Effect of Irradiation on Adenosine Triphosphate Catabolism in Skeletal Muscles

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Objective: *To evaluate the effect of irradiation on adenosine triphosphate (ATP) catabolism in skeletal muscles.*
Methods: *Thirty-six inbred female mice were used in the study. The animals were randomly divided into five irradiated groups and one unirradiated group as control. The left hind legs of the mice in irradiated groups were exposed to a single-dose γ-irradiation of 60 Gy. The concentrations of adenosine monophosphate (AMP), adenosine diphosphate (ADP) and ATP in the irradiated gastrocnemius muscles were determined by high-performance liquid chromatography.*
Results: *In the early periods after irradiation, a significant reduction of the three examined substances was observed in the irradiated gastrocnemius muscle compared with the controls ($P < 0.05$), whereas the concentrations of AMP, ADP, and ATP in the irradiated legs recovered to the normal level 30 days after irradiation.*
Conclusion: *ATP catabolism in skeletal muscle was influenced by radiation in the early stage. This early disorder of ATP catabolism in the irradiated skeletal muscle might be one of the potential factors that initiate the morphological and functional changes of the muscle in late periods after irradiation.*

Key words: *γ-irradiation, trismus, ATP catabolism, skeletal muscle, HPLC*

Trismus is a well recognised late complication of radiotherapy for head and neck cancers[1]. The result is limitation of mouth opening, with major effects on nutrition, dental hygiene, swallowing and phonation[2]. Radiation-induced trismus develops as a result of damage to the temporomandibular and skeletal muscles of mastication, especially the pterygoid muscles[3,4]. However, little evidence of radiation damage to pterygoid muscles could be supplied due to the difficulty of establishing the animal model. Thus, the prevention and treatment of radiation-induced trismus seems to be just based on clinical experience and practice[5,6].

Interestingly, several experimental studies on the hind legs of both mice[7] and rats[8] found that radiation could lead to skeletal muscle contracture and/or atrophy. Other studies, which mostly focused on the radiation-induced late fibrosis of the skeletal muscle, suggested that ischemia resulting from vascular lesions might be the main reason for the late muscle injury after irradiation[9-11]. However, the early effects of radiation on the skeletal muscles, including the pterygoid muscles, has not been sufficiently studied and its mechanism is still unknown.

Based on the skeletal muscle injury of the irradiated mouse leg, and by use of high-performance liquid chromatography (HPLC), this study attempts to investigate the early disturbance of adenosine triphosphate (ATP) catabolism in the irradiated skeletal muscles and to

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obtain some insight into how to prevent trismus after radiotherapy for head and neck cancers.

Materials and Methods

Agents

Standards of adenosine monophosphate (AMP), adenosine diphosphate (ADP) and ATP were obtained from Amresco (Solon, Ohio, USA). HPLC-grade perchloric acid ($HClO_4$), methanol, potassium bicarbonate ($KHCO_3$), potassium dihydrogen phosphate (KH_2PO_4), ethylenediamine tetraacetic acid (EDTA), tetrabutylammonium hydroxide (TBAH) and other reagents were purchased from Beijing Chemical Reagents Company (Beijing, China).

Mice

The experiments were conducted with the permission of the Animal Care & Welfare Committee of Peking University Health Science Center.

Thirty-six inbred specific pathogen-free C_3H/He 12-week-old female mice, purchased from the Vital River Laboratory Animal Technology Co. Ltd (Beijing, China), were used in this study. The animals were randomly divided into five irradiated groups and one unirradiated group, with six mice in each group. The mice were housed six in one cage and received food pellets and drinking water *ad libitum*.

Irradiation

In order to induce leg contracture, a single dose of 60 Gy was given to the left hind leg. The irradiation technique was in accord with the method of Stone[12]. Briefly, the left leg was irradiated at a focus to skin distance of 0.5 m and a dose rate of 12.8 to 13.3 Gy/min, using ^{60}Co source (School of Basic Medical Sciences, Peking University Health Science Center). The field was about 3 cm in diameter, while the reminder of the body was shielded with a 3 cm thick lead block. The right leg was used as a unirradiated control.

Muscle samples preparation

The skeletal muscle samples of the unirradiated group were prepared 1 day before irradiation, while the irradiated ones were prepared on 1, 3, 7, 14, and 30 days after irradiation. The mice were anesthetised using an intraperitoneal injection of 1% sodium pentobarbital (60 mg/kg body weight). After removing the skin of the leg, the gastrocnemius muscle was rapidly excised, freeze-clamped, and stored in liquid nitrogen. Then the mice were sacrificed by cervical dislocation. Frozen muscle was rapidly weighed and then extracted by homogenising in 1 ml ice-cold 0.4 mol/L $HClO_4$ (containing 1 mmol/L EDTA). After 10 min in ice, the acid extract was centrifuged at 12,000 rpm for 10 min and then 0.7 ml of the supernatant was neutralised with 0.175 ml of 2.1 mol/L $KHCO_3$. After complete reaction, the neutralised extract was centrifuged to precipitate the insoluble potassium perchlorate and the 0.5 ml supernatant was stored at −80 °C or used for analysis immediately.

HPLC analysis for AMP, ADP and ATP

AMP, ADP and ATP in the skeletal muscle were assayed using an HPLC procedure[13] modified as follows. The HPLC instrument (Waters, Molford, MA, USA) was equipped with a Waters 717 plus autosampler, Waters 2487 dual γ absorbance detector, Waters 1525 binary HPLC pump, computer and software (Millennium 32) for instrument control, data collection and analysis. Chromatography was performed on a reverse-phase 250 mm × 4.6 mm analytical column (5 μm particle size, C-18 Symmetry, Waters) and operated at room temperature. The injection volume of both the standard and sample preparations was 5 μl. The mobile phase consisted of a mixture of 110 mmol/L KH_2PO_4 and 10% methanol, adjusted to pH 6.5 with TBAH. The flow rate was 1 ml/min. Eluted compounds were detected by absorbance at 254 nm and quantified by comparison with standards of known concentrations.

In order to make standard curves for these three substances, 10.91 mg AMP, 9.28 mg ADP, and 12.89 mg ATP were exactly weighed and dissolved in 1 ml of double-distilled water. Then, eight standards were obtained by geometric proportion deliquation.

Precision and recovery tests were applied to assess the stability of this system. Under the HPLC analytical conditions described above, the same muscle sample was assayed consecutively five times. Then the coefficients of variability (CVs) of retention time and peak area were computed. After chromatographic separation, the peak areas of one standard solution and one sample ($A_{standard}$ and A_{sample}) were obtained. Then 0.25 ml standard solution was added into the 0.25 ml sample just assayed. The peak area of the mix solution ($A_{standard+sample}$) was obtained and the recovery rate was computed as follows:

$$\text{Recovery rate} = A_{standard+sample}/0.5(A_{standard}+A_{sample})$$[13]

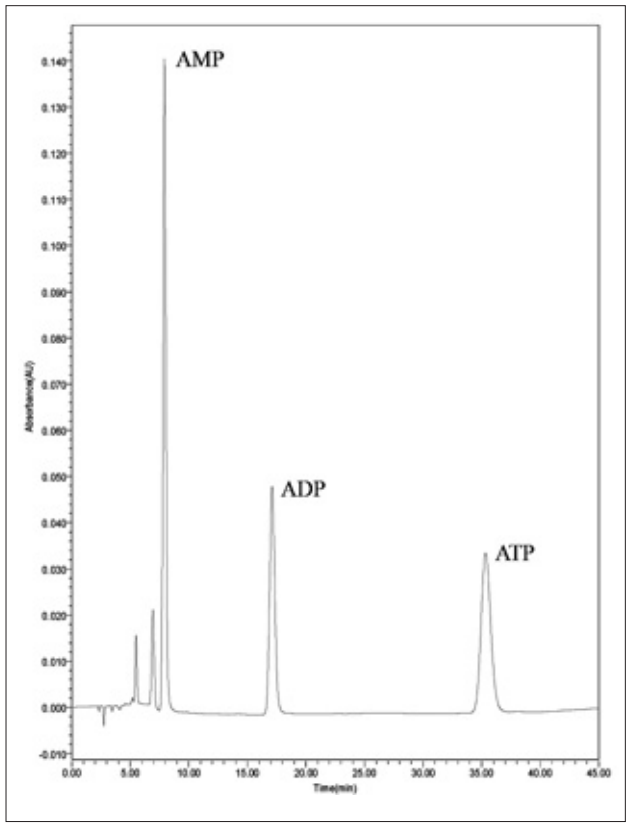

Fig 1 Separation of a standard mixture. Injection volume, 5 µl; column, Waters Symmetry (5 µm, 250 mm × 4 mm); flow rate, 1.0 ml/min; mobile phase, 110 mmol/L KH_2PO_4 and 10% methanol adjusted to pH 6.5 with TBAH; detection, UV 254 nm.

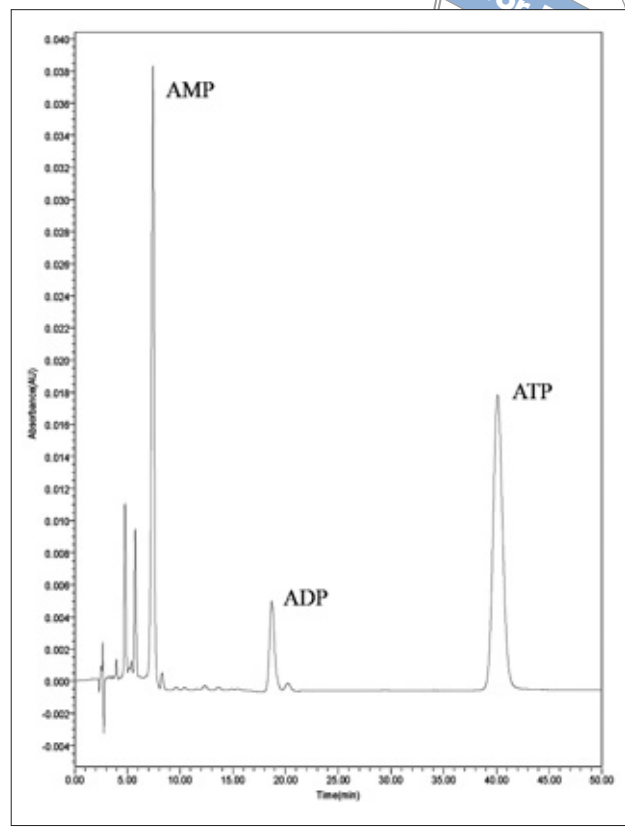

Fig 2 Separation of a random sample prepared by the method described previously. AMP, ADP and ATP can be well separated from other components in the skeletal muscle.

Statistical analysis

Calculations were performed with the software package SPSS 11.0 for Windows (SPSS Int., Chicago, IL). A paired *t* test was applied to compare the difference of leg AMP, ADP and ATP concentrations between the left and right legs. Data are expressed as mean plus/minus standard error of the mean (SEM). The criterion for significance was $P < 0.05$.

Results

Standard curves

The HPLC system used in this study is able to separate AMP, ADP and ATP in the standard mixtures. The peak areas of AMP, ADP and ATP were highly correlated with their concentrations at the absorbance wavelength of 254 nm. The coefficients of determination R^2 of AMP, ADP and ATP were 0.9982, 0.9997 and 0.9998, respectively. The chromatogram from a standard mixture of these three substances is shown in Fig 1.

Precise test

The retention time and peak area of these three substances in the same muscle were little changed; the CVs were all smaller than 4%.

Recovery rate test

The recovery rates of AMP, ADP and ATP were 109.86% ± 5.3%, 103.98% ± 7.6% and 100.08% ± 9.5% respectively.

AMP, ADP, and ATP concentrations

Figure 2 shows a typical chromatogram with the separation of a randomly chosen sample. The separation was clear and it was easy to identify the peaks. Under the conditions described above, the concentrations of AMP, ADP and ATP in the muscles of unirradiated legs were 3.66 ± 0.10 mmol/kg ww, 0.88 ± 0.04 mmol/kg ww and 5.14 ± 0.14 mmol/kg ww (millimoles per kilogram wet weight muscle tissue) respectively, which were similar

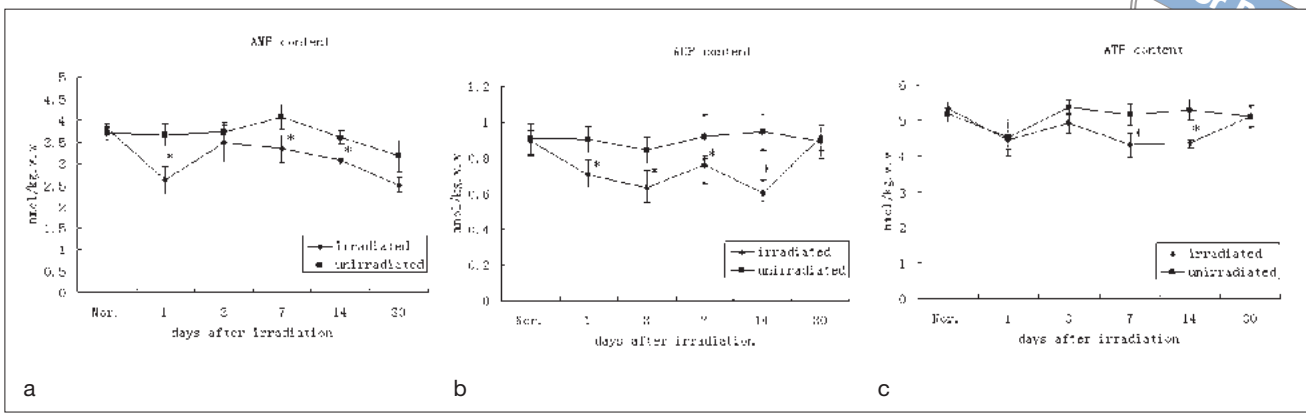

Fig 3 Changes in AMP (a), ADP (b) and ATP (c) concentrations of the skeletal muscle after irradiation. There were decreases in the three analysed substances in the early days after irradiation, while in the 30-day group the concentrations were close to the controls. Nor.: unirradiated group. *$P < 0.05$.

to the results reported by Ally and Park.[13] However, the concentrations were 3.16 ± 0.13 mmol/kg ww, 0.77 ± 0.04 mmol/kg ww and 4.80 ± 0.13 mmol/kg ww, respectively, in the irradiated muscles. The decrease in AMP, ADP and ATP was 15.8%, 12.5% and 6.6%, respectively.

In this study, the 1-, 3-, 7- and 14-day groups after irradiation were considered to represent the early effects of irradiation on the skeletal muscle. As shown in Fig 3, no significant difference was found between the left legs and right legs in the unirradiated group for AMP, ADP and ATP, whereas all three of the assayed substances of the irradiated gastrocnemius muscle reduced to a lower level than the contralateral leg in the 1-, 3-, 7-, and 14-day groups. The radiation-induced decreases in AMP in the 1-, 7- and 14-day groups, ADP in all of the early groups, and ATP in the 7- and 14-day groups were statistically significant ($P < 0.05$). In the 30-day group, no significant difference in ADP and ATP was found between the irradiated and unirradiated legs; and the decrease in AMP in the irradiated legs was also not significant. The radiation led to decreases in AMP, ADP and ATP in the early groups, while the concentrations of the three substances in the 30-day group were very close to the controls.

Discussion

The reported incidence of trismus after radiotherapy to the head and neck regions ranged from 5% to 16%[14,15]. After re-irradiation, an even higher incidence, between 17% and 41%, has been reported[16]. As radiation dosage to the pterygoid muscles increased, the maximal mouth opening decreased[4]. A significant reduction in the cross-sectional area of the medial pterygoid muscles on the high-dose side after unilateral radiotherapy was also found[17]. To alleviate the symptoms of trismus, several treatments were tried, such as a mobilisation exercise[18], hyperbaric oxygen[19] and oral administration of pentoxifylline[2]. However, the effects of therapeutic interventions have hardly been investigated and the normal tissue structures involved in trismus, including the pterygoid muscles, have lacked study[20]. The difficulty in establishing an animal model of trismus induced by radiation is probably one of the main reasons. In the present study, an animal model of radiation-induced leg contracture was used to simulate the trismus after radiotherapy for head and neck cancers. The changes in ATP catabolism in gastrocnemius muscle may also give some insight into the pterygoid muscle injury early after radiotherapy.

The results of this study demonstrate that local irradiation to the left hind legs of the mice leads to a decline in ATP level in the early days compared with the control legs. It is widely accepted that muscle contraction depends on the energy supplied by ATP hydrolysis to inorganic phosphate (Pi) and ADP, which makes the myosin filament go through a conformational change of working stroke and interact cyclically in a rowing motion with an actin filament[21]. Thus, if the ATP concentration is reduced to a lower level, the muscle would stay in a state of rigor without contraction and relaxation[22]. Though high-dose radiation could lead to a 20–30% decrease in the skeletal muscle ATP content[23], the results of this study show that the influence of a clinical dose of radiation on the ATP content of skeletal muscle was not serious enough to arouse ATP-deleted rigor, which is different from a previous report[24]. Since there was a simultaneous decline of both ADP and AMP contents in the corresponding groups, the ATP consumption may accelerate in order to resist and prevent radiation injury. How-

ever, this early alteration inevitably leaves the radiation-involved muscle with a hidden danger, which may lead to a late response such as muscle atrophy and wasting[25]. The reason for the recovery of ATP content in the 30-day group may be that the satellite cells surrounding the irradiated muscle fibers could be recruited and available for the compensatory higher production of ATP[26].

Early damage to muscle energy metabolism by radiation may lead to poor performance of the skeletal muscle, such as atrophy, fatigue, necrosis and even cell death. The radiation may primarily attack energy metabolism rather than the contractile mechanism with a manifestation of decreased ATP level after irradiation of the dissected sartorius or gastrocnemius muscles[23]. The decline of the activity of creatine kinase due to γ-irradiation might be one of the reasons for the radiation-induced ATP reduction[27]. Based on morphological studies, significant changes in the number, size and structure of mitochondria in the muscle cell after irradiation could also reduce the production of ATP[28]. On the other hand, local γ-irradiation with 15 Gy could not affect the concentrations of phosphocreatine, ATP, glycogen and lactate in the gastrocnemius muscle[29]. Another study[30], which focused on the effect of *in vivo* heart irradiation on myocardial energy metabolism, also found that a 20 Gy irradiation did not affect the adenosine nucleotide concentrations after short intervals (24 h and 2 months). So, it can be argued that the dosage plays a crucial role in the radiation-induced muscle injury, but the early injury induced by irradiation to the skeletal muscle is difficult to find in the literature.

As described above, a lower content of ATP might result in the inability of the muscle to relax, which was coincident with the clinical observation after radiotherapy[31]. Since leg contracture was lightly affected by clinical dose irradiation in the early stage[7], the decrease of ATP content and the disturbance of its catabolism might only be a prelude to the late radiation effects on the skeletal muscle. In addition, this could be just the reason why most patients show symptoms of skeletal muscle dysfunction several years after radiotherapy[32]. The skeletal muscle was considered to be one of the radiation-resistant tissues especially in the early period, because, with a clinical dose, there usually were no morphological changes which could be observed by light microscopy in the skeletal muscle[33]. The energy metabolism disorders may happen earlier than the histological changes of skeletal muscle after irradiation. Hence, early interventions for the occurrence of energy metabolism disturbance after radiotherapy would potentially prevent the late complications of irradiation to the skeletal muscle, including pterygoid muscles.

In conclusion, radiation could influence the concentrations of AMP, ADP and ATP in skeletal muscle. This early disorder of ATP catabolism in the irradiated skeletal muscle might be one of the potential factors to initiate the morphological and functional changes of the muscle in late periods after irradiation.

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