Melatonin reduces oxidative stress in erythrocytes and plasma of senescence-accelerated mice

Introduction

Aging is the progressive accumulation of changes associated with or responsible for the increasing susceptibility to disease and death, which accompanies advancing age. The nature of the aging process has been the subject of considerable speculation. Accumulating evidence now indicates that the sum of the deleterious free radical reactions going on continuously in all cells and tissues constitutes the aging process or is a major contributor to it. This process can be modified by genetic and environmental factors. In mammalian systems, free radical reactions are largely those involving oxygen [1]. According to the free radical theory of aging, the oxidative reactions that occur in the organism generate free radicals as a by-product, and these cause multiple lesions in macromolecules (nucleic acids, proteins, and lipids) resulting in aging. This theory explains not only the mechanism of aging per se and the life span, but also a wide variety of age associated pathologies such as cancer, degenerative diseases, and other age-associated phenomena [1, 2]. Moreover, the free radical theory of aging supports the use of antioxidants for counteracting these effects and a number of studies have shown that antioxidants can reduce oxidative damage improving the quality of life [3–9]. On the other hand, other studies are contradictory [3, 10, 11] or do not provide convincing evidence that antioxidant administration can slow aging [12].

Melatonin is one of the molecules known as a ‘geroprotector’, which means capable of ‘preventing aging’. It acts as a direct scavenger of free radicals, detoxifying both reactive oxygen and nitrogen species, and indirectly increasing the capacity of melatonin to extend life span or to improve the prooxidant actions [18–23]. Thus, the results about the potent antioxidant, under some conditions, it may have AMK, are also excellent free radical scavengers [16, 17]. Although a majority of the studies confirm that melatonin is a potential antioxidant, under some conditions, it may have prooxidant actions [18–23]. Thus, the results about the capacity of melatonin to extend life span or to improve the parameters of oxidative stress are still unclear [2], but clinical trials have demonstrated that exogenous melatonin is safe and may be useful for managing several conditions [24–26].

Various studies are currently being performed using the senescence-accelerated mouse (SAM) model to clarify the mechanisms that underlie the aging. There are two types of SAM strains: senescence-prone inbred strains (SAMP) and senescence-resistant inbred strains (SAMR). The pathological phenotypes in SAMP8 mice show decreased learning ability, memory loss, impaired immune response,
abnormal circadian rhythms, loss of activity, hair loss and lack of glossiness, skin coarseness, periorbital lesions, increased lordokyphosis, and early death. SAMR1 mice, on the other hand, show nonthymic lymphoma, histiocytic sarcoma, and ovarian cysts [27]. In 1999, data from SAM strains reared under conventional conditions showed that the median survival time of SAMP mice was 9.7 months, 40% shorter than that of the SAMR strains (16.3 months). The grading score of the senescence of SAMP strains at 8 months of age was 7.97, about twice that of SAMR (3.94) [27]. SAMP strains also have a much higher incidence of amyloidosis (77.9%) than SAMR strains (32%) [28].

Based on these data, the main goal of the present study was to determine the oxidative balance in blood of SAMP8 and SAMR1 mice, and to assess whether long-term melatonin treatment may counteract the age-dependent oxidative stress.

Material and methods

Animals

Sixty-four SAMP8 mice (32 males and 32 females) and 64 SAMR1 mice (32 males and 32 females) of 1 month of age were obtained from the University of Granada’s facility (Spain). The progenitors were purchased from Harlan Ibérica S.L. (Barcelona, Spain). They were all housed and maintained in a room at 22 ± 2°C with automatic light cycles (12-hr light/dark) and a relative humidity of 40–60%. Food (Panlab rat chow, Panlab, Barcelona) and treated tap water were offered ad libitum throughout the study. The procedures applied in the study were approved by the Ethics Committee of Animal Research, ‘Rovira i Virgili’ University (Tarragona, Spain), and they were carried out according to the Spanish Government Guide and the European Community Guide for animal care.

Chemicals

Melatonin was purchased from Sigma (St Louis, MO, USA). Other reagents were of the highest quality available and obtained from commercial sources.

Treatment

After a quarantine period of 7 days, 16 SAMP8 male mice, 16 SAMP8 female mice, 16 SAMR1 male mice, and 16 SAMR1 female mice were treated with melatonin for 9 months. Melatonin was dissolved in absolute ethanol and added to the drinking water in a final ethanol concentration of 0.066% in feeding bottles protected from light. A fresh melatonin solution was prepared twice a week (Monday and Thursday) depending on the water consumption and the weight of the animals so that they obtained a melatonin dose of 10 mg/kg/day. For example, at the end of the study, for the SAMP8 female mice, their mean weight was 32.3 ± 3.4 g, their water consumption was 5 mL/day and the melatonin concentration in the drinking water was 0.065 mg/mL. Treatment began when animals were 1 month old. The remaining mice (four control groups) received 0.066% ethanol in their drinking water.

Tissue samples

At the end of the period of treatment, mice were anesthetized with an i.p. injection of ketamine-xylazine (100–10 mg/kg, respectively) dissolved in 0.9% saline. Blood was obtained by heart puncture of each animal until exsanguination. Blood from half of the mice was put into a tube with sodium–ethylene diamine tetraacetic acid, and the other half was put into tubes with sodium–heparin 1% so that there was enough sample to determine all the parameters (therefore, n was 8 in each group). Plasma and erythrocytes were obtained and the following parameters were determined.

Biochemical analyses

In erythrocytes and plasma, reduced (GSH) and oxidized (GSSG) glutathione were determined by the Hissin and Hilf method [29]; TBARS were determined with the Buege and Aust [30] method modified for fluorimetric detection with the Richard et al. [31] method. In erythrocytes, total GST activity was determined by the Habig et al. [32] method using 1-chloro-2,4-dinitrobenzene as substrate; CAT activity was determined by the Cohen et al. [33] method; SOD activity by the Misra and Fridovich [34] method; GPX selenium-dependent and selenium-independent (GPX) and GR by the method of Wheeler et al. [35], and the hemolysis by the method of Farrell et al. [36]. Finally, hemoglobin (Hb) was determined in whole blood using a commercial kit (QCA, S.A., Amposta, Spain) based on the Drabkin reagent.

Statistical analysis

Statistical analyses were performed with the spss program. Normality was tested by the Kolmogorov–Smirnov test, and mean comparisons were made by Student’s t-test. Results are expressed as mean values ± S.E.M. The level of statistical significance for all tests was P < 0.05.

Results

Table 1 shows the results of the oxidative stress parameters between the male and female SAMP8 and SAMR1 control mice. Signs of greater oxidative stress are apparent in senescent mice of both sexes. In comparison with SAMR1 males, the GST activity and the GSH levels of SAMP8 males were 25% and 32% lower, respectively, and they had twice as much GSSG and TBARS in erythrocytes. In plasma, GSH was almost three times lower. To compensate for these imbalances, their GPX and GR activities were 92% and 52% higher, respectively.

Comparisons between SAMP8 and SAMR1 female control groups indicate that the SAMP8 females had lower erythrocyte GSH (57%) but more than twice as much plasma GSH. In erythrocytes, SAMP8 had GSSG (71%) and TBARS (28%) higher than SAMR1, and plasma TBARS were almost four times higher than SAMR1. In turn, the GPX and GR activities were greater in SAMP8 than in SAMR1 (83% and 30%, respectively).
In the groups of male mice treated with melatonin (Table 2), erythrocytes of the SAMP8 males hemolyzed 30% less than SAMR1, and SAMP8 males had 38% less plasma GSSG than SAMR1. Compared with SAMR1, SAMP8 mice had lower GST activity (35%), lower erythrocyte and plasma GSH content (23% and 51%, respectively), and higher erythrocyte GSSG (95%) and plasma TBARS (23%). The only compensatory mechanism displayed in SAMP8 mice was the increased GR activity (81%).

Regarding female mice treated with melatonin (Table 2), SAMP8 had higher erythrocyte GSH (32%), lower plasma TBARS (37%), and higher plasma GSSG than SAMR1 mice. GR activity was increased (30%), and CAT and GPX were decreased by 24% and 36%, respectively, in SAMP8 mice compared with SAMR1.

When the mice treated with melatonin were compared with controls, it was observed that melatonin caused significant changes in some parameters, which suggest lower oxidative stress or greater antioxidant status than untreated mice. Figs 1–6 show the parameters that were most affected by the treatment. The same pattern can be observed for all parameters, i.e. a reduction of oxidative stress because of melatonin treatment. Thus, Fig. 1 shows lower erythrocyte SOD activity in males (60%) and females (25%) of SAMR1 and in males of SAMP8 strains (42%) in melatonin-treated mice. Fig. 1 also shows that SOD activity was lower in males than in females, although in the control SAMR1 group these differences were not significant.

Fig. 2 documents an increase in plasma GSH in all groups treated with melatonin, with significant differences in SAMR1 males and females, and in SAMP8 males. In SAMP8 and SAMR1 males, melatonin induced a strong increase in plasma GSH. Comparing plasma GSH between males and females, both control and treated SAMR1 males and treated SAMP8 males had higher plasma GSH than the corresponding females. Plasma GSH was also higher in control SAMP8 females than in males.

Table 1. Oxidative stress parameters in blood of senescence-accelerated mice (SAMP8) and SAMR1 control mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SAMP8</th>
<th>SAMR1</th>
<th>SAMP8</th>
<th>SAMR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysis (%)</td>
<td>95.88 ± 18.10*</td>
<td>85.25 ± 3.70</td>
<td>79.69 ± 10.39</td>
<td>85.06 ± 3.84</td>
</tr>
<tr>
<td>Glutathione S-transferase (GST) (µmol/min/g Hb)</td>
<td>3.30 ± 0.87*</td>
<td>4.45 ± 1.09†</td>
<td>2.58 ± 0.50</td>
<td>2.20 ± 0.39</td>
</tr>
<tr>
<td>Glutathione (GSH) er (µmol/g Hb)</td>
<td>5.34 ± 1.18†</td>
<td>7.86 ± 1.42</td>
<td>3.30 ± 0.67*</td>
<td>7.74 ± 1.53</td>
</tr>
<tr>
<td>Oxidized glutathione (GSSG) er (µmol/g Hb)</td>
<td>1.89 ± 0.19*</td>
<td>0.94 ± 0.23†</td>
<td>2.84 ± 0.62*</td>
<td>1.66 ± 0.37</td>
</tr>
<tr>
<td>GSSG/GSH</td>
<td>0.36 ± 0.07†</td>
<td>0.12 ± 0.04†</td>
<td>0.88 ± 0.24*</td>
<td>0.22 ± 0.06</td>
</tr>
<tr>
<td>Thiobarbituric acid reactive substances (TBARS) er (nmol/g Hb)</td>
<td>3.35 ± 1.69*</td>
<td>1.67 ± 0.36†</td>
<td>2.97 ± 0.66*</td>
<td>2.32 ± 0.45</td>
</tr>
<tr>
<td>Catalase (CAT) er (nmol/min/g Hb)</td>
<td>46.02 ± 10.63†</td>
<td>52.11 ± 5.52†</td>
<td>77.80 ± 18.40</td>
<td>61.95 ± 9.47</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPX) er (U/g Hb)</td>
<td>230 ± 54*</td>
<td>119.57 ± 37.40</td>
<td>211 ± 38*</td>
<td>115.50 ± 23.05</td>
</tr>
<tr>
<td>Glutathione reductase (GR) er (U/g Hb)</td>
<td>5.78 ± 1.38*</td>
<td>3.80 ± 0.91†</td>
<td>6.89 ± 1.05*</td>
<td>5.27 ± 0.80</td>
</tr>
<tr>
<td>Superoxide dismutase (SOD) er (U/g Hb)</td>
<td>1473 ± 182†</td>
<td>1864 ± 637</td>
<td>2225 ± 270</td>
<td>2428 ± 617</td>
</tr>
<tr>
<td>GSH pl (nmol/mL)</td>
<td>10.72 ± 3.26*</td>
<td>30.16 ± 4.18†</td>
<td>22.35 ± 9.70*</td>
<td>9.34 ± 2.18</td>
</tr>
<tr>
<td>GSSG pl (nmol/mL)</td>
<td>24.20 ± 3.33</td>
<td>35.23 ± 13.75</td>
<td>22.60 ± 2.31*</td>
<td>31.95 ± 6.71</td>
</tr>
<tr>
<td>GSSG/GSH pl</td>
<td>2.40 ± 0.68*</td>
<td>1.19 ± 0.50†</td>
<td>1.11 ± 0.27*</td>
<td>3.50 ± 0.69</td>
</tr>
<tr>
<td>TBARS pl (nmol/mL)</td>
<td>1.94 ± 0.42†</td>
<td>1.79 ± 0.15*</td>
<td>5.17 ± 1.31*</td>
<td>1.30 ± 0.26</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E.M. er, erythrocytes; pl, plasma; *P < 0.05 comparison between SAMP8 and SAMR1; †P < 0.05 comparison between males and females.

Table 2. Oxidative stress parameters in blood of SAMP8 and SAMR1 mice treated with melatonin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SAMP8</th>
<th>SAMR1</th>
<th>SAMP8</th>
<th>SAMR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysis (%)</td>
<td>60.69 ± 16.4**†</td>
<td>85.93 ± 3.66</td>
<td>90.62 ± 4.07</td>
<td>73.83 ± 30.64</td>
</tr>
<tr>
<td>GST (µmol/min/g Hb)</td>
<td>2.64 ± 0.31*</td>
<td>4.05 ± 1.21</td>
<td>3.14 ± 0.58</td>
<td>3.23 ± 0.72</td>
</tr>
<tr>
<td>GSH er (µmol/g Hb)</td>
<td>5.64 ± 1.44**†</td>
<td>7.32 ± 1.47†</td>
<td>3.26 ± 0.80*</td>
<td>2.46 ± 0.80</td>
</tr>
<tr>
<td>GSSG er (µmol/g Hb)</td>
<td>2.50 ± 0.43*</td>
<td>1.28 ± 0.51†</td>
<td>2.93 ± 0.42</td>
<td>2.06 ± 0.52</td>
</tr>
<tr>
<td>GSSG/GSH</td>
<td>0.45 ± 0.06**†</td>
<td>0.17 ± 0.07†</td>
<td>0.96 ± 0.30</td>
<td>0.87 ± 0.13</td>
</tr>
<tr>
<td>CAT er (nmol/min/g Hb)</td>
<td>2.27 ± 0.46†</td>
<td>1.98 ± 0.50</td>
<td>1.67 ± 0.51</td>
<td>2.03 ± 0.30</td>
</tr>
<tr>
<td>GPX er (U/g Hb)</td>
<td>47.16 ± 17.30</td>
<td>61.14 ± 12.72</td>
<td>56.67 ± 11.7*</td>
<td>74.28 ± 16.57</td>
</tr>
<tr>
<td>GR er (U/g Hb)</td>
<td>183 ± 59</td>
<td>207 ± 52†</td>
<td>187 ± 51*</td>
<td>292 ± 77</td>
</tr>
<tr>
<td>SOD er (U/g Hb)</td>
<td>6.73 ± 1.47**†</td>
<td>3.71 ± 0.79</td>
<td>4.78 ± 0.73*</td>
<td>3.70 ± 0.58</td>
</tr>
<tr>
<td>GSH pl (nmol/mL)</td>
<td>858 ± 268†</td>
<td>741 ± 170†</td>
<td>1856 ± 337</td>
<td>1829 ± 374</td>
</tr>
<tr>
<td>GSSG pl (nmol/mL)</td>
<td>33.85 ± 5.67**†</td>
<td>69.75 ± 15.1†</td>
<td>25.49 ± 2.84</td>
<td>23.99 ± 6.14</td>
</tr>
<tr>
<td>GSSG/GSH</td>
<td>16.23 ± 2.08**†</td>
<td>26.03 ± 5.13†</td>
<td>24.40 ± 2.21*</td>
<td>19.42 ± 3.99</td>
</tr>
<tr>
<td>TBARS pl (nmol/mL)</td>
<td>0.49 ± 0.07†</td>
<td>0.39 ± 0.10†</td>
<td>0.97 ± 0.13</td>
<td>0.83 ± 0.16</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E.M. er, erythrocytes; pl, plasma; *P < 0.05 comparison between SAMP8 and SAMR1; †P < 0.05 comparison between males and females.
Melatonin lowers oxidative stress in SAM mice

Fig. 1. Superoxide dismutase levels in erythrocytes of senescence-accelerated mice (SAMR1) and SAMP8 mice treated with and without melatonin. Data represent mean ± S.E.M. n = 8. *P < 0.05 compared with values in the control group; †P < 0.05 compared with values in the female group.

Fig. 2. Reduced glutathione levels in plasma of SAMR1 and SAMP8 mice treated with and without melatonin. Data represent mean ± S.E.M. n = 8. *P < 0.05 compared with values in the control group; †P < 0.05 compared with values in the female group.

Fig. 3. Oxidized glutathione levels in plasma of SAMR1 and SAMP8 mice treated with and without melatonin. Data represent mean ± S.E.M. n = 8. *P < 0.05 compared with values in the control group; †P < 0.05 compared with values in the female group.

Fig. 4. Thiobarbituric acid reactive substances levels in plasma of SAMR1 and SAMP8 mice treated with and without melatonin. Data represent mean ± S.E.M. n = 8. *P < 0.05 compared with values in the control group; †P < 0.05 compared with values in the female group.

Fig. 5. Thiobarbituric acid reactive substances levels in erythrocytes of SAMR1 and SAMP8 mice treated with and without melatonin. Data represent mean ± S.E.M. n = 8. *P < 0.05 compared with values in the control group; †P < 0.05 compared with values in the female group.
Fig. 3 shows that melatonin administration significantly reduced plasma GSSG in SAMR1 females and in SAMP8 males compared with controls. In comparison with females, males also had more plasma GSSG, except in the case of senescent females treated with melatonin. The calculated GSSG/GSH ratio was also lower in SAMR1 mice of both sexes (results not shown).

Melatonin also significantly decreased plasma TBARS in SAMR1 males and SAMP8 females (Fig. 4), and erythrocyte TBARS in SAMP8 females (Fig. 5). Senescent females treated with melatonin had lower lipid peroxidation than males, both in plasma (Fig. 4) and in erythrocytes (Fig. 5). Control SAMR1 females presented less TBARS in plasma (Fig. 4) and more TBARS in erythrocytes (Fig. 5) than the corresponding males. Control SAMP8 males presented lower plasma lipid peroxidation levels than the corresponding females (Fig. 4).

Finally, melatonin treatment increased erythrocyte GPX activity in SAMR1 mice of both sexes but had no effect on senescent mice (Fig. 6). There were no differences between the sexes in this parameter.

**Discussion**

Our results report for the first time the blood changes in oxidative stress markers and the effect of chronic melatonin administration on them in SAMP mice. In our study, we administered melatonin in drinking water throughout a 9-month period beginning during the first month of life. In other studies, melatonin had been administered orally or injected in a wide range of doses, different animal species and ages, length of treatment, and time of administration (day/night). Concerning the effect of melatonin administration on animal survival, Anisimov [2] reviewed the results of various studies that were very different; only one of these studies was carried out with female SAM mice (SAMP1 and SAMR1), which were treated with melatonin at the half of the dose used in the present study, at night in drinking water, from three months old until they died a natural death. In this study, no effect was observed on the mean life span.

In the present SAMR1 female group treated with melatonin, two mice presented subcutaneous tumors. In the SAMR1 female control group, one mouse had a subcutaneous tumor while another had a tumor in the lung. These data match those observed by other authors [27] and show a greater tendency for tumorigenesis in the SAMR1 strain, which is one of its phenotype characteristics. The appearance of tumors in our experiment, was independent of the treatment of melatonin, because they were observed in both control and treated animals. The cause of these phenotypic changes in SAM strains may relate to the fact that the AKR/J mouse strain, progenitor of the SAM strains, has high levels of endogenous ecotropic murine leukemia virus (MuLV), which can cause a variety of changes in mice, deficits in learning and memory and other senescence-related changes in SAMP mice [37, 38]. Although some authors reported an increase of the incidence of tumors in mice of different strains and both genders after melatonin treatment [39–41], most of the studies confirm the oncostatic properties of the indoleamine [42–45]. The lack of significant differences between control and melatonin-treated groups in our experimental model in terms of tumorigenesis may be related to the timing of tissue collection. Perhaps maintaining mice for a period longer than 10-month differences between melatonin-treated and untreated mice would have appeared.

Body weight at 10 months showed no significant differences between controls and the melatonin-treated mice in any of the groups studied. These data match those from studies carried out in mice of different strains and ages by other authors, who concluded that treatment with melatonin does not affect the body weight of mice [2, 46].

Our results clearly indicate that SAMP8 have greater oxidative stress than SAMR1. The parameters most involved were those related to both erythrocyte and plasma glutathione, the GR and GST enzymes, which use GSSG and GSH as substrate, respectively, the GPX which reduces peroxides by converting GSH into GSSG, and the levels of lipid peroxidation. GSH, a major cellular antioxidant, has various biological functions, one of which is to protect cells from damage by substances such as reactive oxygen species, free radicals, and reactive metabolites. It is a scavenger of free radicals per se, and it also acts as a substrate for antioxidant and detoxifying enzymes such as GPX and GST. Our data show that erythrocyte GSH was lower and erythrocyte GSSG was higher in SAMP8 mice than in SAMR1. These data agree with the low GSH content found in many tissues in old animals, including liver, kidney, blood, and brain [47–49]. Erythrocyte TBARS increased in both male and female control SAMP8 mice, indicating greater oxidative stress that might depend on the deficit of GSH in these groups. In the melatonin-treated groups, however, erythrocyte TBARS did not show significant differences between SAMP8 and SAMR1, perhaps because of the antioxidant effect of the melatonin. Moreover, GST activity was reduced in SAMP8 with respect to SAMR1 in control and treated males. Together with the lower level of GSH, the substrate of this enzyme, this result means that oxidative stress was greater in males than in females.
Some compensatory mechanisms were found in our study. The increase in erythrocyte GSSG in the four groups of SAMP8 induced GR to reduce the GSSG and convert it into GSH. This is the reason for the increased activity of GR in these groups of mice with respect to SAMR1. GPX, another enzyme responsible for converting hydrogen peroxide and lipid peroxides into inoffensive molecules, increased in both male and female SAMP8 mice, but only in controls. In the melatonin-treated groups, this enzyme was not induced in mice with accelerated aging, perhaps because of the antioxidant effect of melatonin.

Our results show that SOD decreased after melatonin treatment. Some authors reported that melatonin increases SOD activity in various tissues of different animal species [13–15, 50], whereas other did not find changes in SOD activity after melatonin administration [51–53]. In a study on SAMP8 and SAMR1 mice, melatonin did not increase SOD activity in the cerebral cortex [54]. The effects that melatonin has on SOD activity may depend on the redox status and the activity of the other antioxidant defense systems. Thus, if the oxidative stress is high and the amount of melatonin available is insufficient to counteract it, then SOD activity will probably increase. However, if there is sufficient melatonin to scavenge free radicals, SOD activity has no need to increase and may even decrease, as we found in our results.

The effect of melatonin on the redox state of GSH observed here is in agreement with that of many other studies that show an increase in GSH and a decrease in GSSG in different tissues: for example, in isolated mitochondria of brain and liver [55–59], in rat gastric mucosa and testicles [60], and the hippocampus, cortex and cerebellum of rats [61–63]. Numerous studies also observed that melatonin decreased lipoperoxidation, either quantifying the TBARS [50, 51, 54, 59, 61–63], as in our case, or other parameters such as ketodienes and conjugated hydroperoxides [52, 55]. Many studies have also described that GPX is another antioxidant enzyme that is increased by melatonin. But only Okatani et al. [54] studied SAM mice and showed that GPX increased in the cerebral cortex of melatonin-treated SAMP8 and SAMR1 mice. In our work, erythrocyte GPX only increased in male and female SAMR1 mice, but not in senescent males and females.

In conclusion, our results show an increase in age-dependent oxidative stress in senescent mice, which chronic melatonin administration was able to improve. The main emphasis of the present study was to evaluate a number of parameters; this made it possible to assess the overall age-dependent oxidative imbalance in the blood. This approach might be easily reproduced in human studies to evaluate antioxidant therapies. Because of the high bioavailability of melatonin after its oral intake and the lack of side effects, an antioxidant supplement with melatonin may be of great interest for reducing age-dependent oxidative damage.

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References


