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Research Article

Sperm Sex Ratio (X: Y Ratio) and its Variations

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Abstract

Sex ratio can be studied at various levels viz., at the time of spermatogenesis i.e. ratio of X and Y bearing sperm (pre-zygotic sex ratio) or at the time of fertilization/early preimplantation embryo (close to primary sex ratio or post-zygotic sex ratio) or at the time of birth (secondary sex ratio). The natural sex ratio at the time of spermatogenesis is expected to be 1:1. In this study we have examined sex ratio in ejaculated spermatozoa (human) as well as epididymal sperm (mouse) to determine proportion of X and Y bearing sperm i.e., pre-zygotic sex ratio. We also examined effects of seasons (temperature; summer vs. winter), diet (vegetarian vs. non vegetarian), profession (professionals vs. laborer) on sperm (pre-zygotic) sex (X: Y) ratio of ejaculated sperm.

The sperm sex ratio was carried out on 813066 human spermatozoa and 10390 mouse spermatozoa. In human, we have found more (52%) X than Y (48%) bearing sperms (421531X: 391535Y or 1.07X: 1Y). In mouse also we observed preponderance of X (55.5%) as compared to Y (44.5%) bearing sperms (1.24X: 1Y). In all sub-groups (season, diet, profession) we have observed more X bearing sperm. Our observations at pre-zygotic sex ratio have shown skewed sex ratio towards female. A probable reason for this could be preferential elimination of Y bearing sperm. This is also supported by the evidence of more aneuploidy with Y-bearing spermatozoa (~1.5 times more with Y bearing sperms).

Keywords: Spermatozoa; Prezygotic sex ratio (X: Y ratio); Season-Diet-Profession Influence

Introduction

Sex ratio is defined as ratio of number of males to females. Worldwide, the human sex ratio at birth is fairly constant with male excess (51.4%) [1]. The natural sex ratio at the time of spermatogenesis is expected to be 1:1 (accordance with Mendelian segregation principle). Theoretically, offspring sex ratio may be attributed to events that occur before fertilization viz., sperm sex ratio or favor selection of Y or X chromosome bearing spermatozoa or events that occur after fertilization such as preferential survival of embryos of one sex or a combination. Sex ratio in sperm was studied initially by Y-body analysis or analysis of chromosome complements derived from fusion of human sperm with hamster oocytes. With this technique Barry Bean [2] demonstrated an excess of X-bearing sperm. However, Chernos and Martin [3] reported that the sex ratios did not differ significantly on fresh as well as cryopreserved sperm. With the recent advances in molecular technologies sexing of sperm is easily possible thus allowing us to study sperm sex ratio in large numbers of sperms rapidly and made possible to test the hypothesis that a sperm sex ratio underlies a live birth sex ratio bias.

Polymerase chain reaction (PCR) technique for sexing sperm was used initially by many [4,5]. Lobel et al. [4] had found a variation in Y chromosome bearing spermatozoa in humans from 41.9% to 56.7%. Chandler et al. [5] studied the variation in sex ratio in ejaculates in bulls and found that X & Y bearing spermatozoa are unequally distributed per ejaculate in a wave pattern. However with the application of X and Y chromosome FISH to sperm allowed us a more accurate assessment of the sex chromosomal complements [6]. In a sperm fluorescent in situ hybridization (FISH) study by Martin et al. [7] mean frequencies of X and Y bearing sperm was found to be 50.1% and 49.0%, respectively. In a study by Mercier et al. [8] on XYY male, an X: Y ratio of 0.78:1 was found in sperm with normal sex chromosome constitution. Similarly Griffin et al. [9] also reported equal Y and X bearing sperm. However, in contrast Halder and Tutscheck [10] was observed unequal sperm sex ratio, with an excess of X bearing sperm. They also observed higher segregation error of Y chromosome in comparison to X chromosome, thus reducing the number of normal Y bearing sperm further. Similarly, Spriggs et al. [11] identified a small but significant excess of X bearing sperm in a study with 50,000 sperm. Sex ratio distortion is also observed in bovine sperm [12], an excess of X bearing sperm (53%) than Y bearing sperm (46%). However, in a recent [13] Rhesus monkeys study did not find any significant difference between X and Y bearing sperm.

Due to above contradictory reports this study was undertaken to examine sex ratio in ejaculated spermatozoa (human) as well as epididymal sperm (mouse) to determine proportion of X and Y bearing sperm i.e., pre-zygotic sex ratio in a very large number of sperms (over 0.8 millions). We have also examined effects of seasons (temperature), diet & profession on sperm (pre-zygotic) sex ratio.

Material and Methods

Men with normal sperm count [14] were enrolled into the study between February 2008 and March 2011. Study group comprised of 50 subjects (80 samples) among which, 10 subjects were enrolled in season based study (provided semen samples 4 times). In season's category, semen sample was taken twice in summers (May and

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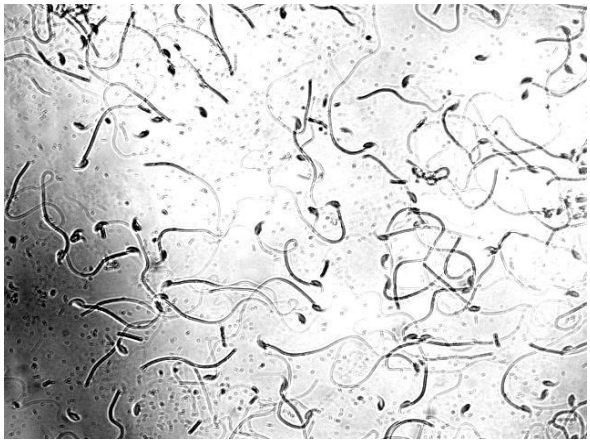

Figure 1: Photomicrograph showing mouse sperms.

August) as well as in winters (December and February). This was done to evaluate the change in sperm sex ratio at the beginning and end of a season and also to find variation in sperm sex ratio according to seasons (temperature). Twenty subjects were included in diet based category; out of them 10 were strict vegetarians and 10 were non-vegetarians (who eat meat, fish etc at least thrice in a week). Another twenty subjects were included for profession based category; out of them 10 were professionals and 10 were daily wage laborer. Age of the volunteers ranged from 21-45 years. Information & biological samples were collected from all cases as per prescribed proforma. In addition we also obtained epididymal sperms from 10 adult male mice.

Institute Animal Ethics Committee & Institute Human Ethics Committee clearance for human & mouse study were obtained (IHEC & IAEC letter nos. T-10/30.01.09 & 485/IAEC/09). Written consent was also obtained from all donors before obtaining semen samples for the study.

Preparation of sperm cells (human) for FISH

Liquefied semen sample was transferred to the 1.5ml micro centrifuge tube and is centrifuged to separate sperm cells and seminal plasma. In the sperm cells pellet 1 ml of phosphate buffer saline (PBS) was added. It was then centrifuged at 8000 rpm for 3mins. The supernatant was removed. This step was repeated 2 more times or when sperm pellet appears clean. Then 1 ml 50mM hypotonic solution (KCl) was added to the sperm pellet, mixed well and incubated at 37°C incubator for 45mins. It was then centrifuged again at 8000rpm for 3mins. About 1 ml fixative (3:1, methanol: acetic acid) was added to the pellet and vortexed and centrifuged at 8000 rpm for 3mins. The supernatant was removed. This procedure was repeated until pellet was whitish and then sperm cells were suspended in fixative and stored at -80°C.

Extraction of mouse spermatozoa

Male mouse (6-8 weeks old) issued from the animal experimental facility, AIIMS. Within 15-20mins of euthanasia epididymis was extracted and placed in petridish containing PBS. Under a zoom dissecting microscope cauda epididymis was gently incised at 2-3 points and left for 3-4mins. This caused release of all live sperms into PBS (Figure 1). PBS along with sperm was collected into 1.5 ml tube & centrifuged at 5000 rpm for 3mins. Supernatant discarded and pellet was given hypotonic treatment for 1 hour. This was followed by 2 washes of fixative and finally stored at -80°C till further use.

FISH (human sperm)

FISH was carried out using centromeric probes for chromosome X (red; Cy3 labeled) & Y (green; FITC labeled). Fixed sperm cell suspension was spinned at 5000 rpm for 5 min and supernatant was discarded. Cells were resuspended in a small volume of fresh fixative (3:1 methanol/acetic acid; amount depends on size of the pellet). About 10-15 µl of fixed cell suspension was dropped on the chilled clean glass slide, ensure nuclei spread and left to dry completely. Slide was then flooded for 10 seconds with 3:1 methanol/acetic acid (for fixation of the cell on the slide) and then 70% acetic acid for 1-2 min. Slide was then left to dry and checked under microscope to ensure presence of optimal density of nuclei. Slide was dehydrated in alcohol series (70%, 90% & 100% ethanol) three minutes in each and air dried at room temperature. Then slides (with sperm nuclei attached) were treated with pepsin (1%) in 0.01N HCL for 20 min at 37°C. Slides were then rinsed twice with bi-distilled water and once with PBS. Nuclei on slides then again fixed with 1% Para formaldehyde in PBS for 10 min at 4°C, rinsed twice with PBS and once with bi-distilled water. Slides were then dehydrated in alcohol series (70%, 90% & 100% ethanol) three minutes in each. About 3-5 µl probe mixture (1000 ng labeled probe in 10 µl hybridization buffer containing 60% formamide, 2X SSC, 10% dextran sulfate) were applied on slide containing test sample/cell, cell and probe DNA were denatured together at 76°C for 6 min. and then incubated overnight (18 hours) at 37°C in hybrite chamber.

The slides then washed for 2 minutes in 50 ml of solution containing 0.4XSSC/0.3% NP-40 at 72°C water-bath and for 1 minute in 50 ml of solution containing 2XSSC/0.1% NP-40 at room temperature. The slides were dehydrated in descending series of 70%, 90%, and 100% ethanol series 3mins each. Then 10 µl antifade solution (Vector, USA) containing DAPI 1 µg/ml 4´6 diaminidino-2-phenylindol (DAPI; Sigma USA; counter stain/nuclear stain) applied to the target area onto slide followed by coverslip. Excess of antifade was removed and sealed with nail varnish to avoid moisture. Then slides are viewed using appropriate filter set on Olympus BX

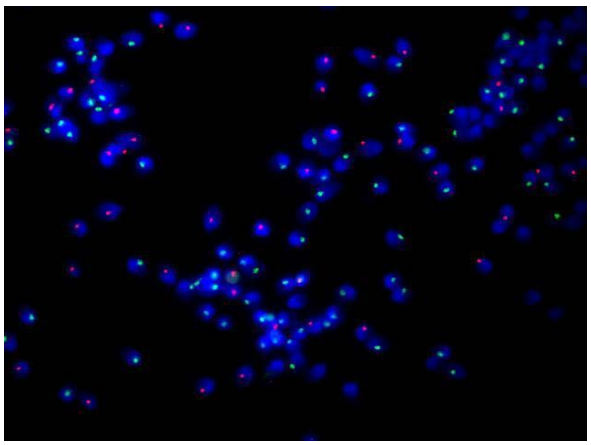

Figure 2: XY FISH on human sperms showing either Y (green) or X (red) sperms.

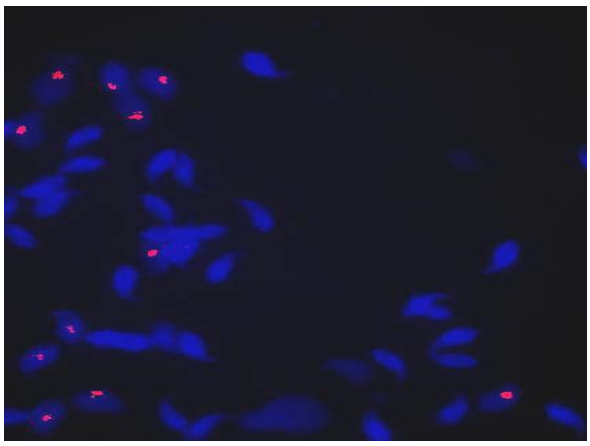

Figure 3: Chromosome Y FISH on mouse sperm showing Y (red signal) and X (no red signal) bearing sperms.

51 fluorescent microscope with a 100 watt mercury bulb using 100X plane appochromatic objective and single band pass filter for DAPI, FITC and TRITC/Cy3 and a dual band pass filter for TRITC and FITC (Olympus Japan). FISH images were captured using FISH Imaging System (Applied Spectral Imaging system & FISH view software version 4.5, Israel). Sperm with red signals were considered as Y bearing sperms and green signals were considered as X-bearing sperms (Figure 2).

FISH (mouse Sperm)

Same as human protocol except slides were incubated in 10mM DTT (Sigma) in a coplin jar for 30mins on ice followed by a dip in milli-Q water at room temperature to decondense sperm nuclei [15] and mouse Y chromosome probe was used. We performed chromosome Y probe-FISH (labeled with Cy3; red) for mouse study. The mouse X probe was excluded from the study due to difficulties in interpretation of results and frequent X probe FISH failure. Sperm with red signals were considered as Y bearing sperm and without any signals were considered as X-bearing sperm (Figure 3).

Results

This study was comprised of 50 subjects (80 semen samples) with normal sperm counts. Cases were divided into three categories as follows:

Seasonal study (temperature effects)

Winter season: samples collected twice in the month of December (beginning of winter) & February (end of winter) on 10 subjects (20 samples).

Summer season: samples collected twice in the month of May (beginning of summer) & August (end of summer) on same 10 subjects (20 samples).

Diet study

There were 10 subjects each from vegetarian diet group (10 samples) and non-vegetarian diet group (10 samples).

Profession

There were 10 subjects each from professional groups (doctor/ engineer; 10 samples) and laborers (daily wage laborer; 10 samples).

Table 1: Sex Ratio in beginning (December) and end (February) of winter season.

SN	Dec X	Dec Y	Total sperm	Dec X/Y	Feb X	Feb Y	Total sperm	Feb X/Y
1	5318	5090	10408	1.045	5125	4924	10049	1.041
2	5672	5844	11516	0.971	5127	4936	10063	1.039
3	5374	4881	10255	1.101	5259	4995	10254	1.053
4	5216	5218	10434	1	5215	4922	10137	1.06
5	5378	4920	10298	1.093	5184	4862	10046	1.066
6	5008	5008	10016	1	5175	4886	10061	1.059
7	5223	4827	10050	1.082	5386	5128	10514	1.05
8	5226	4799	10025	1.089	5214	4901	10115	1.064
9	5248	4812	10060	1.091	5411	4670	10081	1.159
10	5418	4700	10118	1.153	5314	4802	10116	1.107
Total	**53081**	**50099**	**103180**	**1.06**	**52410**	**49026**	**101436**	**1.069**

Statistical significance of Sperm FISH data for winter (n=10)

Month	X bearing sperm Median (min-max)	Y bearing sperm Median (min-max)	p-value	X/Y ratio Median (min-max)
Dec	5283 (5008-5672)	4900.5 (4700-5844)	**0.0072**	1.086 (0.97-1.15)
Feb	5214 (5125 - 5411)	4911.5 (4670-5128)	**0.0084**	1.06 (1.039-1.159)
p-value	0.1736	0.879		0.9397

(Analysis by Wilcoxon rank-sum (Mann-Whitney) test and Wilcoxon signed-rank test)

Table 2: Sex Ratio in the beginning (May) and end (August) of summer season.

SN	May X	May Y	Total sperm	May X/Y	Aug X	Aug Y	Total sperm	Aug X/Y
1	5313	4953	10266	1.073	5471	4699	10170	1.164
2	5296	4890	10186	1.083	5314	5222	10536	1.018
3	5229	4831	10060	1.082	5030	4999	10029	1.006
4	5231	4774	10005	1.096	5121	4901	10022	1.045
5	5193	5041	10234	1.03	4982	**5070**	10052	0.983
6	5282	4847	10129	1.09	5440	4862	10302	1.119
7	5383	4824	10207	1.116	5143	4897	10040	1.05
8	5446	4862	10308	1.12	5222	4816	10038	1.084
9	5334	4800	10134	1.111	5223	4999	10222	1.045
10	5439	4632	10071	1.174	5190	4899	10089	1.059
Total	**53146**	**48454**	**101600**	**1.097**	**52136**	**49364**	**101500**	**1.056**

Statistical significance of Sperm FISH data for summer (n=10)

Month	X bearing sperm Median (min-max)	Y bearing sperm Median (min-max)	p-value	X/Y ratio Median (min-max)
May	5304.5 (5193-5446)	4839 (4632-5041)	**0.0051**	1.093 (1.03-1.174)
Aug	5206 (4982-5471)	4900 (4699-5222)	**0.0093**	1.0475 (0.983-1.164)
p-value	0.0821	0.0887		0.0587

(Analysis by Wilcoxon rank-sum (Mann-Whitney) test and Wilcoxon signed-rank test)

Statistical significance of Sperm FISH data for total winters and summers (n=20)

Season	X bearing sperm Median (min-max)	Y bearing sperm Median(min-max)	p-value	X/Y ratio Median (min-max)	X/X+Y Median (min-max)
Winter	10585.5 (10183-10799)	9885 (9482-10780)	**0.0051**	1.071 (1.002-1.129)	0.517 (0.5-0.53)
Summer	10583.5 (10175-10784)	9693.5 (9531-10112)	**0.0051**	1.0825 (1.006-1.117)	0.52 (0.502-0.528)
p-value	0.8206	0.3256		0.4270	0.3841

(Analysis by Wilcoxon rank-sum (Mann-Whitney) test and Wilcoxon signed-rank test)

Table 3: Sperm Sex Ratio calculated for Vegetarian diet.

Samples	X bearing sperm	Y bearing sperm	Total sperm	X/Y ratio
1	5375 (53.3%)	4709 (46.7%)	10084	1.14
2	5591 (53.4%)	4887 (46.6%)	10478	1.14
3	5221 (52%)	4817 (48%)	10038	1.08
4	5246 (51.8%)	4873 (48.2%)	10119	1.08
5	5284 (52.4%)	4794 (47.6%)	10078	1.10
6	5437 (52.1%)	4989 (47.9%)	10426	1.09
7	5237 (52.1%)	4822 (47.9%)	10059	1.09
8	5137 (51.2%)	4898 (48.8%)	10035	1.05
9	5222 (51%)	5010 (49%)	10232	1.04
10	5331 (52.5%)	4827 (47.5%)	10158	1.10
Total	**53081 (52.19%)**	**48626 (47.81%)**	**101707**	**1.09**

Table 4: Sperm Sex Ratio calculated for Non-Vegetarian diet.

Samples	X bearing sperm	Y bearing sperm	Total sperm	X/Y ratio
1	5290 (52.5%)	4794 (47.5%)	10084	1.10
2	5429 (51.5%)	5107 (48.5%)	10536	1.06
3	5171 (51.4%)	4887 (48.6%)	10058	1.06
4	5262 (52.5%)	4759 (47.5%)	10021	1.11
5	5200 (51.2%)	4948 (48.8%)	10148	1.05
6	5123 (51.2%)	4888 (48.8%)	10011	1.05
7	4998 (49.9%)	5010 (50.1%)	10008	1.00
8	5211 (51.5%)	4899 (48.5%)	10110	1.06
9	5335 (52.5%)	4822 (47.5%)	10157	1.11
10	5246 (51.8%)	4888 (48.2%)	10134	1.07
Total	**52265 (51.6%)**	**49002 (48.4%)**	**101267**	**1.066**

Statistical significance of Sperm FISH data for diet (n=20)

Diet type	X bearing sperm Median (min-max)	Y bearing sperm Median (min-max)	p-value	X/Y ratio Median (min-max)
Vegetarian	5265 (5137-5591)	4850 (4709-5010)	**0.0051**	1.088 (1.042-1.144)
Non-Vegetarian	5228.5 (4998-5429)	4888 (4759-5107)	**0.0069**	1.0635 (0.998-1.106)
p-value	0.2121	0.3634		0.2263

(Analysis by Wilcoxon rank-sum (Mann-Whitney) test and Wilcoxon signed-rank test)

Table 5: Sperm Sex Ratio calculated for Professions.

Professional	X bearing sperm	Y bearing sperm	Total sperm	X/Y ratio
1	5130 (51%)	4921 (49%)	10051	1.04
2	5231 (51.6%)	4911(48.4%)	10142	1.07
3	5111 (50.9%)	4923 (49.1%)	10034	1.04
4	5176 (51.5%)	4880 (48.5%)	10056	1.06
5	5335 (52.5%)	4834 (47.5%)	10169	1.10
6	5246 (51.7%)	4901 (48.3%)	10147	1.07
7	5375 (53.3%)	4708 (46.7%)	10083	1.14
8	5437 (53.6%)	4700 (46.4%)	10137	1.16
9	5591 (54.3%)	4702 (45.6%)	10293	1.19
10	5223 (52.1%)	4811 (47.9%)	10034	1.09
Total	**52855 (52.26%)**	**48291 (47.74%)**	**101146**	**1.09**

Sex chromosome specific (XY) Fluorescent In-Situ Hybridization (FISH) was carried out in all cases using centromeric chromosome X and Y probes (Figure 2). About 10,000 sperm nuclei were counted in each semen samples and X/Y ratio (sex ratio) was calculated. Overall sex ratio was observed as 1.076:1. Tables 1-7 are showing details of FISH results in various groups.

We observed that the number of X bearing sperms were significantly more than the number of Y bearing sperm in all groups and irrespective of subgroups viz., winters or summer (p=0.0051). We did not find any statistically significant difference in median values of X/Y ratio between summer and winter (p=0.4270). However, we observed more X bearing sperm in winter, although statistically insignificant (p=0.8206).

Table 6: Sperm Sex Ratio calculated for Laborers.

Laborers	X bearing sperm	Y bearing sperm	Total sperm	X/Y ratio
1	5339 (52.2%)	4894 (47.8%)	10233	1.09
2	5211 (51.6%)	4882 (48.4%)	10093	1.07
3	5340 (53.3%)	4680 (46.7%)	10020	1.14
4	5010 (50.1%)	4998 (49.9%)	10008	1.00
5	5200 (51.2%)	4950 (48.8%)	10150	1.05
6	5171 (51.6%)	4859 (48.4%)	10030	1.06
7	5284 (52.3%)	4822 (47.7%)	10106	1.10
8	5331 (52.5%)	4823 (47.5%)	10154	1.11
9	5234 (52.1%)	4820 (47.9%)	10054	1.09
10	5437 (52.2%)	4988 (47.8%)	10425	1.09
Total	**52557 (51.8%)**	**48716 (48.2%)**	**101273**	**1.08**

Statistical significance of Sperm FISH data for Profession (n=20)

Professions	X bearing sperm Median (min-max)	Y bearing sperm Median (min-max)	p-value	X/Y ratio Median (min-max)
Professionals	5239 (5111-5591)	4857 (4700-4923)	**0.0051**	1.078 (1.038-1.189)
Laborers	5259 (5010-5437)	4871 (4680-4998)	**0.0051**	1.088 (1.002-1.141)
p-value	0.8501	0.4963		0.7913

(Analysis by Wilcoxon rank-sum (Mann-Whitney) test and Wilcoxon signed-rank test)

Table 7: Showing statistical significance of total human sperm data analysis (n=80).

Total X bearing sperm (Mean ± SE)	Total Y bearing sperm (Mean ± SE)	p-value	X/Y ratio
421531 (7025.517 ± 323.980	**391535** (6525.583 ± 306.508)	**0.0001**	**1.076:1**

(Analysis by Paired t- test)

The number of X bearing sperm were found to be significantly more than the number of Y bearing sperm in both vegetarian (p=0.0051) and non-vegetarian diet group (p=0.0069). We observed more X bearing sperm in vegetarian than non-vegetarian diet group (p= 0.2121), but statistically not significant.

The number of X bearing sperm also were found to be significantly more than the number of Y bearing sperm in professional group (p=0.0051) as well as in laborer group (p=0.0051). However difference in median values of X/Y ratio between two subgroups were statistically insignificant (p=0.7913).

In mouse FISH study also we observed significantly more (p=0.0051) X bearing sperms than the number of Y bearing sperms (Table 8).

Discussion

The study on the pre-zygotic/sperm sex ratio was carried out on 813066 human spermatozoa and 10390 mouse spermatozoa. At the pre-zygotic level in human study, we have found more (52%) X than Y (48%) bearing sperms (421531X: 391535Y or 1.07X:1Y). We have also observed more X in almost all samples (76/80) irrespective of subgroups viz., season, diet or profession. In mouse (epididymal sperms) also we observed preponderance of X (55.5%) as compared to Y (44.5%) bearing sperms (1.24X:1Y). We have also taken into

Table 8: Y-Fish Result on 10 Adult Male Mice Sperm Samples.

Mice	X bearing sperm (sperm without Y signal)	No. of Y bearing sperm (sperm with Y signal)	Total sperm	X/Y
1	562 (55.4%)	452 (44.6%)	1014	1.24
2	589 (55.3%)	476 (44.7%)	1065	1.24
3	594 (56%)	466 (44%)	1060	1.27
4	581 (55.8%)	460 (44.2%)	1041	1.26
5	587 (56.6%)	451 (43.4%)	1038	1.3
6	578 (56.3%)	449 (43.7%)	1027	1.29
7	567 (55.75%)	450 (44.25%)	1017	1.26
8	545 (53.1%)	481 (46.9%)	1026	1.133
9	569 (54.4%)	476 (45.6%)	1045	1.19
10	590 (55.8%)	467 (44.2%)	1057	1.26
Total	**5762 (55.45%)**	**4628 (44.55%)**	**10390**	**1.24**

Statistical significance of Sperm FISH data for mice (n=10)

X bearing sperm Median (min-max)	Y bearing sperm Median (min-max)	p-value	X/Y ratio Median (min-max)
579.5 (545-594)	463 (449-481)	**0.0051**	1.26 (1.133-1.3)

(Analysis by Wilcoxon signed-rank test)

account the effect of various environmental factors like temperature, diet, profession on human sperm sex ratio. In all sub-groups as well as in mouse we have observed more X bearing sperm. A probable reason for this could be presence of meiotic drive elements which could lead to incapacitation or fragmentation of Y bearing sperm or preferential elimination of Y bearing sperm through apoptosis mechanism. This is also supported by the evidence of more aneuploidy with Y-bearing spermatozoa (~1.5X more with Y bearing sperms; 192 aneuploid Y sperms and 124 aneuploid X sperms). This is also we observed in our previous study [10]. This indicates more X bearing sperms available for fertilization.

We have investigated influence of season (temperature) on sperm sex ratio. Seasonal (temperature) variation in sex ratio has been observed in variety of vertebrates viz. frog, fishes, lizard, birds, reptiles, etc [16-18]. In fishes it was shown that male population increases on increase in temperature of water. In human also temperature based variation in secondary sex ratio was observed [19]. According to the findings of Cagnacci et al. [20] sex ratio at the time of conception showed a seasonal rhythm, higher in 3 months of peak (September, October, November) than in 3 months of nadir (March, April, May). We wanted to check whether such skewing originates at sperm level, so we carried out the sperm sex ratio study in two different time points viz. winter and summer. In winters, we also tried to find out the change in sperm sex ratio at beginning (December) as well as at end (February) of each seasons. Similarly, we investigated the sperm sex ratio in beginning (May) and end (August) of summer. Approximately 10,000 sperm were counted per individual after performing XY-FISH on sperm nuclei. We observed more X bearing sperm (210773) than Y bearing sperm (196900) in both winters and summers (more so at beginning i.e. December: 53081 & May: 53146 than at the end i.e. February: 52410 & August: 52136). However, sex ratio (X/Y) change at the beginning and end of winter as well as summer was statistically insignificant. The X/Y ratio in December, February, May, August was 1.06:1, 1.069:1, 1.097:1, 1.056:1 respectively; which is just reverse of secondary sex ratio (sex ratio at birth) i.e., male:female as 1.07:1. When the two seasons i.e. winters and summers as a whole were considered then also our data showed statistically insignificant, however X bearing sperm were significantly more than Y bearing sperm. Thereby we can say that there seems to be no difference in sperm sex ratio upon seasons.

We have also investigated influence of diet with sperm sex ratio. The origin of the study was the study of Lloyd et al. [21] who reported that butchers (who themselves eat more non-vegetarian food) bore more sons. We speculated that the skew originated at the spermatozoa level. However we found significantly more X bearing sperm than Y bearing sperm in all groups. Mean X/Y ratio for vegetarian category was 1.09:1 and for non-vegetarian category was 1.067:1. Inter-categorically X/Y ratio didn't differ in spite the fact that there were more X bearing sperm in the vegetarian category. We observed that diet (vegetarian or non-vegetarian) do not seem to cause an effect on sperm sex ratio.

Our third group for sperm sex ratio was with profession i.e., professionals vs. laborers. We selected these two groups, as there are several studies reported which seem to show an association of paternal profession and birth sex ratio [22-25]. Literature suggests that work place exposure (to different chemicals) can cause a shift in sperm sex ratio [26-28]. We selected two extreme groups, one who do more physical work and the other who are indulged in mental work like educated professionals (scientist, doctor, etc). We observed more X bearing sperm than Y bearing sperm in both categories. Mean value of X/Y ratio in professionals and laborers was 1.09 and 1.08 respectively. We couldn't find statistically significant difference in 2 groups.

In all the three study subgroups i.e. seasons, diet and profession we observed significantly more X bearing sperm (210773, 105346 and 105412 respectively) than Y (196900, 97628 and 97007 respectively) bearing sperms. In totality of human sperm data, X:Y ratio was 1.076:1 (or can be represented as Y:X :: 0.929:1). Thus it seems unlikely that variation in gametic sex ratio is an important contributor to excess of males (1.07 male: 1female) observed at birth. Our results are supported by various other studies [7,11] as well as contradicted by some [8] who have observed more Y bearing sperm whereas Griffin et al. [9] found equal ratio for X and Y bearing sperms.

During our study we have also observed Y bearing sperms with more sex chromosome aneuploidy (192 aneuploid Y sperms and 124 aneuploid X sperms i.e., 1.5 times more). More aneuploidy in Y bearing sperm was also observed in some other study [10] in which they observed higher segregation error with Y chromosome thus further reducing normal Y bearing sperm concentration. This work was extended on mouse epididymal sperm. Our mouse data also suggest a surfeit of X bearing sperm (5762 i.e. 55.5%) as compared to Y bearing sperm (4628 i.e. 44.5%).

This study finds excess of X bearing sperms both in human and mouse. This outcome cannot be considered as chance as interpretation was derived from study of over 0.8 millions of sperms. A probable reason for this could be presence of meiotic drive elements which could lead to incapacitation or fragmentation of Y bearing sperm or preferential elimination of Y bearing sperm through apoptosis mechanism. Stalked eye fliers often have an extreme sex ratio due to meiotic drive element on the X chromosome [X^d]. Male carriers of [X^d] show a decrease of meiotic drive intensity and represented as

resistant Y chromosome [Y^m]. Resistant Y chromosome [Y^m] when paired with [X^d] cause the transmission of predominantly Y sperm [29]. Similarly, in fruit fly an X linked meiotic drive has been reported. Males that are carriers of X linked drive produce an abundance of female offspring caused by a deficiency of Y bearing sperm. Non-disjunction of Y chromatids during meiosis II results in failure of non disjunctioned (YY) spermatids to develop into functional sperm [30]. High sex ratio distortion has also been shown to run in families [12]. Szyda et al. [12] looked for recombination rate of pseudoautosomal region of the sex chromosome and found a significant skew in the X and Y chromosomes. He proposed two hypotheses for this deviation. The most straight forward hypothesis was that recombination can produce a certain combination of alleles that are detrimental to Y – sperm viability or preferential to X-sperm viability. Y recombinant products were being lost at some stage during spermatogenesis. The alternative hypothesis was that there may be X specific genes located near the bovine pseudoautosomal region, that when expressed affect the viability of the Y bearing sperm. Similarly, in the mouse, deletions on the Y-chromosome long arms (MSYq) leads to the up- regulation of multiple X- and Y-linked transcripts in spermatids. Two suspected genes are the X-linked multi-copy gene Xmr and its counterpart MSYq-linked Sly, which are up- and down-regulated, respectively, in the testes of MSYqdel males [31] leading to distortion of sex ratio.

Conclusion

Our results at pre-zygotic/sperm level study have shown skewed sex ratio towards X/female. In contrast to pre-zygotic/sperm sex ratio, secondary sex ratio (sex ratio at birth) all over the world shows an excess of males. Hence, we conclude that the mechanisms that cause skewing of secondary sex ratio is not through altered sperm/prezygotic sex ratio. We also conclude that excess of X bearing sperm in spermatogenesis is real (almost all samples i.e., 76/80 have similar findings) and thus have biological basis.

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