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Via email: sww123@fda.gov.tw

Dear Ms. Wang,

Thank you in advance for your time. On behalf of People for the Ethical Treatment of Animals (PETA) and our 6.5 million members and supporters worldwide, I provide below scientific critiques on the animal tests recommended by the Taiwan Food and Drug Administration's (TFDA) guideline on anti-fatigue health claims for foods. We urge TFDA to reevaluate the guideline and remove the requirements, suggestions, and acceptance of animal tests.

Background

The TFDA published a guideline on experiments that applicants need to perform to substantiate anti-fatigue health claims¹ for foods in 2003.² In the guideline, TFDA specified that applicants should submit existing literature in support of the foods of interest and conduct the recommended human tests or animal tests in addition. However, if the literature isn't substantial enough, applicants must conduct both the human and animal tests.

The guideline recommended two animal tests: the forced swim test (FST) and treadmill running test (TRT). For the FST, experimenters are to feed mice or rats the test foods 2-11 times of the equivalent quantity of what is recommended for human consumption, starve the animals for 12-24 hours, drop them each in individual tanks or beakers filled with water, and time how long it takes for the animals to either drown to death or remain under water for eight consecutive seconds. Experimenters can add weight in the form of lead coils to the animals to "speed up the process", and if the animals learn to float, experimenters can stir the water to make the animals struggle more. For the TRT, experimenters are to feed rats the test foods 2-11 times of the equivalent quantity of what is recommended for human consumption, put the animals on treadmills equipped with electrified plates, force them to run with increasing speeds and slopes, and time how long it takes for the animals to choose being repeatedly electrocuted over continued running. For both the FST and TRT, animals are killed at the end and dissected. At least four groups (one control and three experimental groups) and more than eight animals per group are required.

¹ The anti-fatigue claims are restricted to physical fatigue during and/or post-exercise per the guideline.

² <https://www.fda.gov.tw/TC/newsContent.aspx?id=19957&chk=2ee0ed20-6f4c-4325-873b-a808a50d8965¶m=pn>

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In addition to the blatant and unacceptable cruelty associated with the FST and TRT, below is a detailed list of scientific limitations of these two tests as well as issues related to using mice or rats to substantiate human anti-fatigue health claims in general.

Scientific Limitations of the Proposed Animal Tests

The guideline referenced only one paper³ for the recommendations of the animal tests, and it was published in 1979. Since then, pathophysiological differences between mice/rats and humans have become more well-known, thereby discrediting these animal tests as inapplicable for translating to the human condition.

The FST has an emotional and psychological component and is hard to control

The FST is stressful for mice and rats as they don't live near water or swim regularly in the laboratories. Stress hormone increases significantly in animals after the FST,⁴ and both acute⁵ and chronic⁶ stress are implicated in muscle fatigue. Stress is also implicated in other types of fatigue, such as mental fatigue.⁷ In addition, there is a large body of literature using the FST to study depression and as a screening tool for antidepressants.⁸ Some argue that the FST measures stress-coping strategy and not depression-like behavior.⁹ Regardless, since the guideline specifically excludes psychological types of fatigue in the definition of anti-fatigue health claims, it would seem that using animals who are susceptible to stress in psychological tests is inappropriate.

With human subjects, researchers can communicate the research objectives and study procedures, monitor the subjects' stress level, and intervene and control the stress level if necessary. With animals however—especially prey animals such as mice and rats—their stress is often uncontrollable as light,¹⁰

³ The first and second citations are the same paper.

⁴ Jameel, M. K., Joshi, A. R., Dawane, J., Padwal, M., Joshi, A. R., Pandit, V. A., & Melinkeri, R. R. (2014). Effect of various physical stress models on serum cortisol level in wistar rats. *Journal of clinical and diagnostic research: JCDR*, 8(3), 181.

⁵ Keller-Ross, M. L., Pereira, H. M., Pruse, J., Yoon, T., Schlinder-DeLap, B., Nielson, K. A., & Hunter, S. K. (2014). Stressor-induced increase in muscle fatigability of young men and women is predicted by strength but not voluntary activation. *Journal of Applied Physiology*, 116(7), 767-778.

⁶ Keller-Ross, M. L., Schlinder-Delap, B., Doyel, R., Larson, G., & Hunter, S. K. (2014). Muscle fatigability and control of force in men with posttraumatic stress disorder. *Medicine & Science in Sports & Exercise*, 46(7), 1302-1313.

⁷ Finsterer, J., & Mahjoub, S. Z. (2014). Fatigue in healthy and diseased individuals. *American Journal of Hospice and Palliative Medicine*®, 31(5), 562-575.

⁸ Chen, L., Faas, G. C., Ferando, I., & Mody, I. (2015). Novel insights into the behavioral analysis of mice subjected to the forced-swim test. *Translational psychiatry*, 5(4), e551.

⁹ Commons, K. G., Cholanians, A. B., Babb, J. A., & Ehlinger, D. G. (2017). The rodent forced swim test measures stress-coping strategy, not depression-like behavior. *ACS chemical neuroscience*, 8(5), 955-960.

¹⁰ Azar, T. A., Sharp, J. L., & Lawson, D. M. (2008). Effect of housing rats in dim light or long nights on heart rate. *Journal of the American Association for Laboratory Animal Science*, 47(4), 25-34.

noise,¹¹ temperature,¹² cage cleaning and transport,¹³ lack of enrichment,¹⁴ social deprivation,¹⁵ human interaction and handling,¹⁶ routine experimental procedures,¹⁷ and even the male gender of experimenters,¹⁸ all can induce stress and affect the animals' well-being and outcome of measurements. A case in point of animals' stress being uncontrollable is that although rotation of a multi-tier caging system is recommended to unify the exposure to lights on the ceiling¹⁹ (as an attempt to unify the animals' stress level), this may actually heighten animals' stress due to increased noise, cage transport, and human handling. In short, in this situation the control group is not a true control and both the control and experimental groups are contaminated with stress confounders.

There are many other factors that can influence the results of the FST.²⁰ The most important one being inter-individual variations: animals of the same strains/characteristics in the same groups/settings can have large variations in the FST outcome measures. In this case, a cross-over and self-controlled design (using the pre-food exposure condition as control in individual animal) is the only way to mitigate the limitation. However, since death is the end point of the proposed FST, this is not feasible.

Species differences in Circadian rhythm

Mice and rats, unlike humans, are nocturnal. The circadian rhythm has a clear effect on the outcome of FST measurements, and the animals are more stressed if they are forced to perform the FST while they are supposed to be asleep,²¹ understandably so. The circadian rhythm also affects metabolism.²² Active periods correspond to higher metabolic rate, and many metabolic substrates and hormones fluctuate in concentration and sensitivity throughout the day. Since mice and rats are nocturnal, their physiological functions are opposite to humans in many aspects. Thus the animal studies cannot be trusted if performed during day time.

¹¹ Baldwin, A. L., Schwartz, G. E., & Hopp, D. H. (2007). Are investigators aware of environmental noise in animal facilities and that this noise may affect experimental data?. *Journal of the American Association for Laboratory Animal Science*, 46(1), 45-51.

¹² David, J. M., Chatziioannou, A. F., Taschereau, R., Wang, H., & Stout, D. B. (2013). The hidden cost of housing practices: using noninvasive imaging to quantify the metabolic demands of chronic cold stress of laboratory mice. *Comparative medicine*, 63(5), 386-391.

¹³ Castelhana-Carlos, M. J., & Baumans, V. (2009). The impact of light, noise, cage cleaning and in-house transport on welfare and stress of laboratory rats. *Laboratory animals*, 43(4), 311-327.

¹⁴ Balcombe, J. P. (2006). Laboratory environments and rodents' behavioural needs: a review. *Laboratory animals*, 40(3), 217-235.

¹⁵ Reinhardt, V., & Reinhardt, A. (2006). *Variables, refinement and environmental enrichment for rodents and rabbits kept in research institutions*. Animal Welfare Institute.

¹⁶ Gouveia, K., & Hurst, J. L. (2013). Reducing mouse anxiety during handling: effect of experience with handling tunnels. *PLoS one*, 8(6), e66401.

¹⁷ Balcombe, J. P., Barnard, N. D., & Sandusky, C. (2004). Laboratory routines cause animal stress. *Journal of the American Association for Laboratory Animal Science*, 43(6), 42-51.

¹⁸ Sorge, R. E., Martin, L. J., Isbester, K. A., Sotocinal, S. G., Rosen, S., Tuttle, A. H., ... & Leger, P. (2014). Olfactory exposure to males, including men, causes stress and related analgesia in rodents. *Nature methods*, 11(6), 629.

¹⁹ NRC [National Research Council]. (1996). Guide for the care and use of laboratory animals. 7th ed.

²⁰ Bogdanova, O. V., Kanekar, S., D'Anci, K. E., & Renshaw, P. F. (2013). Factors influencing behavior in the forced swim test. *Physiology & behavior*, 118, 227-239.

²¹ Bogdanova, O. V., Kanekar, S., D'Anci, K. E., & Renshaw, P. F. (2013). Factors influencing behavior in the forced swim test. *Physiology & behavior*, 118, 227-239.

²² Jha, P. K., Challet, E., & Kalsbeek, A. (2015). Circadian rhythms in glucose and lipid metabolism in nocturnal and diurnal mammals. *Molecular and cellular endocrinology*, 418, 74-88.

Circadian misalignment occurs, for example, in human shift workers and mice and rats in the laboratories who are interrupted or forced to perform tasks during day time. Short term circadian misalignment (three days and a half) is enough to reduce insulin sensitivity in skeletal muscle,²³ which can affect its energy utilization and subsequent performance. Further, insulin induces glycogen storage, an important fuel source for exercise, and circadian misalignment reduces this process. If insulin insensitivity progresses to insulin resistance and hyperinsulinemia, this would drastically affect metabolism in general and substrate utilization for both basic maintenance and exercise.

Species differences in muscle physiology

Even in the perfect situation where none of the limitations described above are present, mice and rat are hugely different from humans in terms of muscle physiology and should not be used. The performance of skeletal muscles is largely determined by muscle fiber types, which is designated by myosin heavy chain (MyHC) protein isoforms expressed within. Mice and rats are complete opposite of humans in terms of MyHC expressions.²⁴ Mice and rats' skeletal muscle is predominantly comprised of muscle fibers expressing MyHC IIB. In contrast, human skeletal muscle does not express this protein isoform but express MyHC I/β. (The overall MyHC isoform abundance in mice and rats is IIB > IIX > IIA > I/β, whereas in humans it is I/β > IIA > IIX.) Muscles expressing MyHC IIB tend to be larger fibers, contract faster, produce larger forces, rich in glycolytic enzymes and tend to run on anaerobic energy system, have low mitochondria and capillary density, and have low resistance to fatigue. Muscles expressing MyHC I/β are the complete opposite, are smaller, contract slower, produce lower forces, rich in mitochondria, capillary, and oxidative capacity, and hence run on aerobic energy system and have high resistance to fatigue.²⁵ (Elite runners have more/bigger muscles expressing MyHC I/β and this can be an adaptive and acquired characteristic.)

The protein synthesis rate is also different between type IIB and I muscle fibers. In response to food deprivation (such as recommended in the guideline), there is a greater decrease in protein synthesis in type IIB fibers compared to type I.²⁶ This is important because it translates to differential muscle function between mice/rats and humans under food deprivation.

Muscle glycogen, expressed relative to total body glycogen, is about ten times lower in mice compared to humans.²⁷ Both mice²⁸ and rats²⁹ have about five to ten times more liver glycogen than muscle

²³ Wefers, J., van Moorsel, D., Hansen, J., Connell, N. J., Havekes, B., Hoeks, J., ... & Boekschoten, M. V. (2018). Circadian misalignment induces fatty acid metabolism gene profiles and compromises insulin sensitivity in human skeletal muscle. *Proceedings of the National Academy of Sciences*, 201722295.

²⁴ Haizlip, K. M., Harrison, B. C., & Leinwand, L. A. (2015). Sex-based differences in skeletal muscle kinetics and fiber-type composition. *Physiology*, 30(1), 30-39.

²⁵ Zierath, J. R., & Hawley, J. A. (2004). Skeletal muscle fiber type: influence on contractile and metabolic properties. *PLoS biology*, 2(10), e348.

²⁶ Goodman, C. A., Kotecki, J. A., Jacobs, B. L., & Hornberger, T. A. (2012). Muscle fiber type-dependent differences in the regulation of protein synthesis. *PloS one*, 7(5), e37890.

²⁷ Kasuga, M., Ogawa, W., & Ohara, T. (2003). Tissue glycogen content and glucose intolerance. *The Journal of clinical investigation*, 111(9), 1282-1284.

²⁸ Pederson, B. A., Cope, C. R., Schroeder, J. M., Smith, M. W., Irimia, J. M., Thurberg, B. L., ... & Roach, P. J. (2005). Exercise capacity of mice genetically lacking muscle glycogen synthase: in mice, muscle glycogen is not essential for exercise. *Journal of Biological Chemistry*.

²⁹ Baldwin, K., Reitman, J. S., Terjung, R. L., Winder, W. W., & Holloszy, J. O. (1973). Substrate depletion in different types of muscle and in liver during prolonged running. *American Journal of Physiology-Legacy Content*, 225(5), 1045-1050.

glycogen, whereas humans have three to eight times more muscle glycogen than liver glycogen.³⁰ Even though it is well documented that adequate muscle glycogen is important to sustain exercise in humans, accumulating evidence shows that muscle glycogen is not even necessary for mice to perform demanding muscle activities. For example, genetically modified mice completely lacking muscle glycogen were able to run on treadmills until exhaustion just like normal mice.³¹ Genetically modified mice with over-accumulated muscle glycogen did not perform any better than normal mice either.³²

Species differences in other aspects of exercise physiology

Nitrate is converted to nitrite and further nitric oxide after ingestion which serves as a potent modulator that increases fatigue resistance, exercise efficiency, and exercise performance.³³ It does so through facilitating vasodilation, angiogenesis, mitochondrial respiration, mitochondrial biogenesis, glucose uptake, and calcium handling. Nitrate is abundant in foods such as leafy greens and beetroots, and one can increase body nitrate and nitrite through foods. In humans, about a quarter of the nitrate circulating in the bloodstream gets taken up by the salivary glands and then concentrated in saliva to about 20 times that in plasma.³⁴ It is then reduced to nitrite by the commensal bacteria in the oral cavity, which further increases the bioavailability of nitrite and nitric oxide. In mice and rats however, nitrate is not concentrated in saliva, and hence much higher doses of nitrate are needed to induce similar physiological effects in mice and rats. To further complicate the matter, mice and rats secrete endogenous nitrate in their upper gastrointestinal tract, but this doesn't happen in humans. These differences are major confounders for any animal study on health foods containing nitrate.

In addition to glycogen, blood fatty acids and blood sugar are also important fuel sources during exercise. However, mice and rats' metabolism of fatty acids and glucose differ from humans significantly. The hepatic enzymes delta-5 (D5D) and delta-6 desaturases (D6D) are important for metabolism of fatty acids. These enzymes introduce double bonds to fatty acid chains and alter their functions. The activity of D5D is inversely related to type 2 diabetes (T2D), and the activity of D6D is directly associated with it.³⁵ Rats have a much higher D5D activity than humans,³⁶ and it is known that rodent models of T2D do not recapitulate human T2D.³⁷ In terms of glucose regulation, significant species differences between mice/rats and humans exist at every level, from gene/protein expression, cellular signaling, tissue and organ, to whole organism level.³⁸ The authors concluded that: "Decades

³⁰ Ivy, J. L. (1999). Role of carbohydrate in physical activity. *Clinics in sports medicine*, 18(3), 469-484.

³¹ Pederson, B. A., Cope, C. R., Schroeder, J. M., Smith, M. W., Irimia, J. M., Thurberg, B. L., ... & Roach, P. J. (2005). Exercise capacity of mice genetically lacking muscle glycogen synthase: in mice, muscle glycogen is not essential for exercise. *Journal of Biological Chemistry*.

³² Pederson, B. A., Cope, C. R., Irimia, J. M., Schroeder, J. M., Thurberg, B. L., DePaoli-Roach, A. A., & Roach, P. J. (2005). Mice with elevated muscle glycogen stores do not have improved exercise performance. *Biochemical and biophysical research communications*, 331(2), 491-496.

³³ Jones, A. M. (2014). Dietary nitrate supplementation and exercise performance. *Sports medicine*, 44(1), 35-45.

³⁴ Montenegro, M. F., Sundqvist, M. L., Nihlén, C., Hezel, M., Carlström, M., Weitzberg, E., & Lundberg, J. O. (2016). Profound differences between humans and rodents in the ability to concentrate salivary nitrate: implications for translational research. *Redox biology*, 10, 206-210.

³⁵ Kröger, J., & Schulze, M. B. (2012). Recent insights into the relation of $\Delta 5$ desaturase and $\Delta 6$ desaturase activity to the development of type 2 diabetes. *Current opinion in lipidology*, 23(1), 4-10.

³⁶ Stone, K. J., Willis, A. L., Hart, M., Kirtland, S. J., Kernoff, P. B. A., & McNicol, G. P. (1979). The metabolism of dihomo- γ -linolenic acid in man. *Lipids*, 14(2), 174-180.

³⁷ Chandrasekera, P. C., & Pippin, J. J. (2014). Of rodents and men: species-specific glucose regulation and type 2 diabetes research. *ALTEX-Alternatives to animal experimentation*, 31(2), 157-176.

³⁸ Chandrasekera, P. C., & Pippin, J. J. (2014). Of rodents and men: species-specific glucose regulation and type 2 diabetes research. *ALTEX-Alternatives to animal experimentation*, 31(2), 157-176.

of research have made it clear why *a priori* application of rodent data to humans is inappropriate and why human-based data must go from being anecdotal to systematic frontline evidence.”

*The limitations specific to the TRT are similar to the FST, including stress, circadian rhythm, and species differences in muscle and exercise physiology, as described above.

A Note on the 3R principles

The 3R principles have a hierarchy. Replacement is the most important, followed by reduction, and last refinement. When we follow the 3R principles, we need to focus on replacement first. Considering that human tests are available and accepted by the guideline, it is clear that non-animal alternatives are available and hence the replacement principle should be followed. Allowing animal tests in this case is in violation of the 3R principles and the Taiwan Animal Protection Act, which clearly states that “[o]ne shall avoid using live animals for scientific application.”³⁹

Conclusion

Animal tests are not fit for purpose to substantiate human health claims and should not be recommended or accepted by the guideline. Further, only human tests can be used to substantiate human health claims. The regulatory agencies in the United States, Canada, European Union, and others do not require or recommend animal tests to substantiate human health claims for foods. They don’t accept animal test results as standalone evidence either.⁴⁰ Given these facts, along with the scientific limitation and cruelty associated with these animal tests, we respectfully urge TFDA to remove the requirements, suggestions, and acceptance of animal tests from the guideline.

Sincerely yours,



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<http://law.moj.gov.tw/Eng/LawClass/LawParaDeatil.aspx?Pcode=M0060027&LCNOS=%20%2015%20%20%20&LCC=2>

⁴⁰ Please see “PETA to Taiwan FDA food health claim animal testing” sent to TFDA on April 18, 2018 for details.