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The Acute Effect of Trimetazidine on the High Frequency Fatigue in the Isolated Rat Diaphragm Muscle

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The objective of this study was to determine the acute effect of trimetazidine (TMZ) on the pre-fatigue, fatigue and post-fatigue contractile characteristics and tension-frequency relationships of isolated rat diaphragm muscle. Muscle strips were taken from the ventral-costal aspects of the diaphragm muscle of rats killed by decapitation. The muscle strips were suspended in organ baths containing Krebs solution, with a gas mixture of 95% O_2 and 5% CO_2 at 37°C and pH 7.35-7.45. After determining the thermoregulation and optimum muscle length the muscles were subjected to direct supramaximal stimulation with 0.05 Hz frequency square pulses for periods of 0.5 msec to obtain control values. After adding 5×10^{-6} and 5×10^{-5} M trimetazidine solution to the respective bath media, the contractile parameters of the muscles were recorded. The contractile parameters were also recorded for both the trimetazidine and trimetazidine-free media after application of the high frequency fatigue protocols. Later, the tension-frequency relationship was determined by applying stimulating pulses of 10, 20, 50 and 100 Hz to the muscle strips. Whilst the twitch tension obtained from the 5×10^{-6} and 5×10^{-5} M trimetazidine media showed numerical increases compared to that of the controls, these were not statistically significant ($p>0.05$). The contraction time exhibited a dose dependent increase ($p<0.001$), whilst the contraction and relaxation rates did not differ significantly. The isometric contraction forces obtained with the different stimulating frequencies showed a significant increase in the tetanic contraction only at 100 Hz ($p<0.05$). A comparison of the pre- and post-fatigue twitch tensions in the trimetazidine media showed the post- fatigue twitch tensions to be significantly higher than those of the pre-fatigue contraction forces ($p<0.05$). In the 5×10^{-6} and 5×10^{-5} M trimetazidine media the increases in the post-fatigue contraction force were 22 and 30%, respectively. These results demonstrated that in isolated rat diaphragm muscle, TMZ significantly limited the mechanical performance decrease during fatigue. It is our opinion that trimetazidine contributed to the observed fatigue tolerance by eliminating the factors of fatigue, due to preservation of intracellular calcium homeostasis, provision of the ATP energy levels needed by ATPase dependent pumps and especially by keeping the intracellular pH within certain limits.

Key words: Trimetazidine (TMZ), Isolated rat diaphragm muscle, Isometric contraction, Fatigue

INTRODUCTION

Trimetazidine (TMZ) is an intracellular anti-ischemic agent with the chemical name of 1-(2,3,4-trimethoxybenzyl)-piperazine dihydrochloride (Albengres *et al.*, 1998; Harpey *et al.*, 1988). It is known to restore the ATP synthesis and mitochondrial permeability, which for msec is the basis of ischemic events (Harpey *et al.*, 1988; Guarnieri *et al.*, 1997). TMZ has been reported to prevent the metabolic changes that follow ischemia or hypoxia, but has no effect under normoxic conditions (Harpey *et al.*, 1988).

Nowadays, despite the increase in the number of transplantations, loss of graft function after transplantation remains an important complication. Although there have been attempts to reduce this complication by the widespread use of hypothermic storage solutions, the development of acute tubular necrosis in the cadaveric kidneys following renal transplantation has been observed

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to be as high as 35-50% (Hauet *et al.*, 1998). With the aim of reducing the injury due to reperfusion, exogen agents, used as preservatives, have shown corrective effects. TMZ, which has both myocardial and renal ischemic reducing properties, has been used in several experimental ischemic models (Hauet *et al.*, 1998; Garnier and Roulet 1985; Rossi *et al.*, 1990). However, studies on the effect of TMZ on the changes in the metabolic and contractile processes, such as hypoxia and ATP depletion and cytosolic acidosis, in the skeletal muscle following fatigue are not seen in the literature. Therefore, this study was designed with the aim of determining the anti-ischemic effect of TMZ, taking into consideration that in the determination of fatigue and tension-frequency curves the skeletal muscle serves as a better model. The intracellular changes occurring post fatigue show similarity with the intracellular changes formed during and subsequent to hypoxia. It has been proved that under hypoxic conditions TMZ showed cytoprotective effect, but the cytoprotective effect in fatigue models has not been examined.

The aim of this study was to determine to what degree TMZ offered tolerability to fatigue by determining the isometric contractile parameters and tension-frequency relationship during the post-fatigue, fatigue and post-fatigue periods in isolated rat diaphragm muscle.

MATERIALS AND METHODS

Tissue preparation

The animal care committee of Çukurova University approved the protocol. A total of 30 male wistar rats, weighing between 220-260 g, were used in this study. The diaphragm muscles of these rats, killed by decapitation, were prepared according to the method described by Kelsen and Nochomovitz (Kelsen and Nochomovitz, 1982). Muscle strips (N=30), 53.4±6.2 mg in weight and 1.8±0.2 cm long, were cut from the ventral-costal regions of the diaphragm. The muscle strips were placed between platinum electrodes in an appropriate order, and kept in isolated organ baths, containing 20 mL of Kreb's solution, at a temperature of 37°C. The Kreb's solution was composed of (as mM): NaCl 118, KCl 4.69, $CaCl_2$ 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.17, $NaHCO_3$ 25 and Glikoz 11.1, with the pH in the organ bath kept between 7.35 and 7.45 and aerated with a 95% O_2 5% CO_2 gas mixture (Fig. 1).

Experimental protocols

After thirty-minutes of thermoregulation and equilibration the muscle length was determined (length giving the *maximum* muscle tension). During the entire preparatory period the muscles were directly stimulated for 20 minutes, supramaximally, by applying pulsatile square frequencies of 0.05 Hz (15-20 V) of 0.5 msec durations. To stimulate

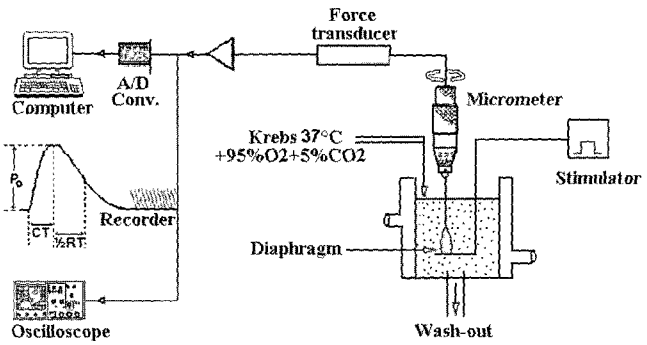

Fig. 1. Plan of the isolated organ bath and recorders. Twitch tension of the isolated rat diaphragm muscle, and the contraction parameters. P_o: Contraction force, CT: Contraction time and 1/2RT: Half- relaxation times.

the muscles and for the recording of the response force transducer (Harvard 50 gram, Edenbridge, England) a Nihon Kohden Stimülatör (SEN-3301 Tokyo, Japan) and isolator (SS-102J) and Harvard Universal Oscillograph (Edenbridge, England), respectively, were used. The high frequency fatigue protocol was designed to have pulse durations of 0.5 msec, pulse frequencies of 40 Hz, pulse trend duration of 8 sec, trend periods of 30 sec and a total application time of 5 minutes (within this time a total of 320 pulses were applied). Five minutes after the tension-frequency determination, fatigue was induced using a similar protocol to that of Baraka, 1974, by stimulation at 40 Hz.

The muscle twitch tension (P_o, g), contraction and half-relaxation times (CT, 1/2RT, msec), and the contraction and relaxation rates (± dP/dt, g/msec) were determined (Fig. 2). Later, with the application of pulse trends of 10, 20, 50 and 100 Hz frequencies, for periods of 200-400 msec, the maximum muscle tension was determined. All of these parameters were repeated in normal (control), 5×10^{-6} and 5×10^{-5} M TMZ (Servier Laboratory, France) containing Krebs solution media.

Data analysis

The twitch tension was calculated in gramsec and the tension-frequency relationship determined. Normalization of the tension-frequency values was achieved by representing the value at each frequency as a percentage of the maximum tetanic value. For the fatigue protocol, values were normalized by expressing the force generated during incomplete tetanus of the first pulse from each of the stimulus trains, at 1.0, 2.0, 3.0, 4.0, and 5.0 min, as a percentage of the first pulse from the first train. With a Digital Storage Oscilloscope (Hitachi VC-6045, Japan), the muscle twitch tension curves obtained were transferred to a computer, through serial cables (RS232C) and with the aid of a program prepared on Quick Basic (BISIP

ver.2.0), and the contractile parameters of the muscle determined. Statistical evaluation: All the results were recorded as the mean ± standard deviation. The pre- and post-fatigue contractile parameters were compared using the paired-t-test (SPSS, Version: 10.0). The significance level was considered as a $p<0.001$.

RESULTS

Effect of TMZ on the pre-fatigue isometric contractile parameters

Table I shows the isometric twitch tension, contraction time, 1/2RT (half the relaxation time) and the contraction and relaxation rates of the isolated diaphragm muscle strips at TMZ concentrations of 5×10^{-6} and 5×10^{-5} M.

In the control group (n=10), after the isolated rat diaphragm muscle attained a stable state, the twitch tension of the muscle after direct stimulation was found to be 15.9±0.9 g. TMZ at concentrations of 5×10^{-6} M and 5×10^{-5} M showed muscle contraction forces of 16.0±1.4 and 16.6±1.1 g, respectively. Comparing the TMZ concentrations with those of the control no significant differences were shown (Table I). In a similar manner, comparison of the durations of contraction and relaxation with those of the controls revealed no significant differences. Comparison of the contraction and relaxation rates with those of the controls showed no significant decrease at either TMZ concentration (Table I).

Effect of TMZ on during fatigue and the post-fatigue contractile parameters

The post-fatigue isometric contractile parameters upon treatment with the 5×10^{-6} and 5×10^{-5} M TMZ medium are shown in Table II. The post-fatigue contraction force (P_f) was 11.1±0.7 g, whereas in the 5×10^{-6} M and 5×10^{-5} M TMZ media they were 19.6±1.0 and 21.0±1.2 g, respectively, which were statistically significant compared with that of the controls ($p<0.001$, Table II, Fig. 2).

The CT_f times formed in the post-fatigue period in the TMZ media showed no significant difference from those of the controls ($p>0.001$, Table II). However, the half relaxation time ($1/2RT_f$) was found to differ significantly between the two groups ($p<0.001$, Fig. 3, Table II). Comparison of the contraction and relaxation rates with that of the controls showed significant increases at both TMZ concentrations ($p<0.001$, Table II). The fatigue in both concentrations was significantly effected by TMZ treatment ($p<0.001$, Fig. 4).

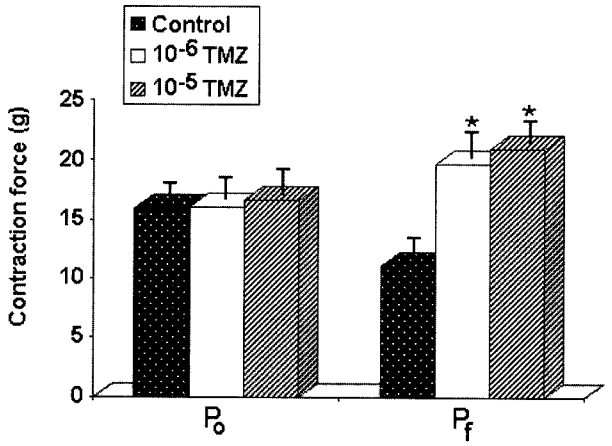

Fig. 2. Effect of TMZ on the isometric contraction forces of the isolated rat diaphragm muscle in the pre-fatigue (P_o) and post-fatigue (P_f). Values are means±SE; n=10 for all contraction forces. Control vs. Group, *P<0.001.

Table I. Effect of TMZ on the isometric contractile parameters of the isolated rat diaphragm muscle in the pre-fatigue period (N=30, mean±SD)

	Twitch tension P_o (g)	CT (msec)	1/2RT (msec)	+dP/dt (g/msec)	-dP/dt (g/msec)
Control (n=10)	15.9±0.9	63.6±3.8	71.4±5.1	0.37±0.01	0.13±0.01
5×10^{-6} M TMZ (n=10)	16.0±1.4	66.0±4.0	74.1±3.2	0.37±0.22	0.12±0.01
5×10^{-5} M TMZ (n=10)	16.6±1.1	66.4±3.2	74.5±4.4	0.38±0.01	0.11±0.01
P	NS	NS	NS	NS	NS

The significance level of the comparison of the TMZ concentration values with those of the control. NS: Not significant.

Table II. Effect of TMZ on the isometric contractile parameters in the post- fatigue period (N=30, mean±SD).

	P_f (g)	CT_f (msec)	$1/2RT_f$ (msec)	$+dP_f/dt$ (g/msec)	$-dP_f/dt$ (g/msec)
Control(n=10)	11.1±0.7	64.9±4.4	87.8±6.5	0.31±0.02	0.10±0.01
5×10^{-6} M TMZ (n=10)	19.6±1.0*	65.0±4.8	70.0±4.1*	0.42±0.01*	0.16±0.01*
5×10^{-5} M TMZ (n=10)	21.0±1.2*	65.3±3.1	71.0±5.7*	0.44±0.01*	0.15±0.02*
p	p<0.001	NS	P<0.001	p<0.001	p<0.001

*p<0.001; shows the significance level of the comparison of the TMZ concentration values with those of the control. NS: Not sign ificant. *: Group 1 versus Group p<0.001.

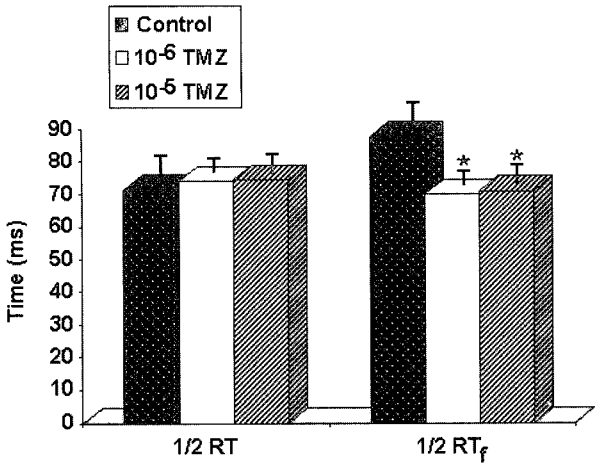

Fig. 3. Effect of 5×10^{-6} and 5×10^{-5}M TMZ on the isometric half relaxation time of the isolated rat diaphragm muscle in the pre-fatigue (1/2RT) and post-fatigue ($1/2RT_f$). Values are showed by means±SE; n=10 for all half relaxation time. Control vs. Group, *P<0.001.

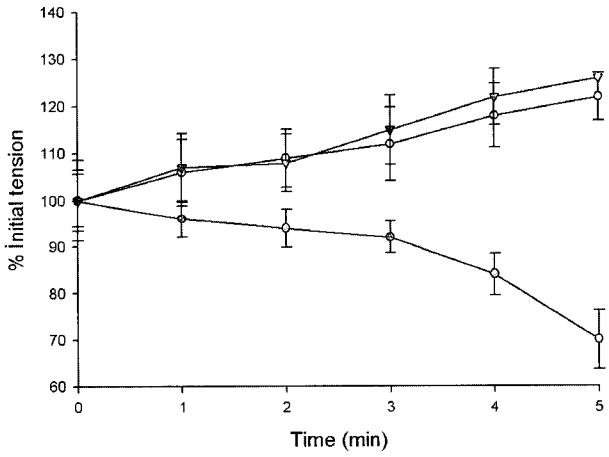

Fig. 4. Effect of TMZ on the tension frequency relationship in the isolated rat diaphragm muscle. Data are the mean ± SD for control (●), 5×10^{-6} M (○) and 5×10^{-5} M TMZ-treated (▼) rats. There were significant differences due to TMZ (n=10, p< 0,001, ANOVA).

Tetanic and tension-frequency relationship in the TMZ media

Fig. 4 shows the isometric twitch tensions formed as a result of the stimulation frequencies in the 5×10^{-6} M and 5×10^{-5} M TMZ bath media. In the TMZ bath media the twitch tensions, obtained by stimulation with frequencies of 10, 20 and 50 Hz, showed no significant differences compared with those of the controls (p>0.001). However, a stimulation frequency of 100 Hz the tetanic twitch tension formed showed a significant increase compared with the controls (p<0.001, Fig. 5).

DISCUSSION

Whether TMZ could induce and provide a beneficial

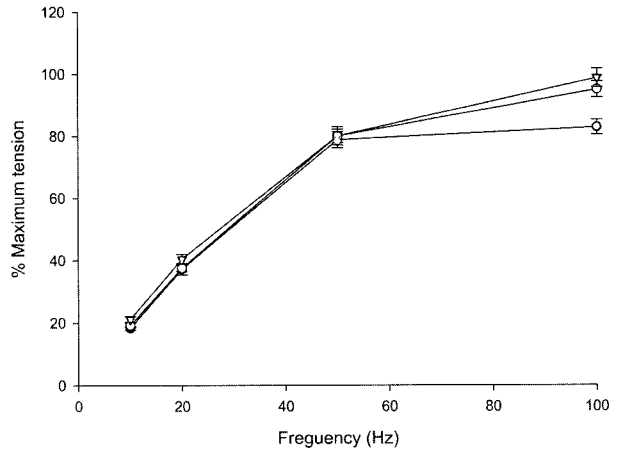

Fig. 5. Effect of TMZ on fatigue of the isolated rat diaphragm muscle. Data are the mean ± SD for control (●) 5×10^{-6} M (○) and 5×10^{-5} M TMZ-treated (▽) rats. There were significant differences due to TMZ (n=10, p< 0,001, ANOVA).

effect on isometric and tetanic contractile characteristics of pre- and post-fatigue were tested in an isolated rat diaphragm muscle. An isolated rat diaphragm muscle was chosen for this study because in the examination of the contraction, relaxation and tetanic contractility properties, taking into consideration the determination of fatigue, the skeletal muscle would serve as a better model. TMZ at concentrations of 10^{-6} and 10^{-5} M where used in this study as 10^{-6} M is within the range of the therapeutic levels observed in the treatment of patients with cardiovascular disorders (Opie and Boucher, 1995).

As is well know, the energy performance of a muscle is determined by the phosphogene, glycogen-lactic acid and aerobic systems (Guyton and Hall, 2000). For this reason, changes in these metabolic processes are expected with muscle fatigue. Muscle fatigue is the result of the decline in the sensitivity of the myofilaments to the Ca^{+2} ions, lactate and protons (H^+ ion concentration), and to the accumulation of metabolites, like inorganic phosphate, as well as changes in the complex intracellular metabolisms, including ATP consumption (Allen and Lee, 1989; Fitts, 1994; Miller *et al.*, 1988; Wilson *et al.*, 1988). Those intracellular changes are also known to have improved postischemic-reperfusion (Albengres *et al.*, 1998; Harpey et al., 1988). Therefore, the effect in fatigue model of TMZ, which is an antiischemic agent, has been investigated.

In this study, the loss in contractile forces (30%) as a result of application of the high frequency stimulation protocol in the TMZ-free medium has been demonstrated to be prevented by the addition of TMZ (5×10^{-5} M). According to our data, of the parameters reflecting the kinetics of contraction, the muscle contraction and relaxation periods were increased by TMZ. The contraction kinetics have been reported to result from the spread of the action

potential within the sarcolemma and T-tubular systems, Ca^{+2} efflux from the sarcoplasmic reticulum (SR), initiation of the stimulationcontraction coupling and the changes in the activation of the reuptake and restoration rates (Allen, 1989; Fitts, 1994; Allen *et al.*, 1992; Murthy et al., 2001).

The increase in the contraction force and the decrease in the 1/2RT duration in the fatigue model formed in the TMZ media, imply increases in the rates of contraction and relaxation. According to the results of this study, it can be inferred that TMZ, by increasing the contraction kinetics, improves the contractile parameters by preventing fatigue. Because it was not possible to measure the intracellular [Ca^{+2}] and pH in our laboratory the interpretation of the changes in the processes involved is limited.

According to our results, rather than the duration of contraction, the 1/2RT time was affected much more by fatigue. The rate at which skeletal muscle relaxes slows as a muscle fatigues (Van Lunteren *et al.*, 1995; Van Lunteren *et al.*, 1997). This has been attributed to several factors; including depletion of height-energy phosphates, intracellular acidosis and alterations in the membranous ionic-conductances, and lowering either one of these conductances enhances the degree of slowing of relaxation during fatigue (Albengres *et al.*, 1998; Opie and Boucher, 1995; Van Lunteren *et al.*, 1997). Relaxation may also be prolonged by increased sarcoplasmic Ca^{2+} due to delayed repolarization. Repetitive contractions and hypoxia both lead to resting membrane depolarization, the former as a direct consequence enhanced K^+ efflux (Van Lunteren *et al.*, 1995; Van Lunteren *et al.*, 1997; Sjogaard, 1991), and the latter possibly as a result of an adenosine-mediated reduction Na-K pump activity (Esau, 1994). The shortening and velocity of relaxation (±dP/dt), which are indications of the performance of contractile muscle, were significantly affected by TMZ. Namely, in isolated rat diaphragm muscle, trimetazidine significantly limits the decrease in the biomechanical performance under fatigue conditions. The maximal shortening depends on the rate of cross-bridge formation and mitochondrial/sarcoplasmic reticular Ca^{2+} release. The velocity relengthening is determined by adenosine triphosphate-dependent processes and the restoration of the diaphragm muscle forces. Some other investigators findings are in support of ours. Hirano et al, in a fatigue model with 30 Hz, attributed the loss of contraction force to the increase in the cytosolic Ca^{+2} ion concentration in addition to the decrease in the sensitivity of the myofilaments to Ca^{+2} ions (Hirano *et al.*, 2000). In another study, which supports our finding of the prolongation of the relaxation period in fatigue, it was observed that reuptake of the Ca^{+2} ions by the Ca^{+2} pump in the SR is impaired in fatigue, and as a result an increase in the intracellular Ca^{+2} ions led to slowing of the relaxation process (Fryer *et al.*, 1995; Westerblad and Allen, 1991; Westerblad and Allen, 1993; Westerblad *et al.*, 1993; Westerblad *et al.*, 1991). In a similar way, the changes in the parameters of contraction, such as the contraction force and the contraction and relaxation periods, should also lead to changes in the contraction and relaxation rates of the muscle. These changes were also recorded in our study.

Another important finding of this study was that whilst no significant difference was noted in the isometric contraction forces between the 10^{-6} M TMZ and 10^{-5} M TMZ-free media at stimulation frequencies of 10, 20 and 50 Hz, an increase in the contraction force was observed in the 100 Hz tetanic stimulation. Muscle fatigue may be defined as the failure to maintain the required or expected force (Fitts, 1994), which can be caused by numerous central and peripheral processes, as mention above (Allen *et al.*, 1989; Fitts, 1994; Miller *et al.*, 1988). However, in human experiments, the association between hypoxia and fatigue (reduction in muscle force) has proved controversial (Hogan *et al.*, 1994; Stansby *et al.*, 1990). Although a few human studies show association (Murthy *et al.*, 2001; Eiken and Tesch, 1984; Kaijser, 1970) between hypoxia and reduced muscle force production, they often involve high-intensity exercise protocols to induce fatigue. Therefore, the cause of fatigue may be confounded by factors other than hypoxia, such as acidosis, which is also implicated in causing fatigue (Fitts, 1994). One of the most important properties of TMZ is the prevention of cellular changes occurring with ischemia and hypoxia without any effect under normoxic conditions (Westerblad *et al.*, 1993; Stary *et al.*, 2003). Rossi *et al.*, showed that the cardiac adenosine triphosphate and phosphocreatinine levels during reperfusion were significantly higher in hearts treated with TMZ than in those of the controls (Rossi *et al.*, 1990). Trimetazidine prevents the depletion of high-energy phosphates, reduces acidosis and restores the synthetic activities of mitochondrial phosphorylation immediately from the beginning of reperfusion (Rossi *et al.*, 1990; Renaud, 1988). Emre *et al.*, showed that chronic treatment of TMZ was found to prevent the reduction in the contraction force caused by fatigue (Emre *et al.*, 2003). In the light of the findings from both our study and understanding from the literature, TMZ is a good anti-ischemic agent. Considering the works of the investigators, widespread use of TMZ as a protective agent for tissue against hypoxia, and its use as an agent in tissue preservation solutions in the future, remain high possibilities.

In conclusion, TMZ can be said to prevent the decrease in the contractility following fatigue, and has been shown to lead to a 22-30% increase in muscle contraction. The anti-ischemic effect of TMZ, by preservation of the cell membrane structure and cellular functions, causes the quick recovery of the ischemic cell energy stores (Guarnieri *et*

al., 1997; Allen *et al.*, 1989). In addition to this, it is our opinion that it also restricts intracellular acidosis, as well as providing effective cellular homeostasis to alleviate the derangements in the transmembrane ion exchange that lead to the accumulation of cytosolic calcium. However, further research in this field will need to be performed.

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