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Evaluation of sperm apoptosis in Iraqi severe oligozoospermic patients using annexin-V by flow cytometry

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Abstract: The present study was conducted in the institute of genetic engineering and biotechnology – university of Baghdad during a period from 15 September 2015 to 10 February 2016. The aim of this study was to detect the apoptotic alterations in sperm of Iraqi patients with severe oligozoospermia depending on annexin V- binding using flow cytometry and study its relationship with some semen parameters. Semen samples were taken from severe oligozoospermic (SOS) patients (n=75) and apparently healthy (AH) subjects (fertile, n=25) by masturbation following 3-7 days of sexual abstinences in Baghdad specialist fertility Centre. Semen samples were evaluated according to the WHO criteria which include volume, count and the percentages of motility, active, normal and abnormal sperm. Sperm apoptosis was determined using annexin V-FITC kit (Aviscera Bioscience, USA) by flow cytometry. The percentages of live sperms were significantly ($p \leq 0.01$) higher in AH subjects than in SOS patients. The percentages of early and late apoptotic sperms were significantly ($p \leq 0.01$) lower in AH subjects than in SOS patients. Apoptotic index values were significantly ($p < 0.01$) lower in AH subjects than in SOS patients. Semen volume significantly ($p < 0.01$) correlated with the percentage of viable sperm ($r = 0.76$) and inversely correlated ($p < 0.01$) with the percentages of early and late apoptosis and apoptotic index. Sperm count was inversely correlated ($p < 0.01$) with the percentages of early and late apoptosis and apoptotic index. sperm motility percentages were inversely correlated ($p < 0.01$) with the percentages of early and late apoptosis and apoptotic index. The percentages of both active and normal sperm were inversely correlated ($p < 0.01$) with the percentages of early, late apoptotic and apoptotic index. In conclusion, our results showed that the percentages of apoptotic and necrotic spermatozoa detected by flow cytometry may be used as biomarkers for the detection of fertility.

Key words: oligozoospermia, sperm apoptosis, annexin-V, flow cytometry.

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Introduction:

Fertility is one of the most tragic marital problems. It was estimated that nearly 8-12% of couples were infertile (Barbara, 2003). Despite advances in the treatment of infertility, the problem could not be satisfactorily tackled so far for varied reasons. Poor semen quality is correlated with low fertilization rate, impaired preimplantation development, increased abortion and elevated incidence of disease in the offspring (Brinkworth, 2000; Jensen *et al*., 2002). In humans, a strong association has been found between abnormal semen parameters and the abortive apoptosis in ejaculated sperm (Sakkas *et al*., 1999; Shen *et al*., 2002). Germ cell apoptosis is physiologically involved in

various stages of mammalian testicular development (Dunkel *et al*., 1997). It plays an important role in regulating germ cell number and eliminating defective germ cells and thus in maintaining normal spermatogenesis (Koji , 2001). The increase in the individual motility and mass activity percentages may be due to the increase in the sperm content of energy (Alhassani *et al.,* 2008).

Various factors have been attributed to the development of apoptosis-like changes such as cryopreservation, heat exposure, radiation, hydrogen peroxide, genetic disturbances, and endocrine disruptions (Aitken and Koppers,2011; Aitken and Baker, 2013). Apoptotic sperms with fragmented DNA and asymmetrical membrane result in poor fertility (Anzar *et al.,* 2015).

The loss of phospholipid asymmetry leading to externalization of phosphatidylserine from the inner leaflet to the outer leaflet of membrane which is considered as a sign of early apoptosis (Tavalaee *et al.,*2014). The anticoagulant Annexin-V preferentially binds to negatively charged phospholipids such as phosphatidylserine (Koopman *et al*., 1994; Martin *et al*., 1995; van Heerde *et al.,* 1995). The conjugate of fluorescein to Annexin V has been possible to use the marker to identify apoptotic cells by flow cytometry. During apoptosis the cells bind Annexin- V prior to the loss of the plasma membrane's ability to exclude propidium iodide (PI). Therefore, by staining cells with a combination of Annexin V and PI it is possible to simultaneously distinguish live, apoptotic and necrotic sperm populations. Previously, two authors were investigated sperm apoptosis, but conflicting results have been obtained (Glander and Schaller, 1999; Oosterhuis *et al.,* 2000). In the first study the percentage of apoptotic sperm in the ejaculate positively correlated with motility, while in the second study a negative correlation was observed between apoptotic cells and sperm motility and concentration.

The aim of this study was to detect the apoptotic alterations in sperm of Iraqi patients with severe oligozoospermia depending on annexin V- binding using flow cytometry and to explore the association between the percentages of early, late apoptosis and apoptotic index with some semen parameters of Iraqi patients with severe oligozoospermia.

Materials and methods:

In this study semen samples were collected from severe oligozoospermic patients (n=75) and apparently healthy subjects (Control, n=25) by masturbation following 3-7 days of abstinences. Semen analysis was done in Baghdad Specialist Fertility Centre. After semen liquefaction for 30 min at 37 C°, semen samples were evaluated according to the World Health Organization (WHO) criteria (World Health Organization, 1999). The semen parameters studied include sperm count, sperm activity, semen volume, sperm motility, normal sperm and abnormal sperm of Iraqi patients with severe oligozoospermia and apparently healthy subjects (fertile).

The evaluation of apoptosis was depended on the translocation of membrane phosphatidylserine and determined by using Annexin V-FITC apoptosis Assay Kit and propidium iodide (Aviscera Bioscience Inc, USA).

Additional material required (EGTA / Hepes content from NaCl, 137 mmol, KCl, 2.68 mmol, Hepes, 10 mmol, $MgCl_2$, 1.7 mmol, Glucose, 25 mmol, pH 7.4) and phosphate buffer saline (Dilute 1 g in 1 liter of deionized water) (Shen, *et al*., 2002). Experimental protocol was liquefaction semen samples for 30 min in incubation at 37C°, collect cells by centrifugation 400g for 12 min ,discard supernatant cells, wash cells with buffer EGTA/Hepes, collect cells by centrifugation 400g for 10 min ,discard supernatant cells , resuspend cells in 1x binding buffer, wash cells with buffer phosphate and buffer saline ,discard supernatant cells , resuspend cells in 195 µl from binding buffer ,add 5µl of Annexin V and 10 µl of propidium iodide to 1000µl of sample ,incubation in dark at room temperature ,add1000 µl from binding buffer to samples ,determine the fluorescence of the cells immediately with a flow cytometer (Ex = 488 nm ;Em = 530 nm).

The Statistical Analysis System-SAS (2012) program was used. Least significant difference (LSD) test was used to compare between means. Chi-square test was used to compare between percentages. Estimate of correlation coefficient between difference parameters in this study.

Results and Discussion:

Annexin-V/propidium iodide (PI) analysis was conducted using flow cytometry in this study to measure the percentage of apoptotic and necrotic sperm and the apoptotic index. This method does not involve enzyme activity and does not require cells to be previously fixed. Also, this assay enables living sperm to be evaluated (Ricci *et al.*, 2002).

The early event of apoptosis of all human cells is the exposure of phosphatidylserine on the outside of the plasma membrane (Ricci *et al*., 2002). Annexin-V preferentially binds to negatively charged phospholipids such as phosphatidylserine (Martin *et al*., 1995; van Heerde *et al.,* 1995). By conjugating fluorescein to annexin-V, it has been possible to use the marker to identify apoptotic cells by flow cytometry. During apoptosis the cells bind annexin-V prior to the loss of the plasma membrane's ability to exclude PI. Therefore, by staining cells with a combination of annexin-V and PI, it is possible to distinguish live, apoptotic and necrotic sperm.

Figure 1 illustrate the annexin-V/PI analysis used to identify the different sperm status. The lower left quadrant of each graph contains annexin-V negative/ PI negative (viable sperm). The lower right quadrant reveal annexin-V positive/ PI negative (early apoptotic sperm). The upper right quadrant represents annexin-V positive/ PI positive (late apoptotic sperm). The upper left quadrant represents annexin-V negative/ PI positive (necrotic sperm). Graph A represents semen sample taken from apparently healthy subject (fertile), while graph B represents semen sample taken from severe oligozoospermic patient.

Table 1 shows the results of sperm status that measured according to annexin-V/ PI analysis using flow cytometry in apparently healthy subjects (fertile) and severe oligozoospermic patients. The percentages of live sperms were significantly ($p \leq 0.01$) higher in apparently healthy subjects than in severe oligozoospermic patients (84.27

versus 15.04 %, respectively). The percentages of early and late apoptotic sperms were significantly ($p \leq 0.01$) lower in apparently healthy subjects than in severe oligozoospermic patients (6.92 and 3.05 versus 34.35 and 44.83 %, respectively).

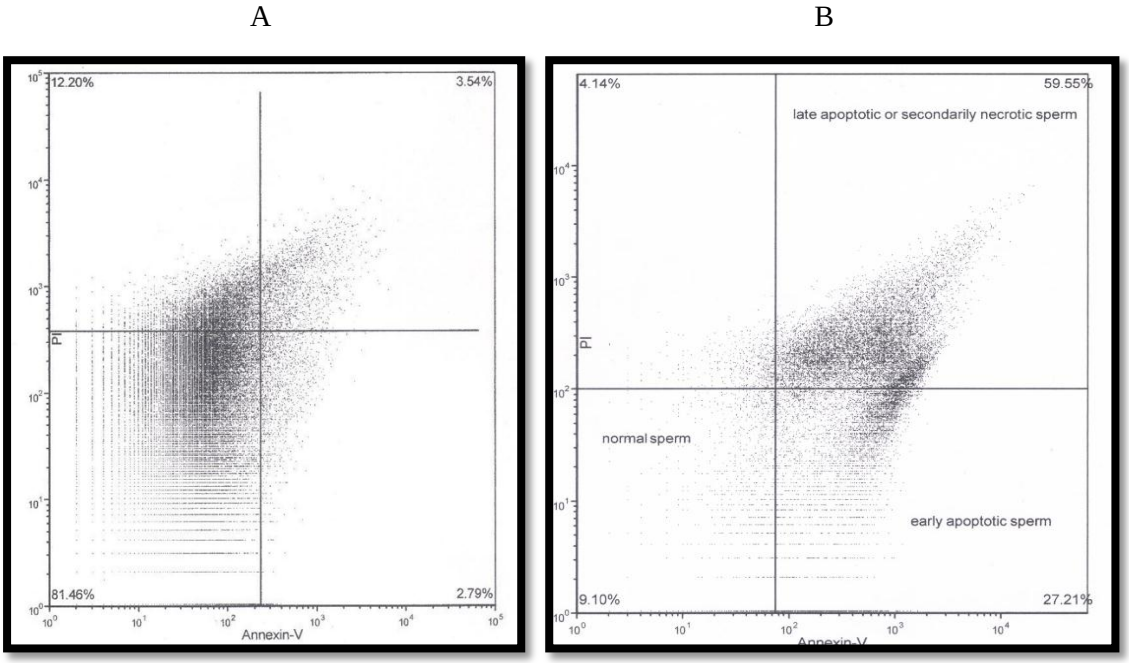

Figure (1): Annexin-V/ PI analysis used for identification of the sperm status in apparently healthy subjects (A) and severe oligozoospermic patients (B).

There were no significant differences between apparently healthy subjects and severe oligozoospermic patients as related with necrotic sperms. Apoptotic index values were significantly ($p<0.01$) lower in apparently healthy subjects than in severe oligozoospermic patients (8.16 versus 278.12, respectively).

Table (1): Staining of sperm by annexin-V (AN-V) and propidium iodide (PI).

Sperm status	AN-V	PI	Control [1]	Patient [2]	*p* value
Live sperm	-	-	84.27 ± 0.77 [3]	15.04 ± 0.92	**
Early apoptotic	+	-	6.92 ± 0.58	34.35 ± 1.51	**
Late apoptotic	+	+	3.05 ± 0.29	44.83 ± 1.78	**
Necrotic	-	+	5.67 ± 0.74	5.57 ± 0.56	NS
Apoptotic index			8.16 ± 0.73	278.39 ± 14.83	**

[1] apparently healthy subjects (fertile). [2] severe oligozoospermic patients.
[3] mean± standard error. NS: no significant **: significant at 0.01 level.

Flow cytometry has become the method of choice for analysis of apoptosis in many cell systems providing fast and accurate analysis for thousands of cells (Telford *et al*., 1994; Darzynkiewicz *et al*., 1997). When the membrane loses its integrity, the cell becomes PI positive indicating that the

cell is necrotic. Using flow cytometry, two PI positive sperm fractions were observed, one stained with annexin-V whereas the other did not stain with annexin-V. Ricci *et al.* (2002) suggest that PI positive / annexin negative sperm may be sperm in the latter stage of apoptosis.

Correlations between semen parameters and the results of flow cytometry assay (annexin-V / PI) are reveal in table 2. This study found that the semen volume significantly ($p<0.01$) correlated with the percentage of viable sperm ($r = 0.76$) and inversely correlated ($p<0.01$) with the percentages of early and late apoptosis and apoptotic index ($r = - 0.53$, $- 0.64$ and $– 0.51$, respectively). Sperm count was significantly ($p<0.01$) correlated with viable sperm ($r =0.95$) and inversely correlated ($p<0.01$) with the percentages of early and late apoptosis and apoptotic index ($r = - 0.70$, $- 0.79$ and $– 0.70$, respectively). Also, sperm motility percentages were significantly ($p<0.01$) correlated with viable sperm ($r = 0.89$) whereas inversely correlated ($p<0.01$) with the percentages of early and late apoptosis and apoptotic index ($r = - 0.67$, $- 0.72$ and $– 0.73$, respectively). In the same way, the percentage of both active and normal sperm were significantly ($p<0.01$) correlated with the percentage of live sperms ($r = 0.93$ and 0.91, respectively), while were inversely correlated ($p<0.01$) with the percentages of early, late apoptotic and apoptotic index ($r = -0.70$, $- 0.77$ and $– 0.71$ for active sperm; $r = - 0.66$, $- 0.75$ and $– 0.69$ for normal sperm). In contrast, the percentage of abnormal sperms was significantly ($p<0.01$) correlated with the percentages of early, late apoptotic, necrotic and apoptotic index ($r = 0.66$, 0.75, 0.75 and 0.69, respectively), whereas was inversely correlated ($p<0.01$) with the percentage of live sperm ($r = - 0.91$).

Table (2): Correlation between semen parameters and results of flow cytometry assay.
[1] annexin-V/ PI **: significant at 0.01 level.

Sperm status / Semen parameter	Live sperm		Early apoptotic		Late apoptotic		Necrotic		Apoptotic index	
	- / - [1]		+ / -		+ / +		+ / +			
	r	p	r	p	r	p	r	p	r	p
Semen volume	0.76	**	-0.53	**	-0.64	**	-0.07	NS	-0.51	**
Sperm count	0.95	**	-0.70	**	-0.79	**	-0.03	NS	-0.70	**
Sperm motility%	0.89	**	-0.67	**	-0.72	**	-0.03	NS	-0.73	**
Active sperm%	0.93	**	-0.70	**	-0.77	**	-0.02	NS	-0.71	**
Normal sperm%	0.91	**	-0.66	**	-0.75	**	-0.06	NS	-0.69	**
Abnormal sperm%	-0.91	**	0.66	**	0.75	**	0.75	**	0.69	**

The correlations between apoptotic index and semen parameters studied on semen samples taken from apparently healthy subjects (fertile) and severe oligozoospermic patients are shown in table 3. No significant correlations were noted between apoptotic index and semen parameters in apparently healthy subjects or severe oligozoospermic patients, severally, whereas as a total samples there were a significant ($p<0.01$) negative correlations between

apoptotic index and semen volume, sperm count, sperm motility, active sperm and normal sperm (r = - 0.51, - 0.70, - 0.73, - 0.71 and – 0.69, respectively). Apoptotic index was positively correlated ($p<0.01$) with the percentage of abnormal sperm (r = 0.69).

Table (3): Correlation between semen parameters and apoptotic index.

Groups semen parameters	Control [1]		Patient [2]		Total samples	
	r	p	r	p	r	P
Semen volume	-0.04	NS	0.22	NS	-0.51	**
Sperm count	-0.21	NS	0.06	NS	-0.70	**
Sperm motility%	-0.27	NS	-0.31	NS	-0.73	**
Active sperm%	-0.28	NS	-0.32	NS	-0.71	**
Normal sperm%	0.02	NS	-0.9	NS	-0.69	**
Abnormal sperm%	-0.02	NS	0.10	NS	0.69	**

[1] apparently healthy subjects (fertile). [2] severe oligozoospermic patients.
NS: no significant **: significant at 0.01 level.

Apoptosis is physiologically programmed cell death that affects single cells without any associated inflammation in the surrounding tissues (Wyllie *et al.*, 1980). Apoptosis is characterized by series of ultrastructural morphological (Wyllie *et al.,* 1980) and biochemical changes (Williams and Smith, 1993). There is chromatin aggregation and cytoplasmic condensation of nuclear and cytoplasmic membranes in apoptotic cells and at the end of this process, the nucleus undergoes fragmentation and the whole cell blebs and fragments into apoptotic bodies (Aznar *et al.,* 2002). Sperm DNA damage and sperm apoptosis have been considered as potentially useful indices of male fertility (Chen *et al.,* 2006). As shown from the results of apparently healthy subjects *versus* severe oligozoospermic patients in this study, male infertility appears to be positively correlated with increased levels of sperm with apoptotic markers (Oehninger *et al.,* 2003; Paasch *et al.*,2003; Wang *et al.,* 2003). In apoptotic cells, the plasma membranes are intact, whereas in necrotic cells, the plasma membranes lose their integrity and become leaky (Vermes *et al.*, 1995). Many published studies were investigated the relationship between traditional semen parameters and apoptosis and the results of these studies were inconsistent (Sun *et al.*, 1997; Gandini *et al.,* 2000; Oosterhuis *et al.*, 2000; Shen *et al*., 2002). In the present study, there were clear correlations between apoptosis and semen volume, sperm count, sperm motility, active sperm, normal sperm and abnormal sperm of semen collected from apparently healthy subjects (fertile) and severe oligozoospermic patients. Chen *et al.* (2006) found inverse associations between percent apoptosis in ejaculated human semen and sperm motility, progressive motility, and morphology. Similar results were reported by several researchers (Sun *et al.*,1997; Barroso *et al.,* 2000; Oosterhuis *et al.,* 2000; Shen *et al.,* 2002). Oosterhuis *et al.* (2000) found a significant inverse correlation between sperm motility and apoptosis by annexin-V assay (r = - 0.289; $p<0.05$) in ejaculated sperm. Some studies found apoptosis positively associated with various forms of abnormal sperm morphology, including

defects of the sperm head, midpiece, and tail (Gandini *et al*., 2000; Shen *et al.*, 2002). In contrast to the results of the present study, Chen *et al.* (2006) found that the percent of apoptosis was not significantly correlated with either sperm concentration or total sperm count. Shen *et al.* (2002) in a study on subfertile men reported that the percentage of apoptosis was significantly positively correlated with both sperm concentration and total sperm count when using the Annexin- V assay. The detection of apoptosis in human sperm could provide further information and may explain more about the causes of fertilization failures (Levy and Seifer-Aknin, 2001). Wang *et al.* (2002) reported that sperm apoptosis by flow cytometry was significantly higher in infertile group than in fertile.

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