Does oxidative stress shorten telomeres *in vivo*? A review

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Abstract

The length of telomeres, the protective caps of chromosomes, is increasingly used as a biomarker of individual health state since it has been shown to predict chances of survival in a range of endothermic species including humans. Oxidative stress is presumed to be a major cause of telomere shortening, but most evidence to date comes from *in vitro* cultured cells. The importance of oxidative stress as a determinant of telomere shortening *in vivo* remains less clear and has recently been questioned.

We therefore reviewed correlative and experimental studies investigating the links between oxidative stress and telomere shortening *in vivo*. While correlative studies provide equivocal support for a connection between oxidative stress and telomere attrition (10/18 studies), most experimental studies published so far (7/8 studies) partially or fully support this hypothesis. Yet, this link seems to be tissue-dependent in some cases, or restricted to particular categories of individual (*e.g.* sex-dependent) in other cases.

More experimental studies, especially those decreasing antioxidant protection or increasing pro-oxidant generation, are required to further our understanding of the importance of oxidative stress in determining telomere length *in vivo*. Studies comparing growing *vs.* adult individuals, or proliferative *vs.* non-proliferative tissues would provide particularly important insights.

Keywords: telomere, oxidative stress, ageing, senescence, antioxidant, DNA damage, review

Introduction

Because of the central function of telomeres in protecting chromosome ends and genome integrity, their study has gained interest in different domains of biology, ranging from cellular biology and epidemiology to ecology and evolutionary biology [1],[2]. It has been shown that telomeres shorten with age in a broad range of organisms [3,4], and more importantly that telomere length and/or shortening rate could predict subsequent survival [4,5]. Consequently, telomere length and/or attrition has been suggested to act as a biomarker of individual 'biological age'. Telomere dynamics has been linked to individual survival prospects, early-life growth conditions and reproductive success, but also to various physiological and psychological stressors. Telomeres are thus thought to be a biomarker of exposure to environmental challenges and individual lifestyle [1,2,6].

Although the pivotal role of telomeres in health and ageing biology is well recognized, our understanding of the physiological determinants of telomere dynamics *in vivo* is still imperfect. For instance, information regarding the *in vivo* effects of oxidative stress on telomere length and/or shortening rate remain limited since most studies conducted so far have used an *in vitro* approach. Yet, most studies on telomere dynamics make the assumption that, because there is an *in vitro* effect of oxidative damage on telomeres, it is also the case *in vivo*. The recent paper by Boonekamp et al. [7] highlights this limitation and the gap that exists in the literature on these *in vivo* effects.

With this review, we aim to provide a clearer picture of the situation by focusing on what we do and do not know about the *in vivo* links between oxidative stress and telomeres. We provide a brief summary of telomere structure and main mechanisms by which telomere length is regulated. We then cover the *in vivo* aspects of the impact of oxidative stress on telomere dynamics. We survey the literature and critically evaluate *in vivo* correlative and experimental studies investigating the link between oxidative stress and telomere length and/or shortening. Finally, we highlight several key parameters likely to contribute to the mixed results published so far, and propose different experimental approaches that should help to provide robust data in future studies.

Telomeres structure and shortening

Telomeres are protective DNA-protein complexes situated at the end of eukaryotic chromosomes, are made of non coding DNA sequences that consist of tandem repeats of a simple sequence of

nucleotides, which is rich in guanine (G) [8]. While the length of telomeres varies between chromosomes and species, the sequence is similar in all eukaryotes, indicating that telomeres are a highly conserved and ancient structure with a significant evolutionary role in protecting genome integrity [9].

The length of telomeres is dynamic and results from a balance between restoration and loss processes. Because DNA replication is a partially incomplete process, each time a cell divides telomeric DNA sequences of the chromosomes are lost, a phenomenon known as the 'end replication problem' [10]. Telomeres can shorten by 30 to 200 bp per cell division, but only 10 bp are thought to be due to the end replication problem in human cultured cells [11]. Oxidative stress leading to DNA damage is thought to be the main factor responsible for the remaining loss [12].

Oxidative stress can arise from the reactive oxygen species (ROS) generated from exogenous sources (UV radiation and pollutants), but the majority of intracellular ROS are thought to arise as a by-product of aerobic metabolism and ATP production in the mitochondria [13]. ROS are highly reactive and will cause oxidative damage to various biomolecules. Such damage can either be prevented by defence mechanisms known as antioxidant defences, or repaired in some cases after they occur. Oxidative stress is thus the result of an imbalance between antioxidant defences and ROS production. Due to their high guanine content, telomeres are thought to be especially sensitive to oxidative damage [14]. If not prevented, the oxidative damage of telomere regions will lead to an accumulation of damage to DNA and exacerbate telomere loss. Although oxidative damage can cause telomere shortening through double stranded breaks to DNA, most telomere loss due to oxidative stress occurs during DNA replication as a result of single-strand DNA damage [12]. As telomeric regions have a low efficiency of single-strand DNA damage repair, telomeres containing such single-strand DNA damage will not be fully replicated at the next cellular division. Therefore telomeres containing such DNA damage will shorten more following the next cellular division since the sequence beyond the damage will be lost [15]. Different mechanisms exist to maintain or restore telomere length, and the main one is telomerase activity, a ribonucleoprotein being able to elongate telomeres [16]. In the absence of restoration, telomere length shortens with each cell division; when the telomeres reach a critical length threshold, they induce a permanent arrest in the cell cycle known as cellular senescence, which may be followed by cell death. Given their role into cellular senescence, telomeres are thought to be also implicated into organismal senescence and ageing [1].

Most of the work looking at the effects of oxidative stress on telomere dynamics has been

conducted *in vitro*. Except for a couple of studies [17,18], most *in vitro* experiments have shown that oxidative stress accelerates telomere shortening [12,15,19]. Oxidative stress is therefore thought to mediate the effects of several environmental factors on telomere dynamics at the organismal level, but surprisingly *in vivo* effects of oxidative stress on telomere dynamics have been relatively poorly investigated, as highlighted in a recent publication [7].

What is the current evidence showing that oxidative stress shortens telomeres *in vivo*?

We searched the published literature using the Web of Science search engine in May 2017, using combinations of the following terms: telomere*, oxidative stress, antioxidant*, oxidative damage, correlation*, experiment*. We identified studies of interest reporting either correlations between oxidative stress markers (without restriction on the nature of the markers) and telomere length and/or shortening, or experimental manipulations of oxidative stress (antioxidant depletion/supplementation) and subsequent measures of telomere length and/or shortening.

Correlative studies**:**

Eighteen studies reported correlative information on the links between oxidative stress and telomeres (Table 1); 8 in humans and 10 in avian species. Overall, 10/18 studies report significant correlations between a variety of oxidative stress marker(s) and telomere length and/or attrition. Studies in human (6/8) were slightly more likely to report significant results than studies in birds (4/10). The methodology used for telomere measurement had no major effect on the outcome, with 2/5 studies using TRF and 7/12 studies using qPCR reporting significant results. Surprisingly, markers of oxidative damage were not more likely (6/14) to be associated with telomere length than markers of antioxidant defences (5/12). In birds, studies looking at telomere shortening were slightly more likely to find significant results than those looking at telomere length *per se* (4/8 *vs.* 1/8). Overall, the correlative evidence remains equivocal in supporting the assumption that oxidative stress contributes to telomere shortening *in vivo*.

Experimental studies**:**

In total, 8 studies used a controlled experimental approach (*i.e.* manipulation of oxidative stress) to investigate the links between oxidative stress and telomeres (Table 2). Two studies used L-buthionine sulfoximine (BSO) treatment to selectively reduce the endogenous levels of glutathione, an important intra-cellular antioxidant. The six other studies used supplementation with various antioxidants either alone or in combination, such as vitamin C and E, Coenzyme Q10

or methionine. Overall, 7/8 studies provide partial or total support for a significant effect of oxidative stress on telomere length and/or shortening rate. The only study not supporting this hypothesis [20] was conducted during embryonic development, when telomerase activity is supposed to be high, and did not show a clear effect on oxidative damage levels either. Still, it is worth noting that the effects of oxidative stress on telomere length are likely to be tissue-dependent [21], and in some cases restricted to particular groups of animals that might be more sensitive to changes in antioxidant defences than others [22,23]. Among the six studies measuring the impact of their treatment on oxidative damage levels, five of them obtained results that were mostly consistent between the effects of the treatment on oxidative damage on the one hand, and on telomere length and/or shortening on the other hand. Overall, the experimental evidence gathered so far mostly support the assumption that oxidative stress contributes to telomere shortening *in vivo*.

Limitations of the current correlative and experimental evidence

Several experimental aspects could explain the heterogeneity of results we found in studies looking at *in vivo* relationships between oxidative stress and telomere length. First of all, the tissues sampled as well as the timing of sampling are key parameters to consider. Indeed, it was shown that increased telomere shortening in response to oxidative stress is likely to be tissue-dependent [21]. However, most correlative studies (13/18) measured oxidative stress markers and telomere length in different tissue types (*e.g.* oxidative stress in plasma and telomere length in DNA isolated from blood cells). This probably precludes obtaining robust information since both variations in telomere length and oxidative stress markers can be tissue-dependent (*e.g.* [24,25]). Similarly, measuring oxidative damage to lipids/proteins but not to DNA is not ideal when testing the effect of oxidative stress on telomere length, since oxidative damage levels to different biomolecules are not necessarily correlated (*e.g.* [26,27]).

The timing of sampling to measure both oxidative stress and telomere length is also a key parameter to take into account. Indeed, oxidative stress levels are likely to vary much more quickly than telomere length. Moreover, most of the effects of oxidative stress on telomere length are supposed to be visible only after the next cellular replication, because single-strand damage are more likely to occur than double-strand breaks, and such single-strand damage will only shorten telomeres during replication [12]. Therefore, the effects of a rise in oxidative stress at a given time point might only be visible on telomere shortening later on. This implies that

166 experimental studies should look at telomere length long enough for replication to happen after
167 the manipulation occurred, but also that correlative studies should wisely choose their sampling
168 timing. For instance, one potential sampling strategy could be to measure 'initial' telomere length
169 and oxidative stress, measure 'final' telomere length later on (ideally considering the timing of
170 cellular division in the target tissue), and then correlate telomere shortening to initial oxidative
171 stress levels. Indeed, since telomere length is likely to be largely determined by inheritance and
172 early-life conditions [28,29], using the rate of telomere shortening will avoid this 'background
173 noise' in a correlation with oxidative stress levels. Accordingly, we show in supplementary material
174 (ESM S1 and S2) using one of our own dataset (data available in ESM S3) that such an approach
175 was the only one revealing a significant relationship between oxidative damage to DNA and
176 telomeres in coal tit (*Periparus ater*) nestlings (information on oxidative stress and telomeres
177 measurements were previously published separately in [30,31]).

178 The life stage at which animals are sampled is a paramount aspect to consider as well. For
179 instance, telomerase is likely to be active during embryo development, and potentially at later life
180 stages in particular tissues in some taxa [3]. This is important to consider, since it could mask the
181 true relationship between oxidative stress and telomere shortening *in vivo*. In addition, during the
182 growth period, the end replication problem during cellular division is likely to be one key driver of
183 telomere shortening, which can have different consequences that researchers should consider.
184 Indeed, the rapid cellular division and the associated end replication problem during growth could
185 reduce the likelihood of finding significant results in correlative studies, because it will decrease
186 the relative proportion of telomere shortening being linked to oxidative stress. Alternatively, rapid
187 cellular division linked to growth could increase the likelihood of detecting significant results in
188 experimental studies by converting rapidly single-strand damage into actual telomere shortening.

189 The nature of the experimental manipulation should also be carefully considered. Indeed, while
190 antioxidant supplementation studies detailed in Table 2 were quite successful in finding significant
191 beneficial effects on telomere length, any non-significant result of such supplementation would be
192 unsurprising in our opinion. Indeed, such antioxidant supplementation is likely to be beneficial
193 only if there is a need for extra antioxidants, but not if animals are not naturally resource-limited
194 [32]. This could explain why in some cases antioxidant supplementation was only beneficial for
195 some specific groups of animals [22,23].

196 Finally, other types of biases, such as statistical bias or publication bias could also skew our
197 understanding of the effects of oxidative stress on telomeres. Indeed, keeping in mind that

198 "correlation is not causation", the lack of significant correlation is definitively not a good support
199 against causation either. Importantly, the type II statistical error (i.e. 'false-negative') thus has to
200 be carefully considered before drawing conclusions about non-significant relationships (as done by
201 [7]), and sample sizes have generally to be very large to limit type II error. The potential bias
202 toward the publication of only significant results is also likely to alter the overall picture found in
203 the scientific literature so far. This is likely to be especially true in experimental studies as their
204 main focus is on the links between oxidative stress and telomere shortening; correlative studies
205 are probably less sensitive to this bias since they are often reporting the correlation between
206 oxidative stress and telomeres as part of other biological information.

207

208 **What should we do to move the field forward?**

209 We believe that only carefully designed experiments will provide a robust answer to the question
210 of the importance of oxidative stress for telomere shortening *in vivo*. Direct manipulation of ROS
211 production or down-regulation of antioxidant defences is undoubtedly a more powerful approach
212 than antioxidant supplementation, since supplementation is only efficient in response to a natural
213 limitation in antioxidant defences. However, manipulating ROS *in vivo* is very challenging as
214 highlighted in a recent review [33]. Still, some experiments using pro-oxidant molecules have been
215 successful in inducing moderate oxidative damage (*e.g.* [34]), and measuring telomere length in
216 such context should provide useful information. The selective down-regulation of the endogenous
217 antioxidant glutathione using L-buthionine sulfoximine (BSO) is undoubtedly one of the most
218 powerful tools available to researchers [21]. This manipulation is highly selective since BSO only
219 inhibits glutathione synthesis and does not affect other cellular pathways. It is also worth
220 mentioning that experimental studies are more likely to reveal a significant impact of oxidative
221 stress on telomeres than correlative studies. Indeed, it is possible that organisms under natural
222 conditions are able in most cases to maintain oxidative stress at a threshold level that does not
223 impact telomeres, while experimentally manipulating oxidative stress could disrupt such balance.

224 Regardless of the kind of experimental manipulation employed, it is important to validate the
225 impact of the treatment on oxidative damage (preferably on DNA) before examining the impact on
226 telomere length and/or shortening. If possible, oxidative damage and telomere length should be
227 measured in the exact same sample type. Investigating the impact of the treatment should be
228 done in several tissues since the most convincing study to date [21] found tissue-specific effects of
229 BSO on telomere length. As mentioned in the previous paragraph, life stage as well as tissue type

could constrain the effects of oxidative stress on telomere dynamics. Conducting the same experiment in both growing and adult individuals and comparing proliferative *vs.* non-proliferative tissues will thus be important, in order to assess the sensitivity of telomeres to oxidative stress at different life stages as well as the importance of cellular division in revealing the impact of oxidative stress on telomere length. Finally, given the various experimental constraints (*e.g.* repeated injections or continuous supplementation in water/food, close monitoring of health state) and ethical considerations, we suggest that such studies should be conducted in captive animals.

Conclusion

The limited number of studies investigating the *in vivo* connection between oxidative stress and telomere dynamics highlights that our understanding of this link still remains incomplete. Although the correlative studies display equivocal results, findings from the limited number of experimental studies conducted so far seem to indicate that oxidative stress affects telomere shortening *in vivo*. Yet, experimental studies are more likely to be susceptible to publication bias as mentioned above. The key to a better understanding of the impact of oxidative stress on telomere shortening *in vivo* will undoubtedly come from robust experimental studies, especially if conducted in a broad range of organisms since between-taxa differences in telomere biology do exist. Finally, when the number of published studies will be sufficient to overcome limitations linked to data heterogeneity, it will be of utmost importance to conduct a quantitative meta-analysis of the relationships between oxidative stress and telomere length *in vivo*.

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Author contributions

AS and SR had the original ideas and wrote the paper

Data accessibility

Data is accessible as an excel file in ESM S3

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Competing interests

Authors declare no competing interests.

Ethical statement

No ethical statement to declare

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Table 1: Summary of correlative studies conducted *in vivo* and testing the relationships between oxidative stress markers and telomere length (TL) and/or telomere shortening (ΔTL). The directions of the correlations are not presented in the table, since they were always in the predicted direction, namely that high oxidative damage were associated with shorter telomeres or faster telomere shortening, while high antioxidant levels were associated with longer telomeres or reduced telomere shortening. Method of telomere length measurement is indicated as quantitative PCR (qPCR), terminal restriction fragment (TRF) or quantitative fluorescence in situ hybridization (qFISH). RBCs refers to red blood cells. TAC refers to measurements of total antioxidant capacity; SOD refers to the antioxidant enzyme superoxide dismutase; glutathione is a major intra-cellular antioxidant; ROMs refers to reactive oxygen metabolites, a marker of overall early oxidative damage.

Species		Sample type (TL vs. OS)	Oxidative stress (OS) markers	TL method	Significant link between OS and TL / ΔTL	Reference
Human	*Homo sapiens*	Leukocytes vs. Urine	Urinary lipid damage	TRF	**TL: YES**	[35]
Human	*Homo sapiens*	Leukocytes vs. Plasma	Plasma TAC	TRF	**TL: YES**	[36]
Human	*Homo sapiens*	White blood cells vs. plasma	Plasma protein damage, Glutathione and SOD	TRF	**TL: NO** overall[1]	[37]
Human	*Homo sapiens*	Monocytes	DNA damage	qFISH	**TL: YES**	[38]
Human	*Homo sapiens*	Leukocytes	Oxidative stress genes polymorphism	qPCR	**TL: YES**	[39]
Human	*Homo sapiens*	White blood cells vs. plasma	Plasma non-enzymatic antioxidants	qPCR	**TL: YES**	[40]
Human	*Homo sapiens*	White blood cells vs. plasma	Plasma vitamins C and E	qPCR	**TL: YES**	[41]
Human	*Homo sapiens*	White blood cells vs. urine	Urinary lipid and DNA damage	qPCR	**TL: NO**	[42]
Great tit	*Parus major*	RBCs vs. plasma	Plasma ROMs and TAC	qPCR	**TL: NO** **ΔTL: YES** (ROMs)	[43]
Common yellowthroat	*Geothlypis trichas*	RBCs vs. plasma	Plasma TAC RBC DNA damage	qPCR	TL: not reported **ΔTL: YES** (TAC)	[44]
King penguin	*Aptenodytes patagonicus*	RBCs vs. plasma	Plasma ROMs and TAC	qPCR	**TL: YES** (TAC + ROMS) **ΔTL: YES** (ROMs)	[45]
King penguin	*Aptenodytes patagonicus*	RBCs vs. plasma	ROMs, TAC and DNA damage	qPCR	**TL: NO**	[46]
Coal tit	*Periparus ater*	RBCs vs. plasma	RBC DNA damage and plasma TAC	qPCR	**TL: NO** **ΔTL: YES** (DNA damage)	[30,31], see ESM
European starling	*Sturnus vulgaris*	RBCs vs. plasma	Plasma lipid damage	qPCR	**TL: NO** **ΔTL: NO**	[47]
European starling	*Sturnus vulgaris*	RBCs vs. plasma	Plasma DNA damage	qPCR	**TL: NO** **ΔTL: NO**	[48]
Zebra finch	Taeniopygia guttata	RBCs vs. plasma	Plasma ROMs + TAC, and RBC DNA damage	qPCR	**TL: NO** **ΔTL: NO**	[49]
Tree Swallow	*Tachycineta bicolor*	RBCs vs. plasma	Plasma ROMs + TAC	TRF	**TL: NO**	[50]
Jackdaw	*Corvus monedula*	RBCs vs. plasma	Plasma ROMs + lipid damage, RBC glutathione	TRF	TL: not reported **ΔTL: NO**	[7]

[1] except for a significant correlation between protein damage and TL in Parkison disease patients only.

Table 2: Summary of experimental studies conducted *in vivo* and testing the effects of antioxidant depletion or supplementation on telomere length (TL) and/or telomere shortening (ΔTL). Method of telomere length measurement is indicated as quantitative PCR (qPCR), terminal restriction fragment (TRF) or quantitative fluorescence in situ hybridization (qFISH), and arrows describe decrease (↘), increase (↗) or non-significant (↔) effects.

Species		Type of study	Tissue type	Oxidative damage	TL Method	Telomere length (TL) / Telomere shortening (ΔTL)	Significant link between OS and TL / ΔTL	Reference
CAST/Ei mouse	*Mus musculus*	**Antioxidant depletion**	12 tissues	↗ **Protein damage**	TRF	↘ **TL** in 5/12 tissues	**YES**, but tissue-dependent	[21]
New Zealand White Rabbit	Oryctolagus cuniculus	**Antioxidant depletion**	Contralateral arteries	↗ **Oxidized glutathione**	TRF	↘ **TL**	**YES**	[51]
Wistar rat	*Rattus norvegicus*	**Antioxidant supplementation**	Heart	↔ Lipid damage ↘ **Protein damage**[2]	TRF	↘ **ΔTL and ↗ TL**	**YES**	[52]
Broiler chicken	*Gallu gallus*	**Antioxidant supplementation**	Lymphocytes	↘ **DNA damage** for vit C and E	qFISH	↘ **ΔTL** for vit E but not vit C	**YES**, but for vit E only	[53]
Blue tit	*Cyanistes caeruleus*	**Antioxidant supplementation**	RBCs	not measured	qPCR	↘ **ΔTL**	**YES**	[54]
Zebra finch	Taeniopygia guttata	**Antioxidant supplementation**	RBCs vs. plasma	↔ Lipid damage[1]	qPCR	↘ **ΔTL and ↗ TL in Females** ↔ TL and ↔ ΔTL in Males	**YES** in females	[22]
Yellow-legged gull	*Larus michahellis*	**Antioxidant supplementation**	RBCs	not measured	qPCR	↗ **TL** in 'bold' chicks ↔ TL in 'fearful' chicks	**YES** in 'bold' chicks	[23]
Yellow-legged gull	*Larus michahellis*	**Antioxidant supplementation**	RBCs vs. plasma	↔ Lipid and protein damage	qPCR	↔ TL	**NO**	[20]

[1] presented in [55]; [2] in the 'recuperated' group at 3 month of age only