Changes in Nitric Oxide Level in Striated Muscles of Rats Following Different Types of Death Running Title: Nitric Oxide Level in Striated Muscles after Death

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Summary

Backgraund: **Nitric oxide is a signal molecule regulating the organism functions in living bodies. The aim of this study was to investigate the NO levels of striated muscles after different types of death in rats.**

Methods: **Nitric oxide levels in the muscles of masseter, triceps, and quadriceps obtained from right and left sides of 24 Spraque-Dawley rats following death were investigated. The rats were divided into three groups as cervical dislocation (control) group, electric shock group, and drowning group. After applying a light anesthesia, the rats were killed by cervical dislocation, electric shock and drowning. The samples were taken immediately and 120 minutes after death.**

Results: **In all muscle types of all groups, NO concentrations were lower in samples obtained 120 minutes after death than in those obtained immediately after death. NO concentrations were lower in the electric shock and drowning group than in the control group for both times.**

Conclusion**: We can conclude that the type of death may affect the occurrence of rigor mortis and NO measurement may give an important clue in evaluation the mode of death.**

Key words: nitric oxide – skeletal muscle – rat, electric shock – drowning

Souhrn

Změny hladiny oxidu dusnatého v příčně pruhovaném svalu potkanů v závislosti na způsobu smrti

Pozadí: **Oxid dusnatý je signální molekula regulující funkce organismu v živém těle. Cílem studie bylo zkoumat hladiny NO příčně pruhovaného svalu v závislosti na typu smrti u potkanů.**

Metody: **Byly zkoumány posmrtné hladiny oxidu dusnatého v musculus masseter, triceps a quadriceps získaných z pravé a levé strany 24 potkanů Sprague-Dawley. Potkani byli rozděleni do tři skupin: skupina usmrcená cervikální dislokací (kontrolní skupina), skupina usmrcená elektrickým šokem a utopením. Po aplikaci lehké anestezie byli potkani usmrceni cervikální dislokací, elektrickým šokem nebo utopením. Vzorky byly odebrány okamžitě a 120 minut po smrti.**

Výsledky: **Ve všech vzorcích všech svalů všech skupin potkanů odebraných 120 minut po smrti byly koncentrace NO nižší než ve vzorcích odebraných okamžitě po smrti. Koncentrace NO ve skupině po elektrickém šoku a po utopení byly nižší v obou intervalech než u kontrolní skupiny.**

Závěry: **Je možno uzavřít, že způsob smrti může ovlivnit nástup rigor mortis a měření NO může dát důležitý klíč při hodnocení způsobu smrti.**

Klíčová slova: oxid dusný – kosterní sval – potkan, elektrický šok – utopení

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INTRODUCTION

Nitric oxide (NO) is a signal molecule regulating the organism functions in living bodies (1–4). Since NO molecule is a small, neutral, lipophylic molecule, it can penetrate biological membranes (5, 6). NO is different from the other mediators in that it can bind covalently to the molecule which it will affect, and it is not stored after diffusion. NO is known as a multifunctional molecule in intracellular and extracellular space. Thus, one can think NO to function in rigor mortis occurrence (7).

Electrical shock to living body results in death causing ventricular fibrillation, respiratory muscle spasm, stroke and burns. Cell membrane destruction is an important feature of electrical shock. 800–1 000 mili volt electric flow disturbs most of the membranes of the cells of human being (8).

Drowning is a kind of death based on anoxia, occurs as a result of aspiration of water instead of air into upper and lower airways, after the reflex apnea period. All organ system may be affected in pathophysiology. Alveolar damages (surfactan disturbance and noncardiogenic pulmonary oedema) are seen. Metabolic acidosis appears due to the anoxia (9).

The key to understanding the apoptotic process is the activation of a set of protease known as caspases (10, 11). Although there is evidence for NO-induced cell death pathways that are caspase-independent, most apoptosis and necrosis pathways do involve caspases (12, 13). The overlapping spectrum of death processes that proceed from apoptosis to eventual necrosis likely involves caspases that can be regulated by NO (12). Although the concentration and source of NO can determine the response of the cell to NO exposure, NO activates or inactivates apoptosis. However effect of NO on skeletal muscle is not so far investigated. Normal muscle expresses two NO synthase isoforms: the neural type nNOS and the endothelial type eNOS. Little is known about the mechanisms, which control NOS expression in skeletal muscle (13).

Rigor mortis is known as the hardening of all muscles following death, being an important post-mortem change for the determination of death itself, the manner, and the time of death (14–18). Actin and myosin are known the essential elements of muscle contraction (19). The energy needed for muscle contraction is provided by ATPase activation, localized on myosin head. ATP is hydrolyzed by the enzyme ATPase and the energy is released. If the ATP is not replaced (ATP insufficiency), actin and myosin combine irreversibly, resulting in rigor mortis following death (2). In this study, we aimed to investigate the possible relationship between NO in certain muscles and rigor mortis occurrence in rats killed with different modes.

MATERIAL AND METHODS

Subject

Spraque-Dawley species rats were used in the study. The rats were fed with similar diet. Twenty-four (12 males, 12 females, weighing 185–270 g) rats were divided into three groups, each containing 8 (4 males and 4 females): cervical dislocation group (controls), electric shock group, and drowning group. Light anesthesia was applied by giving di-ethyl ether. Rats were killed with neck dislocation, by giving alternate electrical shock, and by drowning.

Neck dislocation was performed by pulling from tail after squeezing the neck of the rat with a hard material, and rats died immediately. In the electric shock group, 220-volt alternating electrical current was given from tail and left anterior extremity and rats died in 3–4 minutes. For drowning, rats were put into water filled container, and pushed down to prevent the coming up of rats. Rats died in 4–6 minutes in this group.

Specimen collection

Immediately after death, the right masseter, triceps, and quadriceps muscles were isolated, and their tendons and fascia were removed. This sample was called as sample 1. After the killed bodies were waited for 120 minutes at supine position at 21-22 °C, other samples were taken from the left side of the animals. The last sample was called as sample 2. The muscle samples were weighed carefully, put into saline, and then frozen just after they were taken. The tissue samples were cut finely and homogenized within 6 ml (0.6 N) of perchloric acid in homogenizer. The resultant homogenates were centrifuged at 3000Xg for 15 min. The pH of 5 ml of supernatant was adjusted to 6 with 5N KOH. The last contents were reserved in deep freezer up to the day of analysis.

NO Analyses

NO concentrations in resultant homogenate were determined with colorimetric method. Very short-lived radical NO is fast oxidized into NO_2 and NO_3, and these two products were measured.

The measurement principle of the method is that NO3 is reduced to NO_2 with nitrate reductase in the presence of NADPH+ H+ (Reaction 1).

1-NO_3 + NADPH + H^+ —NR→ NO^2 + NADP+ + H_2O

The produced NO_2 enters a reaction with sulphanylamide and N-(1-naphtyl)-ethylenediamine dihydrochloride (Griess reactive), and produce violet colored diazo dye (reaction 2).

2-NO_2 + sulphanylamide and N-(1-naphtyl)-ethylenediamine dihydrochloride → diazo. The absorbency of colored complex is measured at 550 nm (20). The results were expressed as mg / g. wet tissue.

Statistical analyses

Values are expressed as means ± SD. The statistical significance of the difference in NO level between groups was determined by Mann-Whitney U test in SPSS for windows program. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

The mean values of NO level of all muscles of all groups was found to be decreased in sample 2, compare to sample 1 (table 1). The difference between the NO level of sample 1 and sample 2 was statistically significant in each type of muscles within groups (table 2). Three muscle samples had different NO levels in all groups. When the NO levels of each sample are concerned the level of NO in masseter muscle was found to be the highest both in sample 1 and 2. When groups are concerned the NO level was highest in control group, and it was lowest in electric shock group. In drowning group the NO level of triceps and quadriceps muscles was lower than that of control group, whereas it was not different in masseter muscle (figure 1).

Tab. 1. NO levels of muscles in all groups (mean ± SD; mg / g. wet tissue)

	CONTROL (N=8)	ELECTRICAL SHOCK (N=8)	DROWNING (N=8)
Masseter 1	106.88 ± 32.4	78.54 ± 26.52	105.40 ±34.01
Masseter 2	63.68 ± 0.63	45.30 ± 27.79	78.04 ± 36.51
Triceps 1	103.49 ± 16.3	44.26 ± 19.29	48.40 ±22.16
Triceps 2	57.76 ± 6.77	24.93 ± 7.09	31.07 ±13.07
Quadriceps 1	97.95 ± 13.70	36.59 ± 20.94	71.95 ±33.90
Quadriceps 2	58.05 ± 17.67	18.75 ± 14.43	38.80 ±13.76

Tab. 2. Statistical evaluation of NO results in three groups and in three muscles (1 and 2 represent sample 1 and sample 2 respectively)

	CONTROL (N=8)	ELECTRICAL SHOCK (N=8)	DROWNING (N=8)
	p	p	p
Masseter $^1/_2$	0,001	0,001	0,029
Triceps $^1/_2$	0,001	0,047	0,021
Quadriceps $^1/_2$	0,001	0,001	0,018

Tab. 3. Statistical evaluation of NO results. The comparisons of any two groups (1 and 2 represent sample 1 and sample 2 respectively)

	CONTROL / ELECTRICAL SHOCK	CONTROL / DROWNING	ELECTRICAL SHOCK / DROWNING
	P	p	p
Masseter1	0,1415	0,7527	0,2076
Masseter2	0,4008	0,5632	0,1152
Triceps 1	0,0011	0,0011	0,5992
Triceps 2	0,0008	0,0008	0,3446
Quadricep 1	0,0011	0,0929	0,0357
Quadricep 2	0,0023	0,0742	0,0357

When control and electric shock group compared, the NO level was found to be lower in triceps and quadriceps muscles in electric shock group both in sample 1 and sample 2. This difference was statistically significant (P<<0.05). When control and drowning group compared, the NO level was found to be lower in triceps and quadriceps muscles in drowning group both in

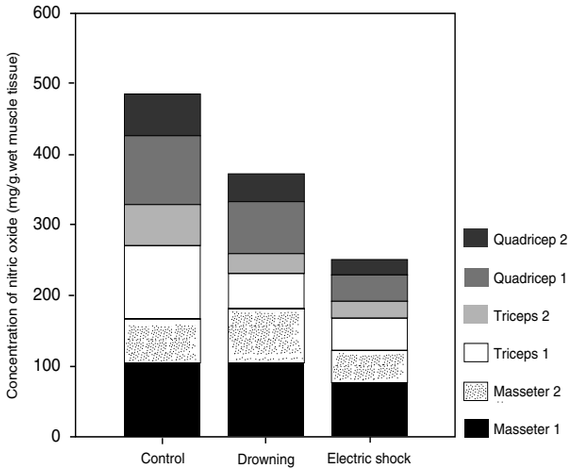

Figure 1. NO levels of all muscles in all groups

sample 1 and sample 2. This difference was statistically significant in triceps muscle (P<<0.05), and near significant in quadriceps muscle (P=0.09 for sample 1, P=0.07 for sample 2). The difference in the level of NO in masseter muscles was not statistically significant between groups. There was no statistically significant difference in NO level of all muscles between electric shock and drowning groups.

DISCUSSION

NO is synthesized from L-arginine by NO synthase (NOS, E.C 1.14.23) in a Ca /calmodulin-dependent manner. The enzyme exists in multiple isoforms, some of which have been purified and characterized: type I from brain, or nNOS; type II from macrophages, or iNOS; type III from endothelial cells, or eNOS(19). In a study on human brain NOS, unexpectedly, more messenger RNA of nNOS was found in human brain. Measurements of NOS activity in human muscle homogenates confirmed a high level of nNOS expression in muscle. The presence of NOS was confirmed in rat skeletal muscle and in many different muscles of various animals (21). Skeletal muscle is one, if not the only, of the few tissues able to express simultaneously all the NOS isozymes. Expression and localization of NOS isoforms are dependent on age and developmental stage, innervation and activity, history of exposure to cytokines and growth factors, and muscle fiber type and species.

In recent years, many studies have been done on cellular effects of NO. NO synthesized endogenously in muscle, is important in regulation of muscle functions. Immediately after death, ATP is no longer produced, resulting in the dysfunction of many cellular functions. As a result, NO synthesis is diminished (22). Our results related to the decreased NO level in muscles in sample 2 support the interpretation mentioned above. On the other hand, an isolated skeletal muscle in resting state produces lees NO, whereas any repeatedly contracting muscle syntheses high level of NO. In the case of our experiments, the dead muscle samples were used. Thus, the diminished NO levels in sample 2 for all groups are expectable results, since membrane transport dysfunction gives rise to low level of L-arginine, molecular oxygen, calcium, resulting in decreased NO synthesis.

Rigor mortis is known as the hardening of all muscles of the body following death, which is an important post-mortem change for the determination of death itself, the manner of death, and the time of death. When the muscles begin to autolysis, the rigor mortis disappears within 18 to 36 hours (21). It can be thought that rigor mortis can be associated with NO level of muscle tissue. There is no study in the literature about the relationship between NO and rigor mortis, or postmortem changes in NO concentration of muscle tissue. Since in each group of rats, a statistically significant decrease was determined in sample 2 when compared to sample 1 for each type of muscle, it can be concluded that NO may have an important role in postmortem changes in muscle tissue. If so, NO concentration in the muscle tissue may also be associated with the type and the time of death.

Marechal and Gailly reported that the effects of nitric oxide (NO) on skeletal muscle fibers can be classified in two groups. In the first, the effects of NO are direct, in the second group, the effects are mediated by cGMP. The general consequence of cGMP – mediated effects of NO is to improve mechanical and metabolic muscle power. On the other hand the general consequence of the direct effects of NO is to 'brake' the contraction and its associated metabolism. The effect on calcium release channels varies, being inhibitory at low and stimulatory at high NO concentrations. These studies suggest that NO level affects the muscle contraction (23). In our study it can be seen that when the muscle contracts too much as in the case of drowning (in the case of drowning rats were debilitated before death), NO level may be affected, and it was decreased in our cases.

Human muscles expressed two different cellular compartments related to NOS (24).Type I NOS (nNOS) was localized in the sarcolemma and in the cytoplasm of all muscle fibers, but more strongly in type I fibers; type III NOS (eNOS) was observed both in the endothelium of larger vessels and of microvessels. The third isoform, type II or iNOS , play s a major role in immune responses to bacteria and tumors, and iNOS gene transcription is induced by endotoxin and cytokines. Northern blot analyses demonstrated a 4.2-kb iNOS mRNA in skeletal muscle fibers. Anti-NOS II antibody labeled type I muscle fibers (25). Muscle extracts, total (26) or particulate (27), had some Ca2+-independent NOS activity, an indication of the presence of iNOS. inducible iNOS is strongly upregulated in skeletal muscle challenged by endotoxin (26).Little is known about the mechanisms which control NOS expression in skeletal muscle. Type I NOS is upregulated by mechanical loading (27), treadmill training (28), chronic electrical stimulation (29), and in aging rats (30). It decreases after denervation , but returns back to a normal level after reinnervation (31). This means that the severity of hypoxia closely related to the level of NO. Since the work in drowning and electrical shock is more than cervical

dislocation, the NO level was found less in drowning and electrical shock groups.

Another possible mechanism can be suggested for this event as follows. NO is synthesized from L-arginine in normal conditions. In the case of death, amino acid transport across the plasma membrane cannot be achieved because of low ATP levels. All membranous activities become defective. The second messengers of hormones, such as Ca/phosphatidyl inositide, cannot work. The intracellular Ca concentration does not increase, resulting in decrease of NO synthesis in the cell. Another cause of NO decline is oxygen lack, since molecular oxygen is needed in NO synthesis.

It can be concluded on the basis of the obtained results that, analysis of NO may be helpful in the estimation of the type and the time of the death, and rigor mortis occurrence may be associated with the manner by which the death occurs.

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