



Micronuclei Formation and Comet Assay in Women with Polycystic Ovarian Syndrome (PCOS)

Hazim Ismail Abdul-Bari AL-Ahmed^{1*}, Rasha Abdul-Hussein Mahood², Shaimaa Yousif Abdul-Fattah¹

¹Biotechnology Research Center, University of AL- Nahrain, Baghdad, Iraq

²Lecturer –Institute of Genetic Engineering and Biotechnology for Postgraduate, University of Baghdad, Baghdad, Iraq

Abstract:

Recently the polycystic ovary syndrome (PCOS) has been linked with DNA damage and genomic instability. Amis of this study to evaluated some parameters of genetic instability such as micronuclei and comet assay score in women with PCOS. Genotoxicity and cytotoxicity of DNA damage were evaluated by measured of micronuclei and comet assay in 25 patients with PCOS and 15 normal menstrual women as control group. The results showed higher significant differences in the level of micronuclei and comet score in patients with PCOS compared with normal women. We concluded that, the genetic instability more occurred in patients with PCOS when compared with control group.

Keywords: MN, comet assay, PCOS

تكوين النويات الصغيرة وفحص المذنب في النساء التي تعاني من اعراض تكيس المبايض المتعدد

حازم اسماعيل عبد الباري^{1*} ، رشا عبد الحسين ماهود²، شيماء يوسف عبد الفتاح¹

¹مركز أبحاث التكنولوجيا الحيوية، جامعة النهرين، بغداد، العراق

²معهد الهندسة الوراثية والتكنولوجيا الحيوية للدراسات العليا، جامعة بغداد، بغداد، العراق

الخلاصة:

في الاونه الاخيره ارتبطت اعراض مرض تكيس المبايض المتعدد مع الضرر الحاصل في المادة الوراثيه وعدم استقرارها، اذ تهدف الدراسه الى تقييم بعض المؤشرات المرتبطه بعدم استقرار المادة الوراثيه مثل تكوين النوى الصغيره وفحص المذنب اذ قيمت السميّه الوراثيه والسميه الخلويه للماده الوراثيه المتضرره عن طريق قياس تكوين النوى الصغيره وفحص الكومت ل25 مريضه مصابه بتكيس المبايض المتعدد و15 امراه ذات دوره شهريه طبيعيه كمجموعه سيطره. اظهرت النتائج ارتفاعاً معنوياً في معدل تكون النوى الصغيره والمذنب للمريضات المصابات بمرض تكيس المبايض المتعدد مقارنة بالنساء الطبيعيات ومن خلال النتائج المذكوره تم الاستنتاج بان عدم استقرار ماده الوراثيه يحدث بصوره اكبر في حاله تكيس المبايض المتعدد مقارنة مع مجموعته السيطره.

Introduction:

Polycystic ovary syndrome (PCOS) condition occurred in about 4-18% of women in reproductive age [1]. PCOS occurred or associated with reproductive condition (anovulation, irregularity of menstrual, hyperandrogenism increase complications of pregnancy). Metabolic condition (increased risk factors for impaired glucose tolerance and type 2 diabetes mellitus) and cardiovascular disease sequelae [2].

*Email: Dr.Hazim_Al_Ahmed@yahoo.com

Some recent studies showed that PCOS associated with insulin resistance, hyperandrogenism and may be lead to cardio vascular disease. DNA strand break elevated in patients with acute coronary syndrome when compared with CAD patients, and increase chromosomal damage and frequency of micronucleus (MN) by using the cytokinesis-block micronuclei (CBMN) assay in patients associated with cardiovascular disease (CVD) and PCOS [3].

Micronuclei are formed as a result of breakage chromosomal or spindle damage and are identical to the main nucleus, varying in their diameter and not linked to the main nucleus [4,5].

Carmina [6] reported that women with PCOS had statistical increase in genomic instability which demonstrated by a significant elevated number of binucleated lymphocytes containing micronuclei, micronuclei total number, elevated proportion of aneuploidy X chromosome signals (2:1 X and 3:1 X) and a lower proportion of normal X chromosome segregation signals (2:2 X) in binucleated lymphocytes than women without PCOS.

Alkaline Comet assay is a very important method for genotoxicity study in cells exposed *in vitro* or *in vivo* to a variety of physical and chemical agents [7]. It provides DNA damage quantitatively in single cells and is established as a valuable tool in fundamental DNA damage and repair studies [7]. The chromosome aberration assay is also a powerful classical cytogenetic tool for genotoxicity testing and can be used as a validation test for Comet assay results [8].

Some studies showed the possibility of relationship between polycystic ovaries and ovarian cancer. DNA Fragmentation or damage is the first step of the carcinogenesis, and susceptibility to cancer, in general, is characterized by high DNA damage. Free radical-mediated DNA damage and impaired antioxidant defense have been implicated as contributory factors for the development of cancer [9].

The Oxidative stress (OS) causes elevation cellular/tissue damage demonstrated by protein oxidation and lipid peroxidation and induce micronucleus formation in bone marrow [10]. The aim of this project to study genetic instability by measuring micronucleus formation and comet score in DNA of the PCOS patients.

Materials and Methods:

Subjects

The study comprised 25 women diagnosed with PCOS and 15 healthy women as controls. Whole blood sample were obtained from some Baghdad Hospitals and physicians private clinics. Each collected blood sample was dispensed into tubes with EDTA for comet assay analysis and into tubes with heparin for MN assay.

Micronucleus Assay:

Procedure:

Lymphocyte cultures were set up in the laboratory by adding 0.5 ml of heparinized blood to 4.5 ml of complete medium Quantum PBL(Proplem-based learning) supplemented with 1% L-Glutamine, 15% fetal calf serum and penicillin (100 U/ul), streptomycin (100 µg/ul) and phytohemagglutinin as mitogen (PHA, 1%) (PAA, Austria). Cells were incubated for 72 h in a 5% CO₂ incubator. 44 h after culture initiation, cytochalasin-B (Sigma, St. Louis, MO, USA) at a final concentration of 4 µg/ml was added to the cultures. The cultures were then centrifuged at 10000 rpm for 10 min. The pellet was re-suspended in hypotonic solution (KCl, 0.087 M, PAA, Austeria) and immediately centrifuged at 10000 rpm for 10 min, and re-suspended in freshly prepared, ice-cold fixative containing methanol: acetic acid (3:1) (Merck, Darmstadt, Germany), left for 20 min at room temperature. The solution was then centrifuged at 10000 rpm for 10 min, and the pellet was re-suspended in freshly prepared ice-cold fixative containing methanol acetic acid (3:1). If the solution was not clear after additional centrifugation, the last step was repeated until a clear solution was obtained. After decantation to reduce the volume to about 1 ml, the pellet was mixed with the remaining fixative and dropped from about 30 cm with a Pasteur pipette onto an ethanol washed slide; the fixative was removed by slight blowing, decantation and air-drying. Subsequently, the slides were stained in 5% giemsa solution for 10 min [11].

Micronuclei were analyzed under a blind fashion using coded slides among 1,000 binuclear cells for each sample using a light microscope with a 1000 objective lens. Scoring criteria was followed as described by Fenech, [12]. All particles in the cytoplasm, with the size smaller than one-third of the main nuclei, round-shaped, and with similar staining characteristics as the main nuclei were scored as micronuclei.

Nuclear division index

The proliferation index was estimated by measuring the nuclear division index according to Lamberti *et al.* [13].

$$NDI = [1(M1\%) \pm 2(M2\%) \pm 3(M3\%) \pm 4(M4\%)]/N$$

$$MN = [1(MN1) \pm 2(MN2) \pm 3(MN3) \pm 4(MN4)]/N$$

NDI=Nuclear division index.

M, 1, 2, 3, 4=Number of nucleate cells

MN, 1, 2, 3, 4=Number of micronucleus in cells.

N= Total number of cells.

Isolation and Counting of lymphocytes:

Lymphocytes were isolated from whole blood with EDTA using the method described by Boyum, [14], using lymphoprep separation fluid. Counting the cells were performed before experiment according to Porakishvili *et al.*, [15] by using trypan blue stain and counting by hemocytometer, then the viability was determined according to the following equation:

$$\text{Viability (\%)} = \left(\frac{\text{Number of Living Cells}}{\text{Total Number of Cells}} \right) \times 100$$

The cells concentration was adjusted to 1×10^6 cell/mL.

Alkaline comet assay:

The electrophoresis conditions used will determine the sensitivity of the assay. Neutral Comet Assay will detect double-stranded DNA breaks, whereas Alkaline Comet Assay will detect single and double-stranded DNA breaks.

Protocol:[15]

Lysis Solution was prepared and cooled at 4°C for at least 20 minutes before use. LM Agarose were melted in a beaker of boiling water for 5 minutes, with the cap loosened. Bottle was placed in 37°C water bath for at least 20 minutes to cool. Cells were combined at 1×10^5 /mL with molten LM Agarose (at 37°C) at a ratio of 1:10 (v/v) and immediately pipetted 50 µl onto Comet Slide. Slides flat was placed at 4°C in the dark for 10 minutes. A 0.5 mm clear ring appears at edge of comet slide area. Slides were immersed in 4°C Lysis solution for 30-60 minutes. Comet Slide were immersed in Alkaline Unwinding Solution for 20 minutes at room temperature or 1 hour at 4°C, in the dark. For the Comet Assay electrophoresis (ES-unit), ~850 mL 4°C Alkaline Electrophoresis Solution was added, slides were placed in electrophoresis slide tray (slide labeled adjacent to black cathode) and covered with Slide Tray Overlay. Power supply was set to 21 volts and voltage was applied for 30 minutes.

Excess electrophoresis solution was drained gently immersed twice in dH₂O for 5 minutes each, then in 70% ethanol for 5 minutes. Samples were dried at 37°C for 10-15 minutes. 100 µl of diluted SYBR Green was placed onto each circle of dried agarose and stained 30 minutes (room temperature) in the dark. Slide was gently tapped to removed excess SYBR solution and rinsed briefly in water. Slides were dried completely at 37°C. Slides were viewed by fluorescence microscopy.

Comet assay scoring:

Fifty randomly selected cells were counted per sample to quantify the comet cell. To determine the comet index (CI), scored range from 1.2 to 2 considered low DNA damage (LD), from 2.1 to 3 medium DNA damage (MD), and up to 3 high DNA damage (HD) [16,17]. The way of quantification by using image analysis software comet score, the analysis software will calculate different parameters for each comet, three parameters were estimated to indicate DNA migration, tail length (distance from the head center to the end of the tail), mean tail moment (product of tail DNA/total DNA by the tail center of gravity) and tail DNA%=100X Tail DNA Intensity/Cell DNA Intensity [18,19].

Statistical analysis:

Data were analyzed by one-way analysis of variance (ANOVA- test), data are presented as means ± SE. The level of significance was $P < .05$. (SPSS version 22)

Results and Discussion:

Results obtained from this study indicated to significant increase in total frequency of MN ; binucleated MN and NDI in patients with PCOS Table-2 and Figure-3. Micronuclei are either whole chromosome or chromosomal fragments, and, hence, increased number of MN reflects clastogenic and/or aneugenic effects in cells. In recent study suggested about both structural and numerical aberration increased in lymphocytes of PCOS patients [22].

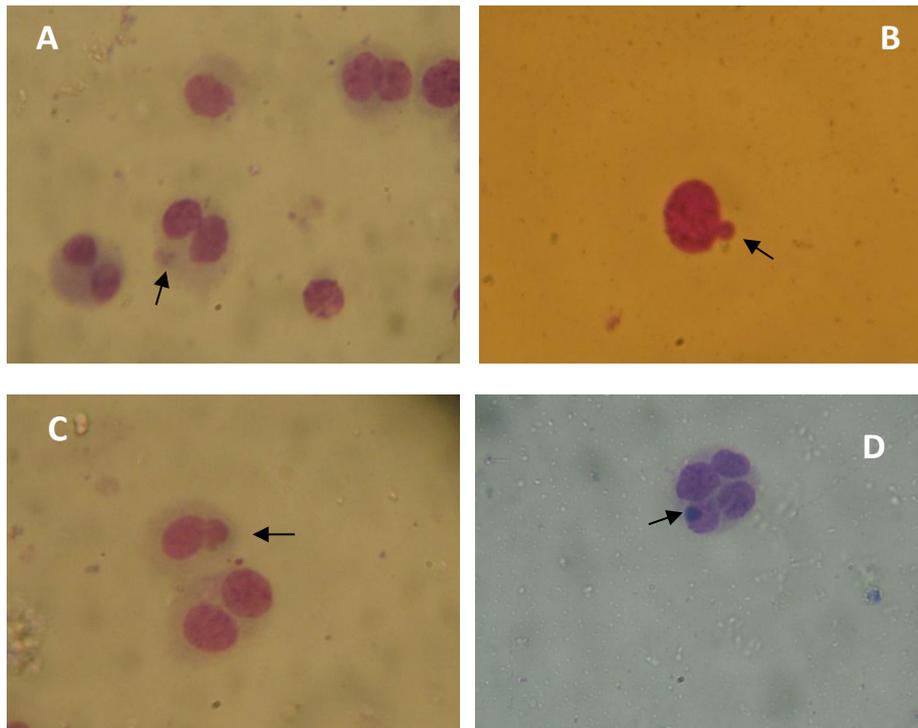


Figure 3- MN in leukocytes of PCOS patients and healthy women

Table 2- Frequency of MN and NDI in leukocytes of PCOS patients and healthy women

Groups	Mean \pm SD			
	Cells with MN	Total number of MN	Binucleated cells	Nuclear division index NDI
PCOS	A 3.27 \pm 0.56	A 4.65 \pm 0.92	A 7.79 \pm 2.06	A 3.05 \pm 0.43
Control	B 1.23 \pm 0.24	B 1.85 \pm 0.41	B 5.25 \pm 1.84	B 1.38 \pm 0.19

This results agreement with some investigators about increased cytogenetic damage of somatic cells in PCOS patients (MN and chromosomal aberrations in lymphocytes) [23,24]. Genetic instability may be having very dangerous consequences for patients with PCOS due to correlation of elevated levels of MN and chromosomal aberration with incidence of cancer [25]. Many studies suggested that conflicting results were published concerning a relation of PCOS and cancer, more scrupulous investigation in this area is certainly warranted [1,3].

Results showed highly score mean comet % of lymphocyte in women with PCOS compared with control group, include: No damage (ND) % , Low damage (LD)% , Medium damage (MD) % and High damage (HD) % as shown in Figures-1,-2.

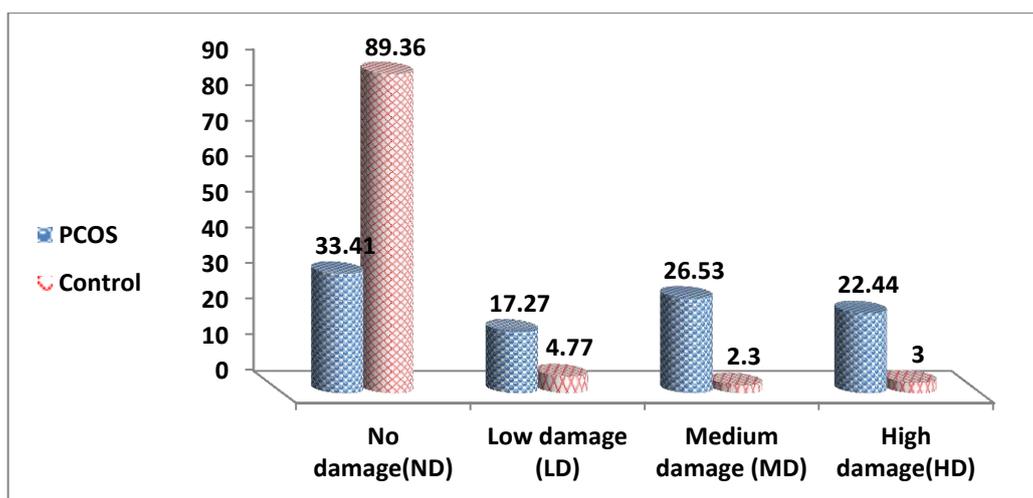


Figure 1- Score mean comet % of lymphocyte in males with RSA and control

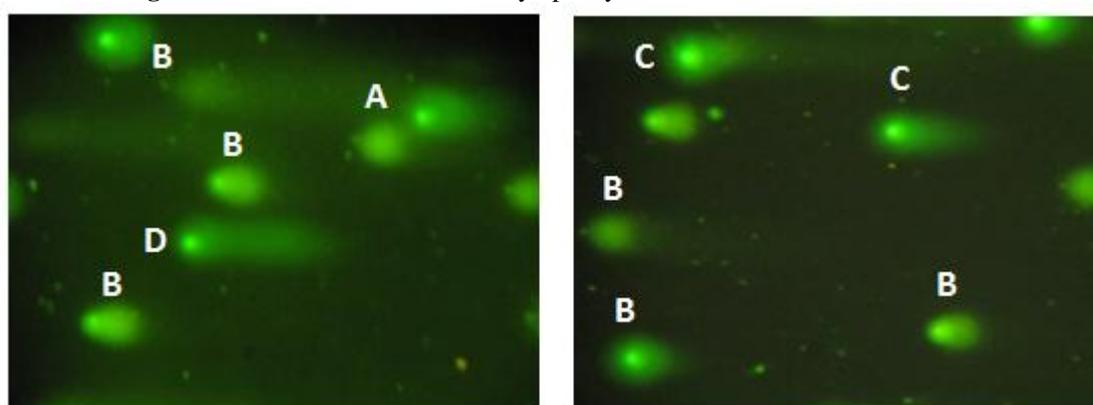


Figure 2- The examples of scoring categories for comet assay (A: Normal; B: Low DNA Damage (LD); C: Medium DNA Damage (MD); D: High DNA Damage (H)) in infertile patients cells.

Results show significant increase in comet assay percentage in women with PCOS compared with control group tail length (8.33 ± 1.16 ; 0.945 ± 0.037), tail DNA% (37.71 ± 5.81 ; 3.61 ± 0.48) and tail mean moment (2.97 ± 0.52 ; 0.075 ± 0.0061) respectively. Table- 1.

Table 1- Percentage frequency of DNA damages in leukocytes of PCOS patients and healthy women using comet assay.

Groups	Mean \pm SD		
	Tail length(px)	Tail DNA (%)	Tail mean moment
PCOS	A 8.33 ± 1.16	A 37.71 ± 5.81	A 2.97 ± 0.52
Control	B 0.945 ± 0.037	B 3.61 ± 0.48	B 0.075 ± 0.0061

Alkaline comet assay allows the detection of DNA damage such as single and double-strand breaks in single cell after acute and/or chronic exposure to a genotoxic agent [20]. Generate or elevated reactive oxygen species (ROS), in PCOS patients due to exposure to a genotoxic agent or another oxidative stress which can cause increase DNA damage [21].

In conclusion PCOS patients have genetic instability with increase in MN formation and elevated level of comet score compared with normal women may be due to exposure to oxidative stress with PCOS which lead to increase DNA damage.

References:

1. March, W.A., Moore, V.M. and Willson, K.J. **2010**. The prevalence of polycystic ovary syndrome in a community sample assessed under contrasting diagnostic criteria. *Hum Reprod*, 25, pp:544-551.
2. Azziz, R., Carmina, E., Dewailly, D. and Diamanti-Kandarakis, E. **2006**. Position statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. *J Clin Endocrinol Metab*, 91, pp:4237-4245.
3. Manikkumar, R. Dinesh Roy, D., Viji Krishnan, T. and Vijayakumar, V. **2013**. Association of DNA damage and dyslipidemia with polycystic ovarian syndrome. *J Med Allied Sci*, 3 (1), pp:15-21
4. Jahan, P., Krishna, K.V. and Rao, V.V. **2009**. The effects of industrial pollution on human genome. *J. Genet. Evol.*, 2, pp: 14-30.
5. Sabuncu, T., Vural, H., Harma, M. and Harma, M. **2001**. Oxidative stress in polycystic ovary syndrome and its contribution to the risk of cardiovascular disease. *Clin. Biochem.*, 34: 407-413.
6. Carmina, E. **2009**. Cardiovascular risk and events in polycystic ovary syndrome. *Climateric*, 12 (Suppl 1), pp:22–25.
7. Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. and Sasaki, Y.F. **2000**. Single cell gel/Comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ Mol Mutagen* 35, pp:206-221.
8. Hartmann A, Plappert U, Poetter F and Suter, W. **2003**. Comparative study with the alkaline Comet assay and the chromosome aberration test. *Mutat Res*, 536, pp:27-38.
9. Venturi, S. **2001**. Is there a role for iodine in breast diseases? *Breast*, 10, pp:379-382.
10. Deepika, M.L.N. , Nalini, S., Maruthi, G. Ranjith, K and Jahan, P. **2014**. Analysis of oxidative stress through MN test and serum MDA levels in PCOS women. *Pakistan Journal of Biology Sciences*, 17(5), pp:574-577.
11. Sahar, M., Hossein, M. and Ashraf, A. **2012**. Increased frequency of micronuclei in lymphocytes of infertile males after exposure to gamma irradiation: a possible sign of genomic instability. *J Assist Reprod Genet*, 29, pp:89–94.
12. Fenech, M. and Morley, A. **1985**. Measurement of micronuclei in lymphocytes. *Mutat. Res.*, 147, pp:29-36.
13. Lamberti, L., Ponzetto, P. B. and Ardito, G. **1983**. Cell kinetics and sister chromatid exchange frequency in human lymphocytes. *Mutation Res.*, 120, pp:193-199.
14. Boyum, A. **1968**. Separation of lymphocyte from blood and bone marrow. *Scand .J.clin .lab.invent* .21(suppl .97), pp:77-89.
15. Porakishvili, V., Kardava, L., Jewell, A., Yang, K., Glennie, M., Akbar, A. and Lydyard, P. **2004**. Cytotoxic CD4 T cells in patients with B cell chronic lymphocytic leukemia kill via perforin mediated pathway. *Haematological*. 89, pp: 435-443.
16. Collins, A.R., Harrington, V., Drew, J. and Melvin, R. **2003**. Nutritional modulation of DNA repairs in a human intervention study. *Carcinogenesis*. 24, pp: 511-515.
17. Al-Jewari, H.S.J. **2010**. *In vitro* Cytotoxic Activity of the L-asparaginase Extracted and Purified from Pathogenic *Escherichia coli* against four Leukemic cell lines. Ph.D. Thesis, Genetic Engineering Biotechnology for Post Graduate Studies, University of Baghdad, Iraq.
18. Gontijio, A., Elias, F., Salvadori, D., Oliveira, M., Correa, L. and Goldberg, J. **2001**. Single cell gel comet assay detects primary DNA damage in nonneoplastic urothelial cells of smokers and Ex smokers. *Cancer Epidemiology biomarkers and prevention*, 10, pp:987-993.
19. Azqueta, A., Shaposhnikov, A. and Collins, A. **2009**. Mutation research genetic toxicology and environmental mutagenesis. *Mutation Research.*, 674, pp:101-108.
20. Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. and Sasaki, Y.F. **2000**. Single cell gel/Comet Assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.*, 35, pp: 206-221.
21. Gewirtz, D.A. **1999**. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol*, 57, pp:727–741.

22. Nersesyan, A., Martirosyan, A., Parsadanyan, G. and Zalinyan, G. **2006**. Chromosomal aberrations level in peripheral blood lymphocytes of women with polycystic ovary syndrome. *J BUON*, 11, pp:477-480.
23. Yesilada E, Sahin I, Ozcan H, Yildirim IH, Yologlu S, Taskapan C.**2006**. Increased micronucleus frequencies in peripheral blood lymphocytes in women with polycystic ovary syndrome. *Eur J Endocrinol*, 154, pp: 563-598.
24. Moran, L.J., Noakes, M., Clifton, P.M., Norman, R.J. and Fenech, M.F. **2008**. Genome instability is increased in lymphocytes of women with polycystic ovary syndrome and is correlated with insulin resistance. *Mutat Res*, 639, pp: 55-63.
25. Daniilidis, A. and Dinas, K. **2009**. Long term health consequences of polycystic ovarian syndrome: a review analysis. *Hippokratia*, 13, pp: 90-92.