Medical Hypotheses 117 (2018) 50–53

Contents lists available at ScienceDirect

Medical Hypotheses

journal homepage: www.elsevier.com/locate/mehy

A mathematical model predicting the individual outcome of IVF through sperm-analysis: The role of the HaloSpermG2® DNA fragmentation test

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ARTICLE INFO

Keywords:
Assisted reproduction
DNA fragmentation
IVF
Semen analysis
Pregnancy rate
HaloSpermG2®
Infertility

ABSTRACT

The present paper reports the results of a pragmatic prospective trial in a group of 38 random infertile couples in whom a battery of semen assays were performed before in vitro fertilisation (IVF). Sixteen couples (42.1%) attained ongoing pregnancy. Using logistic regression analysis only the result of the Oxisperm® ($P = 0.047$) and the HaloSperm G2® for DNA fragmentation ($P < 0.0001$) were significantly associated with the occurrence of pregnancy, whereas neither the conventional semen characteristics, nor the outcome of multiple other tests were significantly related ($P > 0.05$). Based on the logistic regression analysis the following formula could be derived: Logit(p) = 6.15–0.407 × (% *halotest*), whereby (p) is the probability of pregnancy, and % *halotest* is the proportion of spermatozoa showing DNA fragmentation in the HaloSperm G2® test. Receiver operating characteristic curve analysis revealed an area under the curve (AUC) of 0.83. In 16 out of 38 couples the IVF outcome, either positive or negative, could unequivocally be predicted, while in the remaining cases the probability of pregnancy was significantly related to the result of the formula. These findings confirm the hypothesis that sperm DNA-fragmentation largely determines the success of IVF.

Introduction

Assisted reproduction, in vitro fertilization (IVF) in particular, has successfully been applied since over 4 decades, and has offered a solution for many thousands of infertile couples. In spite of several technical improvements, the success rate per initiated cycle remains relatively low, even after the transfer of selected embryos. One of the reasons for this may be the poor fertilizing potential of spermatozoa, the quality of which is impaired by diseases such as varicocele, or infection of the accessory sex glands. Also external factors play a pivotal role in causing genetic, and/or epigenetic, and/or oxidative alterations of sperm DNA inducing DNA fragmentation [1].

Many studies have emphasized the poor capacity of the conventional sperm characteristics to predict the outcome of IVF, whereas tests of oxidative stress on DNA and of DNA fragmentation may have a stronger predictive power [2,3]. The majority of these tests are, however, rather complicated and time consuming, sometimes poorly reproducible, and difficult to implement in the sperm lab. Hence, their routine use in couples undergoing assisted reproduction remains limited [4].

In the present paper we have evaluated the predictive capacity of a large set of conventional and advanced tests on spermatozoa in relation to the outcome of IVF, by means of a pragmatic prospective cohort trial. We have deduced a mathematical formula that allows for the calculation of the probability of individual couples to attain ongoing pregnancy.

Materials and methods

Patients

Patients were 38 random couples presenting at the Centre for Reproductive Medicine of the municipal community hospital Jan Palfijn in Ghent and who were treated by IVF. Both partners had been fully investigated and, if applicable, treated for causal factors contributing to their infertility problem. The median age of the male partners was 35 years (range 24 years–44 years). There were no complementary inclusion criteria.

After having been fully informed orally, all patients signed an informed consent. The ethical committee of AZ Jan Palfijn Gent approved the study.

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https://doi.org/10.1016/j.mehy.2018.05.021
Received 6 April 2018; Accepted 25 May 2018

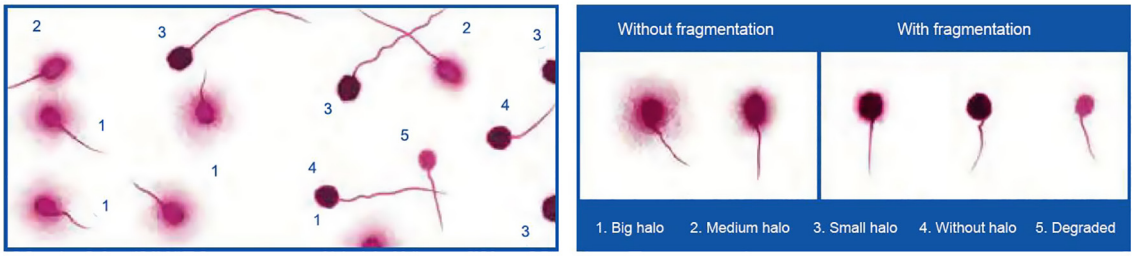

Fig. 1. Overview of cells stained with HaloSperm G2®. A possible view under the microscope, different possibilities of halos. Spermatozoa that have intact DNA produce a halo (nr. 1 and 2). Spermatozoa with DNA fragmentation produce a small halo or no halo at all (nr. 3 and 4). Spermatozoa with degraded DNA are lightly stained, without producing a halo (nr. 5). (Figure provided with the HaloSperm G2 kit).

Semen analysis

Routine semen analysis was performed by highly trained technicians in agreement with the WHO-guidelines [5] including measurement of ejaculate volume, sperm concentration, progressive motility and morphology [6], and the concentration of so-called round cells.

Oxidative burden (ROS) was estimated using several tests including chemiluminescence (area under the curve), resazurin reduction measured by spectrophotometry [7], and the OxiSperm® test (Sperm Oxidative Stress Test, Halotech, Spain) measured by spectrophotometry.

Tests of *DNA integrity* included the acidified aniline blue staining [8], the acridine-orange test [9,10], and the HaloSperm G2® test (HT-HSG2; Selinion Medical, BM's-Hertogenbosch, The Netherlands).

The latter test used a commercial kit, provided by Halotech DNA. The test was performed within 3 h after the semen sample was produced, as described in the manufacturer's instructions. The detailed description of the method can be found in Appendix A. The DNA in the sperm head becomes visible in bright field microscopy after double staining. Spermatozoa without fragmented DNA form DNA loops that appear as halos. Fragmented DNA does not form loops and consequently no halo is seen (Fig. 1).

Five hundred spermatozoa were evaluated, and the % spermatozoa with fragmented DNA *(%halotest)* was calculated. Degraded spermatozoa were also counted as positive for DNA fragmentation.

Statistical analysis

Statistics used the MedCalc program (MedCalc, Ostend, Belgium) [11] to perform logistic regression analysis with stepwise elimination, and to assess receiver operating characteristic curve plots [12].

Results

Logistic regression analysis was performed with the occurrence of ongoing pregnancy as dichotomous dependent variable. There was no significant relation with sperm concentration (P = 0.16), progressive motility (P = 0.54) or morphology (P = 0.92), nor with the concentration of round cells (P = 0.94). Neither was there any significant relation with chemiluminescense (P = 0.067), the resazurin reduction (P = 0.74), the acidified aniline blue staining (P = 0.14), or the acridine orange test (P = 0.21).

There was a borderline significant relation with the result of the OxiSperm® test (P = 0.047). However, there was a highly significant relation between the occurrence of pregnancy and the result of the HaloSperm G2® test, with P < 0.0001. Receiver operating characteristic (ROC) curve analysis reveals an area under the curve (AUC) of 0.83.

Based on the logistic regression analysis the following formula was derived predicting the probability of pregnancy (p):

$$\text{Logit(p)} = 6,153 - 0.407 \times (\%halotest)$$

The logit(p) value can be back transformed to the predicted probability of pregnancy (p) by using the formula below.

$$logit\,(p) = \ln\left(\frac{p}{1-p}\right)$$

$$p = \frac{1}{1 + e^{-logit\,(p)}}$$

Alternatively the logit table can be used to estimate the probability of pregnancy from the logit(p) value (Table 1), or by means of the MedCalc software, or it can be derived with approximation from Fig. 2.

The results of the *%halotest* in the groups with or without pregnancy are plotted in Fig. 3. It can be seen that the semen samples with a high level of fragmentation, and *%halotest* in excess of 16, were exclusively associated with failure to attain pregnancy (n = 8). Semen samples with *%halotest* lower than 11 occurred only in the couples who did get pregnant (n = 8).

Discussion

It should be emphasized that the present study is based on a limited number of observations. However, the pregnancy rate in this group is similar to that registered in 1267 couples treated by IVF in the same

Table 1
Logit(p) back transformation table.

p	logit(p)	p	logit(p)	p	logit(p)	p	logit(p)
0.01	−4.5951	**0.26**	−1.0460	**0.51**	0.0400	**0.76**	1.1527
0.02	−3.8918	**0.27**	−0.9946	**0.52**	0.0800	**0.77**	1.2083
0.03	−3.4761	**0.28**	−0.9445	**0.53**	0.1201	**0.78**	1.2657
0.04	−3.1781	**0.29**	−0.8954	**0.54**	0.1603	**0.79**	1.3249
0.05	−2.9444	**0.30**	−0.8473	**0.55**	0.2007	**0.80**	1.3863
0.06	−2.7515	**0.31**	−0.8001	**0.56**	0.2412	**0.81**	1.4500
0.07	−2.5867	**0.32**	−0.7538	**0.57**	0.2819	**0.82**	1.5163
0.08	−2.4423	**0.33**	−0.7082	**0.58**	0.3228	**0.83**	1.5856
0.09	−2.3136	**0.34**	−0.6633	**0.59**	0.3640	**0.84**	1.6582
0.10	−2.1972	**0.35**	−0.6190	**0.60**	0.4055	**0.85**	1.7346
0.11	−2.0907	**0.36**	−0.5754	**0.61**	0.4473	**0.86**	1.8153
0.12	−1.9924	**0.37**	−0.5322	**0.62**	0.4895	**0.87**	1.9010
0.13	−1.9010	**0.38**	−0.4895	**0.63**	0.5322	**0.88**	1.9924
0.14	−1.8153	**0.39**	−0.4473	**0.64**	0.5754	**0.89**	2.0907
0.15	−1.7346	**0.40**	−0.4055	**0.65**	0.6190	**0.90**	2.1972
0.16	−1.6582	**0.41**	−0.3640	**0.66**	0.6633	**0.91**	2.3136
0.17	−1.5856	**0.42**	−0.3228	**0.67**	0.7082	**0.92**	2.4423
0.18	−1.5163	**0.43**	−0.2819	**0.68**	0.7538	**0.93**	2.5867
0.19	−1.4500	**0.44**	−0.2412	**0.69**	0.8001	**0.94**	2.7515
0.20	−1.3863	**0.45**	−0.2007	**0.70**	0.8473	**0.95**	2.9444
0.21	−1.3249	**0.46**	−0.1603	**0.71**	0.8954	**0.96**	3.1781
0.22	−1.2657	**0.47**	−0.1201	**0.72**	0.9445	**0.97**	3.4761
0.23	−1.2083	**0.48**	−0.0800	**0.73**	0.9946	**0.98**	3.8918
0.24	−1.1527	**0.49**	−0.0400	**0.74**	1.0460	**0.99**	4.5951
0.25	−1.0986	**0.50**	0.0000	**0.75**	1.0986		

(Reproduced from: Schoonjans. Manual to the MedCalc statistical program).
Note: logit(p) values lower than the −4.5951 correspond with p < 0.01 (or < 1%), and values higher than 4.5951 correspond with p > 0.99 (or > 99%).

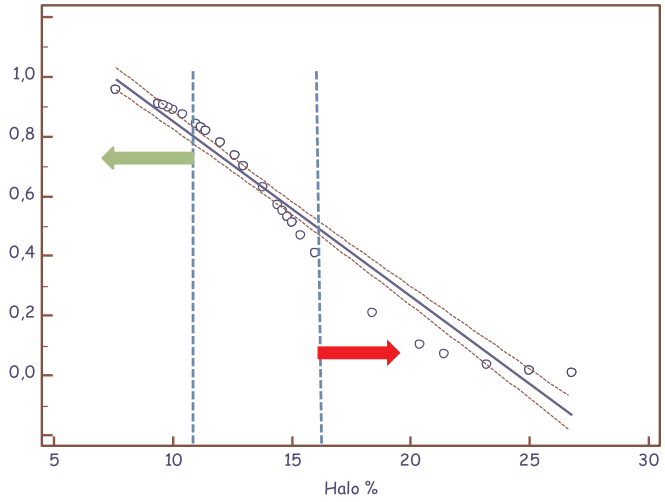

Fig. 2. The relation is depicted between the HaloSperm test result (in % on the horizontal axis) and the predicted probability of successful result of IVF (between 0 and 1.0, on the vertical axis), as deduced from the formula: logit (p) = 6,153–0.407 × (%*halotest)*. The regression line and its 95% confidence intervals are shown. The graph allows for the approximate estimation of the probability of pregnancy after IVF based on the result of the halotest. The vertical lines indicate the limits below, respectively above which the result of the halotest was found to always be associated with successful (green arrow), respectively failed (red arrow) IVF.

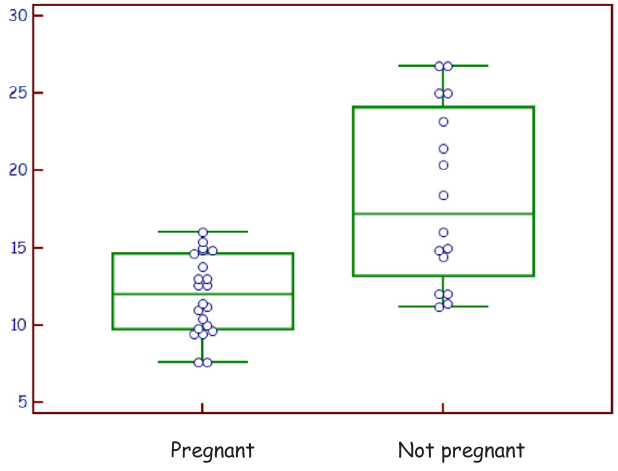

Fig. 3. Box and whisker plot of the %halotest (on the vertical axis) in the couples who did (pregnant) or did not (not pregnant) attain pregnancy (Mann-Whitney test for independent samples: $P < 0.0001$).

centre in 2017, suggesting that the couples selected at random probably were representative of the entire patient population. Much attention was given to the quality of the technical laboratory work, and all tests were submitted to analysis of inter- and intra assay variability (details accessible in the master thesis of A. Messiaen [13] and to be published separately).

The present findings are in agreement with published reports sustaining the importance of DNA damage as a limiting factor of success in IVF, with or without intra cytoplasmic sperm injection (ICSI) [14,15], though not confirmed by other investigators [16]. These discrepancies may be related to the sensitivity and reproducibility of tests used [17,18].

The approach taken in the present study, particularly the statistical method of logistic regression analysis, has revealed the critical importance of implementing the result of the HaloSperm G2® test into a formula that allows for the calculation of the individual probability of IVF being successful or not. In fact, the extreme values of the calculated score are unequivocally related to the occurrence or not of pregnancy in the subsequent IVF cycle. Intermediate logit(p) scores indicate the relative probability of success [19]. In the latter cases, the interaction with possible female factors may be of pivotal importance.

In spite of the limitations of the present pilot trial, some interesting aspects may be highlighted. First it should be explored in a future study, including a larger number of couples, whether it makes sense to perform IVF using sperm with a high degree of DNA damage, as evidenced by the HaloSperm test and logit(p) score. Excluding such couples for IVF may rather dramatically improve the success rate, reducing the cost per take-home baby [20]. Also, this score may be used as a marker of the possible favourable effect of treatment of the infertile man [2] which would be associated with increased probability of successful IVF.

Acknowledgements

The authors express their gratitude to prof. Kaan Osmanagaoglu MD. PhD. for his financial support and technical supervision of the laboratory techniques. Also to Lieve Declercq for collecting the data, to Frederik Mahieu and Tahira Verheecke for their excellent input into the execution of the laboratory tests, and Frank Schoonjans for his statistical advice. The study was part of the thesis for master in biomedical sciences (2016–2017) at the University of Brussels of Alexine Messiaen, with prof. Kaan Osmanagaoglu as promotor and prof. Ellen Goossens as co-promotor.

Conflict of interest

The authors report no conflict of interest.

Appendix A

Detailed description of the HaloSperm G2® method (Messiaen, 2017): 100 µl agarose was melted at 95 °C and placed for five minutes at 37 °C. The agarose was mixed with 50 µl of the semen sample that was standardised to a maximum concentration of 20×10^6 spermatozoa/ml. Eight microliter of this mixture was placed on a microscope slide provided with the kit. The slide was covered with a coverslip and put at 4 °C for 5 min. The coverslip was then removed and a solution was applied on the slide for 7 min to denature fragmented DNA. This solution was discarded by tilting the slide, and a lysis solution was applied for 20 min removing sperm proteins and membranes. After this lysis solution was removed, the slide was fixed by rinsing with a mixture of 70% water and 100% ethanol. The first staining was added to the slide for 10 min, followed by the second staining for an additional 10 min.

The double staining coloured the DNA in the sperm head, which becomes visible in bright field microscopy. Because of the denaturation and lysis, spermatozoa without fragmented DNA form DNA loops that become visible as halos (Fig. 1). If the DNA was fragmented, no loops will be formed and consequently no halo is seen.

Five hundred spermatozoa were evaluated, and the % spermatozoa with fragmented DNA (% halotest) was calculated. Degraded spermatozoa were also counted as positive for DNA fragmentation (Fig. 1).

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.mehy.2018.05.021.

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