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Original Article

How to train a mouse-methodological issues in pre-clinical exercise oncology

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Abstract: We point at several challenges that current exercise oncology rodent models face, which call their human-relevance into question: the vast majority of pre-clinical studies in exercise oncology treat "physical exercise" as a primitive concept without further analysis or qualification, and their results are based on dosages that no human can endure. The lack of analysis and qualification together with the dosage mismatch conceal the fact that rodents do not run like humans. Consequently, while these pre-clinical studies may yield insights into potential biological mechanisms underlying the systemic effects of physical exercise on cancer, the applicability of this knowledge to preventive interventions in healthy humans and the ability to translate it to practical therapies in the critically ill remain limited. We propose an alternative exercise rodent model that has better chances of meeting these challenges.

Keywords: Exercise oncology, rodent models, dose-response, human relevance, translational research

Introduction

Recent years have seen a significant increase in literature on pre-clinical studies aiming to probe the effects of physical exercise on a wide range of human disease models. The common overarching methodology of these studies is straightforward: one hypothesizes (or identifies through epidemiological data) a potential association between physical exercise and reduced disease morbidity or mortality in humans, and sets out to probe a potential mechanism behind this association in respective rodent disease models, by comparing sedentary and exercised rodents and by identifying potential cellular or molecular pathways that may underlie the preventive or therapeutic effects of physical exercise on that disease.

Focusing on cancer as a representative example, in this review we would like to point at several challenges to these studies that, in our mind, call into question the conclusions one would like to draw therefrom. The problem is *not* the usual one - that of using the biology of a model organism to obtain knowledge about the biology of humans; admittedly there is currently no way around the use of rodents in pre-clinical studies within the workflow of biomedical research, and physical exercise studies in disease rodent models are no different in this respect than any other rodent study that generates biological data for biomedical research. The problem, rather, is that the vast majority of these pre-clinical studies of physical exercise treat "physical exercise" as a primitive concept without further analysis, and without qualifying or quantifying it according to the different *types* of exercise that can be compared with respective human-relevant dosages. The threefold lack of analysis, qualification and quantification conceals the fact that rodents do not run like humans. Consequently, while these pre-clinical studies may yield insights into potential biological mechanisms underlying the systemic effects of physical exercise on cancer, the applicability of this knowledge to preventive interventions in healthy humans and the ability to translate it to practical therapies in the critically ill remain limited.

In what follows we shall review some of the history of exercise rodent models and the current state-of-the-art in this field. While not exhaustive, the short review will be sufficient to demonstrate the challenge we believe exists for such exercise models in general. We shall then suggest how to meet this challenge by identifying the constraints that are missing from the majority of current studies. In a nutshell, our conclusion is that if one would like to draw practical knowledge on prevention, diagnostics, therapy or prognosis for humans from pre-clinical studies of physical exercise and disease, and in particular exercise oncology, one must train a rodent to run with a human-relevant dosage. In the final section we suggest such a rodent exercise model that satisfies the above constraints and can meet this challenge.

Aerobic exercise and cancer

With hundreds of studies to date, it is widely believed that regular physical exercise reduces risk of cancer incidence [1, 2]. In breast cancer, for example, cohort studies and case-control studies estimated a 20% d 30% risk reduction, respectively [3]. In colon cancer there is strong and consistent evidence from multiple meta-analyses that physical activity is associated with a significant 24% risk reduction [4]. Similarly, evidence for 28% risk reduction exists for pancreatic cancer [5], 10% risk reduction in prostate cancer [6], 19% in ovarian cancer [7], and 23% in lung cancer [8]. While these results are derived from small case studies hence may seem inconclusive, a recent meta-analysis of 12 prospective epidemiological studies comprising a total of 1.44 Million individuals found a significant risk reduction in 13 types of cancer with self-reported leisure time physical activity of moderate level, equivalent to 150 weekly minutes of intensive walk [9]. Other epidemiological findings include lower prevalence of breast cancer and cancers of the reproductive system in athletes versus non-athletes [10, 11], and lower recurrence rates in breast and colon cancer survivors who exercise regularly [12, 13].

While these results are consistent with the hypothesis that aerobic exercise slows tumor progression, they do not allow us to quantify said impact. As far as we know, to date there exists only one study which directly associates aerobic fitness with solid tumor progression rates in humans, quantifying the effect in 14 invasive ductal carcinoma (IDC) patients, all with T1-T2 tumors before any treatment [14]. The study found a statistically significant association between aerobic fitness (measured with blood lactate concentration during an incremental pedaling session, adjusted for age and rest heart rate) and tumor doubling times (in days): the more aerobically fit were the subjects, the slower was their tumor growth. No association was found in that study, which focused solely on early stage IDC, between growth rate and tumor grade, patients' BMI, or the IDC molecular subtype.

For obvious reasons, besides the problem of quantifying dose-response effects in the critically ill, mechanistic underpinning for this hypothesis in humans is also hard to obtain. Most of the evidence must thus come from pre-clinical studies in rodent models.

Pre-clinical exercise oncology

The perception that aerobic exercise effects tumor progression in animal models dates back to reports from the 1940s. In 1943, Rusch & Kline [15] referred to previous works that indicated an inverse relation between tumor growth and caloric intake in animals [16-19], or body weight in humans [20]. They hypothesized that any intervention that influences energy supply could also impact the growth of tumors. The two subjected male mice to forced exercise and controlled caloric feedings. One group of mice was exercised continuously for 16 hours and rested 8 hours, and a second exercised for 2 hours and rested for 1 hour during the course of a 24 hours period (the intense exercise resulted in severe exhaustion among the animals, and no IACUC would allow such a study today).

Since the 1940s there has been an exponential increase in PUBMED indexed articles on aerobic exercise and cancer in rodent models, from single digit numbers to more than 1100 a year in 2015. The evidence accumulated cuts across different types of solid tumor and different rodent models, and is consistent with said hypothetical impact of aerobic exercise on tumor progression. The modality of aerobic exercise used in these models varies between voluntary running wheels, forced swimming, and forced running using a rodent treadmill.

More than two-thirds of these studies demonstrated growth inhibition as a result of training [21, 22].

Since mice are natural runners, voluntary running wheels dominated the research landscape up to the early 1990s and present the easiest experimental design to implement exercise training. In these experiments, mice are allowed to run freely in a wheel placed inside of the standard mouse cage. The number of wheel rotations is recorded electronically and data on the frequency, distance, and average velocity can be calculated. There are two types of voluntary wheels: the saucer-shaped wheel and the regular wheel. The saucer-shaped option has a larger cage footprint and mice tend to run more with this setup. In this scenario, the standard wire top lid for food delivery can be replaced by a food container on the floor of the cage [23]. For the regular voluntary wheel, no extra arrangements are required, but mice might not run as much as in the saucer-shaped version because of the extra space they now have inside the cage. On average, a cage of mice can run 5 to 7 km per night on a voluntary wheel, with an average velocity of 4 m/min that can increase up to 12 m/min [24]. Strain, gender and age should be considered in the design of the experiment, as males run more than females, older mice run less and younger mice, and, for example, CB6F1 mice run more than C57BL/6 mice [23].

Similar differences were observed in forced swimming [25]. In this scenario, mice are placed individually in a closed transparent tank with water and their escape related mobility behavior is measured. To allow comparisons between different experimental set ups, volume and temperature of water (23-25°C) must remain constant. In order to mask potential loud noises that could alarm the animals, a white noise generator is often selected. While only an option in the voluntary wheel scenarios, the use of video recording is mandatory in the swimming scenarios, as the experiment usually involves multiple animals. Video tracking allows the researcher to detect the mobility behavior and its variation: immobile, mobile or highly mobile, which can be translated to floating, swimming and escaping/climbing behaviors. Clearly, such data is not precise and can only give us qualitative information on the exercise dosage in each swimming pattern. After the experiment is complete, it is indispensable to dry the animals and use a heat lamp to (not exceeding 32°C) to prevent hypothermia [25].

Voluntary wheel running requires almost no effort from the experimenter, while forced swimming is heavily time-consuming. A compromise, which has become the dominant in the field, is the forced treadmill. This set up requires specialized equipment consisting in individualized tracks with treadmills. For this kind of experiment, the exercise is introduced slowly to the mouse, and the experimenter controls the velocity and duration.

To induce constant running behavior, researchers use a small stick to push the animals whenever they refuse to run. If the mouse continues to avoid running, a transient and weak electric stimulation is used. If the mouse still doesn't run, it is removed from the treadmill and reintroduced later. Stadelmann *et al*. [26] reported that even the best mice runners had to be motivated with electric stimulation up to three different days. Most treadmill exercise protocols require mice to for 60 min a day at 14 m/min, 5 day/week [27], which, as we shall see below, exceeds the threshold of what mice could do in a stress-free environment.

Methodological issues

Despite the promise to perform dose-response studies in rodents in a more controlled fashion relative to humans, and the obvious advantage of gaining mechanistic insights, we believe current pre-clinical studies in exercise oncology should be read with caution: there are inherent limitations to the quantification of the amount of aerobic exercise in such models, and to their translation to human setting. We emphasize that these limitations are germane to all current rodent exercise studies and exist over and above the general problem of translating from the biology of the rodent to the biology of humans; rather, they stem from the mere fact that rodents do not run like humans.

Single vs. group caging

Housing conditions impact animal behavioral and biological responses, and inappropriate housing conditions can affect the experimental results by inducing additional stress [28-30].

Same-sex grouping must be guaranteed, and conditions should be reported in much details as possible, as small deviations between experimental conditions could yield large discrepancies in results. In the voluntary wheel set up, for example, single caging allows better quantification of potential dose-response relations, but may not be the best choice for rodent exercise studies, which would depend on the rodent and its sex. Male rats, for example, have higher corticosterone levels under crowded conditions [31], thus for them single caging is a must. But female rats behave in the opposite way. For them group housing is essential, as higher levels of corticosterone are detected when they are individually housed [30]. In contrast, single housing of mice should almost always be avoided [32, 33]. And yet most, if not all voluntary wheel experiments do not employ sophisticated tracking equipment, and so the data they collect is restricted to the cage level [34]. In other words, in these experiments it is the *cage* that exercises, rather than the individual mouse, and it is hard to extract from the data any useful individual measures of distance, velocity and duration.

Voluntary vs. forced exercise and the stress dilemma

Treadmill exercise allows group caging of mice *and* dosage quantification. However, researchers who rely on the rodent treadmill are often confronted with the dilemma of using the electric grid shocker to encourage mice to run. The shocker may be effective for controlling the exercise dosage and ensuring compliance, but it induces additional stress to the animals, and often masks the desired response [35]. Indeed, while not often reported, in some studies the repeated electric shock may eventually kill some of the rodents, and researchers commonly acknowledge this adverse stimulus and attempt to correct it by exposing *all* animals to the same amounts of shock [36]. Notably, almost no treadmill exercise oncology study reported the stress level of the animals (by, e.g., measuring cortisol levels in feces). Lacking such reports, it is hard to draw unequivocal conclusions from those studies. Admittedly, physical exercise does induce the release of several stress hormones in humans who go above over 50%-60% of their aerobic capacity [37]. Therefore, exercise interventions either in humans or animals will have some level of stress involved. Our point, however, is that one should aim for minimizing and controlling such stress, rather than augment it with additional uncontrolled stress from environmental sources such as single caging or electric shock.

Human dosages of exercise

The standard measure for aerobic fitness is the VO_2max test [38]. To score high in this test, humans can choose two different exercise regimes, endurance training or high intensity interval training (HIIT). Both have documented health benefits, and there is an ongoing debate on their relative merits [39-45]. Regardless of this debate, however, the ability and preference of humans who are non-athletes to persist in the former is much greater than in the latter [42, 43], and when doing so, the HIIT dosage humans are comfortable with is quite limited. This fact, we shall argue, raises serious questions about the applicability of data generated from current exercise oncology pre-clinical studies to humans.

Traditionally, endurance training consists of moderate or low intensity exercise (up to 70% of maximal heart rate) for an extended time span, while HIIT involves repeated short bouts of high intensity exercise (up to 90-95% of maximal heart rate). Since "lack of time" is the most popular reason humans give for not meeting the minimum exercise activity recommendations [43], and since both HIIT and endurance lead to similar improvements in the VO_2max test, HIIT appears to be more attractive time wise to humans in modern society.

This appearance notwithstanding, HIIT has several high-risk factors that may overweigh its benefits. Since it involves reaching close to maximal heart rate, its applicability to the general public is questionable, in particular in vulnerable subpopulations such as the elderly or the critically ill. Indeed, data from clinical HIIT studies have been generated from short-term designs, executed in laboratory settings and performed in selected patients. Thus, contrary to endurance training, the *general* safety of HIIT has so far not been well established. In addition, special attention must be given to correct warm-up and cool-down procedures. These procedures may be able to reduce the risks involved in chronic HIIT training [44, 45], but

their implementation requires strict adherence.

The problem, however, is that even if HIIT were proven to be completely safe and clinically applicable to *all* humans, healthy or otherwise, it would still remain *an addition to* the common repertoire of human endurance exercise. And the reason for this is that when given a choice between endurance and HIIT as their chronic exercise regime, elite athletes, let alone untrained humans, prefer the former and not the latter, spending 80% of their time on endurance and only 20% on HIIT [46-49]! Chronic HIIT is simply harder to implement, requires more preparation (warm up) and recovery times (cool down), and, in short, not the way the average human trains. Contrast that with the typical mouse behavior of voluntary running in bouts of 1-2 minutes [50], and you get the major obstacle in generalizing pre-clinical exercise oncology data to humans.

On mice and men

Despite the growing dissatisfaction with the applicability of the mouse model to human disease [51], it remains the model of choice in biomedical research. We have nothing to add here to this ongoing debate which has traditionally revolved around the difference (or lack thereof) in the biology of a model and its target. Our point in this paper is different: even if the relevant biology of mice and men could be shown to match in some specific domains, there is a stark contrast in the way mice and humans run. To repeat, the problem is not a *behavioral* one, but a problem of mismatched dosage. A mouse could perform HIIT all night long, every night, from infancy to old age [23], while humans will drop to the ground after 30 minutes of HIIT, and can repeat the experience at most twice a week, with a sharp decrease in age-related performance [52]. Left to its elements, a mouse will rarely run voluntarily for more than 1-2 minutes [50] in speeds quite unparalleled in humans [22], while the average human prefers to run for an extended period of time in low to moderate intensity [48]. As a result, pre-clinical rodent exercise studies that are based solely on the voluntary wheel and which report health benefits of "exercise" in mice should be read with caution. At most they can be seen as probing potential *in vivo* mechanisms, the translation of which to humans as *dose-response* guidance for prevention or therapy is severely limited.

To make this point bluntly, suppose one finds that mice that exercised for 5 weeks on a voluntary wheel had a specific signaling pathway activated that enhances antitumor immune response. One then has gained knowledge about a potential mechanism by which "exercise" inhibits disease progression, but in order to translate this knowledge to practical intervention in humans, one must either (1) adjust the experimental set up to human-relevant dosages by testing the effects of said mechanism in mice which are exposed to the voluntary wheel only few times a week for only a short duration, or (2) make humans run with mice-like dosages. As far as we know, option (1) has never been considered in the pre-clinical exercise oncology literature and so the reported positive effects of exercise in mice which are potentially relevant to humans could be highly exaggerated. As for option (2), well, good luck...

The forced running wheel

To better harness the benefits of physical exercise for the prevention and management of human disease, we need a quantifiable exercise model in which mice *volitionally* run in human-relevant dosages, without the additional stress incurred from "incentives" such as electric shock. We believe such a model exists. We have developed it in our lab, tested its robustness for 2 years, and showed its efficacy in slowing mammary tumor growth [53]. The model is based on a "shock-free" forced running wheels, and on a training protocol that slowly and incrementally trains mice over a period of 8 weeks to continuously run in low to moderate intensity, up to a velocity of 12 m/min, but for increasingly longer periods of time, up to 26 minutes each session. The apparatus houses 4 mice, one per wheel, and in principle can be used to control and quantify endurance training for an individual mouse. To avoid stress, we trained the mice with no a-priori goal. Instead, we implemented the following rule: when a mouse would show first signs of exhaustion by freezing or clinging to the rungs, the velocity would be lowered until the mouse would begin running again. This rule ensured the mice kept running continuously for longer

and longer periods with slowly increasing velocities, adjusting the intensity level to the ability of the lowest performing mouse. In the 8th and final week the mice ran for 26 minutes a day, spending 1 min at 6 m/min, 1 min at 8 m/min, 22 min at 10 m/min, and 2 min 12 m/min.

The exercise dosage we induced may seem low compared with most exercise oncology studies, but our data show that the model leads to higher concentration of slow twitch muscles in the trained mice relative to their sedentary controls, and to better muscular endurance, based on a comparison of blood lactate concentration kinetics during a short exercise period, while maintaining cortisol levels constant during the training period. Importantly, this seemingly low dosage was sufficient to induce systemic effects on the immune system of healthy trained mice relative to their sedentary controls, and led to 17% slower doubling time of an aggressive mammary tumor, and 33% longer survival rates in trained vs. sedentary mice, while allowing us to identify a potential underlying mechanism of antitumor immune response that was enhanced by the training.

The most important point is that the dosage we induced was significantly more human-relevant than any voluntary wheel study, and required no adverse stimulus. Studies are underway to compare this exercise model and its effect on disease progression to the standard voluntary wheel model, and our hope is that future preclinical studies would use the forced running wheel as their model of choice, as its translational relevance is likely to be higher than current existing rodent exercise models.

Conclusion

We believe we have identified a serious problem in current exercise oncology pre-clinical studies that generalizes to many exercise rodent disease models, namely, that the current usage of two widespread experimental setups-the voluntary wheel and the electric shock treadmill-precludes any practical dose-response translation to humans. We might gain a lot of insight from these experimental setups on *potential* biological and molecular mechanisms underlying the effects of physical exercise on disease, but we cannot harness this knowledge to improve patient outcomes in humans.

What is needed is an alternative experimental setup that induces a type of exercise and a respective dosage that are both translatable to humans, that does so in a stress-free intervention, and that is quantifiable and controllable. We have demonstrated that such a model exists, and that it can be used to establish human-relevant dose-response effects of aerobic exercise on tumor progression.

Disclosure of conflict of interest

None.

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