long-term experiments on the effects of zeatin on ASF-2 cells, a maximum concentration of up to 200 μM was used.

Long-term effects of zeatin treatment

Replicative lifespan, morphology, and cell yield. Figure 3 shows the longevity curves for ASF-2 cells treated with various doses of zeatin throughout their replicative lifespan *in vitro*. In this series of experiments untreated control ASF-2 cells achieved CPDL 27.85 before becoming irreversibly growth arrested after 286 days of continuous serial passaging. This much replicative lifespan of 27.85 CPDL in this series of experiments is here considered as 100% lifespan completed. Under the same conditions, ASF-2 cells continuously grown in the presence of 40, 80, and 200 μM zeatin underwent 28.04, 28.27, and 27.96 CPDL, respectively.

Age-related changes in cellular morphology, cell size, cytoskeletal organization, and various biochemical parameters are well established markers of youthfulness and senescence in the Hayflick system of cellular aging *in vitro*. In the

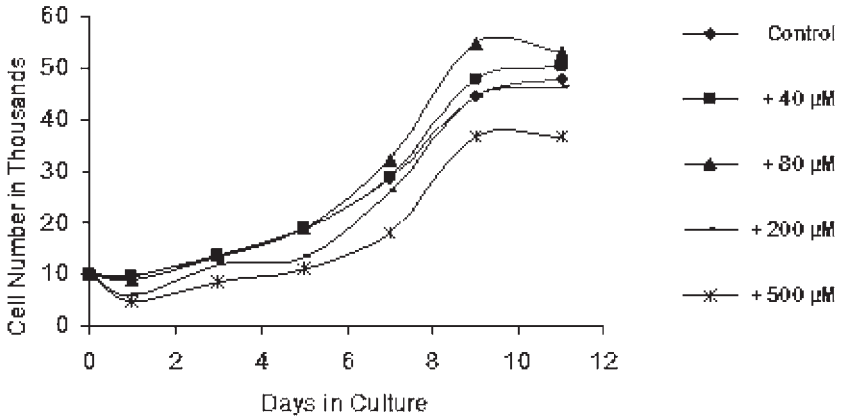

FIG. 2. Effect of zeatin on one-step growth of human skin fibroblasts.

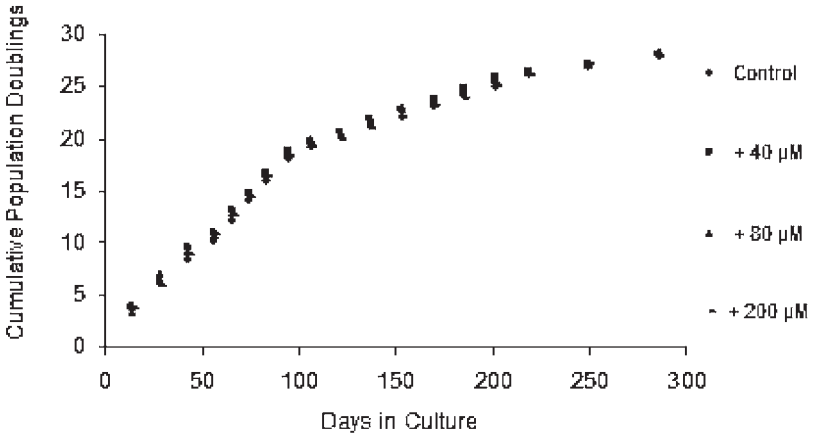

FIG. 3. Effect of zeatin on long-term growth and longevity of human skin fibroblasts.

present series of experiments, some of these changes were compared between untreated and zeatin-treated ASF-2 cells throughout their replicative lifespan. Figure 4 shows a morphological comparison between early passage young cultures (<30% lifespan completed) and late passage senescent cultures (>90% lifespan completed) grown in the absence or presence of 40, 80, and 200 μM zeatin. In untreated control cultures, there was a dramatic change in the morphology of cells from thin, long, spindle shaped cells arranged in fingerprint-like arrays (Fig. 4A) to highly enlarged, disorganized, vacuolated and sometimes, multinucleated senescent cells full of intracellular debris (Fig. 4B). In comparison, whereas zeatin treated young cells appeared to be very much similar to the untreated cells (Fig. 4C,E,G), late passage senescent cells treated with zeatin had much better appearance and structural integrity than untreated cells (Fig. 4D,F,H). There was a significant preservation of youthful morphology in cells treated with 40 and 80 μM zeatin, but the most dramatic effects could be observed in cells grown in the presence of 80 μM zeatin (Fig. 4F). Zeatin-treated cells maintained their spindle shape and arrangement in fingerprint-like arrays and avoided any significant enlargement, vacuolation and accumulation of intracellular debris.

Since one of the main youth preserving effects of zeatin was the maintenance of young morphology of cells, this was further confirmed by the data obtained for cell yield in confluent cultures, which is an indirect measure of cell size (Fig. 5). Early passage untreated young confluent cultures had a cell yield in the range of 15–20,000 cells per cm^2, which was progressively reduced to about 6–9,000 cells per cm^2 in the mid-passages, and to about 5,000 cells per cm^2 in senescent cultures. In comparison, cell cultures maintained in the presence of 40 and 80 μM zeatin generally had a higher cell yield both at early passages (more than 15,000 cells per cm^2) and at late passages (more than 10,000 cells per cm^2).

Cytoskeletal organization. Alterations in the cytoskeletal organization are one of the crucial age-related changes during cellular aging *in vitro*. Figure 6A shows three typical fluorescence staining patterns seen for actin in ASF-2 cells. These types are termed here as "actin staining type (AST) 1, 2, and 3," respectively. AST1 is the diffused staining pattern of actin homogenously dispersed throughout the cell with little or no polymerization. AST2 is the mixed and heterogeneous pattern of actin staining where both a diffused distribution and string-like polymerized pattern of actin can be observed. AST3 is the highly polymerized rod-like staining pattern observed most commonly in enlarged and senescent cells. These three patterns of actin staining can be observed in cell cultures at all passage levels, but their relative proportions change during serial passaging and cellular aging *in vitro*. Visual identification and counting of the number of cells with dif-

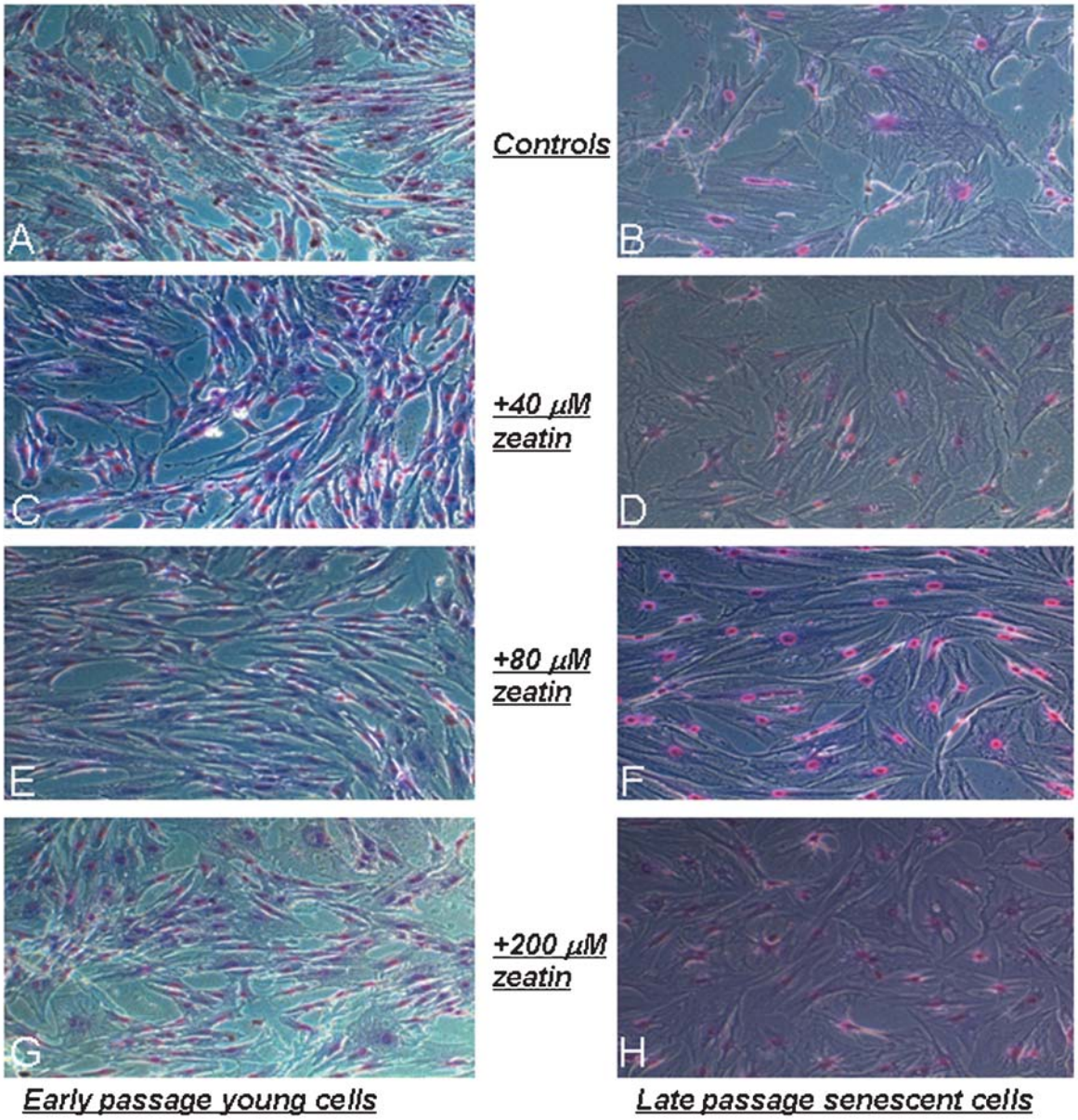

FIG. 4. Effect of zeatin on the morphology of serially passaged cultures of human skin fibroblasts. Original magnification, ×20.

ferent ASTs, using a flourescence microscope, shows that untreated control cultures at early passage had 68% AST1 cells, and 32% AST2 cells with no detectable AST3 cells (Fig. 6B). On serial passaging, the proportion of different AST in late passage senescent cultures changed to 8% AST1, 67% AST2 and 32% AST3, respectively (Fig. 6C).

Zeatin treatment of ASF2 cells significantly prevented these age-related alterations in actin polymerization. Figure 6B shows that early passage cultures grown in the presence of 40, 80, and 200 mM zeatin had significantly increased proportion of AST1 cells (78–94%) and reduced proportion of AST2 cells (5–20%). Similar trends in the maintenance of youthful actin staining patterns could be seen in late passage senescent ASF-2 cultures maintained in different concentrations of zeatin throughout their replicative lifespan in vitro. Figure 6C shows

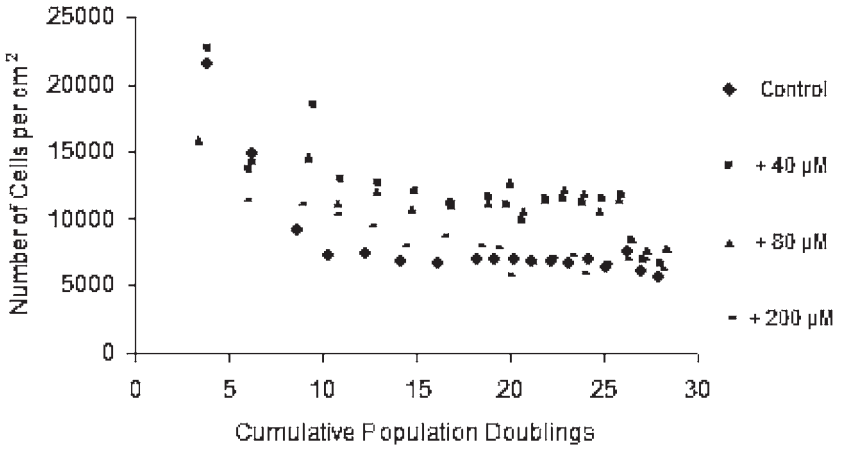

FIG. 5. Effect of zeatin on cell yield of serially passaged cultures of human skin fibroblasts.

that zeatin-treated late passage cultures had higher proportion of youthful AST1 cells (30–60% vs. 8% in controls) and reduced proportion of near senescent AST cells (35–55% vs. 67% in controls), and senescent AST3 cells (2–10% vs. 32% in controls).

H_2O_2 decomposing ability. Antioxidative effects of zeatin were tested by determining the H_2O_2 decomposing ability of crude extracts prepared from treated and untreated young and senescent ASF2 cells. Figure 7 shows that early passage young cells grown for 3 PD in the presence of different concentrations of zeatin had significantly increased ability to decompose H_2O_2. However, this increase was not strictly dose-dependent. For example, whereas early passage young ASF-2 cells treated with 40 and 80 μM zeatin had an increase in their H_2O_2 decomposition ability by 59% and 236%, respectively, this increase was only about 27% in 200 μM–treated cells. Serial passaging of ASF-2 cells did not result in a decrease in H_2O_2 decomposing ability of late passage senescent cells as compared with early passage young cells, and zeatin-treated senescent cells maintained higher H_2O_2 decomposing abilities even at the end of their lifespan *in vitro.*

In addition to the above experiments, we had also determined the effects of zeatin on the induction of apoptosis both on its own and in the presence of other stresses, such as ethanol and H2O2. Table 2 gives the results of the effects of zeatin treatment on the inhibition of apoptosis in young and senescent cells. There was no additional induction of apoptosis by zeatin treatment (40–200 mM) in young and senescent cells (1–5% spontaneous apoptosis). On the other hand, whereas 10% ethanol or 500 mM H2O2 treatment for 3 days induced apoptosis in more than 80% young and senescent cells, zeatin-treated cells resisted these stresses and only 10–15% cells became apoptotic (Table 2).

DISCUSSION

The results obtained in this series of experiments provide evidence for zeatin as another example of cytokinins having gerontomodulatory, youth preserving and anti-aging effects on human skin fibroblasts. Previously, work done in our labs had shown that kinetin had powerful anti-aging effects on human skin fibroblasts.[4] Those studies were followed up by a thorough and systematic testing of kinetin for its suitability and marketing as a component of numerous skin care products.[28,29] (also see www.senetekplc.com). Our new data on zeatin suggest that it may be another cytokinin with a potential for development into anti-aging and health care products. Similar studies on other cytokinins and their derivatives may discover other compounds with applications in aging prevention, intervention and therapy.

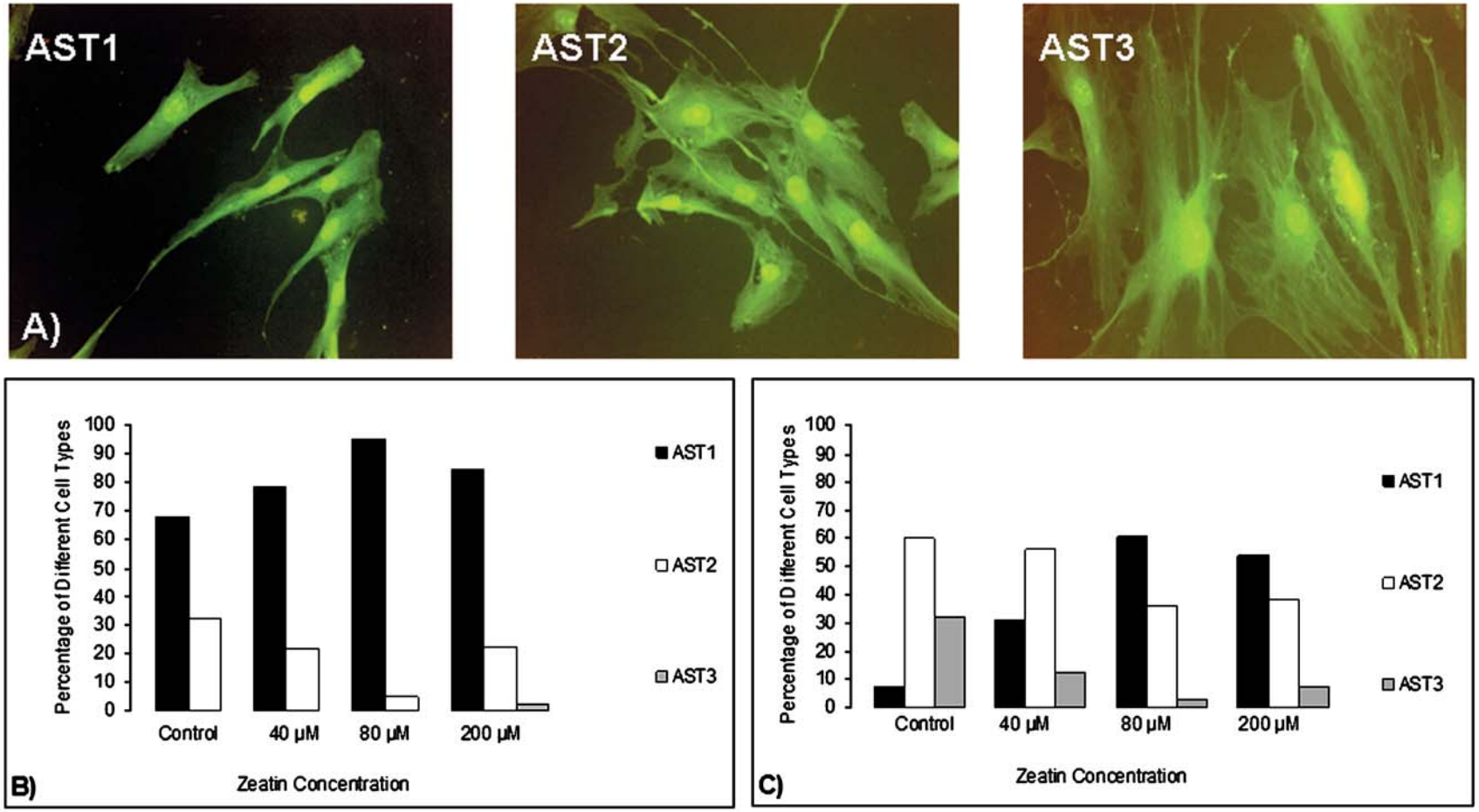

FIG. 6. Effect of zeatin on the actin staining pattern and the proportion of actin staining cell types (AST) in serially passaged human skin fibroblasts. Original magnification, ×40.

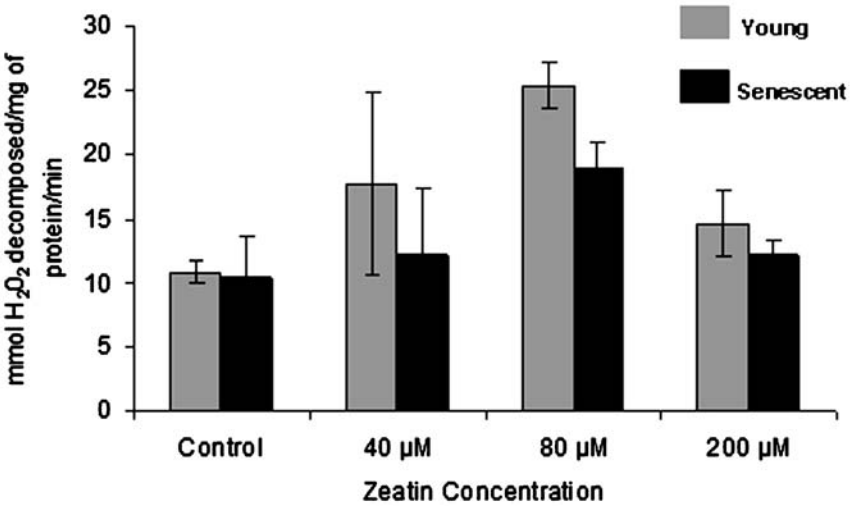

FIG. 7. Effect of zeatin on hydrogen peroxide decomposing ability of serially passaged human fiboroblasts.

Our studies on the short-term and immediate effects of zeatin have ruled out any negative and toxic effects in terms of cell attachment, cell proliferation, cell survival, cytoskeletal organization, and cellular growth up to 200 μM concentration. Higher concentrations of zeatin do affect cell survival and growth, but it should be pointed out that such negative effects at high doses can be observed universally for antioxidants, hormones, growth factors, and other chemical entities. The important point is to determine the biologically effective and beneficial window of concentration, which in the case of zeatin appears to be between 40 and 200 μM, and is comparable to the previously reported range for kinetin.

The optimal concentration of zeatin for its anti-aging and youth preserving effects on human skin fibroblasts is between 40 and 80 μM, which prevents, reduces and delays the onset of age-related cellular and biochemical alterations. The main biomarkers of cellular aging that we have studied in this series of experiments include cell enlargement, accumulation of cellular debris, increased actin polymerization, decreased antioxidative ability and reduced stress resistance. Zeatin treatment of human cells not only slows down their aging in terms of modulating above mentioned biomarkers, these effects are achieved without interfering with the genetic regulation of their cellular proliferative capacity or the Hayflick limit. This fact is underlined by our observations that zeatin treatment of human skin fibroblasts neither induces cell proliferation on a short-term basis nor does it increase the maximum proliferative lifespan (CPDL) on a life long basis. Therefore, one could infer that zeatin does not have any potentially harmful and carcinogenic effects on human cells and is not expected to upset any genetic mechanisms of proliferation regulation, such as telomere loss and DNA methylation.[30,31]

TABLE 2. EFFECT OF ZEATIN ON THE INDUCTION OF APOPTOSIS IN THE PRESENCE OF OTHER STRESSES

	Percentage of apoptotic cells[a]	
Treatment	*Young cells*	*Senescent cells*
No treatment	1–5	1–5
Ethanol (10%)	85–90	25–30
H_2O_2 (500 μM)	50–55	70–80
Zeatin 40 μM	1–4	1–4
+ Ethanol (10%)	75–80	65–75
+ H_2O_2 (500 μM)	5–10	10–15
Zeatin 80 μM	1–5	1–4
+ Ethanol (10%)	65–70	20–25
+ H_2O_2 (500 μM)	15–20	10–12
Zeatin 200 μM	1–5	1–4
+ Ethanol (10%)	70–80	20–25
+ H_2O_2 (500 μM)	15–20	10–15

[a]Percentage range of apoptotic cells was determined by fluorescence microscopic count of at least 500 cells in each condition in two separate experiments.

The most dramatic effects of zeatin on human skin fibroblasts observed in these studies are in terms of maintaining the youthful appearance of cells and enhancing their antioxidative ability. Since age-related cell enlargement and disorganization is mainly due to the accumulation of debris consisting of oxidatively and glyoxidatively modified proteins,[32,33] one can assume that zeatin treatment of cells affects this change either by enhancing the turnover of abnormal proteins through proteasomal and lysosomal pathways and/or by preventing the formation of abnormal proteins during aging. Direct evidence in support of any of these mechanisms with respect to the mode of action of zeatin awaits further investigations. However, our previous studies on the molecular mechanisms of action of kinetin have shown that kinetin, which also has similar anti-aging affects on human cells, protects DNA and proteins against oxidative and glyoxidative damage.[7,8] Therefore, it will be interesting to find out whether zeatin also works through the same antioxidative pathways of defence and maintenance. There is some indication from our observations on the enhanced ability of zeatin-treated cells to decompose H_2O_2 that zeatin stimulates cellular antioxidative pathways involving catalase and glutathione peroxidase. Further studies are required to fully understand the antioxidative mode of action of zeatin on human cells.

Another significant effect of zeatin on human skin fibroblasts undergoing aging *in vitro* is the prevention of actin polymerization into rigid rod-like structures which influence intracellular molecular flow and interactions.[34,35] Early passage young cultures are comprised of a majority of cells with homogenously distributed diffused pattern of monomeric forms of actin, as visualized by staining pattern termed AST1 described in the results above. Although one can observe even in early passage cultures some AST3 type cells, it is the progressive shift in their proportion from AST1 in young cultures to AST2 and 3 in late passage cultures that typifies the cellular aging process. Several of the age-related alterations in cellular biochemistry and physiology, such as protein synthesis, modification, localization and degradation, and entry into the cell cycle are negatively associated with the rigidity of the cytoskeleton.[34,35] The presence of zeatin in the cell culture medium significantly reduces this age-related shift in actin rigidity, which may have beneficial effects on cellular functioning and performance. However, it will be important to know what happens to other components of the cytoskeleton, especially microtubules which are involved in the regulation of cell cycle and cell proliferation. Furthermore, what effects zeatin has on the synthesis, modification and turnover of total and specific proteins, such as collagen and other extracellular matrix components also remains to be determined. Our present series of experiments opens up a whole range of important lines of investigation that will not only lead to attaining a complete understanding of the biological effects and molecular mechanism of action of zeatin, but also will facilitate identifying appropriate intracellular and extracellular targets where zeatin and other related compounds may have beneficial health care applications.

Since the effects of zeatin reported here are very much similar to the previously reported effects of kinetin, some comments on a comparison between kinetin and zeatin are warranted. Although studies performed on kinetin and zeatin are more than ten years apart and hence making any comparisons between their efficacies cannot be definitive, it appears that zeatin and kinetin are very much alike in their anti-aging effects on human cells. However, there are a few important differences between kinetin and zeatin treatments. For example, on a short-term basis, human skin fibroblasts can tolerate higher concentration of zeatin (200 μM) as compared with 100 μM of kinetin. On a long-term basis, although cells can be maintained at up to 200 μM zeatin throughout their lifespan, the optimal anti-aging effects are seen at 80 μM, which is the same as in the case of kinetin. Interestingly, most dramatic differences between zeatin and kinetin were observed when their respective ribosides are used. Whereas kinetin-riboside is highly toxic for cells even at 10 μM concentration,[4] zeatin-riboside had no toxic effects even up to 500 μM, as tested by 3-day MTT survival assay *(data not shown)*. At present, we have no data for the long term effects of zeatin-riboside on human cells.

Finally, from a mechanistic point, it is intriguing that a DNA-based modified adenine (kinetin) and a tRNA-based modified adenine (zeatin) have similar anti-aging and youth preserving biological effects when supplied from outside to human skin fibroblasts. Further research on the molecular basis of the modes of action of kinetin, zeatin and other cytokinins and their derivatives is required to unravel their modes of action and to compare and contrast their biological properties.

ACKNOWLEDGMENTS

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