J Physiol Biochem (2011) 67:35–42
DOI 10.1007/s13105-010-0046-2

ORIGINAL ARTICLE

Oxidative stress in relation to telomere length maintenance in vascular smooth muscle cells following balloon angioplasty

Gonen Ozsarlak-Sozer · Zeliha Kerry · Goksel Gokce · Ismail Oran · Zeki Topcu

Received: 4 March 2010 / Accepted: 14 September 2010 / Published online: 6 October 2010

Abstract Telomeres are specialized DNA–protein complexes found at the tips of linear chromosomes. In this study, we investigated the effects of oxidative stress on telomeric length distribution of proliferating vascular smooth muscle cells following balloon injury in single or combined treatment of rabbits with either buthionine sulfoximine or taurine. Exposure to oxidative stress increased the balloon injury whereas taurine treatment significantly diminished L-buthionine-sulfoximine-related intimal hyperplasia. Our results also showed that both variables had a significant influence on mean telomeric length distribution.

Keywords Oxidative stress · Telomere length · Balloon angioplasty · Atherosclerosis

G. Ozsarlak-Sozer · Z. Kerry · G. Gokce
Department of Pharmacology, Ege University,
35100 Izmir, Turkey

I. Oran
Department of Radiology, Faculty of Medicine,
Ege University,
35100 Izmir, Turkey

Z. Topcu (✉)
Department of Pharmaceutical Biotechnology,
Faculty of Pharmacy, Ege University,
35100 Izmir, Turkey
e-mail: zeki.topcu@ege.edu.tr

Introduction

Oxidative damage is an important factor in the accumulation of DNA damage. It can lead to mutagenesis and carcinogenesis among other biological effects [9]. Increased levels of reactive oxygen species (ROS) are found in atherosclerosis in all layers of the affected artery wall [24, 30, 38]. Oxidative damage was also proposed to have an impact on telomeric DNA ends as the accumulation of ROS can cause an accelerated telomere shortening [7, 36]. Telomeres are specialized G-rich repeat sequences, playing an important role in the maintenance of genomic stability by preventing the natural ends from being recognized as damaged DNA [1, 3, 4, 13, 19]. Telomeric repeats are generated by a specialized reverse transcriptase, called telomerase, which is composed of an RNA component, serving as a template for the addition of telomeric repeats, and a protein component, which is the telomerase reverse-transcriptase catalytic subunit (Tert) [28]. The sensitivity of telomeric DNA ends to oxidative stress was attributed to their high guanine content [23, 24, 36]. As the single strand breaks at telomeric DNA cannot undergo a repair process, 8-oxo dG as a result of reactive oxygen species causes telomere shortening through replication [36]. Ataxia telangiectasia (A-T), an autosomal recessive disease, is an example of the relation between oxidative stress and telomere shortening [31].

ROS-related damage is known to be a reversible process induced by a number of antioxidant agents. We previously reported that balloon angioplasty in iliac arteries resulted in intimal hyperplasia due to proliferation of the smooth muscle cells, and small-size telomeric restrictional fragments were evident in injured arteries [22]. In the present study, we investigated the effects of oxidative stress on telomeric length regulation of proliferating vascular smooth muscle cells after balloon injury. We employed buthionine sulfoximine (BSO) to generate oxidative stress and taurine as the reversing agent by either single or combined treatment of the experimental animals with these two agents. Buthionine sulfoximine is a selective inhibitor of γ-glutamyl cysteine synthetase, which is responsible for the synthesis of

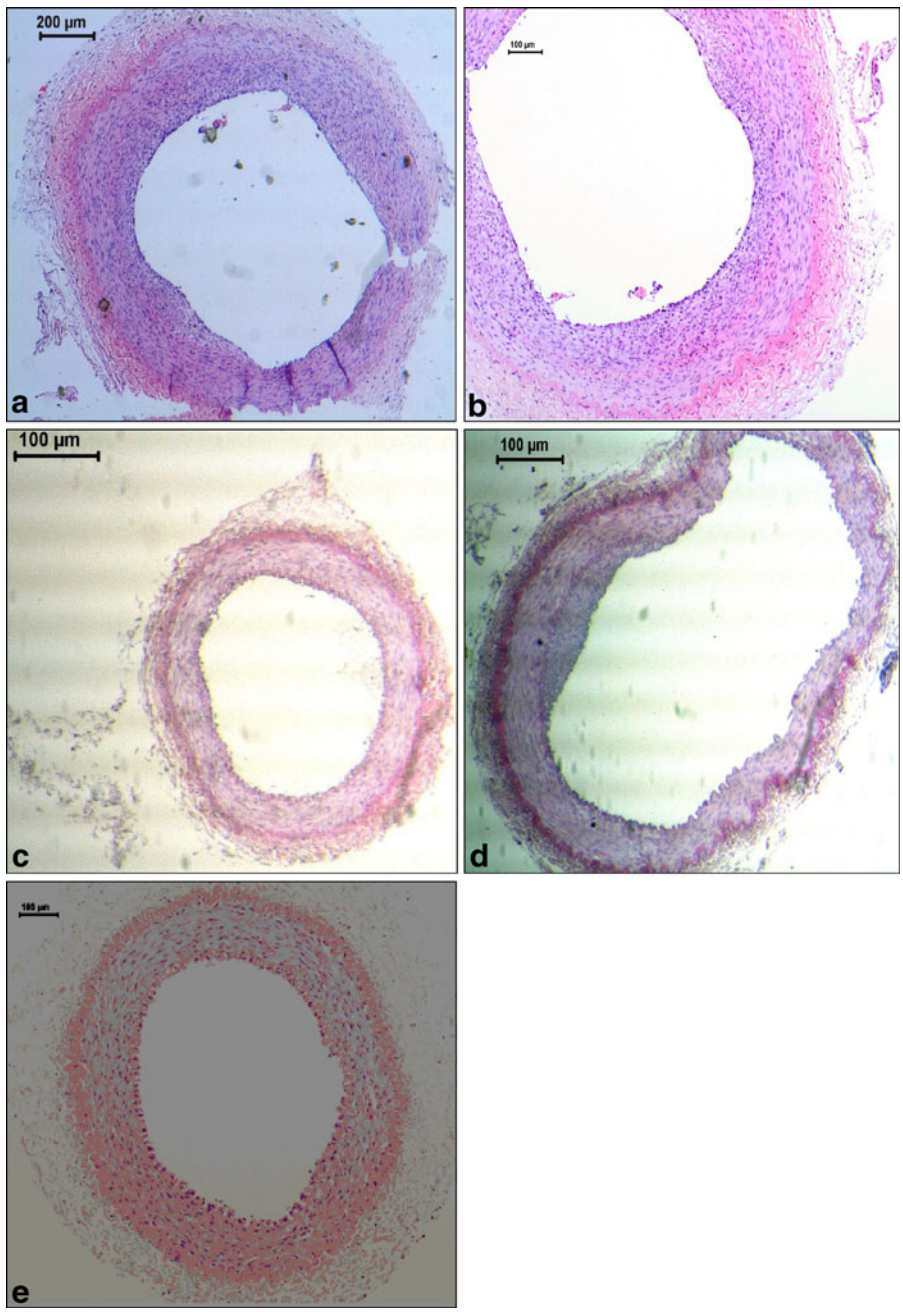

Fig. 1 Hematoxylin–eosin-stained iliac artery tissue sections: **a** only balloon-injured, **b** balloon-injured+BSO-treated, **c** balloon-injured+taurine-treated, **d** balloon-injured+BSO- and taurine-treated, **e** uninjured and untreated contralateral artery (×10 magnification)

glutathione (GSH) [34, 37]. Taurine, on the other hand, is involved in a variety of biological processes such as bile salt formation, osmoregulation, immunomodulation, diabetes, atherosclerosis, and oxidative stress inhibition [5, 21]. The effects of these agents were monitored by determining GSH concentrations, the ratio of [GSH] to [GSSG], glutathione peroxidase (GPx) activity, and telomeric restriction fragment (TRF) length, as well as morphometric and histochemical analyses.

Materials and methods

General

Telomeric length analyses were performed in a total of 12 New Zealand white rabbits (2.5–3.0 kg) of both sexes. Briefly, experimental animals were anesthetized with pentobarbital (25 mg/kg, iv), and balloon angioplasty was performed in the iliac artery of rabbits with a Fogarty 2.5-mm balloon catheter four times with 6-, 8-, 4-, and 10-atm pressure for 1 min [22, 29]. Whole injured and uninjured contralateral arteries (sham) were isolated for morphometric, immunohistochemical, and telomeric length analyses on day 14. Experimental animals were treated with BSO (75 mg/kg/day) (group I), taurine (10% *w/v* of total water intake) (group II), both BSO and taurine (group III). Group IV animals did not receive further treatment following balloon angioplasty. Blood samples were drawn on days 0 and 14 to determine selected oxidative stress markers, namely [GSH], [GSH]/[GSSG], and GPx activity. The animals were anesthetized with pentobarbital (25 mg/kg, iv) on the 14th day of treatment to excise iliac arteries (~100 mg) for morphometric, immunohistochemical, and telomeric length analyses. The study was approved by the Ethics Committee of Ege University, Izmir, TR. All the animals received care according to the criteria outlined in the "Guide for Care and Use of Laboratory Animals" prepared by the National Academy of Science, also adopted and implemented by Ege University, Izmir, TR.

Histological and immunohistochemical analyses

Histological and immunohistochemical analyses were carried out as described [21]. Four-micron-thick samples were analyzed through immunohistochemical staining using an antibody against proliferating cell nuclear antigen (PCNA) (PC10 clone, M0879, Dako, Glostrup, Denmark) [6, 27].

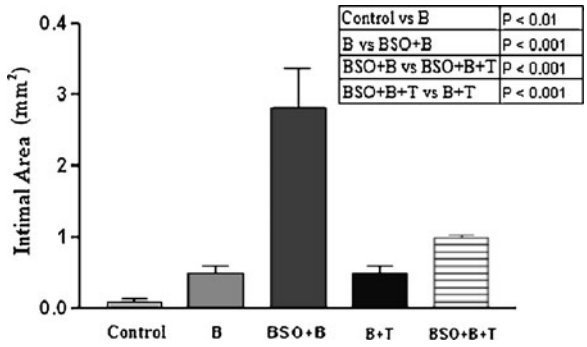

Fig. 2 Intimal area (mm^2) after balloon injury (*Control* uninjured and untreated contralateral artery, *B* balloon-injured, *BSO+B* balloon-injured+BSO-treated, *B+T* balloon-injured+taurine-treated, *BSO+B+T* balloon-injured+BSO- and taurine-treated)

Biochemical analyses

Total GSH concentration, the ratio of reduced and oxidized glutathione (GSH/GSSG), and the activity of GPx were determined using Bioxytech kits, namely GSH-420, GSH/GSSG-412, and GPx-340 (Oxis, USA), respectively, according to the manufacturer's specifications.

Analyses of telomeric restriction fragments

Total DNA was isolated using "Qiagen DNeasy® Tissue Kit" according to the manufacturer's specifications (QIAGEN, Haan, Germany). Following

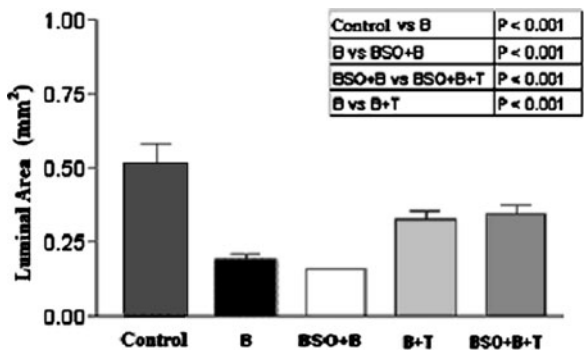

Fig. 3 Luminal area (mm^2) after balloon injury (*Control* uninjured and untreated contralateral artery, *B* balloon-injured, *BSO+B* balloon-injured+BSO-treated, *B+T* balloon-injured+taurine-treated, *BSO+B+T* balloon-injured+BSO- and taurine-treated)

spectrophotometric (UV-1208, Shimadzu, Kyoto, Japan) quantification of isolated DNA, comparable amounts of DNA (~5 μg) dissolved in Tris–ethylenediaminetetraacetic acid (EDTA) (10 mM Tris–Cl, 1 mM EDTA, pH 8.0) were fragmented using 10 U of each of *Hin*fI and *Rsa*I enzymes at 37°C for 2 h in a total volume of 20 μL. Reaction products were analyzed on 1% agarose gels in TBE buffer (5 V/cm) and photographed under UV light after staining with ethidium bromide (EtBr) (0.5 μg/mL). Southern transfer and hybridizations were carried out as described [18, 25]. Transferred DNA was cross-linked to the membrane (HYBOND, Escondido, CA, USA) in a UV cross-linker (Vilber Lourmat, Cedex, France) for hybridization with the telomeric probe labeled with digoxigenin (digoxigenin 3′-end-labeled 5′-CCTAAA-3′). Membranes were visualized using an X-Ray developer (Kodak X-Omat, Plano, TX, USA). The signal strength of the DNA bands were quantified from gel photographs using Bio-Rad Multianalyst (ver. 1.1), and mean telomeric fragment lengths of digested samples were calculated according to the following equation:

$$\sum(\mathrm{ODi})/\sum(\mathrm{ODi/Li})$$

where ODi and Li stand for the signal intensity and the length of the given fragment, respectively [12,

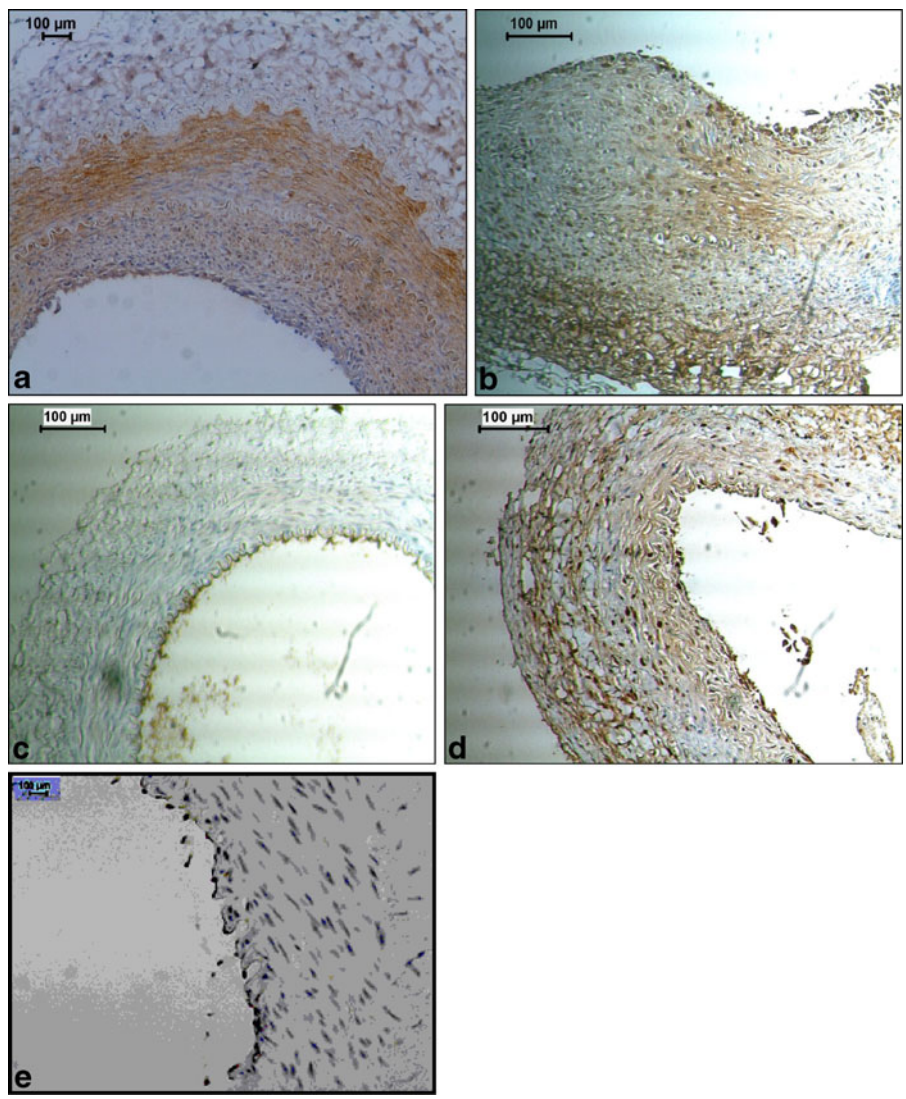

Fig. 4 PCNA staining after balloon injury: **a** balloon injury, **b** balloon injury+BSO, **c** balloon injury+taurine, **d** balloon injury+BSO+taurine, **e** uninjured, untreated contralateral artery (×10 magnification)

32]. All reactions were carried out in DNase-free 1.5-mL microcentrifuge tubes. To check for reproducibility, all reactions were repeated at least once. The values of the injured arteries were given as percentage of the uninjured arteries [15].

Statistical analysis

Values are represented as mean ± standard error. One-way analysis of variance was carried out using GraphPad Prism Software (Ver 3.0), and the significance of differences between the groups was assessed using Bonferroni test. Significance was defined at 95% confidence interval.

Results and discussion

We used the balloon angioplasty model to study oxidative stress in relation to telomeric length regulation of proliferated vascular smooth muscle cells. Figure 1 shows representative vascular smooth muscle segments obtained from balloon-injured iliac arteries (Fig. 1a) treated either with BSO (Fig. 1b) or taurine (Fig. 1c). The result of treatment when the animals received both agents is given in Fig. 1d. Balloon angioplasty resulted in a remarkable thickness of intimal area, which was further increased by BSO treatment. We then measured intimal area of individual samples to quantify the detected intimal thickness of the samples (Fig. 2). As seen in Fig. 2, taurine treatment reversed the intimal thickening together with BSO. Our results were attributed to balloon angioplasticity, as there was no difference between the treatments among uninjured arteries. On the other hand, our analyses on luminal area to a considerable degree supported the results we obtained with intimal area (Fig. 3). As was to be expected while balloon angioplasticity resulted in remarkable thickness of intimal area (Fig. 2), it also caused a significant decrease in luminal area (Fig. 3). However, as seen in Figs. 2 and 3, taurine treatment significantly inhibited BSO-induced intimal thickening whereas no differences were observed in luminal area. Taking these findings into consideration, our results suggests that the effect of BSO together with balloon injury seems to be more pronounced in intimal thickening than in luminal area.

Figure 4 gives the results of immunohistochemical analyses to detect the proliferation in the smooth muscle cells as a consequence of treatments in the same order as Fig. 1. We employed antibody against PCNA as proliferation indicator and detected positive staining in the nuclear area following balloon injury, independent of either BSO or taurine treatment. However, there was no staining in uninjured arteries (Fig. 4). We also detected smooth muscle-specific α-actin expression in our samples (data not shown).

Measurement of oxidative stress parameters in blood samples of days 0 and 14 is summarized in Table 1. As seen in Table 1, BSO diminished the GSH concentration, while this agent, when applied together with taurine, significantly reversed this

Table 1 Levels of oxidative stress parameters

GROUPS	Glutathione concentration (μM)		GSH/GSSG		GPx (μmol/NADPH/min/ml)	
	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14
Control (n=3)	1,111.3±67.96	1,052±1,098.1[a]	176.99±3.49	166.49±1.97[e]	2.80±0.03	2.76±0.02[e]
BSO (n=3)	1,098.1±66.18	431.2±34.23[b]	166.96±2.4	78.85±7.79[b]	2.72±0.04	1.64±0.12[f]
BSO+taurine (n=3)	1,072.1±102.96	1,049.9±53.57[c]	172.55±5.05	156.21±5.50[c]	2.76±0.07	2.65±0.09[c]
Taurine (n=3)	1,259.8±60.32	1,036.7±56.63[d]	175.56±3.74	175.19±2.92[d]	2.69±0.10	2.80±0.04[d]

[a] Control day 14 vs BSO day 14 (p<0.05)

[b] BSO day 0 vs BSO day 14 (p<0.001)

[c] BSO day 14 vs BSO+taurine day 14 (p<0.01)

[d] BSO day 14 vs taurine day 14 (p<0.001)

[e] Control day 14 vs BSO day 14 (p<0.001)

[f] BSO day 0 vs BSO day 14 (p<0.01)

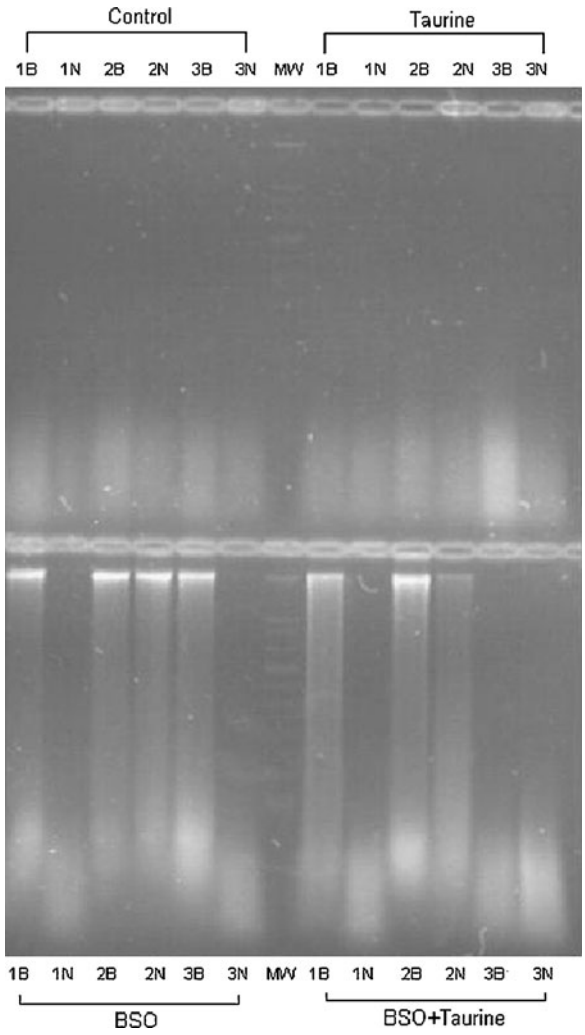

Fig. 5 Southern blot of *Hin*fI and *Rsa*I fragments hybridized with telomeric oligonucleotide probe (*mw* molecular weight marker, *B* balloon-injured artery, *N* balloon-uninjured contralateral artery)

effect ($p<0.001$). Likewise, the ratio of [GSH] to [GSSG] was decreased upon BSO treatment with a considerable reversal by taurine ($p<0.01$) (Table 1). Finally, BSO-related [GSH] depletion decreased the activity of GPx (Table 1) while BSO treatment together with taurine normalized the depleted [GSH], [GSH]/[GSSG], and decreased GPx activity (Table 1).

Our previous study showed no detectable telomerase activity following balloon angioplasty [22]. Absence of telomerase activity is a common property of the vast majority of adult somatic cells [8, 14, 26]. We, therefore, analyzed telomeric restrictional fragment length distribution using Southern blot to determine mean telomeric length. Southern blot is a highly sensitive methodology in estimation of desired fragments transferred from agarose gels, provided that hybridization of the membrane is carried out using specific probes in stringent conditions [25]. We used digoxigenin-labeled telomere-specific oligonucleotide of 15 mer as telomere-specific probe. The isolated DNAs were fragmented using the restriction enzymes, *Hin*fI and *Rsa*I, and applied on 1% agarose gel. A representative gel, accommodating fragmented telomeric DNAs, is given in Fig. 5. High molecular weight DNA samples were detectable at the upper left and upper right panels of the gel due to uneven sample loading (Fig. 5). However, the small-sized DNA molecules representing telomeric DNA repeats were visualized throughout the groups (Fig. 5). Although the DNA content of the gels were transferred by Southern blot, we used the negatives of the EtBr-stained gel photos to calculate the average band intensities to eliminate any variation due to underexposure or overexposure of the X-rays. Our results showed a significant decrease in the length of mean telomeric fragments upon BSO treatment (72.3% vs 56.7%, for control and BSO groups, respectively) ($p<0.01$) (Table 2). Taurine effectively reversed the decrease in telomere shortening (56.7% vs 66.7%, for BSO and BSO+taurine groups, respectively) ($p<0.05$) (Table 2). The MTF obtained in taurine-treated group was no different from that of the control samples (72.3% and 77.0% for control and taurine groups, respectively) (Table 2).

Table 2 Relative mean telomere fragments of control, BSO, BSO+taurine, and taurine groups following balloon injury on day 14

	Relative mean telomere fragment (%) (MTF)
Groups	Injured artery vs uninjured artery
Control ($n=3$)	72.3±1.4
BSO ($n=3$)	56.7±2.03[a]
BSO+Taurine ($n=3$)	66.7±2.6[b]
Taurine ($n=3$)	77.0±2.1[c, d]

[a] BSO vs control $p<0.01$

[b] BSO vs BSO+Taurine $p<0.05$

[c] BSO vs taurine $p<0.001$

[d] BSO+taurine vs taurine $p<0.05$

In conclusion, our study showed that exposure to oxidative stress increased balloon injury, whereas taurine treatment significantly diminished intimal hyperplasia. This result was consistent with our previous study [10]. We also report that there was a remarkable shortening of telomere length after balloon injury. In vivo oxidative stress accelerated this shortening of the telomere length, and antioxidant taurine partially reversed the shortening. Several studies have shown that senescence can be triggered either by telomeric DNA instability resulting from telomere erosion (replicative senescence) or following exposure to multiple types of stress (stress-induced senescence), such as oxidative stress [33], DNA damage, and mutagenic stress [2, 35]. In vitro, chronic oxidative stress results in increased cellular turnover that promotes telomere erosion and senescence of endothelial [16] and vascular smooth muscle cells [17, 20]. Previously, it was shown that glutathione depletion by buthionine sulfoximine induces oxidative damage to DNA in organs of rabbits [11]. Our findings suggest that, in vivo, telomeres in vascular smooth muscle cells shorten after balloon injury, which may result in a replicative senescence and shortening of the telomeres. This process is accelerated by oxidative stress, which may result from an oxidative damage to telomeric DNA, and prevention of cellular senescence with antioxidant agents like taurine may be a novel therapeutic target in atherosclerosis. The mechanisms of cellular senescence caused by telomeric erosion and possible oxidative damage to telomeric DNA will be included within the scope of our future research.

Acknowledgement Our research was supported by grants from The Scientific and Technological Research Council of Turkey (SBAG-K-70, ZK) and Ege University Research Found (04ECZ012, GOS). This study was part of a Ph.D. thesis submitted to the Institute of Health Sciences of Ege University, Izmir, Turkey, by Ozsarlak-Sozer.

References

1. Bechard LH, Butuner BD, Peterson GJ, McRae W, Topcu Z, McEachern MJ (2009) Mutant telomeric repeats can promote runaway recombinational telomere elongation. Mol Cell Biol 29:626–639
2. Ben-Porath I, Weinberg RA (2005) The signals and pathways activating cellular senescence. Int J Biochem Cell Biol 37:961–976
3. Blackburn EH (2005) Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. FEBS Lett 579:859–862
4. Blackburn EH, Gall JG (1978) A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in tetrahymena. J Mol Biol 120:33–53
5. Bouckenooghe T, Remacle C, Reusens B (2006) Is taurine a functional nutrient? Curr Opin Clin Nutr Metab Care 9:728–733
6. Bravo R, Macdonald-Bravo H (1987) Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. J Cell Biol 105:1549–1554
7. Cattan V, Mercier N, Gardner JP, Regnault V, Labat C, Maki-Jouppila J, Nzietchueng R, Benetos A, Kimura M, Aviv A, Lacolley P (2008) Chronic oxidative stress induces a tissue-specific reduction in telomere length in CAST/Ei mice. Free Radical Biol Med 44:1592–1598
8. Cong YS, Wright WE, Shay JW (2002) Human telomerase and its regulation. Microbiol Mol Biol Rev 66:407–425
9. Evans MD, Dizdaroglu M, Cooke MS (2004) Oxidative DNA damage and disease: induction, repair and significance. Mutat Res 567:1–61
10. Gokce G, Ozsarlak-Sozer G, Oran I, Oktay G, Ozkal S, Kerry Z (2008) The role of oxidative stress in hyperplasia: modulation of LOX-1 expression by taurine, 77th EAS Congress, 26–29 April 2008, Istanbul. Atheroscler Suppl 9 (1):58–58
11. Gokce G, Ozsarlak-Sozer G, Oktay G, Kirkali G, Jaruga P, Dizdaroglu M, Kerry Z (2009) Glutathione depletion by buthionine sulfoximine induces oxidative damage to DNA in organs of rabbits in vivo. Biochemistry 48:4980–4987
12. Harley CB, Futcher AB, Greider CW (1990) Telomeres shorten during ageing of human fibroblasts. Nature 345:458–460
13. Kim H, Chen J (2007) C-myc interacts with trf1/pin2 and regulates telomere length. Biochem Biophys Res Commun 362:842–847
14. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PLC, Coviello GM, Wright WE, Weinrich SL, Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011–2015
15. Kojima H, Miyazaki H, Shiwa M, Tanaka Y, Moriyama H (2001) Molecular biological diagnosis of congenital and acquired cholesteatoma on the basis of differences in telomere length. Laryngoscope 111:867–873
16. Kurz DJ, Decary S, Hong Y, Trivier E, Akhmedov A, Erusalimsky JD (2004) Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. J Cell Sci 117:2417–2426
17. Matthews C, Gorenne I, Scott S, Figg N, Kirkpatrick P, Ritchie A, Goddard M, Bennett M (2006) Vascular smooth muscle cells undergo telomere-based senescence in human atherosclerosis: effects of telomerase and oxidative stress. Circ Res 99:156–164
18. McEachern MJ, Haber JE (2006) Break-induced replication and recombinational telomere elongation in yeast. Annu Rev Biochem 75:111–135
19. McEachern MJ, Krauskopf A, Blackburn EH (2000) Telomeres and their control. Annu Rev Genet 34:331–358

20. Muller M (2009) Cellular senescence: molecular mechanisms, in vivo significance, and redox considerations. Antioxid Redox Signal 11:59–98
21. Oudit GY, Trivieri MG, Khaper N, Husain T, Wilson GJ, Liu P, Sole MJ, Backx PH (2004) Taurine supplementation reduces oxidative stress and improves cardiovascular function in an iron-overload murine model. Circulation 109:1877–1885
22. Ozsarlak-Sozer G, Kerry Z, Oran I, Gokce G, Tosun M, Bechard L, Reel B, Yasa M, Lebe B, Topcu Z (2009) Telomeric restriction analysis of vascular smooth muscle cells following balloon angioplasty in rabbits. J Physiol Biochem 65:243–250
23. Rhodes D, Fairall L, Simonsson T, Court R, Chapman L (2002) Telomere architecture. EMBO Rep 3:1139–1145
24. Richter T, von Zglinicki T (2007) A continuous correlation between oxidative stress and telomere shortening in fibroblasts. Exp Gerontol 42:1039–1042
25. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York
26. Shay JW, Gazdar AF (1997) Telomerase in the early detection of cancer. J Clin Pathol 50:106–109
27. Shinohara M, Kawashima S, Yamashita T, Takaya T, Toh R, Ishida T, Ueyama T, Inoue N, Hirata K-i, Yokoyama M (2005) Xenogenic smooth muscle cell immunization reduces neointimal formation in balloon-injured rabbit carotid arteries. Cardiovasc Res 68:249–258
28. Shippen-Lentz D, Blackburn EH (1990) Functional evidence for an RNA template in telomerase. Science 247:546–552
29. Strauss BH, Chisholm RJ, Keeley FW, Gotlieb AI, Logan RA, Armstrong PW (1994) Extracellular matrix remodeling after balloon angioplasty injury in a rabbit model of restenosis. Circ Res 75:650–658
30. Taniyama Y, Griendling KK (2003) Reactive oxygen species in the vasculature: molecular and cellular mechanisms. Hypertension 42:1075–1081
31. Tchirkov A, Lansdorp PM (2003) Role of oxidative stress in telomere shortening in cultured fibroblasts from normal individuals and patients with ataxia-telangiectasia. Hum Mol Genet 12:227–232
32. Topcu Z (2000) Densitometric quantification of DNA topoisomers in ethidium bromide-stained agarose gels and chemiluminescence-detected X ray films. Acta Biochim Pol 47:835–839
33. Toussaint O, Medrano EE, von Zglinicki T (2000) Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. Exp Gerontol 35:927–945
34. Vaziri ND, Wang XQ, Oveisi F, Rad B (2000) Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats. Hypertension 36:142–146
35. Voghel G, Thorin-Trescases N, Farhat N, Nguyen A, Villeneuve L, Mamarbachi AM, Fortier A, Perrault LP, Carrier M, Thorin E (2007) Cellular senescence in endothelial cells from atherosclerotic patients is accelerated by oxidative stress associated with cardiovascular risk factors. Mech Ageing Dev 128:662–671
36. Von Zglinicki T, Pilger R, Sitte N (2000) Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. Free Radical Biol Med 28:64–74
37. Watanabe T, Sagisaka H, Arakawa S, Shibaya Y, Watanabe M, Igarashi I, Tanaka K, Totsuka S, Takasaki W, Manabe S (2003) A novel model of continuous depletion of glutathione in mice treated with L-buthionine (S, R)-sulfoximine. J Toxicol Sci 28:455–469
38. Zalba G, Beaumont J, San JG, Fortuño A, Fortuño MA, Díez J (2000) Vascular oxidant stress: molecular mechanisms and pathophysiological implications. J Physiol Biochem 56:57–64