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Balance of the Sexes: Addressing Sex Differences in Preclinical Research

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Preclinical research is fundamental for the advancement of biomedical sciences and enhancing healthcare. Considering sex differences in all studies throughout the entire biomedical research pipeline is necessary to adequately inform clinical research and improve health outcomes. However, there is a paucity of information to date on sex differences in preclinical work. As of 2009, most (about 80 percent) rodent studies across 10 fields of biology were still conducted with only male animals. In 2016, the National Institutes of Health implemented a policy aimed to address this concern by requiring the consideration of sex as a biological variable in preclinical research grant applications. This perspective piece aims to (1) provide a brief history of female inclusion in biomedical research, (2) describe the importance of studying sex differences, (3) explain possible reasons for opposition of female inclusion, and (4) present potential additional solutions to reduce sex bias in preclinical research.

INTRODUCTION

Preclinical research is invaluable for the advancement of the biomedical field, especially for directly informing clinical research. Much of our understanding of the mechanisms of disease and treatment processes begin at the preclinical level. For example, animal models are pivotal for the understanding of the pharmacological processes involved in testing and predicting treatment effects that are difficult to assess in humans due to safety and ethical issues. Studies have shown that men and women experience diseases differently and benefit from treatment differently [1-4]. However, preclinical biomedical research remains heavily reliant on male cells and animals [5,6]. A recent policy by the National Institute of Health (NIH†) aimed to address this concern by mandating the consideration of using female cells and animals in preclinical research. This perspective discusses the importance of considering sex as a biological variable in preclinical research and proposes additional solutions to reduce sex bias in preclinical biomedical research.

While the need for female inclusion in preclinical research has become more important, the progress for inclusion has in some ways remained stagnant. In 2009, a study estimated that most rodent studies (about 80 percent) across 10 fields of biology were still using only male animals [6]. This statistic is similar to that observed in 1990 attesting that, historically, this sex bias did not change over a 20-year period [7-9]. Further, women continue to experience adverse drug effects which might have been prevented by representation in preclinical research as well as in early-phase drug clinical trials [10,11]. Some biomedical fields *have* begun to include the examination of sex differences in preclinical research, such as neuroscience, pain/analgesia research and cardiovascular health investigations [12-17]. This perspective piece will primarily provide examples from the field of neuroscience, due to the authors' expertise.

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†Abbreviations: NIH, National Institute of Health; FDA, Food and Drug Administration; APA, American Psychological Association; ORWH, Office of Research on Women's Health; GAO, General Accountability Office; NSF, National Science Foundation; IRB, Institutional Review Board; IACUC, Institutional Animal Care and Use Committee.

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Recognizing that sex and gender are terms often used interchangeably, the differences described in the current article are specific to either sex or gender as defined by the American Psychological Association (APA). Sex is defined as a person's biological status (male or female) and gender as the attitudes, feelings, or behaviors associated with a person's biological sex (boy/man or girl/woman) [18].

Important Milestones

Several drugs have shown the need for modification of federal policies related to preclinical testing such as in the case of thalidomide. Thalidomide is a drug developed in the 1950s to treat nausea, or "morning sickness," in pregnant women that led to the death of approximately 2,000 children and to serious birth defects in 10,000 children. Evidence found in the 1980s showed that *in vitro* testing on female human tissue could have predicted the danger that thalidomide posed [19,20]. This noteworthy example demonstrates the importance of studying females in preclinical research.

In 1986, the NIH promoted the inclusion of women in NIH-supported biomedical and behavioral clinical research and subsequently, in 1990, established the Office of Research on Women's Health (ORWH) to increase the representation of females in research. Three years later, the 1993 NIH Revitalization Act mandated the inclusion of women in clinical research. The United States Food and Drug Administration (FDA), one month after the NIH Revitalization Act was passed, removed its prohibition on women of childbearing potential participating in clinical trials. While this guideline did allow for female participation, it did not impose mandates on the involvement of women in clinical research [21]. The prior FDA ban on female inclusion likely contributed to the current paucity of female participation in preclinical and clinical trials despite the most recent NIH efforts to include women. While marginal efforts were being made to include females in clinical research, efforts in preclinical research remained very slow.

Finally, in May 2014, the NIH announced that it would begin to rollout a plan for the consideration of the use of female animals, tissues, cells, and cell lines in preclinical research [22]. This policy took effect on January 25th, 2016, such that grant applications submitted on this date and thereafter required the consideration of female inclusion. This NIH initiative now requires researchers "to report their plans for the balance of male and female cells and animals in preclinical studies in all future applications, unless sex-specific inclusion is unwarranted, based on rigorously defined exceptions" [11]. This marked the first time in 20 years that the NIH intervened forcefully on the matter of sex bias in research. In 2009, the Canada Institute on Health Research implemented a similar policy requiring researchers to explain their choice for inclusion or exclusion of both sexes in their research [23]. In 2012, in addition to authorizing a similar policy, the European Commission funded nine national agencies across Europe to enhance gender/sex equality in research and mandated the report of gender/sex-based analyses in scientific communications and publications [24].

Sex Bias in Preclinical Research

Preclinical research studies have generally either (1) failed to report the sex of experimental cells/animals, (2) excluded female cells/animals entirely, (3) included an insufficient number of female cells/animals, or (4) excluded sex as a biological variable in their analyses when both sexes were included [25]. One review study examining surgical research reported that 22 percent of animal studies did not report the sex of the animals. Of the papers that did specify sex, 80 percent studied only males, 17 percent studied only females and 3 percent studied both sexes. Only 1 percent of the studies reported sex-based results, including reports of not finding any sex-related differences [25]. Reporting findings of no sex-related differences is just as important as reporting sex-related differences. Nonetheless, our current body of knowledge on sex-related differences is obscured by a lack of inclusion and reporting in publications [26]. This exclusion not only risks harming individuals but also hinders the opportunity to discover critical information for improving healthcare.

In 2001, the General Accountability Office (GAO) reported that eight out of ten drugs that were removed from the market by the FDA posed a greater health risk to females than males [27]. A prominent example relating to the neglect of females in preclinical and clinical research involves the sleeping aid zolpidem (Ambien™), initially marketed in 1992. Millions of women were prescribed doses of zolpidem within the dosing guidelines and many reported "morning-after" effects including impaired alertness during driving [13]. However, it took 21 years for the FDA guidelines regarding zolpidem to reflect decreased dosing strategies for women. In 2013, the FDA changed the dosage guidelines for females because similar doses as those prescribed for men posed greater health risks in women [28], due to sex-related differences in clearance rates [29]. If the pharmacodynamics of zolpidem had been tested in male and female animals prior to entering clinical trials, sex-related differences in behavioral effects may have been identified sooner [29,30], and multiple adverse drug reactions in women may have been avoided.

Another example in which sex bias in preclinical research may have increased the risk of undesirable outcomes in clinical research is in the pharmacological testing of the libido-enhancing medication flibanserin (Addyi™), for women [31]. In preclinical investigations of flibanserin's effects on serotonin receptors, behavior, and cognition, almost all animals were male despite the drug being developed for use in women [32,33]. This sex bias carried over into pharmacokinetic testing of flibanserin in which the participant breakdown included 28 male and 10 female healthy volunteers [34]. Moreover, to investigate the potential interaction of flibanserin and alcohol with re-

spect to increasing the potential risk of fainting in Phase I clinical trials, 25 participants underwent administration of both flibanserin and alcohol at varying doses [35]. Of those 25 participants, *only two* were female [36]. In the study of such a sex-specific therapy, the selection of animal and human participants should have been more carefully chosen to adequately represent the target population.

Opposition to Female Inclusion

Researchers who have opposed worldwide efforts to examine sex-related differences have argued that the implementation of female inclusion is very complicated and should be addressed with caution. In particular, some researchers have cited complexities with respect to accounting for menstrual cycle phases or fluctuating sex steroid hormones [37]. While the complexity of any scientific problem is not a valid reason for the lack of its examination, other arguments include the practicalities of mandating sex difference examination in all areas of biomedical research. These arguments claim that cell and animal models do not adequately reflect sex-related differences in humans. While considering multiple social factors relating to sex differences may be complicated in preclinical research, cells and animals still serve to illuminate biological aspects of human disease [38], as they provide a valid approximation of human biological phenomena [39].

Additional arguments include unnecessary duplication of data resulting in a decrease in progress/productivity, and an increase in time, money, and space used [7,23,37,40]. Some researchers have even described the sex-related differences initiative as burdensome or as an obstruction of innovation [37]. Klein, however, proposed a counter-argument stating that the examination of sex-related differences in the preclinical phase will actually save time and money in the clinical trial phase [40]. Further, preclinical examination of sex differences could decrease medical costs of treating those with adverse effects to a drug. By examining sex differences early in the preclinical trial phases, burdens related to conducting more costly clinical trials and treatment of those with adverse drug effects may be reduced.

While the practicalities of the equal inclusion of female animals in preclinical work may consume more resources [37], the use of female cell cultures may pose different complications. Every cell has a sex, and its cellular function is influenced by its sex and genome [38]. However, it may be challenging to trace cell lines appropriately to identify their sex due to chromosomal mutations and intra-species contamination [7,37,41]. Sex-related differences exist at many levels and are often ignored as major disease modifiers in patients [42]. In the case of some non-primary cell lines, it may be justified to not consider or report sex in publications. However, primary cell lines *do* have an identifiable sex, and the sex should, therefore, be reported.

Potential Impact on Public Health

In the era of genomics and personalized medicine, studying sex-related differences at the preclinical level is crucial for determining the effectiveness of treatment [38]. The current overreliance on male cells and animals in preclinical research has led to several instances in which adverse drug reactions were initially underappreciated in human females, "including life-altering, disfiguring surgical complications, birth defects in babies and onset of chronic disease" [27]. The reliance on male cells and animals in preclinical research generates an environment vulnerable to missing important observations at early stages of experimentation that relate to the health of girls and women. Studying sex-related differences is essential for disentangling various mechanisms underlying sex-related differences in human diseases and promoting the health of both males and females.

For example, sex-related differences in preclinical research may have implications for public health outcomes related to tobacco smoking. Males tend to smoke for the reinforcing effects of nicotine, whereas females tend to smoke to regulate stress and mood [43,44]. In general, females also have a harder time quitting than males [45]. Nicotine replacement therapy, the first line of treatment for tobacco smoking, is more effective in males compared to females [44]. Unfortunately, preclinical studies have not identified the neurobiological bases for these sex-specific differences in treatment. However, by measuring smoking-induced dopamine release in the brain, Cosgrove and colleagues found that the dopaminergic response to the cigarette was sexually dimorphic [46]. These differences in dopamine release patterns could help us identify important biomarkers for more personalized smoking-cessation medications.

Assuming that the same regulatory pathways involved in diseases and disorders operate similarly in both sexes occurs too often in the scientific literature. Cell and animal studies can uncover differences and inform clinical trials by identifying new treatment target possibilities in females. By accounting for the entire population, as opposed to just half, we can advance scientific understanding of sex-related differences, decrease the frequencies of adverse drug reactions in females, and potentially improve the lives of millions of people.

Additional Solutions

In order to address sex biases in preclinical research and publications, the United States should consider implementing additional federal policies. Other major research institutes, such as the National Science Foundation (NSF), could follow NIH's efforts to balance the sexes by implementing similar policies requiring researchers to address potential sex differences. Furthermore, journals could require the analysis and reporting of sex differences in publications, following the European model. Sex should always be included as a biological variable in analyses and the findings should be reported regardless of whether or

not a sex difference was found. This will increase awareness of the sex bias concern in preclinical research and begin to address the problem.

Information on the sex of cells and subjects is often neglected in publications, particularly in preclinical research studies. One way to mandate the inclusion of cell and animal sex identification and sex analyses in publications is through author guidelines provided by world-leading publishing companies such as Elsevier and Nature. Just as all journals require authors to report Institutional Review Board (IRB) or Institutional Animal Care and Use Committee (IACUC) approval of research and/or the demographic breakdown of the experimental participants, they should also require authors to report subjects' sex. Further, sex should be considered a biological variable of interest similar to other demographic features such as age, race/ethnicity, and years of education. Reporting no sex-related difference is just as important as reporting one.

While several journals have dedicated their focus to sex/gender differences such as *Gender Medicine* and *The Biology of Sex Differences*, the study of sex differences should be integrated into all studies of biomedical research instead of being treated as its own field of study. This will lead to a drastic expansion of the sex differences literature in the biomedical field. One potential problem that may arise from the booming of sex differences research is an exaggeration, misrepresentation, or misinterpretation of findings [30]. Thus, publications could report not only whether or not the sexes differ but also, by how much they differ, using accurate statistics [47].

Another possible way to ameliorate sex bias in preclinical research is local monitoring of sex differences research at each institution through the IACUC. This will assure that researchers plan to examine sex differences in their protocols. It will also assure that sex differences are being addressed in currently funded projects.

CONCLUSIONS

Among the science community, there is an increasing awareness of the importance of examining sex differences [30]. However, females are often not considered throughout the entire research pipeline. This makes it impossible to determine if the health outcomes we observe in male cells and animals also apply to female cells and animals. By requiring equal representation of sexes in grant applications, the NIH has made a step towards decreasing sex bias in biomedical research. However, the applications of sex differences research in all projects can be difficult and should be approached with appropriate rigor to ensure quality, originality, and productivity are not compromised.

Efforts through other national funding agencies, publishing companies, and educators may enhance our body of knowledge on sex differences in preclinical research. Requiring grant applications to address sex differences is not enough to advance sex differences forward into publications, especially in preclinical work. Journals should begin mandating the report of sex in author guidelines. It should be mandatory to take sex into account, just as it is to account for effects of age, race, and animal strain. Researchers should always consider sex as a fundamental factor of biological influence.

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