Cytogenetic Studies on Goat Blood Lymphocytes: Cell Cycling

دراسات خلوية وراثية على خلايا دم الماعز اللمفاوية: دورة الخلية

E. K. Shubber , Z. MT Jaffer , A. Abdul-Kareem , M. I. AL-Tememi.
Ministry of Science and Technology
* Biology Dept./ College of Science for Women/ University of Baghdad
**Biotechnolgy Research Center/Al- Nahrain University

اسماعيل كاظم شبر ، زينب محمد طاهر ، اروى عبدالكريم * ، مجيد ارشيد التميمي**
وزارة العلوم والتكنلوجيا
* كلية العلوم للبنات / جامعة بغداد
**مركز بحوث التقنيات الاحيائية/جامعة النهرين

Abstract:
Peripheral blood lymphocytes from goats (local breed) were cultivated in RPMI-1640 medium containing 15µg/ml of BudR 20 µg/ml of PHA for different times (12, 24, 36, 48, 60, 72 and 96) hrs. to determination the cell cycle duration. Blastogenesis was appeared post first 12hr of cultivation followed by first mitoses post 24 hrs. of culture initiation. The second and third cell cycling lasted 22 and 21 hrs, respectively. Effects of 6-thioguanine, methotrexate , colchicine and tamoxifen on cell cycle progression were investigated. Goat cells were found to be resistant to tamoxifen and MTX and sensitive to 6 TG and colchicine, which could be use as genetic markers to chick cellular genome integrity. Priming of goat blood lymphocytes was achieved by treating the blood with PHA for 24hr. Such treatment increased the *in vitro* growing period of derived lymphoblasts with short cycling time. However, PHA was found to be a promoting factor for initiation of blastogenesis and cell divisions in goat blood lymphoblasts. These techniques: Genetic markers, cytogenetic analysis cell cycling and lymphoblast explantation are crucial processes for nuclear transplantation processes.

المستخلص :
زرعت خلايا الدم اللمفاوية المعزولة من الماعز المحلي، في الوسط الزرعيRPMI-1640 الحاوي على 15 مايكروغرام/مل من مادة الـ BudR و20 مايكروغرام/مل من الـ PHA وقد تمت تنمية الزروعات على فترات مختلفة 12 ، 24 ، 36 ، 48 ، 60 ، 72 و96 ساعة لقياس فترة دورة الخلية. ظهرت الخلايا في طور الاورمة بعد 12 ساعة من فترة بداية الحضن بينما ظهر اول انقسام خلوي في الساعة 24 ، وكانت الفترة للدورة الثانية والثالثة

تتراوح ما بين 22 و21 ساعة على التوالي . تمت دراسة تأثير المركبات الكيميائية التالية : 6-ثايوكوانين ، الميثوتركسيت ، الكولجسين والتاموكسيفين المعروفة بتأثيرها التثبيطي لدورة الخلية . أظهرت بعض من خلايا الماعز مقاومة للميثوتركسيت والتاموكسفين بينما كانت تلك الخلايا حساسة للثايوكوانين والكولجسين . لذا يمكن استخدام هذه كمؤشرات الوراثية لكشف عن سلامة المحتوى الوراثي لخلايا الحيوان . استخدمت طريقة المعاملة بمادة الـPHA لمدة 24 ساعة لاستزراع الخلايا اللمفية المولدة من دم الماعز، حيث استمرت تلك الخلايا بالنمو والانقسام لمدة 45 يوماً، وكانت مادة الPHA مادة محددة لتحفيز الخلايا على التكاثر والانقسام ، او التوقف في طور ما قبل الاورمة (G0) . تعتبر تقنيات التحليلات الخلوية الوراثية ، استزراع خلايا اللمفاوية المولدة من الدم استخدام المؤشرات الوراثية واحتساب دورة الخلية الاسس الرئيسية في عملية نقل الانوية والكلونة .

Introduction:

Mammalian cell cycle is defined as the ordered sequence of events by which cells grow, copy their DNA and divide, which ensures that each new cell is born with a full set of genetic instructions in somatic cells or with half the genetic content of their progenitors as in sperms and eggs [1]. The phases of the cycle each have an abbreviated name. G1 (Gap) is the period immediately after cell division; S phase is the next stage, during which the cell synthesis a copy of its DNA. The gap between the end of S. phase and the onset of mitoses is known as G□. Cells may also leave the cycle all together and spend long periods without dividing stands Go. The entire cycle, from one division to the next in mammals takes anything from 10-24hrs. [2, 3].

For the cloning biologists, the cell cycle had to remain just an uncomfortable awareness. At the time no biologist understood detail how the cell cycle worked, or how it control it- and still less why the phase of the cell cycle would affect the outcome of nuclear transfer [4]. The cell cycle is complicated and esoteric. It requires full-time dedication. The science and technology of cloning need input from a different branch of biology to study cell cycles [5]. It was postulated that the fate of transferred karyoplasts depends very much on the maturation promoting factor (MPF) content of the cytoplasts. If the nuclei are diploid-meaning in G1 or G0, they can be then safely transferred whether the MPF content of the receiving cytoplast is high or low. But if they are not diploid if they are in S or G□, then they cannot be transferred safely into high MPF environment [6]. They can be transferred into low MPF environments without chromosomal damage although.

In this work, we attempted to study goat cell cycling in order to reach the point of a proper stage for nuclear transfer using blood lymphocyte as a model.

Materials and Methods:-

Goats: Ten local breed goats (AL-Shami and Arabi: 8 females and 2 males) from the animal unit at the center were included in this investigation. All animals were healthy at the time of testing.

Cytogenetic analysis:

Blood lymphocytes were cultured in RPMI-1640 medium for blastogenesis, cell divisions and replication under optimum conditions. [7] , [8] , [9] . Two cultures were initiated from the blood samples of each animal. Briefly, 0.5ml of heparinized blood was inoculated in 4.5ml medium containing 10% foetal calf serum (Gibco, USA) , 0.2ml (20µg/ml) of PHA (IAEC, Iraq), and 15µg/ml of BudR. The cultures were kept in the dark at 37°c for 68 hrs. Colchicine (Sigma) was added at concentration of 10µg/ml for 3hr. before harvesting. The

cells were treated with 0.075M KCL, fixed with methanol: acetic acid (3:1) and spread on clean-wet slides. The blood lymphocyte transformation which expressed as blastogenesis was counted as number of blasts per 1000 cultivated cells (BI). The mitotic activity that expressed as mitotic index (MI) was counted as percentage of cells at mitoses per 1000 cultured cells (i.e. interphase nuclei). For each animal 10-15 well spread metaphase were examined for chromosome counting.

Identical slides from each culture for the same animal were stained with DAPI -stain [10] for counting the percentage of the cells at 1st , 2nd or 3rd metaphases for counting the replicative index (RI) [11].

$$RI = \frac{1\times \%M1 + 2\times \%M2 + 3\times \%M3}{100}$$

Standardization of colchicine treatment:

A: Different concentration of colchicine (10, 20, 30, 40 µg/ml) (From Sigma Company) was added to blood cultures of 3 animals at last 3hrs. Of incubation period 72hrs. and slide were prepared and BI and MI were counted.

B: At a concentration of 10µg/ml of colchicine, the treatment times were either 2 or 3hrs. and both BI and MI were counted in slides which stained with Giemsa.

Cell Cycle progression:

Blood taken from the same animals that been examined before in the above experiment using colchicine at 10 µg/ml for last 3hr was used in this experiment. Starting at 12, 24, 36, 48, 60, 72 hrs. And 96 hrs. of incubation time BI, MI and RI were counted [12] . DNA/RNA ratio was determined after 24, 48 and 72 hr following method of [13].

Cell Cycle blocking:-

Three agents: Methotrexate, 6-thioguanine and tamoxifen which known to block cell cycling at different phases were used in this experiment. Different concentrations of each agent were added to cultures that initiated from the same animal independently. BI and MI were counted in 72 hrs. Cultures as described before [14, 15].

Explantation of lymphoblasts:

Priming with PHA: Blood samples from same animal were divided into 3 parts. 1st was inoculated as whole blood (0.5ml in 4.5 ml medium), 2nd and 3 rd parts treated with 0.1ml PHA and incubated for 3 and 24 hrs. after centrifugation at 500 rpm. 0.5 ml of each was added into 4.5ml medium with BudR to complete the incubation period for 72hrs. Preliminary results showed that priming the cells for 24hrs. gave encouraging data, thus this procedure was applied on 7 goat blood samples.

Results and Discussion

Arresting of goat blood lymphocytes at mitoses was achieved by using colchicine (Sigma) at a concentration of 10 µg/ml however, increase of the concentration up to 20, 30 or 40µg/ml showed a significant ($P<0.01$) reduction in the blastogeneseis and no mitoses could be observed in those cultures. These results might due to its inhibition for the microtubules formation [16].

Further, it might be indicated that goat lymphoblasts or lymphocytes are very sensitive to colchicine. This drug is acting on the disassembly of microtubuline of spindle fiber, and resistance to colchicine as reported by other investigators, that it belong to amplification of protein with polypeptide p 22 that controlled by a gene located on chromosome number 4 [17] .

Thus colchicine resistant could be added as a biomarker for detection of genome integrity. Using 10µg/ml of colchicine in other set of experiment for determination of the mitotic activity of blood cells as a function of time resulted in an increase in the number of mitoses for most tested animals reach 2-5times at 3hrs. than that at 2hrs. without affecting the blastogenesis (Table 1).

Table (1)*: Effect of colchicine treatment on goat blood lymphocyte division *in vitro*.

Sample No.	Incubation time (hr)	Blastogenic index (%)	Mitotic index (%)
1	□	24.0	2.0
	3	27.0	4.0
2	□	12.9	3.0
	3	12.2	8.0
3	□	8.8	1.0
	3	8.6	5.0

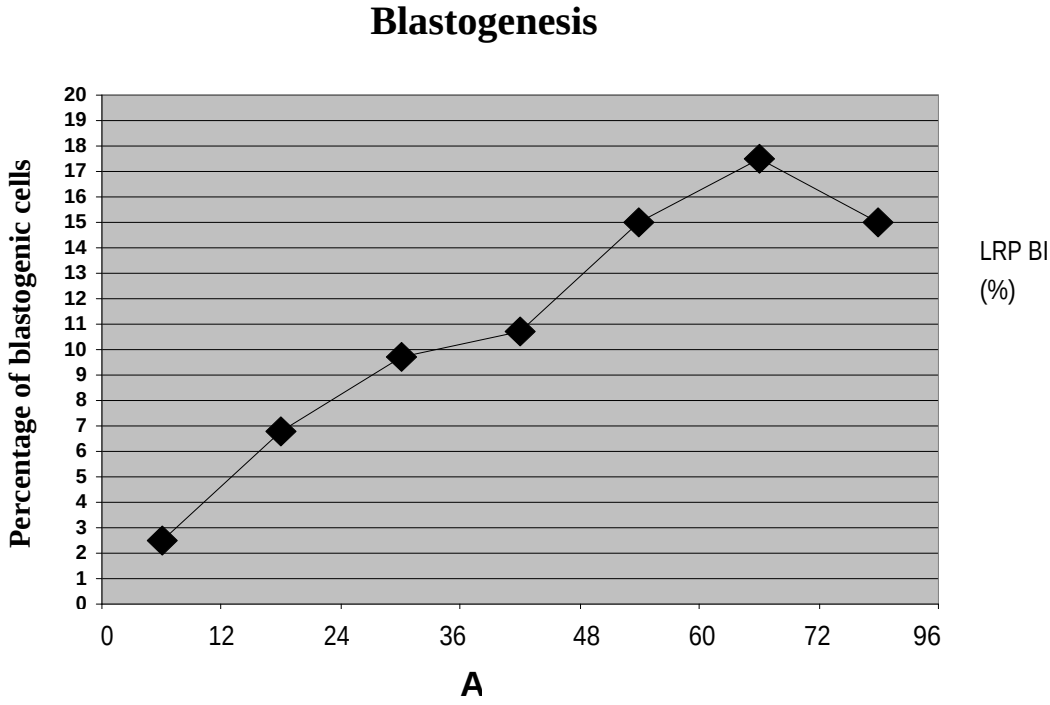
- **Using 10µg/ml of clochicine (Sigma).**

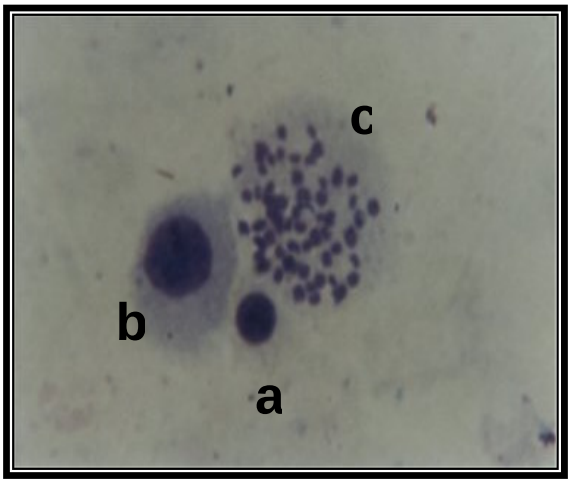
The colchicine concentration (10µg/ml) and selective time of treatment 3hrs. From one source of colchicine was followed for next experiments.

Cell Cycle duration:-

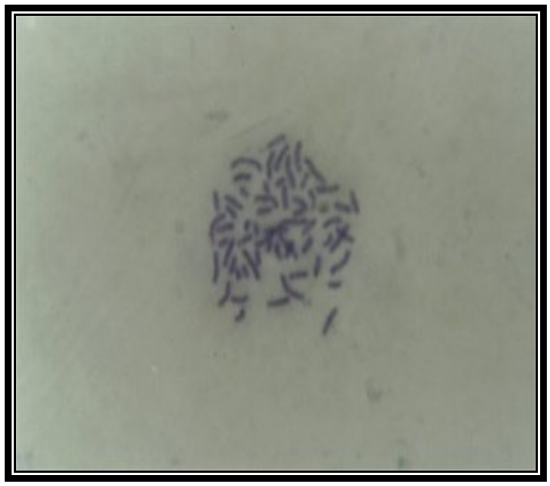
For determination of goat blood cell cycling, blood samples from 3 animals were grown in complete RPMI- medium containing 20µg/ml of PHA,15µg/ml of BudR 10µg/ml heat-inactivated fetal calf serum and antibiotics. Sampling of the cells was performed after (6, 12, 24, 36, 48, 60, 72 and 96) hrs.

Three hours treatment with colchicine was included with those times. As a function of incubation time, a blast-forming blood lymphocytes had started to appear with increased percentage after 12 hrs. of incubation up to 72 hrs. After which they showed a decline Fig.(1) .





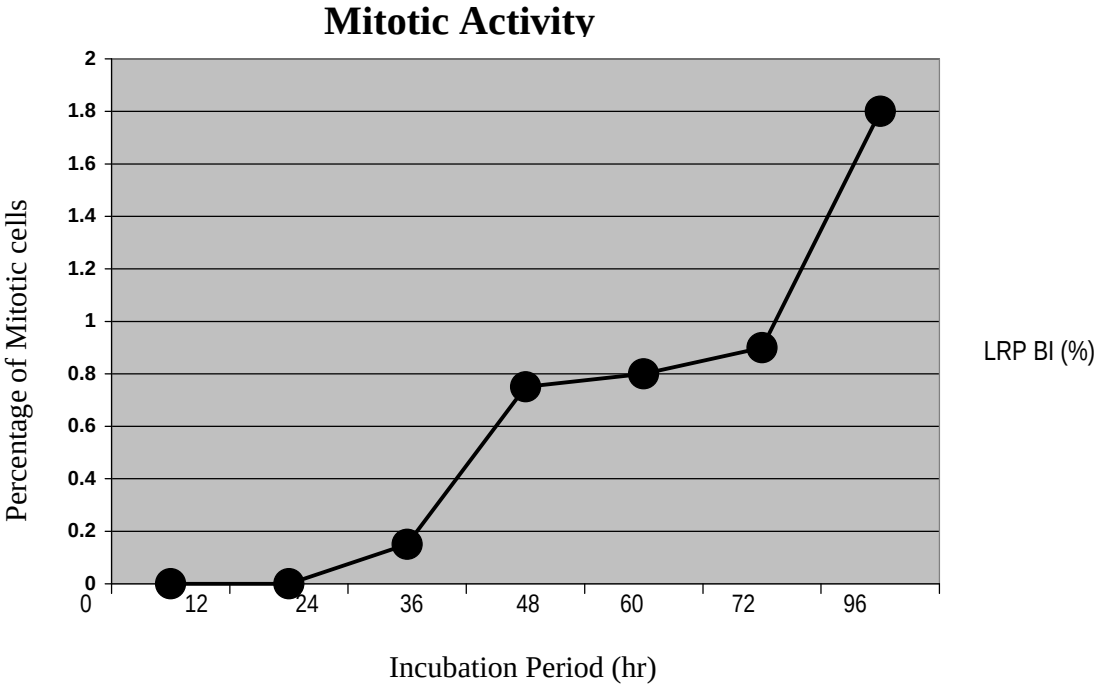
Goat Blood Cells
a) Lymphocytes. b) Lymphoblasts. c) Mitotic cells
B

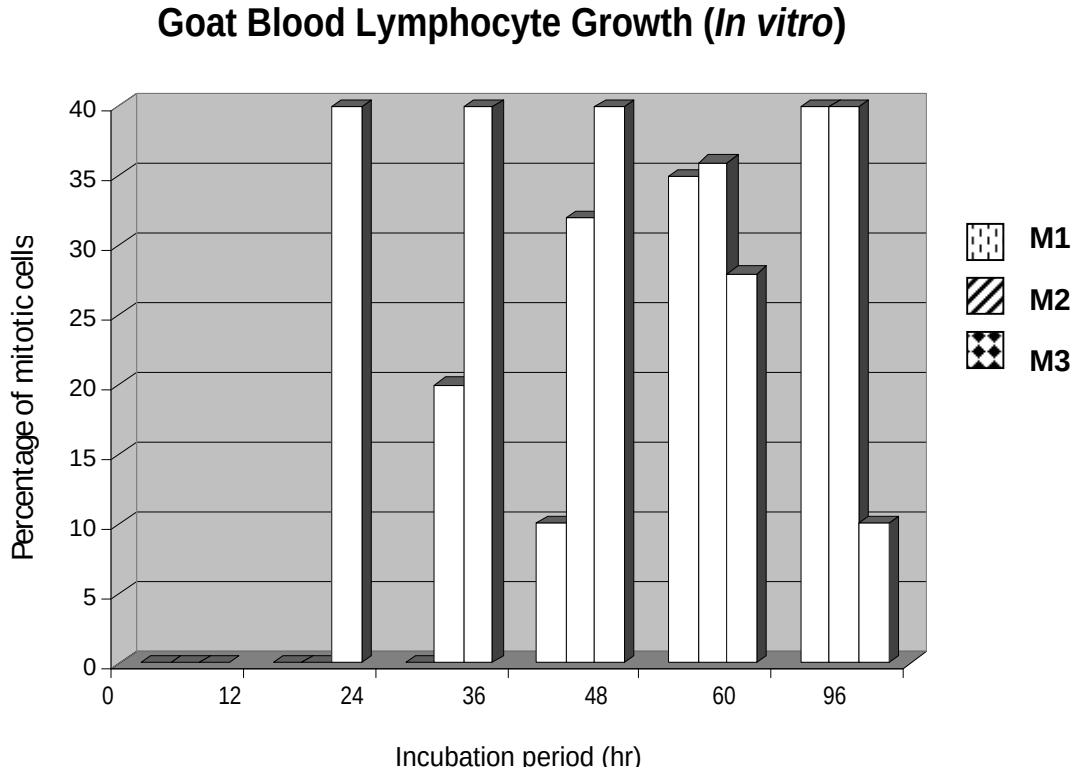


Goat Blood Lymphocyte with 60 Chromosomes
C

fig (1): Blastogenesis of goat blood lymphocytes

First cells at mitotic phase were shown post 22-24 hrs. (i.e. 10 hrs. post blastogenic cells appearance). The ratio of mitotic cells was increased as a function of incubation time. Second metaphase cells were released during 36-48 after 60hrs. of incubation .While 3rd metaphase cells started to appear. Thus the duration of goat lymphocyte cycling in vitro is ranging as 24hrs. for the first cell cycling as first cultivation , although the 2nd and 3rd cell cycling time could be estimated to be 22-21hrs.Fig. (2-3). However, cultural conditions such as type and concentration of PHA , colchicines , serum , medium , blood storage , labeling with BUdR techniques are playing crucial role (s) in the determination of cell cycling [18] , [19] [20] [16] .





Goat Blood Lymphocyte cycle Progression (*in vitro*)

Goat Blood Lymphocyte Priming

In order to examine the influence of PHA priming on cellular growth performance BI, MI as well as cell cycle progressions were counted in cultures of whole blood (W?) and Leukocyte rich plasma (LRP) of the same blood samples with equal (approximately) inoculated number of cells. The results are presented in table (2).

Table (2): Whole blood versus leukocyte rich plasma cultivation of goat lymphocytes *in vitro.*

Sample No.	Blasto Index		Mitotic Index		WB				Cell Cycle Progression LRP			
	WB	LRP	WB	LRP	M1	M2	M3	RI	M1	M2	M3	RI
1	21.75	30.2	2.5	1.01	60	32	8	1.48	40	30	30	1.90
□	33.80	29.5	1.0	0.47	39	27	34	1.95	25	20	55	2.30
3	15.30	23.0	0.80	0.55	64	24	12	1.44	20	30	50	2.30
4	16.56	23.3	1.20	0.90	31	23	46	215	20	30	50	2.30
5	26.10	37.7	1.26	0.99	58	22	20	1.62	14	72	14	200
6	29.20	30.6	1.35	1.20	31	31	38	1.97	15	46	29	1.94
7	25.00	36.3	1.55	0.78	31	37	32	2.01	33	35	32	1.89

Seven samples from 7 animals were included in this experiment. Five animals (71.5%) were exhibiting higher blastogenesis in LRP than in W? cultures, although, their cells had lower mitotic activity when they were primed. It is interesting to note that, the replcative activity of these LRP cells were significantly ($P<0.01$) higher than those in W? cells. These results may suggest that cellular components of erythrocytes in W? play an important role (s) for the proliferation of the lymphocytes such as cellular protein from RBC acting as stimulants for lymphocyte growth [16] , [20].

It is also suggested that long duration of PHA treatment might lead to activate many biochemical events, including early membrane- related phenomena inverse synthesis of phospholipids, increased permeability to divalent cations, RNA DNA and protein synthesis [21].

Lymphocytes were the major cell constituent of leukocyte rich plasma and the increasing of their priming by elongation of duration of PHA treatment from 3 to 24hr cause a cerement of their culture duration from 15 up to 45 days *in vitro*. When they were cultivated in presence of PHA, however, growing of the cells in PHA- deficient medium caused a reduction in their blastogenesis and mitotic activity (i.e. render them at G0-G1 phase. Similar observations were seen with human blood lymphocytes [22], suggested that PHA playing crucial roles for promoting lymphoblast cycling under these experimental conditions.

Thus priming of blood lymphocytes could reach after 24hrs. treatment with PHA in whole blood at 37°c followed by cultivation as lymphoblasts in complete culture medium and re-incubation for another 48h to complete 72h culture. These cells, however, were PHA dependent. i.e. presence of PHA was conditional for their continuous growth. Although,

withdrawal of PHA from the medium render the cells at G1 with no blastogenesis or mitotic activity which is being required at this phase for nuclear transfer techniques [23].

Cell Cycle Controls:

There are two major factors are controlling the cell cycling in general. Intrinsic factors, which regulate cell movement from one phase into other such as cyclins and cyclin - dependent kinase (CDKs) which they are directly involved in cell cycle regulation and must be periodically expressed at appropriate time [24]. Other factors are extrinsic regulators, either promoter such as PHA, or metaphase promoting factor [16],[18],[25] or blocked the cell cycling such as anticarcingenic drugs antimetabolites: methotrexate (MTX) and 6-thiognanine (6TG) [26] . Tamoxifen as another anticancer antisteroidal drug blocks cell cycling between G2/M phases [15]. Those promoting or blocking agents should be present in the culture medium to express their activity

Cell Cycle Controls:

To investigate the sensitivity of measuring goat blood cell cycling *in vitro* , cell cycling promoting agents PHA , and blocking agents such as methotrexate , 6- thioguanine and tamoxifen were tested in vitro in 72 hrs.cultures initiated from 3-4 animals each (Table 3).

Table (3): Sensitivity of goat blood cells to cell cycle Inhibitors.

Concentration (µg/ml)	Blastogenic index (%)	Mitotic index (%)	Number of resistant cells 1000 cells
Methotrexate			
1. 0	14.5	0.7	-
0.5	12.0	0.6	6
1.0	11.4	0.4	4
5.0	17.8	0.5	5
10.0	8.4	0.4	4
20.0	1.0	0.0	0
2. 0	18.8	1.8	-
0.5	6.3	1.3	13
1.0	1.7	1.2	12
5.0	1.2	0.0	0
10.0	0.8	0.0	0
3. 0.0	21.0	3.0	-
0.5	12.0	0.0	0
1.0	9.8	0.0	0
5.0	10.0	0.0	0
10.0	1.0	0.0	0
6- Thioguanine			
1 0.0	18.8	1.2	-
1.0	14.2	0.0	0
10.0	11.0	0.0	0
20.0	6.3	0.0	0
2 0.0	26.0	1.0	-
1.0	25.0	0.0	0
10.0	10.5	0.0	0
20.0	12.0	0.0	0
3 0	21.0	0.3	-
10.0	10.5	0.0	0
20.0	5.2	0.0	0
4 0	18.4	0.3	-

10.0	10.0	0.0	0
20.0	6.3	0.0	0
Tamoxifen			
1 0.0	20.8	1.5	-
0.5	33.3	0.8	8
1.0	28.0	0.6	6
5.0	20.3	0.5	5
10.0	18.9	0.2	2
2. 0.0	30.5	1.0	-
1.0	25.0	0.6	6
5.0	19.0	0.3	3
10.0	10.0	0.2	2
Colchicine			
10	24.0	0.2	
90	8.8	0.0	
30	3.3	0.0	
40	104		

1. Treatment with PHA:

Goat blood lymphocytes priming and growing of leukocytes in rich plasma or in blood and long-duration growing of lymphoblasts were solely dependent on PHA presence, data presented in (Table 2) and preceding section. However, no cell could grow under such conditions in PHA -deficient medium.

2. Treatment with 6-Thioguanine:

It was necessary to determine the concentration of 6 TG that perturbs the cell cycle progression for controlling cycling of the cells and selecting of mutant cells. Different concentrations 1,10 and 20 µg/ml of 6 TG were tested in blood lymphocytes isolated from 4 animals .Significant reduction of blastogenesis in dose-dependent pattern was observed ,with complete inhibition of cellular division (Table 3). 6 TG is acting as anti metabolites against guanine in DNA- synthetics pathway .This activity is solely dependent on presence of hypoxanthine Guanine phosphoribosyl transferase (HPRT) enzyme. Cells which were deficient in HPRT were resistant to 6TG [27]. Comparing to human lymphoblastiod cells [22] or blood lymphocytes [9] , goat blood cells were highly sensitive to 6 TG. Thus these cells have no mutational events at hprt locus.

3. Treatment with Methotrexate (MTX):

Three animals which were tested for 6 TG effects were included in this assay . MTX at concentration of 0.5,1,5,10, and 20 µg/ml were investigated . A concentration-dependent reduction in cellular blastogenesis and division was noticed. Blood cells from those three animals exhibit different response, some of them were resistant to MTX at concentration of 1-10µg/ml. Those resistances were estimated from the presence of cells at metaphase at toxic level of MTX [28] which was $5\text{-}10\times10^{-3}$ events/ generation /cell at 1.0 µg/ml (Table 3) . [29] [30].

This resistance to MTX is usually associated with mutation at dihydrofolate reductase DHFR loci. As might be expected from its action in blocking DNA synthesis, MTX is active primarily against cells in the S-phase of the cell cycle [31]. A mechanism of cell resistance to MTX might be resulted from poor ability into transport the drug into the cell

(Bertino *et al* 1985). Also, over production of DHFR enzyme is a marker of MTX-resistance cells which resulted from a mutational events at this loci that altering its affinity to MTX [32] ,[29].

4. Treatment with Tamoxifen (TAM):

Different concentration of TAM (0.5, 1.0, 5.0 and 10.0 µg/ml) were tested in their activity to block cell cycling. The results were dedicated in Table 3. Two goats, one was resistant to MTX and the other was sensitive to MTX, but both of them were sensitive to 6TG. TAM treatments showed a significant reduction in both BI and MI in concentration-dependent patterns although; there were TAM-resistant cells at toxic concentration of 10µg/ml [15] with level of 2×10^{-3} events/ generation /cell. TAM as an anti-estrogen acts as anti transcription agent which influencing cellular protein production leading to blocking G2/M cell passing [33]. This resistance phenomenon might add another biomarker to be use for checking the genome integrity of somatic cells before initiation of nuclear transfer techniques. Conventional cell cycle analysis is still highly informative field [34] based on the analysis of chromosome differential staining from dividing cells by modifying the technique making it more accurate for cell analysis that help in the differential response of the cells to drugs.

References

1. Young, S. (1992). Dangerous dance of the dividing cells New Scientists .51:23-25.
2. Smith, J.A. and Martin, L. (1973). Do cell cycle? Proc. Natl.Acad.Sci.70:1263-1267. Of Oncological and Palliative Care. In "Medicine: Principles and Practis" 8 th (Eds) By C. Haslett, ER Smith JF (1999). Principles Chilvers, JAA Hunter and NA Boon. Edinberg, London. Pp 1049-1064.
3. Murray, A.W, and Kirschner, M.W. (1991). What control the cell cycle? Sci. American. March 34-41.
4. Wilmut, I. and Campbell, K. (2000). Megan and Morag In "The Second Creation " Eds by I. Wilmut , K. Campbell and C.Tudge .Headline Book Publishing , Irland PP205-230.
5. Wilmut,I Schnieke, A.E., McWhir, J., Kind, A.J and Campbell, K.H.S. (1997). Viable offspring derived from fetal and adult mammalian cells. Nature 385:810-813.
6. Tudge, C: (2000) Keith and Cell cycle .In "The Second Creation" (Eds) I Wilmut ., K.Campbell and C. Tudge. Headline Book Publishing Irland .PP 179-204.
7. Crossen , P.E.(1982). SCE in lymphocytes: IN " Sister Chromatid Exchange ". ed by A.A Sandbery .Liss New York . pp. 175-194.
8. Shubber, E.K., Altaif, K.I., AL-Khateeb ,G, et al (1992). Cytogenetic studies on blood lymphocytes from sheep infected with F. gigantica and treated with Albendazol. Iraqi J. Vet. Med. 15:10-24.
9. Shubber, E.K., Jaffer, Z.M.T., Abdul-karim, A, and Sebah MI (2001) .Culturing of goat blood lymphocytes *in vitro* for cytogenetic analysis. Proc. 1 st Sci. Conf. National Board Biotech. Res. Baghdad September 18-19 pp11-23.
10. Lin, M., and Alfi ,O . (1976). Detection of SCE by 4,6diamidino -2- phenyl indole fluorescence. Chromosoma 57:219-225.
11. Lamberti, L., Ponzetto, P.B., and Ardito, G. (1983). Cell kinetics and SCE frequency in human lymphocytes Mutation Res. 120:193-199.

12. Shubber, E.K., Altaif .K.I., AL-Khateeb , G, et al (1992). Cytogenetic studies on blood lymphocytes from sheep infected with F. gigantica and treated with Albendazol. Iraqi J. Vet. Med 15:10-24.
13. Dell'Anno, A., Dunivald, G, C.A ., KoK, A., and Danovaro, Tudge C: (2000). Keith and Cell cycle .In "The Second Creation" (Eds) I Wilmut ,K.Campbell and C. Tudge .Headline Book Publishing Irland .PP 179-204.
14. Armstrong ,R.D., Vera, R., Synder, P and Cadman, E (1983). Cellular enzymes and protein inhibitors. Biochem. Biophys. Res Commun. 109:595-602.
15. Vincenzo, R.D., Scamlioa, G., Panici, P.B,et al (1996) Modulatory effect of tamoxifen and ICI 182,780 on Adriamycin resistance in MCF-7 human breast cancer cells. Int.J. Cancer 68:340-348. Murray, A.W., and Kirschner, M.W. (1991). What control the cell cycle? Sci. American. March 34-41.
16. Shubber, E.K., and AL-Alaak, B.M.A. (1986). Spontaneous chromosome aberrations and SCE in human lymphocytes the Nucleus 29:92-98.
17. Kopnin, B.P.. And Gudkov, A.V. (1983). Gene amplification in mammalian somatic cells resistant to colchicines. Biochem. Oncol. 28:865-871
18. Nowell, P. C. (1960). Phytohaemagglutimine: an initiator of mitoses in culture of normal human leukocytes. Cancer Res. 20: 462-467.
19. Shubber, E.K. (1984). Effects of cultural conditions: Serum, Incubation time and blood storage on spontaneous frequencies of SCE and CA in human blood lymphocytes. 3 rd Int. Congress on cell Biol. Aug. 1984. Tokyo Japan.
20. Kubbies, M.D., Schindler, H., Hoehn, H and Rabinovitch, P.S. (1985). Cell cycle kinetics by Hochest flow cytometry: An alternative to the cell differential metaphase labeling technique. Tissue Kinetics 18:55-562.
21. Stites, D.P. (1976). Laboratory methods of detecting of cellular immune function. In "Basic and Clinical lmmunol" Eds by HH Fundenbery, D. P. Stites, J. L. Galducell and J. V Wells. Long Medical Publication pp 318-322.
22. Shubber, E.K., Jaffer, Z.M.T., Nada, S.M., and Karam, A.H. (1998). Induction of chromosomal anomalies and gene amplification in human cells by anti-cancer drugs. The Nucleus 41:120-127.
23. Campbell, K.H.S., Ritchie, W.A and Wilmut, I (1993). Nuclear cytoplasmic interaction during the first cell cycle of nuclear transfer reconstructed bovine embryies :Implications for DNA synthesis and development Biol.Reprod. 49:933-942.
24. Dynlacht .B.D. (1997). Regulation of transcription by protein that control the cell cycle . Nature 389:149-152.
25. Wilmut,I. and Campbell K. (2000). Megan and Morag In "The Second Creation " Eds by I. Wilmut ,K. Campbell and C.Tudge .Headline Book Publishing , Irland PP 205-230.
26. Smith , J.f. (1999). Principles of Oncological and Palliative Care . In : Medicine Principles and Practics" 8th (eds). By C.Haslett , E.R. Chilvers , JAA Hunter and NA Boon . Edenberg, London .pp 1049-1064.
27. Caskey ,C.T and Kruch, G.D. (1979). The HPRT Iocus. Cell 16:1-9.
28. Szyblaska, E.H., and Szyblaski, W. (1962). Genetics of human cell line. Proc. Natl. Acad. Sci. (USA) 60:2026-2030.
29. Shubber. E.K.. Auda, H.M., Jaffer. Z.M.T., and Abdul Rahman. M.H. (1999). Phenotypic expression of three genetic markers in human lymphoblastoid cell (GM-7254) treated with MMC.The Nucleus 42:122-130.

30. Jaffer, Z..M.T., Shubber, E.K., Amash, H.M (2001). Cytogenetic analysis of Chinese hamster lung fibroblasts spontaneously resistance to methotrexate. The Nuclens 44:28-35.
31. Blyer, W.A (1987). The clinical pharmacology of MTX. Cancer 41: 36-51.
32. Barakat, R and Li, W (1993). Intrinsic resistance of cervical sequamous cell carcinoma cell lines to MTX as a result of decreased accumulation of intracellular poly glutamates. Gynecol. Oncol. 51:54-60.
33. Katzenellen-bogen, B.S. (1991). Antiesterogen resistance: mechanisms by which breast cancer cell undetermined the effectiveness of endocrine therapy J.Natl .Cancer Inst .83:1434-1435.
34. Mondal, N.K., and Chakrabarti, S. (2007). A simpler, cheaper and quicker method to study somatic chromosome from goat. Cytologia. vol. 72:419-425.