

Relationship between sperm chromatin status and ICSI outcome in men with obstructive azoospermia and unexplained infertile normozoospermia

M. R. SADEGHI¹⁾, N. LAKPOUR²⁾, H. HEIDARI-VALA³⁾, MAHSHID HODJAT³⁾,
N. AMIRJANNATI³⁾, H. HOSSAINI JADDA³⁾, SIMA BINAFAAR³⁾,
M. M. AKHONDI³⁾

¹⁾Monoclonal Antibody Research Center

²⁾Nanobiotechnology Research Center

³⁾Reproductive Biotechnology Research Center

Avicenna Research Institute (ARI), ACECR, Tehran, Iran

Abstract

This study was done to evaluate the effect of sperm source on chromatin integrity and ICSI outcomes. One hundred and thirteen samples containing epididymal aspirates of 57 obstructive azoospermic men and 56-ejaculated semen of normozoospermic men were included in this study. Sperm chromatin status was evaluated by Chromomycin A3 (CMA3), Aniline Blue (AB) and Toluidine Blue (TB). Fertilization rate and embryo quality were recorded. In epididymal group the percentage of sperms stained with AB, CMA3 and TB were significantly higher compared to ejaculate group while fertilization rate (60.6% vs. 74.04%) was significantly lower. However, embryo quality was not significantly different between two groups. In addition, abnormal sperm chromatin condensation and DNA fragmentation were not correlated with fertilization rate and embryo quality. Our results highlight the role of epididymis in sperm maturation and confirm that ICSI using ejaculated sperm is the gold standard for treatment of infertile men.

Keywords: azoospermia, embryo quality, fertilization rate, PESA, sperm chromatin integrity.

Introduction

Since the microinjection techniques have been innovated, some long-standing male infertility such as azoospermia and oligoasthenoteratozoospermia find the chance to conceive their own biological offspring. Indeed, intracytoplasmic sperm injection (ICSI) can bypass some biological barriers in fertilization process and alter circumstances to ward successful fertilization, presumably. A few numbers of sperm need for ICSI can be retrieved from different origins with a wide variety of maturity and quality [1]. It was proved that ICSI outcomes including fertilization rate, embryo quality, chemical and clinical pregnancy and finally the rate of take home babies highly related to sperm quality [1].

Nowadays, different methods have been used to retrieve sperm with a range of exclusive properties and drawbacks. Precutaneous epididymal sperm aspiration (PESA) is a simplified and reputable surgical method for sperm retrieval in men with obstructive azoospermia (OA), specially [2]. Prior to ICSI introduction in 1993, some cases of pregnancy using in vitro fertilization (IVF) with epididymal spermatozoa have been reported [3, 4]. In fact, irreversible obstructive azoospermia is the first nominate for PESA [5, 6]. Although PESA is a less invasive technique [7–9] with acceptable efficiency but there are some controversies about its results in comparison with sperm retrieved by other methods and

ejaculated spermatozoa. In despite of its simplicity and routinely applications, some studies showed that PESA has less diagnostic value, so that at least 20% of PESA attempts encounter to failure to retrieve spermatozoa [10].

DNA integrity/maturity is the main factor in sperm quality to take high fertilization rate, good embryo quality and healthy ongoing pregnancy. Sperm is passed from epididymis with transition time 2–6 days [11] and during this time most of its functional abilities is acquired in contact with epithelial cells and luminal environment in epididymis, therefore it is suggested that application of epididymal retrieved spermatozoa for *in vitro* fertilization may be accompany with lower outcome in comparison with ejaculated spermatozoa. On the other hands, part of this difference may be rise from sperm aging due to long stay in efferent duct, epididymis and vasa deference of obstructive azoospermic men [12].

To date, ICSI outcome such as embryo quality/pregnancy rate and their correlations with sperm source and sperm retrieval method has been faced with many controversies. Silber SJ *et al.* reported that sperm obtained from the caput epididymis can result in high pregnancy rate [13], but other researchers [14] have not confirmed these results.

On the other hand, some surveys have shown that

fertilization rate in ICSI using ejaculated spermatozoa is higher than non-ejaculated spermatozoa; however there are some studies with conflicting results [15–18]. With regard to above controversies and few numbers of studies on relationship between sperm source, DNA integrity/maturity, fertilization rate and ultimately embryo quality and also in order to better comprehend these correlations in obstructive azoospermic men, we conducted this study on infertile men attending to Avicenna Infertility Clinic (AIC) in Tehran, Iran.

Materials and Methods

Patients

One hundred and thirteen samples containing epididymal aspirate of 57 obstructive azoospermic men and 56 ejaculated semen of normozoospermic men of infertile couples submitted for ICSI program at Avicenna Infertility Clinic (AIC) were included in this study. Samples from couples accompany with female factor infertility were excluded from the study. Written informed consent for using the excess epididymal aspirates and semen was obtained from the patients. The research proposal was approved by Ethical Committee of Avicenna Research Institute (ARI).

Sample collection and preparation

In brief, in normozoospermic men, semen samples on the day of oocyte retrieval were collected on-site by masturbation and allowed to liquefy at 37°C for 30 minutes before analysis. Each sample was divided in two aliquots to be used for semen analysis and sperm chromatin evaluation and another part was used for sperm processing to inseminate oocytes *via* ICSI. The semen analysis was done according to WHO guidelines [19]. Swim up method was done as previously described [20]. In azoospermic men, percutaneous epididymal sperm aspiration (PESA) was done to retrieve sperm as has been previously described [21]. Aspirated spermatozoa were incubated for 45 minutes in 37°C and 5% CO₂ until run time ICSI procedure and chromatin assay.

ART procedure

Controlled ovarian hyperstimulation was done according to the long luteal suppression protocol which use a GnRHa (Superfact, Ferring, Germany) and combination of HMG (Menogon, Ferring, Germany) or recombinant FSH (Gonal-F, Serono, Switzerland). Inducing ovulation was done when at least three follicles had a diameter of ≥ 18 mm, using 10000 IU HCG (Choriomon, IBSA, Switzerland). Oocytes were retrieved by transvaginal ultrasound guided follicle aspiration. The oocytes containing cumulus cells were collected from clear follicular fluid. Granulosa cells were detached from collected oocytes using enzymatic (Collagenase, Sigma) and mechanical digestion. ICSI procedure was carried out as previously described [22]. Luteal phase support was done using intravaginally and intramuscularly Progesterone (Osveh, Iran) administration.

The oocytes fertilization was evaluated by the

presence of two pronuclei (2PN) in fertilized oocytes at 16–18 hours after the injection. Embryos grading was performed according to their morphology at 48–72 hours after insemination [23]. Briefly, embryo classification was as follows: grade A – without fragmentation and regular cells; grade B – lower than 25% fragmentation; grade C – between 25 and 50% fragmentation, and grade D – higher than 50% fragmentation. The embryos were transferred either at 48–72 hours after ICSI, according to treatment plan of the patients.

Sperm chromatin assays

Toluidine Blue staining (TB)

Fresh semen was washed three times in Phosphate Buffered Saline (PBS, pH 7.4). Thick smears of washed spermatozoa were prepared on pre-cleaned degreased slides (product: 7201, Surwin Plastic Enterprises, China) and allowed to air-dry for 10 minutes. The air-dried smears were fixed for at least 30 minutes in ethanol–acetone (1:1) at 4°C and allowed to dry for a few minutes prior to staining for 10 minutes at 0.05% Toluidine Blue (Merck, Germany) in staining buffer. The staining buffer was containing 50% citrate phosphate (McIlvaine buffer, pH 3.5) [24]. After staining, the slides were gently rinsed in a stream of distilled water and air dried for a few minutes. Ultimately, slides were observed under light microscope using a 100 \times oil immersion objective. Sperm head with intact chromatin were light blue and those of fragmented chromatin and abnormal compaction were deep violet (purple). For each sperm sample, at least 200 sperm per each slide were counted and recorded as percent of sperm with light blue and violet head [25].

Aniline Blue staining (AB)

After preparation and fixation of smears, air-dried fixed smears were stained for 7 minutes with 0.5% Aniline Blue in PBS buffer. The pH of staining solution is important and should be adjusted to 3.5 by adding acetic acid [26]. Slides were gently rinsed in a stream of distilled water and air dried for few minutes at room temperature. Ultimately, the slides were observed under light microscope using a 100 \times oil immersion objective. Sperm head with intact chromatin were colorless and immature sperm with excessive histone were blue. For each sperm sample, at least 200 sperm per each slide were counted and recorded as percent of sperm with blue and colorless head.

Chromomycin A3 staining (CMA3)

Air-dried fixed smears were stained for 20 minutes with 100 μ L CMA3 solution (0.25 mg/mL CMA3 at McIlvaine buffer, containing 10 mM/L MgCl₂). Following of staining, the slides were gently rinsed in PBS (pH 7.4) and air-dried in room temperature. For each sperm sample at least 200 sperm per each slide were counted as abnormal chromatin (bright green) or intact (dull green or colorless) for CMA3 by fluorescence microscope (Zeiss, Germany) with a 100 \times oil immersion objective [27].

Some semen samples were treated with hydrogen

peroxide 100 μ M for 10 minutes, before staining with Toluidine Blue, Chromomycin A3 and Aniline Blue stains that these treated samples were used as the positive control.

Two expert laboratory staff evaluated the semen specimens individually. Both intra-assay and inter-assay coefficient of variation of TB, AB and CMA3 staining and semen analysis were lower than 10%.

Statistical analysis

In this study, fertilization rates was calculated for each cycle as the number of cleavage embryos divided by the number of retrieved mature oocytes. SPSS software (Version 13, SPSS Inc., USA) was used for statistical analysis. One-Sample Kolmogorov–Smirnov Test showed that variables of age, Aniline Blue data, Toluidine Blue data, percentage of CMA3-positive sperm and fertilization rate were normally distributed but number of metaphase II oocyte, number of embryos and number of embryos with grade A, B and C were not

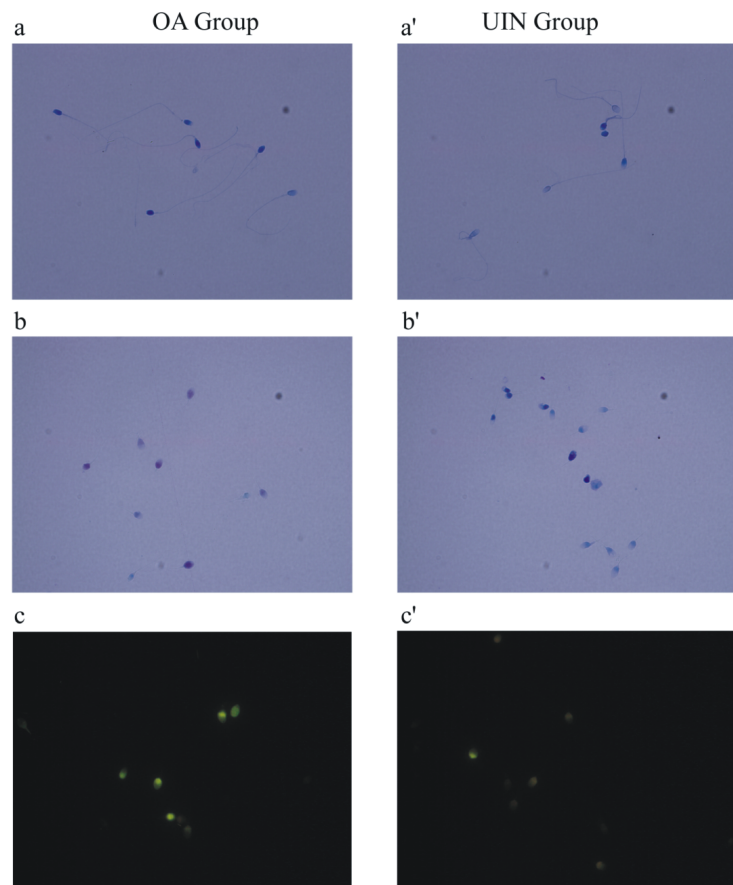
normally distributed. For comparing mean variables that were normally distributed between two groups (ejaculated sperm and PESA), Independent sample *t*-test and for comparison of variables which were not normally distributed non-parametric Mann–Whitney *U*-test were used. Pearson's correlation coefficient was used for parametric correlations and Spearman's rank correlation used for non-parametric correlations. *P*-values less than 5% were considered statistically significant.

Results

Sperm cytology based on chromatin health and quality

Sperm chromatin assays by three cytological tests on smears prepared from ejaculate and PESA are presented in Figure 1.

Figure 1 – Evaluation of sperm chromatin status by Aniline Blue (a, a'), Toluidine Blue (b, b') and Chromomycin A3 (c, c') stainings in obstructive azoospermia (OA) and unexplained infertile normozoospermia (UIN). Aniline Blue staining (a, a'): sperm head with intact chromatin were colorless and immature sperm with excessive histone were dark blue; Toluidine Blue staining (b, b'): sperm head with intact chromatin were light blue and those of fragmented chromatin and abnormal compaction were deep violet (purple); Chromomycin A3 (c, c') staining: sperm head with abnormal chromatin were bright green and those with intact chromatin were dull green or colorless.



The sperm having dark blue head are immature (Figure 1, a and a'), and were stained due to possessing persistent histones, while mature spermatozoa are seen as colorless. Sperm with a light blue head (Figure 1, b and b') have condensed chromatin and deep violet (purple) represent sperm with uncondensed chromatin. Moreover, in Figure 1 (c and c'), bright green sperm head represent abnormal chromatin due to incomplete protamination and maturity, while intact sperm chromatin is observed as dull green or colorless. During spermiogenesis, histones are replaced by protamines in guanine-cytosine (GC)-rich area of sperm DNA. CMA3

as a fluorochrome dye competes with protamines for these areas.

Patient's characteristics

Patient's characteristics according to source of retrieved sperm were summarized in Table 1.

The mean age of men was not different between ejaculate and PESA groups (34.73 ± 7.06 vs. 37.29 ± 7.02).

As has been shown in Figure 2, mean of chromatin abnormality (AB staining results), DNA fragmentation (TB staining results) and percentage of CMA3-positive sperms were significantly higher in epididymal compared

to ejaculated spermatozoa (35.45 ± 16.27 , 41.54 ± 13.42 and 52.66 ± 16.38 vs. 27.45 ± 12.51 , 30.05 ± 11.51 and 36.07 ± 11.91 , respectively; $p < 0.001$).

Table 1 – Characteristics of patients according to source of retrieved spermatozoa

Variable	Ejaculate	PESA	P-value
Age [years]	34.73 ± 7.06	37.29 ± 7.02	0.088
No. of retrieved oocytes (MI)	8.21 ± 4.8	8.16 ± 4.03	0.59
Total no. of embryos	5.86 ± 3.76	5.65 ± 3.31	0.99
Fertilization rate [%]	74.04 ± 23.75	60.62 ± 27.26	0.006*
Total number of embryos with:			
Grade A	2.88 ± 3.41	2.77 ± 3.4	0.95
Grade B	2.34 ± 2.23	2.01 ± 1.88	0.29
Grade C	0.75 ± 0.21	1.04 ± 0.37	0.41
Chromatin assays:			
Chromatin abnormality (AB [%])	27.45 ± 12.51	35.45 ± 16.27	0.008*
DNA fragmentation (TB [%])	30.05 ± 11.51	41.54 ± 13.42	0.000*
Chromatin immaturity (CMA3 [%])	36.07 ± 11.91	52.66 ± 16.38	0.000*

Variables were presented as mean \pm SD; * – $p < 0.05$.

Fertilization rate

Fertilization rate was significantly higher in ejaculate group in comparison with PESA group (74.04 ± 23.75 vs. 60.62 ± 27.26 , $p < 0.01$) (Figure 2). However, total number of embryos had not statistically different distribution between two groups (5.65 ± 3.31 vs. 5.86 ± 3.76 for PESA and ejaculate group, respectively).

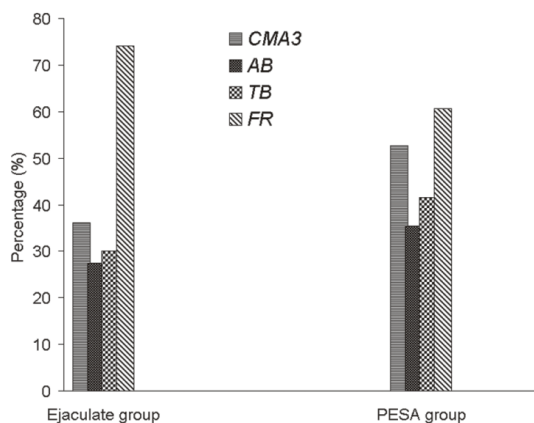


Figure 2 – Sperm chromatin status and fertilization rate in ejaculate and PESA group. AB: Aniline Blue staining results, TB: Toluidine Blue staining results, CMA3: Chromomycine A3 staining results, and FR: Fertilization rate. All data are mean \pm SD.

On the other hand, no significant difference was not found at total number of embryos with grade A, grade B and grade C between two groups (Table 1).

Correlations of sperm staining by AB, TB and CMA3 with fertilization rate and embryo quality were evaluated. There was no significant correlation between chromatin condensation (AB and CMA3 results) and DNA fragmentation (TB) with fertilization rate and good embryo quality (embryos with grade A and B) in both ejaculate and PESA groups ($p > 0.05$) (Table 2).

Table 2 – Correlations of chromatin condensation and DNA fragmentation with ICSI outcomes in both ejaculate and PESA groups

ICSI outcomes	Ejaculate group			PESA group		
	AB	CMA3	TB	AB	CMA3	TB
Fertilization rate [%]	-0.13, 0.33	0.09, 0.5	0.02, 0.87	0.17, 0.28	-0.05, 0.78	0.05, 0.75
Number of embryos:						
Grade A	-0.1, 0.46	0.02, 0.83	-0.11, 0.41	0.06, 0.7	0.29, 0.09	0.09, 0.58
Grade B	-0.07, 0.6	-0.2, 0.13	-0.22, 0.1	-0.03, 0.8	-0.14, 0.42	-0.09, 0.57

Numbers in the table are Pearson's correlation coefficient or Spearman's rank correlation and p-value (r, p-value) respectively.

Discussion

This study has more focused on DNA integrity/maturity of retrieved sperm from epididymis and ejaculated sperm and possible correlation between these parameters with ICSI outcomes in the both groups. Retrieved sperm from epididymis result in good fertilization rate following ICSI procedure. However, since sperm DNA integrity can influence the quality of embryo and ART outcomes [28–30], and probability offspring health [10, 12], so evaluating of DNA integrity is inevitable. According to our results, abnormal chromatin condensation (AB and CMA3 results) and DNA fragmentation (TB results) were higher in epididymal sperm in comparison with ejaculated spermatozoa. These results are in agreement with previous reports on DNA damage [31] and chromatin condensation [31–33]. In normal status, chromatin condensation is continued during sperm journey from caput to cauda epididymis [34]. Thus, epididymis is a major site for sperm chromatin maturity. In obstructive azoospermic men, sperm for a long time stay in epididymis and aging process is occurred [12, 31]. As a result, sperm functions, especially their DNA integrity are reduced by reactive oxygen species (ROS) and/or cell aging [12]. In addition, the activity of endonuclease enzymes in both epididymal and ejaculated sperm is triggered by exposures to exogenous DNA resulted from destructed spermatozoa, consequently, DNA fragments in epididymis increase progressively DNA fragmentation level [12, 35]. As well, due to incomplete sperm maturation in testis, maturation process is completed in epididymis, therefore epididymal retrieved spermatozoa is more susceptible to damage by deleterious factors [33]. Above-mentioned reasons justify increased abnormal chromatin maturity and DNA damage in PESA group in comparison with ejaculate group.

The first epididymal sperm that applied in *in vitro* fertilization (IVF) procedure resulted in low fertilization [10]. Consequently, animal experiment suggested that microinjection could be more effective, which achieved better success rates [10]. The outcome of ICSI using surgically retrieved sperm is related to cause of azoospermia and quality of retrieved spermatozoa [36]. Even the preference of surgically retrieved sperm from epididymis to testis is controversy. In the present work, we continued our research on effect of sperm origins in ICSI outcomes.

Fertilization rate was higher in ejaculate group compared to PESA group ($p < 0.01$), while good embryo quality were not different (number of embryos with grade A and B). To date there is controversial results about relation of sperm origin with fertilizing ability and consequently fertilization rate and embryo quality. Some studies have shown higher fertilization rate with ejaculated sperm vs. epididymal sperm (in agreement with our study) but similar pregnancy rate [37, 38], whereas other studies showed similar fertilization rate (in contrast with the results of our study) and pregnancy rate [15–18]. In addition, another group of studies demonstrated similar fertilization rate (in contrast with the results of our study) but high pregnancy rate with ejaculated sperm [39, 40]. In relation to embryo quality, previous two studies showed ejaculated sperm has better embryo quality than epididymal sperm [41, 42]. According to aforementioned studies and our study fertilization rate, pregnancy rate and embryo quality were either similar or higher in ejaculate group in comparison with epididymal group, therefore it seems the ICSI outcome is better using ejaculated vs. epididymal sperm.

Although this study showed that fertilization rate and DNA integrity/maturity were higher in ejaculate group than in PESA group, but no significant correlation was found between chromatin condensation and DNA fragmentation with fertilization rate and embryo quality in both PESA and ejaculate group. Similar to our results, Sakass D *et al.* [43] and Zini A *et al.* [44] found no significant correlation between chromatin condensation and DNA fragmentation with fertilization rate in ejaculated sperm. In contrast, more studies showed negative associations between frequency of sperm DNA fragmentation and fertilization rate and embryo development [45–48]. To our knowledge, the present study is first work that has investigated effect of DNA integrity/maturity on PESA/ICSI outcomes in obstructive azoospermia. Normal sperm chromatin integrity has important role in sperm fertilizing potential. Some studies showed a predictive threshold of 27% for sperm DNA fragmentation to result in a successful pregnancy in IVF/ICSI cycles [49, 50]. In contrast, other studies indicated that specimens with a high rate of DNA damage could result a successful ongoing pregnancy [51–53]. Sperm DNA damage is more valuable in predicting IVF than ICSI outcomes [50, 54, 55]. Therefore, this may be one of reasons that we did not find any correlation between chromatin condensation and DNA fragmentation with ICSI outcomes.

Raw semen contained, immature germ cells, mature live and/or dead sperm. Evaluations of DNA integrity/maturity in present study were performed on raw semen. While, before ICSI procedure, motile sperm with normal morphology was retrieved by sperm processing methods; in addition, ICSI operator tries to select a motile sperm with normal morphology for injection. Therefore, evaluated sperm for chromatin assay in raw semen may not show chromatin integrity of the sperm population selected for ICSI procedure in both ejaculate and PESA group. This may also be the second reason

for the lack of correlation between sperm chromatin condensation and DNA fragmentation with ICSI outcomes. According to study by Ramos L *et al.* that showed motile sperm obtained from ejaculate and epididymis did not differ in terms of sperm DNA damage [31], it is suggested that the effect of sperm chromatin integrity on ICSI outcomes be evaluated on selected motile sperm from ejaculate and epididymal aspirate. Unlike our expectation, increased fertilization rate with ejaculated sperm in comparison with epididymal sperm is not related to low rate of DNA fragmentation and abnormal DNA integrity; but it may be related to another parameter that should be clarified by more investigations in future. There is a point here that in obstructive azoospermia, obstruction for a long time leads to remodeling of caput epithelium to alter proximal microenvironment to partially functional for sperm maturation [14]. Sperm retrieved from this region may have better quality than other regions of epididymis, so, it is recommended that sperm be retrieved from caput region for ICSI treatment in obstructive azoospermic men.

✎ Conclusions

Our study shows that abnormal sperm chromatin condensation and DNA fragmentation are significantly lower in ejaculated sperm in comparison with epididymal sperm. In addition, fertilization rate is higher in ejaculated sperm vs. epididymal sperm while embryo quality including number and grade of embryos is not different between two groups. There is no significant correlation between sperm chromatin condensation and DNA fragmentation with fertilization rate and embryo quality in both ejaculate and PESA groups. This lack of relationship may be because that best quality sperm were selected in ICSI procedure, but sperm chromatin assay as a diagnostic test was performed on raw semen containing sperm with diverse qualities prior to ICSI cycles for infertile men. Increased fertilization rate in ejaculated sperm in comparison with epididymal sperm is not related to decrease abnormal sperm chromatin condensation and DNA fragmentation in ejaculated sperm and may be related to a series of other factors that are associated with sperm epididymal maturation. This highlights the role of epididymis in sperm maturation and confirms that ICSI, using ejaculate sperm, is generally as the gold standard for the treatment of male infertility.

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Corresponding author

Mohammad Mehdi Akhondi, PhD, Reproductive Biotechnology Research Center, Avicenna Research Institute, P.O. Box 19615–1177, Tehran, Iran; Phone +98 21 22432020, Fax +98 21 22432021, e-mail: Akhondi@Avicenna.ac.ir

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