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Application of light emission diode irradiation during sperm swim-up selection skewed the primary sex ratio and improved bovine sperm quality

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Trials to skew the primary sex ratio or preselecting offspring with a predefined sex have been considered to be of interest for a number of years. Several methods and techniques have been used to this purpose. The present study evaluated the effects of red Light Emission Diode (LED) radiation on primary sex ratio and qualitative parameters of bull sperm during the swimup method. Fresh semen samples of four Holstein bulls were used in these experiments. Two experimental groups were designed (with or without LED radiation). The LED radiation group received red LED irradiation during the swim-up procedure. The photo-stimulation pattern consisted of sequential light-exposure periods (the light phase for 10 min followed by the dark phase for 10 min and the final light phase for 10 min). In each experiment, 5 tubes (#1 to 5) were considered for the swim-up procedure. The sperms were collected at 5, 10, 20, 30 and 60 min of swimming up time. Samples were analysed by Computer Assisted Sperm Analysis (CASA) and quantitative real-time PCR. Results of CASA parameters show significant differences between the two experimental groups for most parameters estimated at 10 and 30 min. Results of the comparative threshold cycle (ΔCt] reveal that the Y-bearing sperm swim faster than the X ones and the percentage of Y-bearing sperm

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in the control group was significantly greater. In conclusion, using red LED during the swim-up method could enhance sperm CASA parameters and increase the Y/X- chromosome bearing sperm ratio. Our results showed that red LED irradiation improves motility of sperm, which in turn can improve fertility. Additionally, the Y/X bearing sperm chromosome ratios are different in different times of the swim-up procedure.

KEY WORDS: bull / primary sex ratio / red light LED / swim up / sperm

Sex pre-selection using sexed sperm has long been a desired technological tool in breeding of cattle and other mammals. Isolation of X-bearing sperm provides an opportunity for breeders to preselect female offspring for heifer replacement and ensures increased effectiveness of multiple ovulation, embryo transfer and in vitro embryo production [Weigel 2004].

There are two well-known categories of sperm sex selection methods, one is based on kinetic or physical properties of sperm and the other one on genomic distinction. Several approaches based on the former category have been attempted with conflicting results and included the use of albumin gradients [Dmowski *et al*. 1979], swim-up techniques [Cesari *et al*. 2006], Percoll gradients [Machado *et al.* 2009] or Sephadex columns [Steeno *et al*. 2009]. Sex selection of sperm by flow cytometry based on genomic differences was reported. Flow cytometry produces an extensive enrichment of X- and Y-chromosome bearing sperm with a 90% accuracy, but safety is a matter of concern due to the exposure of cells to mutagenic DNA stains, high pressure, ultraviolet light and electrical charge [Maxwell *et al.* 2004]. On the other hand, this method requires special equipment for sperm processing, so sexed sperm doses are too expensive and the fertility of sexed sperm is lower than that of unsexed sperm, because of lower sperm numbers per dose and damage to sperm cells in the sexing process [Morrell *et al.* 2011].

The swim-up procedure is the simplest sperm preparation method which isolates as much motile sperm as possible (>90%) and eliminates both dead sperm and cryoprotectant agents [Henkel *et al.* 2003]. Several studies have been conducted to separate X- and Y-bearing sperm using the swim-up method, based on the different motility of X- and Y-bearing sperm [Flaherty *et al.* 1996]. Motility is an important parameter in sperm fertilization ability, since it leads to the penetration of the zona pellucida and delivery of the male's genetic material to the oocyte [Suarez and Ho 2003]. Motility of sperm can be affected by red Light Emission Diode (LED), which has been proposed as a sperm motility stimulator [Yeste *et al*. 2016]. New studies indicate that red light irradiation could improve sperm motility and fertilizing ability in several species [Chow *et al.* 2016, Preece *et al.* 2017].

We hypothesize that red light irradiation has a positive impact on sperm motility and it might alter the ratio of X- to Y-bearing sperm during the swim-up method. The objective of the present study was to evaluate effects of red LED on the primary sex ratio and quality parameters of bovine sperm during the swim-up method in a CO2 incubator at 37°C.

Materials and methods

Semen collection

Fresh semen was collected from four 3- to 5-year-old Holstein AI bulls from the Abbas Abad Animal Breeding Center in the Khorasan Razavi province, Iran. Semen collection was performed 4 times weekly. A total of 16 semen samples (4 samples from each bovine) were collected using an artificial vagina. Semen was analyzed microscopically and macroscopically and only regular semen were used. Each sample was divided into 3 parts and each test was repeated 3 times.

Swim-up and sampling

Guidelines of Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes were followed in sampling and dealing with animals.

Each semen sample was divided into 5 subsamples and each test was repeated three times. The swim-up procedure was run for the control and the treatment group separately. The swim-up procedure was performed following an established protocol [Parrish and Foote 1984]. The treatment group received red LED irradiation during the swim-up process, but the control group was not exposed to red LED light. The photo-stimulation pattern followed the protocol presented by Yeste *et al.* [2016] with sequential light-exposure periods (the light phase for 10 min followed by the dark phase for 10 min and the final light phase for 10 min, wavelength window: 620-630 nm).

In each experiment, five tubes (#1 to 5) were considered for the swim-up procedure. Specimens in these 5 tubes swam up for 5, 10, 20, 30 and 60 min (one tube for each time point), within the defined time, afterwards 0.1 mL of the supernatant was collected. The volume of 0.01 mL of the sample was assayed immediately with the Computer-Assisted Sperm Analysis (CASA) system (HFT co. Tehran, Iran) for the analysis of sperm parameters and the remaining 0.09 mL were stored at -20°C until DNA extraction and Real Time PCR analysis.

DNA extraction and Real-Time PCR

Total DNA from each sample was extracted using the Salting out protocol [Kholghi *et al.* 2014]. Nucleic acid concentration was measured in a NanoDrop spectrophotometer (NanoDrop ND-1000, Logan, UT, USA). The quality of extracted DNA was assessed by electrophoresis in 1% agarose-gel containing Ethidium Bromide. Quantitative PCR was performed using the SYBR Green supermix. A pair of specific primers were used for each selected gene. Selected genes were the Sex Determining Region Y (*SRY*) located at the end of the Y-chromosome short arm as the Y-chromosome marker gene (GenBank accession no. EU581861.1), and Proteolipid Protein (*PLP*) located at the end of the X-chromosome long arm as the X-chromosome marker gene (GenBank accession no. AJ009913.1). A pair of primers designed to amplify a 79 bp DNA fragment of the pseudoautosomal region or the Proteinase

Activated Receptor (*PAR*) gene (GenBank accession no. AC234910.2). The *PAR* gen was considered as the reference gene to compare the amount of *SRY* and *PLP* gene expression. All primers are shown in Table 1.

Table 1. Specific primers of *PLP, SRY* and *PAR* genes

Accession number	Sense primer sequence 5′→3′	Anti-sense primer sequence 5′→3′	Length (bp)	Tm (°C)
AJ009913-1	GAGGGAGGGTGGATCATAGA	CCTCTGGGACCTTCAACAAT	90	60
EU581861-1	CTCAGACATCAGCAAGCAGC	GTAGTCTCTGTGCCTCCTCA	89	60
AC234910-2	GCCATCACATCTGAGACCAC	GACTCAGCATCTCGAAGCAA	79	60

Real-Time PCR was performed on a Sequence Detection System (Corbett, RotorGene 3000) using the SYBR Green qPCR Master Mix (Ampliqon, Cat. No. A323406). DNA of each sample was run in duplicate along with no template control (NTC) for every assay and primer in separate wells for the three genes of interest. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers.

The PCR mixture contained 1 µL (50 ng) of template, 0.5 µL (10 pmol) forward primer, 0.5 µl (10 pmol] reverse primer and 4 µL RealQ Plus 2x Master Mix (Ampliqon, Cat. No. A323406), then made up with sterile water to a final volume of 10 µL in 0.1 mL microtube. Amplifications for genes were performed by an optimized protocol recommended by the producer (15 min at 95°C, 45 repeated cycles of two steps at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s) – Table 2.

Table 2. Time and temperature conditions of Real-Time PCR

Stage	Phase	Time (sec)	Temperature (°C)	Cycle number
Holding	1	15	95	1..
Cycling	denaturation	30	95	
	annealing	30	60	45
	extension	30	72	

Statistical analyses

The data obtained from qPCR were analyzed according to the method of Livak and Schmittgen [2001] with the *PAR* gene serving as the reference gene for the estimation of the initial Ct values. The mean Ct value was calculated for *PAR* and each of the two studied genes (*PLP* and *SRY*), while the ΔCt value was determined for each gene using the following formula:

$$\Delta Ct = Ct_{(\text{target gene})} - Ct_{(PAR)}$$

After calculation of ΔCt for all the samples taken from cattle sperm, the expression status of the *PLP* and *SRY* genes relative to *PAR* was estimated using the following formula [18]:

copy number of chromosome (X- or Y-chromosome) = $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct\ (\text{target gene}) - \Delta Ct\ (PAR)}$

Finally, the ratio of *PLP* and *SRY* was considered as the ratio of the X and Y chromosomes, respectively.

The normal distribution of the analyzed traits was verified by the GLM Procedure and statistically analyzed by the Student's t-test ($P \leq 0.03$). These computations were performed using the SAS computer software version 9.1 (SAS Institute Inc., Cary, NC, USA).

All sperm motility data (CASA data) were analyzed by the nonparametric Mann-Whitney U test because the normality test (Shapiro-Wilk) with $p<0.05$ failed.

Results and discussion

Effects of treatment on CASA parameters

Results of CASA sperm analysis indicate significant differences in most parameters estimated at 10 and 30 min. Sperm Density, mostly A class, which was faster, Straightness, Linearity, Wobble, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), beat-cross frequency (BCF) and mean angular displacement (MAD) were significantly different at 10 and 30 min of the experiment compared to the control. Sperm density or the number of sperm per mL of samples were significantly greater at 5, 10 and 30 min of the experiment in the treatment group. Four parameters showed significant differences for the samples that were taken at 20 and 60 min of the experiments. Detailed results of CASA parameters are listed in Table 3.

Effect of treatment on sperm sex ratio

All semen samples were assessed for the sperm sex ratio at the beginning of the experiment. The mean ΔCt value in the *SRY* and *PLP* genes was used to examine the ratio of X / Y-chromosome bearing sperm and to calculate the percentage of each population of sperm. Results of quantitative Real-Time PCR data showed no marked deviation from the expected 1:1 ratio at the beginning of swim-up (0 min). Samples in the control group indicated a much higher percentage of the *SRY* gene at all time points of the test, at a slight increase of X-bearing sperm with the passage of test time. In the treatment group as shown in Table 4, the percentage of X-bearing sperm significantly increased relative to the control at all time points, but the percentage of Y-bearing sperm was still higher than that of X-bearing sperm at 5, 10 and 20 min of the experiment. In the treatment group there was no difference between percentages of X-and Y-bearing sperms at times of 30 and 60 min of the experiment.

The comparative CT method was used to assess fold change difference of the SRY and PLP genes in the treatment group relative to the control. There was a 6.8-fold lesser enrichment of the SRY gene at 10 min of the experiment, which was significantly different compared to the control. A significantly greater enrichment of the PLP gene

Table 3. CASA parameters estimated in bovine semen control (C) and treatment (T) groups

Time (min)	Group	Sperm density (10^6/mL)	Percentage of class A sperm	Density of class A sperm	Mean straightness	Mean linearity	Mean wobble	Mean VCL	Mean VSL	Mean VAP	Mean ALH	Mean BCF	Mean MAD
	C	10.605	41.455	1.885	61.975	47.390	59.220	39.850	29.075	32.250	2.030	3.185	12.565
5	T	15.460	50.425	6.680	69.570	51.005	59.605	55.130	39.510	43.145	2.485	4.285	20.425
	P value	0.020	0.885	0.885	0.885	0.885	0.885	0.885	0.885	0.885	0.885	0.885	0.885
	C	30.155	30.155	3.200	47.240	34.295	47.785	34.935	21.565	25.445	1.975	2.160	12.425
10	T	56.280	56.280	9.190	64.295	52.625	65.090	53.560	38.135	43.645	2.515	4.225	16.940
	P value	0.020	0.030	0.030	0.030	0.030	0.311	0.030	0.030	0.030	0.311	0.029	0.029
	C	36.720	28.765	10.375	46.610	29.955	44.840	43.850	20.615	26.835	2.930	2.700	20.655
20	T	18.825	41.090	8.350	59.660	41.270	57.135	51.560	29.790	35.685	2.930	4.040	22.760
	P value	0.885	0.030	0.885	0.030	0.030	0.311	0.311	0.030	0.311	0.885	0.311	0.311
	C	25.315	22.085	4.915	46.535	27.445	45.610	34.925	15.045	20.225	2.510	2.240	16.545
30	T	41.490	38.640	12.235	57.755	38.980	55.670	56.580	28.540	36.300	3.655	4.250	26.045
	P value	0.030	0.030	0.030	0.311	0.0310	0.030	0.030	0.030	0.030	0.311	0.030	0.030
	C	38.860	20.280	7.325	47.215	26.565	44.645	51.715	17.315	25.960	3.870	2.620	26.050
60	T	36.330	32.260	12.260	53.540	34.840	51.270	50.070	23.780	30.960	3.320	3.455	22.990
	P value	0.885	0.030	0.311	0.311	0.030	0.030	0.885	0.030	0.311	0.311	0.311	0.311

* Fresh semen samples from four different Holstein bovines were used for this study.
**Semen samples in the treatment group received red LED light irradiation during the swim-up procedure and samples in the control group with no light exposure.
The photo-stimulation pattern consisted of sequential light-exposure periods (the light phase for 10 min followed by the dark phase for 10 min and the final light phase for 10 min.

Table 4. Δct analysis of *SRY* and *PLP* genes

Time (min)	Group	Δct *SRY* (mean±SEM)	Δct *PLP* (mean±SEM)	*SRY/PLP* (mean±SEM)	*SRY* %	*PLP* %	P value
0		1.720±0.067	1.577±0.055	0.917	52.149	47.850	0.154
5	C	0.882±0.133	4.687±0.111	5.314	84.334	15.665	0.0001
	T	1.170±0.146	4.090±0.098	3.495	78.006	21.993	0.0001
	P value	0.1978	0.0071				
10	C	0.457±0.094	3.712±0.132	8.122	89.279	10.720	0.0001
	T	3.302±0.241	2.335±0.163	0.707	58.513	41.4864	0.0161
	P value	0.0001	0.0006				
20	C	1.385±0.125	3.625±0.106	2.617	72.462	27.537	0.0001
	T	3.200±0.091	2.592±0.083	0.81	55.248	44.751	0.027
	P value	0.0001	0.0003				
30	C	2.677±0.021	3.025±0.240	1.129	61.603	38.396	0.0001
	T	4.297±0.063	2.415±0.249	0.562	55.773	44.226	0.1289
	P value	0.2006	0.0003				
60	C	3.842±0.192	4.500±0.070	1.171	54.018	45.981	0.0185
	T	2.852±0.073	2.705±0.168	0.948	51.959	48.040	0.4534
	P value	0.0030	0.0001				

*Fresh semen samples from four different Holstein bovines were used for this study.
**Semen samples in the treatment group received red LED light irradiation during the swim-up procedure and samples in the control group with no light exposure.
The photo-stimulation pattern consisted of sequential light-exposure periods (the light phase for 10 min followed by the dark phase for 10 min and the final light phase for 10 min).

Table 5. Fold change comparison of Real Time PCR data in treatment (T) relative to control (C) group

Time (min)	Gene	Δct (C) (mean±SEM)	Δct (T) (mean±SEM)	Fold change
5	sry	0.882±0.133	1.170±0.146	-1.17
	plp	4.687±0.111	4.090±0.098	1.51
10	sry	0.457±0.094	3.302±0.241	-6.84
	plp	3.712±0.132	2.335±0.163	2.68
20	sry	1.385±0.125	3.200±0.091	-3.47
	plp	3.625±0.106	2.592±0.083	2.05
30	sry	2.677±0.021	3.025±0.240	-1.21
	plp	4.297±0.063	2.415±0.249	3.79
60	sry	3.842±0.192	2.852±0.073	2.01
	plp	4.500±0.070	2.705±0.168	3.52

Fresh semen samples from four different Holstein bovine were used for this study.
*Semen samples in treatment group received LED red light irradiation during swim up and samples in control group with no light exposure.
Photo-stimulation pattern was done with sequential light-exposure periods (light phase for 10 min followed by dark phase for 10 min and final light phase for 10 min).

was found at 5, 10, 20, 30 and 60 min of the experiment in relation to the control. We observed on average 1.1-, 3.4- and 1.2-fold lesser enrichment of Y-bearing sperm for semen samples taken at 5, 20 and 30 min, respectively. Mean enrichment of X-bearing sperm was 1.5-, 2.68-, 2.05-, 3.7- and 3.5-fold greater at 5, 10, 20, 30 and 60 min of the experiment, respectively. Table 5 shows results of quantitative real time PCR data in detail.

The objective of the present study was to evaluate effects of red LED on the primary sex ratio and quality parameters of bovine sperm during the swim-up protocol. According to the fold change analysis of quantitative Real Time PCR data, 2.6-fold greater enrichment of X-bearing sperm and 6.8-fold minor enrichment of Y-bearing sperm were observed within 10 min of irradiation compared to the control. This trend was observed at 5, 20 and 30 min to a lesser extent. The analysis of the CASA system also showed significant differences for most estimated parameters within 10 and 30 min of irradiation in relation to the control. Sperm density or the number of sperm per mL of samples significantly increased at 5, 10 and 30 min of the experiment in the treatment group, which was in accordance with light exposure time. As it is obvious in both groups Y-bearing sperm can swim faster and our results confirm this regularity, but the application of red LED can gradually correct this skewness and there was no such difference at 30 and 60 min of the experiment in the treatment group. One of the interesting findings in the present study is related to the increment of X-bearing sperm from 10.7% in the control to 41.4% in the treatment group within 10 min of irradiation. Based on this result we assume that red LED has more effect on X-bearing sperm motility and if light exposure continues for 15 min or more this skewness might fade sooner.

The skewed sex ratio of IVF embryos is an issue of great concern. Alteration of the sex ratio toward male embryos has been demonstrated by many authors [Avery *et al.* 1991, Lonergan *et al.* 1999] and several other studies also confirmed this phenomena in bovine and porcine IVF embryos [Gutirrez-Adan *et al.* 2001, Torner *et al.* 2014]. Several mechanisms have been proposed for the skewed sex ratio of males and females in *In Vitro* Production of Embryos (IVEP), as factors causing superiority of Y-chromosome bearing sperm over X-chromosome bearing sperm for fertilization of the oocytes, culture conditions that influence development of embryos according to their sex and faster development of male embryos than females, which are more resistant to various stress conditions in vitro [Edwards *et al.* 2001].

Methods for shifting the sperm sex population toward the desired sex would be a useful approach for skewing sex of IVEP embryos [Parati *et al.* 2006]. Therefore, by shifting the sex ratio of a sperm population toward X samples using a modified swim-up method it might be possible to reduce the deviation towards males without compromising embryo development in the case of IVEP embryos. Separation of motile and non-motile sperm populations using the swim-up technique has long been used in IVF laboratories. Several studies reported separation of sperm sex populations using this technique and its modification [Roca *et al.* 2016]. Different swimming speeds

between two populations of sperm (because of the greater DNA content of X-bearing than Y-bearing sperm) have been proposed as the cause of this phenomenon that can be used for sperm sex pre-selection [Cesari *et al.* 2006]. Based on this statement, some authors described that the supernatant in the swim-up procedure contained more Y-sperm, which is in agreement with the results of the present study [Check *et al.* 1994].

According to results of the CASA parameter analysis, significant differences in most CASA parameters were observed between the two study groups at 10 and 30 min of irradiation, which was in accordance with the pattern of light exposure. Sperm density was the only parameter that was significantly different between the two groups within 5 min of irradiation. There were significant differences for most CASA parameters (i.e. Sperm Density, Density of faster Sperm, Straightness, Linearity, Wobble, VCL, VSL, VAP, BCF and MAD) at 10 and 30 min of light exposure. Results of CASA were statistically similar for the most estimated parameters in samples at the time of 20 and 60 min of the experiment, which is logically due to the dark period pattern of the experiment design. In the study of comparative sperm motility Penfold *et al*. [1998] concluded that there is no difference between swimming speed of X and Y-bearing sperm. However, they did not evaluate the effect of time and light on motion parameters. Results of quantitative Real-Time PCR data in the present study indicate that Y-bearing sperm reached the surface of the swim-up medium faster, but with the passage of time the percentage of X-bearing sperm increased. The result of the present study also indicated that applying red light significantly increases sperm density within 10 min. We assume that 10 or more probably 15 min of the swim-up protocol with red LED light would be enough to reach the amount of sperm needed for IVF and to correct this skewness. Previous studies found that motion parameters of mouse, human, bovine and ram sperm are enhanced by the application of incoherent light, i.e. visible light at a wavelength range of 400-800 nm [Zan-Bar *et al.* 2005]. CASA parameters estimated in the case of sperm motility in the present study are consistent with the results of other studies. Data in our study clearly demonstrate that red LED light has an evident impact on enhanced enrichment of X-bearing sperm, which is probably due to the effect of light photons on the motion parameter. Previous studies on human sperm had found that progressive motility of sperm can be improved using near IR irradiation at 940 nm [Singer *et al.* 2009] and following red-light at 647 nm [Shahar *et al.* 2011]. It was also shown that the swimming speed of human sperm was enhanced using 905 nm light [Firestone *et al.* 2012]. A recent study on boar sperm using red LED light found increased curvilinear velocity (VCL) in CASA [Yesta *et al.* 2016]. In turn, Zan-Bar *et al*. [2005] enhanced the 'in vivo' fertilizing ability of ram sperm using 660 nm red light. The mitochondria in the neck region of the sperm cell provide energy for its progressive motility. Several other studies suggested that increasing intracellular Ca^2 levels and inducing nitric oxide production are the reasons for improving motility in irradiated sperm [Rodriguez 2005].

Since the motility of sperm is affected by red LED irradiation, it might have an impact on the separation of X- and Y-bearing sperm using the swim-up technique.

Several authors attempted to skew the primary sex ratio through the swim-up procedure and its modification, but to the best of the authors' knowledge this is the first study using LED irradiation during the swim-up process to separate sperm by sex. The results of quantitative Real Time PCR data in the present study showed a 2.6-fold greater enrichment of X-bearing sperm and a 6.8-fold lesser enrichment of Y-bearing sperm at 10 min of irradiation compared to the control (Tab. 5). Results of fold change analyses indicate that the level of enrichment for the PLP gene was significantly greater at all time points of the experiment and the amount of SRY gene enrichment was the lowest at 10 min of the experiment in the treatment group, although the percentage of Y-bearing sperm was still higher than that of X-bearing sperm at this time point. Based on the results of the present study we assume that continuing light exposure for 15 min or more might be helpful to correct the primary sex ratio skewness or even increase it toward the X-bearing sperm.

Several studies reported that the swim-up or modified swim-up technique can separate sperm according to sex. Khatamee *et al.* [1999] reported that intrauterine insemination using the bottom or the top layers of separated semen was successful for selecting sex in the human. The swim-up method was found to be effective, leading to 90.9% [Rawlins *et al.* 1998] or 86% of male births [Check *et al.* 1989] using IVF. Greater enrichment of Y-bearing sperm using the swim-up method before insemination resulted in 5 male infants [71%] out of 7 birth [Dmowski *et al.* 1979]. However, neither of these studies evaluated the primary sex ratio on sperm cell. Some studies failed to skew the primary sex ratio using the swim-up method. In this study for the effect of the swim-up process on bovine semen there was no evidence of the sex ratio imbalance from the expected 1:1 ratio [Machado *et al.* 2009]. In agreement with this finding, Amadesi *et al.* [2015] found no significant effect of swim-up on the ratio of X- and Y-bearing spermand commercial semen of the same bulls was analyzed immediately after thawing and after swim-up using a real-time polymerase chain reaction method developed and validated in our laboratory. Calving data relative to single bulls did not reveal any significant deviation between genders from the theoretical 1:1 for none of the bulls, being the mean values of male and female calves born 52.1±2.80% and 47.9±2.71%, respectively. Thereafter, calving events of bulls were classified and analyzed according to four classes of years: 2009 (n=13,261). Many other studies evaluated the swim-up supernatant and reported no differences between X- and Y- bearing sperm proportions, thus concluding that it is an ineffective technique for skewing sex ratios for sex preselection [Van Dyk 2001] blinded study. Setting: University hospital laboratories. Patient(s.

Many IVF laboratories use the swim-up technique to separate motile sperm. Different swim-up time points failed to select or enrich either the X or Y bearing sperm [De Jonge *et al.* 1997]. According to research studies, the swim-up process itself cannot skew the primary sex ratio, but some authors reported sperm sex skewness using a swim-up modification. Different motility stimulators, centrifuge forces and protocols were used to achieve this goal. Finally, according to the results of both the

present and previous studies flow cytometry currently is the only available technology for separating X- and Y-bearing sperm for commercial use with 90% accuracy. The swim-up procedure still remains a cheap and simple technique applied in IVF laboratories; nevertheless, modification of this technique applying red LED light for 10 or more probably 15 min would be helpful to gather enough sperm for IVF and to correct primary sex ratio skewness.

Conclusion

Long-term incubation of sperm has a detrimental effect on sperm function. Using red light irradiation for 10 or more probably 15 min during the swim-up method would be possible to gather enough amounts of sperm for IVF without compromising sperm function and to correct primary sex ratio skewness. Our ongoing project on the sex ratio of IVF embryos using this new modified swim-up protocol will ratify the effectiveness of this technique for correcting secondary sex ratio skewness.

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