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Comparison of Rat Fetal Sex Determination Using Placental gDNA and mRNA *via* qRT-PCR

Anthony L Su [1,2] and Rita Loch-Caruso[1*]

[1]*Department of Environmental Health Sciences, School of Public Health, University of Michigan, Ann Arbor, MI 48109-2029, USA*

[2]*Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*

[*]**Corresponding author:** Rita Loch-Caruso, Department of Environmental Health Sciences, School of Public Health, University of Michigan, Ann Arbor, MI 48109-2029, USA, Tel: 734-936-1256;Fax: 734-936-7283;E-mail: rlc@umich.edu

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Abstract

A growing need exists to consider fetal sex as a biological variable and accurately assess sex-specific effects. Among the multiple methods used to determine fetal sex, quantitative real-time polymerase chain reaction (qRT-PCR) of Sry (sex-determining region Y) with genomic DNA (gDNA) is commonly used in addition to use of methodologies such as transcriptomics and detection of Barr body. However, Sry messenger RNA (mRNA), a product of SrygDNA, has not been previously assessed for sex determination. Using placental samples from timed-pregnant Wistar rats at gestational day (GD) 16, this study assessed the compatibility of Sry detection using gDNA versus mRNA to determine fetal sex. Samples used in this current study come from a larger study that investigated trichloroethylene (TCE) reproductive toxicity and potential modulation by N-acetyl-L-cysteine (NAC) and aminooxyacetic acid (AOAA). In 90 out of 91 samples, the sex classification determined by gDNA matched the sex classification determined by mRNA analyzing Sry (Sry/B2m) values. For both gDNA and mRNA, statistically significant differences in Sry/B2m values between males and females were observed with samples considered in totality and when samples were separated by treatment groups (all comparisons were $p<0.01$ or below, and all but two comparisons were $p<0.001$ or below). Finally, the validity of using SryCq values to determine fetal sex and the B2m reference gene were also discussed. Together, this study suggests that determination of fetal sex in Wistar rats can be accomplished using Sry measurements in gDNA or mRNA with highly compatible results.

Keywords: gDNA; mRNA; qRT-PCR

Introduction

With increased emphasis of sex as a biological variable that influences disease, behavior, and other outcomes [1], the accurate determination of fetal sex in placental samples of otherwise unknown sex is increasingly important. Multiple methods are available to determine sex of a biological sample, each with its limitations and strengths. Among the multiple methods to determine sex of a biological sample, the newest technologies provide novel opportunities and methods for sex determination. For instance, single-cell RNA sequencing has provided precision over more traditional transcriptomic approaches, including microarrays and RNA-sequencing, to allow for the determination of sex of a single cell versus a population of cells [2]. Transcriptomic approaches can provide ample information, but considerations including cost and project purpose may limit use of a transcriptomic approach to determine sex.

Traditional approaches that are less expensive than the aforementioned "-omics" technologies are available to determine sex, albeit with their own limitations and other considerations. In samples not limited to mammals, the detection of sex-specific genes, such as Sry (sex-determining region Y), can be performed using Southern blot, nested polymerase chain reaction (PCR), or quantitative PCR methods. Consideration of these approaches include execution difficulty and the retrieval of only genomic, not protein-level, information. Detection of enzymatic activities that are known to differ by sex, including activity of CYP3A4, CYP2B6, and methyltransferases [3,4], can be used but care should be taken to establish thresholds and consider other factors that may influence enzymatic activity. Therefore, in both above-mentioned approaches, appropriate controls should be used to rule out false positive or false negative readings.

In mammalian samples, the detection of the Barr body, detection of the H-Y antigen, or consideration of fetal germ cell development (only in humans) has been used to attempt to determine the sex of the individual [5-12]. The Barr body is formed from a silenced X chromosome and is only present in female samples [5]. However, it may not always be detected in females [6, 9, 10] and its absence, therefore, could yield a false reading of sex. In contrast, the H-Y antigen is theoretically only present in males, appears as early as the eight-cell stage, and can differentiate males from females, but suffers from a complex detection method and may not be sufficiently accurate because false positive and negative findings have been reported [7,11,12]. Assessment of events in fetal germ cell development in humans can be used to determine sex. As an example, retinoic acid signaling is specific to females [8]. The timing of events in fetal germ cell development can also be assessed to determine sex. An example of this is the case of mitosis, which happens in both sexes but only after migration in the case of males.

The Sry gene, found exclusively on the Y-chromosome in normal cases, can be assessed through messenger RNA (mRNA) levels or through quantities of genomic DNA (gDNA). Although both types of assessments have been used, assessment of Sry in gDNA[13-19], which can be performed using gel electrophoresis [16,19], is more common than assessment of Sry mRNA abundance [20,21]. A prior report that measured Sry mRNA abundance in mouse used a PCR assay for Aard (alanine and arginine rich domain) expression to determine sex [22], but the validity of using Sry mRNA specifically to determine sex was not considered. Similarly, a prior report measured variation in Sry mRNA expression in mouse across time using RNA-sequencing, but no comparison to gDNA was made [23]. mRNA assessments benefit from evaluation of DNA that is actually transcribed, that is, by assessing the Sry mRNA that codes for the SRY protein responsible for sexual dimorphic developmental processes. These processes include the formation of a SRY and steroidogenic factor 1 (SF-1) protein complex that binds to sry-box 9 (SOX-9) enhancer regions to upregulateSOX-9 expression [24] and facilitate Sertoli cell differentiation and testis development [25]. Although some studies may also have available mRNA but not gDNA[22, 23], benefits exist for

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assessment of Sry from gDNA as opposed to mRNA. Unlike detection of gDNA, detection of mRNA requires the synthesis of complementary DNA (cDNA) from RNA. An additional benefit to assessing gDNA as opposed to mRNA is that gDNA is more stable because it lacks a reactive hydroxyl group found in RNA.

Despite the variety of approaches used to determine sex, few publications compare methods side-by-side. In this report, we used two methods and showed that the determination of sex using placental samples from timed-pregnant Wistar rats yielded similar results whether the approach was through assessment of gDNA or mRNA of Sry. This approach was used for the Wistar rats exposed to trichloroethylene (TCE), a common environmental contaminant, in combination with potential TCE metabolism modulators, including N-acetyl-L-cysteine (NAC) [26-31] and aminooxyacetic acid (AOAA) [29,30,31], according to Figure 1. Samples from all experimental groups were included and appropriately analyzed to ensure treatments did not affect sex classification. Because mRNA is transcribed from DNA, we hypothesize that Sry detection from mRNA and gDNA will be congruent and successfully differentiate between male and female fetuses.

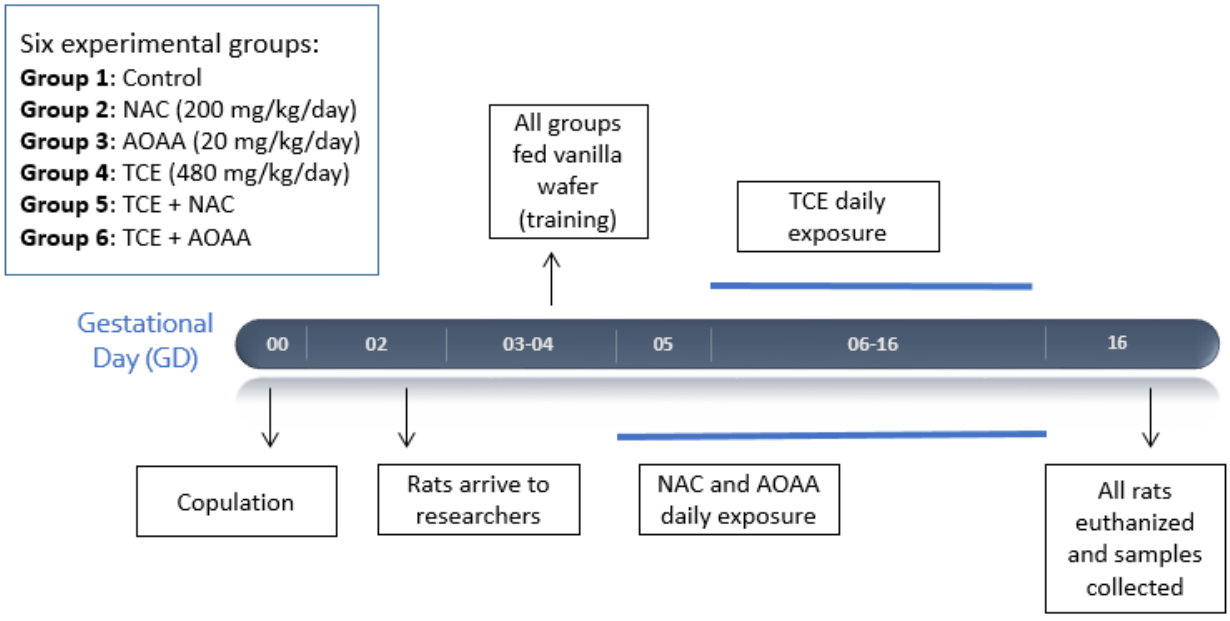

Figure 1: Treatment schedule of the timed-pregnant Wistar rats. With gestational day (GD) 0 designated as day of copulation, rats arrived on GD 2, were trained to eat the vanilla wafer on GD 3, and were euthanized on GD16. The following dosages of each chemical were used: 200 mg NAC/kg/day, 20 mg AOAA/kg/day, 480 mg TCE/kg/day.

Materials and Methods

Chemicals and reagents

TCE, NAC, and AOAA (purchased as O-(Carboxymethyl)-hydroxylamine hemihydrochloride) were from Sigma-Aldrich (St. Louis, MO). Vanilla wafers (Nabisco) were purchased locally.

Timed-pregnant Wistar rats

Timed-pregnant Wistar rats between 60 to 90 days of age were purchased from Charles River Laboratories (Portage, MI). The day of copulation was designated asgestational day (GD) 0. The rats were transported to the University of Michigan School of Public Health animal facility on GD 2. Rats were individually housed in a controlled environment with a 12-hour light/dark cycle and provided with standard rat chow (Purina 5001) and water ad libitum.

Exposures

The placental samples used in this analysis were obtained from a toxicology study in which rats were exposed to TCE alone or in combination with chemical modulators of TCE metabolism as shown in Figure 1 using the vanilla wafer method of exposure developed by Seegal et al. [32]. The TCE dosage of 480 mg/kg/day was chosen because similar dosages induce oxidative stress and selective neurodegeneration in rats [33-35]. Importantly, one of those studies used timed-pregnant Wistar rats exposed from GD 6 to GD 16 [34]. The dosage of 480 mg/kg/day is also within an order of magnitude of the Occupational Safety and Health Administration (OSHA) Permissible Exposure Level (PEL) for inhalation exposure over an eight-hour work day [36]. The NAC dosage of 200 mg/kg/day was chosen because that is representative of effective in vivo repeated NAC exposures [37-39], including one study performed on pregnant Sprague-Dawley rats [37]. The AOAA dosage of 20 mg/kg/day is a moderate value in the range of effective AOAA dosages performed in prior in vivo rat studies [40, 41]. Assignment of rats to treatment group within each arrival (batch) was done randomly and is indicated in (Supplementary Table 1). This research was approved under the Animal Welfare Assurance Number A3114-01, andthe IACUC approval number PRO00006721.

Placental sample collection

Placental samples were collected for RNA and gDNA extraction on GD 16.For RNA extraction, placental samples obtained after rat euthanasia were stored in RNAlater (Qiagen, Germantown, MD) overnight at 4°C. The next day, the RNA later was removed, and the placenta samples were stored at -80°C until RNA extraction. For gDNA extraction, the placental samples were snap-frozen in liquid nitrogen and then archived at -80°C until gDNA extraction.

Extraction of gDNA and RNA

The gDNA was extracted from placental tissue using a NucleoSpinTM Tissue kit (Machery-Nagel, Bethlehem, PA) according to the manufacturer's instructions. To extract RNA, placental tissue was first homogenized using a FastPrep-24 tissue and cell lyser (MP Biomedicals, Solon, OH). Homogenization occurred in RLT Buffer PLUS (Qiagen, Germantown, MD) containing 1% (v/v) 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Homogenized placenta was then subject to RNA extraction using an RNeasyPlus Mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Concentration and purity of DNA and RNA were verified using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). DNA was stored at -20°C and RNA was stored at -80°C until further analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR) of Sry

To synthesize cDNA from the RNA samples, an iScriptTMcDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) was used with a Bio-Rad CFX ConnectTM Real-Time System according to the manufacturer's protocol. The protocol on the Real-Time System was as follows: (1) 25°C for 5 minutes, (2) 42°C for 30 minutes, (3) 85°C for 5 minutes, and (4) cool down to 4°C. The cDNA was stored at -20°C until further use. The gDNA extracted from placenta was diluted in elution buffer (5 mMTris/HCl, pH 8.5) as appropriate to ensure that a given volume resulted in the same mass of gDNA added into a given qRT-PCR mixture.

qRT-PCR was performed using a total 25 µL mixture consisting of the following: 60% (v/v) SsoAdvancedTM Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.32 µM of each (forward and reverse) primer, and either 40 ng of cDNA template or 50 ng of gDNA. Beta-2-microglobulin (B2m) served as the reference gene. Primer sequences and sources are listed in (Supplementary Table 2). All primers were synthesized and made by Integrated DNA Technologies(Skokie, IL).

Samples were analyzed in Hard-Shell® 96-well plates (Bio-Rad Laboratories, Hercules, CA) using a Bio-Rad CFX ConnectTM Real-Time System with the following protocol: (1) 95°C for 10 minutes, (2) 95°C for 15 seconds, (3) 60°C for 1 minute, (4) repeat 39 times steps 2 and 3, (5) 95°C for 1 minute, (6) 65°C for 2 minutes, (7) 65°C to gradual increase to 95°C, stopping at every 0.5°C interval for 5 seconds each. Analysis was performed using the ΔΔCt method [42], and all samples were run and analyzed in duplicate. From this procedure, we obtained Cq (also known as Ct) values [43] (and SEM), which are proportional to the number of amplification cycles necessary to achieve a threshold fluorescence and inversely proportional to the logarithm (base 2) of cDNA or gDNA template quantity [42]. We also obtained Sry(Sry/B2m) values for each sample. Although these normalized values are traditionally referred to as expression values in the case of mRNA [42], because gDNA is not expressed [44], these values will be referred as Sry (Sry/B2m) values or Sry/B2m abundance values when gDNA is included in the relevant text. Unlike the SryCq value, these Sry (Sry/B2m) values for both gDNA and mRNA take normalization by B2m into account.

Statistical analysis

Because each sample was run in duplicate, mean Sry and B2mCq values and Sry expression values were obtained for each sample. Statistical analysis was performed on the mean Cq and expression values. The SEM of Cq and expression values are displayed in Supplementary Tables 3 and 4 and served to ensure accuracy of pipetting. Rat 17, Placenta 3, which had a strong Sry signal from gDNA analysis and no Sry signal from mRNA analysis, was excluded from all statistical analysis requiring sex classification. Statistical tests included student's two-tailed unpaired t-test, two-tailed Mann-Whitney test, and one-way ANOVA followed by Tukey's post-hoc comparison of means, as appropriate. The Mann-Whitney test was selected as a non-parametric test for data that failed to meet assumptions for parametric analysis based on its suitability for analysis of data sets with tied and zero values. Statistical tests are described in figure legends, and $p<0.05$ was considered statistically significant. SryCq values ≥ 40 automatically received a corresponding zero, or N/A, Sry/B2m value.

Results

Use of Sry (Sry/B2m) values to identify sex in placental gDNA and mRNA

The sex determined through Sry(Sry/B2m) mRNA expression matched the sex determined by Sry(Sry/B2m) valueing DNA for 90 out of 91 samples (Supplementary Table 3). There was one exception, which was positive for Sry in gDNA but negative for Sry mRNA expression (Rat 17, Placenta 3). Because we could not classify the latter placenta as male or female, this placenta was excluded from all subsequent statistical analyses comparing values between sexes. Among the 90 samples with concordant SrygDNA and mRNA findings, gDNASry(Sry/B2m) values were 832.0% higher in males compared to females ($p<0.0001$) (Table 1). Similarly, mRNA Sry (Sry/B2m)expression values were all above zero for males but were all zero, resulting from SryCq values all ≥ 40, for females ($p<0.0001$). Furthermore, for both gDNA and mRNA analyses, the Sry(Sry/B2m) value range for female did not overlap with the Sry(Sry/B2m) value range for male. Implicit from the lack of overlapping values, cut-offs could be assigned (Males: >0.05 and Females: <0.05 in the case of gDNA; Males: >0.01 and Females <0.01 in the case of mRNA) though these cannot be assumed to be universal for all studies.

Analysis	Sex	Mean ± SEM	Range	Cut-off	*p* value
gDNA	Male	1.362 ± 0.1323	0.10017 to 6.36962	>0.05	<0.0001

gDNA	Female	$1.635 \times 10^{-3} \pm 4.868 \times 10^{-4}$	N/A (or 0)[b] to 0.01917	<0.05	
mRNA	Male	1.716 ± 0.4422	0.07279 to 20.19149	>0.01	<0.0001
mRNA	Female	N/A (or 0)	N/A (or 0)[b] to N/A (or 0)[b]	<0.01	

Table 1: Sex determination for rat placenta based on Sry (Sry/B2m) values for gDNA and mRNA analyses with all treatment groups combined. [a]Descriptive statistics are shown and highlight that no values overlapped between males and females. Hence, cut-offs were determined as indicated. Two-tailed Mann-Whitley tests were used to determine p values in the male and female comparisons. N=46 and 44 for male and female samples, respectively, for both gDNA and mRNA samples. [b]A value of N/A (or 0) is derived from the corresponding SryCq value of ≥ 40.

Analyses of Sry (Sry/B2m) values across and by treatment groups for gDNA and mRNA

Because the placentas were collected from rats receiving different treatments, we also analyzed the results by treatment group to ensure that treatments did not affect sex classification. Using the sex classified by both gDNA and mRNA, Table 2 contained the breakdown of sex as a function of treatment groups. Each treatment group contained at least six males and at least four females. Within treatment group, the differences in Sry(Sry/B2m) values between males and females were significant for all comparisons (p value range of 0.0001 to 0.0095 for gDNA and p value range of <0.0001 to 0.0095 for mRNA) (Table 3).

Therefore, Sry(Sry/B2m) sex differences remained significant when analyzing by treatment group with decreased sample size, suggesting the suitability of using Sry(Sry/B2m)values for determining sex for subsets of a population, as well. Additionally, no statistically significant differences were detected in Sry(Sry/B2m) values when investigating across treatment groups for a given sex for both mRNA and gDNA (Figures 2A-2C).

	Number of placenta (Number of dams)	
Treatment	**Male**	**Female**
Control	6 (4)	12 (4)
NAC	10 (4)	6 (4)
AOAA	6 (3)	4 (3)
TCE	9 (4)	7 (4)
TCE+NAC	8 (4)	8 (4)
TCE+AOAA	7 (4)	7 (4)
Total	46 (23)	44 (23)

Table 2: Distribution of rat placenta by treatment group and sex classification based on Sry/B2m.a[a]Each dam within a treatment group came from a different batch. For 90 total samples (46 male+44 female), sex determined *via* gDNA analysis matched the sex determined *via* mRNA analysis.

Analysis	**Treatment**	**Male Sry/B2m (Mean ± SEM)**	**Female Sry/B2m (Mean ± SEM)**	***p* value[a]**
gDNA	Control	1.420 ± 0.1460	$7.258 \times 10^{-4} \pm 2.951 \times 10^{-4}$	0.0001
gDNA	NAC	1.085 ± 0.1583	$3.752 \times 10^{-3} \pm 3.086 \times 10^{-3}$	0.0002
gDNA	AOAA	1.886 ± 0.9149	$9.275 \times 10^{-4} \pm 5.243 \times 10^{-3}$	0.0095
gDNA	TCE	1.673 ± 0.1342	$3.356 \times 10^{-3} \pm 1.022 \times 10^{-3}$	0.0002
gDNA	TCE+NAC	1.158 ± 0.1826	$1.199 \times 10^{-3} \pm 7.722 \times 10^{-4}$	0.0002
gDNA	TCE+AOAA	1.090 ± 0.1489	$5.600 \times 10^{-4} \pm 1.852\ 10^{-4}$	0.0006
mRNA	Control	1.867 ± 0.5535	0	<0.0001
mRNA	NAC	0.9734 ± 0.2341	0	0.0002
mRNA	AOAA	1.319 ± 0.4043	0	0.0095
mRNA	TCE	1.271 ± 0.3541	0	0.0002

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mRNA	TCE+NAC	4.441 ± 2.304	0	0.0002
mRNA	TCE+AOAA	0.4455 ± 0.08977	0	0.0006

Table 3: Sry (Sry/B2m) values within each treatment group for gDNA and mRNA analysis of rat placenta. [a]*p* values were computed using the two-tailed Mann-Whitney test to compare males and females. Sample sizes are shown in Table 2.

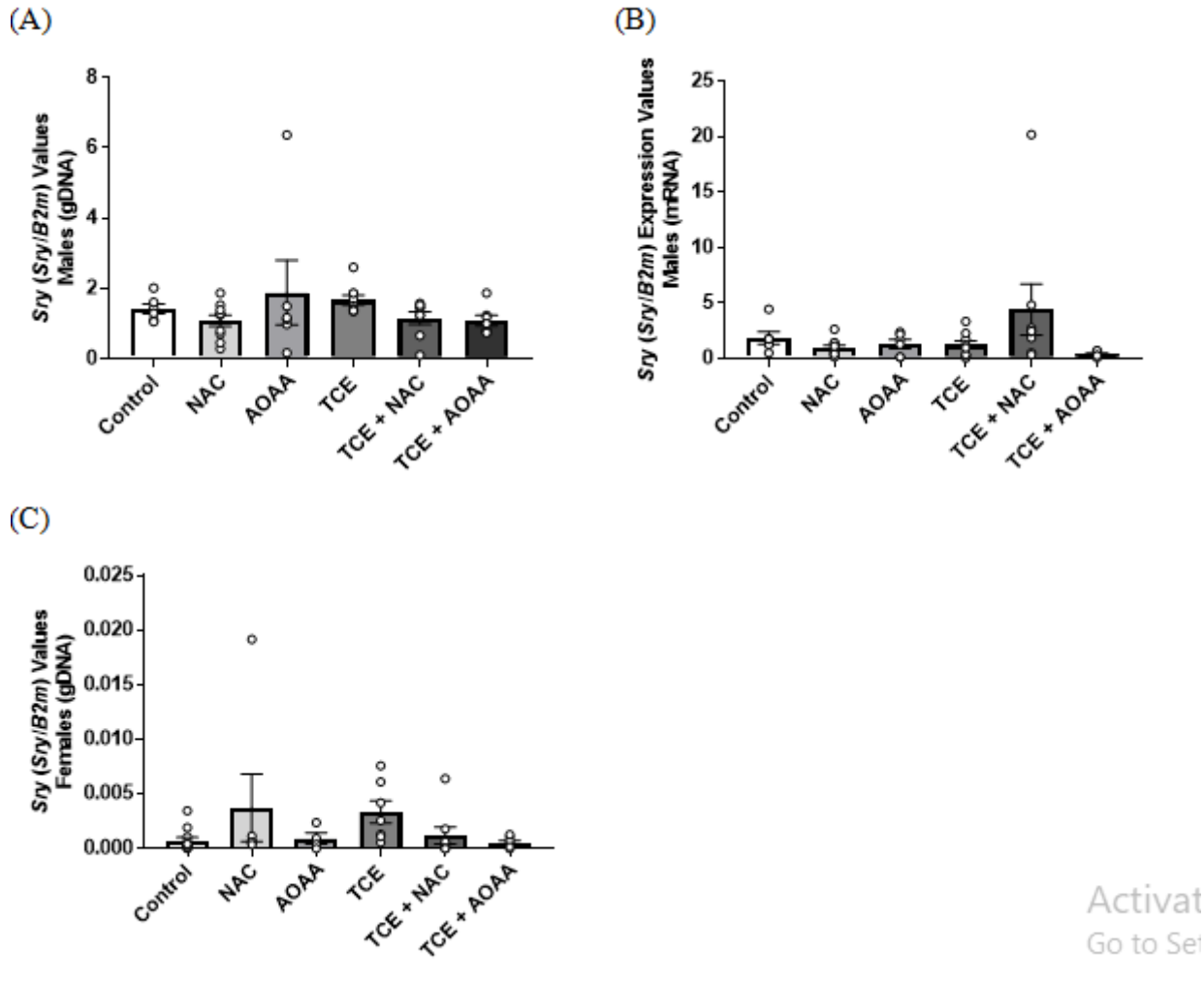

Figure 2: Sry/B2m values across different treatment groups separated by sex for gDNA and mRNA. Sry/B2m values for (A) male gDNA samples, (B) male mRNA samples, and (C) female gDNA samples from rat placenta. Note the different range of units of the y-axis across the different graphs and that no values between males and females overlapped. Statistically significant treatment effects were not detected with one-way ANOVA. Columns represent mean ± SEM, with individual data points shown. There is no graph for female mRNA because all female mRNA Sry/B2m values were zero. Sample sizes are shown in Table 2.

Use of SryCq values to identify sex for placental gDNA and mRNA

To provide insight for studies not using a traditional reference gene or using a reference gene other than B2m, we evaluated non-normalized SryCq mRNA and gDNA values as a metric for distinguishing between male and female samples, as well. As a method for classification of sex, this would require that the nucleic acids underwent accurate measurement and pipetting to assure equal amounts of genetic material in the sample wells.

Evaluation and analysis of SryCq values were performed on the basis of sex classification determined by the cut-offs described in Table 1. For mRNA, all classified female samples had SryCq values that were not determined (ND) (listed as "N/A" in the Bio-Rad CFX program and applied to sample readings ≥ 40) whereas all males had a defined

SryCq value (Table 4 and Supplementary Table 4). Although statistical analysis could not be performed because SryCq values were not determined for all females, there was an obvious difference between male and female SryCq values. In contrast, all males had an existent SryCqgDNA value and most females also had an existent SryCqgDNA value. A statistically significant difference was observed between males and the subset of females with SryCqgDNA values (excluding four females with undetermined Cq values) ($p<0.0001$). However, there was an overlap in range between male and female SryCq values for this gDNA analysis (the highest SryCq value for males was 31.08; the lowest SryCq value for females was 30.27, and three females had a SryCq value below 31.08) (Table 4). Thus, whereas SryCq values were observed to be different between males and females for both mRNA and gDNA,SryCq values without normalization to a reference gene could lead to false sex classification. Overall,SryCq values could benefit in accuracy from normalization or assessment of a secondary metric, although the reliability of SryCq value assessment was especially true in our mRNA analysis where all females had undetermined SryCq values.

Analysis	Sex	SryCq (Mean ± SEM)	SryCq Range	*p* value[b]
gDNA	Male	24.56 ± 0.3201	21.92 to 31.08	<0.0001
gDNA	Female	34.52 ± 0.3609[c]	30.27 to N/A[a]	
mRNA	Male	33.92 ± 0.2431	30.32 to 37.17	N/A[a]
mRNA	Female	N/A[a]	N/A[a]	

Table 4: Rat placental SryCqgDNA and mRNA non-normalized values with all treatment groups combined.[aa]The Bio-Rad CFX program denoted Cq values [b]ThegDNA data were analyzed using atwo-tailed unpaired t-test to compare male and female Cq values (excluding four female samples with undetermined SryCq values). For mRNA samples, there was no overlap between male and female Cq values, but statistical analysis was not performed because all female samples had undetermined SryCq values (. Sample sizes are indicated in Table 2.[c]This particular mean ± SEM calculationexcluded the four female samples with undetermined SryCq values.

Analyses of SryCq values across and by treatment group for gDNA and mRNA

Analyses of SryCq within and across treatment groups were conductedto determine the suitability of SryCq values for sex determination specific to treatment groups. Because all females had undetermined SryCq mRNA values and all males had defined SryCq mRNA values, this was true for any given treatment group, also (Table 5), and the SryCq mRNA values classified males and females for all treatment groups consistent with Sry (Sry/B2m) analysis. For gDNA samples, differences between sex were statistically significant for all treatment groups (p value range from <0.0001 to 0.0066; Table 5). Furthermore, the mRNA-derived SryCq values for male samples did not statistically differ across treatment groups (Figure 3B). However, several significant differences were observed between treatment groups for non-normalized male and female SryCqgDNA values (Figures 3A and 3C, respectively), in contrast to the lack of treatment-related differences when treatment groups were combined (Table 4). Nonetheless, sex differences within each of the treatment groups (Table 5) were still observed. This further indicates that investigation of Sry data normalized to B2m (i.e., using a reference gene), in which no statistical differences were observed (Figures 2A-2C), may be more suitable for avoiding treatment-related discrepancies in sex classification.

Analysis	Treatment	Male SryCq Value (Mean ± SEM)	Female SryCq Value (Mean ± SEM)	*p* value[b]
gDNA	Control	23.18 ± 0.2562	34.89 ± 0.5491	<0.0001
gDNA	NAC	24.70 ± 0.4034	34.55 ± 1.031	<0.0001
gDNA	AOAA	26.98 ± 1.136	34.70 ± 1.768[c]	0.0066
gDNA	TCE	22.51 ± 0.1446	32.02 ± 0.4172	<0.0001
gDNA	TCE+NAC	25.53 ± 0.9616	35.32 ± 1.011[d]	<0.0001
gDNA	TCE+AOAA	25.03 ± 0.488	35.76 ± 0.735[c]	<0.0001
mRNA	Control	34.05 ± 1.013	N/A[a]	N/A[a]
mRNA	NAC	33.97 ± 0.5436	N/A[a]	N/A[a]
mRNA	AOAA	33.36 ± 0.5146	N/A[a]	N/A[a]
mRNA	TCE	34.00 ± 0.3888	N/A[a]	N/A[a]
mRNA	TCE+NAC	33.76 ± 0.5704	N/A[a]	N/A[a]

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mRNA	TCE+AOAA	34.28 ± 0.7675	N/A[a]	N/A[a]

Table 5: Rat placental SryCq values within each treatment group for non-normalized gDNA and mRNA. [aa]The Bio-Rad CFX program denoted Cq values .[b]p values comparing males to females were computed using unpaired two-tailed t-tests. Sample sizes are shown in Table 2.[c]These particular mean ± SEM calculations excluded one female sample with undetermined SryCqvalues. [d]This particular mean ± SEM calculation excluded two female samples with undetermined SryCq values.

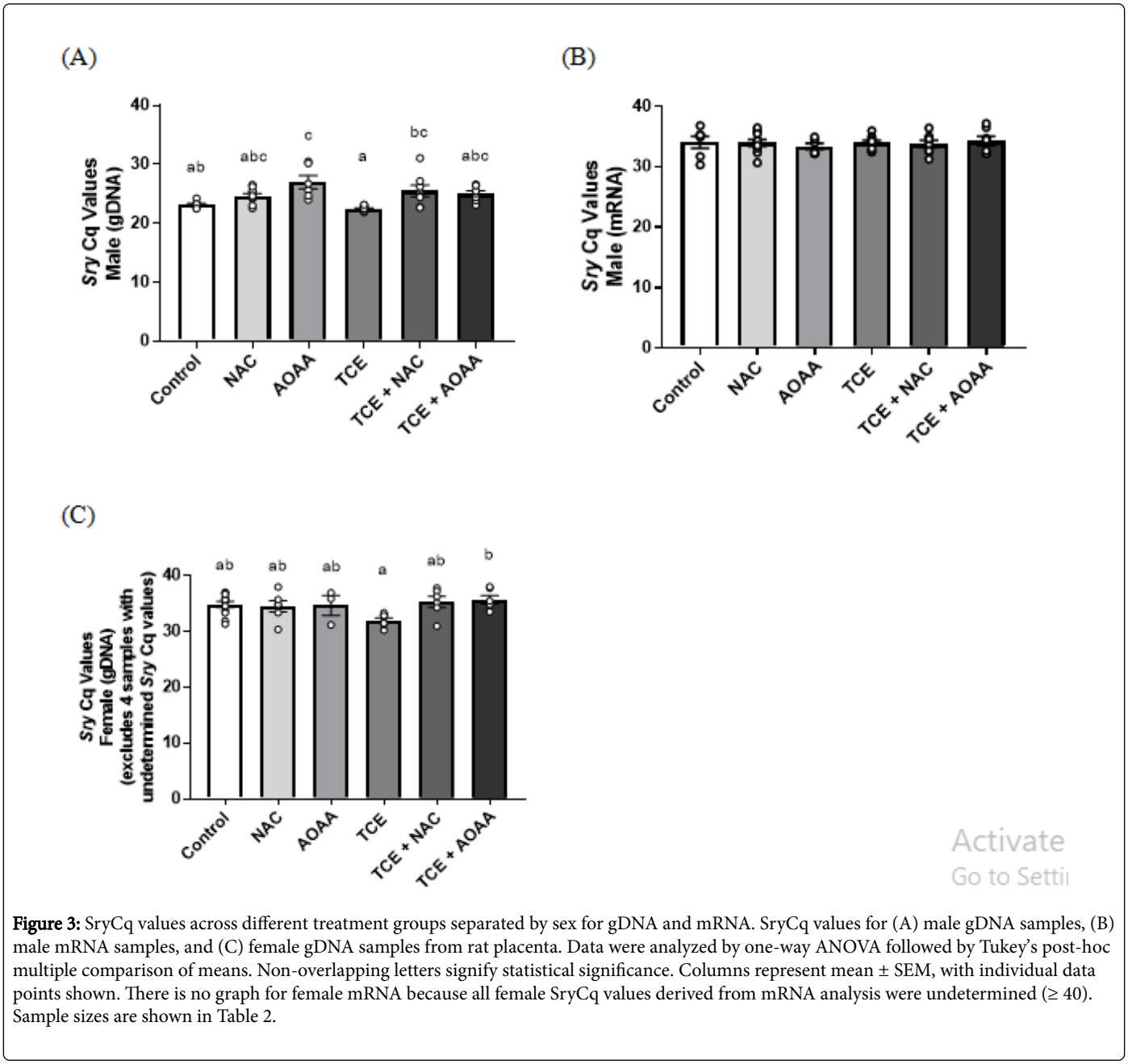

Figure 3: SryCq values across different treatment groups separated by sex for gDNA and mRNA. SryCq values for (A) male gDNA samples, (B) male mRNA samples, and (C) female gDNA samples from rat placenta. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc multiple comparison of means. Non-overlapping letters signify statistical significance. Columns represent mean ± SEM, with individual data points shown. There is no graph for female mRNA because all female SryCq values derived from mRNA analysis were undetermined (≥ 40). Sample sizes are shown in Table 2.

The validity of B2m as a reference gene

B2m was used as the reference gene in this project. Presence of B2m ensured that nucleic acid was present in the samples and that lack of SryCq or expression was not due to degraded nucleic acid but rather because the sample was female. We obtained strong Cq values for B2m for all samples, particularly in the case of mRNA where the Cq for B2m averaged 19.94 (the Cq for B2m in the case of gDNA analysis averaged 25.37). No significant differences between sexes were observed for B2mCqgDNA or mRNA values whether combined or separated by treatment group (Tables 6 and 7, respectively). Supplementary Table 4 contains the results by individual sample.

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Analysis	Sex	SryCq (Mean ± SEM)	SryCq Range	*p* value[a]
gDNA	Male	25.58 ± 0.2372	23.44 to 30.44	0.1879
gDNA	Female	25.16 ± 0.2108	22.71 to 28.34	
gDNA	Male and Female	25.37 ± 0.1598	22.71 to 30.44	
mRNA	Male	19.79 ± 0.1734	17.87 to 23.42	0.2417
mRNA	Female	20.11 ± 0.2103	18.13 to 24.19	
mRNA	Male and Female	19.94 ± 0.136	17.87 to 24.19	

Table 6: Rat placental B2mCq values for gDNA and mRNA with all treatment groups combined.[a]*p* values were computed using the two-tailed Mann-Whitney test to compare males and females. Sample sizes are shown in Table 2.

Analysis	Treatment	Male B2m Cq Value (Mean ± SEM)	Female B2m Cq Value (Mean ± SEM)	*p* value[a]
gDNA	Control	24.51 ± 0.2894	24.08 ± 0.2391	0.2996
gDNA	NAC	25.67 ± 0.3888	25.34 ± 0.4829	0.6062
gDNA	AOAA	27.32 ± 0.8443	26.31 ± 1.009	0.4663
gDNA	TCE	24.03 ± 0.1258	24.11 ± 0.2303	0.7481
gDNA	TCE+NAC	26.18 ± 0.6365	26.11 ± 0.4115	0.9316
gDNA	TCE+AOAA	26.17 ± 0.2715	26.13 ± 0.3380	0.9178
mRNA	Control	20.94 ± 0.4439	20.36 ± 0.3831	0.3738
mRNA	NAC	19.31 ± 0.2810	19.73 ± 0.2664	0.3310
mRNA	AOAA	19.56 ± 0.1875	19.37 ± 0.2849	0.5635
mRNA	TCE	19.30 ± 0.2362	19.56 ± 0.1880	0.4332
mRNA	TCE+NAC	20.55 ± 0.6467	20.53 ± 0.7690	0.9893
mRNA	TCE+AOAA	19.43 ± 0.2447	20.47 ± 0.6602	0.1638

Table 7: Rat placental B2mCq values within each treatment group by sex for gDNA and mRNA. [a]p values were computed using the two-tailed Mann-Whitney test to compare males and females. Sample sizes are shown in Table 2.

We also investigated B2mCq values across treatment groups, separated by sex (Figure 4). Whereas multiple comparisons within gDNA analysis were statistically significant (Figures 4A and 4C), only one comparison within mRNA analysis was statistically significant (Figures 4B and 4D).Although these differences exist, they should not be overemphasized. Despite these differences among B2mCq values across treatment groups (particularly for gDNA more than mRNA), the Sry (Sry/B2m) values yielded classification of male versus female for both gDNA and mRNA analyses in which both analyses agreed with each other for 90 out of 91 samples. Although B2mCq differences could have contributed variability to theSry (Sry/B2m) values, because classification of sex based on Sry (Sry/B2m) values led to gDNA and mRNA agreement for 90 out of 91 samples with clear cut-offs, inclusion of B2m as a reference gene increased validity of sex classification based on Sry.

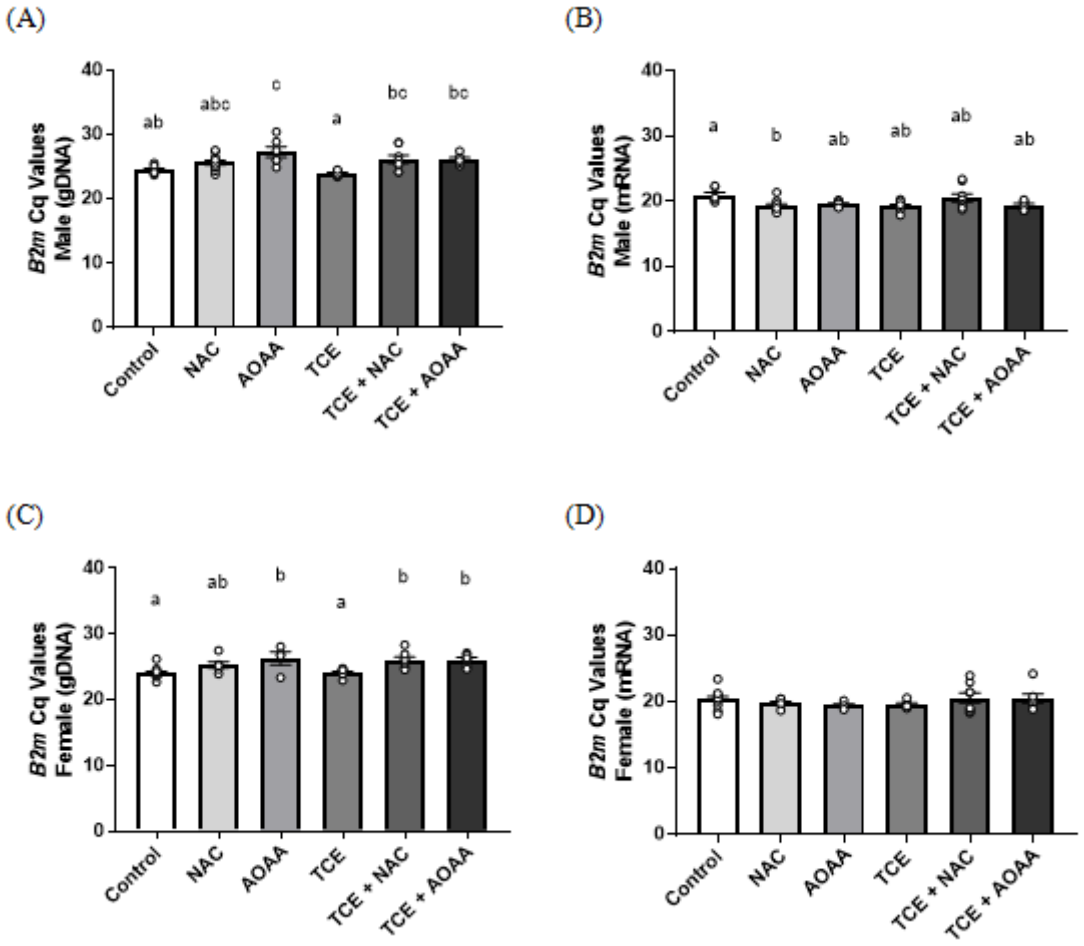

Figure 4: B2mCq values across different treatment groups separated by sex for gDNA and mRNA. B2mCq values for (A) male gDNA samples, (B) male mRNA samples, (C) female gDNA samples, and (D) female mRNA samples from rat placenta. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc multiple comparison of means. Statistical significance is denoted by non-overlapping letters. Columns represent mean ± SEM, with individual data points shown. Sample sizes are shown in Table 2.

Discussion

We identified qRT-PCR as a method for fetal sex determination producing remarkably similar results whether analyzing gDNA or mRNA for Sry. A strength of our study comes from assessment of Sry (Sry/B2m) values, SryCq values, and validity of our reference gene, B2m, for both gDNA and mRNA samples. This allowed inferences about the suitability of each metric, particularly Sry (Sry/B2m) values or SryCq values for sex determination, aspects of which have been used in sex determination from gDNA in prior reports.

The most useful metric used in the present study to determine sex was the Sry (Sry/B2m) metric. Assessment of this metric allowed sex classification in which the classification *via* gDNA matched the classification for mRNA for 90 out of 91 of the samples. For both mRNA and gDNA, no Sry (Sry/B2m) values classified as male overlapped those classified as female. We were also able to establish cut-offs of Sry (Sry/B2m) values by sex. Finally, the Sry (Sry/B2m) metric also benefits from assurance of being normalized to a reference gene.

The use of only SryCq values to evaluate sex has advantages and limitations. Xiang et al. (2016) evaluated the ratio of AR to Sry to determine sex in Wistar rats and observed that the SryCq values were much lower in males compared to females [19]. In our mRNA analysis, all our females had non-defined SryCq values (anything ≥ 40) whereas males had SryCq values ranging from 30.32 to 37.17 (Table 3), clearly distinguishing the difference between male and female placentas. The difference in gDNASryCq values between sexes was evident despite three females having a lower SryCq than the male with the highest SryCq value. Overall, SryCq mRNA values could be used to determine sex because these values were always undetermined for the female placentas.

Although B2M/B2m is a relatively common reference gene used for placental mRNA analysis [45-47], some considerations arose from our study. First, the most rigorous qRT-PCR experiments would benefit from use of three or more reference genes [48]. However, the quantity of samples we used and the analysis of both gDNA and mRNA limited our ability to include additional reference genes.

Additionally, because we were not primarily interested in the degree of Sry expression, the use of additional reference genes for this study was not as relevant as for an experiment assessing degree of gene expression. Secondly, although a few differences in B2mCq values were detected between treatment groups, particularly for gDNA, our B2mCq values were strong. Furthermore, our use of Sry (Sry/B2m) values, which were normalized to B2m, resulted in sex classification in which mRNA and gDNA analysis agreed for 90 out of 91 samples.

A strength of our study is the analysis of samples with treatment groups combined as well as separated by treatment group. This allowed us to determine that the Sry (Sry/B2m) and SryCq value differences were upheld for the totality of samples and by treatment group. Therefore, not only do we recommend the use of Sry (particularly (Sry/B2m)) values from either gDNA or mRNA analyses in determining fetal sex, but we also suggest that Sry values from either gDNA or mRNA analyses are relevant to both a population with varied treatments and a more homogenous population. Importantly, this is of interest to researchers determining placental sex in a population that is totally unexposed or another more homogenous population compared to the totality of the samples we used in our analysis. A worthy future direction could be to see the applicability of this in different strains of rats (i.e., Wistar rats versus Sprague-Dawley rats) or different species (i.e., mice).

One of our placentas had a strong Sry signal from gDNA analysis but lacked a Sry signal from mRNA analysis. The possibility exists that the gDNA corresponding to Sry had not been transcribed to mRNA at the time of rat euthanasia, GD 16. Alternatively, the placenta may have lacked crucial components of the machinery required to transcribe SrygDNA into mRNA. A less likely explanation is that only a portion of the placenta contained SrygDNA or mRNA; that is, the distribution of Sry across given placenta may have been uneven. However, we analyzed separately various portions of the stored placenta for gDNA and mRNA and arrived at the same classification each time. Additionally, because this placenta was the only placenta out of 91 placentas in which sex was not definitively assigned because of differing gDNA and mRNA analysis classifications, the aforementioned phenomenon is unlikely to be widespread in Wistar rat SrygDNA or mRNA.

The current study is not without limitations. The first limitation is that the current study investigated placental gDNA and mRNA at only one time point, GD 16. In mice, Sry mRNA varies in abundance as a function of gestational age [23]. Particularly, in a pool of mice gonadal tissue, Sry mRNA expression for male samples are at a low level on 10.5 days post coitum (DPC), peak on 11.5 DPC, and decreases back to a low level on 12.5 DPC [23]. The second limitation is that the current study used only two genes, Sry and B2m. To improve rigor and reproducibility, further studies could include additional genes with relevance to both males and females, such as Kdm5c and Kdm5d, and additional gestational ages.

Conclusion

We established that gDNA or mRNA from placenta of Wistar rats at GD 16 allowed similar sex classification when assessing Sry (Sry/B2m) values. This was true whether the samples were separated by treatment group or not. Assessment of SryCq values by themselves indicated that the male values were significantly different from the female values regardless of separation by treatment group. However, because of some overlap between male and female SryCq values, limitations and alternative approaches should be kept in mind if using only the SryCq metric for sex determination. Assessment of B2mCq values indicated that our reference gene provided strong and low Cq values, particularly for mRNA. As a whole, we have established that sex classification *via* Sry mRNA, which has not been assessed in conjunction with SrygDNA previously, was a valid method of sex classification compared with SrygDNA, the method used more extensively in sex determination in prior studies.

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Competing Interests Statement

The authors have no competing interests to declare.

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