RESEARCH ARTICLE

IMMUNO-HISTOCHEMICAL EXPRESSION OF MCL-1 IN TESTICULAR BIOPSY OF PATIENTS WITH AZOOSPERMIA.

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Abstract

Background:- Azoospermia is the medical condition of a man not having any sperm in his semen. Myeloid cell leukemia-1 (Mcl-1) is a member of the Bcl2 family of proteins involved in regulation of apoptosis. It is an inhibitor of programmed cell death.

Objective:- Assess the immunohistochemical expression of MCL-1 in testicular biopsies in azoospermic patients and to correlate this expression with risk factors age, fertilization rate, quality of embryo and histopathological changes.

Patients and methods:- This cohort study included 60 patients with azoospermia, 15 male with obstructive type and 45 males with non-obstructive type. Those patients were randomly selected form the population of infertile couples who regularly visits the High Institute for Infertility Diagnosis and ART and from Kamal Al-Samarai Hospital center of fertility.

Result:- Mean histological H&E score was significantly higher in the obstructive group, 8.67 ±0.75 versus 4.82 ±2.60 (P<0.01). Additionally, mean MCL1 immunohistochemical score was significantly higher in the obstructive group than non-obstructive group, 2.73 ±0.46 versus 0.84 ±0.95 (P<0.001). Histological H and E score was highly significantly correlated with the immunohistochemical MCL1 score (r = 0.863, P<0.001). MCL1 score showed no significant correlation with the risk factors whereas it showed significant negative correlations with FSH, LH and prolactin and significant positive correlation with serum testosterone. Sperm isolation rate was significantly determined by higher histological and MCL 1 scores.

Conclusion:- Histological H&E score and MCI-1 immunohistochemical score were significantly higher in the obstructive group than non-obstructive one, reflecting that apoptosis is higher in non-obstructive azoospermia and may have a role in its pathogenesis.
Introduction:
Azoospermia is defined as the lack of sperm in male ejaculate and is suggested to be present in about 1% of all males and to account for 10-15% of the causes of infertility in men\(^{(1)}\). It is classified into two major forms: either obstructive or non-obstructive. The presence of organic obstruction in the reproductive male pathways distal to the testes is the pathognomonic pathophysiology in obstructive type \(^{(2)}\). Non-obstructive azoospermia (NOA) is often regarded as a non-medically manageable reason of infertility in men. These patients, who make up to 10% of global infertile male subjects, have abnormal spermatogenesis as with consequent azoospermia. The establishment of in vitro fertilization using intracytoplasmic sperm injection (ICSI) as a mainstay treatment modality made a number of these male patients to be successful biologic fathers a child via surgically retrieved sperm from the testis. The obstacle, however, is to facilitate their spermatogenic activity to enable the acquisition of sperm in their ejaculate or to improve the opportunity of a successful sperm retrieval from the testis for ICSI \(^{(3)}\). An accurate diagnosis of azoospermia and thorough evaluation of the patient to diagnose the cause of infertility are needed to guide appropriate treatment options and to identify the associated cost advantages, risks and hope for treatment success. The development of intracytoplasmic sperm injection (ICSI) as an effective therapy for profound male factor infertility has become a definite treatment for the majority of male reproductive tract abnormalities \(^{(1)}\). During spermatogenesis, there is a need of germ cell removal by apoptosis in order to maintain healthy germ cell development and to reach a normal sperm outcome. In cases of improper spermatogenesis with azoospermia, apoptosis of testicular sperm has also been a target of debate in the published literature\(^{(4)}\). The anti-apoptotic myeloid cell leukemia factor 1 (Mcl-1) was discovered based on its increased expression during cell commitment toward differentiation in a human myeloid leukemia cell line. The rapid induction and destruction of Mcl-1 has been proposed as a molecular mechanism for cell commitment in a human myeloid leukemia cell line. The level of significance was considered at 0.05.

Patients and Methods:
This prospective cohort study included 60 patients with azoospermia, 15 male with obstructive type and 45 males with non-obstructive type. Those patients were randomly selected from the population of infertile couples who regularly visit the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies and from Kamal Al-Samarai Hospital center of fertility and IVF (Baghdad / Iraq). Testicular biopsies of azoospermic patients were obtained and fixed with modified Zenker (fixative) and then processed routinely to produce paraffin blocks. From each paraffin block, 2 sections of 5 µm thickness were taken: one section was stained with H&E to study histopathological changes and the other was stained immunohistochemically for MCL-1 monoclonal antibody. All male patients were subjected to hormonal assays including: FSH, LH, testosterone and prolactin, whereas female partners were subjected to assessment of FSH, LH, progesterone and estradiol.

Statistical analysis was performed using SPSS version 22. Data were presented as number and percentage for categorical variables or as mean ±SD for numerical variables. For the analysis of numeric variables, independent student test was used in case of normal distribution and Mann Whitney U test in case of non-normal distribution. Chi-square test and Fischer Exact tests were used to study association between any two categorical variables. Correlation and regression analysis was used between immunohistochemical and histological scoring variables. The level of significance was considered at 0.05.

Results:

General characteristics of the Azoospermic men and their female partners are shown in tables 1 and 2.

| Characteristic                      | Obstructive (N = 15) | Non-Obstructive (N = 45) | Total (N = 60) | P*       |
|------------------------------------|----------------------|--------------------------|---------------|----------|
| Age (mean ±SD) years               | 34.07 ±6.82          | 33.07 ±7.84              | 33.32 ±7.55   | 0.661†   |
| Smoker N (%)                       | 4 (26.7)             | 15 (33.3)                | 19 (31.7)     | 0.755*   |
| Varicocele N (%)                   | 3 (20.0)             | 8 (17.8)                 | 11 (18.3)     | 1.000*   |
| RGUTI N (%)                        | 3 (20.0)             | 1 (2.2)                  | 4 (6.7)       | 0.073*   |
| Chemical Toxin N (%)               | 2 (13.3)             | 7 (15.6)                 | 9 (15.0)      | 1.000*   |
| Physical Toxin N (%)               | 3 (20.00)            | 10 (22.2)                | 13 (21.7)     | 1.000*   |
| Congenital anomaly N (%)           | 1 (6.7)              | 1 (2.2)                  | 2 (3.3)       | 1.000*   |

†Independent samples t-test *Corrected Chi-square test; RGUTI: recurrent genitourinary tract infections
Table 2: General characteristics of female partners.

| Characteristic               | Group 1                 | Group 2                 | Total       | P     |
|-----------------------------|-------------------------|-------------------------|-------------|-------|
| Age (mean ±SD) years        | 29.00 ±4.61             | 27.60 ±6.12             | 28.30 ±5.37 | 0.485*|
| Infertility type            | 12/3                    | 15/0                    | 27/3        | 0.244†|
| (Primary/Secondary)         |                         |                         |             |       |
| Duration (mean ±SD) years   | 7.20 ±2.78              | 5.60 ±4.27              | 6.40 ±3.64  | 0.234*|
| Weight (mean ±SD) kg        | 68.07 ±8.72             | 66.73 ±9.51             | 67.40 ±8.99 | 0.692*|
| FSH (mean ±SD) IU/ml        | 7.04 ±1.76              | 7.19 ±1.08              | 7.11 ±1.43  | 0.785*|
| LH (mean ±SD) IU/ml         | 5.51 ±1.46              | 5.93 ±1.09              | 5.72 ±1.29  | 0.381*|
| Progesterone (mean ±SD) IU/ml | 15.89 ±3.81            | 15.73 ±3.71             | 15.81 ±3.70 | 0.912*|
| Estradiol (mean ±SD) IU/ml  | 157.93 ±59.05           | 115.00 ±44.71           | 136.47 ±55.90 | 0.042**|

Group 1: Female partners of males with obstructive azoospermia; Group 2: Female partners of males with non-obstructive azoospermia; *Independent sample t-test; † Fischer Exact test; ** Mann Whitney U test

Mean serum hormonal levels with corresponding ranges were presented in table 3 and figure 1. Comparisons revealed the following: serum FSH, LH and prolactin were significantly higher in the non-obstructive group whereas serum testosterone was significantly lower in the non-obstructive group.

Table 3: Comparison of mean serum hormonal levels between obstructive and non-obstructive azoospermia patients.

| Hormone  | Group          | Mean ± SD | Range     | P     |
|----------|----------------|-----------|-----------|-------|
| FSH      | Obstructive    | 5.96 ±1.06| 4.5 - 8.1 | 0.001*|
|          | Non-Obstructive| 12.81 ±8.37| 2.4 - 38.6|       |
|          | Total          | 11.10 ±7.84| 2.4 - 38.6|       |
| LH       | Obstructive    | 5.29 ±1.18| 3.2 - 7.2 | 0.017*|
|          | Non-Obstructive| 7.58 ±3.63| 1.6 - 18  |       |
|          | Total          | 7.01 ±3.34| 1.6 - 18  |       |
| Prolactin| Obstructive    | 7.75 ±3.62| 4.7 - 20  | <0.001†|
|          | Non-Obstructive| 12.56 ±3.76| 0.55 - 21.4|       |
|          | Total          | 11.36 ±4.25| 0.55 - 21.4|       |
| Testosterone| Obstructive | 22.20 ±1.39| 20.1 - 24.6| <0.001*|
|          | Non-Obstructive| 3.09 ±2.58| 0.86 - 15.5|       |
|          | Total          | 7.87 ±8.67| 0.86 - 24.6|       |

*Mann Whitney U test; †Independent samples t-test

Figure 1: Bar chart showing comparison of mean serum hormonal levels between obstructive and obstructive azoospermia groups.
Mean histological H and E score was significantly higher in the obstructive group, 8.67 ±0.75 versus 4.82 ±2.60 (P<0.01). Additionally, mean MCL1 immunohistochemical score was significantly higher in the obstructive group than non-obstructive group, 2.73 ±0.46 versus 0.84 ±0.95 (P<0.001), as shown in table 4 and figure 2.

**Table 4:** Comparison of H&EScore and MCL1Score scores between obstructive and obstructive azoospermia groups.

| Score     | Group          | Mean ± SD | Range   | P*   |
|-----------|----------------|-----------|---------|------|
| H&E Score | Obstructive    | 8.67 ±0.75| 7.5 - 10.0| <0.001|
|           | Non-Obstructive| 4.82 ±2.60| 1.0 - 9.0|      |
|           | Total          | 5.78 ±2.83| 1.0 - 10.0|      |
| MCL1Score | Obstructive    | 2.73 ±0.46| 2.0 - 3.0| <0.001|
|           | Non-Obstructive| 0.84 ±0.95| 0.0 - 3.0|      |
|           | Total          | 1.32 ±1.19| 0.0 - 3.0|      |

*Mann Whitney U test

**Figure 2:** Bar chart showing comparison of H&EScore and MCL1Score scores between obstructive and non-obstructive azoospermia groups.

Histological H and E score was highly significantly correlated with the immunohistochemical MCL1 score (r = 0.863, P<0.001), and regression analysis showed that adjusted R² was 0.891 which means that the regression model can explain perfectly 89.1% of the correlation between the two parameters, figure 3.

**Figure 3:** Correlation between H and E scores and MCL1 scores in all patients.
MCL1 score showed no significant correlation with age, smoking, varicocele, recurrent genitourinary tract infections, exposure to chemical and physical toxins and the presence of congenital anomaly, whereas it showed significant negative correlations with FSH, LH and prolactin and significant positive correlation with serum testosterone, table 5.

**Table 5:- Correlation between MCL1 score and other variables.**

| Variable        | r    | P      |
|-----------------|------|--------|
| Age             | -0.006 | 0.966* |
| Smoker          | 0.057 | 0.634† |
| Varicocele      | 0.022 | 0.857† |
| RUTGI           | 0.088 | 0.465† |
| Chemical Toxin  | -0.030 | 0.803† |
| Physical Toxin  | -0.035 | 0.770† |
| Congenital anomaly | 0.171 | 0.154† |
| FSH             | -0.548 | <0.001* |
| LH              | -0.379 | 0.003* |
| Prolactin       | -0.429 | 0.001* |
| Testosterone    | 0.607 | <0.001* |

*Spearman correlation; †Kindall's Tau_b correlation

Sperms were successfully isolated from 30 patients (50%) and the isolation rate was significantly higher in the obstructive azoospermia group than in the non-obstructive azoospermia group, 100% versus 33.3% (P<0.01). Sperm isolation rate was not affected by age of the patient, but it was significantly related to lower FSH, LH and prolactin and higher testosterone level and also it was significantly determined by higher histological and MCL 1 scores, as shown in table 6.

**Table 6:- Factors predicting positive sperm isolation.**

| Characteristic       | Positive (N = 30) | Negative (N = 30) | P          |
|----------------------|------------------|-------------------|------------|
| Obstructive/non-obstructive (N) | 15/15 | 0/30 | <0.001* |
| Age (mean ±SD)       | 33.40±8.22       | 33.23±6.96       | 0.933**    |
| FSH (mean ±SD)       | 9.09±8.29        | 13.10±6.93       | 0.001†     |
| LH (mean ±SD)        | 6.49±3.89        | 7.52±2.64        | 0.028†     |
| Prolactin (mean ±SD) | 10.03±4.41       | 12.69±3.70       | 0.014**    |
| Testosterone (mean ±SD) | 12.60±9.92    | 3.13±2.82        | 0.001†     |
| ScoreH&E (mean ±SD)  | 8.28±0.73        | 3.28±1.68        | <0.001†    |
| ScoreMCL1 (mean ±SD) | 2.40±0.50        | 0.23±0.43        | <0.001†    |

*Chi-square test; **Independent samples t-test; † Mann Whitney U test

Successful fertilization was seen in all cases with positive sperm isolation, in other words fertilization rate was 100% with regard to sperm isolation.

Among those 30 patients with positive fertilization, pregnancy was encountered in 7 patients (23.33%). It was not statistically associated with type of azoospermia (P=0.388), also it was not affected by age, serum hormonal level, and histological and MCL 1 scores, table 8.

**Table 8:- Factors predicting positive pregnancy outcome.**

| Characteristic       | Positive (N = 7) | Negative (N = 16) | P          |
|----------------------|------------------|-------------------|------------|
| Obstructive/non-obstructive N | 5/2  | 10/13 | 0.388*      |
| Age (mean ±SD)       | 32.86±6.69       | 33.57±8.76       | 0.825†     |
| FSH (mean ±SD)       | 7.46±5.26        | 9.58±9.05        | 0.750†     |
| LH (mean ±SD)        | 6.33±4.79        | 6.54±3.70        | 0.432†     |
| Prolactin (mean ±SD) | 8.64±3.81        | 10.45±4.57       | 0.280†     |
| Testosterone (mean ±SD) | 17.01±10.12     | 11.26±9.68       | 0.091†     |
| ScoreH&E (mean ±SD)  | 8.64±0.75        | 8.17±0.70        | 0.109†     |
| ScoreMCL1 (mean ±SD) | 2.71±0.49        | 2.30±0.47        | 0.057†     |

*Corrected Chi-square test; **Kindall's Tau_b correlation; † Mann Whitney U test
Histological sections of patients with non-obstructive azoospermia revealed several microscopic patterns: absence of seminiferous epithelium and negative immunoreactivity to MCL-1 (figure 4), Sertoli cells-only with corresponding negative MCL-1 IHC (figure 5), or various stages of incomplete spermatogenesis with a range of weak to strong MCL-1 IHC expression (figures 6), whereas obstructive azoospermia showed full spermatogenesis with strong immunoreactivity to Mcl-1 (figure 7).

Figure 4: Seminiferous tubule hyalinization.

A. Section of testicular tissue of patient with non-obstructive azoospermia showing Seminiferous tubules surrounded by thickened and hyalinized membranes and absence of seminiferous epithelium (score 1 according to Modified Johnson scoring system), the tubules are lined with fibroblasts (black arrows), with interstitial fibrosis (red arrows). H&E, 20x.

B. Section of testicular tissue of the same case showing negative immunohistochemical expression of Mcl-1 (score 0). 400x.

Figure 5: Sertoli cells-only syndrome.

A. Section of testicular tissue of patient with non-obstructive azoospermia showing seminiferous tubules lined by Sertoli cells-only (arrows), with absence of germ cells (score 2). H&E, 20x.

B. Section of testicular tissue of the same case showing negative immunohistochemical expression of Mcl-1 (score 0). 400x.
Figure 6: Germ cell maturation arrest.
A. Section of testicular tissue of patient with non-obstructive azoospermia showing seminiferous tubules lined by sertoli cells and spermatogonia (arrows), (score 3). H&E. 20x.
B. Section of testicular tissue of the same case showing positive brown nuclear, cytoplasmic, membranous expression of Mcl-1 of sertoli cells and spermatogonia lining seminiferous tubules (arrows); of weak intensity (score 1+). 400x.

Figure 7: Normal spermatogenesis.
A. Section of testicular tissue of patient with obstructive azoospermia showing full spermatogenesis in the seminiferous tubules (arrows), (score 10). H&E. 20x.
B. Section of testicular tissue of the same case showing positive brown nucleus, cytoplasmic, membranous expression of Mcl-1 in all of the cells lining seminiferous tubules (arrows); of strong intensity (score 3+). 400x.
Discussion:-
Histopathology and immunohistochemistry scores
The results of the current study showed that Mean histological H and E score was significantly higher in the obstructive group, 8.67 ±0.75 versus 4.82 ±2.60 (P<0.01). Additionally, mean MCL1 immunohistochemical score was significantly higher in the obstructive group than non-obstructive group, 2.73 ±0.46 versus 0.84 ±0.95 (P<0.001).

The process of spermatogenesis is based on the equilibrium between proliferation, and apoptosis of germ cells. Throughout various stages of spermatogenesis there should be a role for apoptosis in order to get rid of abnormal spermatogenic cells, and hence maintaining the ratio of germ cell to Sertoli cell with in certain normal range for the propose of lifelong continual spermatogenesis (Razi and Malekinejad, 2015)60. Nonetheless, when there is excessive apoptosis, there will be severe reduction in sperm production. Apoptosis is regulated by several modulators; some working as anti-apoptotic (Bcl-2, Bcl-x long, Bcl-w, Mcl-1, A1/Bfl1, and Nr13) while others enhance apoptosis (Bax, Bcl-x short, Bak, and Bok) (Hardwick and Soane, 2013) (9).

MCL-1 is unique among pro-survival BCL-2 family members in that it is essential for embryonic development and for the survival of multiple cell lineages in the adult including: lymphocytes, hematopoietic stem cells, neutrophils, and neurons. Moreover, MCL-1 is frequently amplified in human cancers and associated with chemotherapeutic resistance and relapse (Kafara et al., 2015) (8).

The significantly lower MCL-1 score in non-obstructive azoospermia type, recorded in the current study, implies that apoptosis is higher in those group and may play a significant role in the pathogenesis of azoospermia. This finding opens the discussion toward the benefit of using some anti-MCL-1 strategy to reduce apoptosis in those patients and subsequently may improve their spermatogenesis. On the other hand the high MCL-1 score, in the present study, was correlated with positive sperm retrieval; hence it can be used as an indicator for success or failure of assisted reproduction in patients with infertility. Hegazy et al. in 2015 (9) investigated the immunohistochemical expression in testicular biopsies obtained from patients with azoospermia and he found out that a strong positive immunoreaction in Leydig cells was observed among all investigated specimens; a moderate reaction was detected in spermatocytes and spermatozoa in cases of normal spermatogenesis and hypospermatogenesis, but a negative reaction was detected in cases of maturation arrest and germ cell aplasia, and concluded that apoptosis was found to be associated with decreased rate of spermatogenesis and that high apoptosis rates may result in azoospermia.

Sperm isolation rate and fertilization rate:-
The present study showed that Sperms were successfully isolated from 30 patients (50%) and that the isolation rate was significantly higher in the obstructive azoospermia group than in the non-obstructive azoospermia group, 100% versus 33.3% (P<0.01); these results were comparable to the finding of other studies in which sperm retrieval rate was higher in obstructive azoospermia in comparison with non-obstructive type (61%) (Moein et al., 2015) (10). It was also, similar to the present study finding, stated by some authors that testicular sperm recovery from azoospermic males with all diagnoses was high (70 to 100%) except non-obstructive azoospermia (31%) (Omurtag et al., 2013) (11). In the current study, sperm isolation rate was not affected by age of the patient, but it was significantly related to lower FSH, LH and prolactin and higher testosterone level and also it was significantly determined by higher histological and MCL1 scores. We agreed with Kalsi et al., 2015 (12) that age of the patient has no significant effect on sperm retrieval rate; however we disagree with him in that FSH level had no significant impact on rate of sperm retrieval. In accordance with the present study findings, it was found that sperm isolation was significantly affected by FSH, LH and testosterone level (Modarresi et al., 2015) (13).

Pregnancy rate:-
Several studies showed that there is no statistically significant difference in clinical pregnancy rates between the two groups (Merchant et al., 2011) (14) and this result solidify the finding of the present study which also found no significant difference in clinical pregnancy rate between obstructive and non-obstructive azoospermia rate. In accordance with the result of the present study it was found that there was no differences were noted in clinical pregnancy rate between obstructive azoospermia and non-obstructive azoospermia groups (Omurtag et al., 2013) (11). In contradiction to the finding of the current study, rate of fertilization was significantly lower with non-obstructive azoospermia (Omurtag et al., 2013) (11). It has also been recorded that fertilization and clinical pregnancy rates was lower in non-obstructive azoospermia (Tehraninejad et al., 2012) (15). However a meta-analysis of surgical sperm retrieved in azoospermic patients concluded that sperm origin does not affect assisted fertilization cycle.
outcome (Kalsi et al., 2011)(16). Another similar result to our finding has been registered by (Tsai et al., 2015)(17) who found no significant difference in clinical pregnancy rate between the two groups.

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