2020

The Effect of Using CRISPR/Cas9 Treatment to Delete the Myostatin Protein In Vivo and In Vitro

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THE EFFECT OF USING CRISPR/CAS9 TREATMENT TO DELETE THE MYOSTATIN PROTEIN IN VIVO AND IN VITRO

by
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A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Biomedical Sciences
in the College of the Medicine
and in the Burnett Honors College
at the University of Central Florida
Orlando, Florida

Spring Term, 2020

ABSTRACT

This thesis proposal shows the efficiency of different methods of Myostatin inhibition by using CRISPR/CAS9. With the data cataloged by this thesis researchers will have a better understanding on what methods are better to achieve their goals. The data were collected by reading multiple scientific articles involving the usage of CRISPR/CAS9 to inhibit the Myostatin protein. The data collected from all the different studies was analyzed in the same categories. The experiment that used CRIPSR/CAS9 on in vitro specimens had a superior Myostatin inhibition over all, therefore presenting higher muscle mass. The method using CRISPR/CAS9 to inhibit the Myostatin in vivo and in vitro depends on what the researcher is trying to accomplish. By reading this thesis researchers can understand that if they choose to use an in vitro treatment the results would be way more severe than using an in vivo application treatment.

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SPECIFIC AIMS

In 2012, Dr. Jennifer Doudna proposed for the first time that CRISPR/cas9 could be used to modify DNA [8]. Since then, the possibilities that this technology brought to the scientific community seem to be endless. One type of research that has brought much attention is the complete deletion of the Myostatin gene (MSTN) using CRISPR/cas9. Myostatin (MSTN) is part of the transforming growth factor beta (TGF-β) superfamily, acting as a negative regulator of muscle mass, related to muscle growth [8]. Therefore, the absence of this gene allows the muscle fibers to grow bigger and stronger. This MSTN treatment has been tried with a variety of animals such as mice [9], rabbits [8], sheep [3], pigs [14], etc. The primary focus of these experiments is to improve the size and weight of livestock animals. Researches seek to be able to increase the meat harvest from one single animal to attend the increasing demand of food in the near future. Most of the researchers seek generating a MSTN knockout animal using *in vitro* techniques. However, not many researches are currently being conducted *in vivo* subjects. The objective of this paper is to compare experiments that use CRISPR/cas9 to delete the MSTN gene using *in vivo* and *in vitro* techniques and determine which one is the most efficient.

BACKGROUND AND SIGNIFICANCE

The world population increases annually by 80 million [14]. Even though this number seems to be decreasing as time passes, the United Nations has issued a graphic that shows the three possible scenarios for world population growth. (Figure 1) [14].

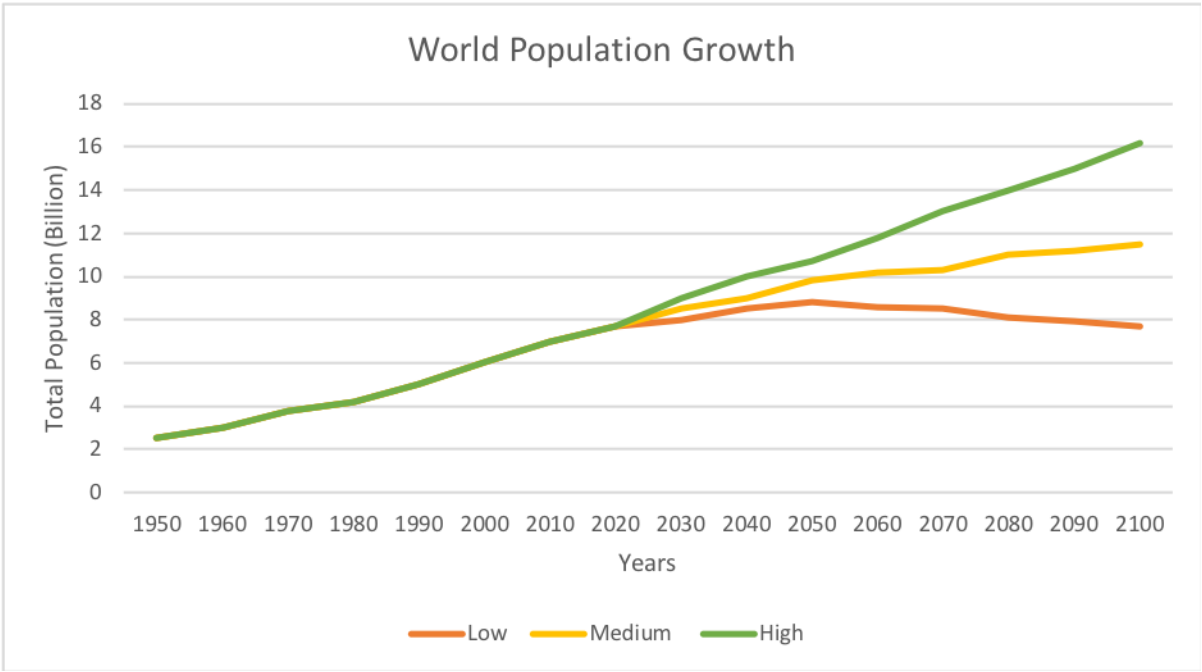

Figure 1: World Population Growth 1950-2100 Source: UN, 2015. 2

The currently world population by the time of this article is 7.7 billion [4]. This number is expected to increase by 2.03 billion in 2050, and 3.5 billion in 2100[14]. The U.N Department of Economics and Social Affairs estimates that high-income countries such as the United States will reach its peak population by 2040. While low and median-income countries in Africa and Asia will show a decrease on its overall growth, but will still be growing according to the median shown on figure 1[14]. China and other extremely populated countries will reach their peak in between 2050 and 2060. With the increasing number of people, the demand for food will also increase. In 2009 the Food and Agricultural Organization of the United States (FAO) stated that

the food production worldwide would have to increase by 70% to meet the demand of the large population by 2050[14]. However, the research done by the FAO has been outdated by the previously mentioned UN research (2012). That shows a smaller growth needed. However, the UN declare that the world food production has to increase by 50% until 2050 to meet the demand. The demand for cereals such as wheat, oats and corn, is projected to reach 3 billion tons by 2050. Meat production must grow from 200 million tons to 470 million tons in 2050 [1].

Agricultural production tripled between 1960 and 2015 [2], due to the technologies developed during the Green Revolution, such as the use of chemical fertilizers and agro-chemicals among other techniques [6]. Thirty years from now, in 2050, more than two-thirds of all people are is expected to be living in urban areas [5]. It has been shown that agriculture has been and will continued to be affected by the changes brought by urbanization [5]. Nowadays, a new array of technologies is being used to increase the efficiency of crops and productions of food. Among others, CRISPR/cas9 edited foods have been used in many areas of the food industry from the development of longer shelf life tomatoes [17], to livestock animals with larger muscle tissue [14].

The types of animals consumed world wide differ regarding on the nation religion, culture, climate and population size. In the US for example, an average person eats 80kg to 60kg of poultry, 60kg to 40kg of pork and 40kg to 20kg of beef per year [11]. Poultry is the most consumed meat in the US due to its affordable price and its reputation of being a cleaner and healthier meat [14]. In China, an average person eats 30kg of Pork, 10kg of poultry, 5kg of beef [10]. Even though the gross amount of pork consumed by an average Chinese is smaller than the

average American, pork meat is 66% of the total meat consumed by a Chinese per year, while pork meat is only 33% of the meat consumed by an Americans. These populational preferences encourage researchers to undergo experiments in different types of animals utilizing CRISPR/cas9 aiming a similar goal, which is to increase the size of the animal. All experiments with the same goal of disabling the MSTN gene therefore increasing the muscle tissue of the animals. On this paper, four specific experiments were analyzed. Three of them seek to better understand the effects of the deletion of the MSTN gene by the introduction of the CRISPR/cas9 treatment on its zygote (*in vitro*) in three different mammals' species: rabbits [8], sheep [3], and pigs [14]. Only one experiment seeks to use CIRSPR/cas9 on adult animals (*in vivo*). However, the purpose of the *in vivo* experiment is to create a treatment that will disrupt MSNT and prevent muscle-wasting syndrome [9] by also utilizing the same technology of CRISPR/cas9.

RESEARCH AND METHODS

Three criteria were chosen to compare the efficiency of each *in vitro* experiment. These criteria include: the overall percentage amount of the successfully breed MSTN (-/-) animals, the overall difference in the body weight of successfully edited animal MSTN (-/-) to wild type MSTN (+/+) and the visual presence of a double muscular phenotype.

The overall percentage amount of the successfully MSTN (-/-) animals will be represented as *OPAS. OPAS* is calculated in each experiment is calculated by the following formula:

$$OPAS_{vitro} = \frac{Number\ of\ born\ animals\ expressing\ MSTN\ (-/-)}{Total\ number\ of\ animals\ born} \times 100$$

To find this information, each of the three research papers were carefully analyzed for information regarding the number of born animals and the number of animals mutated. Although all the experiments used CRISPR/cas9 to inhibit the myostatin gene loci, it is important to emphasize that each experiment was conducted in a different way, altering the stage where CRISPR was introduced and possibly the efficiency of *OPAS*. In the first experiment, using rabbits as research model, the cas9 mRNA and sgRNAs were micro injected in to 158 zygotes. The fertilized zygotes already containing the CRISPR/cas9 complex were then injected in to the oviducts of the female rabbits [8]. The second experiment, using sheep as research model, went a little further with the use of *in vitro* fertilization. Cast 9 mRNA was injected in to 216 fertilized zygotes that were cultivated into 53 blastocysts [3]. These blastocysts were later transferred to the female recipients by a surgery procedure assisted by laparoscopy was done to place the embryos in the cranial side of the ipsilateral uterine horn to the corpus luteum [3]. The

experiment using Chinese Erhualian pigs as a model was the most complex in terms of creating the *in vitro* zygote. Porcine Fetal Fibroblasts were isolated from 33-day-old fetuses. Then it was cultured in high glucose Dubelcco's modified Eagle medium supplemented with 10% FBS, a mixture of penicillin and streptomycin and non-essential amino acids that later undergo electroporation to become a single cell colony [14]. Cas9 mRNA is injected in these cultures before undergoing electroporation. Target regions on single cells colony are amplified by PCR [14]. To create homozygous mutated MSTN (-/-) KO pigs, the single cells colonies are pooled as donor cells for somatic cell nuclear transfer (SCNT). The overall percentage amount of the successfully breed MSTN (-/-) for each experiment is: Erhualian pigs have a OPAS of 86.95%, sheep have a OPAS of 36.36% and rabbits have a OPAS of 80% (Figure 2).

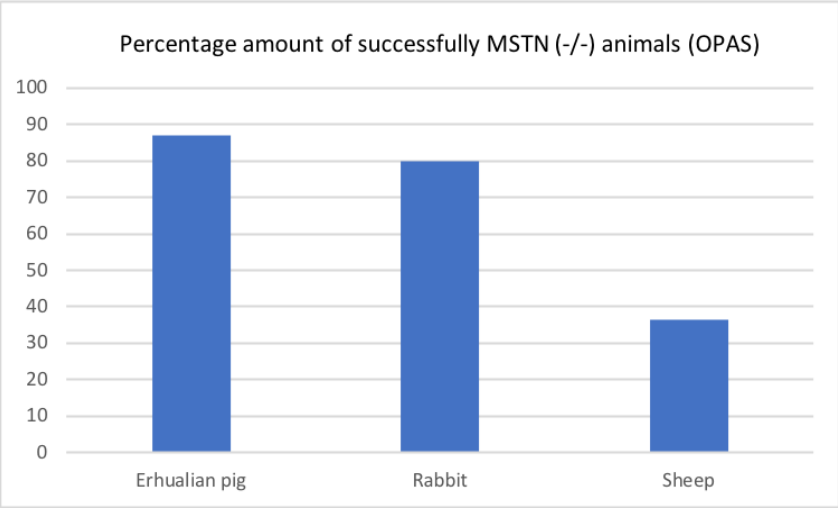

Figure 2: Percentage of OPAS among the animals in each study.

For the sake of this thesis proposal, the success of one experiment on the OPAS category was simply measured by the amount percentage. In the Erhualian pigs experiment, 10 females received an average of 200 fertilized embryos, but only five became pregnant [13]. It is true that from the 23 pigs born, 20 were successfully mutated (hence OPAS 86.95%), but researches do

not have the ability to determine if unsuccessful fertilization of the other 5 females is related with the CRISPR/cas9 induced mutation, or to other possible problems. Such as the rabbit experiment, from the 20 pups born, all 20 where successfully mutate. Still, four of them where MSTN (+/-) that is, presented only one copy of the gene deleted and the other present. As for the sheep experiment, 29 females were recipients of the blastocysts. From the 29, only 19 became pregnant giving birth to 22 lambs. From the 22 lambs, only 10 had a mutation and from the 10 mutated only 8 were homozygous MSTN (-/-).

The second criteria determined to analyze the efficiency of the CRISPR/cas9 treatment is the overall difference in the body weight of successfully edited animal MSTN (-/-) to wild type MSTN (+/+). Unfortunately, for this research, the experiment involving Erhualian pigs failed to provide the amount in kg of both homozygous negative mutated pigs and the wild type, making it impossible to calculate the percentage difference from the two types. However, this information was successfully provided in both other experiments. The rabbits were weighted throughout a period of 2 months. Although the report includes pictures of the three types of rabbits, it only provided weight information of MSTN (+/-) and Wild Type (WT). Both types of rabbits (WT and MSTN (+/-)) had similar weights until to the third week of life. After the fourth week, the mutated rabbits started to show significant increase of the body weight. Within 8 weeks results show that the MSTN (+/-) were 15% to 30% heavier than the Wild Type in total body mass [8]. The muscles of the tongue (T), heart (H), gluteus maximus (G) and vastus lateralis (V) also were extracted for comparison. The only one from the four mentioned above that has a significant increase is the gluteus Maximus, with an average increase of 35% heavier

than the WT [8]. Heterozygous MSTN (-/-) rabbit showed a tongue 30% bigger than the WT. The article did not state any type of information regarding the difference between the size of the jaw on both phenotypes. Myostatin is a protein that controls inhibition only in muscles. Since the jaw is made of bones, cartilage and muscle tissue, it is not expected for the entire jaw to grow. The researcher did mention that MSTN (-/-) rabbits appears to be normal, healthy and had no reproduction problems [8]. Therefore, we can presume that the increase of the tongue size does not interfere much on the life of the animal.

The sheep were weighed throughout the first 60 days of life. All three types had the similar weight at birth. After the first 15 days of life, the weight from the three types of sheep analyzed started to differentiate [3]. Body weight of MSTN -/- sheep were in average 20% to 30% heavier than the WT. While some heterozygous sheep MSTN (+/-) showed some increase of body weight, some heterozygous did not showed the same result and end up having similar weight than wild type animals [3]. It is important to mention that extremes from both types of sheep have their weight compared to come to the previous analysis, this being, the heavier WT sheep has a similar weight of the lightest heterozygous MSTN sheep.

Taking an average estimate with all the information provided by the three articles, it is possible to assume that an animal that displays the successful inhibition of the MSTN gene (+/- or -/-) will have at least 15% increase on its body mass within the first year.

Third criteria analyzed the visual presence of a double muscular phenotype. This is important to differentiate the volume difference between wild type and MSTN (-/-). The visualization of the

double muscular phenotype helps to predict that the animal meat actually grew bigger, instead of just become heavier. Fortunately, all three experiments provided clear pictures from animals where the double muscular phenotype is easily visible. The experiment that used rabbits as research model provided pictures from the wild type animals and the two types of mutated animals. In the picture stated above, it is possible to spot the difference between the three types of phenotypes. It also makes possible to understand why the double negative homozygous MSTN gene is the preferable to create animals which the purpose is only to have their meat harvested.

In conclusion, the rabbit experiment had an 80% efficiency to generate MSTN (-/-) and 15% to 30% heavier MSTN (-/-) than WT. The sheep experiment had 36.36% efficiency to generate MSTN (-/-) and 20% to 30% heavier MSTN (-/-) than WT. Also, the Erhualian experiment pigs had 86.95% efficiency to generate MSTN (-/-) but an unknown increase of the weight of MSTN (-/-) than WT.

The focuses of this thesis is to determine which CRISPR/cas9 treatment is the most efficient between *in vitro* and *in vivo.* Therefore, the three criteria used to compare the past three *in vitro* experiments are also used to qualify the *in vivo* experiment, even though they do not share the same purpose. As mentioned in the beginning, the in vivo experiment did not share the same method of research as the other three in vitro. However, the goal of the four experiments were similar since all four wanted to increase muscle mass. The in vivo rat experiment looked for a way to treat the genetic disease Duchenne Muscula dystrophin. Duchenne Muscula dystrophin is

a genetic disease that causes muscular dystrophy and lack of muscle control [9]. It affects the DMD gene, which is responsible for creating the Dystrophin protein [9]. Dystrophin main function is to connect the cytoskeleton of a muscle fiber to the ECM through the cell membrane. The researchers used a technique called adeno-associated virus (AAV). CRISPR/cas9 complex targeting the DMD gene was placed inside a virus capsule [9]. Then, the serum with the virus already containing the CRISPR/cas9 complex was injected in to the mice muscle [9]. The exact number of animals used in the mice experiment is not delivered in the paper. The author only mentions that groups of six-week-old male mice are used where each group contains 6 mice [9]. Still, the amount of groups analyzed is also not shared. Yet, the author describes the overall efficiency of the genome editing in muscle tissue as low (5%) [9]. For the purpose of this thesis proposal, OPAS for the mice *in vivo* treatment will be 5%. Second criterion is the overall difference in the body weight of successfully edited animal MSTN (-/-) to wild type MSTN (+/+). Again, the author fails to share the exact weigh of the mice studied, but describe that the average weigh of the gastrocnemius and soleus muscle increased by 9% [9]. The third criterion is not met since the article did not provide any picture, making it impossible to determine if there is a visible expression of the double muscular phenotype.

CONCLUSION

After carefully analyzing all the results for each category, the results are the following:

The overall percentage amount of the successfully MSTN (-/-) animals (OPAS) for in vitro treatments is higher than the in vivo treatment by 62.7% (figure 9).

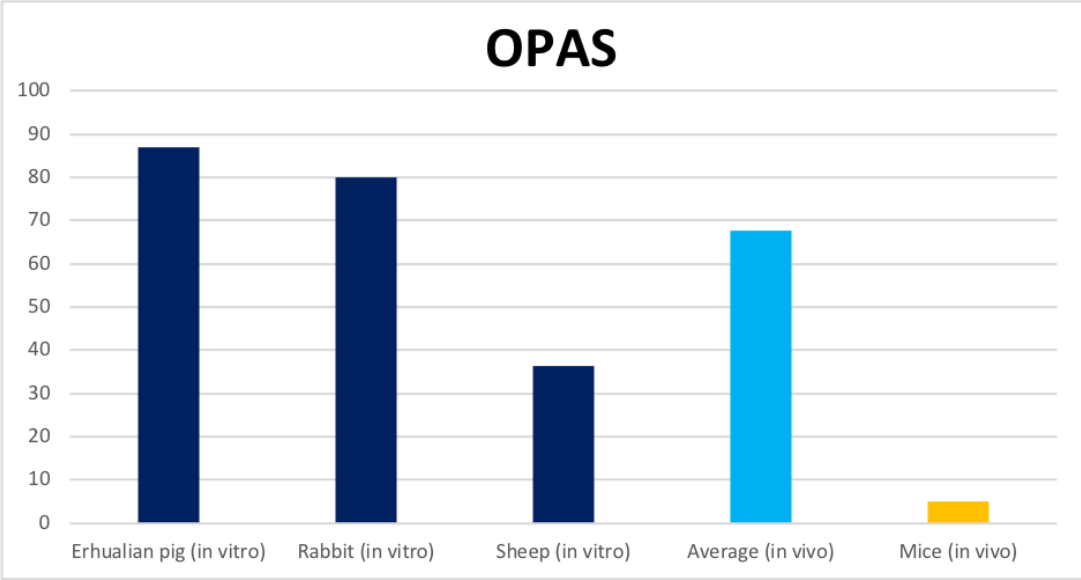

Figure 3 Comparison of OPAS Between Species Presented in the study

The average of the overall difference in the body weight of successfully edited animal MSTN (-/-) to wild type MSTN (+/+) for the *in vitro* experiments (estimating that the Erhualian pigs fall in the same average as the sheep and the rabbit) is an increase of 23.3% on the body weight. While the experiment *in vivo* had an average of 9%. Lastly, from the four experiment selected only three provided pictures of the animals showing the double muscular phenotype. However, the focus of these criteria was to prove that the increase of weight also means an increase of volume,

and not increase of density. It is possible to estimate that the mice treated with CRISPR/cas9 from the in vivo experiment would likely show some increase on its muscle size. It is crucial to understand that the even though the muscle growth from the vivo treatment were extremely lower than the vitro. The vivo experiment was successful on its goal, which was to help to restore dystrophy expression and improve muscle function on subjects with Duchenne Muscula dystrophin [9].

As far as the aim for this thesis, the results show that if the goal is creating bigger animals, scientist would have a higher rate of success if generating these animal from zygote, instead of applying treatment to an existent livestock farm. In order to decide if those animals would be cost benefice for business, other factors should be taken in consideration. If MSTN KO animals are shown to be efficient, these animals could also help to stabilize the CO2 emissions generated by livestock. Livestock is responsible for up to 14% of all greenhouse emissions [13]. An average ruminant produces from 250 to 500 liter of methane per day [13]. When bigger animals are created using CRISPR/cas9, the demand for a higher amount of livestock numbers decrease since more meat can be harvest from one single animal. With that, the total number of livestock would stop increasing, while the production of meat would still be able to grow.

One factor that has to be taken in consideration is the amount of food a MSTN KO animal will need to keep increasing its size, compared to the amount of food consumed by a WT animal. Muscle tissue consumes energy even at it is rest [7]. Humans spend in average 54.4 kJ per kg at rest [7]. With that said, the higher the amount of muscles an animal has, higher the energy necessity, bigger the amount of food require to maintain this animal. Since none of the

experiments cited in this thesis allowed the animals to achieve adult phase, not much is known about their diet and how much food they need to maintain muscle growth, further research is needed in this area.

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