The prevalence of hyperinsulinaemia among normozoospermic donors at Medfem Clinic, South Africa.

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Summary:

The aim of this study was to investigate the prevalence of hyperinsulinaemia in a group of normozoospermic donors and the influence of insulin levels on in vitro fertilization (IVF) outcome. Fasting insulin and two hour post eating insulin levels were determined for a group of thirty four sperm donors. They were divided into three groups according to their insulin profiles. The association between insulin levels and embryo quality was determined in a clinical study for the different groups. The prevalence of raised insulin in the donor group was 44% with a 95%

confidence interval of 27.2% and 62.1%. Donors with normal insulin levels produced more good quality blastocysts in the IVF program than those with abnormal insulin levels. These differences were not statistically significant (P=0.8638). Embryo quality in an IVF program may be influenced by male insulin levels. The role of insulin in male fertility needs to be investigated.

Key words: fasting insulin, two hour post eating insulin, embryo quality, hyperinsulinaemia.

Introduction

The prevalence of hyperinsulinaemia and insulin resistance is on the rise in Western societies (Li *et al*., 2006). A sedentary lifestyle, unhealthy diet, and increased body mass index (BMI) predisposes a person to hyperinsulinaemia and insulin resistance. Poor insulin activity and chronically elevated blood insulin levels are associated with a number of serious diseases such as hypertension, obesity, ischemic heart disease, dislipidaemia and non-insulin-dependent diabetes mellitus (NIDDM) (Cosford, 1999; Reaven, 2004). Male sub-fertility is also on the increase and it has been postulated that lifestyle can contribute to the marked decline in semen quality observed (Carlsen *et al*., 1992; Giwercman *et al*., 1993).

The causative relationship between hyperinsulinaemia and above mentioned diseases can be understood on the basis of the anabolic effects of insulin. Insulin together with amino acids and glucose, play a crucial role in the modulation of gonadotrophin-releasing hormone (GnRH) secretion and testicular function in animals (Bacetti *et al*., 2002). Hyperinsulinaemia has other hormonal effects in humans as well. Insulin causes an increase in the production of free androgens by reducing sex-hormone binding globulin (SHBG) (Nestler *et al*., 1992). Excessive

free androgens are linked with visceral obesity and infertility (Cosford, 1999). Elevated androgens in turn contribute to insulin resistance.

Various hormonal mechanisms have been suggested as mediators of lifestyle effects on insulin resistance. Diets high in saturated fats and trans-fatty acids have been shown to decrease cell membrane fluidity and decrease insulin receptor binding and hormone responsiveness, thus promoting insulin resistance. Micronutrients further affect cellular insulin balance, with some researchers stating that the primary defect of insulin-resistance is intra-cellular imbalance of calcium and magnesium (Anderson, 1997).

The combination of a high omega-6 essential fatty acid intake, as in the typical western diet, with hyperinsulinaemia is a potent stimulus to inflammation. This pro-inflammatory state increases the levels of cytokines such as tumor necrosis factor and free radical production (Ceriello *et al*., 1991; Paolisso, 1993). Spermatozoa are particularly susceptible to damage by free radicals, due to their high content of unsaturated fatty acids and relative lack of cytosolic antioxidant protection (Aitken *et al*., 2003a,b).

Tobacco smoking has been found to increase insulin resistance (Kong *et al*., 2001; Facchini *et al*., 1992), while chronic stress has been suggested to promote insulin resistance by increasing adrenalin and cortisol levels (Sapolsky, 1996).

Alterations in carbohydrate homeostasis, such as diabetes, have been associated with disturbances of the functional activity of the reproductive system in laboratory animals. This not

only involves the hypothalamo-pituitary axis, but also the gonads (Bacetti *et al*., 2002; Nestler *et al*., 1992). Research evidence indicates that, at least in animals, insulin plays a role in the maintenance of spermatogenesis and testicular endocrine function, where it can prevent spermatogenic abnormalities to some extent (Gondos & Bavier, 1995). If similar effects exist in the context of human male reproduction, the rising rates of NIDDM may well pose a significant problem to human fertility.

Despite these warnings regarding the potential negative impact of hyperinsulinism, the effect of insulin levels on male reproductive health has received little attention to date.

Previous studies concentrated on the influence of insulin-dependent diabetes on semen parameters and the associated decline in male fertility (Bacetti *et al*., 2002; Handelsman *et al*., 1985; Vignon *et al*., 1991). However the influence of hyperinsulinaemia on semen parameters and male fertility has not been documented to date. The aim of this study was to investigate the mean concentrations of fasting and 2 hour post-meal serum insulin levels as well as the prevalence of hyperinsulinaemia in a healthy group of men. The effect of insulin levels on embryo quality in an IVF program was also investigated. Insulin sensitivity and secretion can be measured by the hyperinsulinemic-euglycemic “clamp” and the insulin response to an intravenous glucose infusion. These tests are often considered the gold standards for assessment of insulin sensitivity and secretion (Lillioja *et al.*, 1993). Standard insulin sensitivity and secretion tests are labor intensive and difficult to perform in a large number of patients. Therefore, this study investigated the use of fasting and 2 hour post-meal insulin levels as simple

indices of insulin sensitivity. The effective use of these indices has already been established in studies by Hanson *et al*. (2000) and Laakso (1993).

Materials and Methods:

Initial assessment of research subjects:

Hundred and fifty one potential donors were screened over a period of 2 years. All the participants were required to provide written informed consent at the onset of the study. Each participant was requested to complete a self-administered lifestyle questionnaire. The aim of the questionnaire was to investigate environmental/lifestyle factors (e.g. tobacco use, alcohol consumption, physical exercise, chronic disease, emotional stress, work place and medication) that might influence semen parameters and /or hormonal levels. Thereafter, each participant's BMI was calculated. A BMI over 25kg/m^2 is defined as overweight and a BMI of over 30 kg /m^2 as obese (WHO, 2000). Only thirty four normozoospermic men (WHO, 1999) with a healthy lifestyle met the minimum requirements for participating as sperm donors. The majority of aspirant donors (77%) were disqualified on the fact that their sperm count and morphology were suboptimal (WHO, 1999).

Insulin and hormonal testing:

A hormonal profile (FSH, LH, SHBG, GH and testosterone*)* was performed on each of the research participants to exclude any non-insulin-related abnormal hormonal influences. The required period of fasting (22H00 the previous evening until 08H00 the next morning) for the fasting insulin blood test was explained to the participants, as well as the 3-5 days of sexual abstinence required for the standard semen analysis. A fasting (basal) insulin level was

measured on the morning of testing (before 10H00) and then repeated 120 minutes after they had a normal breakfast. Insulin, FSH, LH, SHBG, GH and testosterone assays were performed on the fasting blood sample using an Immulite® Automated Analyser. The research participants were divided into three groups according to their I_0 and I_{120} insulin levels. Group GG (Good, Good) consisted of participants with normal fasting and I_{120} levels, group GB (Good, Bad) had normal I_0 but abnormal I_{120} and group BB (Bad, Bad) had abnormal I_0 and I_{120}. The cut-off value for normal basal insulin levels is ≤ 9 µIU/ml and for 120 min post eating 6- 20 µIU/ml *(Azizi and Tariq, Immulite Technical Data)*. The Insulin ranges for the different Groups are indicated in Table 1.

Table 1. Insulin ranges for Donor Groups

Insulin Groups	Fasting Insulin µIU/ml (I_0)	Post Eating Insulin µIU/ml (I_{120})
Group GG (n=13)	0 - 9	6 - 20
Group GB (n=10)	0 - 9	> 20
Group BB (n=11)	> 9	>20

Semen Analysis:

Semen samples were obtained from each research participant after the recommended 3-5 days of sexual abstinence. All the samples were assessed by conventional light microscopic semen analysis to determine liquefaction, semen volume, sperm concentration and motility according to the World Health Organization recommendations (WHO, 1999). Sperm morphology was

assessed according to the Tygerberg Strict Criteria (Menkveld *et al*., 1990), while chromomycin A_3 (CMA_3) was assessed according to the method of Esterhuizen *et al*. (2000). Chromomycin A_3 is used to assess mature DNA packaging in the sperm head with a normal cut off value of ≤40%. Only normozoospermic males were allowed to participate in the study. Four to six sperm samples were collected and frozen in the month following the semen analysis and blood tests. The samples were frozen with FertiPro SpermFreeze™ (FertiPro N.V., Belgium) according to the method recommended by the manufacturers. The sperm samples were stored in liquid nitrogen for three months quarantine period where after the research participants were re-tested for HIV. The sperm samples of HIV-negative participants were then released for use by infertility patients.

In Vitro Fertilization (IVF):

The controlled ovarian hyperstimulation of IVF patients was achieved after GnRH suppression with Lucrin, using recombinant FSH (Gonal F®, Serono) and Luveris®. The ovarian response was monitored by ultrasound and serum E_2. The patients were triggered for aspiration after 36 hours with Ovidrel® when two or more follicles were >18 mm. The frozen sperm samples were thawed on the day of aspiration and prepared using a one step wash and swim-up technique (Mahadevan & Baker, 1984). The oocytes were inseminated with 200 000 motile sperm three to four hours after aspiration and checked for fertilization 19 hours later. The embryos were cultured to the blastocyst stage in Cook Medium (Cook, 2007). A maximum of two blastocysts were replaced on day 5. Embryo development and quality was noted for each patient. Day 5 blastocysts were evaluated using a scoring system similar to the one described by Gardner and Schoolcraft (1999). This system grades blastocysts according to blastocoel size, appearance and

size of the inner cell mass, trophectoderm and thickness of the zona pellucida. Excess blastocysts were vitrified with VitriFreeze^{TM} (FertiPro) vitrification medium and stored in liquid nitrogen for replacement in a future cycle. Pregnancy was confirmed by serum human chorionic gonadotrophin (hCG) levels thirteen days after embryo transfer. In cases of positive serum HCG levels, a clinical pregnancy was confirmed with ultrasound two weeks later. The outcome of the pregnancy was noted and clinical pregnancy rates were determined.

Statistical Analysis:

Stata Statistical Software: Release 10, College Station, TX, StataCorp was used for statistical analysis of the data (StatCorp, 2007). The results of the three participant groups (GG, GB and BB) were compared for difference in embryo quality and pregnancy outcome by means of the Chi-square test.

Results:

Spermatology:

Thirty four sperm donors participated in the study. Their ages ranged between 19 and 27 with a mean age of 21. The semen parameters in the three different insulin groups were all in the specified normal limits (WHO, 1999). The average sperm count for all the semen donations

Table 2: Mean spermatology data for the different donors

D0N0R	COUNT± SD	MOTILITY	MORPHOLOGY	CMA_3
1	31.6 ±5.27	59.2 ± 4.55	14.2; ±1.48	42.6±1.82
2	107.66 ±8.66	60 ± 3.06	13.33 ± 1.15	30.67 ± 1.15
3	88.75 ±30.24	66.75 ±6.24	13.5± 2.52	32.25±1.89
4	41.2 ±14.22	59.6±6.88	13.8±1.79	44 ± 1.41
5	78.6 ± 21.14	63.4± 10.97	15.4 ± 1.52	41.6 ± 1.82
6	41.33± 9.67	60.16 ± 5.67	14.67 ± 0.82	31.83 ± 0.98
7	48.5 ± 12.47	54.6 ± 10.24	12.8 ± 2.68	38.2 ± 1.79
8	47.25 ± 30.46	61.25 ± 9.46	14.5 ± 0.58	30 ± 1.63
9	33.9 ±11.34	65 ±5.44	16 ±1.34	35 ± 2.63
10	51.33 ± 21.13	58.33 ±10.41	12.67 ± 2.31	39.33± 1.15
11	87.26 ± 1.62	61.00 ± 5.29	14.00 ± 1.52	35.67 ± 1.15
12	58.0± 17.44	65.67 ± 1.15	14.67 ± 0.58	29.33 ± 1.15
13	44.38 ± 22.50	74.4 ± 11.46	13.8 ± 0.84	26.4 ± 2.61
14	31.92 ±13.86	53 ±9.90	17.5 ±3.0	34.75 ± 0.96
15	36.75 ± 7.89	63.75 ±2.5	15.0 ± 2.0	41.75 ± 3.5
16	68.0 ± 12.59	61.0 ±5.48	14.0 ± 1.87	40.8 ± 2.28
17	62.0 ± 25.71	56.67±2.89	15.33 ± 1.15	38.67 ± 1.25
18	76.25 ± 8.83	60.2 ± 0.22	14.2 ± 1.41	40.5 ±3.54
19	48.3 ± 19.80	61.25 ± 4.79	16.5 ± 1.91	34.5 ± 1.91
20	48.25 ± 5.38	65.4 ±12.25	15.3 ± 1.15	31.25 ±1.5
21	64.5 ± 2.12	62.5 ± 3.54	14.5 ± 0.71	37.2 ± 1.41
22	66.25±15.59	58.75 ± 8.54	15.6 ± 0.82	43.75 ± 1.5
23	28.9± 16.70	63.33± 2.89	15.33 ± 1.15	35.67± 2.31
24	84.6 ± 32.60	68.33 ±2.89	17.33±1.15	30.33 ± 2.31
25	59.63 ± 27.90	60.7 ±4.08	13.75 ± 0.5	33.25 ± 2.63
26	58.25 ± 16.62	55.8 ± 4.08	15.75 ±1.26	42.25 ± 2.63
27	108.25 ± 18.95	65.4 ± 4.08	14.25 ± 3.10	35.25 ± 4.43
28	96.25 ± 23.27	61.25 ± 2.5	33.5±2.94	15.75±1.26
29	45.33 ± 19.73	58.33± 2.89	15.33±1.15	34.9±3.61
30	51.25 ± 5.25	63.75 ± 7.5	16.25 ± 1.5	35.25±2.22
31	44.225±10.91	52.5± 5.0	17.25± 0.96	34±1.83
32	86.6 ± 12.17	61.67±10.41	15.33 ± 1.15	31.67±1.53
33	82.67± 20.03	58.33±10.41	13.33 ± 3.06	37.67±2.08
34	73.5±23.85	63.75± 4.78	15.25±0.96	33.5±3.70

during the first month of recruitment was 79.6 million/ml, with an average motility of 62.7%, normal morphology of 14% and normal CMA_3 of 36.3%. The mean spermatology (± SD) is presented in Table 2. All the participants tested HIV negative throughout the study period.

Lifestyle:

An analysis of the completed lifestyle questionnaires indicates that all of the participants followed a healthy lifestyle. None of the participants smoked tobacco products and the majority (80%) exercised three to four times a week. Seventy three percent of the participants indicated that they regularly use alcohol; the average consumption was 2 units (e.g. 2 glasses of wine /2 beers /2 tots or 50 ml spirits), 3.45 times a week. Most of the participants (86.7%) eat healthy meals three times a day and had maintained a stable weight for the past 5 years. Forty six percent of the participants used vitamin supplementation and none of them were using prescription medication on a regular basis. None of the participants were frequently exposed to hazardous chemicals like pesticides, paints, environmental oestrogens or other endocrine disruptors. They did not wear tight clothing and occupational exposure to high temperatures/ radiant heat as in the case of bakers, furnace workers, welders etc. was ruled out. Studies have indicated that afore mentioned factors may result in spermatogenesis failure or a greater risk of infertility (Mieusset & Bujan, 1995a,b; Setchell, 1998; Strohmer et al., 1993).

Two (22.2%) of the participants in Group BB with abnormal insulin levels changed their lifestyle after they were informed of their abnormal fasting insulin levels. They started to eat regular meals and took up sport activities. Their basal insulin levels were normal 3 months after adopting a healthy lifestyle and the sperm of both achieved pregnancies.

Insulin, BMI and hormonal assays:

About halve of the participants (n=19) had normal basal insulin levels (range 2-23 µIU/ml). The prevalence of raised insulin (>9 µIU/ml) was 44% with a 95% confidence interval of (27.2%; 62.1%). This indicates that the point estimated prevalence is large despite the small sample size and the wide interval estimate for the prevalence. The prevalence for abnormal post-meal insulin (I_{120}) (≥ 20µIU/ml) was 55.9%. Thirteen participants had normal I_0 and normal I_{120}; they were allocated to group GG. Ten participants were allocated to group GB with normal I_0 but abnormal I_{120}, while group BB consisted of eleven participants with abnormal I_0 and I_{120}.

All the participants had normal LH-values. The prevalence of abnormal FSH-values and SHBG-values were very low for both parameters, namely 2.94%. Five of the participants (14.7%) had abnormally high (>94.8*)* free index testosterone values. The spermatozoa of one of these five participants achieved a pregnancy in the IVF program. Also, four of these five participants had lower than normal GH levels (<0.1 ng/ml). In the case of one participant this coincided with a low SHBG-value (11 nmol/l) and a high BMI of 31.2. The sperm of this particular participant did not achieve a pregnancy in the IVF program.

A summary of the data for the different hormonal parameters is presented in Table 3.

Table 3. Hormonal and BMI values of the donors

Variable	Normal Range	Mean, standard deviation, minimum and maximum values	Abnormal Hormonal Prevalence (%)	95% Confidence interval
Basal Insulin (I_0)	0-9 µIU/ml	8.76 ± 5.08 (2-23)	44.10	(27.2; 62.1)
Post Eating Insulin (I_{120})	6-20µIU/ml	23.41 ± 14.16 (5.1-61)	55.90	(37.9; 72.8)
FSH	0.7-11.1 mIU/ml	3.56 ± 2.03 (0.6-8.6)	2.94	(0.07; 15.33)
LH	0.8-7.6 mIU/ml	3.66 ± 1.65 (1.1-7.6)	0	
Free Testosterone	14.9-94.8	69.36 ± 24.83 (32-127)	14.7[a]	(31.1; 49.5)
GH	0.1-1 ng/ml	0.61 ± 0.86 (0.01-2.5)	20.60	(8.7; 37.9)
SHBG	13-71 nmol/l	27.61 ± 10.63 (11-61)	2,94	(0.07; 15.33)
BMI	18.5-25.0 kg/m^2	25.93 ± 5.68 (19.9-42.5)	35.3[b]	(19.8; 53.5)

[a] Four of these five participants also presented with low GH values, while the semen of one of the participants achieved pregnancy.

[b] Six of these participants fall in group BB (Bad,Bad)

Twelve of the 34 participants (35.3%) had a BMI above 25 kg/m^2. The sperm samples of seven of these participants were used in the IVF program and five achieved pregnancies.

Six (66.7%) of the participants in Group BB had a BMI above 25 kg/m^2. Five of the six (83.3%) participants with abnormal insulin values and high BMI had low GH levels. Although not statistically significant (p=0.075), a much higher proportion of the overweight donors in Group BB have abnormal insulin values. This finding was less prominent in groups GG and GB where only three participants in each group (23.1 and 37.5 % respectively) had a high BMI of >25 kg/m^2. This was not statistically significant (p=0.95). Groups GG and GB tend to have normal BMI while among the overweight donors the majority belong to the BB group.

IVF outcome:

The semen samples of some of the participants were not tested in the IVF program since they were not chosen by the infertility patients participating in the IVF program. The frozen semen samples of 22 participants were used to fertilize 508 oocytes of 67 women in the IVF program (Table 4). Poor responders that produced less than five ova and women above the age of 38 years were excluded from the study. The average age of the female participants were 34 years. Azoospermia and endometrioses were the main causes of infertility in the IVF program.

Table 4: Summary of IVF data

Donor Group	Group GG (Normal fasting and I_{120} levels)	Group GB (Normal I_0 but abnormal I_{120})	Group BB (Abnormal I_0 and I_{120})
Number of donors	8	7	7
Number female patients with transfers	26	19	22
Female's mean age	35.4±2.4	34.3±4.2	33.6±4.3
Number mature ova	200	134	174
Number of fertilized ova	168 (84.0 %)	93 (69.4%)	120 (68.9%)
Cleavage rate	161 (95.8%)	90 (96.7%)	118 (98.3%)
Good quality embryos	60 (37.3%)	29 (32.2%)	37 (31.3%)
Mean number embryos transferred	1.96	1.73	1.73
Biochemical pregnancy	13[a] (50.0%)	5[b] (26.3%)	5[b] (22.7%)
Clinical pregnancy	8[c] (30.7%)	5[c] (26.3%)	4[c] (18.1%)
Multiple pregnancy per transfer	3 (11.5%)	0 (0%)	2 (9.1%)

[a] The biochemical pregnancy in Group GG was significantly higher than groups GB and BB (P=0.04)

[b] The clinical pregnancy in the three groups were not statistically significant different (P=0.54)

The overall fertilization rate in the IVF program for the research participants was 75%. Five twin pregnancies were obtained from Group GG and Group BB.

The different insulin groups were compared with respect to embryo development. The incidence of 2-4 cell embryo development on day 2, 6-8 cell embryos on day 3 and blastocyst formation on day 5 of culture were assessed. The data analysis was adjusted for clustering, i.e. multiple patients assigned to each of donor. No statistically significant differences were found between the groups for day 2 development (χ^2 =0.55; *P*=0.58), day 3 (χ^2= 0.61; *P*=0.54) or day 5 development (χ^2= 0.14; *P*=0.86).

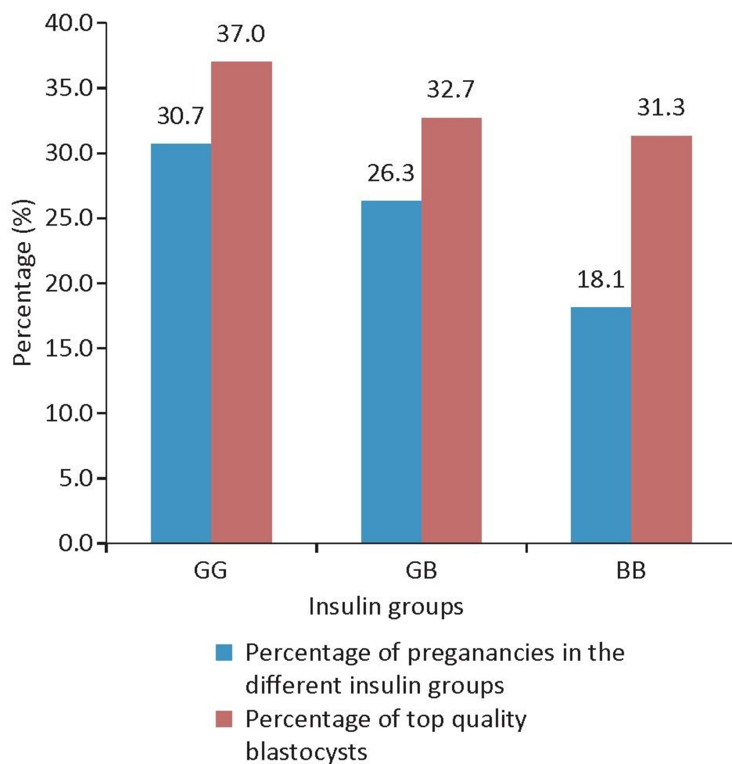

Fig. 1 Pregnancy and blastocyst outcome for the different groups.

The research participants with normal I_0 levels (Group GG and GB) had a slightly higher percentage of top quality blastocysts (A and B scores) on day 5. Group GG = 37.0% and Group GB= 32.7% were higher than the abnormal insulin group (Group BB = 31.3%) (Figure 1). Group BB and GB were comparable for the outcome of the biochemical pregnancy rate but

Group GG showed a significant increase (P=0.04). The clinical pregnancy rate in Group GG (30.7%) and GB (26.3%) were higher than that of Group BB (18.1%). However, these clinical differences were not statistically significant (P=0.54).

Discussion:

The rapidly increasing trend in hyperinsulinism is associated with adverse outcomes for male fertility. It may not be practically/clinically feasible to assess insulin resistance in assisted reproductive technology (ART) patients using a standard insulin assay because of costs and labor constraints. Fasting and 120 minute post-meal insulin levels may help to identify patients at risk. The specific focus on fasting hyperinsulinism has the advantage that it is a simple way to counteract the pathophysiological abnormalities and clinical syndromes that occur more commonly in insulin-resistant subjects. Insulin sensitivity can be effectively improved through pharmacological (e.g. Metformin and Avandia) and non-pharmacological approaches (Knowler *et al*., 2002; Pan *et al*., 1997; Tuomilehto *et al*., 2001). The positive effects of a healthy diet, exercise and lifestyle changes to decrease high insulin levels are well known, and were also demonstrated in two participants of this study who adjusted their lifestyles.

The research participants who presented with normal fasting insulin levels had more top quality embryos and a higher positive pregnancy outcome. The poorer embryo and pregnancy results of Group GB (high post meal insulin) suggest that the trend towards hyperinsulinism may already affect reproductive effectiveness.

A high prevalence of increased BMI and low GH levels were noted in group BB.

An increase in BMI and prevalence to obesity has been reported in the Western world (Mokdad *et al.*, 2001). Some studies suggest that low GH levels may have an influence on insulin sensitivity (Johannsson *et al.*, 1999; Larson *et al.*, 1995). Women with polycystic ovarian syndrome (PCOS) have been found to present with impaired GH response to several stimuli (Lee *et al.*, 1993; Piaditis *et al.*, 1995). This syndrome is characterized by anovulation, hyperandrogenism, obesity and infertility (Villa *et al.*, 2000). Hyperinsulinaemia is present in a considerable percentage of these subjects (Mokdad *et al.*, 2001). Insulin resistance and the resultant hyperinsulinaemia play a key role in the pathogenesis of women suffering from PCOS. In women with PCOS, the hyperinsulinaemia has a direct pathophysiological role because insulin synergizes with LH to stimulate excess androgen production (Poretsky *et al.*, 1999). A BMI >25 kg/m^2 has been reported to be associated with reduced fertility in women and men (Jensen *et al.*, 2004; Zaadstra *et al.*, 1993).

A higher number of good quality blastocysts and pregnancies were obtained in this study from donors with normal fasting insulin levels, suggesting an association between hyperinsulinaemia and IVF outcome. Therefore, it is necessary to further investigate the role of insulin and the fertility profile of men with hyperinsulinaemia. In the mean time, infertile males falling into this category should be advised to take their BMI, diet and lifestyle into consideration as healthy adaptations may enhance their IVF outcome.

We postulate that fasting and 120 minutes post-meal insulin values can be predictive of the onset of hyperinsulinaemia and that further investigations are needed to establish the relationship between hyperinsulinaemia and male fertility.

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