Telomerase measurements in percutaneous testicular cell aspiration can predict the outcome of a subsequent therapeutic testicular biopsy

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Abstract

Introduction & Purpose: The inability of the testicular size, peripheral follicle stimulating hormone (FSH) levels and testicular histological images to predict the presence of spermatozoa (SZA) in males with non-obstructive azoospermia (NOA) is well-known. It has been suggested (Human Reproduction 14:3041) that a cut-off telomerase assay value (TAV) equal to 42 units detected in therapeutic testicular biopsy (TTB), bears high diagnostic accuracy in predicting the presence of SZA in TTB. But in order for the TAVs to be clinically significant, these must be performed on testicular cells recovered via a minimally invasive procedure like percutaneous testicular cells aspiration (PTCA). For this reason, we investigated the role of PTCA in predicting the presence of SZA in a subsequent TTB.

Materials & Methods: 23 NOA-males underwent PTCA. TAVs were measured on the PTCA-sample. TTB followed and TTB-sample was studied for SZA.
Results: Men positive for SZA in TTB (n=12) had statistically significant higher TAVs in the PTCA-sample (51.9±21.2) compared to men negative for SZA in TTB (N=11, 12.0±10.0). All 8 men with TAV equal or greater than 42 units in their PTCA-sample were positive for SZA in TTB. Among the 15 men with TAVs lower than 42 units, 4 men were positive for SZA in TTB, whereas, 11 men were negative.

Conclusions: Telomerase assays in PTCA-samples have a diagnostic accuracy equal to 82.6% in predicting the presence of SZA in a subsequent TTB. A minimally invasive procedure such as the PTCA can be employed in selecting NOA-men exhibiting higher probability of being positive for SZA in the subsequent TTB.

Key words
spermatozoa (SZA), non-obstructive azoospermia (NOA), telomerase assay value (TAV), therapeutic testicular biopsy (TTB)

Introduction
The most complex problems of non-obstructive azoospermia (NOA) males in assisted reproduction techniques (ARTs) are: a) the inability of the peripheral hormone levels (such as FSH, LH, testosterone) to identify the presence or absence of spermatozoa (SZA) in the testicular tissue, b) the inability of the testicular size to predict the presence or absence of SZA in the testicular tissue, c) the inability of the testicular composition to predict the presence or absence of SZA in the testicular tissue and d) the inability of an SZA-negative histological image of the testis to predict the real presence or absence of SZA in the testicular tissue. Thus, it is of clinical significance to discover a new parameter, either molecular or biochemical, diagnostically accurate for NOA-males in the prediction of SZA presence or absence in the testes. In other words, such a parameter will a) encourage a number of NOA-sufferers to undergo therapeutic testicular biopsy (TTB) for the identification and recovery of SZA and b) protect the sub-population of NOA-sufferers manifesting low probability to be positive for testicular SZA from being subjected to an unnecessary and potentially harmful surgical procedure on the testis.

Telomerase acts as an intracellular reverse transcriptase (RT) which extends the telomeres. Telomeres are specialized structures, present at the ends of eukaryotic chromosomes that seem to preserve chromosome stability. Telomeres stabilize and protect the chromosome ends and inhibit genetic material rearrangements occurring on chromosomal breaks. The telomere length, comprising TTAGGG sequences, gradually shortens due to cell division (S phase). Telomerase activity is high in embryonic stem cells (ES cells) and decreases in somatic
tissues during development and differentiation. Several studies suggest that spermatogonia, primary spermatocytes, secondary spermatocytes and round spermatids in mice, rats and humans are positive for telomerase activity, whereas testicular and epididymal SZA, i.e. gametes not processed by mitosis or meiosis, are negative for telomerase activity (Yamamoto et al. 1999a). Telomerase inactivates in the male gamete during the spermatid conversion into spermatozoon (Yamamoto et al., 1999b).

**Materials & Methods**

**Group A**

NOA-men were subjected to percutaneous testicular cells aspiration (PTCA). Telomerase measurements were performed on the recovered cells via the Sensitive Quantitative Telomerase Assay (SQTA). Following a period of more than 6 months, all 23 men underwent TTB. The TTB-recovered testicular tissue was microscopically investigated for SZA identification.

**SQTA**

The SQTA was performed according to the Hisatomi method, as previously described (Hisatomi et al., 1997), on PTCA samples, twice on each sample. Frozen PTCA samples (weighing less than 1mg for each assay), were homogenized in 0.1 ml of frozen CHAPS lysis buffer (TRAP-eze™ kit; Oncor Inc.–Kyowa Co., Tokyo, Japan) and were incubated for 30 minutes on ice. Next, the samples were centrifuged at 12000 g for 20 minutes at 4°C. The supernatants were rapidly stored at -80°C.

The protein concentration was measured and a test tube from each aliquot of extract containing 1 μg protein was used for each telomerase activity measurement. The test tubes with the aliquots of the extract were incubated with 0.1 ng Cy-5 labelled TS primer (TRAP-eze™). Subsequent to their 30-minute incubation at 30°C, a polymerase chain reaction (PCR) was carried out at 94°C (30 s), 60°C (30 s) and 72°C (45 s) for 30 cycles. The external control was TSR8 (TRAP-eze™) as a positive control (Hisatomi et al, 1997). Assisted by an automated DNA sequencer (ALFred™
DNA Sequencer; Pharmacia Biotech, Uppsala, Sweden), we evaluated the telomerase activity according to a previously described mathematical formula (Hisatomi et al, 1997). The TAVs were expressed as TPG Units (μg protein). The quantification of telomerase activity was realized on the following formula (x 100):

**SZA isolation from TTB-testicular tissue**

TTB-samples of 23 men were trice washed with normal saline. The seminiferous tubules were then washed with Dulbecco’s phosphate buffered saline (Sigma Co, St Louis, MO, USA) containing 5.6 mM of glucose and 5.8 mM of sodium lactate and then they were dissected into small pieces. The temperature of the samples throughout the procedure was 5°C. A dissecting microscope (Olympus SZ-STS, Olympus, Tokyo, Japan) was employed to facilitate the dissection of each TTB-sample into small pieces for the avoidance of damaging small blood vessels. The duration of each TTB-specimen dissection was approximately one hour. Following the dissection, every TTB-sample was filtered via a 20-30 μm pore filter. The filtrate was collected, centrifuged and the sedimented cells were placed back in Dulbecco’s phosphate buffered saline (Sigma Co, St Louis, MO, USA) containing 5.6 mM of glucose and 5.8 mM of sodium lactate. Drops of the sedimented cells were observed on an inverted microscope (IX-70, Olympus, Tokyo, Japan) connected to a computer (Apple Computers Inc., Cupertino, CA, USA). SZA were identified in 12 men and they were frozen.

**Results**

NOA-men positive for SZA in TTB (n=12) exhibited statistically significantly higher (P<0.05; Wilcoxon test) telomerase activity (mean ± SD: 51.9±21.2) in their PTCA-samples compared to NOA-men negative for SZA in TTB (n=11, 12.0 ±10.0). All 8 men with TAV equal or greater than 42 TPG Units/microgram protein in their PTCA-samples were positive for SZA in TTB. Among
the 15 men with TAV lower than 42 TPG Units/microgram protein, 4 men were positive for SZA in TTB, whereas, 11 men were negative for SZA in TTB. Consequently, the investigation of the telomerase activity in PTCA-samples in predicting the presence/absence of SZA in a subsequent TTB demonstrated:

- Overall diagnostic accuracy equal to 82.6 %
- Sensitivity equal to 66%
- Specificity equal to 100%
- Positive predictive value equal to 100%
- Negative predictive value equal to 73%

**Discussion**

Diagnostic testicular biopsy, combined with subsequent histological images, is of great clinical significance and serves for the differential diagnosis between an obstructed male reproductive system and a primary testicular damage in azoospermic men. On the contrary, the importance of the diagnostic testicular biopsy in ART-managed NOA-sufferers is limited, given a considerable rate of men with Sertoli Cell-Only Syndrome (SCOS)-compatible
histological image detected in testicular biopsy, present testicular foci of spermatogenesis up to the spermatozoon stage in TTB-samples (Yamamoto et al., 1999a). Thusly, the diagnostic testicular biopsy cannot identify NOA-males who are positive for foci of haploid cells in their testes and can be good candidates for ARTs. In addition, FSH peripheral concentrations, testicular composition and size cannot be considered as reliable parameters in the prediction of testicular SZA presence in NOA-men. In the present study, we evaluated the role of telomerase activity testing in PTCA-samples for the prediction and identification of NOA-males sub-population with SZA foci in TTB.

The difference in TAVs between NOA-men positive for testicular SZA and NOA-men negative for testicular SZA may be attributed to the large number of telomerase-positive testicular cells (such as spermatogonia, primary spermatocytes, secondary spermatocytes, and round spermatids) per testis weight unit in NOA-men positive for testicular SZA. This difference can be additionally explained by the fact that males with SZA testicular foci have focally more active spermatogenesis and subsequently more stem cells mitoses and consequently increased numbers of primary SZA. The conclusive result is that men with non-obstructive azoospermia and testicular SZA foci demonstrate a greater number of primary spermatocytes per testis weight unit when compared to NOA-men negative for testicular SZA foci. It should be stressed that primary spermatocytes constitute the main source of testicular telomerase activity. Moreover, men with non-obstructive azoospermia and testicular SZA foci exhibit higher telomerase activity in their PTCA-samples contrary to NOA-men who are negative for testicular SZA foci caused by the presence of round spermatids which are also telomerase-positive cells. In addition, given than men with non-obstructive azoospermia and testicular SZA foci may have a defect in their cellular capacity to undergo their first meiotic division, the probability that a further qualitative or quantitative defect is present in the primary spermatocyte nuclear telomerase cannot be ruled out. Thus, the diminished telomerase activity in men with non-obstructive azoospermia and testicular foci of spermatogenesis up to the stage of primary spermatocyte albeit not to the spermatozoon, may have a defect in their cellular capacity to undergo their first meiotic division, the probability that a further qualitative or quantitative defect is present in the primary spermatocyte nuclear telomerase cannot be ruled out. Thus, the diminished telomerase activity in men with non-obstructive azoospermia and testicular foci of spermatogenesis up to the stage of primary spermatocyte can further be attributed to the lower telomerase activity per primary spermatocyte in NOA-men negative for testicular SZA. The aforementioned hypothesis is strongly supported by experiments in mice, proving that primary spermatocytes cell populations isolated from healthy experimental animals have higher TAVs against primary spermatocytes cell populations isolated from experimental animals with primary testicular failure (Yamamoto et al., 1999b).

The results of the current study are of considerable clinical significance because:

1. Men with telomerase activity in PTCA-samples equal to or higher than 42 TPG Units/microgram protein should be encouraged to undergo TTB in order to participate in ARTs.
2. Men with telomerase activity in PTCA-samples lower than 42 TPG Units/microgram protein should not be encouraged to undergo TTB in order to participate in ARTs.

3. It is true that PTCA can induce complications like testicular haematoma, testicular arterial supply decrease and inflammation, among others. However, PTCA has fewer serious complications in comparison to TTB, is less expensive, does not require hospitalization and does not reduce the testosterone peripheral levels.

Conclusions

Telomerase activity measurements in PTCA-samples bear an overall diagnostic accuracy equal to 82.6% in the prediction of presence/absence of SZA in a subsequent TTB. NOA-men may be advised that TAVs greater than 42 TPG Units/microgram protein in PTCA-samples, may give them a higher probability to be positive for testicular SZA in a subsequent TTB. So, telomerase activity measurements in PTCA-samples seem to be significant in recognizing the NOA-men sub-population positive for testicular SZA in a subsequent TTB.

In view of the above, the use of a less invasive technique such as percutaneous testicular cells aspiration with subsequent telomerase activity assay on the retrieved cell specimen may represent a tool facilitating the identification of those NOA-men with higher probability of being positive for SZA in a subsequent TTB.

Περίληψη

Εισαγωγή & Σκοπός: Είναι γνωστή η αδυναμία του μεγέθους του όρχεος, των επιπέδων της ωοθυλακιοτρόπου ορμόνης (FSH) και των ιστολογικών εικόνων των όρχεων να προβλέψουν την παρουσία σπερματοζωαρίων (SZA) σε άνδρες με μη αποφρακτική αζωοσπερμία (NOA). Έχει προταθεί ότι τιμή cut-off της δοκιμασίας τελομεράσης (TAV) ίση με 42 μονάδες που ανιχνεύεται σε θεραπευτική βιοψία όρχεων (TTB), έχει υψηλή διαγνωστική ακρίβεια στην πρόβλεψη της παρουσίας του SZA σε TTB. Αλλά για να είναι κλινικά σημαντικό το TAV πρέπει τα κύτταρα των όρχεων να έχουν ανακτηθεί μέσω μιας ελάχιστα επεμβατικής διαδικασίας όπως είναι η διαδερμική αναρρόφηση των κύτταρων των όρχεων (PTCA). Για το σκοπό αυτό διερευνήθηκε ο ρόλος της PTCA στην πρόβλεψη της παρουσίας του SZA σε μεταγενέστερη TTB.

Υλικά & Μέθοδοι: 23-NOA άνδρες υποβλήθηκαν σε PTCA. Τα TAVs μετρήθηκαν στο δείγμα PTCA ενώ τα TTBF και TTBs μελετήθηκαν για SZA.
Αποτελέσματα: Οι άνδρες που είχαν θετική ανίχνευση SZA στην TTB (n = 12) είχαν στατιστικά σημαντικά υψηλότερες TAVs στην PTCA του δείγματος (51,9 ± 21,2) σε σύγκριση με τους άνδρες αρνητική ανίχνευση SZA (N = 11, 12,0 ± 10,0). Και οι 8 άνδρες με TAV ίση ή μεγαλύτερη από 42 μονάδες στο PTCA-δείγμα ήταν θετικοί για SZA στην TTB. Μεταξύ των 15 ανδρών με TAVs κάτω από 42 μονάδες, 4 ήταν θετικοί για SZA στην TTB, ενώ 11 άτομα ήταν αρνητικοί.

Συμπεράσματα: Η δοκιμασία τελομεράση στα PTCA-δείγματα έχουν διαγνωστική ακρίβεια ίση με το 82,6% στην πρόβλεψη της παρουσίας του SZA σε μεταγενέστερη TTB. Μια ελάχιστα επεμβατική διαδικασία όπως η PTCA μπορεί να χρησιμοποιηθεί για την επιλογή ασθενών NOA καθώς παρουσιάζουν μεγαλύτερη πιθανότητα να είναι θετικό για SZA στο μετέπειτα TTB.

Λέξεις ευρετηριασμού

Σπερματοζώαρια (SZA), μη αποφρακτική αζωοσπερμία (NOA), τιμή δοκιμασίας τελομεράσης (TAV), θεραπευτική βιοψία όρχεων (TTB)

References

