Green tea extract and its major polyphenol \((-\)epigallocatechin gallate improve muscle function in a mouse model for Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is a severe muscular dystrophy that affects males with a high incidence (1:3,500 male births). The disease is characterized by progressive muscle wasting starting in early childhood. DMD patients usually die by age 30, when the disease attacks the cardiac and respiratory muscles.

DMD is due to mutations in the dystrophin gene, causing the complete absence of the corresponding protein. Dystrophin is a large cytoskeletal protein located at the inner face of the plasma membrane in normal skeletal muscle fibers. It binds to both a membrane-associated glycoprotein complex and to the F-actin network, allowing the formation of a physical continuum between the extracellular space and the muscle cell inner compartment (for review, see Ref. 6). It is assumed that this provides protection of muscle fibers against mechanical stress caused by contractile activity (32). Apart from its well-established structural function, evidence has recently emerged that presents dystrophin as a more general player in muscle cell architecture and signaling. Accordingly, the dystrophic condition is associated with a wide variety of cellular dysfunctions that comprise membrane instability, dysregulation of Ca\(^{2+}\) homeostasis, increased susceptibility to oxidative stress, enhanced proteolytic activity, and impaired energy metabolism (6). Altogether, these deleterious events lead to muscle fiber death, followed by activation of self-repair processes. Activated cells from the immune system, mainly macrophages and lymphocytes, invade the sites of necrosis and constantly re-release reactive oxygen species, causing further damage to the surrounding muscle tissue (33, 38). As the dystrophic process persists, the regenerative capacity of the muscle becomes exhausted and results in the replacement of functional muscle mass by fibrotic and adipose tissue.

Since the identification and characterization of the dystrophin gene nearly 20 years ago, considerable efforts have been made to replace or correct the mutated dystrophin gene by means of gene- or cell-mediated approaches (29). However, these approaches have not yet led to a therapy for this disease, mainly because of safety issues concerning vectors and limitations with targeting all the muscles of the body. To date, the only treatments that improve the life span and the quality of life of DMD patients are still symptomatic; they consist of surgery, kinesitherapy, ventilatory assistance, and pharmacological interventions. Currently, the only drugs proposed to DMD patients are the glucocorticoids prednisolone and deflazacort (25). Their action on inflammation, promotion of muscle-specific gene expression, correction of dysregulated Ca\(^{2+}\) homeostasis (22, 24), and activation of the calcineurin/nuclear factor of activated T cells (NF-AT) pathway (40) might explain their therapeutic effects. However, in some instances, the DMD patients treated with steroids could suffer from side effects inherent to this class of molecules. Moreover, the therapeutic benefit regarding improvement of life expectancy and quality of life of DMD patients are not universally recognized (25). Therefore, the currently available compounds used in therapy are unsatisfactory.

Consequently, enlarging the range of drugs for palliative treatment of DMD patients still represents a major issue (15, 20). Ongoing clinical trials are assessing the potential of a variety of compounds to counteract some of the dysfunctions mentioned above. These include immunosuppressive and anti-inflammatory agents, antioxidants, and molecules that mimic the effects of dystrophin. For example, it has been shown that the antioxidant N-acetylcysteine, the tyrosine kinase inhibitor K609, and the anti-inflammatory drugs ciclosporin A and prednisolone improve residual force and endurance in a mouse model for Duchenne muscular dystrophy (21, 22). In addition, the use of drugs that stabilize the dystrophin-glycoprotein complex, such as the caspase-3 inhibitor z-VAD-fmk, also improves muscle function in dystrophic mice (5). These results demonstrate that diet supplementation of dystrophic mouse models with green tea extract or \((-\)epigallocatechin gallate) improves muscle function and quality.

Green tea extract and its major polyphenol \((-\)epigallocatechin gallate) improve muscle function in a mouse model for Duchenne muscular dystrophy.
inflammatory drugs, such as cyclosporin A (11) or pentoxifylline (PTX) to block tissue infiltration by activated lymphocytes, mitochondria protecting agents to prevent apoptosis, creatine to improve muscle energy balance and help correct calcium dysregulation (23, 31), or antioxidants to counteract oxidative damage (8, 15).

Concerning antioxidant therapy, green tea extract (GTE) appears to be a promising candidate. Green tea is the first beverage consumed worldwide. It is very popular in Asia, where it has been known for centuries as a healthy beverage. Green tea has been examined intensively over the past 20 years for its medicinal properties (16, 27). These include its potential as antioxidant (16, 28), anticancer (7), anti-inflammatory (12), antibacterial (4), antiviral (13), antifibrotic (49), hypolipidemic (48), or cardioprotective agent (34). In addition, GTE has been shown to protect the brain (17, 41) and heart (3, 45) from ischemia-reperfusion-induced damage. Finally, it modulates cellular Ca²⁺/ handling (19).

GTE refers to the hot water-soluble fraction of unfermented green tea leaves. Its major constituents are catechin polyphenols, but it also contains theaflavin, vitamins, amino acids, and caffeine. Among the polyphenols, (−)-epigallocatechin gallate (EGCG) is the most abundant, accounting for 30–50% of total polyphenols, and is known to convey most of the beneficial properties associated with green tea consumption (7, 16, 46).

We have reported previously (8) that GTE reduces necrosis in the extensor digitorum longus (EDL) muscle of mdx mice, the most commonly used animal model for DMD. In the present study, dystrophic mice from the mdx⁵Cv strain (18) were given two doses of decaffeinated GTE or a dose of pure EGCG for either 1 wk or 5 wk. Using histological examination of leg muscles and functional recordings of the triceps surae contraction, we found that GTE and EGCG protect the hindlimb muscle of dystrophic mice from massive necrosis and greatly improve muscle force and resistance to fatigue.

MATERIALS AND METHODS

Test compounds and food pellets. A decaffeinated polyphenol-enriched fraction of GTE (Suphenon DCF-1) was a kind gift from Taiyo Kagaku (Yokkaichi, Japan). It is hereinafter referred to as GTE. It was composed of 90% polyphenols, and residual caffeine was 0.07%. By reference to total polyphenols or total catechins, its content in EGCG was 34.7%, or 47.8%, respectively. EGCG (purity >99.9%) was purchased from Yixin Pharmaceutical (Zhejiang, China), and PTX was from Sigma (Buchs, Switzerland). GTE, EGCG, and PTX were incorporated into standard rodent food by Kliba-Nafag (Kaiseraugst, Switzerland). Control diet consisted of standard rodent food processed the same way without addition of substance.

Animals and treatments. The study was performed on dystrophic mdx⁵Cv mice (18) and their genetically matched normal counterparts, C57BL/6J mice. Breeding pairs of dystrophic mdx⁵Cv animals were kindly provided by Dr. Serge Braun (Transgene, Strasbourg, France) with the agreement of the Jackson Laboratory (Bar Harbor, ME). C57BL/6J mice were originally purchased from Charles River (Iffa Credo, France). The colonies were thereafter maintained in our animal facility. Animals were housed in plastic cages containing wood granule bedding, maintained on 12:12-h light-dark cycles, and allowed free access to food and water throughout the study. All of the procedures involving animals were performed in compliance with the Swiss Federal Veterinary Office’s guidelines, based on the Swiss Federal Law on Animal Welfare, and were approved by the Cantonal Veterinary Service.

Groups were assembled from litters of 3-wk-old animals. In most cases, two litters received the same diet, such that each group finally contained 7–10 animals. Mdx⁵Cv mice were fed beginning at weaning for either 1 or 5 wk on a control chow (referred as “untreated” group) or on chow containing GTE (0.05% or 0.25% wt/wt) or EGCG (0.1% wt/wt). On the basis of a study by Granchelli et al. (15), PTX (0.1% wt/wt) was chosen as a positive control. A group of normal C57BL/6J mice fed control chow was also included for comparison.

Mouse weight and food consumption. Mice were weighed twice a week. Food consumption was monitored only for animals studied over the 5-wk period. Known amounts of food pellets were delivered, and the amount of remaining pellets was determined every week. Food consumption was calculated as the mass of pellets consumed per gram of body weight per day. The calculation was based on the mouse weight at the end of each week of treatment.

Isometric force recordings. At the end of the treatment period, animals were anesthetized by injections of a mixture of urethane (1.5 g/kg ip) and diazepam (Valium, 5 mg/kg ip). The Achilles tendon of the right hindlimb was exposed and linked to a force transducer coupled to an Watanabe Linear Corder Mark III chart recorder (type WTR331). The knee joint was firmly immobilized. Two fine steel electrodes were inserted into the triceps surae muscle (comprising the fast twitch, glycolytic gastrocnemius and plantaris muscles, and the slow-twitch, oxidative soleus muscle). Square wave pulses, 0.5 ms in duration, were delivered via a stimulus controller. Stimulation voltage and muscle length were adjusted to obtain maximum isometric twitch force. The optimal muscle length was determined. A phasic twitch was then recorded and the absolute peak twitch tension (Pt), the time to peak (TTP), and the time for half relaxation from peak (RT₁/₂) were measured.

After a 3-min pause, muscles were subjected to a tetanization assay using 200-ms bursts of increasing frequency (from 20 to 100 Hz in increments of 10 Hz) with one burst every 30 s. Successive tetanic contractions were used to construct force-frequency curves. The strongest response (usually obtained at 90 or 100 Hz) was taken as the absolute optimal tetanic tension (P₀).

Finally, after another 3-min pause, muscles were submitted to a fatigue assay for 5 min: frequency was set to 60 Hz, and 60 stimuli were delivered, each consisting of a 2-s train of tetanic stimulation and a 3-s rest. The maximal tension was usually obtained during the first five stimulations. The amplitude of the response then decreased as the stimuli were repeated. The residual tetanic tension was expressed as percentage of maximal response. Absolute phasic and tetanic tensions (in mN) were converted into specific tensions (in mN per mm²) after normalization for the total muscle cross-sectional area (CSA). The CSA (in mm²) was determined by dividing the triceps surae muscle mass (in mg), by the product of optimal muscle length (in mm) and d, the density of mammalian skeletal muscle (d = 1.06 mg·mm⁻³).

Tissue and plasma sampling. The triceps surae from the stimulated leg was carefully dissected and weighed for the calculation of CSA. The soleus and EDL from the left, nonstimulated leg were dissected, embedded in tissue-freezing medium (Jung), and frozen in liquid nitrogen-cooled isopentane. The blocks were stored at −80°C until processed for histology. Blood was collected from the thoracic cavity after the aorta has been cut. Plasma was prepared and stored at −80°C until measurement of total antioxidant potential.

Histological evaluation. Transverse sections (10 µm thick) of soleus and EDL muscle were obtained with a cryostat (model HM 560M, Microm, Volketswil, Switzerland), collected on SuperFrost Plus slides, and stained with hematoxylin and eosin according to classical procedures. High-resolution pictures were taken with an AxioCam MRc digital camera (Zeiss, Feldbach, Switzerland) coupled to an inverted microscope (Zeiss Axiovert 35M). One cross-section was analyzed from each of the 7–10 animals per group. The necrosis-regenerated surface (NRS) was determined using Metamorph software (Visitron Systems, Puchheim, Germany) by measuring the surface of all but healthy tissue from the entire cross-section of the
muscle. The whole surface of the cross-section was also measured. The NRS was ultimately expressed as the percentage of the total cross-sectional surface. The samples were coded and analyzed by an observer who was blinded to the study details.

**Determination of total antioxidant potential in plasma.** The total antioxidant potential of plasma was assessed with a commercial kit (Total Antioxidant Status, Randox Laboratories, Ardmore, UK) according to the manufacturer’s instructions. Total antioxidant potential (in mmol/l) was determined by comparison with a control serum of known antioxidant status (Randox).

**Statistical analysis.** Results are expressed as means (SD) of the values, except in Figs. 5 and 6 where, for clarity, data are shown as means ± SE. Statistical analysis was performed using a two-tailed unpaired Student’s t-test with the untreated mdx<sup>5Cv</sup> group used as reference for comparison to any other group. Differences with P values ≤ 0.05 were considered significant.

**RESULTS**

**Effects of GTE, EGCG, and PTX on mouse weight and food intake.** Three-week-old mice had a mean body weight of 8.6 (SD 0.7) g. As illustrated in Fig. 1A, animals that received 0.25% GTE and 0.1% EGCG were slightly lighter than untreated mdx<sup>5Cv</sup> mice when they entered the protocol. Then, the mean body weight was similar in all groups during the next 4 wk. During the fifth week of treatment, untreated mdx<sup>5Cv</sup> mice gained weight more rapidly than the other animals. The mice became significantly heavier than the normal mice and the mdx<sup>5Cv</sup> mice treated with low-dose GTE or PTX (Fig. 1A). Such an augmented body weight of mdx mice compared with normal animals has been observed by others (36).

After the first week of treatment, the food consumption index varied from 0.146 (untreated mdx<sup>5Cv</sup>) to 0.206 g/g body wt/day (normal mice); the index of the groups supplemented with the test substances ranged within these values (Fig. 1B). The food consumption index normalized thereafter toward a value of ~0.150 g/g body wt/day. This corresponds to a daily drug intake of 75 and 375 mg/kg body wt for those receiving 0.05% and 0.25% GTE, respectively, and 150 mg/kg body wt for those receiving 0.1% EGCG and 0.1% PTX.

**Effects of GTE, EGCG, and PTX on plasma total antioxidant potential.** The total antioxidant potential was measured in the plasma of both treated and untreated animals. No significant difference was found between groups after 1 wk of diet supplementation (Fig. 2). However, after 5 wk of dietary intervention, all four treated groups presented a slight but significant increase in plasma antioxidant potential (Fig. 2). Values (mmol/l) ranged from 1.72 (SD 0.16) for untreated mdx<sup>5Cv</sup> animals to 2.35 (SD 0.55) for animals that received EGCG or PTX. Treatment with either dose of GTE elevated the plasma antioxidant potential to ~2.10. Normal mice had an antioxidant potential of 1.85 (SD 0.35).

**Effects of GTE, EGCG, and PTX on muscle histology.** Transverse sections were obtained from EDL and soleus muscles and stained with hematoxylin and eosin. Healthy and regenerated muscle fibers were readily identified by the presence of either peripherally or centrally located nuclei, respectively (Fig. 3). Foci of necrotic fibers were delineated by the presence of densely packed, infiltrated immune cells, and newly formed regenerating myotubes were recognizable as...
small-caliber cells with enhanced eosinophilia and large centrally located nuclei (Fig. 3, A and D).

The fractions of the muscle affected by necrosis and regeneration are shown in Fig. 4. EDL muscles from 4-wk-old untreated dystrophic mice exhibited ~30% NRS (Fig. 3, A and D). In contrast, in mice fed for 1 wk the high-dose GTE, EGCG, or PTX diet (Fig. 3, B and E; Fig. 4), the part of necrotic EDL muscle dropped to 17–20%. This corresponds to 31–42% of protection compared with untreated mdx5Cv mice. Soleus muscle was more affected, with a NRS value of 45–53% in all groups after 1 wk of dietary intervention. At this stage, centrally nucleated fibers with an already fully differentiated appearance contributed little to the NRS. The largest part of NRS was due to necrotic fibers, infiltrated immune cells, and newly formed regenerating myotubes.

After 5 wk of dietary supplementation, the NRS values were similar in all groups of dystrophic mice, regardless the treatment. NRS values reached 56–64% for the EDL muscle and 67–71% for soleus muscle (Fig. 4). At that time, these fractions consisted mainly of fully regenerated centrally nucleated fibers with few signs of necrotic fibers or infiltrating immune cells (Fig. 3, F and G).

Effects of GTE, EGCG, and PTX on triceps surae cross-sectional area. After 1 wk on a standard chow, the CSA of normal triceps surae was significantly lower than the one from mdx5Cv mice (Table 1). This is indicative of hypertrophy of the dystrophic muscles, as reported by Sacco et al. (36). Remarkably, with the exception of the low-dose GTE, all of the test compounds diminished the triceps CSA to near-normal values. In 8-wk-old animals, the difference between normal and dystrophic muscle was no longer seen. Mdx5Cv mice receiving low-dose GTE or PTX, however, had decreased CSA values.

Effects of GTE, EGCG, and PTX on isometric force parameters. Table 1 shows the specific tension levels developed by triceps surae muscles. This parameter is considered to be physiologically more relevant than absolute tension that is influenced by changes in absolute muscle weight and optimal length. At both time points, untreated mdx5Cv muscles displayed a highly significant lowered peak twitch tension compared with normal mice (P< 33.2 vs. 60.2 mN/mm², and 35.3 vs. 67.4 mN/mm², after 1 and 5 wk, respectively). After 1 wk, low-dose GTE increased P< by 22%, but this was not significantly different from the untreated counterpart. High-dose GTE, EGCG, and PTX supplementation for 1 wk markedly

Fig. 3. Histological examination of skeletal muscle. Whole transverse cross-sections of selected muscles were stained with haematoxylin and eosin. The illustration shows extensor digitorum longus (EDL) muscle from dystrophic mice fed the control diet (A, D, and F), or the 0.25% GTE diet (B, E, and G). For comparison, an EDL muscle from a normal mouse shown in C. Low magnification views (A–C) after 1 wk of diet supplementation; bar: 0.5 mm. Higher magnification views after 1 wk (D and E) or 5 wk (F and G) of diet supplementation; bar: 100 μm. Muscles from mice that received GTE for 1 wk show less necrotic lesions than mice fed standard chow. After 5 wk of supplementation, muscles from treated and untreated mice displayed similar histology.
The surface of the muscle presenting necrosis-regenerated features after diet supplementation for 1 wk (A) or for 5 wk (B) was measured and expressed as the percentage of total muscle cross-sectional area. Necrosis of the EDL muscle from mice fed high-dose GTE, EGCG, and PTX for 1 wk was significantly reduced compared with untreated animals (**p ≤ 0.01; ***p ≤ 0.001). NRS, necrosis-regenerated surface.

Table 1. Isometric contraction parameters and cross-sectional area of triceps surae muscle

<table>
<thead>
<tr>
<th></th>
<th>TTP, ms</th>
<th>RT1/2, ms</th>
<th>Ptot, mN/mm²</th>
<th>P0, mN/mm²</th>
<th>P/P0 (×1000)</th>
<th>CSA, mm²</th>
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<tbody>
<tr>
<td></td>
<td>n (M/F)</td>
<td>mean (SD)</td>
<td>Δ(%) SS</td>
<td>mean (SD)</td>
<td>Δ(%) SS</td>
<td>mean (SD)</td>
</tr>
<tr>
<td>1-Wk Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated mdx⁵Cv</td>
<td>10 (5/5)</td>
<td>39.7 (3.6)</td>
<td></td>
<td>27.5 (2.3)</td>
<td></td>
<td>33.2 (4.2)</td>
</tr>
<tr>
<td>0.05% GTE</td>
<td>7 (5/2)</td>
<td>40.6 (2.2)</td>
<td>+2.2</td>
<td>27.1 (1.5)</td>
<td>-1.6</td>
<td>40.6 (11.1)</td>
</tr>
<tr>
<td>0.25% GTE</td>
<td>7 (3/4)</td>
<td>40.7 (2.2)</td>
<td>+2.5</td>
<td>27.9 (1.2)</td>
<td>+1.3</td>
<td>53.0 (10.8)</td>
</tr>
<tr>
<td>0.1% EGCG</td>
<td>8 (4/4)</td>
<td>43.1 (3.9)</td>
<td>+8.7</td>
<td>32.3 (8.8)</td>
<td>+20.8</td>
<td>64.4 (19.9)</td>
</tr>
<tr>
<td>0.1% PTX</td>
<td>8 (5/3)</td>
<td>39.1 (2.0)</td>
<td>-1.4</td>
<td>25.4 (2.4)</td>
<td>-7.6</td>
<td>55.0 (10.3)</td>
</tr>
<tr>
<td>Untreated C57BL/6</td>
<td>7 (4/3)</td>
<td>41.4 (2.2)</td>
<td>+4.4</td>
<td>29.1 (2.0)</td>
<td>+6.0</td>
<td>60.2 (10.5)</td>
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<tr>
<td>5-Wk Treatment</td>
<td></td>
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<tr>
<td>Untreated mdx⁵Cv</td>
<td>8 (4/4)</td>
<td>41.4 (2.7)</td>
<td></td>
<td>37.9 (5.3)</td>
<td></td>
<td>35.3 (5.2)</td>
</tr>
<tr>
<td>0.05% GTE</td>
<td>9 (2/7)</td>
<td>40.7 (3.6)</td>
<td>-1.7</td>
<td>29.1 (3.1)</td>
<td>-23.2‡</td>
<td>50.9 (5.2)</td>
</tr>
<tr>
<td>0.25% GTE</td>
<td>9 (4/5)</td>
<td>39.5 (3.5)</td>
<td>-5.0</td>
<td>27.6 (3.0)</td>
<td>-27.2‡</td>
<td>50.1 (7.5)</td>
</tr>
<tr>
<td>0.1% EGCG</td>
<td>8 (4/4)</td>
<td>37.4 (1.3)</td>
<td>-9.7‡</td>
<td>24.4 (1.9)</td>
<td>-35.7 §</td>
<td>56.3 (5.6)</td>
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<tr>
<td>0.1% PTX</td>
<td>8 (4/4)</td>
<td>38.3 (1.1)</td>
<td>-7.5*</td>
<td>26.2 (1.5)</td>
<td>-31.0</td>
<td>50.4 (8.6)</td>
</tr>
<tr>
<td>Untreated C57BL/6</td>
<td>7 (5/2)</td>
<td>41.1 (2.6)</td>
<td>-0.6</td>
<td>27.5 (1.9)</td>
<td>-27.5‡</td>
<td>67.4 (9.7)</td>
</tr>
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Values are means (SD); n, no. of mice per group. GTE, green tea extract; PTX, pentoxifylline; EGCG, epigallocatechin gallate. The size of each group is given with distribution of male (M) and female (F) in parentheses. Electrically evoked isometric contractions of the triceps surae muscles were recorded. Peak twitch tension (P₀), time to peak (TTP), time for half-relaxation from the peak (RT₁/₂), and optimal tetanic tension (Pt₀) were determined. P₀ and Pt₀ were normalized against the cross-sectional area (CSA) of the muscle to express the specific forces. For each test condition, the difference from untreated dystrophic mice is expressed as percent change (Δ%). Mouse strain, type, and duration of diet supplementation are indicated in the left columns. See RESULTS for details. *p ≤ 0.05; †p ≤ 0.01; ‡p ≤ 0.001 refers to statistical significance (SS).
effect was clearly seen after 1 wk, and after 5 wk, the recruited forces in all four groups of supplemented mdx<sup>5Cv</sup> animals became significantly higher than those in untreated mdx<sup>5Cv</sup> mice, approaching values of their healthy untreated controls.

**Effects of GTE, EGCG, and PTX on contraction and relaxation kinetics.** Time to peak (TTP) and time for half relaxation from the peak (RT<sub>1/2</sub>) (Table 1) were determined from optimal phasic twitch traces. TTP was ~40–41 ms for both normal and untreated dystrophic animals, at either age. Diet supplementation for 1 wk did not significantly alter this parameter. Feeding dystrophic animals for 5 wk with GTE, EGCG, or PTX diminished the TTP by 2 to 10%, indicating that contraction was faster for treated than for untreated animals. Statistical significance was reached in animals that received EGCG and PTX but not GTE (Table 1).

Four-week-old normal and dystrophic animals fed a standard diet had similar RT<sub>1/2</sub> values (Table 1). No significant alteration was seen upon supplementation, although EGCG increased this value by 22% when compared with untreated mice. By contrast, triceps surae muscle from normal 8-wk-old animals relaxed much faster than those of age-matched dystrophic animals. Interestingly, the RT<sub>1/2</sub> value of mdx<sup>5Cv</sup> mice treated with any of the test substances was considerably diminished compared with untreated mdx<sup>5Cv</sup> animals, falling to values similar to those of normal animals (Table 1).

**Effects of GTE, EGCG, and PTX on resistance to fatigue.** The resistance of triceps surae muscle to repetitive tetanization was assessed in a fatigue assay. As shown in Fig. 6, upon tetanization, the tension was preserved for <1 min. Then, it abruptly decreased over 1–2 min. Finally, the curves reached a plateau, with a value corresponding to the residual tension after tetanus.

Four-week-old untreated animals from either normal or dystrophic strains presented similarly similar patterns of fatigue and similar residual force at the end of the assay (Fig. 6). Yet, the values of the normal muscles were always above the values of the dystrophic muscles. Low-dose GTE, EGCG, and PTX...
did not notably alter the pattern of force diminution, although force output tended to be lowered compared with untreated animals. Animals fed a high-dose GTE diet, however, were slightly but significantly more fatigable (Fig. 6). After dietary intervention for 5 wk, the responses presented almost an inverted image of the 1-wk intervention data: normal animals became the least resistant, untreated dystrophic mice were more resistant than normal ones, and, with the exception of low-dose GTE, all supplementations conferred the dystrophic animals an increased resistance to fatigue. Residual force at the end of the fatigue protocol ranged from only 18% of maximal tension for normal mice to ~24% for untreated dystrophic mice, and 31, 32, and 36% for mdx<sup>scv</sup> mice given PTX, high-dose GTE, and EGCG, respectively (Fig. 6). Thus the test substances made the dystrophic muscles 33–50% more resistant to fatigue compared with untreated dystrophic muscles.

**DISCUSSION**

The specific aims of the present study were 1) to examine the effects of GTE and its major polyphenolic component, EGCG, on tissular and functional alterations inherent in the dystrophic condition, 2) to investigate whether these substances improve muscle quality and functional properties, and 3) to establish whether these substances exhibit therapeutic potential for a symptomatic treatment of DMD.

We have shown previously that GTE given for 1 wk at 0.05% (wt/wt) to mdx mice reduced EDL muscle necrosis by ~35% (8). Herein, we investigated the effects of a 5 times higher dose of GTE (0.25% wt/wt) and of EGCG at a dose corresponding to the EGCG content in 0.25% GTE. On the basis of a study by Granchelli and colleagues (15), we used PTX as positive control because it was the most efficient of a panel of 27 pharmacological interventions, improving whole body tension of 10-wk-old mdx mice by 51%. Our test substances were given via the diet fed to 3-wk-old dystrophic mice, either for 1 wk to investigate the effects on the onset of the massive wave of muscle degeneration occurring at 3 to 4 wk of age, or for 5 wk, to document the effects over a period that also causes regeneration, known to be completed by ~8 wk of age.

Up until now, few studies (5, 8, 10, 26, 37) have dealt with the effects of GTE on skeletal muscle function. Moreover, to the best of our knowledge, the potential of EGCG in muscle wasting paradigms had never been investigated.

Our current findings can be summarized as follows. First, test substances delayed muscle necrosis in EDL muscle but not in soleus muscle. Second, important structural and mechanical properties of the dystrophic muscle were corrected to near-normal values by the test substances. This was determined by quantifying CSAs of triceps surae muscles, RT<sub>T1/2</sub> of phasic twitch, specific phasic twitch tensions, twitch-to-tetanic ratios, and force-frequency relationships. Finally, test substances altered the key mechanical parameters of dystrophic muscle in a way expected to confer therapeutic benefit, with the most important being improvements of force output and of muscle resistance to fatigue. Overall, EGCG was slightly more efficacious compared with GTE. This was unexpected because EGCG is better protected from oxidation when associated with other polyphenols, as in GTE, than when given alone (47). This might reflect contrasting effects of different polyphenols on specific cell functions. Alternatively, there might be a competition between different polyphenols for cellular targets on which EGCG has a prominent effect.

PTX and polyphenols from GTE have a bitter taste, which might influence food consumption and alter growth rate. However, as shown in Fig. 1A, this was not the case. The mean body weight was unchanged upon GTE, EGCG, and PTX supplementation. The slight changes in body weight during the fifth week of treatment might be due to an uneven distribution of males and females. Dystrophic animals increased their food consumption within the first week of supplementation but their body weight remained unchanged (Fig. 1B). This could reflect an increased locomotor activity or exploratory behavior. This improvement could also be due to better utilization of fat via β-oxidation because GTE has been reported to stimulate lipolytic activity in vitro (48) and in vivo (26). However, in the latter study, animals were exposed to endurance exercise, whereas in our study, the animals were kept sedentary.

The increased muscle activity of mice around weaning age is thought to trigger the first massive wave of degeneration in the dystrophic mdx mouse. In most mouse limb muscles, wasting starts by the end of the third week of life, and is normally followed by efficient regeneration, which is completed ~7–8 wk of age (43). The degeneration process continues thereafter but at a much slower rate.

In this study, we found that food supplementation with GTE, EGCG, or PTX for 1 wk afforded protection of a substantial fraction (up to 42%) of EDL muscle fibers. However, the soleus muscle escaped from this protective effect. Such a differential sensitivity of EDL vs. soleus muscles toward test substances was noted in previous studies (8, 31) and is likely to be due to different physiological functions: the EDL is a fast-twitch muscle solicited for locomotion, whereas the slow-twitch soleus muscle is involved in posture maintenance and is therefore permanently stimulated. As a result, the soleus is more predisposed to activity-induced damage. In fact, necrotic fibers are already present in soleus muscle of 3-wk-old dystrophic mice (Ref. 31, and Dorchies OM, unpublished observation), i.e., at the time chosen to start the dietary supplementation. This peculiar feature is probably the reason why dystrophic soleus muscle is insensitive to therapeutic intervention. In contrast, EDL muscle, which presents a later onset of necrosis and sporadic contraction, can be expected to be more sensitive to protective drugs.

The protective effects were no longer seen after 5 wk of treatment, suggesting that GTE, EGCG, and PTX delayed necrosis but were not able to durably counteract the pathological process. Probably, the extent of necrosis and its rapidity of progression overcame the beneficial effect of the test substances. Murine and human pathogenesis differ mainly with respect to the regenerative capacity of the muscle: It is quite efficient in mice but is rapidly exhausted in humans. In mice, the first massive wave of necrosis is followed by a second phase, lasting for most of the life of the animal, and is characterized by slow, chronic degeneration (33). This period is associated with low chronic oxidative stress that resembles the slowly progressive human dystrophic condition. If GTE and EGCG are protective through their antioxidant properties, we hypothesize that they would be efficient during this phase characterized by chronic oxidative stress.
The NRS values found for EDL and soleus muscles of untreated mice were lower than in our previous studies (30% vs. 40–45% for EDL, and 50% vs. 70–75% for soleus, respectively) (8, 31). The differences in genetic background (C57BL/10 for the commonly used mdx mouse vs. C57BL/6 for the mdx<sup>5Cv</sup>) as well as the improvement of the method used for histological analysis are likely to account for this slight discrepancy. No sign of fibrosis was seen, probably because the animals were too young for the development of this feature. In fact, in 8-wk-old animals, almost all of the tissue accounting for the NRS values corresponded to centrally nucleated fibers, suggesting that successful regeneration occurred after degeneration of initial fibers. This was not altered by any of the test compounds, indicating that neither GTE nor EGCG impairs muscle regeneration.

Isometric force recordings were consistent with efficient regeneration and maturation of the muscle. The only ambiguous observation consisted of the increased fatigability in 4-wk-old dystrophic animals treated for 1 wk with 0.25% GTE. However, this could be explained by the muscle protection itself. Actually, the EDL muscle is composed almost exclusively of fatigable fast-twitch type II fibers (~80% type IIX and 20% type IIX fibers (1)). Type IIX fibers have been reported to be more susceptible than other fiber types to cell death (33). Thus a substantial fraction of fibers protected by GTE treatment should be composed of fatigable type II fibers. Similarly, the triceps surae muscle consists also mainly of type II fibers (gastrocnemius and plantaris muscles represent >90% of the triceps surae mass). Thus it is reasonable to suggest that the fast-twitch component of the triceps surae benefited from protection similar to that found for EDL muscle. That protection of the triceps surae muscle upon GTE intake should result in increased fatigability compared with the untreated condition.

Specific twitch tension, twitch-to-tetanic ratio, and pattern of frequency-dependent tetanic tension generation were all improved after both 1 and 5 wk of treatment. Moreover, the fact that muscles from dystrophic mice fed a supplemented diet displayed quasi-restoration of these parameters suggests that the muscle phenotype of treated dystrophic animals became similar to that of normal muscle. The rate of contraction and the tension developed during myofiber contraction are mostly dictated by the nature of myosin heavy chains expressed. Therefore, the possibility that supplemented dystrophic animals could display a distribution pattern of these myosins similar to that found in normal animals might explain this functional improvement. By contrast, the increased resistance to fatigue in 8-wk-old mice treated with GTE or EGCG for 5 wk might be explained by an accumulation of fatigue-resistant type I and type IIA fibers. Identification of myosin isoforms should shed light on the molecular basis underlying improved force output and resistance to fatigue. In fact, the fatigue assay we used is very drastic. Although the mice were kept circulating and ventilating throughout the experiment, we observed that the stimulated muscles never recovered after the protocol (not shown) over a period up to 1 h after the end of the fatigue assay. Under the experimental conditions used, fatigue could hardly be attributed to limitation in glucose or oxygen availability. This strongly suggests that diminution of tension output during the fatigue assay is the consequence of structural damage rather than energetic exhaustion of the muscles. Consequently, the increased resistance to fatigue seen upon dietary supplementation of dystrophic animals might be due to improved structure of either muscle membrane or muscle contractile apparatus.

All three substances investigated are known to display anti-inflammatory action (12). However, our interest in GTE and EGCG as candidates for a palliative treatment of DMD relies primarily on their antioxidant potential found in both vitro and in vivo studies (16).

Whether GTE and EGCG exerted their beneficial effects on dystrophic muscle by acting as antioxidants cannot be concluded directly from this study. However, previous and current in vitro work in our laboratory support convincingly that polyphenols from GTE are indeed able to protect cultured skeletal muscle cells against reactive oxygen species-mediated toxicity. GTE dose-dependently counteracted free radical-induced damage of C2C12 myotubes challenged by tert-butylhydroperoxide (8). Moreover, using primary cultures of mouse skeletal muscle cells, we have established that treatment with either GTE or EGCG protected dystrophic myotubes against hydrogen peroxide-mediated cell death (Dorchies OM, Wagner S, Waldhauser K, Buetler TM, and Ruegg UT, unpublished observations). In addition, we show herein that GTE or EGCG intake caused a significant increase in plasma antioxidant potential. Finally, antioxidants are believed to modulate the redox state of regulatory thiol moieties of myosin light chains, allowing production of optimal tension (2). Thus it appears likely that GTE and EGCG act via their antioxidative activity in vivo. This action is consistent with the protection seen at the tissue level in 4-wk-old EDL muscles.

Nonetheless, if the only contribution of GTE and EGCG were to protect muscle tissue from oxidative stress-induced damage, no phenotypic differences would exist between treated and untreated dystrophic muscle after regeneration. In disagreement with this option are our results on animals treated for 5 wk: whereas histology was similar in all groups of dystrophic animals regardless the treatment, functional force recordings revealed multiple changes in muscle mechanical properties. This strongly suggests that GTE and EGCG exert direct actions on skeletal muscle fibers. In agreement with this hypothesis, ongoing work in the laboratory suggests that GTE positively influences myogenesis by promoting the formation of myotubes from myoblasts and by enhancing the expression of several muscle-specific proteins, including myosin heavy chains, sarcomeric α-actinin, and dystrophin. Whether dystrophic mice respond to GTE supplementation by overexpressing structural proteins such as those identified in vitro has not been investigated yet. In this context, overexpression of utrophin, a dystrophin homolog capable of compensating the absence of dystrophin (39), is an important issue to address. Because dysregulation of cellular calcium homeostasis is involved in the downstream events leading to the death of dystrophin-deficient myofibers (14), it is tempting to speculate that the dietary interventions interfered with this process. Accordingly, dietary intervention with compounds able to correct that dysregulation in mdx mice provided some benefit (35). Interestingly, dystrophic animals that received EGCG for 5 wk presented an increased contraction rate, which could result from an improved excitation-contraction coupling. In addition, muscles from GTE- or EGCG-treated dystrophic animals relaxed significantly faster compared with untreated dystrophic animals. The sarco(endo)plasmic reticulum Ca<sup>2+</sup>-ATPase, which
pump Ca\(^{2+}\) ions from the cytosol back into the sarcoplasmic reticulum, is a critical determinant in muscle relaxation. The improved relaxation rate after GTE and EGCG treatment might result from an increased expression and/or increased activity of the sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPase pumps. GTE has already been reported to interfere with Ca\(^{2+}\) homeostasis in platelets (19) and in chromaffin cells (30). Our results suggest that GTE interferes with calcium homeostasis also in skeletal muscle.

Compared with EGCG, the others polyphenols from green tea (e.g., epicatechin, epigallocatechin, and epicatechin gallate) are less antioxidant and have been attributed fewer biological effects. To our knowledge, EGCG is the only polyphenol from GTE for which a membrane receptor has been identified (42). Binding of EGCG to this receptor results in growth inhibition of cancer cells independently of its antioxidant action. The use of other purified polyphenols in future experiments should help determining the relative contribution of antioxidant and non-antioxidant effects of GTE and EGCG.

In conclusion, we found that GTE and its major constituent EGCG were efficient in protecting the fast-twitch EDL muscle from necrosis. It is likely that the same kind of protection occurred in the fast-twitch part of the triceps surae muscle that is composed of gastrocnemius and plantaris muscles. Although the definitive mechanisms of action remain to be clarified, these substances exerted marked effects on muscle mechanical properties after 1 and 5 wk of treatment. Aside from a likely direct action of GTE and EGCG as antioxidant agents, preliminary in vitro results suggest specific action of these substances on skeletal muscle cells. Thus, we hypothesize that GTE polyphenols behave as multitarget agents that are capable of positively altering several of the downstream consequences of dystrophin absence (20, 44). As such, these compounds clearly deserve further consideration for their potential as a palliative treatment for DMD patients.

Additional characteristics make GTE and its polyphenols candidates of unique therapeutic interest because they are orally active, have well-documented pharmacokinetics, pharmacodynamics, and toxicology showing no or little associated side effects, and are readily available at low cost. These characteristics could hopefully preclude the time-consuming processes for usual drug development and provide the patients and their families with a low-cost noninvasive medical treatment.

On the basis of pharmacokinetic data on humans (9, 21), it can be extrapolated that plasma levels similar to those in our mice can be achieved by drinking ~5 liters of green tea per day. Of note, the intake of Asian regular green tea drinkers is ~2–3 liters. However, drinking such volumes can be bypassed because formulations of purified green tea polyphenols are already available as tablets. Indeed, such preparations could be used in the near future in clinical trials of DMD patients designed to investigate whether these encouraging results also apply to humans. A first trial is foreseen under the auspices of the Association Française contre les Myopathies.

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REFERENCES

GREEN TEA EXTRACT IMPROVES DYSTROPHIC MUSCLE FUNCTION


