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14. ABSTRACT: Skeletal muscle atrophy due to bed confinement or cast-immobilization represents a serious medical problem in the military as well as in the civilian population. The increased protein degradation and decreased protein synthesis during muscle immobilization is extremely debilitating. In order to restore proper function to the atrophied muscle, reloading is required. Muscle reloading unfortunately results in a significant amount of oxidative damage. Therefore, means to minimize muscle damage during this period of reloading would also be extremely advantageous for proper recovery. Our interest is to investigate how the heat shock proteins (hsp), a family of proteins present in all mammalian cells, is able to protect muscle tissue against muscle atrophy. The hsps are able to regulate protein homeotasis but also protect against oxidative stress, apoptosis and imflammation. We have demonstrated that the hindlimb muscles of hsp70 overexpressing transgenic mice exhibit improved structural and functional recovery after a 7-day immobilization and 7-day recovery protocol as compared to control mice.

15. SUBJECT TERMS
Heat shock proteins, Skeletal Muscle, Atrophy.

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INTRODUCTION

Skeletal muscle atrophy is a response to disuse that occurs during immobilization due to injury, muscle unloading, aging, starvation and a series of other disease states (cachexia, etc) (1). Recent studies have demonstrated that patients with muscle atrophy/weakness have a greater burden of illness, require higher resource use and treatment that results in higher healthcare costs (2, 3). Therefore, it is evident that therapeutic strategies to ameliorate and reduce the recovery period following muscle atrophy would be important to lessen patient suffering and the economic burden associated with this condition. It is well known that slow muscles of the hind limbs such as soleus are more prone to atrophy than fast muscles. During muscle atrophy there is a marked loss of muscle mass, a reduction in fiber size, accompanied by a decrease in muscle protein content, a reduction in force and power and increased fatigability (4). Among these atrophic events, it has been speculated that programmed cell death or apoptosis plays a role in the reduction of muscle fiber size. Some other investigators have shown that the muscle fiber decrease during atrophy is a direct result of a quick shift from apoptosis to necrosis or what has been called the aponecrosis theory (5). This has led to the notion that therapeutic or preventive regimens that may delay or stop the process of apoptosis in muscle atrophy may prevent the loss of post mitotic myocytes which would be a beneficial outcome in patients undergoing immobilization or bed confinement. Subsequent to a short-term of muscle unloading, the period of muscle reloading is known to induce histological damage such as sarcomere lesions and infiltration of inflammatory cells (6). In addition, it has been shown that during muscle reloading oxygen radicals are generated due to the increased oxygen consumption in the recovering muscle leading to oxidative stress that results in muscle damage. Interestingly, recent studies have shown that a heat treatment during immobilization is able to reduce oxidative stress and protect muscle mass (7). This last study implicated the increase in hsp70 and hsp27 to the observed reduction in oxidative stress and preservation of muscle mass during immobilization. It is important to point out that a heat treatment or increase in temperature does more than just induce the expression of the heat shock proteins, therefore a direct relationship between the observed reduction in oxidative stress and improved muscle mass and the increased presence of the hsp70 and hsp27 is not fully established. Although, this last point has been clearly demonstrated with the use of transgenic mice solely expressing one of the hsps as we have previously shown (8, 9).

Studies using the increased expression of one single heat shock protein in a transgenic mouse line have demonstrated the presence of protection against skeletal muscle injury (8). For example, increased expression of hsp70 has been shown to protect skeletal muscle against lengthening contraction-induced damage and to facilitate rapid recovery (10). Also, the role of certain heat shock proteins on reactive oxygen species (ROS) production in injured skeletal muscle has been recently studied. Several reports have shown that hsp25 may play an important role in protecting skeletal muscle against the damaging effects of ROS (11) and that the increased presence of hsp70 in skeletal muscle prevents cellular damage during age-related increases in oxidative stress (10). We have found that expression of these protective proteins induced by hsp90 inhibiting compounds such as: radicicol, 17-AAG, celastrol and alvespimycin (17-DMAG) protect against frostbite injury (unpublished results). This last compound has recently been used extensively in clinical trials and has shown no or minimal adverse reactions besides having the advantage of being water-soluble which eliminates the need to use organic solvents (12). As we have found, alvespimycin also known as 17-DMAG (17-(dimethylaminoethylamino)-17-demethoxygeldanamycin) applied soon after frostbite injury is able to protect against severe muscle damage (13).

Others have also shown that during muscle disuse a group of transcription factors known as FoxO factors are activated (14). These factors are responsible for the expression of the atrophy-related genes or atrogenes which decrease muscle mass. Interestingly, several studies have shown that the heat shock proteins (hsp) seem to be able to mitigate the effects of muscle atrophy. Studies using whole-body hyperthermia before and during muscle disuse showed that increased expression of the hsps attenuated the decrease in muscle mass (7, 15). Studies using the transfer of the heat

shock protein 70 (hsp70) by electroporation into skeletal muscle demonstrated that it is possible to protect against muscle atrophy during muscle immobilization (16). Subsequent studies have now used more physiological appropriate models such as genetically modified mice. One study demonstrated that in a heat shock factor -1 (HSF-1) knock-out mouse, where decreased expression of the heat shock proteins resulted in poor recovering of skeletal muscles submitted to immobilization (17). Futhermore, our transgenic mice overexpressing the hsp70 gene exhibits improved structural and functional recovery of skeletal muscles after muscle disuse (9). In addition, a study where administration 17-AAG (17-(allylamino)-17-demethoxygeldanamycin), an inhibitor of hsp90 that induces hsp expression in rats showed an attenuated increase in the markers for protein degradation and an increase in markers for protein synthesis indicating a potential protective effect against muscle atrophy (18).

Recent studies have revealed interesting issues related to muscle homeostasis that concern the process of muscle atrophy. A review of studies on the function of the small heat shock proteins (sHSP) concludes that these proteins play a more general role in muscle structure that solely binding actin (19). This calls for a closer look of how sHSPs may be involved in muscle atrophy. Another interesting study performed in humans has shown that during short-term disuse muscle atrophy, most of the muscle mass loss is due to a decline in muscle protein synthesis and not to muscle degradation (20). Therefore, this would imply that the protective effect of the heat shock proteins against muscle atrophy is mainly dependent on the HSPs role in perserving muscle protein synthesis.

KEYWORDS: Heat shock proteins, skeletal muscle, atrophy, hsp90 inhibitors.

ACCOMPLISHMENTS:

In this second year of our research project, we have encountered a delay in the progress of our research program. Mainly, the department's Aurora Scientific muscle contraction setup has needed some serious upgrading. Presently, we finished all of the needed upgrading and now we are in the process of setting it up for our upcoming muscle contraction experiments. Nonetheless, even when we have not yet started our muscle contraction experiments, we have accomplished some of the objectives we proposed in our Statement of Work (see below).

The present research project expands on our previous work that demonstrated that over-expression of the heat shock protein 70 (hsp70) in a transgenic mouse is able to minimize the damage due to skeletal muscle atrophy. We have now set out to demonstrate that alvespimycin (17-DMAG) a compound that induces the expression of the heat shock proteins when administrated during cast immobilization may be a possible means of protecting against skeletal muscle atrophy.

One of the aims of our research project is to establish a reliable model of cast-immobilization in a mouse hind limb. This aim was been achieved by using a model previously used by us (9). In order to confirm that our model is resulting in muscle atrophy, we examined the effects of our cast-immobilization by assessing morphologically the muscle tissues of the left hind-limb. Figure 1 in this annual report shows the effect of muscle atrophy resulting in a 14 day period of cast-immobilization on the left hind-limb of a mouse's tibialis anterior (TA) that was injected with a saline solution every 3 days during the 14 day immobilization protocol (Figure 1A). The TA tissue in Fig. 1A shows significant clear areas (arrows) an indication of significant tissue lost. In contrast, Fig.1B shows the TA submitted to the same protocol but injected with 10mg/kg of 17-DMAG every 3 days during the 14 days of immobilization. In this last case, no significant loss of tissue is observed. Therefore, pointing to the fact that in the present of the heat shock inducing compound 17-DMAG, skeletal muscle does not appreciatively develop significant muscle atrophy.

We also confirmed the effect of 17-DMAG on the muscles of the left hind-limb during cast-immobilization by measuring the weight, and therefore mass of the muscles following cast-immobilization and normalizing it by the final body weight of the animal. Figure 2 shows our results on soleus muscles. Our results show that treatment of mice left hind-limb with saline or 17-DMAG without cast-immobilization shows no major difference in soleus weight. In contrast, while soleus muscle of mice treated with saline during a 7 day cast-immobilization show a decrease in weight, the soleus muscle of mice treated with 17-DMAG are significantly preserved. In addition, Figure 3 shows our results when analyzing the weight of TA muscle following cast-immobilization. Figure 3A presents the results obtained following a 7 day cast-immobilization where clearly a marked loss of weight or mass of the TA muscle results in the presence of saline, while in the presence of 17-DMAG, the TA suffers a lesser loss of weight or mass. Figure 3B presents similar results obtained following a 14 day cast-immobilization.

Our previous results last year showed that in the presence of 17-DMAG during our cast-immobilization atrophy model, the muscle atrophy marker, MuRF1 expression is significantly reduced in the muscles of the hind-limb. Our present results now show that FOXO3a which is responsible for the activation of MURF1 is also significantly reduced in expression during cast-immobilization in the presence of 17-DMAG. Figure 4 shows the Western blot of protein extracts from TA muscles submitted to 7 days of cast-immobilization. FOXO3a expression was normalized to the expression of GAPDH. As seen in Fig. 4, the expression of FOXO3a is significantly reduced when 17-DMAG is injected into the hind-limb during the cast-immobilization. This result confirms what has been previously reported by other investigators (16).

We are also interested in the effect that the increase expression of the heat shock proteins induced by 17-DMAG treatment has on the anti-oxidant proteins such as superoxide dismutase (SOD1). Therefore, we have now examined the effect of 17-DMAG treatment during cast-immobilization on SOD1 expression. Figure 5 presents the results we obtained in tibialis anterior muscle of left hind-limb subject to a 7 day cast-immobilization in the presence of saline, where the expression of SOD1 was significant increased (fig. 5, lanes 3 & 4) while when treated with 17-DMAG

there is an absence of any increase in expression of SOD1 (fig. 5, lanes 5 & 6). This result indicates that the heat shock proteins induced by 17-DMAG treatment block the increase in expression anti-oxidant proteins such as SOD1 due to muscle atrophy, potentially because of the heat shock proteins ability to reduce oxidative stress.

In the interest of exploring the potential role of other small heat shock proteins, we examined the expression of the small heat shock protein HSPB7 or cvHSP. Figure 6 presents our results in gastrocnemius (GAS) muscle of left hind-limb submitted to a 7 day cast-immobilization. In Fig. 6 we can observe that in the presence of17-DMAG the level of expression of HSPB7 is increased (lanes 3 & 4) as compared to the GAS muscle treated with saline (lanes 1 & 2) instead. This increase in this small HSP potentially plays a role in protecting the muscle fibers, since it has been established that it is involved in the maintenance of the contractile apparatus and cytoskeleton of muscle cells (19). Another point of interest concerning the expression of the heat shock proteins by 17-DMAG is the induction of the hsp70 co-chaperone, hsp40. This heat shock protein plays an important role since it is required for the proper function of the hsp70. In Figure 7, we explored the expression of hsp40 in the presence or absence of 17-DMAG during a 7 day cast-immobilization. As expected hsp40 is constantly expressed but it's expression is increased by the presence of 17-DMAG.

These results confirm that our research project is on the right track and promises that our subsequent studies should prove our hypothesis that 17-DMAG induction of the heat shock proteins can minimize the deleterious effects of muscle atrophy due to cast-immobilization.

Statement of Work

Specific Aim 1 (specified in proposal)	Timeline	Site 1	Progress
Major Task 1 All of the animals to be used in this research project will be the **CB6F1 hybrid mouse strain**, in order to connect this study to our previous study (Miyabara et al. 2012). Commercially available at ENVIGO. Total of animals = 180	Months		Done 100%
Subtask 1:IACUC approval; ACURO approval	1-3	Dr. Mestril	Done
Subtask 2: Determining the concentration of 17-DMAG that induces the heat shock proteins. One experiment involving 30 mice (see Table below).	3	Dr. Mestril	Done 100%
Subtask 3: Test 17-DMAG as a means of minimizing muscle atrophy following 7 days of hind-limb cast-immobilization. Five experiments for a total of 150 mice (see Table below).	9	Dr. Mestril	Done 100%
Milestone Achieved: Determination of appropriate concentration of 17-DMAG to preserve muscle morphology and function after immobilization.	15		Done 100%
Specific Aim 2 (specified in proposal)			
Major Task 2 Total of animals = 108			
Subtask 1: Determine anti-oxidant enzyme activity during following cast-immobilization. Experiment involving 18 mice repeated once for a total of 36 mice (see Table below).	4	Dr. Mestril	100% Done
Subtask 2: Determine levels of inflammatory cytokines following cast-immobilization. Experiment involving 18 mice repeated once for a total of 36 mice (see Table below).	4	Dr. Mestril	50% Done
Subtask 3: Determine interaction of heat shock proteins with both anti-oxidant enzymes and inflammatory cytokines. Experiment involving 18 mice repeated once for a total of 36 mice (see Table below).	4	Dr. Mestril	50% Done
Milestone Achieved: Determine potential mechanism or mechanisms how the heat shock proteins minimize muscle atrophy following cast-immobilization	12		67% Done

Specific Aim 3 (specified in proposal)			
Major Task 3 Total of animals =108			
Subtask 1: Determine how effective 17-DMAG is upon prolonged cast-immobilization. One experiment involving 54 mice (see Table below).	6	Dr. Mestril	50% Done
Subtask 2: Determine how long the protective effect of 17-DMAG last after cast-immobilization. One experiment involving 54 mice (see Table below).	6	Dr. Mestril	
Milestone Achieved: Determine the persistance of anti-atrophy effect of the heat shock proteins during and following cast-immobilization	12	Dr. Mestril	

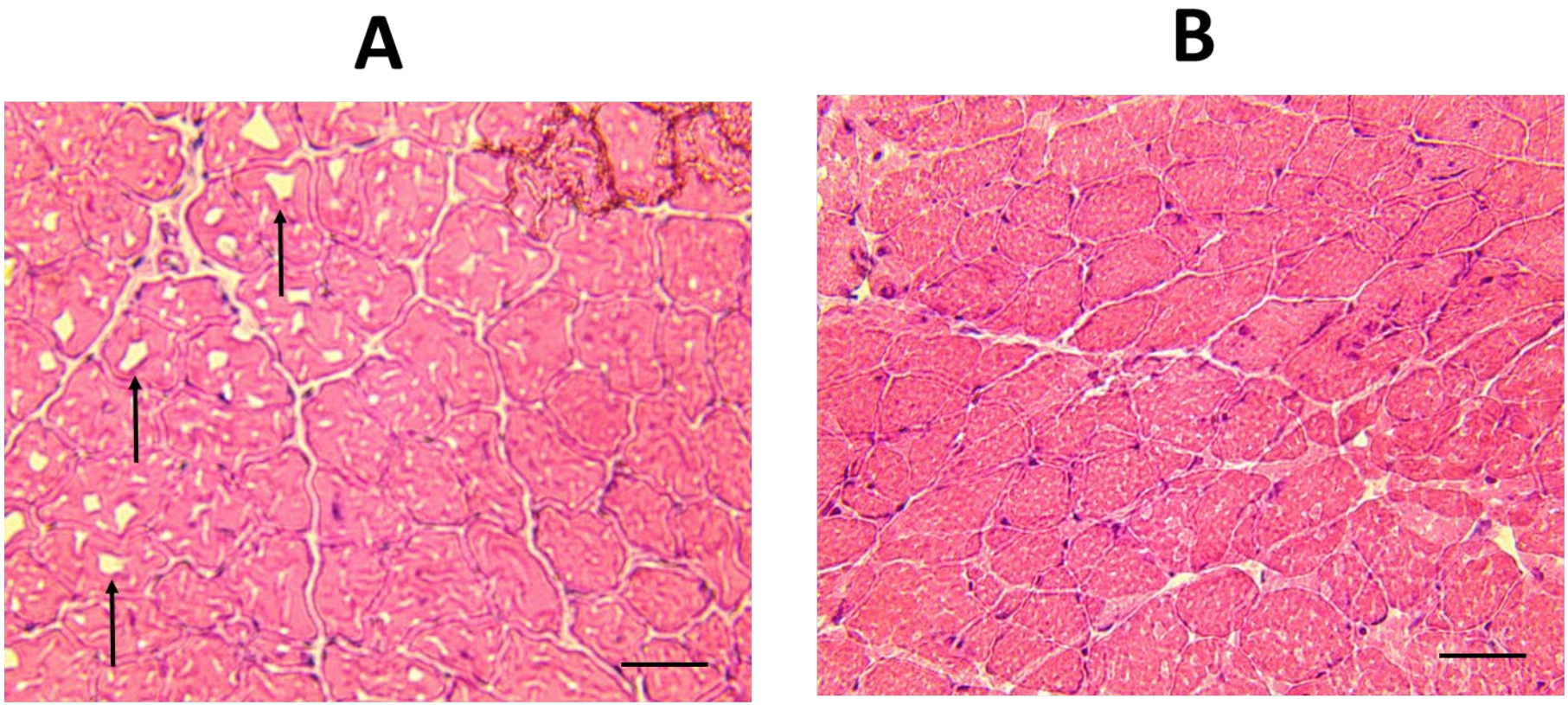

FIGURE 1. Morphological analysis using hematoxylin-eosin staining of tibialis anterior (TA) muscle cross-sections of mice left hind-limb. A) TA of mouse injected with saline and submitted to cast-immobilization for 14 days. B) TA of mouse injected with 17-DMAG and submitted to cast-immobilization for 14 days. Arrows indicate areas of muscle loss during cast-immobilization. Bar: 50 μm.

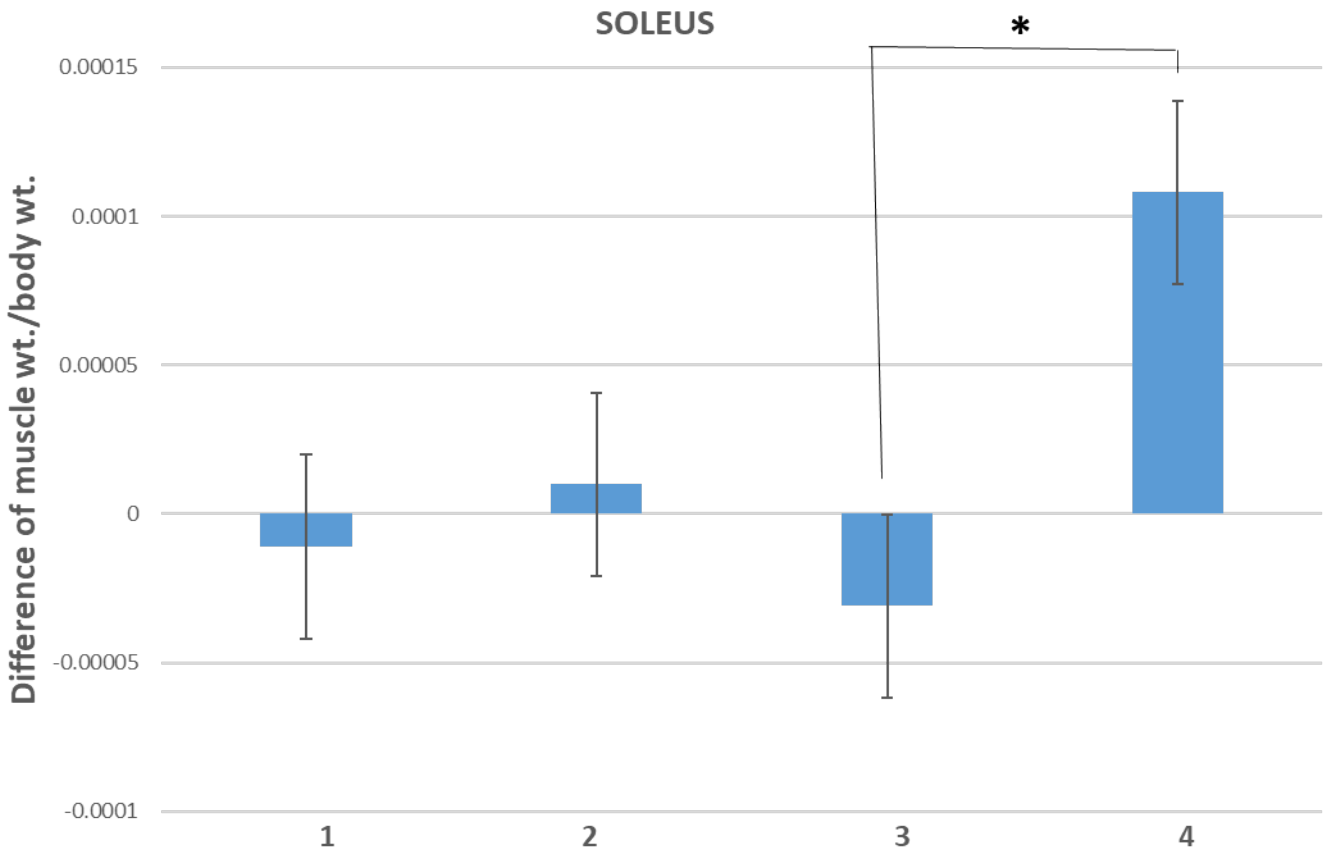

FIGURE 2. Soleus muscle weight from the left hind-limb of mice that was normalized to final body weight. 1) Soleus from control mice treated with saline. 2) Soleus from control mice treated with 17-DMAG. 3) Soleus from mice treated with saline and submitted to left hind-limb cast-immobilization for 7 days. 4) Soleus from mice treated with 17-DMAG and submitted to left hind-limb cast-immobilization for 7 days. Each group consisted of 3 animals, *$p<0.05$.

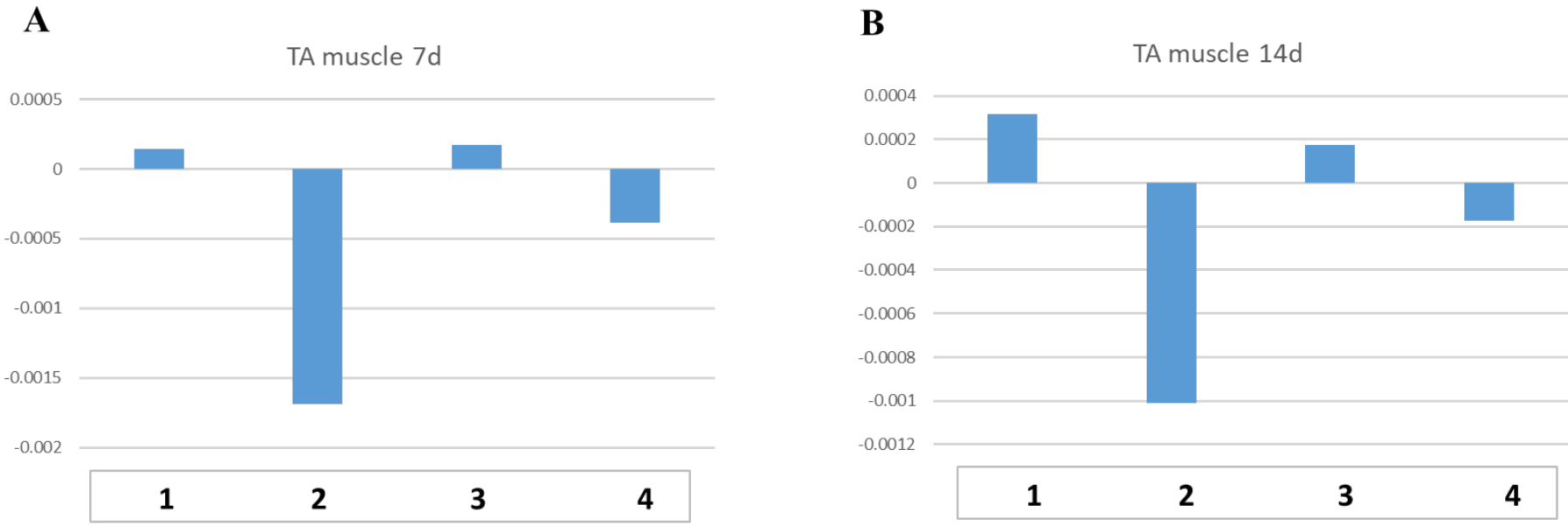

FIGURE 3. Tibialis anterior (TA) muscle weight from the left hind-limb of mice that was normalized to final body weight. In **A**: 1) TA from left hind-limb mouse treated with saline and not casted. 2) TA from left hind-limb mouse treated with saline and submitted to left hind-limb cast-immobilization for 7 days. 3) TA from left hind-limb mouse treated with 17-DMAG and not casted. 4) TA from left hind-limb mouse treated with 17-DMAG and submitted to left hind-limb cast-immobilization for 7 days. In **B**: 1) TA from left hind-limb mouse treated with saline and not casted. 2) TA from left hind-limb mouse treated with saline and submitted to left hind-limb cast-immobilization for 14 days. 3) TA from left hind-limb mouse treated with 17-DMAG and not casted. 4) TA from left hind-limb mouse treated with 17-DMAG and submitted to left hind-limb cast-immobilization for 14 days.

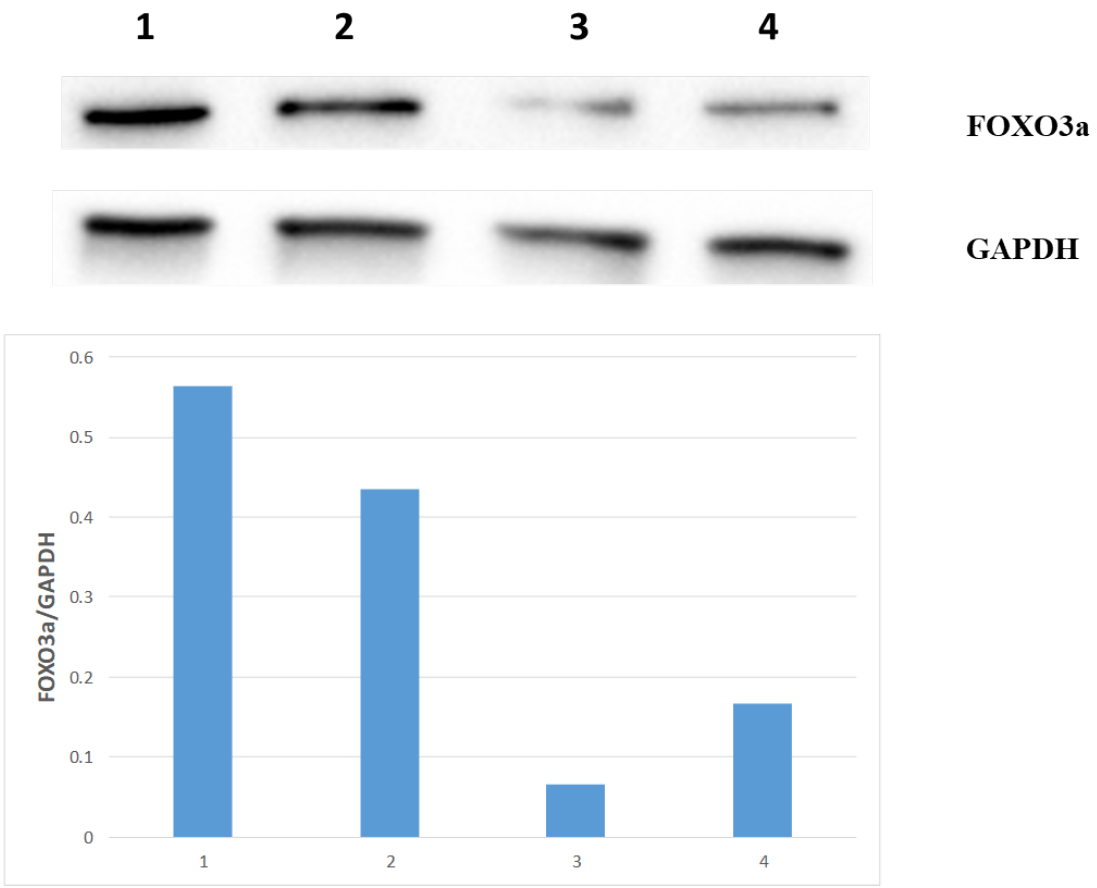

FIGURE 4. Western blot of protein extract from Tibialis Anterior (TA) of the left hind-limb from mice submitted to cast-immobilization for 7 days. Lanes 1 & 2 are from the left hind-limbs of mice injected with saline and submitted to cast-immobilization for 7 days. Lanes 3 & 4 are from the left hind-limbs of mice injected with 17-DMAG and submitted to cast-immobilization for 7 days. The blot was reacted with antibodies against FOXO3a and GAPDH. Histogram shows the relative amount of FOXO3a expressed in each tissue sample normalized to GAPDH.

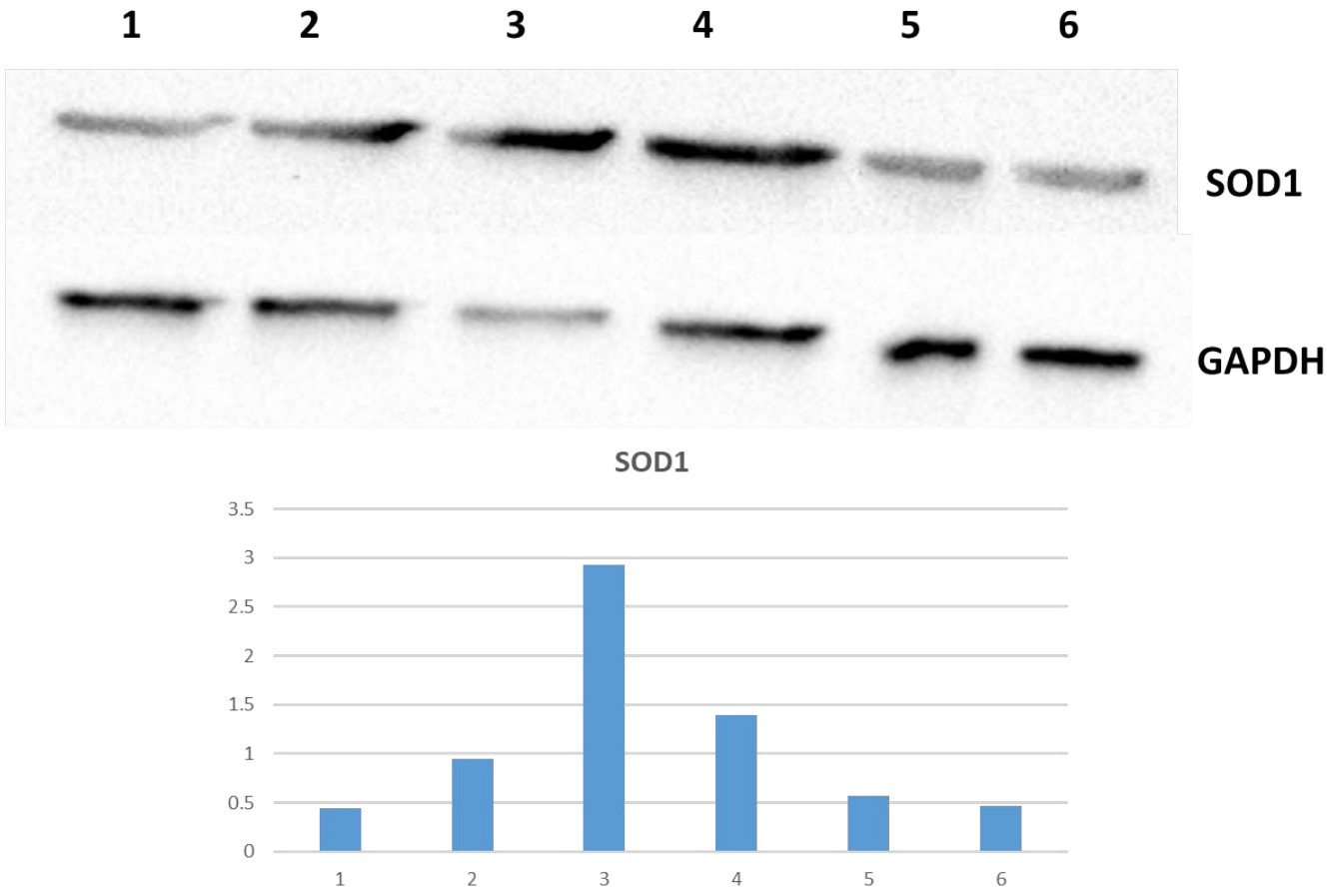

FIGURE 5. Western blot of Tibialis Anterior (TA) muscle protein extracts. Lane 1: Protein extract from left hind-limb injected with saline and not casted. Lane 2: Protein extract from left hind-limb injected with 17-DMAG and not casted. Lanes 3 & 4: Protein extracts from left hind-limb injected with saline and submitted to left hind-limb cast-immobilization for 7 days. Lanes 5 & 6: Protein extracts from left hind-limb injected with 17-DMAG and submitted to left hind-limb cast-immobilization for 7 days. The blot was reacted with antibodies against SOD1 and GAPDH. Histogram shows the relative amount of SOD1 expressed in each tissue sample normalized to GAPDH.

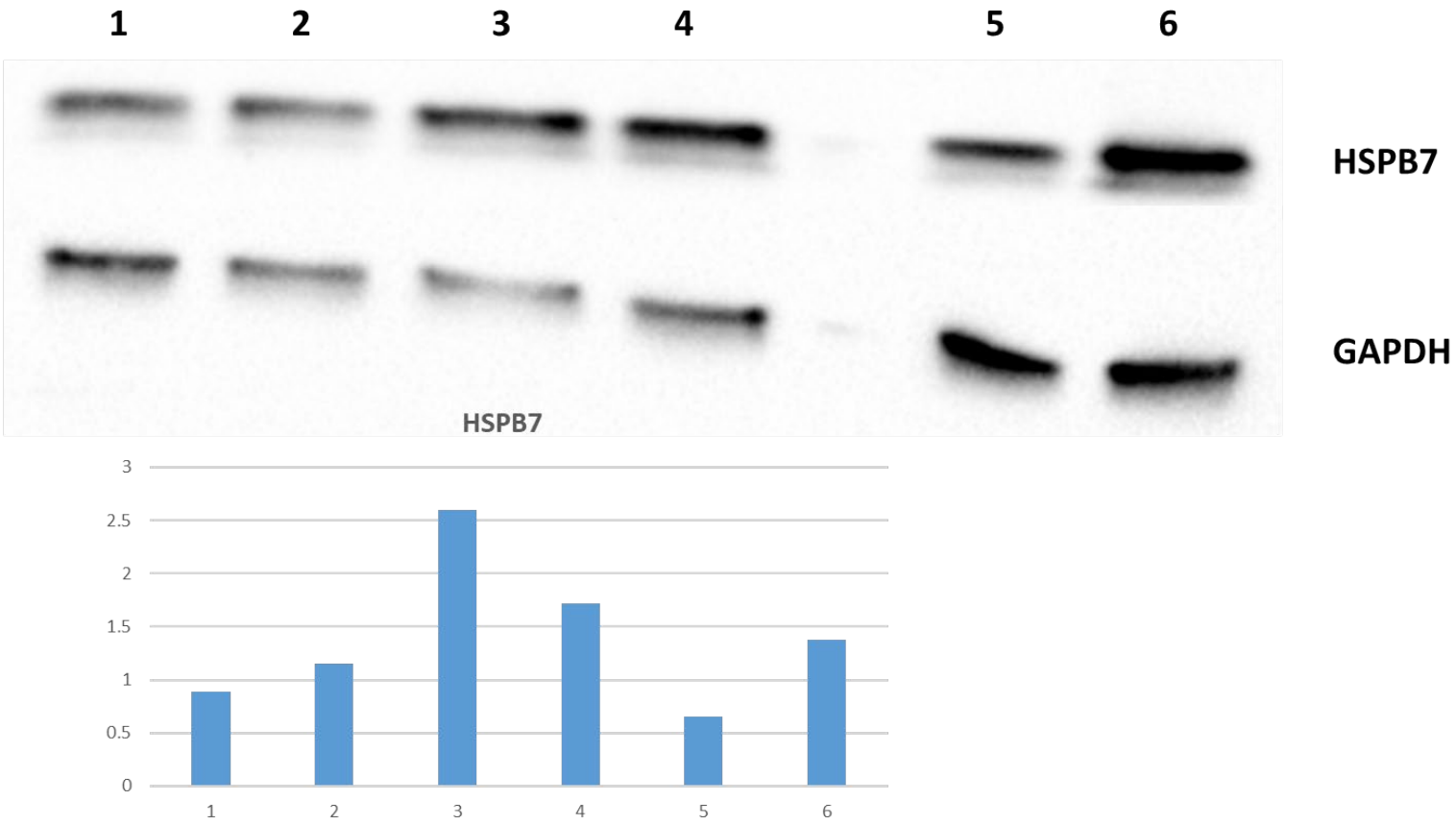

FIGURE 6. Western blot of Gastrocnemius (GAS) muscle protein extracts. Lanes 1 & 2: Protein extracts from left hind-limb injected with saline and submitted to left hind-limb cast-immobilization for 7 days. Lanes 3 & 4: Protein extracts from left hind-limb injected with 17-DMAG and submitted to left hind-limb cast-immobilization for 7 days. Lane 5: Protein extract from left hind-limb injected with saline and not casted. Lane 6: Protein extract from left hind-limb injected with 17-DMAG and not casted. The blot was reacted with antibodies against HSPB7 (cvHSP) and GAPDH. Histogram shows the relative amount of HSPB7 expressed in each tissue sample normalized to GAPDH.

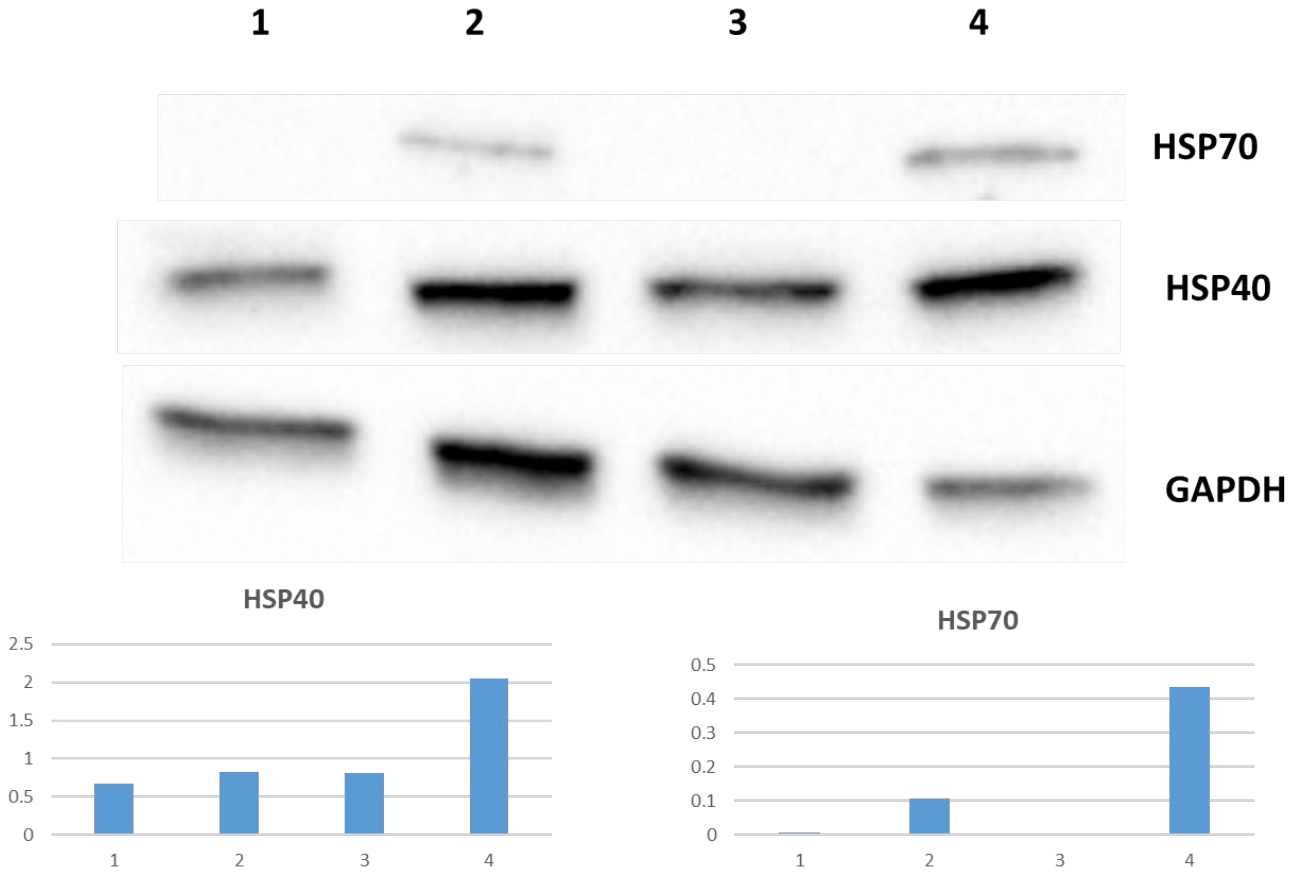

FIGURE 7. Western blot of Gastrocnemius (GAS) muscle protein extracts. Lanes 1: Protein extract from left hind-limb injected with saline and submitted to left hind-limb cast-immobilization for 7 days. Lanes 2: Protein extract from left hind-limb injected with 17-DMAG and submitted to left hind-limb cast-immobilization for 7 days. Lane 3: Protein extract from left hind-limb injected with saline and not casted. Lane 4: Protein extract from left hind-limb injected with 17-DMAG and not casted. The blot was reacted with antibodies against HSP40, HSP70 and GAPDH. Histogram shows the relative amount of HSP40 and HSP70 expressed in each tissue sample normalized to GAPDH.

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IMPACT

The results obtained in this second year confirm that our research project is on the right path and promises that our subsequent studies should prove our hypothesis that 17-DMAG induction of the heat shock proteins can minimize the deleterious effects of muscle atrophy due to cast-immobilization. Therefore, this could develop into a therapeutic strategy to ameliorate and reduce the recovery period following muscle atrophy which would lessen patient recovery and the economic burden associated with this condition.

CHANGES/PROBLEMS:

As mentioned above in this second year of our research project, we have encountered a delay in the progress of our research program. Mainly, the department's Aurora Scientific muscle contraction setup has needed some serious upgrading. Presently, we finished all of the needed upgrading and now we are in the process of setting it up for our upcoming muscle contraction experiments.

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PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS: None.

SPECIAL REPORTING REQUIREMENTS: None.

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