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Husbandry and Handling of D2-*mdx* mice, choice of outcomes

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1. OBJECTIVE

The D2-*mdx* mouse model for Duchenne muscular dystrophy has gained popularity in the field, due to their more severely affected muscle function and histopathology when compared to the classic BL10-*mdx* model. This document describes recommended procedures for the care of these mice aimed to standardize husbandry and key outcome measures.

2. HUSBANDRY AND HANDLING

In compliance with the 3Rs and standards of animal care, mice should be housed in a well-maintained and pathogen-free environment with controlled light cycles (12 hr light, 12 hr dark), room temperature (~21^{o}C), humidity (~50%) and air flow. Furthermore there should be minimal noise, vibrations, traffic flow and odors (e.g. perfumes or chemicals). Animal welfare should be monitored on a daily basis to identify any health issues. While veterinary staff must be available in case consultation is required. Cage bedding, food and water need to be rephreshed as appropriate. Cage enrichment, like shreddable nesting material along with a plastic shelter is highly recommended to minimize stress.

Mdx and D2-*mdx* mice are sensitive to stress. Consequently, first litters are commonly being eaten by new mothers in response to stress. Therefore, attention should be paid to disturb breeding pairs as little as possible. Litter sizes of D2-*mdx* mice, consisting out of 4 pups on average, are much smaller than those of BL10-*mdx* mice. Very rarely litters of 7-8 pups are born, while more commonly they consist of only 1-2 pups. Contrastingly to the BL10-*mdx* model, it can take up to 2-3 months before D2-*mdx* couples become pregnant from their first litter. Subsequent pregnancies occur however within a shorter timespan.

Pups can be weaned from 3-4 weeks of age onwards. D2-*mdx* mice are relatively small in comparison to BL10-*mdx* mice at the age of weaning (typically, 4 – 5 weeks old D2-*mdx* mice exhibit a mean body mass of ~ 10 grams vs. ~ 20 grams of BL10-*mdx*). Therefore, extra attention should be paid to allow weanded mice easy access to water and food. Food pellets should be provided on the floor of the cage. Optionally, food pellets soaked in water or a gel food/hydration source such as HydroGel could be provided in addition. This should however be done consistently to minimize variations in nutrition from litter to litter. Water bottles should have a longer spout to ensure easy accacibility by the pups. Individual housing of animals should be prevented and the use of buddy animals is advised as solution.

When D2-*mdx* mice are purchased from JAX® (4 – 5 weeks is the minimum available age range), an acclimatization period of 1 week is necessary. Male mice need to be segregated in cages (max. 4 – 5 animals *per* cage) without mixing original litters, to avoid phenomena of social distress, fighting, wounding and ultimately killing.

Proper handling is of utmost importance while managing with *mdx* mice. Investigators need to be aware that dystrophic mice are very susceptible to various sources of stress, either due to manipulation (e.g. scruffing) or social dynamics (e.g. subordination), which can ultimately lead to

an increased mortality (1, 2). D2-*mdx* mice are even more delicate, considering the phenotypical features associated with their genetic background, particularly their smaller size in the first weeks of age. Therefore, operators should be encouraged to use a tunnel or cupped hand to pick up mice, while avoid unnecessary and prolonged manipulation. Daily well-being monitoring should be less invasive as possible and routine husbandry procedures as cage cleaning should be organized on a weekly basis. Lastly, experimental procedures should be performed in dedicated rooms, separate from the animal housing rooms.

Randomization, blindedness and gender

Mice of the same litter should be randomized over different groups for therapeutic experiments or natural history studies. Experimentors should be blinded for the experimental groups. Because the phenotype of *mdx* is mild the animals can be bred homozygously. For BL10-*mdx* mice males and females perform equally for most functional tests. Still, we recommend using only males. When both genders are used, this should be randomized over different groups. For D2-*mdx*, females outperform males on functional tests and show less fibrosis (3). Therefore, for this model using only males is strongly recommended to reduce variation and improve the chance of picking up a treatment effect.

3. GENOTYPING

To identify mice, the ears could be clipped or a hole could be punched. Toe clipping is highly discouraged, as this will interfere with *in vivo* assessments of muscle function. Small ear or tail biopsies could be subsequently used for DNA isolation to determine the genotype of the mice by PCR. To avoid distress to the animals, this is done at a young age (<4 weeks). DNA can be isolated with a large variety of methods, which vary in impurity of the isolated DNA. High purity can be obtained using commercially available kits (e.g. Puregene Genomic DNA Purification Kit, Qiagen). On the other hand, crude lysates also appear very suitable for routine genotyping and provide an attractive and cheaper alternative. The protocols provided on the Jackson Laboratories website are recommended (http://jaxmice.jax.org/support/genotyping/dna-isolation-protocols.html). It is of utmost importance to regularly check the genotype of in-house D2-*mdx* colonies. In addition to confirming the exon 23 mutation in the *Dmd* gene, one should also check for alterations in the *Ltbp4* and *Abcc* genes that underlies the more severe phenotype. The following protocol could be used to do so.

Nuclease-free ddH_2O	12.4 µl
5x Phire buffer	4 µl
dNTP mixture (2.5 mM each: dATP, dCTP, dGTP, dTTP)	0.4 µl
Forward primer (10 pmol)	1.0 µl
Reverse primer (10 pmol)	1.0 µl
Phiretaq DNA polymerase	0.2 µl
Total volume	19 µl

19 µl mix and 1 µl 5x diluted DNA

Primers (listed 5'- 3') for *Ltbp4*:
Forward: ATTCCCGCAACCAACCTCTGATTCCTATGG
Reverse: TCTTTACCAGGCTTTCTGCCTACTCGTCG
WT product: 294 bp
DBA product: 258 bp

Primers (listed 5'-3') for *Abcc*:
Forward: TGTATCTCCAGGCTCGAGTG
Reverse: GGTACCAAGTGACACGACAG
The PCR product will be 192 bp.

PCR conditions:
98°C 30 sec

98°C 5 sec
52°C for *Ltbp4* and 62°C for *Abcc* 5 sec 30 cycles
72°C 10 sec

72°C 1 min
4°C hold

Program time is about 45 min.
Run 5 µl on a 3% agarose gel.

ExoSap treatment for *Abcc* PCR products only:
Mix 5 µl of PCR product with 2 µl ExoSAP-IT
Incubate 15 min at 37°C
Incubate 15 min at 80°C to inactivate
Prepare for Sanger sequencing: 7 µl product + 2 µl H2O + 1 µl F primer (10 pmol)
Mice will show C/C, T/T or C/T for mutation in exon 14 (in Bl6 C and in DBA T).

4. STANDARD ASSESSMENTS

The assessment of the D2-*mdx* mouse involves the evaluation of muscle strength, motor coordination, cardiac function and muscle inflammatory state based on functional assays that reveal differences between this strain and the DBA/2J. To assess muscle strength, the assays include grip strength measurements (GSM, TREAT NMD Protocol DMD_M.2.2.001), voluntary wheel and exhaustion assay (TREAT NMD protocol DMD_M.2.1.001). When planning studies that include voluntary wheel, it is important to consider the higher sucsceptibility of the D2-*mdx* mice to stress due to handling, which can influence the behaviour of these mice and reduce their activity levels.

To evaluate the individual muscle force, *in situ* studies or *ex vivo* force measurements allow for a specific assessment of certain muscles and have proven very valuable to study drug efficacy (TREAT NMD Protocol DMD_M.1.2.002 and DMD M.2.2.005). Consistently, the D2-*mdx* shows lower specific force both *in situ* and *ex vivo* and higher percent drop following consecutive eccentric contractions. For the cardiac assessment, echocardiogram or magnetic resonance imaging can be performed longitudinally. In contrast with the BL10-*mdx* model, the D2-*mdx* mice show an earlier onset of cardiomyopathy, making it a particularly useful outcome measure to test treatment efficacy in this model (4). The cardiac phenotype can be detected as early as 19 weeks of age. One crucial factor to consider is that the D2-*mdx* model shows a partial restoration of cardiac function at later ages. A study that compared echocardiography at 7, 28 and 56 weeks of age showed that although the D2-*mdx* presented a cardiac phenotype at early ages (28 weeks of age), they showed restoration of the ejection fraction and fraction shortening later in life (4, 5). This late cardiac improvement should be considered when designing longitudinal studies and interpreting data of older age mice.

In addition, the D2-*mdx* model presents higher inflammation levels in the skeletal muscles than the DBA/2J wildtypes, and this can be studied *in vivo* with optical imaging (4). This is evident by a higher cathepsin activity in the fore and hindlimbs at different ages, indicative of inflammation. Optical imaging using the IVIS system can be studied in a longitudinally fashion. A longitudinal study showed that inflammation decreases significantly with age, with mice at week 7 showing significantly higher cathepsin B activity when compared with the DBA/2J mice, but this inflammation was reduced significantly by 56 weeks of age (4). The evidence of phenotype differences due to age stresses the importance of using age-matched controls during efficacy studies for all the functional tests performed.

4.1 Body weight

Body weight (g) can be monitored on a weekly basis to obtain insights on animal growth. For correct measurements, ensure that the scale is calibrated on a regular basis and that it is leveled before each use. Differences in body weight between D2-*mdx* mice and wildtypes have been detected from the age of 4 weeks onwards. Overall, D2-*mdx* mice are lighter than age and gender matched DBA2/J wildtypes. These differences become more apparent with age. Contrastingly, the classic *mdx* mice on a C57BL/10ScSnJ or C57BL/6J genetic background are significantly heavier than age and gender matched wildtypes (Figure 1). This contrast is predominantly caused by apparent muscle atrophy in the D2-*mdx* model compared to muscle hypertrophy in the classic *mdx* model (3-6)

There is a lot of variation in absolute values within strains between labs. Potential reasons for this are differences in husbandry, diets, litter size, sourcing of the animals (i.e. Jackson versus in-house breeding) or genetic drift.

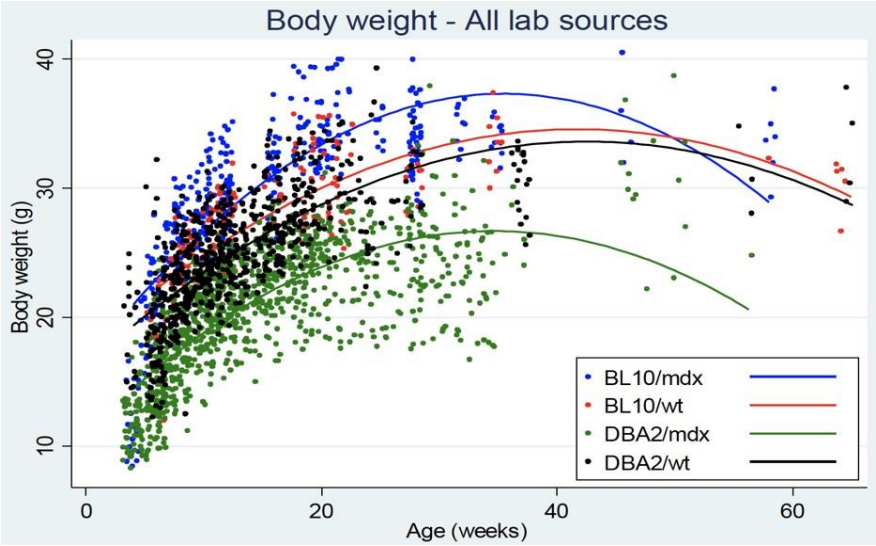

Figure 1. Body weight assessed for D2-mdx, BL10-mdx and genetic background matched wildtype strains. Overall D2-mdx mice are smaller, while BL10-mdx mice are heavier compared to age and gender wildtypes.

4.2 Lean and fat mass

In addition to body weight, one could gain more insight in body composition by non-invasively assessing lean and fat mass. This can be done in a longitudinal manner with a DEXA-scan or EchoMRI. While D2-*mdx* mice have a lower lean mass, their fat mass is higher compard to BL10-*mdx* mice.

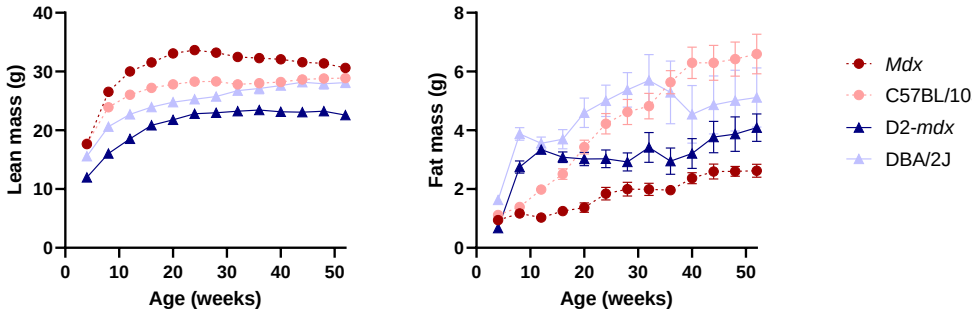

Figure 2. Body composition assessed with EchoMRI. Throughout life, mdx mice have lower fat and higher lean mass levels than wildtype mice. Body composition in D2-mdx mice is more comparable to wildtype.

4.3 Muscle weight

The weight of isolated muscles is also reduced significantly in D2-*mdx* mice (3, 7). To allow correct measurements, carefully dissect muscles and organs, and ensure that the entire muscle is collected by cutting at tendons or as close to the insertion point as possible. Other tissues like fat and overlying muscles should be carefully removed. In case of weighing the heart, excess blood should be removed with a tissue prior to weighing. Also try to avoid tissue dehydration by working quickly and only exposing tissues to the air that are about to be harvested.

Although muscle weights could be compared directly, we advise to represent them as a percentage of total body weight (i.e. g (muscle)/ g (total body)) to compensate for the overall smaller size of D2-*mdx* mice.

4.4 Selection of muscles for histological and molecular analyses

The extend in which different skeletal muscles are affected in D2-*mdx* mice varies, but follows the general pattern seen in the BL10-*mdx* model. Overall, the diaphragm is the most severely affected skeletal muscle, with extensive fibrosis, inflammation and calcification from the age of 10 weeks onwards. The severity in which limb muscles are affected differs significantly between muscles(3). While the tiabialis anterior is largely spared, the gastrocnemius, quadriceps and triceps are one of the most severely affected limb muscles. Based on the individual experimental goals and designs particular muscles could be selected. However, analysis of multiple muscles is strongly advised.

5. BIOMARKERS

When withdrawing blood for storing plasma or serum, it is important to bear in mind that different types of tubes can influence protein abundance. If possible standardize the type of tube. When storing samples ideally aliquot them because freeze thaw cycles also influence protein/lipid/metabolite abundance and mRNA integrity.

5.1 Creatine Kinase

CK levels are highly variable between mice, and they tend to decrease with age. Variability is influenced by multiple factors, including activity, stress, circadian rhythm, the method of blood collection, age and gender of the mice. It is therefore important to always obtain blood for CK measurements with the same method, at the same time of day before mice are handled or undergoing functional tests. Collection of blood on monday morning when the mice are least stressed due to lower exposure to humans in the weekend is advised. CK levels are influenced by freeze thaw cycles but also not very stable by 4 degrees. Ideally levels are measured on the same day as collection.

5.2 Dystrophin levels

Dystrophin is difficult to measure since it is a low abundant protein in humans and in mice. Measuring restored dystrophin levels (lower than wild type levels generally) is even more challenging. We refer the reader to a general overview of measuring dystrophin in clinical trial samples - many of the considerations also apply to measuring dystrophin in animal models (8).

When measuring dystrophin restoration, Western blotting is the most quantitative way to measure abundance. This can be done by western blotting (9) or with capillary western blotting (10). It is important to take along a proper standard curve that includes samples with protein amounts below and above the expected amount. Furthermore, since dystrophin levels vary between different muscles, the wild type reference should always be from the same muscle. Western blotting will only inform you about the abundance of the protein, but not about the

location and whether the associated glycoprotein complex is reformed. For this immuno-fluorescence analysis has to be performed.

5.3 Gene expression

qPCR provides the option to measure the expression of genes involved in different pathways (e.g. inflammation, fibrosis, regeneration etc). Here, stable housekeeping genes are used to normalize the expression of target genes. Housekeeping genes should have a stable expression that should not differ between the strains and treatment groups of the study. Before performing a qPCR, a set of housekeepinggene candidates should be evaluated for its applicability for each of the muscles that will be analysed as part of the study. It is commonly observed that housekeeping genes that are stable for a particular muscle, or age, for the BL10-*mdx* model are unsuitable for the D2-*mdx* model. It also frequently occurs that housekeeping genes which are applicable for certain muscles and treatmentgroups at a young age, are unstable when used in older mice.

When designing primers for qPCR, ensure that the primer pairs are located in adjacent exons to avoid amplification of genomic DNA. It is recommended to use primers that generate products of 60-200 base pairs, with a melting temperature of 80-85^{o}C. Primer efficiency and melting peaks should be investigated for each newly designed primer pair.

6. STATISTICAL CONSIDERATIONS

The statistical analysis of the assessments described in this SOP have certain considerations that must be taken into account for a valid statistical analysis. These considerations differ among assessments.

General considerations

Common statistical methods used on continuous measurements, such as student's t-tests, linear regression, and analysis of variance (ANOVA) require that the outcome under study is drawn from a normal distribution. Before any statistical analysis is performed, normality of the outcome should be tested by either a normality test, a visualization of the distribution, or preferably both. There are several easily implemented normality tests available, such as the Shaprio-Wilk test, the Anderson-Darling, the Kolmogorov-Smirnov test. If the outcome under study is not normally distributed, the researcher must either perform a normalizing data transformation or use a statistical method that does not require the outcome to be drawn from a normal distribution (i.e., a non-parametric method).

Body weight, lean and fat mass

Body weight and lean and fat mass is typically measured multiple times per mouse over the length of the study, thus all statistical analyses must account for the dependence of these repeated assessments per mouse. This is achieved by using methods specifically designed for repeated measurements such as repeated measures analysis of variance (RM-ANOVA) or mixed models for repeated measures (MMRM). While these analysis models are more complex than simple

comparisons, they provide a true assessment of effect and yield important characteristics of the effect. These statistical models allow one to compare the outcome between study arms over the length of the study, determine the rate of change of the outcome over the repeated assessments (typically time), and to determine if the rate of change in outcome differs between the study arms; all while accounting correctly for the fact that repeated assessments on each mouse are related to each other by virtue of their measurement. A typical analysis set up for either a RM-ANOVA or MMRM is to include main model terms for study arm and time and a study arm * time interaction term. These terms provide several pieces of information:

- Study arm term – tests whether there is a difference in outcome between study arms over all time points combined.
- Time term – tests whether there is a difference in outcome over time for all study arms combined.
- Arm*interaction term – tests whether the change in outcome over time differs between the study arms.

If the interaction term is not statistically significant, it can be removed from the model and a new model containing only terms for study arm and time can be performed. In this case, there is no need to compare the outcome between study arms at any particular time point as the magnitude of difference in outcome between study arms remains the same at all time points and is defined by the study arm regression coefficient. If, on the other hand, the interaction term is statistically significant, one must compare the outcome between study arms at each time point to determine the magnitude difference between them. This is done by performing appropriate comparisons between study arms at each time point and adjusting the resulting p-value for multiple comparisons to account for the number of comparisons performed. There are several methods of p-value adjustment for multiple comparisons that can be used, but the method chosen should be one that controls the family-wise error rate such as the common Bonferroni, Sidak, Tukey HSD, or Dunnett's methods. Avoid methods that control the false discovery rate, such as the common Benjamini-Hochberg method.

An additional consideration that occurs with body weight is its often quadratic nature. Given a long enough study length where mice age considerably during the study, body weight does not increase in a linear fashion but instead increases steeply at younger mouse ages and then levels off over time. This describes a quadratic relationship (i.e., a curve over time) and can clearly be seen in Figure 1 where body weight increases at different rates over age. The presence of a quadratic relationship should be tested in you model of choice and included if statistically significant. To test this relationship, the time variable in the study is included as a separate squared term in the model.

Creatine Kinase

CK can be measured once or multiple times throughout the study. If measured multiple times on the same mice, the considerations of repeated measruements described for body weight above must be taken into account. If CK is measured only once, a simple comparison between study arms can be done using the appropriate statistical test.

CK levels typically have a very high variability leading to a very skewed distribution and non-normality. If CK levels are in fact normally distributed, or if a data transformation can be applied to make them normally distributed, then a student's t-test (if two study arms) or one-way ANOVA (if three or more study arms) can be performed to determine if levels significantly differ between study arms. If CK levels are not, or cannot be made, normally distributed, then a non-parametric test must be used. For comparisons of two study arms, a Wilcoxon rank sum (MannWhitney U-test) is appropriate. For comparisons of three or more study arms, a Kruskal-Wallis test is appropriate. Note that, just as with a one-way ANOVA, a significant p-value from a Krukal-Wallis test only tells one that CK levels are significantly different between at least two of the study arms, however it does not indicate which study arms are significantly different. That must be determined by *post-hoc* comparisons between the study arms of interest with an adjustment of the resultsing p-values for multiple comparisons using a method to control the family-wise error rate.

Dystrophin level

Dystrophin levels are typically only measured once per mouse removing the need to account for repeated measurements. However, like CK levels, dystrophin level often come from a non-normal distribution and require non-parametric statistical tests (i.e., Wilcoxon rank sum or Kruskal-Wallis tests). In certain cases, the dystrophin level for a study arm may be zero or undetected for all mice in that study arm. If this is the case, as is often found in untreated affected mice, there is an unusual situation where we have a study arm with non-zero dystrophin levels that we want to compare to a study arm with all zero dystrophin levels. Here we can use a one-sample comparison test (i.e., Wilcoxon one-sample signed rank test) to compre the non-zero dystrophin levels in one study arm to a constant value of zero. If the non-zero dystrophin levels in the study arm being compared happen to be normally distributed, then we can use a one-sample t-test to compare mean dystrophin levels to a constant value of zero.

Gene expression

The analysis of gene expression levels can vary significantly based on the purpose of the study. In general, gene expression levels can be non-normally distributed, thus the need for either non-parametric statistical tests or a data transformation (log-transformation) to normalize the values. Note that if a data transformation is applied, normality of the transformed values should be assessed rather than assumed.

Regardless of the purpose of the study, the statistical methods used to compare gene expression between study arms are the same methods described in this SOP for other outcomes. Student's t-tests, linear regressions or ANOVAs if expression levels are normally distributed and Wilcoxon rank sum or Kruskal-Wallis tests if expression levels are not normally distributed.

The purpose of the study impacts the analysis of gene expression levels at the interpretation phase of analysis. Each expression level that is compared between study arms yields a separate statistical test, each with an unadjusted p-value assessing whether a significant difference is observed or not. If the purpose of the study is to compare gene expression levels between study arms for a short list of candidate genes, the unadjusted p-values resulting from that small number of statistical

tests can be reasonably interpreted as is. An adjustment for the number of statistical tests performed is not typically required if that number of statistical test (i.e., number of genes tested) is small. If the purpose of the study is to compare gene expression levels among study arms for a large list of genes, we must address the issue of multiple testing. Performing a large number of statistical tests impacts the type I error rate of the analysis and thus we must account for the number of statistical tests performed by adjusting the resulting p-values from each statistical test. For these circumstances, one can control the false discovery rate (FDR) using the common Benjamini-Hochberg adjustment method. This method adjusts each p-value so that overall the number of potential false positives is set to a reasonable level. It is highly recommened that researchers performing large-scale gene expression studies seek out the advice of a statistician with experience in these types of analyses as they can be very challenging to perform and often require specialized software.

Sample size considerations

This SOP describes several outcomes, each of which can exhibit a different effect size between study arms and a different level of variability, both of which highly impact the sample size necessary to detect significant differences. Consequently, greater variability in an outcome and a smaller expected difference between study arms both require a larger sample size. It is recommended that a sample size calculation be performed when possible for a given outcome of interest to ensure that the correct number of mice are used in each study arm.

7. NATURAL HISTORY AND SAMPLE BIOBANKING

Just like with patients, knowing the disease course of the animal models used is crucial to properly plan experiments. Notably a large natural history study in BL10-*mdx* and D2-*mdx* is currently ongoing. Here, muscles and organs will be isolated at different timepoints and biobanked.

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