The Investigation of Damage in the Muscle Tissue with the Oxidant/Antioxidant Balance and the Extent of Postmortem DNA Damage in Rats

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Abstract: The relationship of the extent of DNA damage and certain components of the oxidant/antioxidant balance in rat femoral muscle tissue with the post-mortem intervals was investigated and examined by making direct comparison with the histopathological findings. The results have shown that the oxidant/antioxidant balance in the striated muscle tissue to remain constant at the 0 hours post-mortem; to shift mildly in favour of the oxidants at 2 hours post-mortem; to shift moderately in favour of the oxidants at 3 hours post-mortem; and, to shift severely in favour of the oxidants at 4 and 5 hours post-mortem. The amount of DNA damaged products was detected to increase at 4 and 5 post-mortem hours when the oxidant/antioxidant balance became severely shifted. The biochemical findings were found to be in full accordance with the histopathological findings. The above-mentioned findings indicate that DNA becomes subjected to degradation at the hours after post-mortem when the oxidants are strongly predominate.

Keywords: Post-mortem interval; muscle; rat; oxidant; antioxidant; DNA damage

1. Introduction

As it is known, there appears under physiological circumstances to be a trade-off in favour of the antioxidants within the oxidant/antioxidant balance in living tissues (Clarkson and Thompson, 2000). Shift in this balance towards the oxidants give rise to a condition called oxidative stress and cellular death (Halliwell et al., 1992; Serafini and Del Rio, 2004). This literature information documents the free radicals to be associated with cellular death. However, tangible information with regard to the course of the oxidant/antioxidant balance during the post-mortem period has yet to be acquired. Despite continuous generation of free radicals in the cells, endogenous antioxidant defence mechanisms protect the tissues from the detrimental effects exerted by the free oxygen radicals (Ames et al., 1993). Not only do the free radicals cause damage the lipids and the proteins, but they also damage DNA, upon insufficiency of the antioxidant defence mechanisms, 8-hydroxyguanine (8-OHGuA), a DNA oxidation product, reflects the DNA damage (Grollman and Moriya, 1993; Malkoc et al., 2012). Essentially, a balance has been reported to be maintained under normal circumstances between the oxidative damage and repair in DNA (Collins et al., 1996). 8-OHGuA has been experimentally demonstrated to increase in concordance with an increase in the oxidants within damaged tissue (Keles et al., 2009; Polat et al., 2010). The aforementioned data indicate presence of a significant correlation of the oxidant/antioxidant parameters with the tissue damage and repair. As it is already known, only the organs which were extracted just after the decapitation process without being subjected to no other process are used as healthy tissues in experimental studies. These healthy tissues correspond to the zero hour of death. Upon a meticulous literature search, we could not encounter any literature data regarding the levels of oxidant/antioxidant parameters and the extent of DNA damage at different post-mortem intervals. An answer to such a question as time of death has been sought in forensic autopsies. To specify reliably the time of death is the topmost duty of the forensic medicine (Henssge and Madea, 2004). A sizable number of factors are capable of obtaining the time of death more complicated (Poloz and O'Day, 2009;
Prieto-Castello et al., 2007). It is for this reason that specifying the time of death has long persisted as one of the most significant challenges of forensic medicine. No study with respect to whether or not a significant correlation existed among the levels oxidant/antioxidant parameters, the extent of DNA damage and the time duration elapsing since death (post-mortem interval) has yet to be encountered during our literature search.

The purpose of our study was to investigate and examine by comparing to the histopathological findings whether or not a significant correlation was present among the levels of oxidant/antioxidant parameters, the extent of DNA damage and post-mortem intervals in the femoral muscle tissue of rats.

2. Material and Methods

2.1. Animals

A total of 60 albino male Wistar rats, with weights ranging from 200 to 210 gr, were obtained from Ataturk University’s Medical Experimental Practice and Research Centre. All of the animals were assigned into six groups, with each group containing a total of ten rats, and were fed at normal room temperature (22°C). Afterwards, they were all decapitated, followed by extraction of specimens from the femoral muscles at 1 hour intervals (0, 1, 2, 3, 4 and 5 hours). The required biochemical and histopathological examinations were performed on the specimens, evaluating the results obtained from each animal group by directly comparing them to each other.

2.2. Procedure of the experiment

The rats were divided into six subgroups (n=10), fed at room temperature (22°C) and then decapitated. The femoral muscle of the dead animals were extracted at intervals of one hour (at zero, first, second, third, fourth and fifth hours) and biochemical examinations were fulfilled. Biochemical results obtained from the animal groups were evaluated after being compared to each other.

2.3. Biochemical analyses of femoral muscle tissue

2.3.1. Superoxide Dismutase (SOD) analysis

SOD activity was measured according to Sun et al (Sun et al., 1988). Estimates were based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with Nitroblue Tetrazolium to form formazan dye. SOD activity was then measured at 560 nm in relation to the degree of inhibition of this reaction; SOD activity is expressed as U/gr. protein.

2.3.2. Malondialdehyde (MDA) analysis

For the MDA assay, tissue samples were homogenised so that each 0.2 gr. gramme of tissue contained 2ml. of a 1.15% KCl solution. MDA was determined by spectrophotometry of the pink-coloured product of the thiobarbituric acid-reactive substances complex (Ohkawa et al., 1979). Thiobarbituric acid- MDA colour complex was measured at 532 nm. Total thiobarbituric acid-reactive substances were expressed as MDA. Results were expressed as μmol/g protein.

2.3.3. Glutathione Peroxidase (GPO) analysis

GPO activity was determined according to the method of Lawrence and Burk (Lawrence et al., 1974). The absorbance at 340nm was recorded for 5 minutes, and the activity was defined as the rate of NADPH oxidation. Results were expressed as U/gr. protein.

2.3.4. Glutathione S-Transferases (GST) Analysis

The supernatant’s GST activity was measured by using 1-chloro-2, 4-dinitrobenzene and GSH as described in Habig et al (Habig et al., 1974). Results were expressed as U/g protein.

2.3.5. Total Glutathione (GSH) analysis

The amount of GSH in the femoral muscle tissue was measured according to the method described by Sedlak and Lindsay (Sedlak and Lindsay, 1968). The absorbance was measured at 412 nm using a spectrophotometer. The results of the GSH level in the liver tissue are expressed as nmol/g protein.

2.3.6. DNA analysis

Femoral muscle tissue was drawn and DNA isolated using Shigenaga et al.’s modified method (Shigenaga et al., 1994). Samples were homogenised at 4 °C in 1ml of homogenisation buffer (0.1 M NaCl, 30 mM Tris, pH 8.0, 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.5% (v/v) Triton X-100) with six passes of a Teflon-glass homogeniser at 200rpm. The samples were centrifuged at 4 °C for 10 minutes at 1000g to pellet nuclei. The supernatant was discarded, and the crude nuclear pellet re-suspended and re-homogenised in 1ml of extraction buffer (0.1 M Tris, pH 8.0, 0.1 M NaCl, 20 mM EDTA) and re-centrifuged as above for 2 minutes. The washed pellet was re-suspended in 300μl of extraction buffer with a wide-orifice 200-μl Pipetman tip. The re-suspended pellet was subsequently incubated at 65°C for 1 hour with the presence of 0.1ml of 10% SDS, 40μl proteinase K, and a 1.9-ml leukocyte lysis buffer.

Then, ammonium acetate was added to the crude DNA sample to give a final concentration of 2.5mol/L, and centrifuged in a micro centrifuge for 5 minutes. The supernatant was removed and mixed with two volumes of ethanol to precipitate the DNA fraction. After centrifugation, the pellet was dried under reduced pressure and dissolved in sterile water. The absorbance of this fraction was measured at 260 and 280nm. Purification of DNA was determined as A 260/280 ratio 1.8.

2.3.7. Histopathological examination
The striated femoral muscles extracted from the rats were fixated with 10% formaldehyde solution. Following routine tissue processing, sections 4µm in thickness were obtained from the paraffin blocks prepared. These sections were then stained with Haematoxylin and Eosin (H&E), and then evaluated under light microscope (Olympus CX 51).

2.4. Statistical analysis
All data was subjected to one-way ANOVA using SPSS 18.0 software. Differences among groups were attained using the LSD option and significance was declared at p< 0.05. Results are means ± SEM.

3. Results
3.1. Biochemical findings
As can be seen in Table 1, the difference between the oxidant/antioxidant parameters at 0 and 1 hours post-mortem was found to be statistically insignificant. While a significant decline only in the activity of GST was evident at 2 hours post-mortem, statistically insignificant elevations in MDA and 8-OHGua amounts were observed at 3 hours post-mortem. All the parameters exhibited a significant increase at 4 and 5 hours post-mortem, compared with 0 hour.

Table 1: Some of the oxidant/antioxidant parameters and 8-OHGua amount at 0-5 hours post-mortem in the femoral muscle tissues in rats. P was found by comparing the values of 1 and 5 hours according to 0 hour values.

<table>
<thead>
<tr>
<th>Postmortem hours</th>
<th>SOD</th>
<th>MDA</th>
<th>GPO</th>
<th>GST</th>
<th>GSH</th>
<th>8-OHGua</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.7±1.9</td>
<td>7.7±1.0</td>
<td>87±11</td>
<td>149±18</td>
<td>113±22</td>
<td>1.36±0.07</td>
</tr>
<tr>
<td>1</td>
<td>15.5±3.5</td>
<td>8.4±13</td>
<td>97±18</td>
<td>126±25</td>
<td>90±21</td>
<td>1.41±0.02</td>
</tr>
<tr>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10±1.1</td>
<td>11±3.1</td>
<td>52±13</td>
<td>87.3±19</td>
<td>81±16</td>
<td>1.48±0.07</td>
</tr>
<tr>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td></td>
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<tr>
<td>3</td>
<td>3.6±0.5</td>
<td>14.7±3.7</td>
<td>40±9</td>
<td>69±7</td>
<td>63±3.8</td>
<td>1.53±0.1</td>
</tr>
<tr>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
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</tr>
<tr>
<td>4</td>
<td>1.6±0.3</td>
<td>16±2.8</td>
<td>33±6.5</td>
<td>26±1.5</td>
<td>16±1.1</td>
<td>3.3±0.14</td>
</tr>
<tr>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.7±0.12</td>
<td>18±3.1</td>
<td>34±3.7</td>
<td>24±0.95</td>
<td>15±.85</td>
<td>3.5±0.1</td>
</tr>
<tr>
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<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
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</tr>
</tbody>
</table>

3.2. Histopathological findings
No remarkable pathology was visualised under light microscopy in the striated muscle tissues at 0 (Figure 1) and 1 (Figure 2) hours post-mortem. Mild swelling (arrow) in the myofibrils was evident (Figure 3), along with presence of a small number of eosinophils and lymphocytes (stars) in the endomysial connective tissue at 2 hours post-mortem. A marked swelling was visualised in myofibrils (Figure 4), accompanied by a more prominent eosinophilic and lymphocytic (stars) infiltration and a mild degree of necrosis (arrow) at 3 hours post-mortem. The 4th post-mortem hour is characterised by the visualisation of sarcoplasmic fragmentation and myofibrillar necrosis (arrow) (Figure 5) which seem to be more pronounced (arrow) at the 5th hour (Figure 6).

Figure 1: The histopathological examination of the muscle tissues no remarkable pathology was visualised under light microscopy in the striated muscle tissues at 0 hours post-mortem.
Figure 2: The histopathological examination of the muscle tissues no remarkable pathology was visualized under light microscopy in the striated muscle tissues at 1 hour post-mortem.

Figure 3: The histopathological examination of the muscle tissues mild swelling (arrow) in the myofibrils was evident, along with presence of a small number of eosinophils and lymphocytes (stars) in the endomysial connective tissue at 2 hours post-mortem.

Figure 4: The histopathological examination of the muscle tissues a marked swelling was visualized in myofibrils, accompanied by a more prominent eosinophilic and lymphocytic (stars) infiltration and a mild degree of necrosis (arrow) at 3 hours post-mortem.

Figure 5: The histopathological examination of the muscle tissues after the 4th post-mortem hour is characterised by the visualisation of sarcoplasmic fragmentation and myofibrillar necrosis (arrow).
whether or not a significant effect ion.

SOD acts by converting the free radicals into harmless compounds before they interact with the molecules of biological significance, or by inhibiting the formation of free radicals from other molecules (Girotti, 1998). The amount of MDA at 4 and 5 hours appeared to be two times as greater as the amount at 0 hour. However, the activity of SOD decreased at 4 and 5 hours post-mortem to a level which was eight times as less as the level at 0 hour, which translates into a severe shift in the oxidant/antioxidant balance (SOD/MDA) against SOD. Excessive decrease in the activity of SOD in our experiment propels one to consider its independency from the increase in MDA, since the SOD activity in an experimentally-damaged tissue decrease under normal conditions to such a level that is half as much as that of otherwise healthy subjects (Dengiz et al., 2007; Kisaoglu et al., 2011; Kurt et al., 2011; Polat et al., 2011). It can simply be inferred from the literature that the average increase of MDA in an experimentally-damaged tissue has not been that vastly different from that observed during further hours after death. MDA occurs through peroxidation of the fatty acids containing three or more double bounds. MDA directly affects ion transfer through the cell membrane, causing the membrane compounds to be cross-linked; furthermore, it also induces such detrimental outcomes as a change in the ionic permeability and enzyme activity (Niki et al., 2005). It was reported in the experimental animal studies that the amount of MDA increased in parallel to the increase in the extent of damage (Celik et al., 2004). Similarly, it was detected again in our study that GPO and GST activities were documented to decrease significantly starting from the 2nd hour post-mortem. SOD, GPO and GST have been known to represent crucial antioxidant enzymes in the living tissues (Halliwell, 1995). A decrease in the activity of GPO lead under normal conditions to a decrease in the amount of reduced Glutathione (GSH) and to an increase in the amount of oxidised Glutathione. In other words, the ratio of GSH/GSSG decreases in case of oxidative stress (Melchiorri et al., 1997; Turan et al., 2013).

Although the activity of GPO was reported to decrease by an average of 2.5 times (compared with healthy tissues) in the experimentally-damaged tissues which were processed immediately after decapitation (Polat et al., 2011), what was found in our study was a decrease in the activity of GPO in the muscle tissues by 2.5 times at 4 and 5 hours post-mortem compared with that at 0 hour. That is to say, prolongation of the post-mortem period did not bring along a noticeable decrease in GPO activity. Conversely, GST and GSH levels were found at 4-5 hours post-mortem to be 6-7 times lower as that at 0 hour. GSTs act by catalysing the conjugation of various endogenous and exogenous compounds with GSH (Reiter et al., 1995). GSH has proved an important antioxidant compound, protecting the cells from oxidative damage (Yilmaz et al., 2012). GSH

**4. Discussions**

In the present study, the femoral muscle tissue of rats was investigated and examined by comparing it to the histopathological findings in an attempt to disclose whether or not a significant correlation was present among the levels of oxidant/antioxidant parameters, the extent of DNA damage and post-mortem intervals (0-5 hours) in the femoral muscle tissue of rats. The results from our experiment documented that both the level and the amount of oxidant/antioxidant parameters as well as the amount of 8-OHGuA in the femoral muscle tissues extracted immediately after the decapitation of the rats to death (0 hours post-mortem) were almost identical as those obtained at 1 hour post-mortem. Thereby, the difference between both groups was found to be statistically insignificant. However, later after death (2, 3, 4, 5 hours), the oxidant parameters displayed a significant increase in contrast to the antioxidants showing a significant decrease. The activity of SOD exhibited a significant decline as of the second hour post-mortem, and the decline reached its peak rapidity at 4 hour post-mortem. On the contrary, post-mortem period beyond the 4th hour brought along no further decline in SOD activity. SOD acts by converting the free radicals into harmless compounds before they interact with the molecules of biological significance, or by inhibiting the formation of free radicals from other molecules (Niki et al., 2005; Halliwell, 1995). A decrease in the activity of GPO lead under normal conditions to a decrease in the amount of reduced Glutathione (GSH) and to an increase in the amount of oxidised Glutathione. In other words, the ratio of GSH/GSSG decreases in case of oxidative stress (Melchiorri et al., 1997; Turan et al., 2013).

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**Figure 6:** The histopathological examination of the muscle tissues the 5th post-mortem hour is characterised by the visualisation of sarcoplasmic fragmentation and myofibrillar necrosis seem to be more pronounced (arrow).
deficiency has been known to induce oxidative stress, hence emerging as the underlying cause of numerous pathological events (Ross, 1988).

Additionally, the amount of 8-OHGua, a DNA degradation product, was measured in our study at the post-mortem intervals (0-5 hours) in the femoral muscle tissue, and the results obtained were compared. It was reported on an experimental basis that the extent of DNA damage advanced as the oxidant/antioxidant balance shifted continually towards the oxidants in the tissues examined soon after decapitation (Polat et al., 2010). In the present study, however, no significant increase in the DNA damage products was observed in the muscle tissue even at 3 hours post-mortem. This contradiction suggests that the amount of 8-OHGua increase more rapidly in the tissues inflicted by experimental damage. 8-OHGua amount was observed to exhibit a significant increase at 4 and 5 hours post-mortem when the amount of the oxidants increased the most significantly. DNA, apart from the membrane lipids, has been armed with a vastly more robust resistance. However, should the free radicals be generated only in the near vicinity of a DNA molecule, the molecule can readily be damaged (Gutteridge, 1995).

Mild histopathological findings became apparent at the 2nd hour after death, when the amount of the oxidants slightly increased, accompanied by a slight decrease in the antioxidant amounts. The histopathological findings indicated that tissue damage deteriorates in conjunction with an increase in oxidant levels and a decrease in antioxidant levels. Such more prominent and severe histopathological findings as striated muscles sarcoplasmic fragmentation and myofibrillary necrosis were visualized at 4 and 5 hours post-mortem when the amount of the DNA damage product displayed a significant increase.

In conclusion, oxidant/antioxidant balance in the striated muscle tissue was documented to remain constant at 0 hours post-mortem; to shift mildly in favour of the oxidants at 2 hours post-mortem; to shift moderately in favour of the oxidants at 3 hours post-mortem; and, to shift severely in favour of the oxidants, against the antioxidants at 4 and 5 hours post-mortem. A significant increase in the amount of DNA damage products was detected at 4 and 5 hours post-mortem intervals when the oxidant/antioxidant balance exhibited a severe shift. Furthermore, the biochemical findings were found to be consistent with the histopathological findings.

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