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REVIEW

Various Aspects of Sex and Gender Bias in Biomedical Research

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Summary

The main role of research in medicine is to provide relevant knowledge which, after successful translation to clinical practice, improves the quality of healthcare. The sex bias which is still present in the majority of research disciplines prefers male subjects despite legislation changes in the US grant agencies and European research programme Horizon 2020. Male subjects (cells, animals) still dominate in preclinical research and it has detrimental consequences for women's health and the quality of science. Opposite bias exists for data obtained mainly in animal models utilizing female subjects (e.g. research in multiple sclerosis, osteoporosis) with skewed outcomes for men affected by these diseases. Either way, scientists are producing results which compromise half of the population. Assumptions that females as cohorts are more variable and another assumption that the oestrous cycle should be tracked in case the females are enrolled in preclinical studies were proven wrong. Variability of male versus female cohorts are comparable and do not only stem from hormonal levels. The widespread prevalence of sex differences in human diseases ultimately requires detailed experiments performed on both sexes, unless the studies are specifically addressing reproduction or sex-related behaviors.

Key words

Sex bias • Biomedical research • Testosterone • Estrogen

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Introduction

Biomedical research has a high value in society. It can provide important information about disease trends and risk factors, outcomes of treatment or public health interventions, and health care. Driven by curiosity, basic research fuels applied science's innovations and connects with clinical studies *via* translational research (McCormick 2001). Collectively, these forms of research have led to significant discoveries, the development of new therapies, and a remarkable improvement in health care (Bates *et al.* 1998). The main objective of the research is therefore improved health and quality of life of the population. However, the question of how much are the research outcomes relevant and valid and to what extent do they apply with the same relevance to both men and women remains largely unanswered.

Non-human female mammals and women have been neglected from research for a long time. The reasons why it happened are many and range from assumption that what was found in males is valid also for females, through worry that female oestrous cycle increases heterogeneity of the studied population (Wizemann and Pardue 2001) to misinterpreted idea of "protection" of women from inclusion to the clinical studies where their health can suffer a harm (Keitt *et al.* 2004). In the past, women of child-bearing age came to be excluded from early clinical trials given birth defects and other negative health outcomes resulting from fetal exposure to certain drugs, which is certainly understandable. But why those practices of exclusion of women of child-bearing age

were extended to female cells, tissue cultures and animal models is not known. Perhaps the reason for male-only animal models is convention and convenience. Perhaps it was suggested that males-only may be seen as an easier and possibly cheaper option than using both sexes (Blanchard *et al.* 1995). Male-only models are very common in many research areas and their use is always adopted by a new-coming generation of scientists even without questioning why this is a male-only sample. The answer would be probably similar to "because we have done it this way for 40 years and it works well".

Although there are some specific areas of interest in which it is entirely appropriate that only one sex should be investigated such as sex-related behaviour or drug interactions with the sex-specific gonadal hormones, it is difficult to justify the exclusion of females or indeed males from many other types of investigation. Without proper sex analysis, we risk claiming a general effect when it only applies to one sex, or that no effect exists whatsoever when there are opposite offsetting effects in the two sexes (Wetherington 2007).

One more trouble for scientists or facing the truth?

The fact that sex and gender bias does exist in biomedical research has been known for some time (Becker *et al.* 2005) but one never cared much, unless there is a personal experience. We are a group of scientists involved in research of respiratory physiology and laboratory animal science. In 2015 we published an epidemiological study that showed that patients affected by chronic hypersensitive cough are mainly postmenopausal women (Song *et al.* 2015). The treatment of this condition remains unknown because, despite tremendous research of mechanisms leading to up-regulation of cough neural pathways, the cause of this particular condition is still not known – therefore we lack causal treatment. The only data about currently known mechanisms of up-regulation of cough neural pathways we had were from animal studies, which were performed exclusively on male guinea pigs for decades. Finding hypersensitive cough syndrome being a female gender issue and not having relevant answers had started a lot of questioning in our research discipline (Plevkova *et al.* 2017*a*, Plevkova *et al.* 2017*b*). In the scientific literature in largest databases we found only three studies in total, that used female guinea pigs, however, the reason for sex selection criteria was not explained, and the sex of laboratory animals was not taken in consideration during the final data analysis (Forsberg *et al.* 1988, Ebihara *et al.* 1996, Ito *et al.* 2002). Since then, we have developed a model utilizing both sexes of guinea pigs for basic cough research without increased internal variability or decreased power of statistical analysis of combined female and male cohorts (Sterusky *et al.* 2020).

Similar experiences are published also by other scientists. After decades of research, mostly excluding females/women, researchers began to realize that differences between male and female subjects are found also beyond the reproductive system and that sex is important biological variable in biomedical research (Lee 2018). Very inspiring is the story of Melina Kibbe, who after experiencing considerable sex bias in her research became an advocate of this issue and leads a national fight against sex bias in research in the United States (Yoon *et al.* 2014).

Gender and sex in research terminology

Our analysis of sex/gender-related bias in scientific literature has shown that the terms "sex" and "gender" are not used correctly by many authors. Even the English language is not always the first language of authors publishing their results in international journals, the language and expression accuracy should be required. Sex is, according to medical databases, classification of studied subjects as male or female according to their reproductive organs and functions assigned by sets of chromosomes. Sex is related to reproductive organs anatomy and physiology. Gender is a person's self-representation as male or female or how that person is responded to by social institutions. It is shaped by environment and experience and refers to socio-culturally constructed norms and identities (Lee 2018, Beery 2018).

Consequences of sex/gender bias

Paradoxically – protection of women's health by not recruiting them to the clinical studies and neglect of female non-human subjects in basic research has led to the negative impact on women's health. There is substantial evidence that sex bias in research is responsible for e.g. failure of designed treatment that works perfectly well for men, but does not work for women. The reported rate of drugs adverse effects is also higher for women, just because these substances were developed and tested *via* the entire research process on

male subjects (Heinrich 2001). A recent paper by Lee (Lee 2018) reports that during the time period from 1997 to 2000, ten prescriptions drugs were withdrawn from the market by the US Food and Drug Administration because they represented greater health risk for women. Four of these ten drugs that were developed and prescribed to treat diseases both in men and women and belonged to the general categories of antihistamines, cardiovascular and gastrointestinal therapies caused severe, life-threatening arrhythmia Torsade de Pointes, mostly in women (Heinrich 2001). It was thought that the reason was a higher rate of prescription of a particular medication to women, however detailed analysis confirmed that drugs that have been withdrawn were prescribed to women and men equally (Hughes 2007) but had more detrimental effects on women. Of course, those adverse effects could be caused by wrong prescription or improper use, however potential sex differences in physiology and pharmacology of these drugs cannot be ignored.

By avoiding both-sexes studies and performing experiments on male-only or female-only animal models basic scientists are limiting their responsibilities to the half of the human population (Mogil and Chanda 2005), e.g. autoimmune diseases such as Grave's disease, systemic lupus and Hashimoto's thyroiditis affect predominantly women by 7 to 10:1 ratio, but this ratio is opposite for other autoimmune diseases such as ankylosing spondylitis, Reiter or Goodpasture syndromes (Fish 2008). These diseases should be definitely studied concerning sex as one of the determining factors, however, Beery and Zucker (2011) report that in their analysis of articles published in 2011 in journals in the field of immunity and immunology failed to specify the sex of studied subjects in 75 % of cases.

Male and female bias in research

According to the outcomes of multiple studies in different fields of research one sex-only studies produce skewed data. Results from male-only versus female-only studies differ considerably e.g. in studies assessing response to medication. It is known that low dose of aspirin has different preventive effects in women and men and that drug such as zolpidem used to treat insomnia requires different dosing in women and men (Soldin and Mattison 2009). Sex differences are found in the outcomes of studies of neurological, cardiovascular and autoimmune disorders (Fish 2008).

Male bias

Is modern research at all its levels from bench to the bedside sufficiently attentive to female subjects? Analysis of scientific papers published in 10 major biological disciplines confirmed previously recognized strong male sex bias (Sechzer *et al.* 1994, Blanchard *et al.* 1995). The study by Beery and Zucker – analysis of scientific articles published in 2011 – showed that male bias is still present in 8 out of 10 surveyed fields including neurosciences, physiology, pharmacology, endocrinology, zoology and to lesser extent behavioural physiology. The ratio of articles reporting on males-only samples versus females-only samples was most skewed for neuroscience (5.5:1), pharmacology (5:1) and physiology (3.7:1) (Beery and Zucker 2011). Similar bias towards the use of male subjects was found in laboratory research of pain, diabetes, cardiovascular diseases and surgical methods (Yoon *et al.* 2014, Flórez-Vargas *et al.* 2016). A recent analysis from 2017 in neuroscience for example points towards decreased rate of omissions of subjects sex reporting, however, the proportion of male-only rodent studies has increased while analysis by subjects sex remains infrequent (Will *et al.* 2017).

The consequences of male-only studies were already mentioned. The higher rate of adverse effects of medication affecting women and withdrawal of drugs from the market due to their detrimental effects on women's health after they have been claimed effective and safe based on research speaks for itself.

Female bias

Logically, if the disease affects both men and women, while the pathogenic process or drug efficacy is studied mainly in male animal models or men in clinical studies (what is most common scenario seen in the sex-biased research) – the data will be skewed and less valid (if not completely invalid) for women. Does the opposite situation exist? Does the female bias lead to the production of skewed data for men?

A female bias was identified in the field of reproductive physiology where females were used 1.6 times more often than males and immunology where the ratio is 2.2:1. 94 % of these reported animals were rodents – mice or rats (Beery and Zucker 2011). The question is whether should the study population (experimental research on animal models, or cell lines) reflect epidemiological data regarding the prevalence and

distribution of the diseases between men and women or should it be equally attentive to both sexes?

An interesting example is multiple sclerosis (MS). MS is a lifelong disease affecting individuals in young to middle adulthood, typically with the dysregulated autoimmune reaction towards neural tissue leading to progressive demyelination. MS affects women approximately three times more than men, thus resulting in less attention to men with MS (Rahn *et al.* 2014). There are recognized sex differences in MS onset and clinical course which is the consequence of natural sex/gender dimorphism. What is not natural is a less effective treatment of MS, which is not only caused by a disease dimorphism but also by outcomes from basic research of MS and early-stage pharmacological studies in MS because they are conducted mainly on models utilizing female sex. Based on detailed studies researchers conclude that e.g. female rodents are superior to males for the study of neuropathic pain-like behaviors associated with MS (Rahn *et al.* 2014). It makes sense if the disease affects predominantly women to use female animal models, but here the scenario repeats but in the opposite direction. Little knowledge is worse than no knowledge and male patients with MS are treated and managed less effectively e.g. differences in safety issues during fingolimod therapy in subjects with MS were detected. In this cohort, female subjects were experiencing mainly infections as an adverse effect of the drug while men were facing liver damage with an elevation of transaminase markers in the serum (Manni *et al.* 2017).

Another brilliant example is osteoporosis. Osteoporosis was considered as solely women's disease. Clinicians believe that the decline in bone density and its complications solely affect postmenopausal women, however, osteoporosis and its complications affect both genders but at different ages and rates. Osteoporosis is four times more common in women than in men, but some evidence indicates that men tend to have more osteoporosis-related complications (Alswat 2017). Most animal models have utilized females and osteoporosis in males has been largely ignored (Simon Turner 2001, Komori 2015). The consequences of this bias are less known pathogenic mechanisms, insufficient screening schemes and very often a failure in the prevention and/or treatment. It was found that antiosteoporotic drugs effective in women do not have the same effect in men (Schwarz *et al.* 2011, Kaufman *et al.* 2013), and in fact, majority of such drugs have been tested on female animal models. From a clinical perspective, a major limitation is the lack of studies about osteoporosis in men. Further and expanded studies of osteoporosis in men across all areas are needed to address this limitation.

Sexual/gender dimorphisms at its various levels

Author of a comprehensive book "Principles of Gender-specific Medicine" Marianne Legato points to the need of accepting gender as a critical and determining factor in understanding human biology, the nature of the human disease and therapy (Legato 2010). It is necessary to understand for basic science and in general, for all research community, that sex differences are not related only to the reproductive system but they do exist beyond it in every single body system (central and peripheral nervous system, gastrointestinal, respiratory, cardiovascular, endocrine etc.) and they are also relevant for majority of human diseases.

Dimorphism is already present at the cellular level. The sex of cells in the cell cultures *in vitro* matters. Just to set some examples – female and male cells respond differently to stressors, they have different responses in processes of proliferation, differentiation and apoptosis (Penaloza *et al.* 2009). These intrinsic differences are hormone-independent but can be further modulated by the presence of hormones. For example, male neurons (XY neurons) are more sensitive to the stress caused by exposure to reactive oxygen species and excitatory neuromediators while female neurons (XX neurons) are more sensitive to stimuli influencing apoptosis (Du *et al.* 2014). Sex of the cells used in the study is not sufficiently reported in scientific papers. Only roughly 23 % of articles in top cardiovascular journals reported cell sex (Taylor *et al.* 2011). Among these studies, more than 60 % used male cells and none exclusively used female cells. This bias is present also in other research disciplines using cell cultures (Lee 2018). Surprisingly, sex of the cells is also ignored by laboratories providing cell lineages for scientific use – the cells are sold without sex specification (Park *et al.* 2015).

Sexual dimorphism was detected even at the level of genetic information – more than 23000 transcripts of mouse genes exhibit male versus female differences ranging from 14 % in the CNS to 70 % in the liver with intermediate values in adipose tissue and muscles (Yang *et al.* 2006). Many of these genes are involved in the pathogenesis of common diseases, in

which susceptibility is sex-biased and single-sex studies cannot provide a complete understanding of how these genes e.g. interact with epigenetic factors or how they are modulated by internal factors of the organism (Pessin and Marts 2005).

Effect of legislation changes, grant agencies and editorial offices policies

Considerable gender bias in clinical and also basic laboratory research and its impact on women's health has been recognized in the past and led to certain legislative changes in funding agencies. National Institute of Health issued a Revitalization Act in 1993 requiring recruitment female participants in federally supported clinical trials in sufficient numbers to enable a reliable analysis of differences among groups (Klinge and Wiesemann 2010). In 1999 the National Academy of Sciences in the US established a committee that consisted of experts in different fields to evaluate and determine the sex/gender differences and their position in research. Their report clearly stated that sex is an important variable and should be considered in designing research projects (National Institutes of Health 2001). Similarly, the European Union programme was issued for researchers giving them a tool and good practical examples regarding enrolment of women and non-human female mammal subjects to the research protocols (Klinge and Wiesemann 2010). Even though these legislation changes did exist, realistically, a significant move towards research protocols that would conduct e.g. basic science studies on both sexes was not registered (Sandberg and Ji 2012).

This is documented in the survey from 2011. Analysis of research papers have shown interesting and very promising trends regarding the change in sex distribution in animal and human studies, since 1909 for animal studies and since 1949 for human studies (Beery and Zucker 2011). While the proportion of studies with unspecified sex of participants decreased to zero in 1999, the percentage of studies on both sexes of human participants is increasing to more than 60 % from roughly 30 % in 1949, which is an excellent shift for human studies. However, the male-only non-human studies (laboratory research on animals, tissues or cell lines) still take 60 % in average, with still having around 20 % of studies where the sex of subjects is either unspecified or specified but not taken into account during data analysis. In the graphs describing trends in the rate of one sex-only studies versus both-sexes studies after implementation of mentioned changes in grant agency policies, it is clear that these legislative changes were effective enough for human studies. Both sexes human studies are the dominant type of studies in this category, however, animal studies were not influenced at all. Males were still dominating in preclinical and basic research studies.

Similar data are available in an article published in 2010 (Zucker and Beery 2010) in which the authors analysed the use of female animal subjects in studies for diseases such as anxiety, depression, thyroiditis, epilepsy, multiple sclerosis, obesity, hypertension, stroke and pain. This study showed that the proportion of female and male rodents in these studies was not equal, female animals were severely under-represented even in diseases which are commonly more prevalent in women. Diseases such depression or anxiety are twice as likely to be diagnosed in women, but fewer than 45 % of animal models used female subjects to investigate hypotheses postulated about these particular disease conditions (Zucker and Beery 2010). One might suggest that perhaps this report is 10 years old, more recent reports e.g. from 2016 are showing that situation has not improved much (Zakiniaeiz *et al.* 2016, Kong *et al.* 2016).

This is a little bit disturbing because the clinical studies are designed and based on the results of basic and preclinical research. Much of our understanding of disease mechanisms and treatment possibilities came from non-human studies performed either on animal models or cell cultures. If these studies are biased from the very beginning, planning, designing, funding and also final realization and analysis of data from clinical studies will be influenced by this bias. Therefore, sex should be considered as an important variable even at the level of basic research.

Gonadal hormones as a problem in animal research

A title of this paragraph has been changed from "ovarian hormones" to gonadal hormones, because of the assumption that only ovarian hormones cause variability and therefore disqualify female cohorts as reliable samples are not valid any longer. For a long time, there was an assumption, that circulating ovarian hormones during oestrous cycle make the data from female animals more variable than data from males. This biases not only the subject selection but also the experimental design, peer review of grants and manuscripts when female

animals are recruited to the study. It is also important to note that sex differences are in any case, incompletely explained by the action of sex hormones alone (Cahill 2006). Therefore why blaming them and using them as an excuse for not enrolling female subjects to research projects?

Whereas there are indeed examples of greater inter-subject variability among female rats in some traits, there are also examples of lack of oestrous cycle-based fluctuations in investigated traits and finally there also examples of greater inter-subject variability in male rats and males of other species (Hughes 2007).

In female rodent subjects, similarly in humans, levels of circulating ovarian hormones fluctuate during the oestrous cycle, which typically lasts around 4-5 days. Therefore, there are phases characterized by high and low concentrations of oestrogen and progesterone (proestrus, oestrus, metestrus phases). This means that in your study cohort you very likely have mice or rats' female subjects with fourfold differences in their hormonal levels. Presuming that these differences would lead to variable, un-interpretable data, scientists chose to avoid this issue altogether and exclude female animals from the research (Wald and Wu 2010). This seemed to be a very practical choice and in years it became dogma.

Sex hormones are "scary" even for the researchers in clinical research. Some authors advocate for separate women cohort sub-groups – population of women on hormonal contraceptives. Of course, this is particularly important mainly in the brain and behavioural studies (Becker *et al.* 2005).

Attempts to reduce the influence of ovarian hormones in animal research have included testing only during diestrus phase of the cycle, when oestrogen and progesterone levels are low (Mora *et al.* 1996) or simply tracking the cycles by evaluation of hormonal levels from blood or vaginal cytometry. In case of variables fully dependent on oestrous cycle a study requires four times more female subjects (in particular cycle phase) to minimize the effect of hormonal levels on given variable. However, these procedures, which increase the cost of the research and are not in alignment with the 3R are now not necessary in all types of research. Increased costs of such research due to the increased number of animals should be compared to the benefits such study can offer – and it is the highest accuracy of the data. Also, the animal welfare bodies should clearly state their pros versus cons to such approach.

In this aspect, consideration of the impact of oestrous cyclic events on traits which are evaluated in the research is critically important, given the differences between the rodent oestrous cycle and human menstrual cycle. This is of particular interest in behavioural studies, e.g. although the changes in levels of sex hormones may follow a similar pattern in both types of the cycle, the expression of female sexual behaviour in rodents is rigidly tied to the onset of oestrus, whereas women are sexually receptive at any stage of the menstrual cycle (Eliot and Richardson 2016).

Several new genetic and epigenetic animal models have increased translational validity, e.g. it is possible to prepare a model that represents human ovarian failure and menopause using Foxl2 deficient mice with accelerated rates of decline in ovarian reserve. Another frequent approach to eliminate the effect of sex hormones is a genetically modified animal that lacks a specific steroid receptor, e.g. ERα knockout mice. However, this is not the best approach since oestrogen effects are not exclusively mediated only by ERα, but it also utilizes alternative signalling pathways (Phillips and Roth 2019).

In 2014, for the first time in the long-lasting era of what we call modern research in meta-analysis of 300 scientific articles that used rodents as a study subjects, it was found that variability in an array of physiological, cellular, hormonal and behavioural data collected from female rodents, regardless of oestrous cycle, did not vary more than the data from males, and in some parameters, the data from males varied even more than data from females (Prendergast *et al.* 2002, Becker *et al.* 2005). These studies also indicate that it is not necessary to track the stage of the oestrous cycle in research unless the primary role of the research is not related to reproduction or effect of substances which primary target is the level of sex hormones.

From the perspective of tracking the hormonal levels no one ever complained about the variability of male cohorts potentially influenced by testosterone fluctuation. There might be an argument for monitoring of testosterone in some investigations if the male animals are not tested at the same time of the day, as changes in concentration of testosterone in rodent vary rhythmically in a trimonal fashion during a 24-hour period (Mock and Frankel 1978). Another important source of testosterone fluctuation and possibly variability in males in rodents is housing type.

Rodents are usually group-housed. Group-housed males fight opposed to females, they establish

dominance hierarchies in which the dominant male monitors and defends the entire cage space, frequently attacks subordinate males, activating sympathetic pathways and stress axis increasing level of cortisol in subordinates. It can be so significant that it causes analgesia in subordinate males (Shansky 2019). The dominant male has five times higher concentration of testosterone than subordinates on average, therefore the data from male subjects could be influenced by hormonal status as well. Testosterone is known to be a powerful neuromediator, and it also influences multiple body functions (McEwen and Milner 2017). So here applies the hormone-based variability in males no one ever complained about so far. While the levels of sex hormones as a source of variability in females are influenced by the oestrous cycle, the level of testosterone is influenced by natural 24 h fluctuation and housing conditions in males. Although sex hormones influence multiple body functions, they do not disqualify male or female cohorts from studies and both cohorts have comparable variability.

Both American and Canadian research funding agencies require from 2016 both-sex studies and although the policies are based on reasonable background, there were worries about increasing costs of the research caused by an increased number of animals, which will be required, increased costs and time-consuming procedures of their hormonal cycles tracking etc. Because according to the data obtained from studies utilizing both mice and rats and their comprehensive meta-analysis, both male and female samples are equally variable, there is no justification for requiring oestrous cycle assessment (or statistical power to evaluate oestrous effects) in females without demanding evaluation of testosterone (or statistical power to account for cage dominance effects) in males. This is not related to the studies of reproduction where, of course, the role of hormones is the key factor.

If the degree of variability in male samples is acceptable for scientific purposes, so is the degree of variability of female samples if female cohorts should be deemed acceptable in research.

Arguments from laboratory animal science

Laboratory animal science develops as quickly as the research itself. It is believed that valid and reliable data can be only obtained from laboratory animals with the high-quality standards, therefore attention is given to the animal care – housing conditions, nutrition, hydration, light-dark cycles preservation, enrichment, microbiological and veterinary controls and many more.

3R principles in animal care and use suggest reduction, replacement and refinement in laboratory research for the sake of animal welfare. Performing experiments now on both sexes requires more subjects to be tested, which is against the main principles of 3R because they suggest using as few animals as possible to obtain valid and statistically powerful data. Regarding the number of animals, there were voices pointing towards the excessively increased count of female animals in reproductive age if it will be confirmed that studied trait/parameter is influenced by the oestrous cycle. In that case, researchers may need four times more female animals as female rodents have a four-stages ovarian cycle (Becker *et al.* 2005). Based on recent analysis, tracking of hormonal cycles, in general, is not necessary as we said before unless the studied parameter is directly influenced by the stage of the cycle. This is related to the disciplines studying reproduction or behavioural studies (Becker *et al.* 2005).

Some authorities of animal welfare bodies may argue, that using more animals is not ethical and not necessary, however, we cannot ignore substantial evidence pointing towards sex as an important variable. Therefore, careful planning of experimental design and validating results by replication is a prerequisite of excellent science.

Another argument will mention financial burden – more money spent on animals, chemicals, more time spent performing experiments etc. From the quick look into this process one may see this reason not to use as many animals as recommended to prevent money waste, but including both sexes at early stages of research will save money and time than testing sex differences in more expensive and lengthy clinical trials. It also prevents even more costly and dangerous situations such as withdrawing drugs after marketing due to unforeseen sex differences in adverse effects. Analysis of sex differences in basic research is likely to save more money in the long-term perspective (Rich-Edwards *et al.* 2018, Lee 2018).

Some authors mention problems with the experimental procedures in e.g. behavioural studies in case both male and female rodents are enrolled. The possibility that if male rodents are tested in experimental apparatus recently used for testing females, their behaviour might be influenced by the presence of

olfactory stimuli left by the female exists. This is because male mice have been shown to discriminate between male and female odours and to exhibit behavioural changes following exposure to female odours within 24 h even after proper cleaning of the apparatus (Kavaliers *et al.* 2003). Again, to address this issue, a blueprint of the experiment is necessary to avoid such a coincidence.

A sex-informed and gender-informed perspective is essential to increase accuracy and to expand the relevance of research is becoming a new research culture. Investigators who wish to include both sexes in their studies are faced with a number of methodological questions, including issues of motivation, subject selection, sample size, data collection, analysis and interpretation. Inclusion of both sexes is more nuanced than deciding that the sample should be equally divided by sex. Also, sex-specific age incidence of disease, reproductive stage, reproductive cycle and environment need to be considered to optimize validity, generalizability and efficacy of study sample. Rich-Edwards et al. in recent paper reviewed principles, which should be followed in case that investigators want to include both sexes to their study (Rich-Edwards *et al.* 2018). Authors of this review recommend considering sex-specific age prevalence of disease to maximize statistical power, consider reproductive stages and cycles, particularly where they may modify the impact of the main exposure being investigated. For basic and preclinical studies, review options for classical gonadectomy, knockouts, or four-core genotype experiments.

Researchers are also encouraged to seek the guidance of experts in animal care & use and animal welfare bodies to address accurately the sex-related questions. An important contribution is also available from the experts in biostatistics who can help with the calculation of appropriate sample size and the number of experimental/control groups per experiment according to the expected power of the experiment, confidence level and confidence intervals of expected results. Free online sample calculators are not always sufficient to calculate sample size properly for more groups and more variables.

According to the ARRIVE guidelines (Kilkenny *et al.* 2010), in reporting an experiment it is necessary to provide details of the animals used, including species, strain, sex, developmental stage, age and weight. To adhere to the principles of precision in science it is also necessary to specify the total number of animals used in each experiment, and the number of animals in each experimental group and also explain how the number of animals was calculated and provision of details of any sample size calculation that was used (www.3rs-reduction.co.uk/html/6_power_and_sample_size.html). Calculation of sample size is one of the important components of the design of any research including animal studies. If a researcher selects a smaller number of animals it may lead to missing of any significant difference even if it exists in population and if a greater number of animals is selected then it may lead to unnecessary wastage of resources and may lead to ethical issues – have you ever thought about fate of female animals which are born in an animal breeding facility, but are neither used for further reproduction nor research purposes?

Recent legislation changes and scientists themselves advocate sex/gender equality in research

Even after decades of effort to integrate sex/gender in biomedical research, the change has been slow. Most recently issued policies of funding agencies such as National Institutes of Health (NIH) and Canadian Institutes of Health Research (CIHR) are stricter when it comes to review of basic research projects, noting sex bias has negative consequences. Similarly, Horizon 2020 which is the largest Research and Innovation Programme in the European Union supports the sex/gender equality in research. A position paper published by European experts and recommendations of NIH and CIHR encourages and guides scientists to use both male and female animal research subjects and consideration of sex as a biological variable (Clayton and Collins 2014). Researchers are required to use both sexes of laboratory animals, or clearly state and defend by reasonable arguments, why they are using male-only or female-only animals in their study.

The future will answer the question of how effective these policies really are and what changes we will observe in the consideration of sex in biomedical research. Scientists who are willing to follow these rules in their grant applications and research reporting can educate themselves in online training modules and also get advice from the documents issued by above-mentioned authorities online. Recent policy changes and recommendations even paraphrasing crucial parts of documents were extensively reviewed elsewhere (Lee 2018).

Conclusions

To change the state-of-the-art, it is necessary to change the attitude and approach of high-quality scientific journals, which publish research results. Journal editors can facilitate innovation through changes in journal policies. In the past, there were only a few journals, whose policies considered this sex/gender issue. For example, Journal of National Cancer Institute in the instruction for authors states "clinical and epidemiological studies should be analysed to see an effect of sex and if there is no effect it should be stated in the results" (Wald and Wu 2010, Beery and Zucker 2011).

Nowadays, prestigious, high-quality journals already in their "instructions for authors" require authors to clearly report the sex/gender of the research subjects including cells, animal models and human, and to analyse data by sex/gender. SAGER (Sex and Gender Equity in Research) initiative of European Association of Science Editors is changing the global standards and quality of reported research by guidelines which needs to be followed in case scientists want to adopt more systemic approach to the sex/gender reporting (De Castro *et al.* 2016).

Conflict of Interest

There is no conflict of interest.

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