A primer on ageing studies in mice: considerations, opportunities and limitations

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Abstract

One of the major challenges currently facing healthcare providers is an ageing population that is spending more time in ill-health. Many ageing individuals have multiple and complex needs which affect the ability to treat them effectively, which also has a significant impact on their own independence and quality of life. There are many aspects of testing interventions to improve health in old age in pre-clinical models; from breeding strategies to measurements of outcomes. Here we provide a brief overview of the major considerations to take into account in such studies and the limitations or challenges we face in these studies.

Introduction

There are many factors during ageing that result in an increased susceptibility to disease and the impact of ageing can vary greatly from individual to individual. Pathways affected or phenotypes exacerbated by ageing (reviewed in [1]) include; metabolism [2], telomeres [3, 4], mitochondrial function [5, 6], increased senescence [7, 8], inflammaging [3, 9], and immunosenescence [3, 10].

Here, we briefly summarise many of the considerations and limitations associated with testing interventions to improve health outcomes that have been discussed and reviewed as part of an EU funded COST action, MouseAGE (www.mouseage.eu/). The future holds many opportunities in the field of ageing research but careful consideration needs to be given to the choice of strain/model, experimental design, and the measurement of outcomes in preclinical studies [11]. This article provides a concise overview into the aspects of study design which should be considered rather than which model is best for certain studies.

Aspects of ageing to model

In modelling the ageing process, researchers need to understand what it is about ageing they wish to measure so we can assess the effectiveness of interventions. With the myriad effects of ageing this can be difficult. Listed below are the main aspects of ageing that need to be considered when assessing the suitability of a model or experimental protocol.

(i) Life and Healthspan

Humans, and the majority of organisms do not age in the same way and there is a variation in in both life and healthspan between individuals [12]. Lifespan was the first convenient measurement of the effects of ageing and this simple measurement has led to great advances in our understanding of the processes and pathways that are fundamental to ageing and can influence ageing itself [13]. Indeed, a large-scale study, the NIA Intervention Study [14, 15], is mining the drug cabinet for new interventions which improve ageing. Assessing when a mouse dies is a straightforward process and does not need complicated equipment or specific expertise. It yields useful data that can be assessed statistically but does not really provide a rich dataset; why an individual mouse dies is just as important, if not more so, than when. An intervention may be affecting a single aspect of ageing, such

as cancer [16], rather than a general improvement in healthspan. Furthermore, in many countries it is not permitted to allow a mouse to become moribund, and it is questionable how ethical it is to allow mice to die or the scientific benefits of these last weeks of life. Thus the cost:benefit analysis of such studies requires rigorous examination and justification. This makes experience in handling and caring for ageing cohorts critical, to ensure ethical endpoints are not exceeded or that cohorts are not compromised due to a confusion between an old mouse and a sick mouse by cage-side staff. The necessity for cage-side staff and researchers to be experienced in handling and caring for aged-mice cannot be overemphasised. This leads on to the concept of healthspan (the proportion of lifespan spent in good health), a more useful measurement as it is more clinically relevant. Whilst the two concepts are interrelated there is not necessarily a correlation that improving lifespan will also improve healthspan, and it may be possible to improve overall health towards the end of life without having a significant impact on lifespan.

(ii) Frailty

Frailty is an easily recognized concept and a useful measure of ageing that is easily understood across disciplines and by lay people. It is the accumulation of deficits associated with ageing. Whilst easily understood, a strict definition is difficult. The deficits affect multiple systems in different ways and, as with multimorbidity, the various components of frailty do not occur at the same time or in the same order in individuals but there is an overall increase in frailty with age in both mice and humans, making this a useful measurement of outcomes. The link between frailty and morbidity is not clear, and there are clear sex differences in humans and mice [17-19], with females tending to have higher levels of frailty but morbidity being higher in males.

(iii) Multi-morbidity

As we age there are common diseases associated with ageing such as cancer, cardiovascular disease, and sarcopoenia because the ageing process affects susceptibility to a wide range of diseases. However, these and other age-related diseases, do not occur in all individuals, in the same order or in the same time frame [12, 20]; they are simply associated with ageing. Therefore, it is important to capture this ageing associated multimorbidity in models of ageing but the defined genetic backgrounds and environment of mouse models does not necessarily reflect the heterogeneity seen in the human population. The vast majority of mouse models are used to study individual diseases or pathways focus on a single disease or pathway and do not necessarily take into account all of the side effects. There are exceptions to this, and the most obvious is to simply age mice. Old mice, like old humans age at different rates, even in inbred colonies, under defined environmental conditions, there can be a large range at the age of death [12]. Inbred strains, because of their restricted genetic diversity, tend to be susceptible to certain diseases as they age and this may limit the range of morbidities they develop. Using outbred mice can overcome this and the ITP utilizes a four way cross to introduce genetic diversity [15]. Mutants exhibiting accelerated ageing, progeria, can display a range of ageing phenotypes associated with ageing but rarely do they capture the whole range phenotypes observed with ageing [21-23] and as yet there is not a ‘standard’ mouse model of multimorbidity which has been characterised in detail and validated.

(iv) Polypharmacy

With multimorbidity comes multiple treatments, which can in turn lead to the additional complication of polypharmacy where the body is exposed to a range of drugs which may or may not interact, but at a minimum come with a range of side effects. This further challenges the resilience of an individual and may lead to additional symptoms arising. Whilst this is generally not a problem in murine studies

it is never the less an important consideration for the translation of findings to the bedside. Some researchers are now developing a polypharmacy challenge which has been shown to result in an increase in frailty in old, but not young, mice [24].

Selection of models

(i) Inbred strains

The advantage of an inbred strain is that a defined genetic background along with a controlled environment results in predictable and reproducible results, simplifying experimental design and reducing animal numbers. However, this is not the case in patients where there is a much greater genetic and environmental diversity. Thus, inbred strains are very useful in interrogating specific pathways, genetically or through interventions, but the specific susceptibilities of individual strains to morbidities must always be born in mind, and the most commonly used strains are not necessarily the best for particular studies. For example, there is a great variation in the lifespan of inbred mouse strains [25], and each strain has its own foibles and susceptibilities, deafness in C57BL/6J mice, vision loss in C3H strains and so on. It must be emphasized that even within inbred strains that there is variation, which increases with age, in many parameters. Of note is the variation in lifespan and frailty within tightly controlled inbred colonies [25] (https://phenome.jax.org/measures/23401).

(ii) Outbred/mixed strains

A straightforward way of overcoming the lack of genetic diversity in inbred strains is to employ mixed genetic background or outbred mice. Obtaining truly outbred mice necessitates significant breeding of well managed colonies. The ITP employs a four-way cross to generate a reliable source of diversity. Other potential sources of diverse genetic background are diversity outbred mice and the collaborative cross [26]. Whilst a genetically diverse background better models the general patient population but adds complications to study design; phenotypic variation and the introduction of genetic variants. The increased variation in phenotypes necessitates increased numbers in studies to ensure there is sufficient power, and the increase in phenotypic variation with ageing, further compounds this. Introducing genetic variations into an outbred population is also difficult as there will always be founders effects whereby DNA flanking the mutation will be co-inherited [27, 28]. Diversity can be re-introduced by breeding but this wound require an extensive amount of time. The raison d'etre of CC lines is to introduce diversity in a controlled manner and the recombinant inbred nature of these lines means for an individual CC line large cohorts are not needed to study the effect of an intervention or genetic variant but the experiment(s) would have to be repeated across the range of lines, thereby increasing the number mice. The CC lines have been successful in dissecting genetic pathways and it would be interesting to begin ageing studies in this resource as is being done with DO mice.

(iii) Mutants or treatments affecting ageing

Ageing mice is expensive and time consuming and so one option is to employ mice with accelerated ageing to carry out intervention studies. These may be genetically modified lines or mice exposed to treatments such as chemotherapy or irradiation, which have been demonstrated to increase senescence and result in accelerated ageing. These have been reviewed elsewhere [22, 29], and whilst each example may not recapitulate every aspect of ageing they are none the less extremely useful in studying certain pathways and some interventions known to improve ageing have been shown to be beneficial in some of them. These also allow the study of interventions in specific pathways such as improving mitochondrial health in POLG mice [30, 31] or studying senolytics in chemotherapy treated mice [32]. Rapamycin, a geroprotector that has been demonstrated to have beneficial effects on ageing, can ameliorate some of the phenotypes observed in a progeroid model [33, 34].

Assessment of ageing in models

(i) Phenotpying ageing mice

As with other studies, strict protocols are required to generate reproducible results [35, 36]. In general, most phenotypic tests applied to younger mice can also be applied to older mice but there may be subtle variations in technique. For example, older mice tend to be more inactive and tests such as gait analysis and rotarod can be more difficult to perform [37]. More detailed tests such as auditory brain stem response for hearing loss will be more informative than simple click box tests. The normal data range for many tests may not have been determined in aged animals, or at least there will not be as an extensive a dataset as there is for younger mice. As with the care of aged-mice, experience with phenotyping aged-mice is critical; not only in their behaviour during tests but the data generated. Data is likely to be more variable and the impact of ageing on individual mice, bearing in mind the variability in the effects of ageing between individuals, may mean some mice are reluctant to undertake tasks, or may look like outliers in some tests, such as grip strength. It is also a scientific and ethical imperative to obtain as much information as possible from aged cohorts even if there is a limited measure of outcomes and plans should include the banking of samples and tissues for future studies, ideally in an accessible biobank [38].

(ii) The Frailty Index

A useful overall assessment of the effects of ageing, in both mice and humans, is the Frailty Index (FI). This is a compilation of measurements that provides an overview of the overall ageing of an individual and is comparable in humans and mice [19, 39-43]. There are a range of FIs that have been developed but it is clear that an increased FI is indicative of an increased risk or morbidity [12, 44]. Interventions which benefit ageing also improve the FI, demonstrating the utility of this measure. The FI gives a richer dataset than simple lifespan, but less so than a comprehensive phenotypic assessment of health. However, the FI is easy to establish and does not require an extensive array of equipment.

(iii) Resilience

An aspect of frailty is the inability, or reduced capacity, to respond to challenges and is reflected as a loss of resilience. Resilience is effectively the inverse of the FI score and reflects the ability of an organism to return to a steady state after a challenge Thus, it is possible to test resilience by challenging individuals and analysing their response. The challenge should reflect the system wide loss of resilience rather than a deficit in a particular organ, but the challenge may be to a single organ. A variety of challenges are available, such as sepsis, cold, or hypoxia but these have yet to be validated in ageing studies and standardised protocols established for use in ageing studies [29, 45, 46].

(iv) Biomarkers of ageing

Specific biomarkers of ageing or frailty would be of obvious use in pre-clinical studies as they provide a measureable, quantifiable and standardised assessment. Specific biomarkers may also allow the stratification of patients or test groups to improve the accuracy of a particular study, or allow the timing of intervention to be based on biomarker levels rather than chronological age. Detailed phenotypic assessment of healthspan will provide similar data but a being able to assess a panel of ageing specific biomarkers will enable cross comparison of studies. Unfortunately, there is a paucity of validated and translatable biomarkers that are easily measurable and indicate the ageing state of an individual [47], but some promising measures are being identified such as Frailty Indices [47-49], and serum biomarkers [50-52]

Study design

i. Strain/model and breeding design

As noted above the variation of phenotypes in mice increases with age, even within inbred colonies, which necessitates larger cohorts and careful power calculations. The source of this variation has not been defined but may be due to several factors, including social structure within cages and breeding structures, as well as stochastic factors. Careful breeding structures need to be employed as diet and age of the breeders can have an impact on subsequent generations [53-55]. Obesity in dams can affect the metabolic status of their offspring [56, 57] and the age of breeding males can have effects on behavioural phenotypes in the offspring [58]. It is also essential that both sexes are studied as there are clear differences in the effects of interventions and the effects of ageing. The effects of rapamycin, a well-studied geroprotector, vary according to sex [59-61], and there are also sex specific differences in other important pathways related to ageing [62].

ii. Environment

Housing conditions can have an effect on the mice. Within their home cage mice are relatively sedentary and have food *ad libitum*, and are therefore prone to obesity. Whilst it can be argued that this is a growing problem in the patient population such a metabolic challenge can influence the outcome of studies as was seen in recent macaque studies on caloric restriction where differing results were obtained depending on the health and diet of the cohorts studied [63-65].

ii. Planning cohorts

In studying interventions in ageing cohorts of mice a decision on whether to carry out a cross sectional study (comparing separate cohorts at different time points) or using longitudinal cohorts (a large cohort of mice are studied over their lifespan) [35]. Longitudinal studies offer the advantage of tracking phenotypes within individual mice, potentially counteracting the inter-individual variation, but mean subjecting mice to repeated rounds of phenotyping which may be stressful or influence subsequent tests. Exercise and strength testing also needs to be carefully considered because of the clear benefits of exercise in ageing. Thus, the design of the phenotypic pipeline needs to be rigorously tested and validated to ensure the repeated testing does not influence the outcome by interacting with the intervention. Carefully controlled experiments should tease out any effect of phenotyping but will involve increased numbers of mice. Cross sectional studies generally allow a battery of tests to be carried out with the added advantage of *post mortem* analyses across the time points. There is no 'one size fits all' experimental plan or ideal cohort size as this will be determined by the variation of the phenotype or outcome within a particular model or strain, life span (which may result in the loss of individuals), effect size of an intervention, and time span of study as just a few examples. Whereas studies in young mice may require groups of 5 to 6 mice to achieve sufficient power, similar experiments in ageing mice may require cohorts of 20 or more. Many of the factors affecting cohort size may not be known and so experience and publications will act as a guide. Buying in cohorts of aged mice may be possible but these are hard to come buy and therefore the time and expensive of breeding aged cohorts needs to be factored into the experimental plan; it is easier to breed younger cohorts again so generally it is best to ensure you have sufficient ageing cohorts breeding as early as possible. It is also important to plan in advance to make sure equipment and staff are available, and in particular that cohorts do not overlap extensively thus creating bottlenecks. The one advantage of aged cohorts is that there is more flexibility in timing as varying the timing of phenotyping by a day or so, or even a week or so, is less likely to have an impact of results than with cohorts of a few weeks of age.

ii. Intervention testing

The time point of intervention will be determined by many factors, primarily the questions being asked or hypotheses tested. For a purely academic study to understand the effect of an intervention and the pathways it influences then a range of time points may be useful as was found with rapamycin, which exerted some of its effects in young mice [16]. As we move more towards preclinical testing then a single time point may be of more relevance, but that time point may be difficult to define. Taking drugs prophylactically to stave off potential morbidities that may occur at some, undetermined, point in the future is potentially contentious and no doubt expensive. Far better to intervene at a defined time point when individuals can be identified that will benefit. Currently, the majority of studies employ a defined age at which the mice are generally considered old but before they enter ill-health. Other studies to look at the ability of interventions to reverse established phenotypes may choose a later time point. However, as already stated, there is a considerably variability in the development of age related phenotypes and age at death even in inbred strains. At a particular age there may be sub groups of mice which are healthy, pre-frail or frail, and therefore phenotypic assessment before treatments may allow the stratification of mice. Ultimately, it may be more accurate to select the time of intervention for individual mice based on biomarkers or a frailty index rather than chronological age, which is most likely to be the case when treating patients, rather than a strict chronological age. The correct timing of intervention will also vary with each individual experiment and indeed there may not be a single 'correct' time or doing regimen and ultimately can only be determined by extensive experimentation. One of the most studied geroprotectors, rapamycin, has been used in a range of dosing regimens.

Summary

Here we have presented an overview of the some of the considerations that are central to the study of ageing interventions in mice and that should be incorporated into study design (Figure 1). Whilst study design will vary according to the hypotheses being tested, the more consensus that can be achieved the more easily results can be compared, and the quicker the field moves forward. It is likely that any intervention will need to be tested in more than one model and utilising a range of outcome measurements to ensure a successful translation into the clinic. All models have their limitations and mice are no exception, despite the advances in our understanding of biology that have resulted from murine studies. They may not recapitulate all aspects of human disease, they eat a a different diet, are nocturnal, are quadrupeds, and so on. In some particular areas, even where mice have been used extensively, studies have failed to deliver advances which may be due to the unsuitability of the model. However, such arguments apply to all models, and indeed some patient cohorts, and a thorough understanding of the aims of an experiment, the protocols, and possibly most important of all, the limitations of the model to be employed is required to generate useful and reproducible data.

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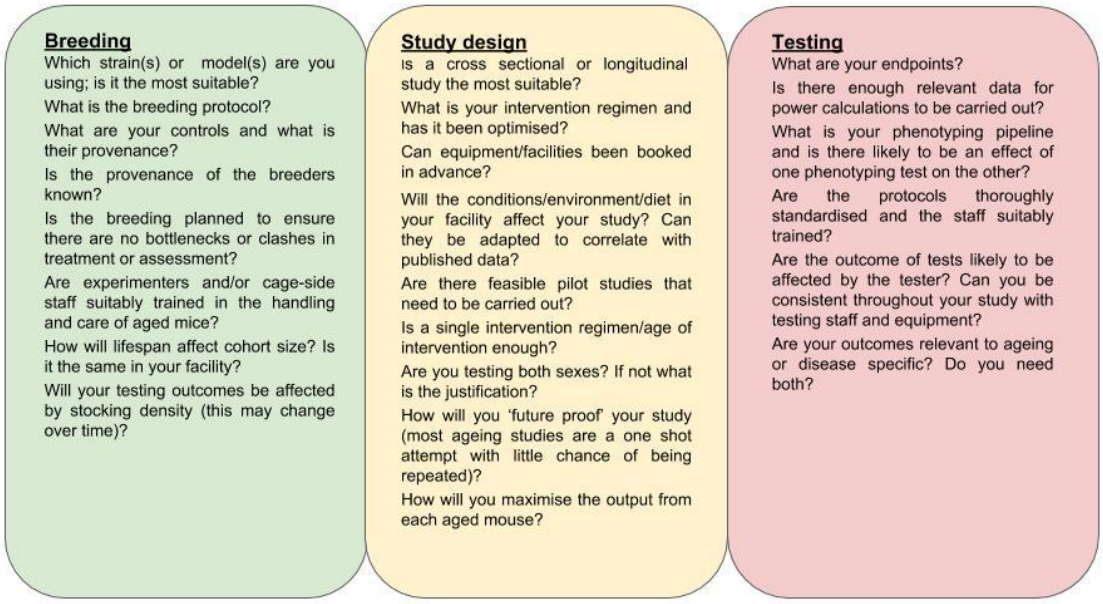
Figure 1.

A summary of some of the key questions that need to be answered before undertaking a project involving ageing mice. Such experiments are costly and time consuming, and as such very detailed, long-term planning is required as it is unlikely that they can be repeated within the confines of an average grant.



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