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The Enhancement of Sperm Retrieval Rate in Cases of Non-Obstructive Azoospermia Via Collagenase IV Enzyme Treatment in Combination with Mechanical Extraction

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Abstract

In cases of non-obstructive azoospermia (NOA), enzymatic digestion and mechanical treatment can be applied for the extraction of vital spermatozoa from the testicular tissues obtained by the microscopic testicular sperm extraction (micro TESE) surgery. Unfortunately, the results have repeatedly proven to be unsatisfactory when using either method separately. This study aimed at *increasing the sperm retrieval rate of testicular sperm extraction (TESE), by using enzymatic digestion using collagenase IV in combination with sperm extraction from a testis tissue with mechanical method. To estimate this, tissue samples were collected from 146 men with NOA who underwent unsuccessful TESE using the mechanical method for sperm extraction. After the micro TESE, the mechanical procedures by mincing and shredding combined with enzymatic digestion using DNaseI and collagenase type IV was applied to the samples. Pathology, testis size and hormones were determined for all patients.The combination of micro TESE and enzymatic digestion can now be foreseen as an effective method to recover spermatozoa.*

Key words: *TESE, Micro TESE, Non-obstructive azoospermia (NOA), enzymatic digestion, collagenase IV*

Introduction

The surgical extraction of spermatozoa for testicular sperm retrieval together with intracyto-plasmic sperm injection (ICSI) has provided a great chance for the treatment of non-obstructive azoospermia (NOA) patients (Chan *et al.,* 2001). ICSI can be performed in cases of male infertility with insufficient sperms in their ejaculate.

The Testicular sperm extraction (TESE) process increases the chance for the detection of rare vital spermatozoa within the testes. The testicular tissue produced from the TESE normally contains sufficient sperms for injection that is usually obtained using mechanical mincing and shredding (Tournaye*et al.,* 1994). Most of the classifications of the tissue of azoospermia cases describe the following patterns; hypospermatogenesis, spermatogenic arrest, sertoli cell only syndrome (SCOS) and tubular hyalinization. The sperm retrieval rate in cases of hypospermatogensis is always very high, but there is a failure in obtaining vital sperms for ICSI in cases with severe testis failure (Seo and Ko, 2001).

A new surgical technique has been recently applied in order to detect the rare spermatozoa called Micro dissection TESE. The optical magnification is now used during the TESE surgery to help in the identification of the areas of testes with intact spermatogenesis that improves the sperm retrieval rates. Seminiferous tubules with active spermatogenesis are usually dilated, whitish opaque in color. The optical magnification can also allow the visualization of the blood vessels, which in turn allow the biopsy incision in

regions with the least blood vascular. Another advantage of the micro dissection TESE over the traditional TESE is to facilitate the removal of minor testicular tissue, avoid intra testicular hematomas, minimizes vascular injury as well as postoperative pain and the possibility postoperative fibrosis (Silber, 1995). Micro TESE technique is much more secure than the traditional TESE however, as far as this paper has determined nospermatozoa were found in up to 53% of the studied patients (Amer*et al*., 2000).

The surgical technique and the *in vitro* tissue preparation procedures have been found to possibly affect the outcome of the TESE/ICSI. The mechanical preparation of the tissue after the TESE is considered a relatively quick approach. On the other hand, in some cases it requires extensive research to find the motile and vital sperms, which consumes a significant amount of time in incubation (Munne and Estop, 1993), this leads to alterations that affect the sperm haploid genome severely and may result in DNA fragmentation. High rates of early stage miscarriages or even poor fertilization may be due to fertilization using sperms with fragmented DNA (Sun *et al.,* 1997). Mechanical separation also causes contamination of testicular tissue suspensions with multiple damaged cells and undesirable residual tissue. Whether the rough extraction method during TESE induces a significant reactive oxygen species (ROS), which is released by damaged cells that results in the impairment of sperm function is an unexplored investigation (Crabbe *et al.* 1997). The enzymatic digestion is another technique applied for sperm preparation from the obtained testicular biopsy (Crabbe *et al.,* 1997). There are different enzymes used for the enzymatic digestion of the testicular tissues. Fischer *et al. (*1996) reported the first pregnancy after ICSI using spermatozoa extracted by collagenase type IA. One of disadvantages of using collagenase type IA is that it requires at least 4 hours for the testicular tissue to be dispersed and consequently it reduces the sperm motility(Ezeh*et al*., 1998). Collagenase type IV is a much more effective type of collagenase in sperm retrieval and requires a lower incubation time. However, the enzymatic digestion using collagenase type IV, which may lead to the formation intercellular bridges in the mammalian testis. The developments in germ cells have been reported to connect together through these formed bridges (Aydos *et al*., 2005). The enzymatic method alone was unable to disperse the germ cells completely. Consequently, the combination of mechanical mincing of the testicular biopsy extracted from the TESE and the enzymatic digestion can overcome this problem and improve the quality of outcome of this surgery (Crabbe *et al*., 1998). In the present study, the effect of the enzymatic digestion using collagenase IV enzyme on the sperm retrieval rates was investigated for the NOA patients who previously underwent failed TESE. To the best of our knowledge, this is the first study to be done using both methods on our Egyptian patients.

Methods

Patient Recruitment

This study was conducted on 146 patients of non-obstructive azoospermia patients. All the subjects in this study had undergone a TESE surgery before with negative results using mechanical methods in the tissue preparation for sperm extraction. The testicular biopsies were obtained from patients referred to Al Nile center for IVF between 2013 and 2014. The mean age of the males in this study was 35.1 ±8.81 years. Subjects used in this study had a full clinical evaluation where examination of the vasa deferentia, epdidymes and testis was carried out. The follicular stimulating hormone (FSH), luteinizing hormone (LH), testosterone hormone (TTE) levels were measured. In addition, the karyotyping analysis for the selected tissues was performed.

Sperm extraction

Sperm extraction from all the samples was performed using the mechanical extraction method combined with the enzymatically

digestion method in order to improve the sperm retrieval rates (Modarresi*et al.,* 2013). After the enzymatic digestion of the testicular tissues sample, they were observed through the inverted microscope. The samples were obtained through micro dissection TESE surgery. Testicular biopsy was obtained through a small incision in the scrotum and testis. Each sample was placed in a petri dish filled with 1ml Ham's F10 medium and was mechanically cut and dispersed by an embryologist. During the testicular intervention, one solution sent for histology examination. Testicular histology was classified into Sertoli cells only (the absence of germ cells in the seminiferous tubules), maturation arrest (an interruption in the development of spermatogonia to mature sperm at the level of spermatogonia, spermatocytes or spermatids), hypospermatogen-sis and complete tubular fibrosis (the tubules were filled with collagen fibers) (Soderstrom, 1986; Ezeh*et al.,* 1998). After biopsy, all testicular tissue pieces were transferred to a petridish filled with Ham's F10 solution, and mature spermatozoa were searched for by mechanical extraction by shredding the biopsy fractions using two fine needles.

After the mechanical extraction of the testes biopsies, the enzymatic digestion was performed. The samples were transferred to a conical tube in the incubation medium containing 25 mg/ml DNAse (Sigma DN25) and 1000 IU/ml of collagenase type IV (Sigma C5138) (Crabbe *et al.*,1998). Commencing incubation at 37 8C for 1 h, the resultant solution was centrifuged for 5 min at 50 xg to remove any remaining tissue residue. Sequentially, the supernatant was diluted with fresh medium, and two more washing steps were done. Finally, the final pellet was then re-suspended in the medium and the presence of free spermatozoa was checked under an inverted microscope.

Statistical Analysis

The results were analyzed on the bases of non-parametric analysis. The r*c cross tabulation that depending on Chie square to test the significance of association between the studied parameters. The significant association was expressed as $P < 0.05$ at $\propto = 0.05$. The age of patients was expressed out using the descriptive statistical analysis. All statistical analysis was performed using statistical package for the social science (SPSS) version 22.

Results

Enzymatic digestion of testicular biopsies was carried out in 146 non-obstructive azoospermia patients with negative result in previous TESE; no spermatozoa have been found in the testicular tissues, using the mechanical method only.The average age of the studied cases was 35.1 ± 8.81 years. The patients then underwent microscopic TESE and after the enzymatic digestion, spermatozoa were found in only 55 out of the 91 patients. According to the cross tabulation of the histopathology (figure1. and table 1a.), the caseswas divided into four categories; Sertoli cell only syndrome (SCO), complete maturation arrest (C1), hypospermatogensis (hypo) and fibrosis (fib). Table 1b show that there is strong significant association between the testicular sperm extraction (TESE) and the histopathology (HP) ($P< 0.01$) with linear association +0.77. On the other hand, the other parameters (testes size, FSH, LH, TTE) were not associated with TESE. According to the testes size, the higher percentage of successful in TESE was recorded in the normal size (50%) and the minimum percentage (25.5%) was recorded in the small size cases (Figure 2 and Table 2.a). cross tabulation of table 2b confirmed that there is no association between the TESE and the testes size ($P>0.05$) that accompanies with a weak linear relationship +0.29.

The levels of the studied hormones (FSH, LH, and TTE) were not associated and/or correlated with the TESE output as shown in table 3b, 4b, 5b ($P> 0.05$).

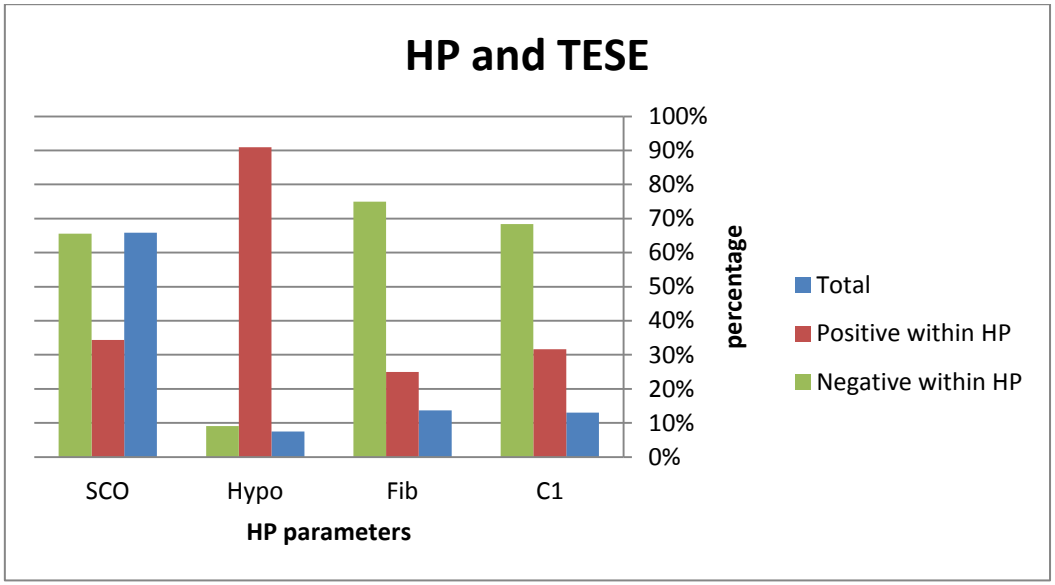

Figure 1. The effect of the different parameter of histopathology on the success of the micro TESE surgery

Table1. The percentages of the testicular sperm extraction in the presence (positive) and absence (negative) of sperms in relation to the histopathology (A). The association between the TESE and HP and the linear correlation *cross tabulation) are represented in table B.

A

			HP				Total
			C1	Fib	Hypo	SCO	
TESE	Positive	Count	6	5	10	33	54
		% within TESE	11.1%	9.3%	18.5%	61.1%	100.0%
		% within HP	31.6%	25.0%	90.9%	34.4%	37.0%
		% of Total	4.1%	3.4%	6.8%	22.6%	37.0%
	Negative	Count	13	15	1	63	92
		% within TESE	14.1%	16.3%	1.1%	68.5%	100.0%
		% within HP	68.4%	75.0%	9.1%	65.6%	63.0%
		% of Total	8.9%	10.3%	0.7%	43.2%	63.0%
Total		Count	19	20	11	96	146
		% within TESE	13.0%	13.7%	7.5%	65.8%	100.0%
		% within HP	100.0%	100.0%	100.0%	100.0%	100.0%
		% of Total	13.0%	13.7%	7.5%	65.8%	100.0%

B

	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	15.476[a]	3	.001
Linear-by-Linear Association	.089	1	.766
N of Cases	146		

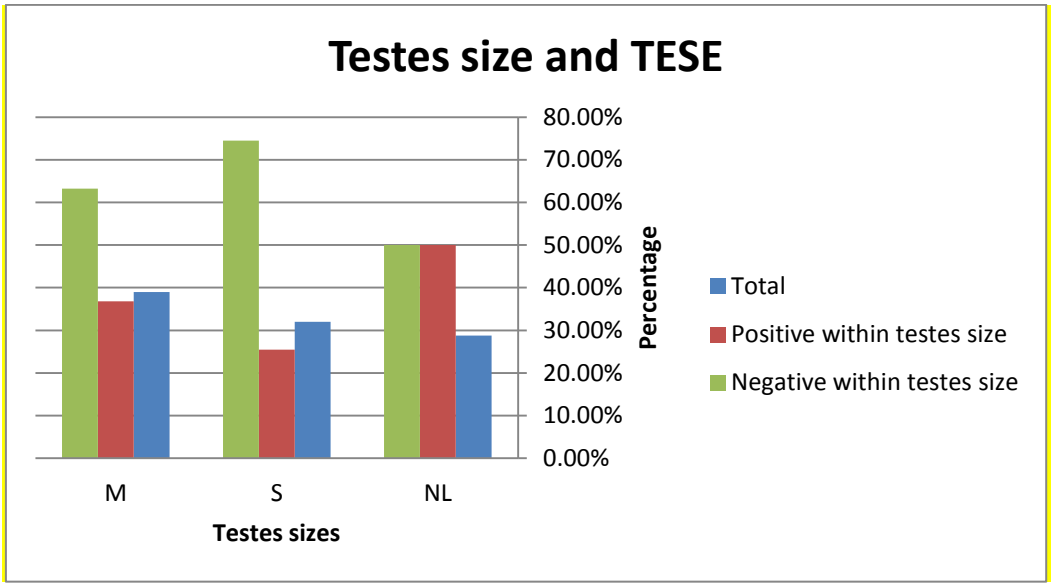

Figure 2. The effect of the different varieties of testes size on the micro TESE result

Table 2. The percentages of the testicular sperm extraction in the presence (positive) and absence (negative) of sperms in relation with the testes size (A). The association between the TESE and testes size and the linear correlation *cross tabulation) are represented in table B.

			Testessize			
			Nl	S	M	Total
TESE	Positive	Count	21	12	21	54
		% within TESE	38.9%	22.2%	38.9%	100.0%
		% within Testessize	50.0%	25.5%	36.8%	37.0%
		% of Total	14.4%	8.2%	14.4%	37.0%
	Negative	Count	21	35	36	92
		% within TESE	22.8%	38.0%	39.1%	100.0%
		% within Testessize	50.0%	74.5%	63.2%	63.0%
		% of Total	14.4%	24.0%	24.7%	63.0%
Total		Count	42	47	57	146
		% within TESE	28.8%	32.2%	39.0%	100.0%
		% within Testessize	100.0%	100.0%	100.0%	100.0%
		% of Total	28.8%	32.2%	39.0%	100.0%

B

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.698[a]	2	.058
Likelihood Ratio	5.743	2	.057
N of ValidCases	146		

Table 3. The percentages of the testicular sperm extraction in the presence (positive) and absence (negative) of sperms in relation to the FSH hormone (A). The association between the TESE and FSH and the linear correlation *cross tabulation) are represented in table B.

A

			FSH			
			NL	H	L	Total
TESE	Positive	Count	24	29	1	54
		% within TESE	44.4%	53.7%	1.9%	100.0%
		% within FSH	50.0%	30.2%	50.0%	37.0%
		% of Total	16.4%	19.9%	0.7%	37.0%
	Negative	Count	24	67	1	92
		% within TESE	26.1%	72.8%	1.1%	100.0%
		% within FSH	50.0%	69.8%	50.0%	63.0%
		% of Total	16.4%	45.9%	0.7%	63.0%
Total		Count	48	96	2	146
		% within TESE	32.9%	65.8%	1.4%	100.0%
		% within FSH	100.0%	100.0%	100.0%	100.0%
		% of Total	32.9%	65.8%	1.4%	100.0%

B

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.526[a]	2	.063
Linear-by-Linear Association	4.301	1	.038
N of Cases	146		

Table 4. The percentages of the testicular sperm extraction in the presence (positive) and absence (negative) of sperms in relation to the LH hormone (A). The association between the TESE and FSH and the linear correlation *cross tabulation) are represented in table B.

A

			LH			
			Nl	H	L	Total
TESE	Positive	Count	40	13	1	54
		% within TESE	74.1%	24.1%	1.9%	100.0%
		% within LH	36.7%	38.2%	50.0%	37.2%
		% of Total	27.6%	9.0%	0.7%	37.2%
	Negative	Count	69	21	1	91
		% within TESE	75.8%	23.1%	1.1%	100.0%
		% within LH	63.3%	61.8%	50.0%	62.8%
		% of Total	47.6%	14.5%	0.7%	62.8%
Total		Count	109	34	2	145
		% within TESE	75.2%	23.4%	1.4%	100.0%
		% within LH	100.0%	100.0%	100.0%	100.0%
		% of Total	75.2%	23.4%	1.4%	100.0%

B

	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.167[a]	2	.920
Linear-by-Linear Association	.095	1	.757
N of Cases	145		

Table 5. The percentages of the testicular sperm extraction in the presence (positive) and absence (negative) of sperms in relation to the TTE hormone (A). The association between the TESE and FSH and the linear correlation *cross tabulation) are represented in table

A

			TTE			
			Nl	H	L	Total
TESE	Positive	Count	46	4	4	54
		% within TESE	85.2%	7.4%	7.4%	100.0%
		% within TTE	36.5%	44.4%	36.4%	37.0%
		% of Total	31.5%	2.7%	2.7%	37.0%
	Negative	Count	80	5	7	92
		% within TESE	87.0%	5.4%	7.6%	100.0%
		% within TTE	63.5%	55.6%	63.6%	63.0%
		% of Total	54.8%	3.4%	4.8%	63.0%
Total		Count	126	9	11	146
		% within TESE	86.3%	6.2%	7.5%	100.0%
		% within TTE	100.0%	100.0%	100.0%	100.0%
		% of Total	86.3%	6.2%	7.5%	100.0%

B

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.229[a]	2	.892
Linear-by-Linear Association	.026	1	.871
N of Valid Cases	146		

Discussion

Few spermatozoa can be extracted from the majority of patients with testicular failure and can be utilized for ICSI. The mechanical method for the extraction of vital suitable sperms for ICSI by mincing and shredding the whole tissue is most common (Schlegel *et al.*, 1997). However, researchers suggested that enzymatic digestion using DNAase and collagenase to loosen the cellular contacts in the tubular walls facilitating release of gametes (Salzbrunn*et al.*, 1996). The enzymatic digestion using collagenase type IV was proved to give a higher yield of spermatozoa with no risk of alteration in the cell membrane composition i.e. no prospect for an increase (Crabbe et al., 1998). Collagenase type IV is probably the best protease to dissociate the testicular tissue, because type IV collagenase is one of the products secreted by the Sertoli cells and its secretion has been identified as possibly playing a role in the translocation of germ cells and spermiation, i.e. the release of mature spermatozoa into the lumen of tubules (Crabbe et al., 1998).

In the present study, a group of men with previously failed TESE underwent a new testicular sperm extraction with enzymatic digestion of testicular tissue, with the aim of

finding at least one spermatozoon. Using this approach, we detected spermatozoa suitable for ICSI in 38% of all NOA cases. In a similar study, it has been shown that enzymatic dissociation made the ICSI possible for 26% of men whose biopsies revealed no spermatozoa during an initial mechanical search (Crabbe *et al.*, 1998). According to the histological type, hypo-spematogenesis had the highest retrieval rate 90%, spermatogenic arrest showed 28 % retrieval rate and in cases of total testicular fibrosis, 18 % of the cases showed areas of spermatogenesis where spermatozoa could be harvested. Interestingly, cases of Sertoli cell showed only a 35 % retrieval rate. Although, the attached cells might be extracted by the rough separation procedure alone, the search time is long. Combining the rough separation with the enzymatic digestion method, sufficient numbers of spermatozoa could be isolated within a relatively short time exposure to open air. However, concerns have been raised regarding the use of enzymes for testicular sperm recovery. Proteases may cause modifications in the structure of cell membrane composition. Moreover, in some cases prolonged incubation time may be required to disperse the testicular tissue, as suggested for collagenase type I and trypsin (Salzbrunn*et al.*, 1996). Our results suggest that when enzymatic digestion with collagense type IV is combined with mechanical mincing, suitable spermatozoa for ICSI can be obtained within a reasonable time period, It provides a superior cell suspension with reduced alterations in cell-membrane composition (Chemes*et al*., 1992).

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