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RUNNING TITLE: SPOT-COUNTING TO STOP-COUNTING

TITLE: FISH ON SPERM: SPOT-COUNTING TO STOP-COUNTING? NOT YET

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CAPSULE:

The reliability and applicability of the CytoVysion SPOT ^{AXTM} workstation for the analysis of sperm chromosome anomalies has been evaluated. The system has potential but we need to overcome some limitations.

ABSTRACT:

Objective: To evaluate the reliability and applicability of the Spot Counting System (CytoVysion SPOT AXTM workstation) that offers an alternative to the tedious manual analysis of sperm FISH.

Design: Manual and automatic analysis were performed and compared.

Setting: Universitat Autònoma de Barcelona.

Patient(s): Twenty-four men who asked for information on fertility showing different seminal parameters.

Intervention(s): A semen sample for each patient was collected and prepared for FISH.

Main outcome measure(s): A dual-color FISH using LSI for chromosomes 13 and 21, and a triple-color FISH with centromeric probes for chromosomes 18, X and Y were employed (Vysis Inc.). Standard FISH analysis was carried out. Automatic analysis was subsequently performed using a Spot AX system.

Result(s): Overall, we performed 120 comparisons. In 116/120 (96.67%), the percentage of anomalies reported using manual counting falls within the incidence detected using the automatic system ($p>0.05$). In the remaining comparisons, statistical differences were detected ($p<0.05$, 4/120; 3.33%). Time consumed by the automatic analysis was always higher than the manual one, being influenced by the characteristics of the preparations.

Conclusion(s): The Spot Counting System has potential, but before the service is ready to be offered, we still need to overcome some limitations associated with the system.

Keywords: Automatic scoring system, FISH, male infertility, spermatozoa.

INTRODUCTION

The screening of aneuploidies in sperm nuclei from infertile patients using fluorescence *in situ* hybridization (FISH) has become a highly demanding task in IVF laboratories. Sperm-FISH studies allow for attributing a genetic cause in some cases of male infertility (1-4). In the last update of our own records, 15% of infertile patients showed significant increases of chromosomal anomalies in spermatozoa, with respect to our control population (unpublished data).

Significantly, increases of sperm anomalies are always moderate, but they are enough to affect the fertility potential of patients. For instance, chromosome anomalies in sperm have been related to significant increases of chromosomal abnormal embryos (5), a significant decrease in pregnancy rates (6) and an increase of miscarriages (7, 8). As a result, sperm-FISH analyses have been revealed as being a useful cytogenetic tool in the classification of patients depending on their risk: patients at risk would present an incidence of chromosomal anomalies higher than the baseline frequency described in controls and should be interpreted as an indication for preimplantational or prenatal genetic diagnosis.

Despite the benefits of performing sperm-FISH studies in infertile patients, some characteristics associated with the methodology have compromised its routine application. The first one is that it is time-consuming. The low incidence of chromosomal abnormalities in sperm makes the analysis of a large number of cells necessary (for clinical purposes 500-2000 spermatozoa), depending on the skill and experience of the scorer, and this will take 30-60 minutes. The second refers to microscope analysis. To limit the potential for inter-scorer differences, interphase spot

analysis depends on the application of strict assessment criteria and the participation of skilled personnel.

One way to overcome both situations could be automation. Although automation has been developed in sample pretreatments and probe hybridization, the analysis of the FISH signals pattern has still remained dependent on manual scoring by a cytogeneticist. A reliable, automated FISH scoring method should result in better cost-effective testing (in terms of time consumed and experienced personnel) and, at the same time, maintaining the accuracy of the results. In this sense, automated systems for scoring numerical and structural chromosomal anomalies have been applied satisfactorily in different human cell-types: uncultured amniocytes (9), lymphocytes (10, 11) and fibroblasts (12). In general, the results obtained in these cases show a coincidence among the data obtained by the manual and the automatic procedures.

To the best of our knowledge, only two papers have explored the application of automated aneuploidy scoring in spermatozoa, (Laser Scanning Cytometry System (13, 14)). Although preliminary because of the low number of samples and probes analyzed, and of its application only in normozoospermic males, both studies agree with the potential of automated systems for the analysis of chromosomal anomalies in human spermatozoa.

The objective of the present study is to evaluate the resolution of a new, automatic FISH-spot counting system, the CytoVision SPOT AXTM workstation (Applied Imaging International, Ltd) in the assessment of numerical chromosomal anomalies in spermatozoa from infertile patients.

MATERIAL AND METHODS

This project has been approved by Ethical Committee on Animal and Human Experimentation of the Universitat Autònoma de Barcelona.

Semen samples from 24 individuals who asked for information on infertility showing different seminal parameters were selected for the study (Table 1). Samples were processed as described previously by our group; details of the sperm fixation, nuclear decondensation and FISH protocol have been described elsewhere (15). Two different probe combinations were used (Aneuvysion Kit, Vysis Inc.; Downers Grove, IL, USA): A triple-color FISH with centromeric DNA probes for chromosomes X (Spectrum Green) Y (Spectrum Orange) and 18 (Spectrum Aqua) and a dual-color FISH with locus-specific probes for chromosomes 13 (Spectrum Green) and 21 (Spectrum Orange). According to the number of the spots, five different chromosomal anomalies for every single patient were assessed: sex chromosome disomies, disomy 13, disomy 18, disomy 21 and diploidy (Table 2). Slides were assessed in a blind manner by two independent researchers using the following approaches:

Manual analysis

Manual analysis was performed as it is done for clinical purposes. Consequently, five-hundred spermatozoa were analyzed at a magnification of 1000x (100x lens) for every patient and probe combination. Analysis was performed using an Olympus BX60 epifluorescence microscope (Olympus SA; Barcelona, Spain) equipped with a triple-band pass filter and specific filters for Aqua, FITC and Cy3 and following the assessment criteria described previously by us (16), in brief:

- Only spermatozoa with a well-defined boundary were evaluated. Overlapping spermatozoa were discarded from the count.

- In cases of disomy or diploidy, signals must be of the same size and intensity.
- In cases of disomy or diploidy, the minimum distance between the 2 signals must be at least the same as the diameter of the signals.

Automatic analysis

One-thousand spermatozoa per patient and probe combination were assessed at a magnification of 600x (60x lens). Analysis was performed using the *Spot AX* software, which is the automatic scanning and capturing version of *Spot for Cytovision* (Applied Imaging; Newcastle, UK) and an Olympus BX60 epifluorescence microscope equipped with specific filters for DAPI, Aqua, FITC and Cy3 coupled to a BX-UCB monotorized system (Olympus SA; Barcelona, Spain). The automatic system consists of three different screens: The *Assay Screen*, which allows for setting up the criteria for analysis and the capture of fluorochromes. The *Scan Screen*, the one that does the automatic analysis by taking pictures of each cell and classifying the cells according to its spot pattern, and the *Review Screen* which reloads and relocates any spermatozoa previously evaluated to manually check the automatic scoring.

The system was set up to achieve two main objectives: to maintain the reliability of the manual analysis, that is, to perform an automatic analysis using the same scoring criteria described by the manual one (Table 3) and, at the same time, to study its applicability for the routine in the laboratory.

System reliability was checked by statistical analyses using the SPSS 14.0 (SPSS Inc.; Chicago, IL, USA) under the advice of the statistical service of the *Universitat Autònoma de Barcelona*. Mean population values of the frequency of abnormal genotypes from manual and automatic analysis were compared using a Wilcoxon test. Results from manual and automatic scoring per patient were compared for every single

genotype using a Chi-square test. The same type of comparison (Chi-square test per genotype) was made comparing the automatic and the manual results, with respect to our internal control population (Table 1). This analysis allows researchers to assess the reliability of the automatic system regarding the classification of the patients as *at-risk* (significant increase of at least one abnormal genotype versus controls) or *not at-risk* patients (percentage of abnormal genotypes within the control values). Results were considered statistically significant when $p<0.05$.

Applicability was directly associated with time consumed and the need of a cytogeneticist for manual re-analysis (cell reclassification). Time consumed by the automatic system was assessed in every single screen: assay, scan and review. Because time will depend, in some way, on sperm density per frame, overlapping sperm and the presence of non-sperm cells, these parameters were evaluated from two pictures taken by phase-contrast microscopy at a magnification of 400x (40x lens). Pearson correlations were performed to assess their effect on time consumed.

RESULTS

Aneuploidy screening was performed in a total of 35,183 spermatozoa, 12,887 manually and 22,296 automatically. Detailed FISH results are given in Table 1.

System reliability

Mean values of manually and automatic results showed significant differences in the case of sex chromosomes anomalies ($p=0.034$). The remaining genotypes did not show significant differences ($p>0.05$) (Table 1).

Overall, 120 comparisons (5 genotypes x 24 patients) were performed. In 116/120 (96.67%), results obtained by the automatic system and those obtained manually were coincident. In the remaining comparisons statistical differences were detected ($p<0.05$, 4/120; 3.33%). In 3 out of the 4 non-coincident genotypes, the difference was in the diploid rates (Cases 396z, 418z and 419z), and figures obtained by manual scoring were higher than the automatic ones. It merits being noted that all three patients showed teratozoospermia (Table 1). The fourth non-coincident genotype was in the disomy 21 rate (Case 279z; Table 1).

From the comparisons of the automatic and manual results with control data, results showed coincidence in 102 out of the 120 genotypes (85%), whereas in 18/120 (15%) results were discordant. Accordingly, out of the 24 patients, 17 (70.83%) were classified in the same way (as *at-risk* or *not at-risk* patients), and 7 (29.17%) were not. Non-coincident patients did not show any preferential alteration of the semenogram.

System applicability

Time consumed by the automatic analysis ~~(Table 4)~~ was always higher than the manual one, which is estimated, based on our experience, to be between 30 minutes and 60 minutes per probe combination. While capture and reprocessing time was quite

homogeneous between probe combination and patients ~~(Table 4)~~, scanning time shows a high degree of heterogeneity, being highly influenced by the characteristics of the preparations (Figure 1). In this sense, a few spermatozoa per frame and the presence of non-sperm cells in the ejaculate clearly increased the time of analysis ($p<0.05$) (Figure 1a and 1b). On the contrary, overlaps showed a negligible effect on time consumed (Figure 1c).

It is important to note that the achievement of accurate results required cell reclassification in all patients and probe combinations.

DISCUSSION

The Spot Counting System, once optimized, can perform sperm-FISH analysis as how it is done manually. Automatic scanning selects and captures the spermatozoa that accomplish the morphological and signal criteria automatically. After that, the system classifies the spermatozoa according to their signal patterns into classes that were predefined by the user. The optimization of the protocol is crucial to exclude those cells that do not accomplish the manual morphological and signal criteria from the final count: overlapping spermatozoa, cells with indistinct margins, abnormally large or small sperm, abnormally long and/or weak fluorescent signals.

The possibility of viewing, editing and reprocessing all the spermatozoa captured by the system for future use is one of their main advantages. The image produced is realistic and the investigator can verify the results further. Moreover, cell location, selection and classification are made in the same way for every single spermatozoon, avoiding the potential for inter-scorer differences in the sperm aneuploidy screening.

The system is less efficient in the identification of diploid sperm than is manual analysis. Thus, the few differences occur mainly for diploidy, finding more in the manual analysis than in the automatic one. One of the critical points of automation is the creation of a DAPI classifier (Table 3). To build our classifier, three parameters were used: Compactness (CMP), Circularity (CIRC) and Area. CMP and CIRC are the result of complex mathematical formulae involving the perimeter or diameter of the cell, the area and the $\prod$ number. The classifier is designed to match, as much as possible, the visual criteria that a scorer would use to consciously discard a cell from any manual scoring. In the case of spermatozoa, the classifier was designed to include small and elongated cells in the count. It is well-known that the heads of diploid sperm

are usually bigger and rounder than that of normal haploid spermatozoa. This fact could affect the ascertaining of diploid sperm using the automatic scope, discarding those spermatozoa from the count that, despite their diploid constitution, display a shape far from the specifications of the classifier. Moreover, it is important to note that patients 396, 418 and 419 are teratozoospermic (Table 1) with a percentage of abnormal sperm-shape of 100%, 94% and 89%, respectively (data not shown). This situation could increase the possibilities of exclusion of diploid sperm from the final count. Obviously, this limitation could be solved making the CMP, CIRC and Area values less restrictive. This would probably increase the system reliability for the identification of diploid sperm, but it would also increase the number of non-sperm cells in the final score (which are very common in infertile patients), significantly increasing the time for reclassification.

Regarding the comparison of the manual and automatic results, with respect to the control population, it showed coincidence in 85% (102/120 comparisons), and coincidence in classifying the individuals as *at-risk* or *not at-risk* in 70.83% (14/24 individuals). There are two reasons that could explain the loss of reliability in relation to the manual vs. automatic comparison. The first one could be related to the comparisons carried out; when three populations are compared (manual and automatic vs. control) instead of two (manual vs. automatic), the likelihood of differences increases. The second reason could be related to the number of spermatozoa analyzed per patient. Without doubt, increasing the cell number to 1000 in the automatic analysis improves the accuracy of the statistical analyses, with respect to the manual assessment (consisting of the analysis of 500 sperm, as is done for clinical purposes). Thus, the need to analyze large populations in studies where phenomena occur with a very low frequency, which is the case of sperm aneuploidies, promotes the need to implement

automatic spot-counting systems, allowing for the analysis of hundreds of cells, and therefore improving accuracy.

Concerning its applicability, two of the situations described in other automatic systems (11, 17) were also observed in the *Spot AX work station*: The need for cell reclassification and the increase in the time consumed for the analysis. Results obtained directly by the system, before any correction, are far from those obtained manually. This situation was observed in each sample and probe combination.

As expected, time consumed was clearly influenced by the number of spermatozoa and by the presence of non-sperm cells (Figure 1). The spot-counting system is designed to capture the cells in a field, independently of the number or their morphological characteristics; a reduced number of spermatozoa or the presence of cells that do not accomplish the conditions specified in the DAPI classifier slows down the capture speed. Taking into account that oligozoospermia and the presence of non-sperm germ cells are common situations in semen samples from infertile patients, the implementation of automatic systems would probably require the selection of the sperm fraction (selection does not alter the frequencies of chromosomal anomalies in the mobile fraction (18) and the application of methods to concentrate the cells into a very limited area (for instance by the use of cytocentrifuge).

Final remarks

Sperm FISH analyses is labor-intensive, time-consuming and requires a skilled cytogeneticist, but at the same time it has become highly demanded in ART-associated laboratories. Technique automation, such as the one described in this paper, has the powerful potential to replace manual microscopic FISH analysis. In any case,

automation will imply reliability of the results and a significant reduction of the time of analysis. Concerning reliability, the spot-counting system has two aspects to be improved, the detection of diploid sperm and the accuracy of the results before reprocessing. Time would be a difficult problem to solve in the analysis of spermatozoa from infertile patients. Nevertheless, given that the scoring can be run automatically overnight, time-of-scorer intervention (capture and reprocessing time) is usually lower than manual analysis. Overall the implementation of the system would represent an optimization of the technician time.

In conclusion, the Spot Counting System has potential, but before the service is ready to be widely offered, we still need to overcome some limitations associated with the system.

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TABLE LEGENDS:

TABLE 1: Manual and automatic comparisons of chromosome disomy and diploidy found in sperm from infertile patients.

TABLE 2: Signal pattern of the different genotypes analyzed.

TABLE 3: Function and values of the different features of the automatic system to simulate the manual scoring criteria. Morphology and signal distances values were independent on the characteristics of the probes while signal size and intensities were clearly dependent.

~~**TABLE 4:** Time consumed in the automatic analyses.~~

FIGURE LEGENDS:

FIGURE 1: Effects of different sample parameters on the time consumed in the analyses (left column FISH X,Y,18; right column FISH 13,21). **A:** Time consumed in the analyses decreases while the sperm concentration per frame increases (X,Y,18: r=0.49/p=0.01; 13,21: r=0.48/p=0.04). **B:** The presence of non-sperm cells significantly increases the time-consumed (X,Y,18: r=0.61/p=0.02; 13,21: r=0.81/p=0.0001). **C:** Negligible (FISH X,Y,18; r=0.42/p=0.034) and null effect of overlapped sperm (FISH 13,21; p>0.05).

Cases	Semenogram[a]	Sex disomy		Disomy 13		Disomy 18		Disomy 21		Diploidy	
		MC	AC	MC	AC	MC	AC	MC	AC	MC	AC
279z	np	0.19	1.01	0.19	0.1	0.19	-	0.94[b]	0.10[b]	0.67	0.46
351z	N	0.19	0.2	0.19	-	-	-	-	0.20	0.19	-
358z	T	0.18	0.12	-	-	-	-	-	0.11	-	0.17
364z	OA	0.16	0.39	-	0.32	-	-	-	0.16	0.09	0.18
366z	A	0.89	0.32	0.18	0.10	-	0.11	-	0.10	0.33	0.26
367z	A	0.69	0.99	0.19	-	-	0.12	-	0.21	-	0.41
384z	OA	0.83	1.15	-	0.11	-	-	0.37	0.23	0.7	0.40
391z	A	0.18		0.19	0.10	-	-	-	-	-	0.05
393z	A	0.39	0.11	-	-	-	-	-	-	-	0.11
394z	A	0.18	0.2	-	-	-	-	-	0.11	-	0.21
395z	AT	-	0.54	-	0.21	-	0.11	-	-	0.95	0.05
396z	OAT	1.34	1.36	-	-	0.38	-	0.19	0.22	1.51[b]	0.32[b]
398z	N	0.19	0.18	-	-	-	-	-	0.11	-	0.05
399z	A	0.86	0.68	0.19	-	-	0.11	-	0.10	0.36	0.22
400z	OA	-	0.21	-	0.11	0.17	-	0.18	0.11	-	-
418z	T	0.17	0.31	-	-	-	0.1	-	0.10	1.3[b]	0.57[b]
419z	T	0.36	0.21	-	0.10	0.18	-	-	-	1.27[b]	0.31[b]
421z	np	0.66	0.77	-	-	-	-	-	0.11	0.09	0.22
423z	A	-	0.36	-	-	-	0.12	-	-	-	0.28
424z	NP	2.33	2.91	-	0.23	-	-	-	0.12	0.09	0.29
425z	OA	0.19	0.98	-	0.11	-	0.11	-	0.43	0.09	0.38
431z	np	-	0.61	-	0.10	-	-	0.19	-	0.28	0.57
432z	A	-	0.12	-	-	-	0.06	-	-	-	0.15
433z	OA	0.19	0.2	0.19	-	0.19	-	-	0.11	0.29	0.16

Mean	-	0.43[b]	0.56[b]	0.05	0.06	0.04	0.04	0.08	0.11	0.30	0.24
SD	-	0.11	0.13	0.09	0.09	0.02	0.01	0.04	0.02	0.09	0.03
Controls Mean±SD	N	0.19±0.07		0.06±0.04		0.03±0.03		0.07±0.04		0.19±0.13	

TABLE 1

MC: manual count; AC: automatic count

[a] Semenogram: N (normozoospermic), T (teratozoospermic), O (oligozoospermic), A (astenozoospermic), np (not provided)

[b] Significant differences ($p<0.05$) between manual and automatic count

Triple FISH X/Y/18	
Genotype	**Signals[a]**
Haploidy	G or R/A
Sex Disomy	
Disomy XY	G/R/A
Disomy XX	G/G/A
Disomy YY	R/R/A
Disomy 18	G or R/AA
Diploidy	
Diploidy 46,XY	G/R/AA
Diploidy 46,XX	G/G/AA
Diploidy 46,YY	R/R/AA
Dual color FISH 13/21	
Genotype	**Signals[a]**
Haploidy	G/R
Disomy 13	GG/R
Disomy 21	G/RR
Diploidy	GG/RR

[a] G= Spectrum green; R= Spectrum orange; A= Spectrum Aqua

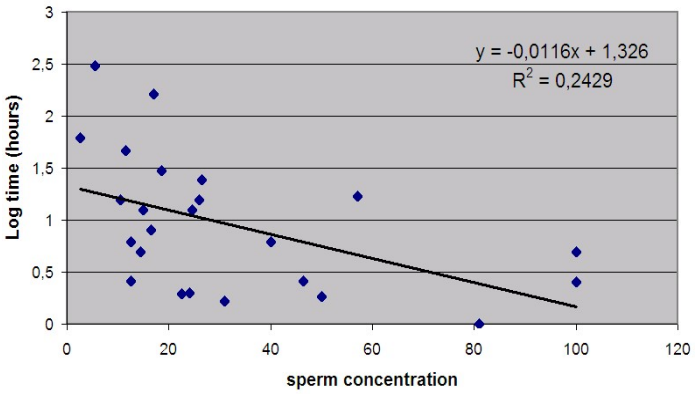
TABLE 2

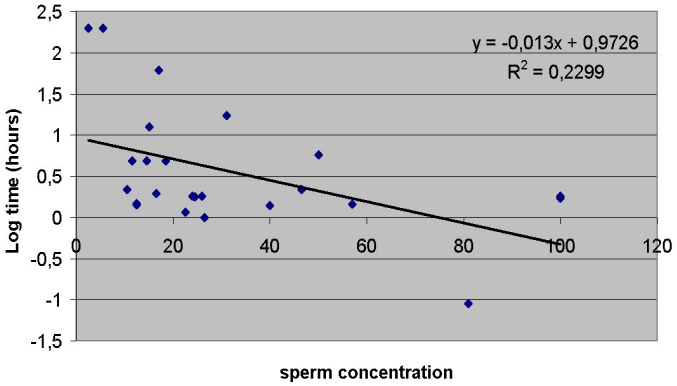
Scoring criteria	Feature	Function	Values		Maxim values
			X,Y,18	13,21	
Sperm morphology	Classifier	Desingned to match the morphological criteria to include a cell in the analysis	2094 vectors		-
	Counterstain threshold	System stringency to determine the DAPI nucleus mask	50		100
	Debris minimum size	Fix the background size	200		
	Boundary dilations	Extends the DAPI mask	4		10
Signal size	Minimum spot area	Range the spot size	4	6	700
	Maximum spot Area	Range the spot size	700		700
	Number of plains	Sets the Z-Stack for the capture	4	6	10
	Spacing	Space between plains	2.1		5
Signal distances	% Spot diameter	Minimal separaction between signals to be considered as independents	200		1000
Signal intensities	Minimum Spot intensity	Minimum signal intensity	50	30	255

	Minimum fusion intensity	If you have two signals of the same color within a nucleus, the weaker signal must be at least this value (%)	20-30	90	100
	Minimum spot intensity after merge				
	Brigh		-	-	128
	Black		-	-	-
	Exposure		-	-	5
	Gamma	Set up the contrast	0	2	3

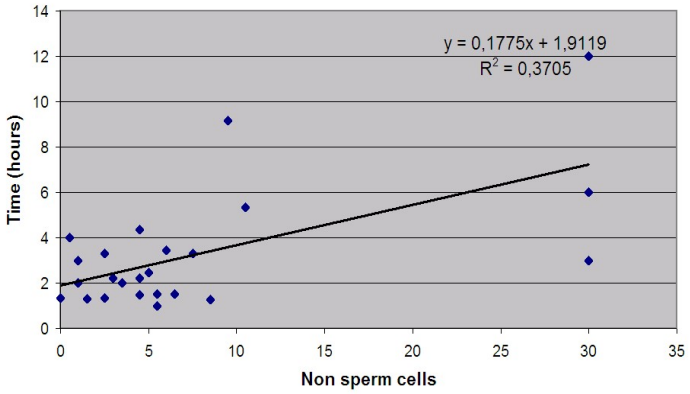
TABLE 3

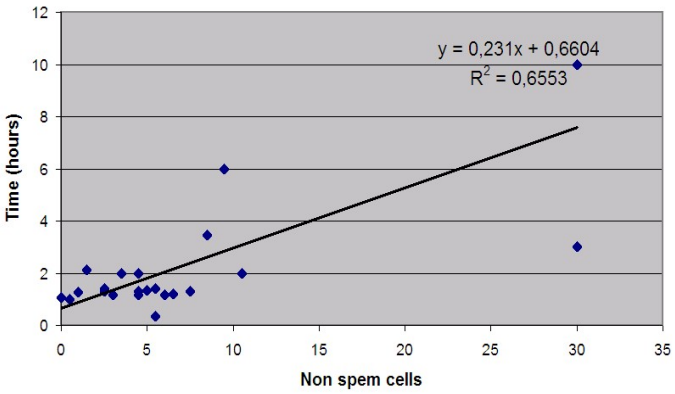
A


y = -0,0116x + 1,326
R2 = 0,2429
Log time (hours)
sperm concentration

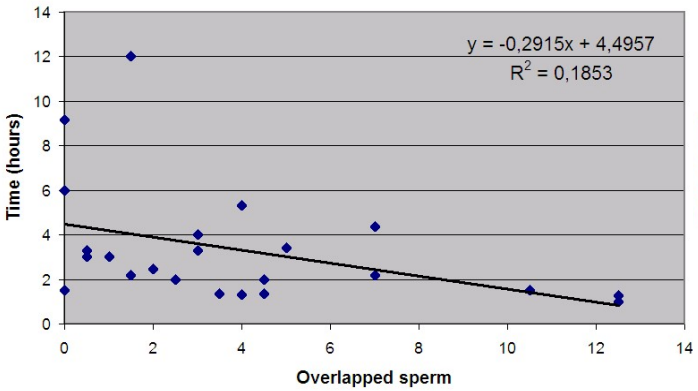

y = -0,013x + 0,9726
R2 = 0,2299
Log time (hours)
sperm concentration

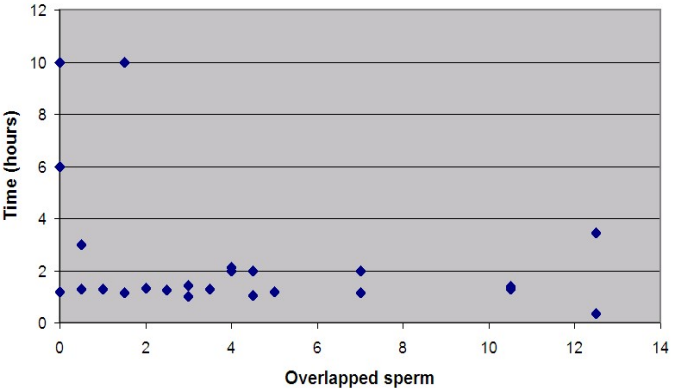
B


y = 0,1775x + 1,9119
R2 = 0,3705
Time (hours)
Non sperm cells


y = 0,231x + 0,6604
R2 = 0,6553
Time (hours)
Non spem cells

C


y = -0,2915x + 4,4957
R2 = 0,1853
Time (hours)
Overlapped sperm


Time (hours)
Overlapped sperm