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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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TABLE OF CONTENTS

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).

KEYWORDS:

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

ACCOMPLISHMENTS:

In this first year of our research project, we have encountered some significant delays in the progress of our research program. Mainly, the animal facility in our Institution suffered a significant amount of flooding at the beginning of 2021. Therefore, the start of the animal work portion of our research project resulted in a delay of approximately 3 months. In addition, due to the consequences of the COVID-19 pandemic many of the parts and supplies we ordered and needed for our research resulted in shipping delays of as much as 6 months. Nonetheless, we have accomplished some of the objectives we proposed for the first 12 months in our Statement of Work.

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 first aims of our research project is to establish a reliable model of cast-immobilization in a mouse hind limb. This aim is achieved by using a model previously used by us (9). In order to confirm that our model is resulting in muscle atrophy, we used the increased expression of a known muscle atrophy biomarker, MuRF1. An example of our confirmation that our model is producing muscle atrophy is shown in Figure 1. Figure 1 shows a Western blot containing protein samples from both gastrocnemius and tibialis anterior muscles from the left hind limbs of mice either submitted to our cast-immobilization model (atrophy) for 7 days or not cast-immobilized (control). The Western blot was reacted with antibodies specific for the atrophy biomarker MuRF1 protein and the protein loading standard GAPDH protein. The level of expression of the atrophy biomarker MuRF1 and GAPDH were scanned. The level of expression of MuRF1 was normalized by the level of GAPDH in order to determine how much atrophy has been by our model of cast-immobilization. As can be seen in Figure 1, both of the muscle types exposed to cast-immobilization exhibit a significant increase of expression of the atrophy biomarker MuRF1. The differences in expression of MuRF1 in the two muscle tissues is most probably due to the different muscle fiber types that make each one of these muscle tissues.

The following section describes our progress in these past months as related to our Statement of Work:

One of the first issues within Specific Aim I or our Major Task 1 is to determine the proper concentration of 17-DMAG that would induce the heat shock proteins in our cast-immobilization model. Our initial results have shown that a concentration of 10mg/kg of 17-DMAG administered during cast-immobilization of the hind limb of mice induces the expression of the heat shock proteins and reduces the expression of atrophy biomarker, MuRF1. Figure 2, shows the level of MuRF1 expression in tibialis anterior muscle, one of the muscles of the hind limb of mice submitted to our cast-immobilization atrophy model and treated with 17-DMAG or saline, which is the vehicle used for dissolving 17-DMAG. As shown in Figure 2, the level of MuRF1 expression is reduced in the presence of 17-DMAG as compared to presence of saline alone. In addition, we have also seen a similar effect of 17-DMAG in the extensor digitorum longus (EDL), another mouse hind limb muscle. Figure 3, shows how the presence of 17-DMAG during the cast-immobilization of a mouse hind limb reduces the level of expression of the atrophy biomarker MuRF1 in EDL muscle as compared to the hind limb treated only with saline.

The reason for the reduction in expression of the atrophy biomarker MuRF1 in the presence of the 17-DMAG is postulated to be due to the induction of the heat shock proteins by this compound. Therefore, we are interested in assessing the effect of 17-DMAG not only on the atrophy markers but also on the expression of the heat shock proteins. In Figure 4, we present the effect of 17-DMAG as

compared to just saline both in the absence or presence of our cast-immobilization model. Figure 4 presents a Western blot of protein extracts from the soleus muscle of the hind limb of mice treated with saline (lane 1), 17-DMAG (lane2), cast-immobilization plus saline (lane 3) and Cast-immobilization plus 17-DMAG (lane 4). The Western blot was reacted with antibodies specific for MuRF1, hsp27, hsp70 and GAPDH. The level of expression for these proteins is shown in Figure 4A that were then scanned and total pixel for each band is used to determine the level of expression for each protein. The scanned levels of MuRF1, hsp27 and hsp70 were normalized by the level of GAPDH expression as a loading standard and the results are shown in the histogram in Figure 4B. As can be noted decreases of the atrophy biomarker MuRF1 coincides with an increase in the expression of the hsp27 and hsp70 caused by the presence of 17-DMAG. This is in agreement with previous results that have shown the protective effects of an increase in the expression of the heat shock proteins against a diversity of cellular injuries.

One of the issues we are interested in studying, proposed in our Specific Aim 2 and is part of Major Task 2 is to determine the involvement of oxidative stress and inflammation during muscle atrophy. One encouraging recent result on this subject is presented in Figure 5, where a Western blot of protein samples from gastrocnemius muscle from the hind limb of mice submitted to cast-immobilization and either treated with 17-DMAG or saline. In this case, we utilized specific antibodies to inducible Nitric Oxide Synthase (iNOS) besides hsp27, hsp70 and GAPDH. Figure 5A shows how cast-immobilization for 7 days increases the expression of iNOS (lanes 1, 2), a protein involved in both oxidative stress as well as inflammatory processes. In contrast, when the hind limb is treated with 17-DMAG during the cast-immobilization (lanes 3, 4), the level of iNOS expression barely detectable. The histogram in Figure 5B shows the quantification of this Western blot and how marked is the effect of 17-DMAG treatment is on the level expression of iNOS.

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.

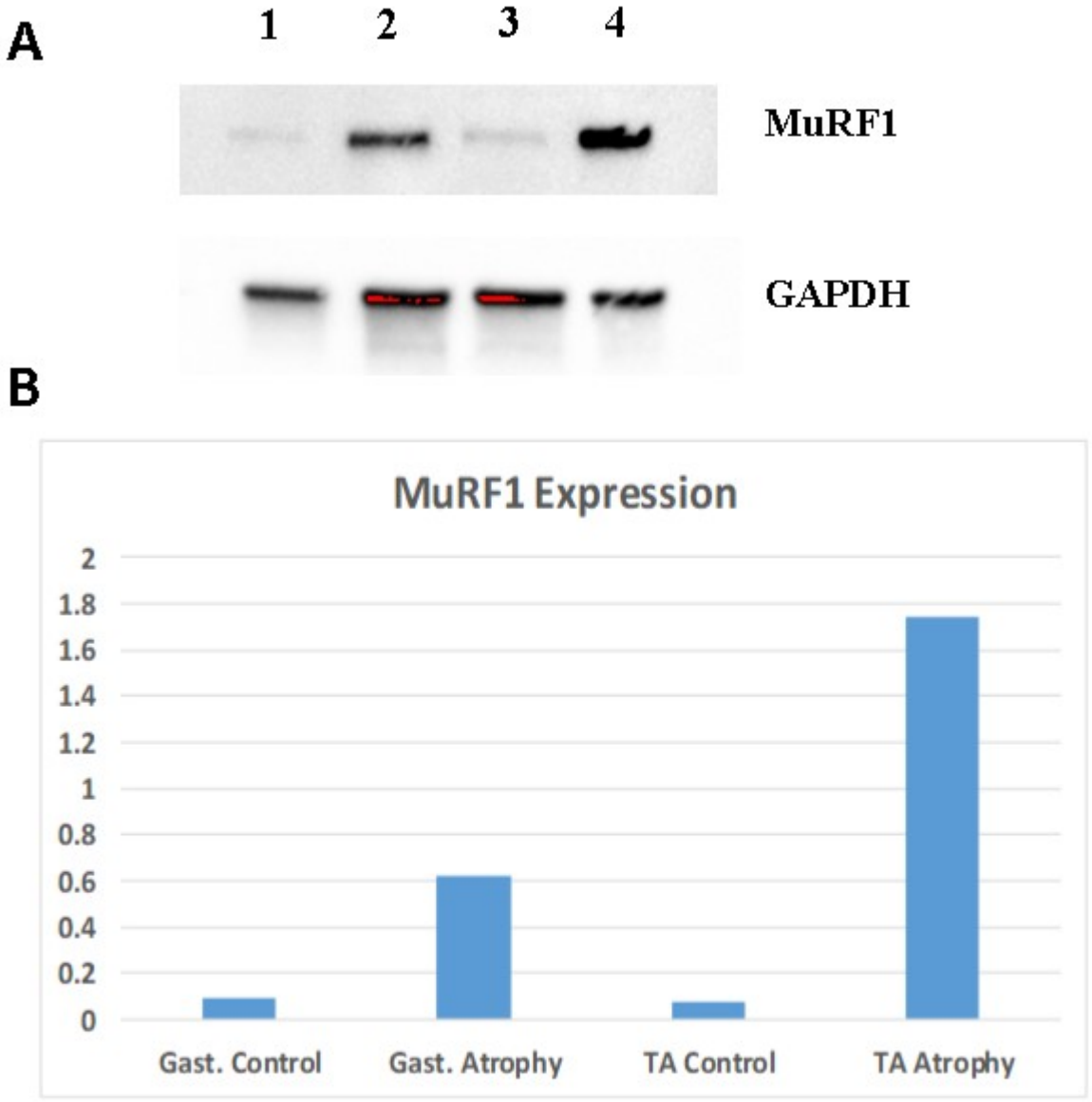


FIGURE 1. (A) Representative Western Blot that shows the increase of the muscle atrophy marker MuRF1 in both mouse gastrocnemius (1, 2)and tibialis anterior (3, 4) muscles. Mice were submitted to cast immobilization (atrophy) for 7 days (2, 4) or left without cast immobilization (control) (1, 3). **(B)** Blots were scanned using UN-SCAN-IT gel 7.1. Total pixels for MuRF1 bands were standardized using GAPDH to correct for loading differences and are shown in this histogram.

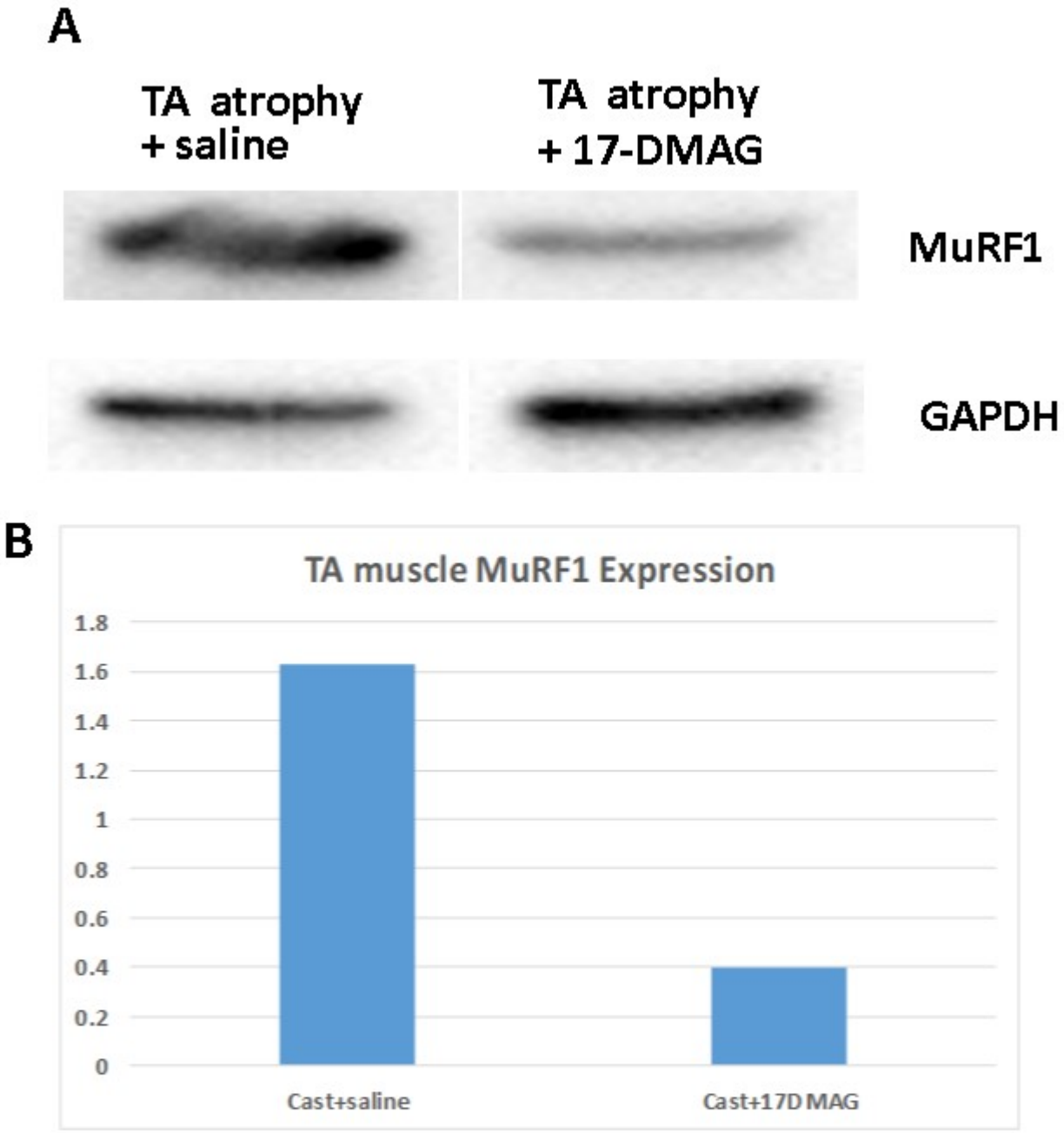
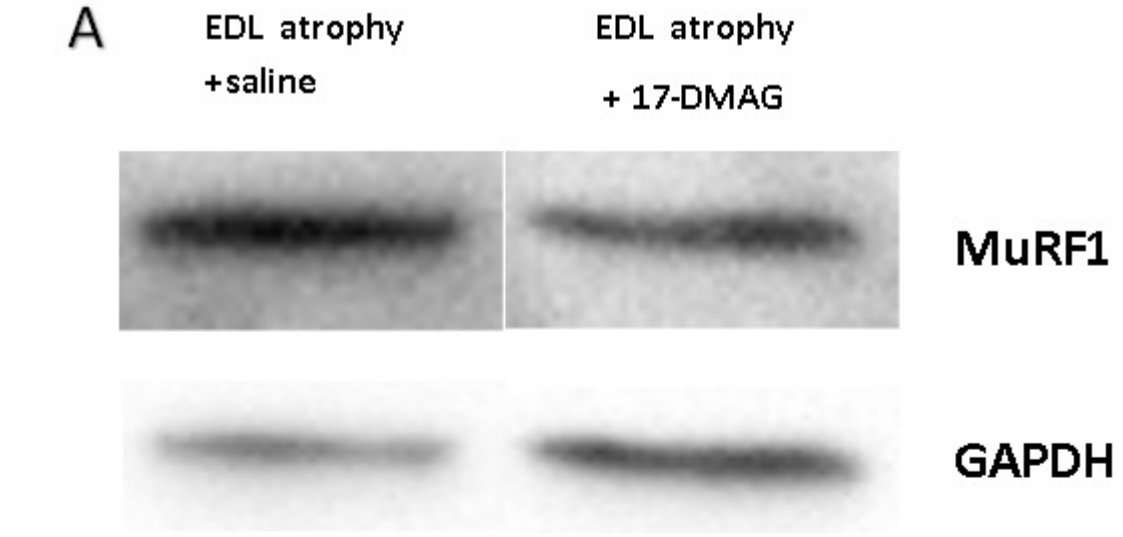


FIGURE 2. (A) Representative Western blot of tibialis anterior muscles of mice submitted to cast immobilization (atrophy) for 7 days and either treated with saline or 17-DMAG. Blot was reacted with MuRF1 and GAPDH antibodies. (B) Blot bands were scanned, total pixels were used to determine level of expression MuRF1 and GAPDH. Level of MuRF1 was standardized by the expression of GAPDH and of expression of MuRF1 in the absence and presence of 17-DMAG is shown in histogram.



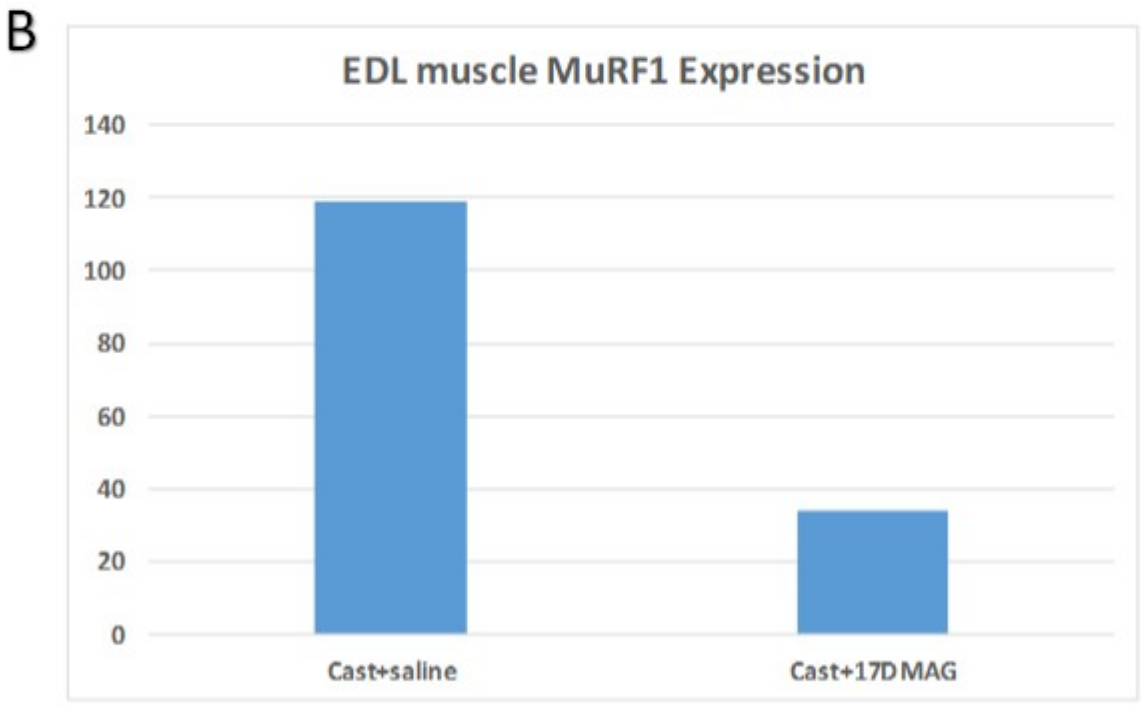


FIGURE 3. (A) Representative Western blot of extensor digitorum longus muscles of mice submitted to cast immobilization (atrophy) for 7 days and either treated with saline or 17-DMAG. Blot was reacted with MuRF1 and GAPDH antibodies. (B) Blot bands were scanned, total pixels were used to determine level of expression MuRF1 and GAPDH. Level of MuRF1 was standardized by the expression of GAPDH and of expression of MuRF1 in the absence and presence of 17-DMAG is shown in histogram.

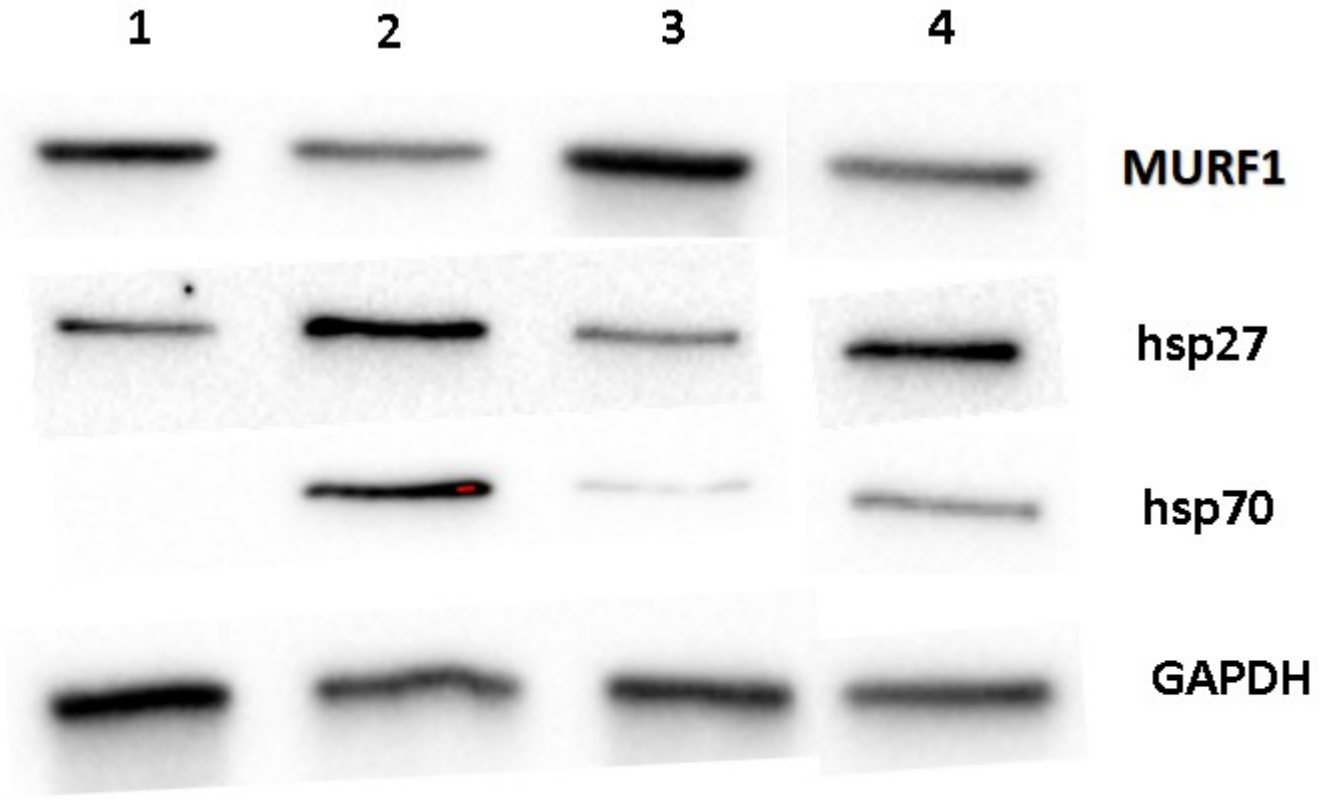


FIGURE 4A. Representative Western blot of soleus muscle proteins of mice. (1) Soleus muscle treated with saline; (2) soleus muscle treated with 17-DMAG; (3) soleus muscle from mice submitted to cast immobilization (atrophy) for 7 days and treated with saline; (4) soleus muscle from mice submitted to cast immobilization (atrophy) for 7 days and treated with 17-DMAG. Blot was reacted with MuRF1, hsp27, hsp70 and GAPDH antibodies.

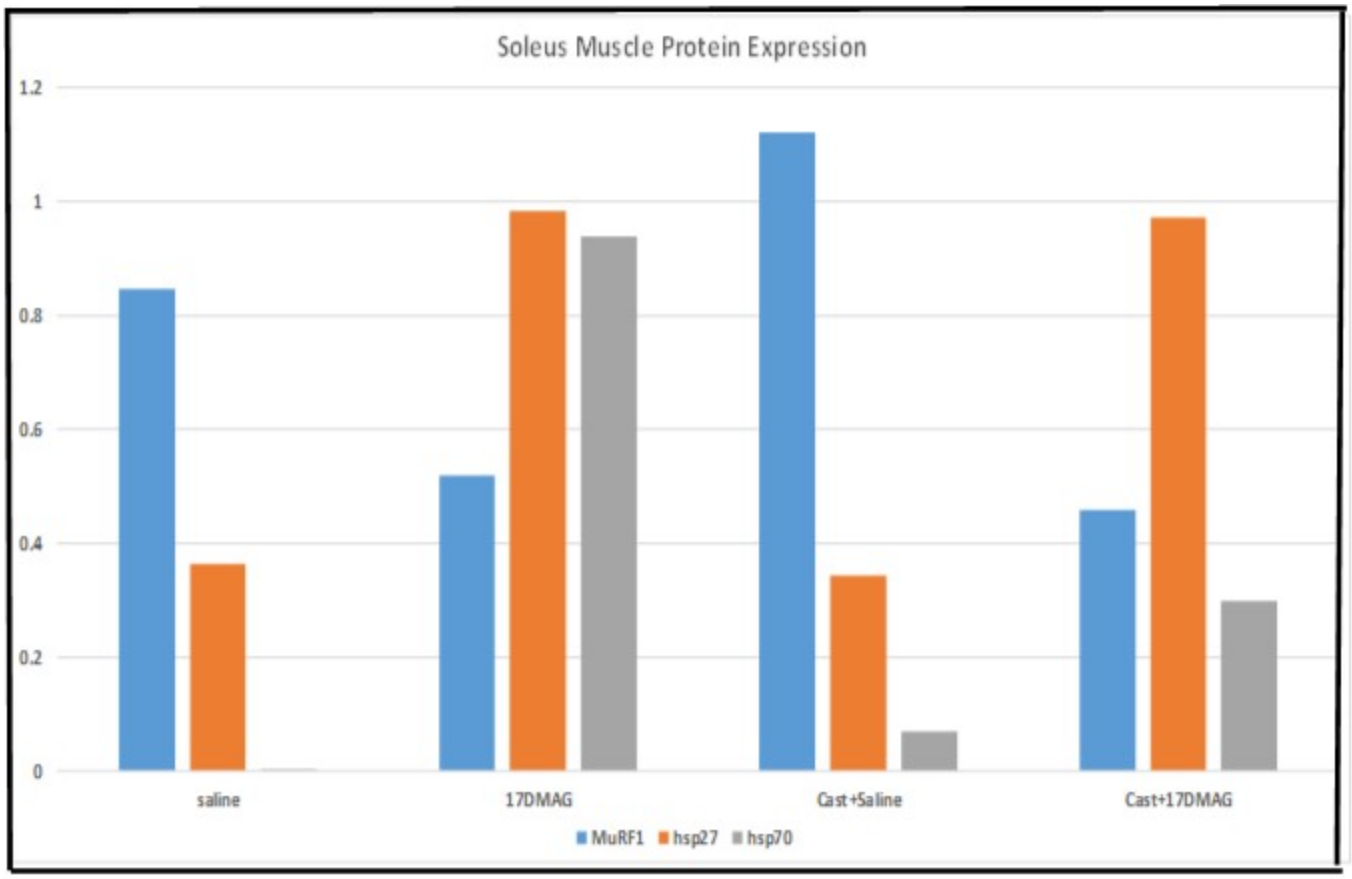


FIGURE 4B. Blot bands from Figure 4A were scanned and total pixels were used to determine level of expression of MuRF1, hsp27, hsp70 and GAPDH. The levels of MuRF1, hsp27, hsp70 were normalized by the expression of GAPDH and the levels of expression of MuRF1, hsp27 and hsp70 in the presence of saline or 17-DMAG in the absence or presence of cast immobilization for 7 days are shown in this histogram.

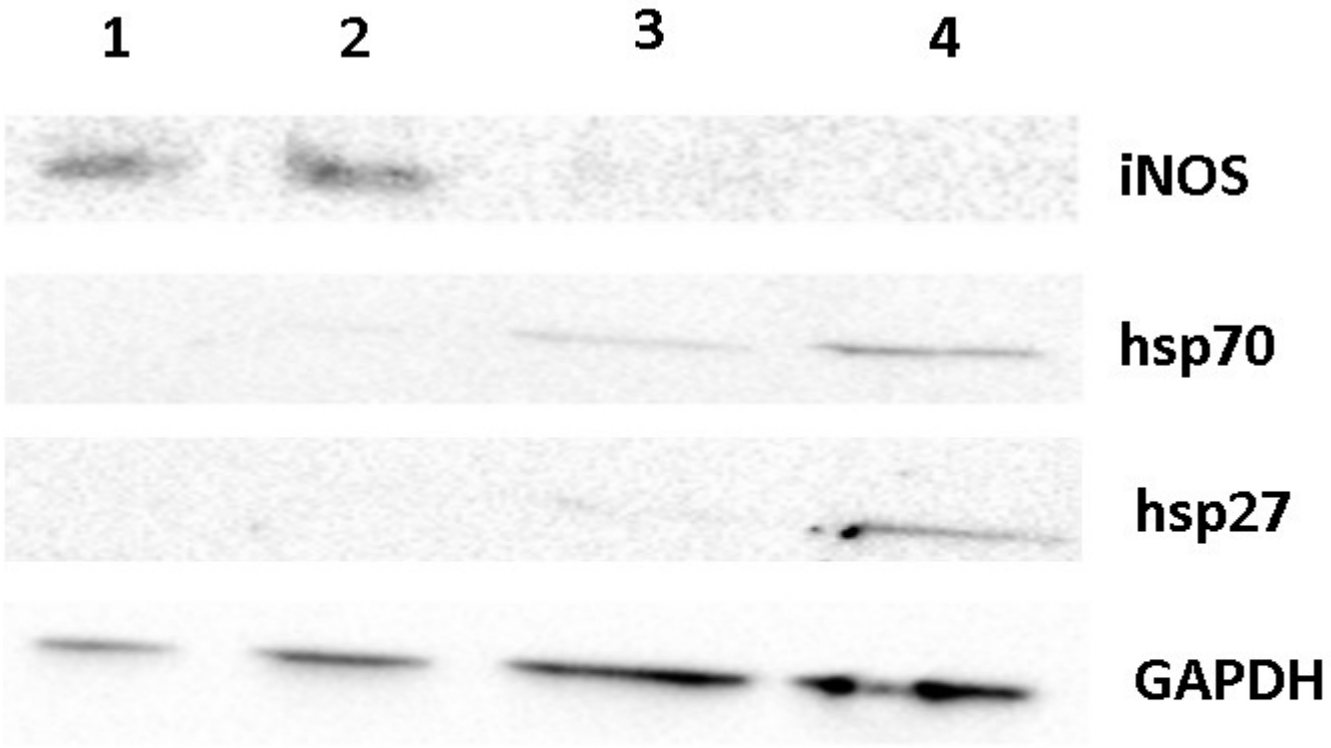


FIGURE 5A. Representative Western blot of gastrocnemius muscle proteins of mice. (1, 2) Gastrocnemius muscle from mice submitted to cast immobilization (atrophy) for 7 days and treated with saline. (3, 4) Gastrocnemius muscle from mice submitted to cast immobilization (atrophy) for 7 days and treated with 17-DMAG. Blot was reacted with iNOS, hsp70, hsp27 and GAPDH antibodies.

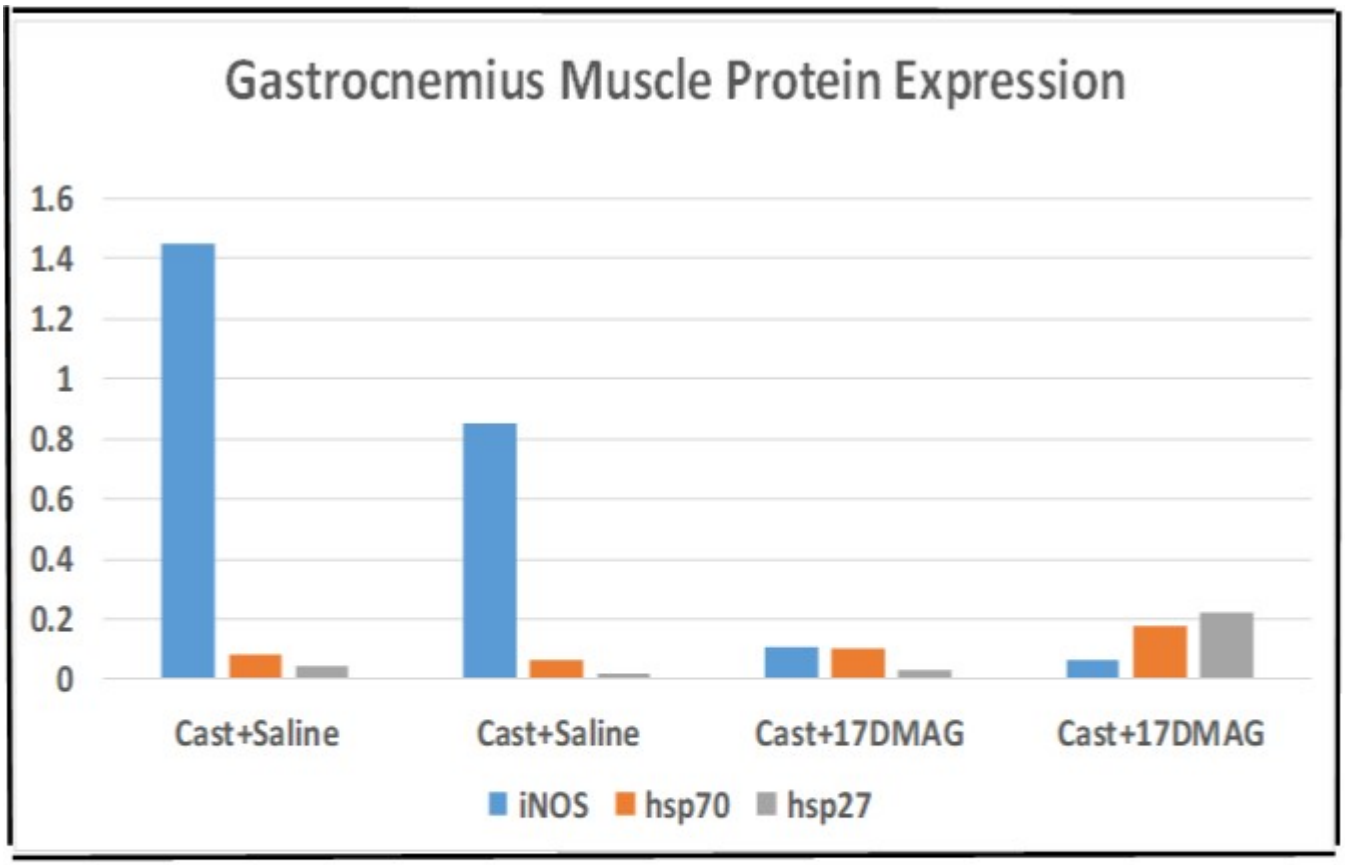


FIGURE 5B. Blot bands from Figure 5A were scanned and total pixels were used to determine level of expression of iNOS, hsp70, hsp27 and GAPDH. The levels of iNOS, hsp70 and hsp27 were normalized by the expression of GAPDH and the levels of expression of iNOS, hsp70 and hsp27 in the presence of saline or 17-DMAG during cast immobilization for 7 days are shown in this histogram.

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IMPACT

The results obtained in this past 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 first year of our research project, we have encountered some significant delays in the progress of our research program. Mainly, the animal facility in our Institution suffered a significant amount of flooding at the beginning of 2021. Therefore, the start of the animal work portion of our research project resulted in a delay of approximately 3 months. In addition, due to the consequences of the COVID-19 pandemic many of the parts and supplies we ordered and needed for our research resulted in shipping delays of as much as 6 months.

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