בחינה בעריכת פירוט פטנט
ביוטכנולוגיה

נבחנים יקרים,

מצורף למטה תיאור של המצאה עליה יש לערוך פירוט פטנט, לרבות תיאור ותביעות.

כדי להקל עליכם, בתיאור הניסויים אין הסבר של השיטות הניסוייות (כפי שתיאור מדעי מחייב). הניחו שכל השיטות שלא פורטו הינם שיטות מוכרות וידועות.

אתם יכולים לכלול בפירוט הפטנט שאתם כותבים שאלות המיועדות לממציא. אך שימו לב, שאלות לא נכונות או כאלה שמעידות על הבנה לקויה בדיני פטנטים יכולות להוריד נקודות.

על התביעות והתיאור הכללי להקיף את כל מה שראוי להגנת פטנט. הגם שאת הפירוט יש לערוך כך שיתאים לדרישות החוק ולנוהל בישראל, אם הדבר רלוונטי יש לכלול גם תביעות שמותאמות לדרישות של חוקי פטנטים אחרים מרכזיים.

בעריכת התביעות יש לתת דגש על עניינים מהותיים ולא להתייחס לעניין אחידות ההמצאה. דהיינו, אם כדי לתבוע את כל מה שראוי להגנה יש צורך לכלול בפירוט תביעות שעל פי הנוהג בישראל או במדינה אחרת יש בהם יותר מהמצאה אחת, אין בכך כדי לפגום בציון. למעשה ההפך הוא נכון: החסרה של אספקטים כאלה תוריד ציון.

הניקוד יינתן לפי המפתח הבא:

- תביעות – 45 נקודות על פי הפירוט הבא:
  - הבנת מהות ההמצאה והגדרה נכונה של ההמצאה בהתחשב בידע הקודם (לא רחב מידי ולא מצומצם מידי) על כל האספקטים הראויים להגנה – 25
  - הקשר בין התביעות לתיאור – 10
  - התאמה לחוקים אחרים – 10

- תיאור כללי – 45 נקודות, על פי הפירוט הבא:
  - מידת התימוכין לתביעות – 15
  - קיומם של הגדרות למונחים שמשמשים בתיאור היכן שצריך (מונחים שדורשים הגדרה) – 10
  - תיאור דרכי הביצוע של ההמצאה – 15

- התרשמות כללית – 10 נקודות

ציון עובר הינו 75.

בהצלחה

Dear Examinee,

In this paper I would like to summarize experiments I have been conducting in the last few years relating to male infertility and means to for treating this male condition.

Today, in the western world about one couple out of five fails to conceive even though they engage in regular unprotected sexual intercourse. In about half of the cases, such failure to conceive is a result of male factors, what is referred to as “male sub-fertility”.

Male fertility can be evaluated by routine semen analysis. Factors that are evaluated include number of sperm cells (spermatozoa) in an ejaculate, the proportion of motile sperm cells or the proportion that is morphologically normal. The probability of conception increases with increased sperm concentration, motility and normal morphology. The routine semen analysis is often not enough to determine the cause for male sub-fertility and additional tests have been developed that provide more information on the fertilization potential of human semen and that include various sperm cell functional tests (Aviv, RJ., 2002, Int J Androl 45:69-75), functional morphology tests as the *"motile sperm organelle morphology examination"* (Baranes, et al, 2002, Int. J Androl. 45:1-8) and tests examining the extent of DNA damage in sperm cells, for example, the sperm chromatin structure assay (Aviv, 2002).

While in some cases the cause of male sub-fertility is known and treatments are available, in others it is not known or even if known treatment is unavailable. While in vitro fertilization (IVF) techniques may be considered in some cases, it is clear that most (if not all) couples would prefer to conceive in a natural way.

DNA has been known to be toxic to sperm cells. Sperm cells from a variety of species have been shown *In vitro* to bind and take up exogenous DNA (Salmani, D. , 1998. Bioassays 20: 955-64. DNA binding is mediated at least in part by CD4 and MHCII molecules present on the surface of sperm cells and is antagonized by the glycoprotein IF-1, present in the seminal fluid. Sperm interaction with extracellular cell free DNA activates intracellular nucleases such as DNase that cleave the sperm genomic DNA, eventually leading to a cell death process which resembles apoptosis.

Bovine DNase I has been used clinically since 1965. It was used in some parts of the world to treat infections caused by DNA viruses such as herpes and adenovirus. DNase degrades the viral DNA to mono and oligo nucleotides and thereby reduced the viral load (Andropov et al, J. Mol. Biol., 1982 123: 23-45). Recombinant human DNase I is used by inhalation to treat cystic fibrosis (CF) patients. In these patients retention of viscous purulent secretions in the airways contributes both to reduced pulmonary function and to exacerbation of infections. Purulent pulmonary secretions contain very high concentrations of extracellular DNA released by degenerating leukocytes that accumulate in response to infection. DNase I hydrolyzes the DNA in sputum of CF patients and reduces sputum viscoelasticity (Shak et al, 1990, Proc Natl Acad Sci USA 87: 9188-92.).

We have now found that high level of cell-free DNA present in men's blood circulation (namely freely circulated DNA) is associated with sub-fertility. We have also found that administration of exogenous cell free DNA reduces sperm quality and causes sub-fertility. We have further found that administering DNase to sub-fertile males improves their fertilization potential. Our findings, thus, elucidate a new cause for male sub-fertility and provide new opportunities for treating this condition. I cannot rule out that in some cases a single administration of DNase may suffice although I believe that repeated injections over a time period, e.g. of several weeks, may be needed.

In the following I will describe the experiments that we have conducted.

***In vivo* effects of cell-free DNA**

(1) High DNA in humans is correlated with male sub-fertility

Blood sample were taken from sub-fertile and fertile men and the DNA level was determined.

The DNA levels in the blood of sub-fertile men were considerably higher than in fertile men (336±97 and 153±41, respectively), indicating a correlation between high blood DNA level and sub-fertility.

(2) Intravenous injection of cell free DNA into male mice impaired their fertility potential

Two groups of 10 male mice received intravenous injections of either 200 μg cell free DNA or phosphate-buffered saline (PBS) over 15 days. The mice were then mated with female mice and the number of offspring was counted in both groups.

Mating with the control mice yielded 6.9±1.7 offspring per female while mating with the DNA injected mice yielded 0.7±2.2 offspring per female.

In addition, the intravenous injection of cell free DNA into mice impaired their sperm cell nuclear quality, as observed by a reduction in sperm cell chromatin stability. Moreover intravenous injection of cell free DNA into mice induced activation of the apoptotic markers Fas receptor (from 17.1% in control mice (PBS-treated) to 91.5% in DNA injected mice), caspas 3 (by 53.5% over control) and DNase activity within their sperm cells (from 5.6 units to 32.6 units).

(3) Cell free DNA injection affects not only the sperm cells but also the testis tissue.

Cell free DNA caused appearance of giant cells and depletion of the tubule epithelium by sloughing of testicular mature and immature germinal cells into the tubule lumen. Cell free DNA injection also caused the appearance of large apoptotic areas and quantification of apoptotic regions in the testis tissue showed that while about 5% of the testis tissue of control mice was apoptotic, 35% of the testis tissue of DNA-injected mice was apoptotic.

It should be noted that spermatogenesis in the testes takes approximately 64 days in humans. Thus, testes impairment caused by DNA is expected to have an impact on sperm cell development over a long period of time and repair of such damage would require prolonged treatment.

(4) Intramuscular administration of DNase

a. I improvement in sperm parameters

Men of 11 couples in which the male was previously identified as being sub-fertile received four times a day 25 mg of bovine DNase I by intramuscular (IM) injection for a period of 7-10 days. The semen analysis revealed the following:

- Sperm density increased by 70% after 8-15 days of treatment compared to pre treatment ($24.28\pm14.73x10^6$ cells/ejaculate vs. $14.31\pm10.15x10^6$ cells/ejaculate respectively, $P < 0.08$);
- a 60% increase in sperm motility was observed after 4-7 days of treatment compared to pre treatment (30.6±12.6% vs. 19±11.3% respectively.
- A 76% increase was observed in sperm progressive motility after 8-15 days of treatment compared to pre treatment (47±33.1% vs. 26.7±23.4% respectively).
- After 8-15 days of DNase treatment the percentage of morphologically shaped sperm cells in the semen of the subjects was elevated by 129% compared to pre-treatment (19.9±12.1% vs. 8.7±15.6% respectively).
- A 24% reduction in the percentage of total head defects was observed after 8-15 days of treatment compared to pre treatment (57.3±17.6% vs. 77.3±13.4% respectively).
- In particular a 73% reduction was observed in the percentage of amorphous heads after 8-15 days of treatment compared to pre treatment (9.4±7.3% vs. 34.5±29.3% respectively); and
- A 60% reduction was observed in the percentage of middle piece defects after 8-15 days of treatment compared to pre treatment (5.4±5.1% vs. 13.5±8.6% respectively).

The morphology of the sperm cells was analyzed also by *motile sperm organelle morphology examination* (MSOME) in 3 subjects. An improvement in a variety of MSOME parameters was observed for these subjects after the DNase treatment as can be seen in Table 1, below. In particular, there is an improvement in the percent of sperm cells with normal nucleus.

Table 1

	Subject No. 1		**Subject No. 2**		**Subject No. 3**		**Average**	
	Before treatment	After 10 days of treatment	Before treatment	After 10 days of treatment	Before treatment	After 11 days of treatment	Before treatment	After treatment
Sperm cells considered for MSOME analysis (%)	90.7	94	95.3	98	83.4	90.7	**89.8**	**94.2**
Sperm cells not considered for MSOME analysis (%)	9.3	6	4.7	2	16.65	9.3	**10.2**	**5.8**

	Subject No. 1		Subject No. 2		Subject No. 3		Average	
	Before treatment	After 10 days of treatment	Before treatment	After 10 days of treatment	Before treatment	After 11 days of treatment	Before treatment	After treatment
Sperm cells with normal nucleus (suitable for IMSI selection, first and second choice) (%)	4	7.3	1.3	5.4	1.6	2.7	**2.3**	**5.1**

b. Effect of in chromatin structure and apoptosis

DNA damage in sperm cells is correlated with impaired conception rates as well as with the health of the offspring. One of the most employed methods to assess this damage is the "sperm chromatin structure assay" (SCSA), which measures the stability of sperm chromatin in cells exposed to acidic media. DNase treatment caused an elevation in sperm cell chromatin, evidenced by smaller DFI (DNA fragmentation index) after 4-7 days of treatment compared to pre treatment (15.1±12.4% vs. 25±19.6% respectively).

In addition DNase treatment caused a reduction in the expression of the apoptotic marker Fas receptor in sperm cell both after 4-7 and 8-15 days of treatment compared to pre treatment (26.5±12.5% and 29.2±17.4% vs. 39.7±23% respectively).

c. Intramuscular administration of DNase increases pregnancy rate

Four out of the females of the 11 couples (36%) became pregnant after an IVF treatment which followed the DNase treatment. The average pregnancy rate in the IVF center in which this trial was conducted is 25% [range 18%-32%]. It should be noted that in the type of couples that were treated in this trial, the rate of pregnancies is considerable lower than the average. Thus, it is clear that DNase treatment improved the chances for pregnancy of ICSI treatments.

d. Discussion

The results noted above show that intramuscular administration of DNase I improved the semen in most of the semen quality parameters. Sperm density was improved by 70% at the end of the experiment. Sperm motility improved by 60% already after 4-7 days of treatment but a reduction in motility occurred later possibly due to an immune response that took place around the seventh day and was observed as a 24 hour fever. Sperm progressive motility improved by 76% at the end of the experiment. The elevation in sperm motility and sperm density improved the fertility potential of the subjects. This may be realized in IVF as well as in intercourse-based conception.

(5) Oral administration of DNase I

a. Results

In order to examine whether oral administration of DNase I can improve semen quality as observed with intramuscular administration, a group of 9 men received orally four times a day 50 mg of DNase I over a period of 16 days. Semen samples were taken and analyzed from the subjects 5 days before the onset of treatment, on the starting day of the treatment, and on days 5, 10 and 17 of the treatment. The semen was analyzed and the following was observed:

- Sperm density (total spermatozoa/ejaculate) increased significantly post treatment compared to pre treatment (103±50x10^6 cells/ejaculate vs. 42±38x10^6 cells/ejaculate respectively). An increase in sperm density over control (control = 100%) was observed already after 5 days of treatment and more dramatically after 10 and 17 days of treatment (235±95 %, 160±72 % and 284±138 % vs. 100% in control respectively).
- In addition, an increase in semen volume was observed after 17 days of treatment (1.3±0.5 ml pre treatment vs. 2.3±0.6 ml after 17 days of treatment). The sperm concentration also increased during the treatment.
- MSOME analysis showed that the percentage of sperm cells having a normal nucleus increased 2 fold already after 5 and 10 days of treatment compared to pre treatment levels.
- There was also an increase in the number of sperm cells with normal nucleus after 10 and 17 days of treatment compared to pre treatment (5.0±3.4x10^6 cells and 5.7±5.1x10^6 cells vs. 1.6±2.1x10^6 cells respectively).
- A decrease in sperm cells with narrow heads was observed after 5, 10 and 17 day of treatment compared to control (84±15 %, 85±15 % and 83±21 % vs. 100% in control, respectively).
- An increase in sperm cell chromatin stability (smaller DFI values) was observed already after 5 days of treatment compared to control (75±35 % vs. 100% respectively). This decreased was more pronounced after 10 and 17 days (65±31 % and 62±33 % vs. 100% respectively).
- The percentage of sperm cells expressing the apoptotic marker Fas receptor decreased after 10 days of treatment compared to pre treatment (10.6±7.5 % vs. 35.4±26.4 % respectively). A further decrease was observed after 17 days of treatment compared to pre treatment (6.4±5.2 % vs. 35.4±26.4 %, respectively).

Among the subjects that participated in the orally administered DNase experiment, only one subject attempted with his female partner to achieve pregnancy through intercourse. This couple had previously failed to achieve spontaneous pregnancies and also in four IVF procedures. Following the DNase treatment the couple achieved spontaneous pregnancy.

b. Discussion

These results show that oral administration of DNase I has the following effects:

1. Increasing semen volume.
2. Increasing sperm cell concentration and density.
3. Improving spermatozoa quality by increasing the fraction of sperm cells having a normal nucleus, increasing chromatin stability and decreasing the proportion of Fas receptor expressing cells.

(6) Administration of DNase I by inhalation

DNase was administered by inhalation to a male subject. 8 mg of DNase was administered in an aerosol formulation, 3-times daily in 4 hours intervals between two consecutive inhalations. This led to elevation in the percentage of normal nuclei that was most profound after 9 and 17 days of inhalation, with an increase of 150% and 1005, respectively, over the level determined 1 day prior to the onset of treatment.

(7) The relationship between Fas receptor expression and caspas 3 activity in sperm cells

One of the known markers of apoptosis is the expression of the Fas receptor.

Injection of cell free DNA to mice leads to a statistically significant elevation in Fas receptor expression on the membrane of sperm cells. Similar increase in Fas receptor expression on sperm cells membrane was observed also following *in vitro* incubation of sperm cells with cell free DNA.

In order to determine whether there is a correlation between Fas receptor expression and male fertility, Fas receptor expression on the surface of sperm cells was determined in fertile and sub-fertile men. The percentage of Fas receptor expressing sperm cells was four fold higher in sub-fertile males than in fertile males (78.8±27.4 vs. 12.7±6.4).

Fas receptor expressing sperm cells from sub-fertile men were separated Fas receptor-expressing (Fas positive) sperm cells and Fas receptor non-expressing (Fas negative) sperm cells. Caspase-3 activity was determined in each of these sperm cell populations. The percentage of caspase-3 activity in the Fas positive sperm cells was 4-fold higher than caspase-3 activity in the Fas negative sperm cells (207.2±39.6 vs. 47.2±10.5).

(8) The relationship between Fas receptor expression fertility

Mice sperm cells were incubated with cell free DNA (which was found to stimulate Fas receptor expression) and then Fas receptor positive sperm cells were separated from non-Fas receptor expressing cells. Four groups of 10 female mice were inseminated with either (i) the Fas positive sperm cells, (ii)

Fas negative sperm cells, (iii) sperm cells that were incubated with cell free DNA but not sorted according to Fas receptor expression, and (iv) control cells that were not exposed to DNA. All inseminations were performed with the same number of sperm cells.

No fetuses were obtained from insemination with the Fas positive sperm cells in contrast to insemination with Fas negative sperm cells or control sperm cells that produced 3±1.4 and 2.7±1.1 average fetuses per female mouse, respectively. Insemination with cells that were incubated with cell free DNA but not sorted gave rise to only 0.3±0.4 average fetuses per female.

(9) The relationship between Fas receptor expression and intra-uterine insemination (IUI) in humans

Semen samples from 72 sub-fertile couples that underwent IUI treatment were examined for Fas expression by immunoflourocense staining with human anti Fas receptor and FACS analysis. 18 cases resulted in a pregnancy while the remaining 54 cases did not result in pregnancy. The frequency of Fas positive sperm cells in the group that achieved pregnancy was statistically lower than than the group that did not achieved pregnancy (22.4%$\underline{+}$18.2 vs. 33.1%$\underline{+}$16.6 respectively).

(10) DNase activity in mouse blood plasma

In order to examine the ability of DNase to reach the blood stream upon oral administration, mice were treated with DNase either by intra peritoneal injection or oral administration and DNase activity was examined after 1 hour. A significant increase in blood plasma DNase activity was observed both in the peritoneal injected and the oral administered groups in comparison to a saline administered control group (14399±4000 and 10853±2150, respectively, vs. 1345±580).