

L-Carnosine reduces telomere damage and shortening rate in cultured normal fibroblasts

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Abstract

Telomere is the repetitive DNA sequence at the end of chromosomes, which shortens progressively with cell division and limits the replicative potential of normal human somatic cells. L-Carnosine, a naturally occurring dipeptide, has been reported to delay the replicative senescence, and extend the lifespan of cultured human diploid fibroblasts. In this work, we studied the effect of carnosine on the telomeric DNA of cultured human fetal lung fibroblast cells. Cells continuously grown in 20 mM carnosine exhibited a slower telomere shortening rate and extended lifespan in population doublings. When kept in a long-term nonproliferating state, they accumulated much less damages in the telomeric DNA when cultured in the presence of carnosine. We suggest that the reduction in telomere shortening rate and damages in telomeric DNA made an important contribution to the life-extension effect of carnosine. © 2004 Elsevier Inc. All rights reserved.

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Telomere is the repetitive DNA sequence that caps each end of a chromosome. Telomere plays important roles in maintaining chromosome stability by protecting chromosome ends from degradation and end-to-end fusion [1]. The DNA replication mechanism in human cells is unable to fully replicate the very end of a linear DNA molecule. Because of this end-replication problem, telomere shortens at each round of cell division in the absence of telomerase, and other mechanisms that compensate the telomere loss. Normal human cells can only divide for a finite number of times, which is referred to as replicative senescence. The shortening of telomere has been suggested to be the mechanism that limits the number of divisions a cell can undergo [2,3].

L-Carnosine (β -alanyl-L-histidine) is a naturally occurring dipeptide and abundant in muscle and nervous tissues in many animals, especially long-lived spe-

cies [4–6]. A striking anti-senescence effect of carnosine was demonstrated by McFarland and Holliday [7–9]. They showed that human diploid fibroblasts grown in 20 mM carnosine had an extended lifespan, both in population doublings (PDs) and chronological time. Carnosine possesses several biological functions, including metal ion-chelating, anti-oxidation, free radical scavenging [10], neutralization of acids, and the inhibition of nonenzymic glycosylation of proteins [4,7,11]. Many studies have been carried out to investigate the effects of carnosine at the molecular or cellular level. However, its effect on telomere has never been reported. Owing to the involvement of telomere in the replicative senescence of human cells, it would be of interest to know what effect carnosine would have on telomere.

In this work, we studied the effect of carnosine on a human fetal lung fibroblast strain (HPF), which was either kept in a continuously proliferating or proliferation-inhibited state. Our results indicate that carnosine can reduce telomere shortening rate possibly by protecting

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telomere from damage. We speculate that this protective effect on telomere may be a major contribution to the ability of carnosine in delaying the replicative senescence of cultured normal fibroblasts.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), X-gal were purchased from Gibco (Grand Island, NY, USA). Fetal calf serum was purchased from Hyclone (Utah, USA). Penicillin and streptomycin sulfate were purchased from Shijiazhuang Pharmacy (Shijiazhuang, China). L-Carnosine (99% purity) was purchased from Sigma-Aldrich (St. Louis, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA) was purchased from Molecular Probes (Eugene, USA).

Cell culture. The human fetal lung fibroblast strain (HPF) was obtained at passage eight from the Cell Bank of the Chinese Medical Academic Science (Beijing, China). Cells were grown in DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin sulfate at 37 °C with 5% CO₂ and 95% air. Serial cultivation was carried out with a split ratio of 1:4 at early and of 1:2 at later passages, respectively. At each passage, cells were harvested by trypsinization, and single cell suspensions were counted by an XB-K-25 Blood Cell Counter to calculate the cumulative population doublings (PDs). Stock solution of 100 mM L-carnosine was made in DMEM, filter-sterilized, and diluted to 20 mM when used. For the detection of accumulation of telomere fragments, cells at the population doubling level of 12 PDs were left confluent for up to 50 days with weekly changes of fully supplemented medium supplied with or without 20 mM carnosine. The control cells were kept in normal proliferation and subcultured at the same time. For tracing telomere length, cells were serially subcultured, and sampled at different population doubling levels for telomere length measurement.

Measurement of telomere fragments and telomere length. Cells were suspended in 0.8% low-melting agarose, 40 mM phosphate-buffered saline (PBS, pH 7.4) and plugs were made in 100 µl plastic mold followed by solidification at 4 °C. Plugs made for the measurement of telomere length were preserved in PBS supplied with 0.1% NaN₃, and stored at 4 °C until use.

For the detection of telomere fragments, the plugs were incubated in lysis buffer (2.5 M NaCl, 100 mM EDTA, 1% sodium lauroylsarcosine, 10% DMSO, 10 mM Tris, pH 10, and 1% Triton X-100 added immediately before use) for 1 h and then in alkaline buffer (333 mM NaOH, 1 mM EDTA, pH 13) for half hour to denature the DNA [12–14]. The two incubations were both conducted at room temperature in dark with gentle agitation.

For the assessment of telomere length, plugs were incubated in ESP solution (0.5 M EDTA, 1% sodium lauroylsarcosine, and 1 mg/ml proteinase K, pH 9.0) for 48 h at 50 °C with gentle agitation. After washing two times with 1 mM PMSF, three times with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), 2 h each, the plugs were then treated with *Hinf*I (Promega, USA) in digestion buffer (10 mM Tris, 10 mM MgCl₂, 50 mM NaCl, and 1 mM DTT) at 37 °C for 4 h. Digestion was terminated by 25 mM EDTA (pH 8.0).

The above agarose plugs, with either alkaline denatured or *Hinf*I digested DNA, were rinsed twice in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), 15 min each, loaded onto 0.8% agarose gel, and electrophoresed at 5 V/cm for 2 h in TAE buffer. After electrophoresis, telomere DNA was blotted onto Hybond-N⁺ membrane and detected using the "Telo TAGGG Telomere Length Assay" kit (Roche, Germany) according to manufacturer's instruction. Briefly, the blotted telomere DNA were hybridized to a digoxigenin (DIG)-labeled probe specific for telomeric repeats and incubated with a DIG-specific antibody covalently coupled to alkaline phosphatase. In some experiments,

the membrane was hybridized with the DIG-labeled minisatellite probe (CAC)₆. After the image was recorded, the probe was stripped and the same membrane was rehybridized with the telomere probe. The immobilized probe was visualized by alkaline phosphatase metabolizing CDP-*Star*, a highly sensitive chemiluminescence substrate. The signals were recorded using ChemiImage 4400 (Alpha Innotech, USA).

Flow cytometry assay of reactive oxygen species. Two microliters of H₂DCFDA at 0.5 mM was added to 4 × 10⁵ cells in 0.5 ml DMEM (pH 7.2), and the cell suspension was incubated at 37 °C for 15 min followed by addition of 10 µl of 500 µg/ml propidium iodide (PI). DCF fluorescence was measured at 530 ± 30 nm on a BD FACScan Flow cytometer. Data were obtained from the PI negative cell population and analyzed with the CellQuest software [15].

β-Galactosidase staining. Senescence-associated β-galactosidase activity was detected according to the method of Serrano et al. [16]. Briefly, the cells were washed twice in PBS (pH 7.2), fixed for 5 min in 0.5% glutaraldehyde (PBS, pH 7.2), and washed in PBS (pH 7.2) supplemented with 1 mM MgCl₂. The fixed cells were incubated in X-gal staining solution (1 mg/ml X-Gal, 0.12 mM K₃Fe[CN]₆, 0.12 mM K₄Fe[CN]₆, and 1 mM MgCl₂ in PBS at pH 6.0) in a humidified incubator at 37 °C for 24 h without CO₂ supply. The cells were then photographed under a microscope (Olympus, Japan).

Results

Characteristics of cells in serial cultivation in the presence and absence of carnosine

The effect of carnosine on the telomere shortening was examined during the lifespan of the HPF cells. Cells continuously subcultured in medium containing 10% fetal calf serum in the presence or absence of 20 mM carnosine were sampled at different population doubling levels until they became senescent. Digestion of genomic DNA with restriction enzyme *Hinf*I generated terminal restriction fragments (TRFs), which were detected by Southern blotting using a telomere-specific probe. The rate of telomere shortening was reflected by the decrease in TRF length, as a function of population doublings the cells had gone through. The results given in Fig. 1 showed that cells grown in medium supplemented with carnosine had an obviously slower rate of telomere shortening compared with the cells grown in medium without carnosine. The length of the TRFs appeared larger than reported data (around 10 kbp). This discrepancy could probably be explained by the way the electrophoresis was done. While the molecular size markers were loaded in solution in the slot, the TRFs were embedded in agarose plug. Therefore, it may take longer for the TRFs to enter the gel bed than for the markers resulting in an overestimation of TRF length.

Similar to the results reported by McFarland and Holliday [8,9], the cells continuously subcultured in medium supplemented with carnosine showed a younger morphology than the cells grown in medium without carnosine when they were approaching the end of their

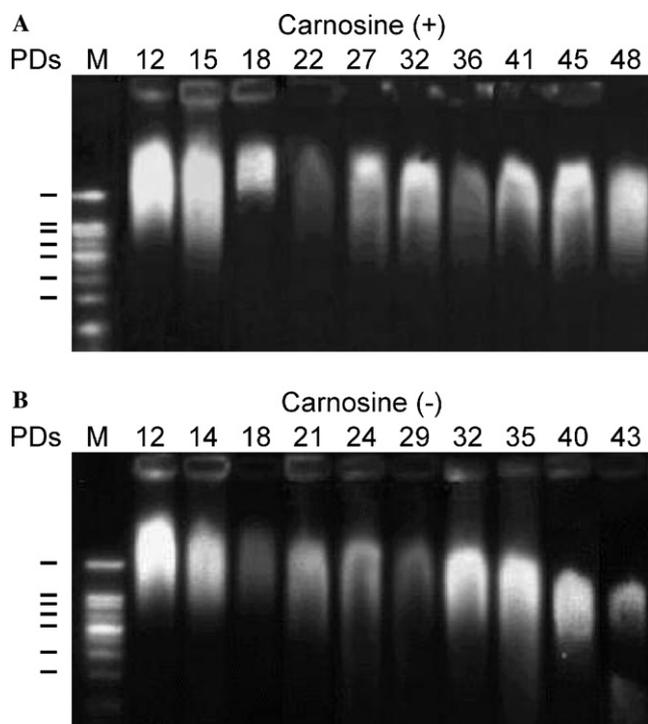


Fig. 1. Carnosine slowed down telomere shorting in HPF cells. HPF cells were serially cultured in DMEM supplemented with 10% fetal bovine serum and with (+) or without (-) 20 mM carnosine. Serial cultivation started at the population of 12 PDs. At the population doubling levels indicated, cells were collected and telomere restriction fragments (TRFs) detected by Southern blotting. M, molecular weight markers (21.2, 8.6, 7.4, 5.0, 4.2, 3.5, and 2.7 kbp from top to bottom).

lifespan. Senescence-associated β -galactosidase (β -Gal) is an established biomarker associated with cellular senescence [17]. When cells at 45 PDs were stained, the culture grown with carnosine showed a lower percentage of β -galactosidase-positive cells than the culture grown without carnosine (Fig. 2). Cells grown in carnosine also exhibited an enhanced proliferative capacity. Within 124 days, the culture grown in carnosine reached a population doubling level of 51 PDs while the one grown in the absence of carnosine only reached 45 PDs (Fig. 3).

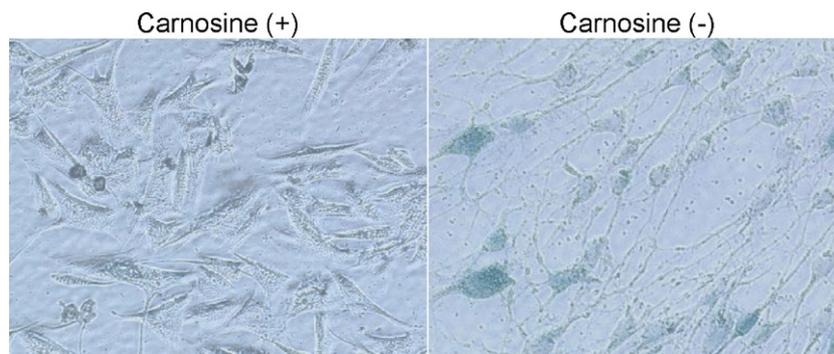


Fig. 2. β -Galactosidase staining of cells grown in the presence or absence of 20 mM carnosine. HPF cells were serially cultured in DMEM supplemented with 10% fetal bovine serum. HPF cells at the population doubling level of 45 PDs were seeded and stained 2 days later.

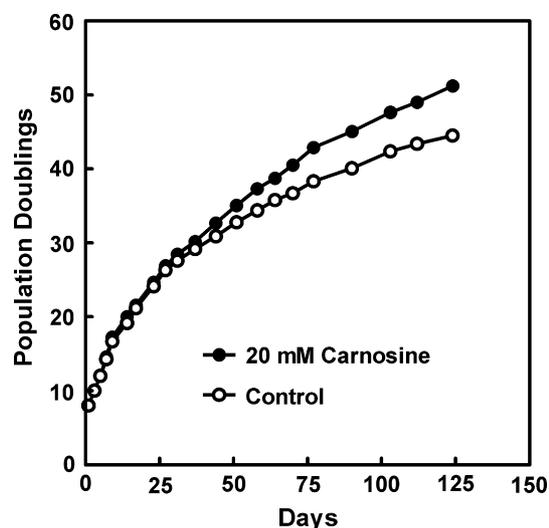


Fig. 3. Carnosine increased the replicative lifespan of HPF cells. Serial cultivation in DMEM supplemented with 10% fetal bovine serum started at 8 PDs and supplement of 20 mM carnosine started at 12 PDs.

Characteristics of cells in confluency in the presence and absence of carnosine

The von Zglinicki [19] group reported that human fibroblasts accumulated single-strand breaks in telomere after extended periods of confluency [18] and suggested that such damage in telomere could be the major cause of telomere shortening. To determine the effect of carnosine on telomere damage, HPF cells at the population doubling level of 12 PDs were kept in confluency to block cell proliferation for 50 days. The production of telomere fragments as a result of strand breaks was detected by Southern blotting after they were separated from the genomic DNA by alkaline denaturation and electrophoresis. As shown in Fig. 4A, weak signal of telomere fragments was detected in the control cells kept in a normal proliferating state. However, when cells were kept at confluency in the absence of carnosine, telomere fragments were detected as a bright smear of

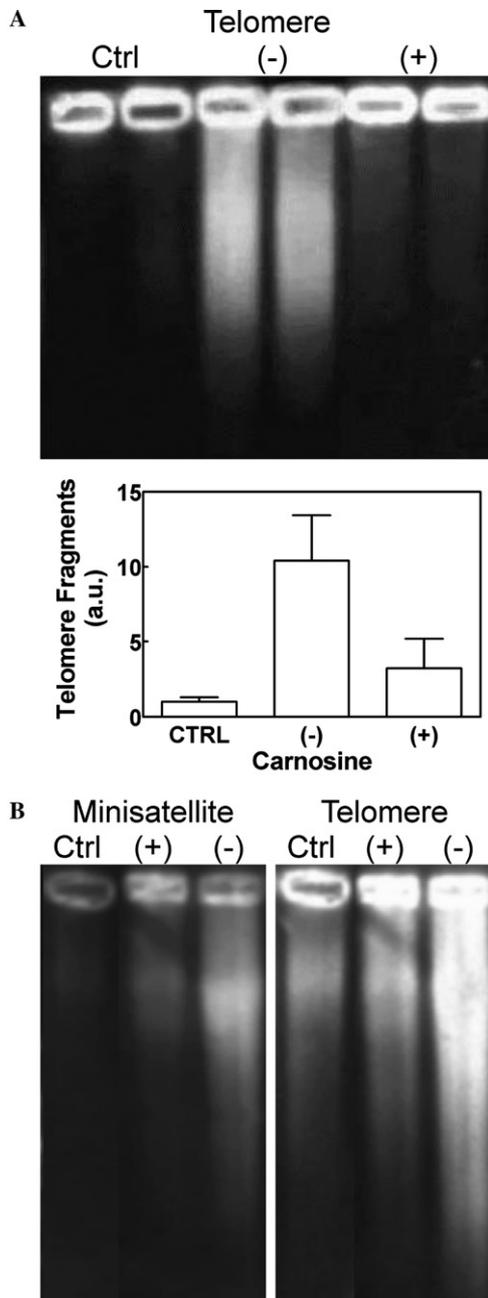


Fig. 4. Effect of carnosine on the production of telomere fragments in proliferation-inhibited cells. (A) Detection of telomere fragments. Data in the lower panel represent the mean values of three independent experiments with standard deviation. (B) Comparison of fragment detection in minisatellite (left panel) and telomere DNA (right panel). HPF cells at the population doubling level of 12 PDs were kept at confluency for 50 days in DMEM supplemented with 10% fetal bovine serum and with (+) or without (-) 20 mM carnosine before they were assayed for DNA fragmentation. Control cells were serially subcultured as usual without proliferation inhibition. The membrane in (B) was first hybridized with the interstitial minisatellite probe (CAC)₆. Then the probe was stripped and the same membrane was rehybridized with the telomere probe. The exposure time was the same for both pictures, which was longer than usual in order to see the minisatellite probe signal. The results are representative of three independent experiments.

signal indicating the production of strand breaks. The supplement of 20 mM carnosine greatly reduced the amount of telomere fragments. As a comparison, we examined the damage at the interstitial minisatellite DNA (Fig. 4B). The signals obtained using the minisatellite probe are much weaker than those obtained using the telomere probe. This result is in agreement with a previous report that shows the frequency of single-strand breaks is several folds higher in telomeres than in minisatellites [20].

Similar to the cells kept in continuous subcultivation, the HPF cells at the end of the 50 day confluency exhibited a better morphology when they were fed with 20 mM carnosine while those cells kept in the absence of carnosine showed a characteristic senescent appearance (Fig. 5). The ROS level was detected by DCF dye after the HPF cells were kept at confluency for 21 days. The DCF assay reflects the ROS level by the intensity of fluorescence signal. As shown in Fig. 6, cells kept at confluency had a much higher ROS level compared to the proliferating control cells. Cells kept at confluency in the presence of 20 mM carnosine showed a reduced ROS level, but it was still higher than that in the proliferating control cells.

Discussion

Our work was intended to examine the effect of carnosine on telomere. We studied the effect of carnosine on the telomere of HPF cells in two different states, i.e., cells in a continuous proliferation and cells in a proliferation-inhibited state. Our results demonstrated that carnosine at physiological concentration remarkably reduced the rate of telomere shortening in the continuously subcultured cells (Fig. 1). This reduction in telomere shortening rate can be explained by the protective effect of carnosine against the telomere fragmentation observed in the cells kept at confluency. Based on the measurement of S1 nuclease-sensitive site, Sitte et al. [18] reported that cells kept in long-term confluency accumulated single-strand breaks in telomeres. They found the cells experienced abrupt shortening in telomere length on releasing into cell cycle and showed reduced proliferative capacity when maintained in continuous subcultivation afterwards. Using a different method, we directly detected accumulation of telomere fragments in cells kept at confluency. Damages to telomere occur during normal physiological metabolisms. When cells were maintained in a continuously proliferating state, the telomere fragments, if not repaired, are expected to be lost during DNA replication, therefore they were kept at a low level that was barely detectable. The loss of telomere fragments would contribute to telomere shortening in addition to the end-replication problem. Keeping cells in confluency allowed such damages to

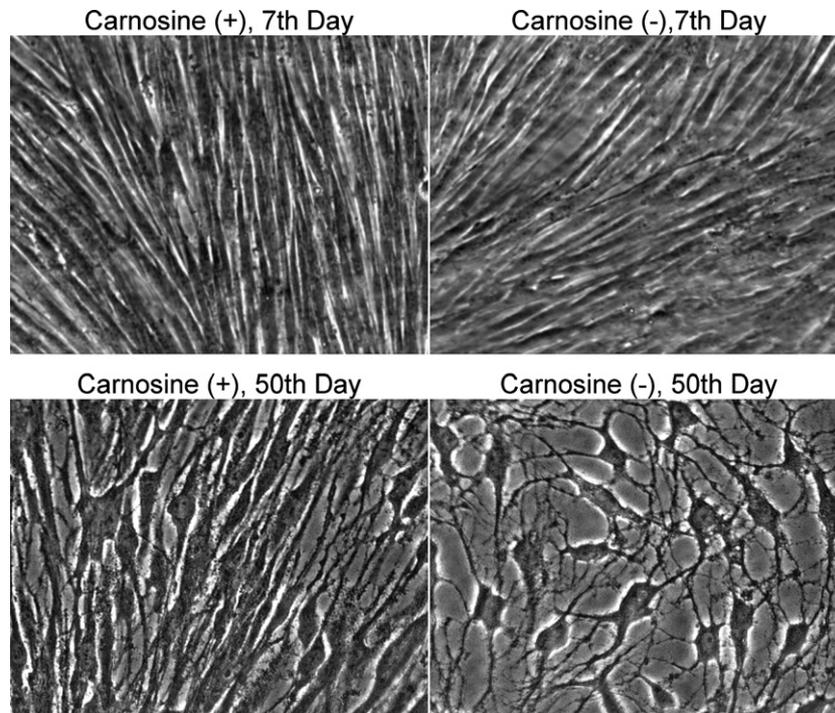


Fig. 5. Effect of carnosine on the morphology of proliferation-inhibited cells. HPF cells at the population doubling level of 12 PDs were kept in confluency for 50 days in DMEM supplemented with 10% fetal bovine serum and with (+) or without (–) 20 mM carnosine. The pictures were taken on the 7th or 50th day.

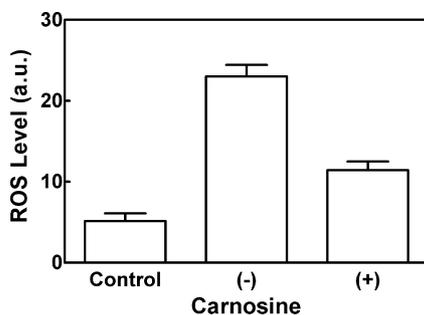


Fig. 6. Effect of carnosine on the reactive oxygen species (ROS) level of proliferation-inhibited cells. HPF cells at the population doubling level of 12 PDs were kept at confluency for 50 days in DMEM supplemented with 10% fetal bovine serum and with (+) or without (–) 20 mM carnosine. Cells were collected at day 21 and assayed as described in the text. Control cells were serially subcultured as usual without proliferation inhibition. Data represent means of two measurements with range.

accumulate to a level that could be detected by the method we used. Our data showed that carnosine is very effective in protecting telomere from damage (Fig. 4). The length of telomeres in proliferation-inhibited cells does not decrease. The accumulation of telomere fragments and appearance of senescent phenotype in the long-term proliferation-inhibited cells observed in this work suggest that senescence can be caused by accumulation of damages in the telomere region without telomere shortening.

Oxidative DNA damage is believed to contribute to the replicative senescence of cultured cells. Enhanced [21] or reduced [22] oxidative stress has been reported to accelerate or delay replicative senescence, respectively. Previous studies suggest that the anti-oxidant/free-radical scavenging property [10] and the protective effect against nonenzymatic glycosylation [23] may be responsible for the anti-senescence effect of carnosine. Our data showed that, compared to the proliferating cells, the cells kept in confluency exhibited an increased level of reactive oxygen species (ROS) (Fig. 6), which might have contributed to the accumulation of telomere fragments in these cells. When carnosine was included in the medium, both the telomere fragmentation and ROS level dropped concurrently, suggesting that the reduction in telomere damage is closely related to the free radical scavenging ability of carnosine.

A previous study of the effect of carnosine on the oxidative DNA damage levels in peripheral blood derived human CD4⁺ T cells has produced inconsistent results with relation to its effect on senescence. While carnosine reduced DNA damage at the early passage and elevated DNA damage at the later passages for the clone either derived from a young or senior donor, the lifespan extension effect was only observed on the clone derived from the young donor [24]. Many studies have indicated that telomere DNA is more susceptible to damages than the bulk sequences of genomic DNA. It has been reported that human telomere insert in

plasmid accumulates 7-fold more strand breakage than control sequence [25]. It has also been reported that the frequency of single-strand breaks is several folds higher in telomeres than in minisatellites or in the bulk genome when cells are treated under different oxidative stresses or with alkylating agents [20]. Based on the production of oxodG, a study has shown that telomere sequences are much more susceptible than nontelomere sequences to damage induced by oxidative stress [26,27]. The preferential accumulation of damages in the telomere sequence may possibly trigger the responses to DNA damage well before the bulk genomic DNA is severely damaged. In this regard, the protection of carnosine on DNA damage may be better reflected by its effect on telomere. Carnosine may delay the onset of replicative senescence by protecting the telomere.

Acknowledgments

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