OFFICE OF RESEARCH ON WOMEN'S HEALTH

Methods and Techniques for Integrating the Biological Variable Sex into Preclinical Research

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BACKGROUND

Biological sex, being female or male, is a fundamental characteristic of biological systems. Sex is a fundamental variable in preclinical biomedical research that underlies drug development research, clinical trials, and prevention approaches. Although the biomedical community has made major progress in human studies—women now account for roughly half of the participants in NIH-funded clinical trials—there has not been a similar pattern in preclinical research. Animal studies have focused primarily on males, and investigators studying cell models have often ignored the sex of the individual from which the cells were obtained. For the most part, considering sex as a biological variable has been a blind spot in biomedical research, leaving critical gaps in our knowledge.

In May 2014, National Institutes of Health (NIH) Director Francis Collins and NIH Office of Research on Women's Health (ORWH) Director Janine A. Clayton announced that NIH will ensure that NIH-funded preclinical research considers females and males.[1] This policy promotes a balanced approach that can be achieved by studying both sexes in NIH-funded preclinical research.

Held on October 20, 2014, the workshop "Methods and Techniques for Integrating the Biological Variable Sex into Preclinical Research" explored how to achieve this goal. View workshop presentations and slides.

WELCOMING REMARKS

Janine A. Clayton, M.D., NIH Associate Director for Research on Women's Health; Director, ORWH

ORWH is nearing its 25th anniversary (in 2015), and the office has made much progress improving women's health in an array of areas. This year has been a banner year for progress, notably the publication in *Nature* stating that NIH will ensure that NIH-funded preclinical research considers females and males.[1] As NIH develops this policy, the agency is listening carefully to stakeholders in academia, societies, at NIH, and elsewhere. The endgame is changing a paradigm toward routine consideration of sex across the research continuum, and those changes will take time. Studying both sexes is not just a women's health issue; it is a call to action for the most rigorous science possible. As stewards of taxpayer money, we can promise nothing less.

The workshop had a dual focus: (1) sharing research results and lessons learned and (2) promoting lively discussion on the importance of considering sex as a basic biological variable. The workshop consisted of four scientific sessions:

- Session I addressed the basic concept of including female and male subjects in studies.
- Session II explored the impact of including or not including sex as a basic biological variable.
- Session III visited specific and practical methods to integrate sex as a variable in research plans and projects.
- Session IV discussed how to introduce a "sex matters" culture across multiple disciplines.

THE IMPORTANCE OF REPRODUCIBILITY IN BIOMEDICAL RESEARCH

Lawrence A. Tabak, D.D.S., Ph.D., NIH Principal Deputy Director

Biomedical research that is rigorous and reproducible must involve approaches that are free of bias. In January 2014, Dr. Collins and NIH Deputy Director Lawrence Tabak published a comment in the journal *Nature* in which they listed crucial design elements that often have been ignored in biomedical research.[2] These elements include blinding, randomization, replication, adequate sample size, and the effect of sex differences.

NIH leadership views the importance of addressing biological sex as a variable as part of the larger NIH effort to ensure rigor and reproducibility in research. Thus, in May 2014, Dr. Collins and Dr. Clayton published another comment, also in *Nature,* announcing that NIH will ensure that NIH-funded preclinical research considers females and males.

Much of the biomedical research canon has been predicated upon a default model subject that is male. In the laboratory, this means male animals, and in the clinic, it refers to the "70 kilogram male." For some time, the results of these mostly single-sex investigations have been used to draw conclusions about both females and males. NIH is addressing this imbalance by developing policies that ensure that sex is considered throughout the continuum of research, from basic to clinical. These actions are based on NIH's obligation to produce the most rigorous science that drives medical advances. However, NIH recognizes that the agency cannot do this alone and is working on multiple fronts with stakeholders in the public and private sectors.

ORWH has developed an S4 initiative (Studying Sex to Strengthen Science) to help investigators who are interested in taking the first step toward considering sex as a biological variable. Several online resources are available, including online courses, a reading room of key publications, profiles of researchers who study female and male biology in preclinical research, upcoming meetings and workshops, and other resources. In addition, ORWH for some time has invested resources in a range of approaches to encourage the study of both sexes. For example, for more than a decade, ORWH has supported the Specialized Centers of Research on Sex Differences (SCOR), which are distributed across the country and are funded in collaboration with the Food and Drug Administration (FDA) and several NIH Institutes and Centers. Each SCOR has an interdisciplinary approach to examining sex and gender influences on a health condition that affects women in particular. Each center straddles projects ranging from basic to clinical, and scientific core facilities are shared.

ORWH also funds administrative supplements that apply a sex or gender lens to existing NIH-funded research projects. Many research projects have been enriched by this funding mechanism. Investigators are using the funds for a variety of purposes, such as to add a second group of animals of the opposite sex to their studies for comparison purposes. Finally, the Science of Sex and Gender in Human Health online course series is rich in content and provides continuing education credits for physicians, pharmacists, nurses, and other allied health professionals. There are some good examples of NIH-funded projects that have considered sex as a biological variable and those that collect and report sex-based data. These include the NIH Common Fund–supported Knockout Mouse Project and the National Institute on Aging's Interventions Testing Program.

Good experimental design and reporting underlies scientific rigor and reproducibility. Tracing back to publications from the mid-1900s, elements of good experimental design include minimizing bias, having precision and general applicability, being straightforward, and having the ability to assess uncertainty appropriately. Research with animals must minimize bias and have adequate sample size based on the research question of interest. Comprehensive reviews of the relevant preclinical literature promote proper use of model systems to further understanding of human health.

One approach that may be especially useful for the consideration of sex as a second independent variable is factorial design, which enables the consideration of sex and another variable without necessitating a doubling in the number of experimental animals. A 2005 experimental stroke study found opposite treatment effects in female and male mice, which is a difference that would not have been detected if the animals had been studied in aggregate. How many more examples like this remain undiscovered?

As a starting point for creating routine consideration of sex as a fundamental variable, investigators need to discuss issues related to the importance and practical details of studying females and males in preclinical research. NIH is convening experts to hear a range of views and to develop scientifically justifiable approaches to accomplish this research. Publications that bring the issue to light[3,4] and workshops such as this one will enrich the conversation.

KEYNOTE PRESENTATIONS

KEYNOTE: Sex Influences on Body and Brain: An Idea Whose Time Has Come

Larry Cahill, Ph.D., University of California, Irvine

The biomedical literature documents significant sex bias in animal and cell research.[5,6] Resistance to studying sex influences is likely to be prompted by several reasons, particularly that females are "too variable and complex" and "the estrous cycle complicates research." A recent meta-analysis, however, examined variability among female and male rodents and concluded that the estrous cycle is not a compelling reason to exclude females.[7]

Some scientists believe that sex differences are not fundamental and that *fundamental* mechanisms should be studied first. A definition of fundamental that implies "shared among all humans" may be flawed but has deep roots in medical history, beginning with the concepts of 16th century English physician William Harvey. A more precise modern definition of fundamental, in the context of living things, might be that females and males are fundamentally similar *and* different. Evolution has borne this out through natural and sexual selection.

Sex influences in biomedicine are ubiquitous and appear throughout body organs and tissues. For example, sex factors in the brain help determine activity and other behaviors, and basic structural features and connectivity differ between the sexes. Molecular mechanisms leading to ischemic cell death are different in females and males.[8] The ApoE-4 allele has been associated with an increased risk of Alzheimer's disease, but recent studies demonstrate that this allelic variant only confers increased risk for females.[9]

Sex influences are clinically meaningful. For example, research has shown sex differences in psychological stress responses such as those in post-traumatic stress disorder. However, even though these studies noted a sex-based difference, publicity related to the findings of the research did not focus on different results in women and men. Drug design and testing are another example. lazaroids were a class of experimental drugs that were tested for use in treating stroke. Data revealed that lazaroids prevented brain damage in men who suffered strokes, but not in women, and the drugs were not approved. This scenario showcases the importance of studying both sexes to understand benefits and harms for both women and men.

KEYNOTE: Gendered Innovations: Analyzing Gender in Preclinical Research

Londa Schiebinger, Ph.D., M.A., Stanford University

Doing research wrong costs lives and money. A 2001 Government Accountability Office report noted, for example, that of 10 drugs withdrawn from the U.S. market because of life-threatening health effects, 8 of those drugs showed greater severity in women.[10] Doing research right, by contrast, has the potential to save lives and money: Every dollar spent on the Women's Health Initiative estrogen plus progestin clinical trial study returned $140 and led to measurable health improvements, including lives saved.[11] These examples demonstrate the importance of considering sex as a variable in research.

A predominant concern among NIH stakeholders is that analyzing sex as a variable will cost more money. Might this question be better addressed nationally instead of in the context of individual research laboratories? In general, some costs might increase, and others might decrease. But even when costs increase, these expenditures should be weighed against additional benefits derived from new insights, improved therapies, and reduced risk of subsequent problems. Many research costs, such as those that cover basic laboratory operations, do not vary with the sex of included animals or cells. Some costs may be expected to decline. For instance, housing female animals may be less expensive than housing male animals.

Adopting a strategy of using sets of animals or cells that are half female and half male instead of all of one sex allows detection of at least some sex influences, namely the largest ones, with no impact on sample size.[12] One helpful online resource for studying both sexes is Gendered Innovations in Science, Health and Medicine, Engineering, and Environment.[13] This project develops new methods of sex and gender analyses and provides case studies to illustrate how these approaches lead to innovative science and technology.

Nomenclature is important to the study of female and male biology. While *sex* refers to biological qualities, *gender* refers to a constellation of sociocultural processes that interact with, and thus influence, biological processes. The precise use of these technical terms will enhance research and enable meta-analyses of published and archived data. Funding agencies and editors of peer-reviewed journals can assist by requiring correct usage of these terms. Some useful definitions, the first two applied to animals only, include:

- Gender norms: researchers' differing attitudes toward and handling of female or male animals. Researchers may act on stereotypes concerning expected female and male behaviors that influence the results of their studies.
- Gender relations: interactions between female and male animals or between female or male researchers and female or male animals. One example is the "observer effect" in pain research, in which rodent stress responses differ when the research is conducted by a woman or a man interacting with either a female or a male animal.[14]
- Gender identities: how humans perceive and present themselves—whether masculine, feminine, straight, or transgender—and how they are perceived by others. This most likely does not apply to rodents.

SCIENTIFIC SESSION I:
THE CONCEPT OF INCLUDING MALE AND FEMALE SUBJECTS IN STUDIES

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Moderator: Cheryl Marks, Ph.D., National Cancer Institute

This session highlighted the importance of including male and female animals and cells in preclinical experiments and identified approaches to performing such studies. The discussions also addressed experimental design, in particular, how to account for variability.

Sex Differences in Preclinical Research

Jill Becker, Ph.D., University of Michigan

When talking about sex differences, it is important to remember that "biology is not destiny." A common fallacy is that brain-based differences alone cause behavior. It is thus important for researchers who study and identify sex differences to use caution in how they describe and explain the results to nonscientists. The picture is complicated; females and males may exhibit sex differences because they secrete different hormones and because females and males have different brains and bodies due to developmental influences.

Differences between females and males may be manifest, and thus be measured, in a number of ways. Qualitative differences include behavioral responses, but the magnitude of such responses can be measured to provide quantitative information. In rodents, one example of a sex-specific behavior is mounting during sex; this behavior may be studied in one sex, since it is more common in males. An example of a distribution of incidence of a given behavior is response to a cue, such as cocaine pellets. More female rats than male rats prefer cocaine pellets to food pellets. In this case, an example of a quantitative difference is the differential response to cocaine access: Females in isolation will work harder for cocaine pellets than will males. Females that are housed with another rat work at about the same rate as do males.

Combinations of qualitative and quantitative differences may also occur, and it is possible that multiple mechanisms can mediate a similar behavioral (or other) outcome. Brain mechanisms for certain amphetamine-induced behaviors are sex specific in rats. The bottom line for advancing research is that if an investigator finds sex differences in a biological mechanism, there is an opportunity for further research in female and male animals to learn more.

Sex in the Dish: Exploring Cellular and Molecular Approaches

Patricia Hurn, Ph.D., University of Texas System

Stroke is a sexually dimorphic disease. Men have more strokes than women when they are young, whereas women have more strokes than men when they are older (older than 85). One well-known animal model of stroke is rodent neuronal cell culture in which oxygen and glucose deprivation, and the subsequent cell death, mimic human physiological insults to brain cells.

Studies have revealed that female and male cells respond differently to oxygen and glucose deprivation and that the effects are mediated at least in part by androgens.[15] Male cells are more vulnerable than female cells. Such experiments rely on accurate sex determination of very young rodents and of cells, which can be typed by PCR analysis to identify the male-specific *SRY* gene.

The scientific precedent for sex specificity in molecular mechanisms of injury calls for investigators to address this issue routinely. Results may have implications for translation, including the development of pharmacological interventions.

Enhancement of Research Findings: The Significance of Well-Designed Experiments

Wei-Jung Chen, Ph.D., Texas A&M Health Science Center

In the past few years, articles have appeared in the popular press questioning the scientific method and the reproducibility and reliability of results in biomedicine. Although these conclusions do not imply willful scientific misconduct, several factors do appear to play a role in perpetuating irreproducibility. These include poor training in experimental design, the lack of disclosure of the basic elements of the experimental methods, and the policies and attitudes of funding agencies, institutions, and publishers.[16] Faculty, mentors, and researchers can help improve experimental design through better education and training.

How can an investigator include sex in his or her experimental design? Doing so requires that sex is treated as an independent variable. Even if sex is not a specific area of interest to an investigator, both female and male subjects should be included to avoid generalization bias.

In addition to proper design, using the appropriate method for data analysis is critical because the wrong approach can alter results. If sex is the only variable in an experimental design, an investigator can perform simple t-tests to assess statistical significance. If sex is one of multiple independent variables, the experimental design may need to be carefully considered.

Factorial design is an approach that can reduce variability by increasing sample sizes. In an experiment with treatment and control groups, as well as females and males, an investigator using a factorial design enables the assessment of interactions among the independent variables of treatment and sex. For example, in a 2 x 3 design, it is possible to use a two-way ANOVA statistical test to interrogate interactions between the two variables of sex and dose. These design features maximize the outcome by controlling variance associated with each independent variable alone and strengthening the outcome from the analysis.

Defining an adequate sample size for an experiment is a difficult concept to address. It relies on consideration of (1) power, or the *real* effect of an independent variable, generally recommended to be 0.8, and (2) effect size, which can be established through preliminary studies and a literature survey of previous work. However, it is important to distinguish between effect size of the phenomenon that is being studied and effect size of sex. An investigator might observe a small effect of his or her experimental manipulation on the specified endpoint but a big effect of sex, or the converse.

One study that analyzed publications in the field of neuroscience, Power Failure: Why Small Sample Size Undermines the Reliability of Neuroscience, identified many studies that were underpowered, and thus, unlikely to present reliable conclusions. Often, increasing the statistical power of a study can be accomplished by controlling for extraneous variability through application of the appropriate experimental design and the accurate application of statistical methods.

SCIENTIFIC SESSION II: WHAT IS THE IMPACT OF INCLUDING OR NOT INCLUDING SEX AS A BASIC BIOLOGICAL VARIABLE?

SCIENTIFIC SESSION II: What is the Impact of Including or not Including Sex as a Basic Biological Variable?

Moderator: Teresa Woodruff, Ph.D., Director, Women's Health Research Institute, Northwestern University

This session explored the impact of incorporating both sexes in studies of disease mechanisms and therapeutics. Presentations also addressed what may be missing when only a single sex is used in preclinical discovery research.

Misunderstandings and Lost Opportunity Are the Cost of Not Including Both Sexes in Preclinical Research

Margaret M. McCarthy, Ph.D., University of Maryland School of Medicine

Scientists discovered sex differences in the brain in 1959.[17] For many years, an assumption held that the sex differences were restricted to just a few brain areas, were relevant to reproduction, and were related to hormones only. Further study in this area over time has revealed a very different picture: Sex differences in the brain are pervasive and are related to hormonal and nonhormonal processes.

Sex differences characterize brain development throughout life, which includes the processes of synaptogenesis and synaptic pruning that occur around birth. Research investigating female and male biology has revealed that striking differences exist in these processes throughout the brain.

> "Sex is like dose, time, and temperature, and so it should be included as a variable in studies."
>
> – Dr. Margaret McCarthy

The prevailing view of synaptogenesis was that physical contact with axons recruited glutamate receptors. While operational in some brain areas, research has shown that synaptic formation in the preoptic area works through a different mechanism, in which α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, a glutamate analog) receptors are critical instead. Further study of the preoptic area has demonstrated that both immune and inflammatory mediators (including immune cell trafficking genes) differ in healthy female and male brains in this brain region.

Microglia are nonneuronal cells; they are the primary immunocompetent cells of the brain. Microglia are derived from macrophages, and they produce and respond to prostaglandins. Not only do microglia mediate synaptic pruning, they participate directly in synapse formation in the preoptic area. Males possess more activated microglial cells than do females.[18]

Beyond the preoptic area, additional brain regions show various sex-specific differences. Newborn females have more new neurons and astrocytes in the amygdala than do same-age males, whereas newborn males

make twice as many new hippocampal neurons as do females. Neurogenesis exhibits sex and age differences. Endocannabinoids are ubiquitous membrane-derived signaling molecules that have been implicated in neural plasticity. Newborn males have a higher endocannabinoid tone in the amygdala. Studies show that sex differences in neurogenesis are secondary to endocannabinoid-induced microglial phagocytosis. Two other examples are hormonally driven differences in two hypothalamic regions: the sexually dimorphic nucleus and the anteroventral periventricular nucleus. These processes are mediated by differential cell death as opposed to cell genesis.

Understanding sex differences benefits both sexes. Research continues to show that disorders with microglia or immune involvement, disorders with sex biases in prevalence or severity, and disorders with developmental origins affect a range of human conditions. Some of these conditions include autism, learning disabilities, Tourette's syndrome, bipolar disorder, and cerebral palsy.

Impact of Not Including Sex as a Variable in Your Research

Farida Sohrabji, Ph.D., Texas A&M Health Science Center

Perils of not including sex as a biological variable include incorrect interpretations, missed opportunities, and failure to recognize different responses to therapy.

Stroke provides an example of a condition that demands a sex-based study approach. The condition presents differently in women and men, with difference in prevalence beginning around menopause. Stroke outcomes are also different in women and men; women experience increased stroke severity as well as increased morbidity and disability post-stroke than do men. Developing a therapeutic strategy for stroke in aging human populations calls for investigators to study the mechanisms underlying age and sex differences in stroke severity. Young females have the best outcomes after stroke, raising questions about what protective factors they may harbor. A seemingly obvious role for estrogen belies the complexity of this hormone and its sometimes unexpected effects in older populations.

Although experimental models of stroke are useful, it is important to note that animal models can only measure severity, not risk. Thus, a clinically relevant model employs middle-aged rats that are closer to the demographic of older women who are at risk of stroke. Studies show that in these female rats, estrogen was not protective; rather, it was toxic. Another unexpected finding related to an apparent inverse correlation between insulin-like growth factor 1 (IGF-1) levels and stroke severity. IGF-1 administered to middle-aged female rats after ischemia appeared to be neuroprotective. But those data did not include males, and further experiments clarified that IGF-1 availability, rather than overall levels of IGF-1, was the pertinent criterion for a worse disease outcome.[19] Looking deeper at the effect of post-stroke IGF-1 treatment, microRNA profiling uncovered the contribution of PI3K-Akt signaling, cell adhesion/extracellular matrix receptor pathways, and T-cell and B-cell signaling.[20] Collectively, the results point to critical targets of IGF-1 in the aging brain.

It is realistic to recognize that sex differences research can be expensive and time-consuming, but it must be regarded as good science. In general, females are not more difficult to work with in a laboratory setting than are males, but variables must be taken into account, including the estrous cycle. Aside from the relevance of studying both sexes in biomedical research for generalizability and applicability, these investigations can also serve as a means to understand mechanisms, develop therapeutic targets, and exclude competing hypotheses.

SCIENTIFIC SESSION III: PRACTICAL METHODS TO INTEGRATE THE BIOLOGICAL VARIABLE SEX INTO RESEARCH PROJECTS

SCIENTIFIC SESSION III: PRACTICAL METHODS TO INTEGRATE THE BIOLOGICAL VARIABLE SEX INTO RESEARCH PROJECTS

Moderator: Cora Lee Wetherington, Ph.D., National Institute on Drug Abuse

This session visited techniques and methods from various research fields that will aid researchers in incorporating both sexes in research. Presentations and discussions also explored how to translate results into tangible and beneficial outcomes.

Toxicology Testing Guidelines Used in Preclinical Research

Susan Makris, Ph.D., National Center for Environmental Assessment, Environmental Protection Agency (EPA)

Testing guidelines, such as those issued by FDA and EPA, are standardized protocols developed through a rigorous process that includes public comment and peer review. Although there are different categories of guidelines, all are typically conducted in accordance with good laboratory practice (GLP) regulations. These GLP requirements include specifications on different aspects of study conduct (as relevant to organization and personnel), facilities, equipment, animal care, standard operating procedures, and other issues. One important note is that GLP specifications may lend confidence in study conduct, but they do not address study design appropriateness or adequacy.

Testing guidelines also address roles and responsibilities of study leadership and of various units such as quality assurance, data documentation, archiving, recording, and reporting. Other requirements relate to toxicology testing, data call-in, test rules, published recommendations or guidance, and negotiated agreements. Toxicity test guidelines evaluate a range of issues, including life stage, duration/route of exposure, species differences, gender differences, structural and functional effects on target organs, and mechanism of action.

The testing of environmental agents (governed by EPA) and pharmaceuticals (FDA) is performed differently. Environmental agents require broad screening studies to identify hazard and dose-response for use in risk assessment. Because of often insufficient information about mode of action and toxicokinetics, rigid adherence to these types of guidelines is typical. Since human exposure assumptions are often based on proposed versus actual use patterns, the overall goal of such guidelines is to avoid or limit human exposure.

Pharmaceutical testing guidelines are based on studies designed to focus on specific questions about safety. Generally, an extensive database of background information is available, leading to flexibility in study design. In the case of pharmaceuticals, human exposure is intentional.

For repeated-dose mammalian toxicity studies, most guidelines specify that both sexes must be evaluated, but some specify the use of either sex, and some do not address sex at all. FDA maintains data on safety assessment of food ingredients, and both females and males should be included in those studies. Only one sex might be used because (1) it reduces the number of animals used, (2) one sex is more responsive to treatments, or (3) human exposure may be anticipated in only one sex.

Inclusion of both sexes in toxicity testing can provide useful information such as sex-related susceptibility. Data from guideline studies can also inform the design of future research.

Sex Chromosome Genes as Proximate Factors Causing Sex Differences in Disease

Art Arnold, Ph.D., University of California, Los Angeles

What is different about females and males, and what role do the differences play in physiology and disease? Identifying causes of sex-specific susceptibility and protection from disease requires establishing a foundation of knowledge of effects of sex-biasing factors on gene networks. These may be hormonally or chromosomally mediated, or they could involve both influences. Dr. Arnold has coined this sum of knowledge the *sexome.*

Although at a genetic level all sex differences result from an imbalance of X and Y chromosomes, there is more to the story. Nongonadal and gonadal effects can be further stratified into sex chromosome effects and hormonal organizational and activational effects, respectively. Collectively, these comprise three classes of proximate factors that cause phenotypic sex differences.

The four-core genotype mouse model enables an investigator to vary sex chromosome complement (XX versus XY) to observe differential effects without confounding hormonal differences.[21] This research tool has led to important insights such as the independent effects of hormones and sex chromosomes on body weight and sex chromosome effects on cardiac ischemia/reperfusion injury.

Integrating Sex and Gender into Preclinical Research

Gillian Einstein, Ph.D., University of Toronto

"The world writes on every body" reflects the notion that gender is not just a self-reflective concept, but it also involves an individual's place in an organizational hierarchy, an individual's environmental influences, and how that individual is treated by others. It is important for investigators to develop and use models that match the human condition. Gender in animals may be interpreted in a different way than it is in humans, in that it may encompass a range of factors. These include environmental conditions, complexity, the model entirety (including role and influence of experimenter), and the notion of disease/condition comorbidity.

Consider various effects of system complexity. One diabetes study showed that sex differences may be exercised or reinforced by commensal microbiota of the host. An extrinsic factor appears to regulate testosterone levels, and perhaps, the sex difference observed in type 1 diabetes mellitus.[22] This study demonstrates the need to consider the whole animal, not just the organ system(s) under evaluation. Another example is experimenter influence. In this study, exposure of mice and rats to male but not female experimenters produced pain inhibition. Male-related stimuli induced a robust physiological stress response that resulted in stress-induced analgesia to a painful stimulus.[23]

> "The world writes on every body."
>
> — Dr. Gillian Einstein

The Canadian Institutes of Health Research (CIHR), via the Institute of Gender and Health, have implemented a number of actions to foster the consideration of sex and gender in biomedical research. These include issuance of a strategic plan that brings together social and biological research, placing mandatory sex and gender questions in CIHR's grant application process, and sponsoring workshops for training bench scientists on how to include sex and gender in biomedical research design, conduct, and analysis. The Intersections of Mental Health Perspectives in Addictions Research Training program at the University of British Columbia brings together investigators conducting qualitative and quantitative research on sex and gender in addiction research. The trainees work in preclinical, clinical, and social-science research. A collaborative graduate program in women's health at the University of Toronto brings together trainees in clinical science and trainees from more theoretical backgrounds. The Canadian Consortium on Neurodegenerative Disease includes a cross-cutting program on Women, Gender, Sex, and Dementia that will work with every team—clinical and preclinical—as well as core facilities, to ensure that sex and gender are incorporated into science as it develops.

SCIENTIFIC SESSION IV: CULTIVATING A CULTURE OF “SEX MATTERS” ACROSS MULTIPLE DISCIPLINES

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Moderator: D. Lee Alekel, Ph.D., National Center for Complementary and Integrative Health

This session addressed a range of issues surrounding "when sex matters." Topics included how the biological variable of sex should be considered in science, how gender fits into the research realm, and when single-sex studies are warranted.

Virginia M. Miller, Ph.D., Mayo Clinic

Every cell has a sex, and thus sex must be considered a biological variable affecting any animal-derived material. Sex should thus always be identified for animals and cells. One issue complicating this action is that the methods sections of scientific papers have become progressively shorter over time and encourage sparse reporting.

It is important to note that although sex must be identified in the context of experimental design, analysis, and reporting, it is not necessary to use both sexes for all experiments in vertebrate animals. For example, excluding one sex of subjects is appropriate when the condition is unique to a single sex and it would do no harm to eliminate the other sex. Another justifiable single-sex study might be one in which a condition occurs more frequently in one sex or presents differently in one sex.

Here are a few questions to help investigators guide experimental design related to considering sex as a biological variable:

- Is there evidence of sex differences in humans in the incidence, prevalence, morbidity, or mortality of the condition or disease of interest? If there is no difference reported in the literature, is this because it has not been studied or reported?
- Is there an experimental model of the condition or of the disease of interest, and if so, does it reflect any sex differences in people?
- Are data lacking from an experimental model in one sex compared to the other sex?

Working effectively across disciplines toward cultivating a "sex matters" atmosphere might be accomplished more readily with new approaches to designing experiments. Forming and working in networks (not silos), sharing or sparing resources, and using a programmatic approach to problem-solving are some ideas. Sharing resources, such as animals, is always important, but it is especially critical in the currently strained fiscal environment.

Kathryn Sandberg, Ph.D., Georgetown University

The following example provides rationale for the importance of studying both sexes. A well-cited 2007 study reporting that infused T cells contribute to hypertension did not publish the sex of animals used.[24] Investigators who attempted to reproduce this result succeeded if they used a male model but obtained opposite results using female T cells and male animals.

Another example relates to hypertension-induced kidney damage. Kidney damage is more severe in male rats than it is in female rats due to the influence of gonadal steroids.[25] Another way to look at this issue is to consider what protects the kidneys of females in this scenario. However, there is no model for the study of chronic kidney disease in females.

Lupus-induced hypertension is another case in which sex differences are apparent. The NZBWF1 mouse model mimics female lupus in which blood pressure increases when a mouse develops lupus. Males of this mouse strain do not develop lupus pathology until very late in life. However, since men also develop lupus, a male model for the disease is needed.

It will be important for NIH and its stakeholders to accompany metrics of success with efforts aiming to induce cultural change in considering sex as a fundamental variable. Short-term measurements might include the number of applications and grant awards for single-sex and dual-sex research and the ratio of the two. Mid-term metrics might include the number of published papers on a single-sex study citing NIH support, the number of papers citing NIH support that use both sexes, and the ratio between the two. The 2011 survey on the sex of nonhuman mammals used in studies in a variety of fields could be repeated in the near future to gauge investigator behavior in performing (publishing) research on both sexes.[26] Another measure would involve finding out how often cell sex is specified in published studies and which sex is most often used. This information could be uploaded to PubMed. Long-term metrics include the number of clinical studies for which the design explicitly addresses sex-specific hypotheses or proposes an aggregated analysis due to lack of sex differences. Another metric could be to track the number of new drugs with sex instructions approved by FDA or to monitor changes in the number of adverse-reporting events affecting one sex.

How can the culture of biomedical research change so that sex matters? Strong resistance is expected, but it should dissipate quickly after a critical mass of believers is realized. Business models exist that have been designed to achieve culture change. For example, the nine-step approach involves three major steps: (1) begin by defining an endpoint, (2) create a team to work toward a common vision, and (3) build a culture of discipline.

CLOSING REMARKS AND CALL TO ACTION

Susan Maier, Ph.D., ORWH, and Janine A. Clayton, M.D., ORWH

Knowledge of mechanisms mediating the development and expression of sex and gender influences on biology will advance science and the translation of knowledge for the benefit of both women and men. Several opportunities offer immediate action toward more routine consideration of both sexes in biomedical research:

- Decrease variability through appropriate experimental design
- Decrease sample size as the knowledge base grows
- Employ factorial design and other methods to analyze multiple variables
- Collect and report data on both females and males
- Understand physiological and pathological mechanisms by studying both sexes in preclinical research, before clinical studies are planned based on a weak evidence base
- Better identify disease targets in cell culture studies and report known characteristics of experimental cells
- Acknowledge variation in results
- Take the context of the experiment into account in both design and interpretation

“Do no harm” is the fundamental tenet of medicine. Beginning with the end in mind, NIH-funded research aims to understand living systems and apply that knowledge to improve human health. Scientists must get as much information as possible out of every experiment. Culture change and hard work will propel the scientific community toward routine consideration of both sexes across the biomedical research continuum.

WORKSHOP DISCUSSION: LESSONS LEARNED

Fundamental biology includes not only that information that is shared, or the same, but also information that is the same and different. Sexual selection is a powerful evolutionary mechanism, and thus sex differences are likely to be of fundamental value to biology. For many years, *fundamental* biology has been male biology, in both preclinical and clinical studies and practice. Studying both sexes is necessary for generalizing results to heterogeneous populations of females and males.

Sex-based research can be a powerful tool for discovery. Studying both sexes can unveil biological truths, new fundamental knowledge, and new biological mechanisms. Basic scientists not attuned to studying both sexes from the hypothesis-generation stage may be surprised to learn that it is possible to find a larger effect size when considering both females and males.

Studying both sexes in preclinical research is good science. It answers questions, advances inquiry, and provides a route to innovative solutions. In the real world of biomedical research, investigators must balance their plans with the resources they have to conduct research. However, this does not permit the omission of key variables, under-powering studies, or failing to collect and report data from both sexes of study subjects. Reporting all data, including negative data, should be the gold standard for biomedical investigation. Unfortunately, that has not been the case for various reasons. Researchers must thus accept that "the absence of evidence is not the same as evidence of absence." As a critical mass of investigators populate the literature with study methods, results, and even raw data on both sexes, the foundation of evidence about the biology of both sexes will grow.

Many different considerations contribute to experimental design, including the selection of an appropriate model system that exemplifies to the best approximation possible a human condition. This includes considering the complexity of the environment, conditions of an experiment, and a reduced reliance on rodent models, in the service of ensuring reproducibility and generalizability of research. Although experimental design (including choice of model) and data analysis are key elements of scientific rigor, more complex statistics may also need to play a role in data analyses.

Growing knowledge of the ways that females and males differ and how different social influences shape biology differently will inform the design and conduct of more experiments that study both sexes. NIH is currently developing a set of materials to enhance reproducibility in biomedical research. These materials will address experimental design, randomization, blinding, and sample-size calculation, among others, including the consideration of sex as a basic variable. Educational messages are most effective early on in an investigator's training. Early-stage investigators may thus be especially open to new approaches to discovery research such as those presented by studying both sexes. Curriculum projects are underway to enrich medical school teaching of the necessity of understanding (and treating) both sexes. ORWH, in cooperation with FDA, offers a free online course, The Science of Sex and Gender in Human Health. These educational efforts will help advance research toward the routine consideration of sex as a basic biological variable.

Any behavioral change is most effective with incentives that stimulate NIH grant applicants to study both sexes in preclinical research across the scientific spectrum, especially in areas traditionally understudied with respect to the effect of sex. Reviewers and publishers will be a vital component of this cultural change.

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