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EVALUATION OF RESAZURIN REDUCTION TEST RESULTS AND THEIR CORRELATION WITH CONVENTIONAL SEMEN PARAMETERS : A LARGE SCALE STUDY*

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SUMMARY

Resazurin reduction test (RRT) was performed on semen samples obtained from 500 patients attending the infertility clinic and 125 proven fertile males. The results of RRT were determined visually using resazurin colour chart. These results were compared with those of sperm parameters analysed by conventional methods to validate the utility of the test in infertility practice. In subfertile specimens (oligozoospermic, asthenozoospermic and oligoasthenozoospermic), the RRT grade ranges from 1 to 4 and in normozoospermic and fertile samples it was from 5 to 11. The highest correlation of RRT grade was with sperm motility ($r=0.924$, $P<0.001$), concentration ($r=0.860$, $P<0.001$), morphology ($r=0.661$, $p<0.01$) and viability ($r=0.656$, $P<0.01$). RRT grade > 5 had a sensitivity of 94.22% for a sperm concentration of > $20x10^6$/ml and > 40% motility, and specificity of 94% for a sperm concentration < $20x10^6$/ml and < 40% motility. These results suggest that the RRT is very simple and reliable test and it can be used in any andrology laboratory as an alternative to the existing semen analysis.

Key words : Asthenospermic; Azoospermic; Oligospermic; Subfertile; Resazurin.

INTRODUCTION

Ten to fifteen percent of couples in the reproductive age are infertile and of these approximately fifty percent are due to male factor (1-3). The conventional semen analysis is still the standard laboratory method for diagnosis of male infertility, despite several descripencies on the results, which could reflect the inaccuracies of the method (4-5). Freund and Carol (6) have reported that 20% of intra-operator error in the routine semen analysis. Descripencies in

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the sperm count with the makler chamber have also been reported (7). Therefore, there is an urgent need to find a reliable and simple screening test to evaluate the quality and fertilizing potentiality of the human spermatozoa.

Glass et al. (1) have reported a simple resazurin test, which has been observed to correlate well with motile sperm. This method was originally described for evaluating the fertilizing capacity of bull semen (8,9) but has recently gained interest for the evaluation of human semen (1, 10-12).

In the past, resazurin test was used to assess the bacterial content of milk (13), microbial content of deepfrozen shrink (14) and quality control of pasturized and ultra high temperature treated starch soup (15).

The objective of the study is to validate the results of resazurin reduction test (RRT) with respect to human semen quality and to test whether these results could be correlated with the semen parameters assessed by conventional methods.

MATERIALS AND METHODS

Sample Collection :

A total of 500 untreated subfertile men (mean age was 32.5 yrs with a range of 24-43 yrs), of which 400 were attending the infertility clinic of Wadia Maternity Hospital and the rest from infertility clinic of our Institute were investigated. The results obtained from these patients were compared with those obtained from a group of 125 normal fertile men (mean age was 27 yrs with a range of 23-37 yrs) who had fathered a child within the past 1 yr. In every case the semen was obtained by masturbation into a wide-mouthed sterile glass container, after a requested period of abstinence ranging from 3-5 days. The samples were brought to the laboratory where the analysis was done within 1 hr of ejaculation. The samples which do not liquify within 45 min after the collection and those contained excess WBC (>10 WBC / high power field) were excluded from this study.

Semen analysis :

The studies were performed in two steps. In the first, each sample was tested for sperm count, motility (forward progressive and slow progressive) morphology and viability by conventional method according to the World Health Organization (16). In the second, the quality of each sample was tested by resazurin reduction test (RRT).

Based on the results obtained, the samples were grouped as follows:

Group A (Oligozoospermic) (n=108)	- Sperm concentration < $20x10^6$/ml, motility > 40%
Group B (Asthenozoospermic) (n=23)	- Sperm concentration > $20x10^6$/ml, motility < 40%

Group C (Oligoasthenozoospermic) (n=104)	-	Sperm concentration <20x10^6/ml motility < 40%
Group D (Azoospermic) (n=44)	-	Nil
Group E (Normozoospermic) (n=221)	-	Sperm concentration > 20x10^6/ml,\ motility >40%
Group F (Proven fertile) (n=125)	-	Sperm concentration ranges between 47-151x10^6/ml, motility ranges between 56-75%.

Resazurin reduction test (RRT) :

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) is a chemical indicator (Fig.1). The basic principle of the RRT is that during its reduction, preceeds through a series of colour changes (8). The reduction of resazurin (blue colour) by metabolically active cells to resorufin (pink colour) offer an assessment of the reducing capacity of semen, which is manifested by a spectrum of colours (Fig. 2).

RRT was performed using the original method described by Glass et al. (1). Briefly, the sodium salt of resazurin (Sigma-R2127) was dissolved in 0.9% physiological saline (50 mg/100 ml) and 50 µl of this solution was added per ml of semen in a glass tube. The tube was placed upright in a switched-off water bath at 48°C and the temperature was monitored with a thermometer. After 1 hr of incubation, the temperature had decreased to 34°C (11). The colour was matched with a chart of 11 colours ranging from pink (grade 11, strongest-reduction of resazurin) to dark purple (grade 1, weakest reduction). True positive were calculated as percentage of samples with count ≥ 20x10^6/ml and motility ≥ 40% produced negative colour in RRT. The sensitivity of the test is defined as its ability to detect the reduction of resazurin from blue to pink and hence equal to (true positive/true positive - false negative x 100). Specificity is defined as ability of the test to detect the absence of change in the colour of resazurin from blue to pink and hence, equal to (true negative/true negative + false positive x 100).

Statistical analysis :

If not stated otherwise, results are presented as means + SD. The statistical significance of difference between the mean values was analysed by Students 't' test (17). Sperman rank correlation analysis was applied for testing the correlation between the resazurin reduction and sperm parameters (18).

RESULTS

Semen characteristics of each group are shown in table 1. Spearman rank correlation analysis of the test is shown in table 2. The positive correlation between the results of RRT and various sperm parameters are shown in figures 1-4. Chemical structures of resazurin and its reduced form, resorufin are depicted in figure 5.

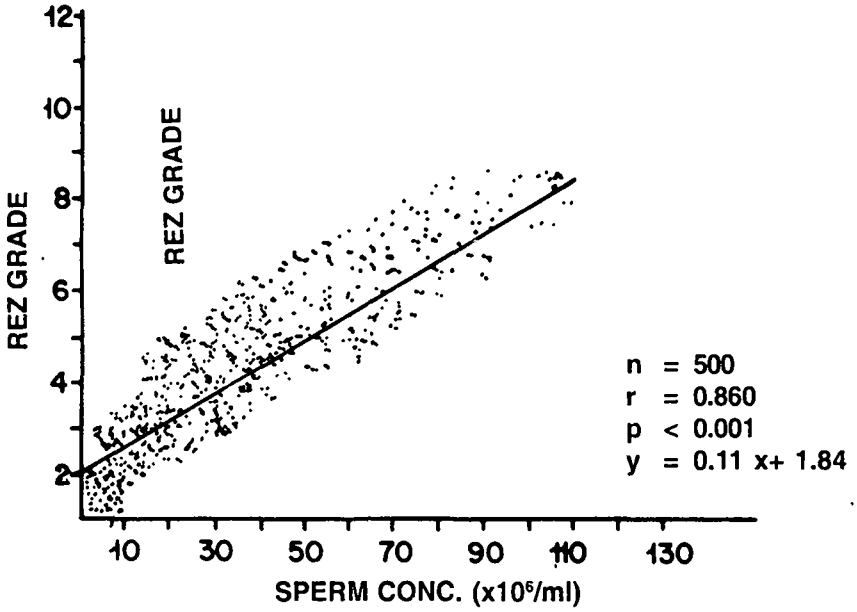

Fig. 1 : Correlation between sperm count and Rez grade

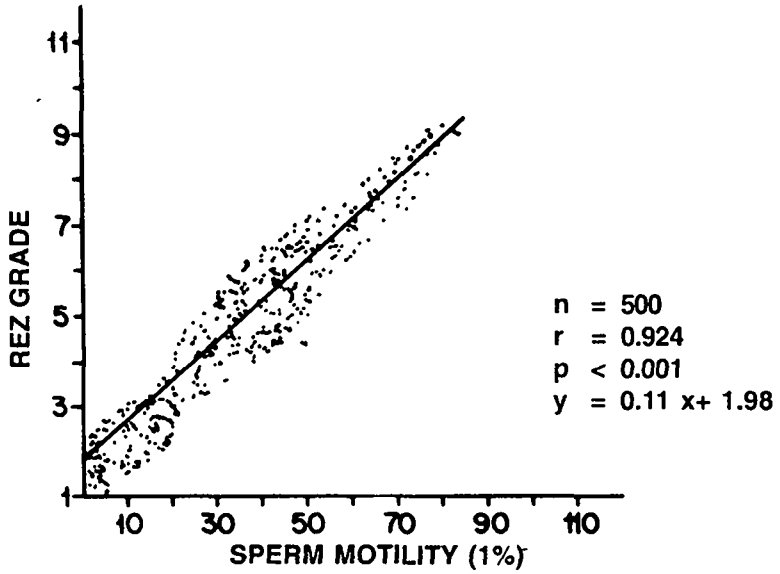

Fig. 2 : Correlation between sperm motility and Rez grade

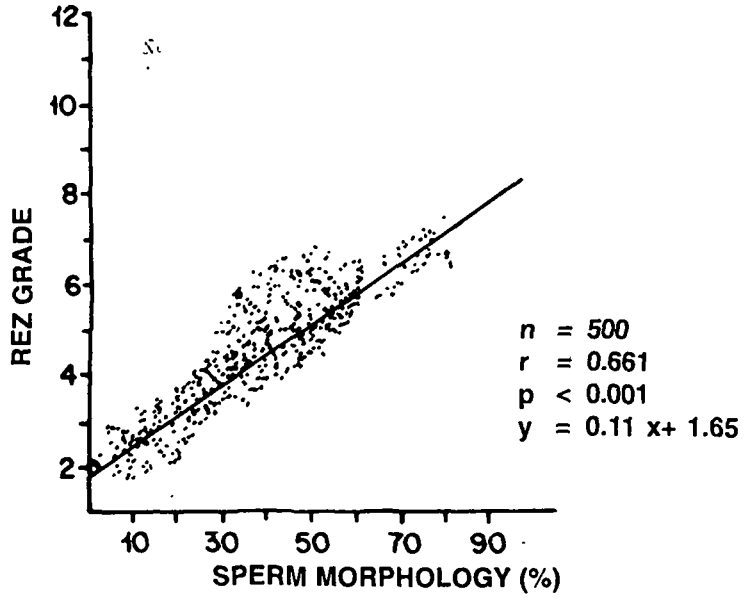

Fig. 3 : Correlation between sperm morphology and Rez grade

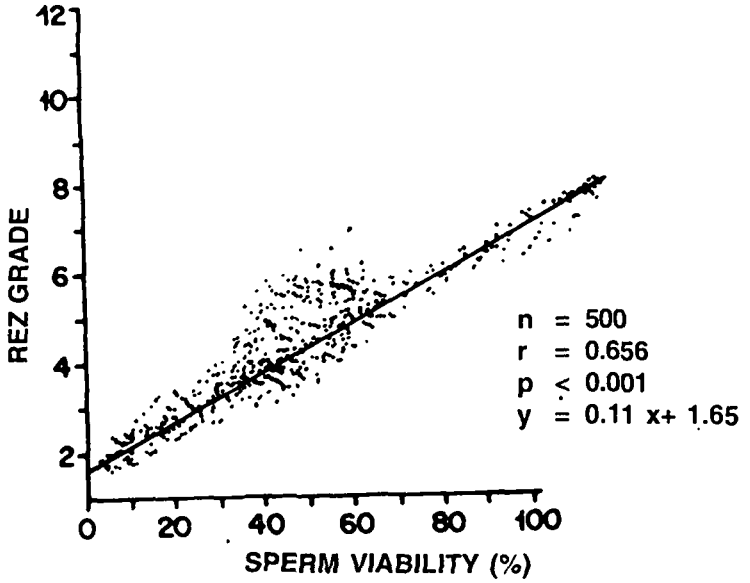

Fig. 4 : Correlation between sperm viability and Rez grade

Of the 500 ejaculates analysed, 108 (21.60%) samples were classified as oligospermic, 23 (4.60%) as asthenozoospermic, 104 (20.80%) as oligoasthenozoospermic, 44 (8.80%) as azoospermic and 221 (44.20%) as normozoospermic. Of the 279 subfertile specimens included in group A, B, C and D, in 191 (68.46%) cases the infertility was identified as due to the male factor alone, and in 88 (31.54%) cases it was due to both male and female factors. Moreover, in 104 (37.23%) subfertile patients, the presence of low sperm count has been associated with poor motility (<40%) (oligoasthenozoospermia), whereas in 156 (56%) cases, the subfertility was associated with morphological defects (<40% normal forms) (teratozoospermic). In 102 (48.10%) oligo - and oligo-asthenozoospermic cases, the patients had poor count and morphology (<40x10^6, <40% normal forms). Overall, in 54% (151/279) of subfertile cases, sperm abnormalities were noticed at acrosomal region and 27% in midpiece and 19% in tail.

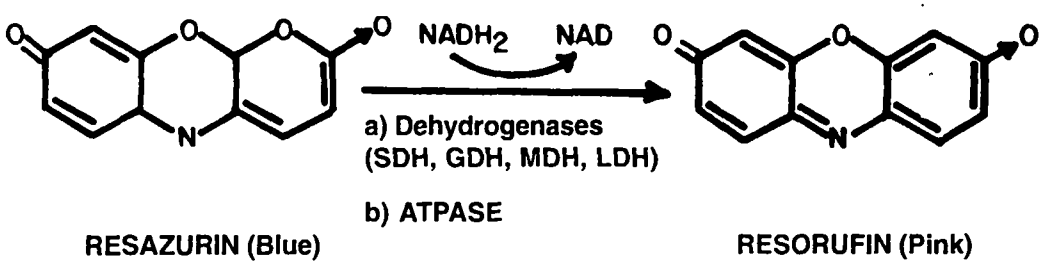

Fig. 5 : Resazurin test : Principle

In patients of group A, B, C and D the semen samples examined showed an RRT reaction with a range of colours from dark purple to dark burgundy (RRT grades 1-4). In patients of group E and F the semen samples showed RRT reaction with a range of colours from red violet to pink (RRT grades 5-11). There was a significant difference in the rate of positive tests between samples which had sperm count > 20x10^6/ml and motility ≥ 40%. 95% (328/346) of the samples with this count and motility fell within the normal range with RRT grade >5. Thus, RRT grade 5 clearly corresponds to the normal semenological values (20x10^6/ml, 40% motility). In addition, this value allowed a good demarcation of two groups of colours.

Analysis of correlation co-efficients for conventional semen parameters and RRT grade revealed a significant correlation for sperm motility ($r=0.924$, $p<0.001$), sperm concentration ($r=0.860$, $p<0.001$), normal morphology ($r=0.661$; $p<0.001$) and viability ($r=0.656$; $p<0.01$). The positive and negative predictive value for the RRT grade >5 and < 5 is 94%.

DISCUSSION

RRT is an *in vitro* diagnostic test used for the assessment of quality and fertilizing ability of spermatozoa in bovine semen (8). Our results confirm the original studies of Glass *et al* (1),

Table - 1 : Characteristics of various semenological parameters

Group	Sperm count (x10⁶/ml)	Motility (%)	Morphology (%)	Viability (%)	RRT grade
A (n=108)	13.98 + 6 40	45.67 + 4.92	37.18 + 5.26	55.36 + 8.40	2.82
B (n=23)	39.30 + 6.92	27.78 +	31.83 + 6.10	37.88 + 4.39	2.22
C (n=104)	7.72 + 3.40	16.60 + 4.55	26.12 + 4.87	27.10 + 5.55	2.07
D (n=44)	-	-	-	-	1.17
E (n=221)	51.19 + 17.04	58.02 + 17 18	46.96 + 8.45	65.69 + 10.11	6 07
F (n=125)	63.76 + 12.30	56.13 + 9.78	58.82 + 11.20	66 99 + 10 12	10.00

Table - 2 : Spearman rank correlation analysis to assess the performance of RRT

Group	Sperm conc. (X10⁶/ml & motility %)	Rez.criterion value	Positive	Negative	Total
A}	< 20 and > 40				
B}	> 20 and < 40	< 5	18	216 *	279
C}	< 20 and < 40				
D}	-				
E}	> 20 and > 40	> 5	323 **	23	346
F}			341	238	625

* consists of 44 azoospermic samples from group D ; ** consists of 125 proven fertile samples from Group F ; Sensitivity = 94.22%; Specificity = 93.53%; The efficiency = 93.89%.

where the authors have shown that RRT results provide an opportunity to differentiate fertile samples from subfertile. The results presented here show that the RRT results correlate well with sperm count, motility, morphology and viability. A good correlation of RRT grade was found with sperm motility, followed by concentration and morphology. Since the reduction of resazurin varies with sperm motility and count, the observed high correlation of RRT grade suggests highest association of the RRT grade with these two most powerful semen discriminating variables (4).

RRT had a positive predictive value of 94.80% for a grade 5 or more, (> 20x10⁶/ml, > 40% motility) suggesting that there is 95% chance for normal sperm activity, and fertility potential is good, if the colour of the sample matches in the positive group. Likewise, the negative predictive power of the test is 93.55%, it means, if the colour match is in the negative group there is 94% chances that the sperm activity is below normal.

For most of the variables, the false positive and false negative groups represent a "greyzone" midway between the true positive and true negative groups. The majority of our false results occurred at RRT grade 4-5 and this range was therefore considered as a borderline result. It was further noticed that the RRT grade > 5 would be the safe criterion value with highest accuracy in differentiating samples with sperm concentration and motility higher than $20x10^6$/ml and 40%, respectively.

The data obtained in the present study suggest that it is possible to identify patients with best qualitative and quantitative parameters of sperm, using RRT. According to Steinberger *et al* (19), patients with sperm counts $\geq 60x10^6$/ml, motility $\geq$ 70%, and $\geq$ 50% normal morphology represent individuals with maximum fertility potential. With these parameters in the male, pregnancy occurs after an average of 1.9 cycles in couples where female fertility potential is also optimal. This maximum fertility potential clearly corresponds to the RRT grades obtained in our fertile group (Table 1). These studies further emphasize that the colour changes of the RRT sharply discriminates not only subfertile samples from fertile but also reveals a putative maximum fertility potential of men. Our findings are consistent with those of Rehaman and Kulal (20).

Interestingly, it was observed that the samples which had similar sperm parameters showed different levels of resazurin reduction capacities, possibly due to the variation in the enzyme activity levels. The enzymes, particularly dehydrogenases, hyaluronidase creatine kinase and adenosine triphosphatase (ATPase) are known to present on sperm and may involve in the conversion of resazurin to resorufin (21-23). The activity levels of these enzymes have been reported to be low in the spermatozoa of subfertile males than the fertile (21-22), Probably, this is one of the many reasons for such differences in the fertility status of the men with similar sperm characteristics. In addition, great variability in semen parameters has been demonstrated among proven fertile fathers (24). Moreover, determination of resazurin reduction in the semen by sperm may reflect a different aspect of sperm function that may not be predicted by conventional methods. To confirm this, the quality and fertilizing ability of few samples were evaluated simultaneously by various sperm function tests (e.g. nuclear chromatin decondensation test, hypo-osmatic swelling test, sperm mitochondrial activity index and test for acrosome intactness) and compared with the RRT results. A close correlation was found between RRT grades and sperm function results (Unpublished data).

The RRT can be performed with relatively small volume of semen and it is easy to perform and requires no major equipment or a trained technician. The requirements for the test are a glass test tube, hot waterbath, a thermometer, the dye and the colour chart. Moreover, the test results can be read with naked eye.

Conventional semen analysis, although provides information about sperm count, motility and morphology, it fails to address the ability of sperm to fertilize the oocyte. This method has been much maligned of late and replaced with very expensive and time consuming assays such as hamster egg penetration test, sperm lateral head displacement test and cervical mucus

penetration test, but these have added more confusion than clarity to the picture. There remains a frustrating discrepancy between the results of these tests (4, 25). For the moment, the RRT will limit their widespread use, because of its simplicity and reliability.

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REFERENCES

1 Glass RH, Ericsson SA, Ericsson RJ, Dronin MT, Marcoux LJ and Sullivan H. (1991). The resazurin reduction test provides an assessment of sperm activity. *Fertil Steril* **56**: 743-747.

2 Fuse H, Okumara M, Kazama T and Katayaina T (1993a). Comparison of resazurin test result with various sperm parameters. *Andrologia* **25**: 153-157.

3 Fuse H, Ohta S and Katayama T. (1993b). Varicocoelectomy : Changes in resazurin reduction reaction. *Arch Andrology* **31**: 49-54.

4 Aitken RJ, Best FSM, Richardson DW, Djwhanbakhch O, Templeton A and Lees MM (1982). An analysis of semen quality and sperm function in cases of oligozoospermia. *Fert Steril* **38**: 705-712.

5 Dunphy BC, Neal LM and Cooke ID (1989). The clinical value of conventional semen analysis. *Fert Steril* **51**: 324-329.

6 Freund M and Carol B (1964). Factors affecting haemocytometer counts of sperm concentration in human semen. *J Reprod Fertil* **8**: 159-153.

7 Ginsburg KA and Armant DR (1990) The influence of chamber characteristics on the reliability of sperm concentration and movement measurements obtained by manual and videomicrographic analysis. *Fert Steril* **53**: 882-889.

8 Erb RE and Ehlers MH (1950). Resazurin reducing time as an indicator of bovine semen fertilizing capacity. *J Diary Science* **33**: 853-864.

9 Dart MG, Mesta J, Crenshaw C and Ericsson SA (1994). Modified resazurin reduction test for determining the fertility potential of bovine spermatozoa. *Arch Androl* **33**: 71-75.

10 Zalata A, Hafez T, Mahmoud A and Comhaire F (1995). Relationship between resazurin reduction test, reactive oxygen species generation and r-glutamyltransferase. *Hum Reprod* **10**: 1136-1140

11 Reddy KVR, Meherji PK, Gokral JS and Shahani SK (1997). Resazurin reduction test to assess the semen quality. *Indian J Exp Biol* **35**: 369-373.

12 Reddy KVR and Bordekar AD (1998). Spectrophotometric analysis of resazurin reduction test and semen quality in men. *Indian J Exp Biol* (In press).

13 Davies JG (1940). The resazurin test. *Diary Industries* **5**: 18-21.

14 Kummerlin R (1982). Technical note : resazurin test for microbiological control of deep frozen shrimps. *J Food Technol* **17**: 513-519.

15 Mattila-Sandholm T, Ah-vehmas T, Wirtanen G, Ronner U and Sandholm M (1991). Automated fluorimetry in quality control of pasteurised and ultra-high temperature treated starch soup. *Int J Food Science Technol* **26**: 325-336.

16 World Health Organization (WHO) (1992) Laboratory manual of the examination of human semen and sperm-cervical mucus interaction, 3rd edn. Cambridge University Press, Cambridge, pp. 20-42.

17 Gupta S (1978). In: Gupta S (ed), *Statistical methods*, Sultan Chand and Sons, 2nd edn. p 53.

18 Zar JH (1984). *Biostatistical analysis*. 2nd edn. Englewood Cliffs, New Jercy, Prentice-Hall, Inc.

19 Steinberger E, Rodriguez-Rigau LJ and Smith KD (1981). The interaction between the fertility potentials of the two members of an infertile couple. In : Frajese G, Hafez ESE Conti C and Fabrini A (eds), *Oligospermia, recent progress in andrology,* Raven Press, New York, USA.

20 Rahaman NA and Kula K (1997). Enlarged spectrum of seminological diagnosis using the resazurin colour reaction; a spectrophotometric application. *Int J Androl* **20**: 17-22.

21 Comhaire FH, Vermeulen L and Schoonjans F (1987). Reassessment of the accuracy of traditional sperm characteristics and adenosine triphosphate (ATP) in estimating the fertilizing potential of human semen in vivo. *Int J Androl* **41**: 453-662.

22 Huszar G, Vigue L and Corrales M. (1990). Sperm creatinine kinase activity in fertile and infertile oligospermic men. *J Androl* **11**: 40-45.

23. Abdul Aziz M, Maclusky N, Bhavnani B and Casper RF. (1995). Hyaluronidase activity in human semen : correlation with fertilization *in vitro*. *Fert Steril* **64**: 1147-1153.

24. Barloov B, Eltes F, Pansky M, Lederman H, Caspi E and Soffer Y (1993). Estimating fertility potential via semen analysis data. *Hum Reprod* **8**: 65-70.

25. Acosta A, Ochinjer S, Morshedi M, Swanson RJ, Scott R and Irianni F (1989). Assisted reproduction in the diagnosis and treatment of the male factor. *Obstet Gynecol Surv* **44**: 1-17.