Detection of mouse spermatocyte's DNA damage in vitro and in vivo using FISH assay

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Abstract

The present study was designed in order to detection DNA damage of spermatocytes in vitro and in vivo in white mice, with explain florescence in situ hybridization FISH assay. The study conducting on seventy (70) male mouse were kept in animals' house in college of Veterinary Medicine in Baghdad University and fed on special pellet and drank on tap water in special bottles, divided into three groups. 1st group; Thirty mice were treated (in vivo) by intra-peritoneal injection with 0.1mg/10gm B.W. of vincristine sulfate weekly for three weeks. 2nd group; Sperms were collected from thirty mice and treated (in vitro) weekly for three weeks with 0.01mg /ml of vincristine sulfate. 3rd group; ten mice treated with intra-peritoneal injection of 0.1ml of distilled water and consider as control group. Results were showed evident of DNA damage in TK (11qE2)/Y gene of spermatocytes, which diagnosed by using florescence microscopy after application of FISH procedure in laboratory. The percentage of green signals and red signals indicated the defect in DNA of spermatocytes, increase percent of each refers to increase damage in chromosomes of sperms. In conclusion, using of vincristine chemotherapy has genotoxic effects on mouse's' sperms in vivo and in vitro.

Key words: FISH assay, mouse, spermatocyte's DNA damage, vincristine sulfate.

الكشف عن تلف الحامض النووي منقوص الاوكسجين في نطف الفأر باستعمال فحص التهجين الموضعي داخل الجسم وفي الانابيب

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الخلاصة

صممت الدراسة الحالية لأجل الكشف عن تلف الحامض النووي منقوص الاوكسجين (الدنا) في النطف داخل وخارج الجسم في الفئران البيضاء ، مع شرح تقنية التهجين الموضعي. اشتملت الدراسة على سبعين فأر، حفظت في البيت الحيواني في كلية الطب البيطري جامعة بغداد وغذيت على بلت خاص وشربت ماء حنفية في قناني خاصة، وقسمت الى ثلاث مجاميع ؛المجموعة الاولى (30 فأر) عولجت بالحقن داخل البريتون بالفنكرستين (0.1 ملغم/ 10 غم من وزن الجسم) (داخل الجسم) اسبوعيا لمدة ثلاث اسابيع ، والمجموعة الثانية (30 فأر) اخذت منها الحيامن وعولجت الحيامن بالفنكرستين (0.01 ملغم/مل) (في الأنابيب) اسبوعيا لمدة ثلاث اسابيع ، والمجموعة الثالثة (10 فأر) عولجت بالماء المقطر(0.1 مل) بالحقن بالبريتون واعتبرت مجموعة سيطرة. أظهرت النتائج وجود تلف الدنا في الجين TK (11qE2)/Y للنطف ، شخص هذ التلف باستعمال مجهر التنوير بعد تطبيق طريقة عمل التهجين الموضعي في المختبر. النسبة المئوية المحسوبة للعلامة الخضراء والعلامة الحمراء اشارت الى تلف دنا النطف في النطف ، وزيادة في نسبتها تشير الى زيادة التلف في كروموسومات النطف. نستنتج من الدراسة ان عقار الفنكرستين له تأثير سام للجينات في الحامض النووي منقوص الاوكسجين لنطف الفأر داخل الجسم وفي الأنابيب.

الكلمات المفتاحية: فحص التهجين الموضعي ، فأر، الضرر في الحامض النووي دنا للنطف ، فنكرستين.

Introduction

Florescence in situ hybridization FISH is a test that maps genetic material in cells used to detection the chromosomal damage directly in spermatocytes, a new applications

for mouse molecular cytogenetic are emerging including the definition of transgenic integration sites in epigenetic studies (1). Detection of nucleotide sequences on examined DNA molecule consists of hybridizing DNA probe to its complementary sequence on chromosomal preparation. Hybridization is formation of a duplex between the complementary (single stranded) sequences of nucleic acid. The nucleic acids required for these applications must be recovered from variety of biological samples including; blood, body fluids, fresh and paraffin embedded tissues. Probes and targets are finally visualized in situ by microscopy analysis. FISH is a new application, for mouse molecular cytogenetic are emerging including the definition of transgenic integration sites in epigenetic studies (2). Also it's used in genetic toxicology in detection numerical and structural chromosomal abnormalities of sperm in expanding number of species, including humans and rodents during gametogenesis or during fertilization, birth development, early aborted fetus, so to identifying several potential paternal risk factors such as age, drugs, life style, environmental and occupational exposure (3,4). Its' also used to assess the sperm of male recovering from genotoxic treatment as in cases of prior repeated in vitro fertilization intracytoplasmic sperm injection ISI failure or recurrent pregnancy loss (5). Hybrids formed between the probes and their chromosomal targets can be detected using a fluorescent microscope, the probe sequence often a piece of cloned DNA is shown in red and the target DNA chromosomes on a glass slide shown in green (6,7). Many chemical drugs which are plants' origin such as vincristine have genotoxic effect on germ cells mainly spermatocytes represented by DNA damage that's interfere with reproduction, and reported to cause many degenerative changes in different organs (8,9). Aims of current study: in order to application a new molecular assay in Veterinary Medicine at first time in Iraq, for detection DNA defects in mouse s' spermato-cytes in vitro and in vivo.

Materials and methods

1-Animals: Seventy (70) mice were used, subdivided to 3rd subgroups as following: 1st Group: Thirty (30) mice were treated (in vivo) weekly for three week with intra-peritoneal injection of 0.1mg/10gm B.W. of vincristine sulfate (8). 2nd Group: Sperms which collected from thirty (30) mice were treated (in vitro) with 0.01mg /ml vincristine sulfate, weekly for three week (10). 3rd Group: Ten mice were treated with intra-peritoneal injection of 0.1ml of distilled water and consider as control group.

2-Drugs: 1-Vincristine Sulfate (VCR) 1mg / Vials, (chemotherapy drug). 2-Halothane (2%): inhalation anesthetic drug.

3-preperation samples: Animals were sacrificed after giving inhalation anesthesia; spermatocytes were collected from 1st and 3rd groups at the end of experiment. Animals submitted to removing of each epididymis and minced with scissors to release the spermatozoa, these were suspended in volume of 1ml (0.9%Nacl), while spermatocytes were collected from 2nd group before experiment, by removing of each epididymis minced with scissors to release

Table (1): The special kits for molecular examination of DNA damage of mouse sperm.

Kits Name	Component	Origin
1-Mouse FISH DNA probe	Test Format Rtu: 100(µl) Of Probe. Store at 2-8C°	KRETECH Diagnostics
2-Tissue Digestion Kit IIKBi-60004	1-LK-104B 2x ssc sol. 2- LK-102B wash buffer I 3-LK-103B Wash buffer II 4-LK-100B pretreat -ment sol.B 5-LK-099B 0.2MHC 6-LK-101B pepsin sol. 7-LK-096B DAPI counter stain (1µ/ml) 8-LK.097B counter stain diluent. Store At 2-8 C	KRETECH Diagnostics

Distributed by KREATECH Amsterdam, NL

the spermatozoa, these were suspended in volume of 1ml (0.9%Nacl) put in sterile test tube and incubated at 37C° with Vincristine sulfate (0.01mg/ml) for three weeks at one dose weekly. After each exposure period, one drop from each sample put on charge slide and allows drying. (10, 11).

4-Special kit: Special kits for sperm mouse were used shown in table (1).

5-Diagnosis: When FISH Procedure was done on each three group's samples according to instructions of manufacturing protocols (KREATECH), slides were examined under Fluorescence Microscopy in dark, through reading two signals, red and green. The number of both signals reflected the size of the damage. Scoring was done by counting the number of red and green signals in 10 high power fields (oil immersion X1000) under florescence microscopy (8, 11, and 12).

6-Statistical analysis

Calculating the percentage of each (red and green signals) was assigned to one of the following scores: score1 (0-5%) score2 (5-20%), score3 (20-40%) score 4(40-95) score 5(<95%) in each animal (13). Statistical analysis depends on evidence of significant differences from the corresponding control assessed by the X^2 test: at $p < 0.05$. (14).

Results

Microscopic examination:

Green and red signals were indicated the defects on the Y chromosome of TK(11qE2) site in the spermatocytes and were seen under the florescence microscope as showed in figure (1-A, B,C) which are refer to the amount of damage on DNA of sperms in vivo and in vitro.

FISH analysis:

The signals (red and green) were calculated in different scores, in each sample as shown in table (2 and 3).

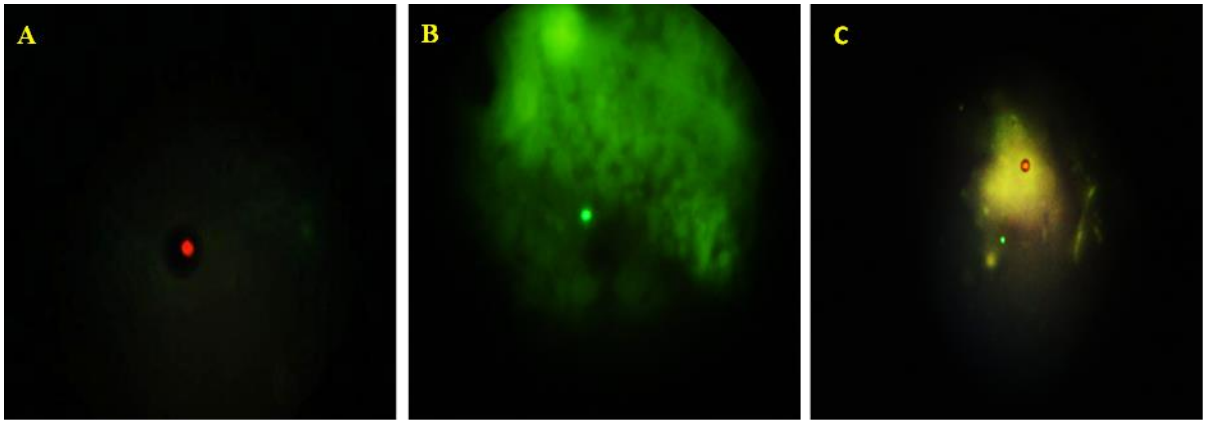

Fig. (1): Microscopic appearances of calculating signals under florescence microscope after application of FISH assay. A: Red signal of control samples, B: Green signal in 2nd group samples (in vitro). C: Red and green signals in 1stgroup samples (in vivo) (X400).

Table (2): Results of TK (11qE2)/Y DNA mouse probe applied by FISH assay on seminal fluid samples of 1st group(in vivo).

1st group	Percentage of spermatocytes with particular level of DNA damage				
n=10	Score 1 (0-5%)	Score 2 (5-20%)	Score 3 (20-40%)	Score 4 (40- 95 %)	Score 5 (< 95%)
1st wk.	5	4	1	0	0 *
2nd wk.	0	2	3	3	2 *
3rd wk.	0	0	4	1	5 *
Control	10	0	0	0	0 *

Table (3): Results of TK (11qE2)/Y DNA mouse probe applied by FISH assay on epididymis samples of 2nd group (in vitro).

2nd group	Percentage of spermatocytes with particular level of DNA damage.				
n=10	Score 1 (0-5%)	Score 2 (5-20%)	Score 3 (20-40%)	Score 4 (40- 95 %)	Score 5 (< 95%)
1st wk.	3	2	2	2	2 *
2nd wk.	0	3	3	1	3 *
3rd wk.	0	0	2	4	4 *
Control	10	0	0	0	0 *

Significantly different from the corresponding control assessed by the X^2 test: * $p < 0.05$.
Scour1(0-5%);Scour 2(6-20%);Scour 3(20-40%);Scour 4(40- 95 %);Scour 5(<95%).

Discussion

Red and green signals indicated the amount of damage in spermatocytes' DNA, increase the number of signals refers to severe defect in chromosomes. This result is agreed with (8).Vincristine injection intra-peritoneal in mouse for several weeks' results in spermatocytes defects' DNA as founds by author (8,13) in vivo. Vincristine causes genotoxic effects on spermatocytes' DNA in vitro due to its capacity to produce damage in genetic material so its affects spermato-genesis during meiotic division and results in marked damage in DNA. That's agreed with (10). The results points that, there are serious spermatocytes DNA defect after exposure to vincristine in vivo and in vitro. This defect is detecting by application of FISH assay at the first time in Iraqi Veterinary Medicine (8), and agreed with the fact that's many chemical drugs offers' their effects in vitro as well as in vivo (15). Other studies on drugs have been linked to gonad and caused direct damage to spermatocytes as well as gametes at lower doses in male than in female (16,17). Male gametes are particularly susceptible to damage by chemical and physical agents (18, 19). Majority of chemical drugs acts on cell cycle phase in order to prevents cancer cells from divided and metastasis but can produce the same effects on healthy cell such as lymphocytes, erythrocytes, spermatocytes, hair follicles, epithelial ling GIT cells and others rapidly dividing cells (20), and agreed with (21) who demonstrated that chemo-therapy are more likely to cause gonad damage by creating an artificial state of spermatogenetic quiescence within the testes to suppress the toxic effects of chemical drugs. Authors (22, and 23) hypotheses' that the gonad toxicity of cancer treatment led to significant increases in testosterone and FSH levels resulting in inhibition of spermatocytes differentiation. An similar experimental study of Vincristine effects' in vivo and in vitro in mice reveals a deterioration in testicular weight and spermatocytes count with defects in spermatocytes morphology due to its' DNA defects (13, 24, and 25), (26, and 27) say that exposure of Spermatogonia and spermatozoa to chemotherapy caused a reduction in their ability to fertilize ova caused by extensive damage to Spermato-gonia. Also agreed with similar study in vivo (28, 29).

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