

# Time-dependent expression of skeletal muscle troponin I mRNA in the contused skeletal muscle of rats: a possible marker for wound age estimation

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**Abstract** To estimate the age of skeletal muscle contusion, the expression of troponin I mRNA in contused skeletal muscle of rats was detected using real-time polymerase chain reaction (PCR). A total of 51 Sprague–Dawley male rats were divided into control and contusion groups, and another nine rats received contusion injury after death. At 0.5, 1, 6, 12, 18, 24, 30, and 36 h after contusion, the rats were killed with a lethal dose of pentobarbital. Total RNA was isolated from muscle specimens using the SV Total RNA Isolation System and reverse transcribed into first-strand cDNA. Sequence-specific primers were then used to conduct real-time PCR to analyze the expression levels of sTnI mRNA. At 0.5, 1, and 6 h after contusion, the expression levels of sTnI mRNA decreased to 78.17% ( $P<0.05$ ), 41.58% ( $P<0.05$ ), and 32.13% of that in the control group, respectively. However, there were no significant changes in the expression levels of sTnI mRNA from 6 to 36 h ( $P>0.05$ ) after contusion when normalized to RpL32 expression. The expression levels of sTnI mRNA in the normal and contused skeletal muscle of postmortem rats were about 70% of that in the control group ( $P<0.05$ ), and no significant changes in the expression levels of sTnI mRNA in the postmortem contusion group were noted among different time points after injury. This result

suggests that determination of sTnI mRNA levels by real-time PCR is useful for the estimation of wound age.

**Keywords** Wound age estimation · Contused skeletal muscle · sTnI mRNA · Real-time PCR

## Introduction

Wound age estimation is one of the most critical issues for forensic pathologists [1–3]. In cases of suspected non-accidental injury, it is often necessary to give an opinion on the age of soft-tissue injuries. Although it has been mentioned that wound age can be estimated by observing skin color changes, there is no general consensus on the duration of coloration at each stage and the exact sequence of color change.

In recent years, many studies on forensic wound age determination have been conducted. Some cytokines have been shown to display regular expressions following the injury. For example, the expression of interleukin (IL-1, IL-6, IL-8, and IL-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) [4–6], intercellular adhesion molecule-1 (ICAM-1) [7], vascular cell adhesion molecule-1 (VCAM-1) [8], and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [3] following injury has been investigated by immunohistochemistry and real-time polymerase chain reaction (RT-PCR).

Since the method for quantitation of specific mRNA by the polymerase chain reaction was developed, the real-time fluorescent quantitative polymerase chain reaction method has been used in many fields. In the present study, we examined the expression levels of skeletal troponin I (sTnI) mRNA in skeletal muscle by real-time PCR to determine whether it can be used as a marker for wound age estimation.

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## Materials and methods

### Experimental skeletal muscle contusion

A total of 60 Sprague–Dawley male rats, around 10 to 12 weeks old, weighing between 250 and 300 g, were used in this study. All procedures were approved by the Animal Center of Shanxi Medical University. The rats were divided into two parts: A total of 51 Sprague–Dawley male rats were divided into control group ( $n=3$ ) and 0.5, 1, 6, 12, 18, 24, 30, and 36 h ( $n=6$ ) contusion groups, and another nine rats were divided into 0.5, 1, and 6 h ( $n=3$ ) groups which received contusion injury after death.

Animals were kept under a 12-h light–dark cycle with free access to food and water. After the rats were anesthetized with ethylether and the right posterior limb was shaved, a depilatory agent (Nair: Carter-Wallace, New York, NY, USA) was applied to remove residual hair [9]. Subsequently, the rats were placed on a foam bed, and a 250-g counterpoise was raised and allowed to fall freely 150 cm through a clear Lucite guide tube onto the right posterior limb of rats [10, 11]. After injury was made, the rats were reared in a cage and fed commercial rat food and tap water ad libitum.

To observe the influence of postmortem RNA degradation, postmortem skeletal muscle contusions were inflicted on the right posterior limb within 30 min after the death of animals, and muscle samples were excised at 0.5, 1, and 6 h after injury.

The experimental procedures were based on the “Principles of Laboratory Animal Care” (National Institutes of Health published no 85-23, revised 1985) and were conducted in accordance with the Guideline for Animal Experimentation established by our university.

### Tissue preparation

At 0.5, 1, 6, 12, 18, 24, 30, and 36 h after contusion, the rats were killed with a lethal dose of pentobarbital (350 mg/kg of body weight intraperitoneal injection). Approximate 100 mg muscle sample was dissected from the right posterior limb, cut into two parts, and immediately frozen in liquid nitrogen. To compare the expression levels of sTnI mRNA between the normal skeletal muscle and injured skeletal

muscle from the same rat, normal muscle samples were taken from the left posterior limb of rats in the 18 h contusion group. In the postmortem contusion group, muscle samples were taken from both posterior limbs.

### Total RNA preparation

Total RNA was isolated from muscle specimens (weighing approximately 50 mg) using the SV Total RNA Isolation System (Promega, Madison, WI, USA) following the instructions provided with the kit. The concentration (ng/ $\mu$ L) of freshly extracted total RNA was quantitated using a UV/visible spectrophotometer (Ultrospec 4300 pro: Biochrom, Cambridge, UK). RNA integrity was assessed using an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) by loading samples onto the Eukaryote total RNA nano-chip [12, 13].

### Real-time fluorescent quantitative PCR

Primers were designed based on sequences obtained from GenBank, which were imported into AlleleID 6 software (Premier Biosoft International) which is designed to generate primer pairs suitable for real-time PCR. The assays setting “SYBR Green Design” were chosen to limit primer sequences to regions of little secondary template structure, and the SYBR Green module was used. Then, the designed primer sequences were validated on BLAST in order to have a high efficiency. Primers were obtained from Takara Biotechnology, and the sequences, length of production, and source sequences are shown in Table 1.

The first-strand cDNA was synthesized using the Prime Script RT-PCR Kit (Takara Biotechnology, Dalian, China) according to the standard protocol. To conduct reverse transcription, 0.4  $\mu$ g of total RNA was used in a reaction volume of 10  $\mu$ L.

Real-time PCR amplification was performed in a 25- $\mu$ L reaction mixture which included 12.5  $\mu$ L SYBR Premix Ex Taq, 9.5  $\mu$ L dH<sub>2</sub>O, 0.5  $\mu$ L (10  $\mu$ M) of each primer, and 2  $\mu$ L cDNA according to the manufacturer’s instructions provided with SYBR Premix Ex Taq TM (Takara Biotechnology). Amplification was performed by one round of predenaturation at 95°C for 10 s, step-cycle mode of 40 rounds of denaturation at 95°C for 5 s, annealing and extension at

**Table 1** Primers used for real-time fluorescent quantitative PCR

Gene symbol	GenBank accession no.	Strand	Nucleotide sequence	Position	Size of PCR products (bp)
sTnI	NM_017185	Sense	5'-TCAAGATGGGAGATGAGGA-3'	29–47	145
		Antisense	5'-AGTTCTGCTTCTCGGATT-3'	146–163	
RpL32	NM_013226	Sense	5'-CACAGCTGGCCATCAGAGTCA-3'	402–422	83
		Antisense	5'-AAACAGGCACACCAAGCCATCTATTC-3'	458–484	

60°C for 20 s. Fluorescence signal was detected at the end of every cycle. All reactions were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA, USA). The results were normalized to the expression level of ribosomal protein L32 (RpL32). The fluorescence curves of PCR products were evaluated using Stratagene MxPro (Stratagene, La Jolla, CA) to yield the expression data of sTnI mRNA relative to RpL32 mRNA. Relative gene expression was calculated using the Stratagene MxPro software.

The synthesized cDNAs were serially diluted (1, 1:10<sup>1</sup>, 1:10<sup>2</sup>, 1:10<sup>3</sup>, and 1:10<sup>4</sup>) in EASY Dilution solution, and 2 µL of each dilution was used for amplification in a reaction volume of 25 µL. Sterile purified water was used as a negative control.

### Statistical analysis

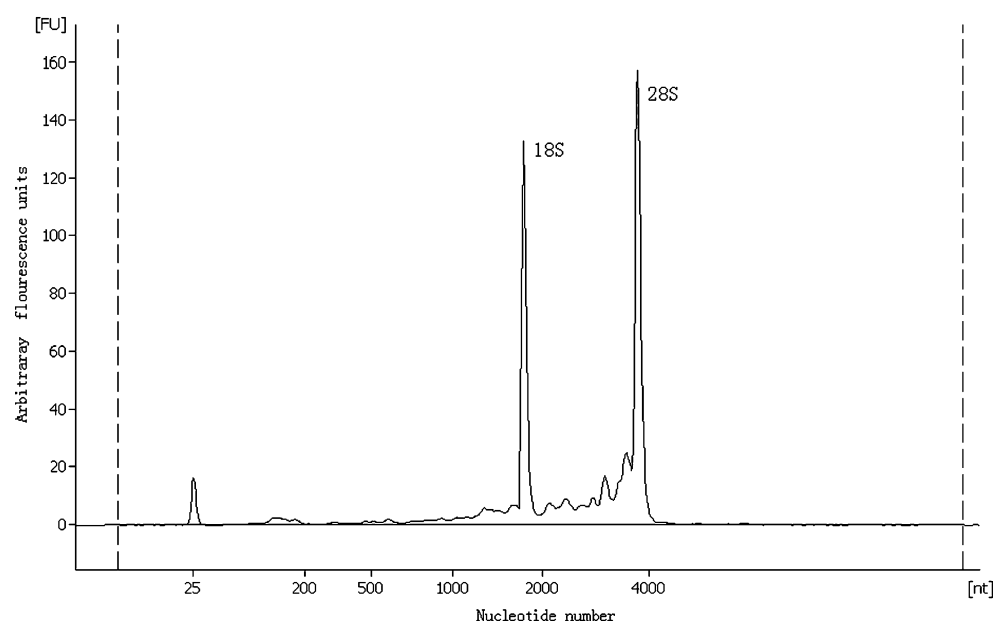
One-way analysis of variance and the Student–Newman–Keuls test were used to compare the survival time after muscle contusion. The *t* test was used to assess statistical significance. When the *P* value was less than 0.05, significant difference was concluded.

## Results

### Integrity of total RNA

All samples were performed on Agilent 2100 Bioanalyzer using the RNA 6000 LabChips Kit. Approximately 0.1–0.4 µg of total RNA was extracted from each milligram wet muscle using the SV Total RNA Isolation System. And

**Fig. 1** Evaluation of total RNA integrity using an Agilent 2100 Bioanalyzer. High quality RNA (RIN=8.8) was obtained from skeletal muscle using the SV Total RNA Isolation System. Approximately 0.1–0.4 µg of total RNA was extracted from each milligram of wet muscle, and the 18/28s peaks are visible clearly. The *first peak* in sample corresponds to the Agilent RNA 6000 Nano Marker, and main ribosomal RNA peaks are indicated



only those RNA with a RNA integrity number (RIN) above 8.0 and with clearly visible 28/18s peaks were used for real-time PCR as shown in Fig. 1.

### Calculation of the amplification efficiency of sTnI and RpL32 genes

The dissociation curves of sTnI and RpL32 genes showed signal cusps, and the dissociation temperatures were 88°C and 84°C, respectively (Fig. 2). As shown in the standard curve (Fig. 3), the amplification efficiency of the two genes was 103.6% and 100.5%, respectively.

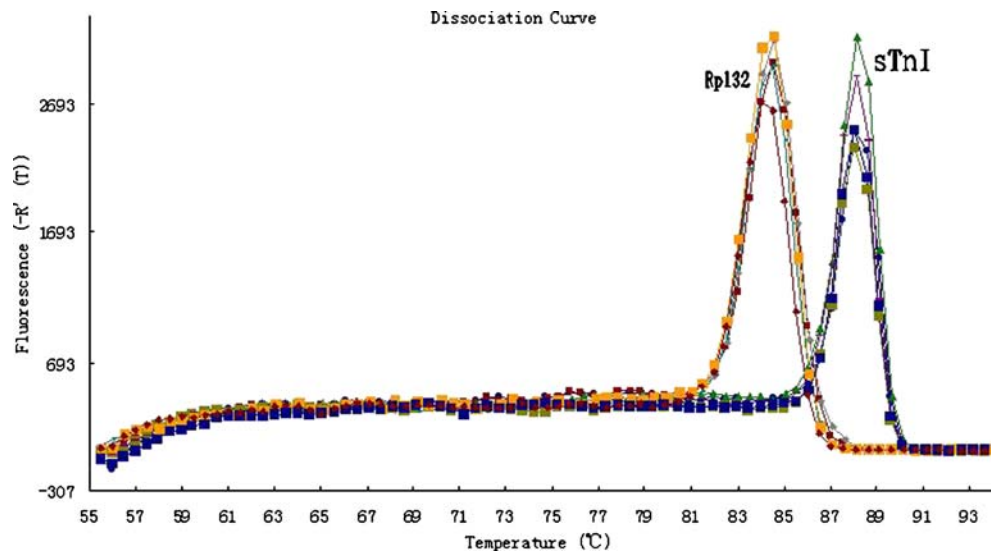
### Validation of RpL32 gene

Figure 4 shows the amplification curve of RpL32 genes in all samples. The *C<sub>T</sub>* value of all samples varies from 17.46 to 18.89; there was no significant differences found between the average *C<sub>T</sub>* among those groups. The results show the RpL32 gene is a suitable normalization gene. Besides, the RpL32 gene has a resembling PCR efficiency with the sTnI gene, which can be concluded from the standard curve (Fig. 3).

### Expression of sTnI mRNA

There was significant difference in the expression levels of sTnI mRNA between the control and the contusion groups when normalized to RpL32 mRNA expression (Fig. 5). At 0.5, 1, and 6 h after contusion, the expression levels of sTnI mRNA decreased to 78.17% (*P*<0.05), 41.58% (*P*<0.05), and 32.13% of that in the control group; however, there were no significant changes in the expression levels of sTnI

**Fig. 2** Dissociation curves of sTnI and Rpl32 using the Mx3005P system. The dissociation curves of sTnI and Rpl32 showed signal cusps, and the dissociation temperatures were 88°C and 84°C, respectively. The results could be explained by that there were no other PCR productions besides our target genes, and there was no primer dimmer



mRNA from 6 to 36 h ( $P>0.05$ ) after contusion when normalized to Rpl32 expression.

Figure 6 shows the difference in the expression levels of sTnI mRNA in the injured skeletal muscle from the right limb and the normal skeletal muscle from the left limb of the same rat. Although no change in the expression levels of sTnI mRNA was observed between the normal skeletal muscle and the control, a significant decrease in the expression levels of sTnI mRNA in the injured skeletal muscle was noted when compared with those in the control and normal skeletal muscle ( $P<0.05$ ).

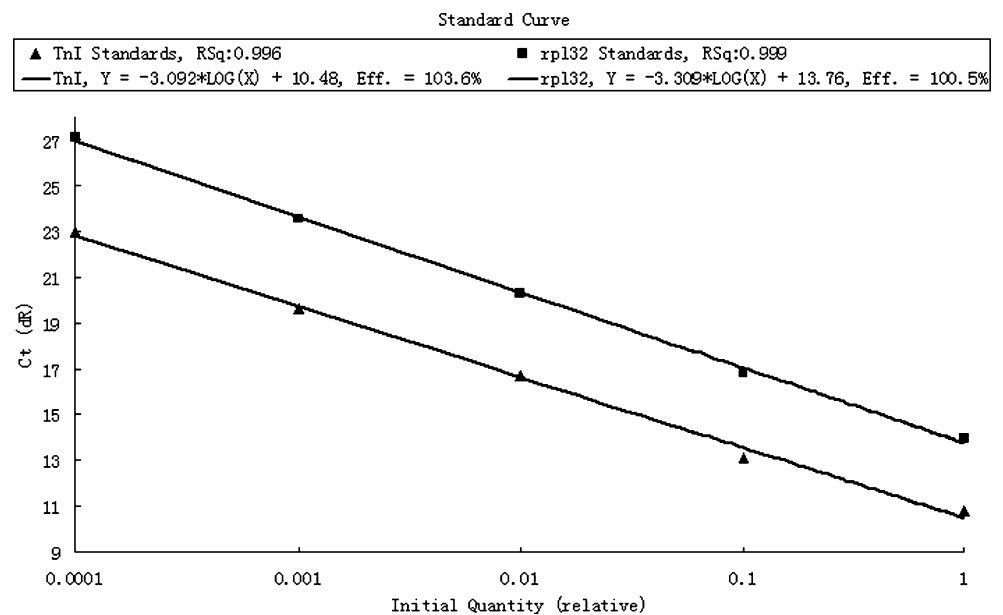
As shown in Fig. 7, a decrease in sTnI mRNA expression in the postmortem contusion group was observed after being normalized to the expression level of ribosomal protein L32 (Rpl32) mRNA. The expression levels of sTnI

mRNA in the normal and contusion skeletal muscle of animals postmortem were about 70% of that in the control group ( $P<0.05$ ), and no significant changes in the expression levels of sTnI mRNA in the postmortem contusion group were noted at different time points after injury.

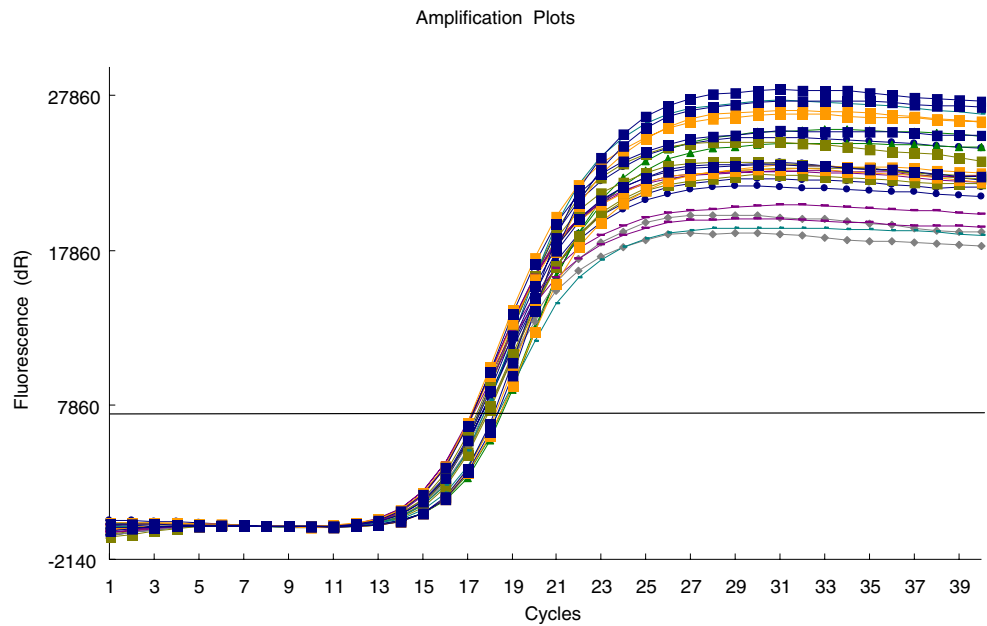
**Discussion**

Wound healing is a complex but spatially and temporally controlled biological process. Generally, it is divided into three phases: inflammatory, proliferative, and maturation [6]. Various kinds of biological mediators such as cytokines and growth factors contribute to the healing process. In previous studies, immunohistochemical assays have been

**Fig. 3** Standard curves of sTnI and Rpl32 genes. The synthesized cDNAs were serially diluted (1, 1:10<sup>1</sup>, 1:10<sup>2</sup>, 1:10<sup>3</sup>, and 1:10<sup>4</sup>) in EASY Dilution solution, and 2 μL of each dilution was used for amplification. As shown in the standard curve, the amplification efficiency of the two genes was 103.6% and 100.5%, respectively



**Fig. 4** Amplification curve of Rpl32 gene. The threshold fluorescence is 6100[Fu]. And the  $C_T$  value of all samples varies from 17.46 to 18.89; there was no significant differences found between the average  $C_T$  among those groups. The results show the Rpl32 gene is a suitable normalization gene



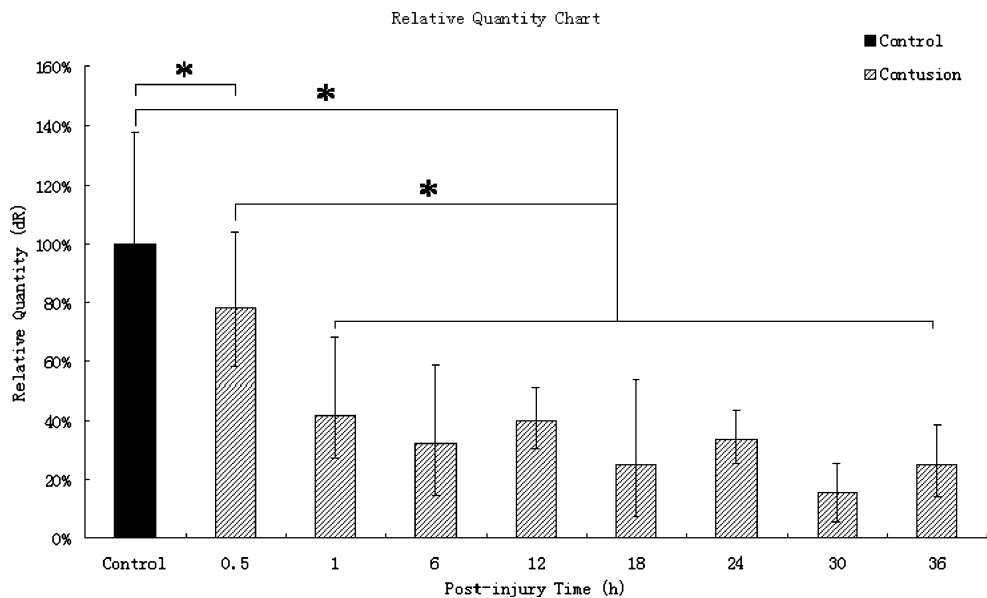
used to detect the expression of many biological markers at different time points after injury [4, 5, 7, 14–19]. However, considering that immunohistochemical assays are not accurate and stable in conducting quantitative analyses, its usage is limited, especially for the estimation of wound ages less than 8 h because the results may be influenced by operator skills [1, 20].

Compared to determination of protein expression by immunohistochemical assays, detection of mRNA by real-time fluorescent quantitative PCR is more suitable for the age estimation of early wounds since the expression levels of cytokine and enzyme mRNAs often vary after injury [20]. It was thought that degradation of mRNA is earlier than that of proteins, especially after death, because of

widely distributed RNases, which makes it difficult to be used in practice. However, recent studies on several selected genes demonstrated that it was possible to isolate total RNA of sufficient quality and quantity from biological stains that are several months or even years old [21–23]. Therefore, mRNA might not be so instable as previously thought and could be used as appropriate markers to determine the age of early wounds. In recent years, some cytokine and enzyme mRNAs have been studied to estimate the age of injuries [1, 3, 10, 20, 24], but there are no reports on the genes of structural protein.

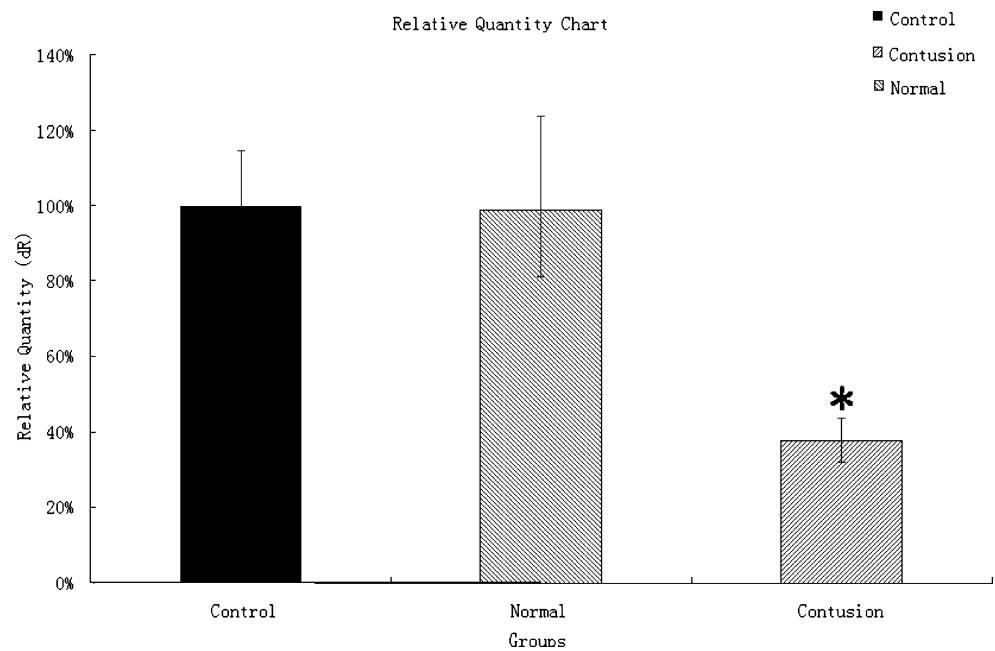
Troponin I (TnI) is one of the thin filament-associated regulatory proteins in muscles. sTnI is a regulatory protein that is only expressed in striated muscle fibers and exists in

**Fig. 5** Real-time PCR analysis of sTnI mRNA expression in contused muscle. A total of 51 Sprague–Dawley male rats were divided into control group ( $n=3$ ) and 0.5, 1, 6, 12, 18, 24, 30, and 36 h ( $n=6$ ) contusion groups. At 0.5, 1, and 6 h after contusion, the expression levels of sTnI mRNA decreased to 78.17% ( $P<0.05$ ), 41.58% ( $P<0.05$ ), and 32.13% of that in the control group





**Fig. 6** Difference in the expression levels of sTnI mRNA between the injured skeletal muscle from the right limb and the normal skeletal muscle from the left limb of the same rat. To compare the expression levels of sTnI mRNA between the normal skeletal muscle and injured skeletal muscle from the same rat, normal muscle samples were taken from the left posterior limb of rats in the 18-h contusion group ( $n=6$ ). There was a significant decrease in expression levels of sTnI mRNA in the injured skeletal muscle when compared with those in the control and the normal skeletal muscle ( $P<0.05$ )



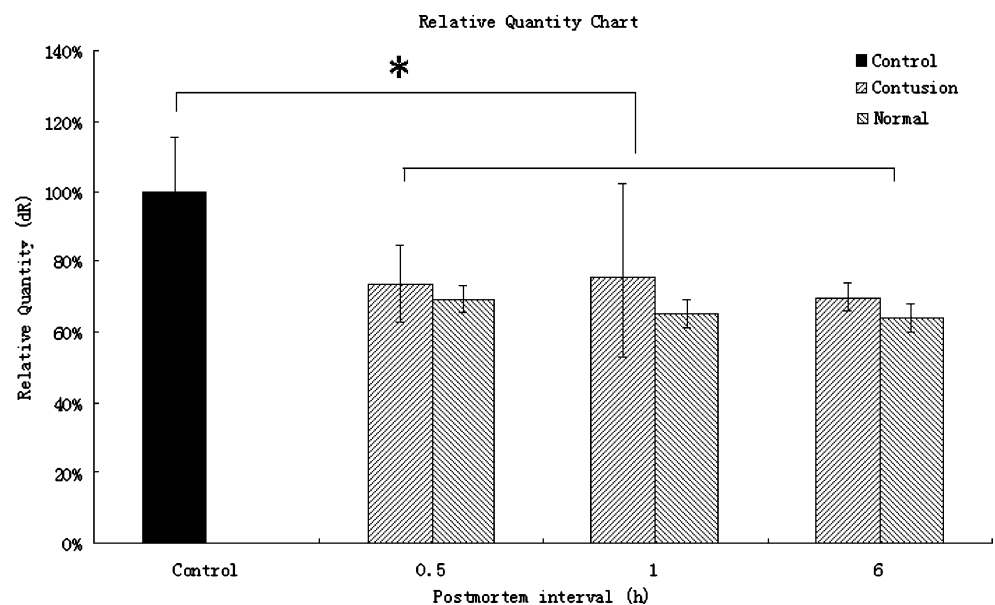
two different isoforms, slow sTnI (ssTnI) and fast sTnI (fsTnI). Recently, some studies have been conducted to determine whether TnI can be used as a marker to estimate the time interval after injury. Using a two-step immunoenzymometric assay and Western blot analysis, Onuoha et al. [25] and Simpson et al. [26] found that the levels of sTnI in the serum from soft-tissue injury patients were higher than those from controls.

In the present study, the expression levels of sTnI mRNA in contused skeletal muscle was determined by real-time PCR. At 1 h after contusion, a rapid decrease in the expression level of sTnI mRNA was noted since the level was only 41.58% of that in the control group. As time after injury increased, the

expression levels of sTnI mRNA gradually became stable. The findings on sTnI mRNA expression are almost identical to the results obtained by Shimada et al. [27], who reported that the expression levels of TnI mRNA decreased following diffuse alveolar damage.

Considering that injury-associated inflammation may affect the entire body, the expression levels of sTnI mRNA in noninjured skeletal muscle from the same contused rat were determined. No changes in the expression levels of sTnI mRNA between the noninjured skeletal muscle and the control were found, suggesting that the noninjured skeletal muscle from the same contused rat could be used as a control to evaluate the contused muscle.

**Fig. 7** Expression levels of sTnI mRNA in the postmortem contusion group. A total of nine rats were divided into 0.5, 1, and 6 h ( $n=3$ ) groups; the contusion on the tissues was made after death within 30 min and sampled after different time breaks. The expression levels in the normal and contusion skeletal muscle of postmortem animals were about 70% of that in the control group ( $P<0.05$ )



Compared to the control group, a significant degradation of sTnI mRNA in the postmortem samples was detected. However, no significant changes in the expression levels of sTnI mRNA in the postmortem samples were observed among different time points after injury, which was inconsistent with the results obtained by Bai et al. [20]. Although it is uncertain what brought about the degradation in sTnI mRNA within 0.5 h after death, it is speculated that different types of mRNA may have different rates of degradation [28] since this process is influenced by many external and internal factors. This phenomenon may influence the results of sTnI mRNA expression less than 0.5 h after injury but has no influence on the expression more than 1 h after injury.

In conclusion, although the data obtained on animal models might not be fully valid on human tissue, investigation of time-dependent expression of sTnI mRNA could help determine the age of early wounds. To enable our results to be used in forensic practice, the comparison of sTnI mRNA expression between the contused skeletal muscle and the normal skeletal muscle should be conducted from the same body since the latter is a good internal control.

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