Cytogenetic Analysis of Goat Blood Lymphocytes cultured *in Vitro*

التحليل الوراثي الخلوي لخلايا دم الماعز اللمفاوية المزروعة خارج الجسم الحي

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Abstract:

The aim of this work is to determine the duration of goat cell cycling *in vitro*.Goat peripheral blood lymphocytes were grown in RPMI-1640 medium containing bromodeoxyuridine (BrdU 10 µg/ml) for 72 h. Blastogenic index (BI), mitotic index (MI), cell cycle progression (CCP) and sister chromatid exchanges (SCE) were determined. Cultured lymphocytes from, whole blood or from leukocyte rich plasma in RPMI-1640 medium containing BrdU showed little differences in BI, MI, but significant differences were seen in cell cycle progression. BI, MI, and CCP from different goat breed were compared. Also, the percentage of lymphocyte blastogenesis, mitoses and cell cycle progression from goat, were compared to those from sheep, and human whichgrown under similar conditions. On successive incubation periods, the cell cycle duration of blood lymphocytes was determined through the mitotic activity. The cells reached first, second and third mitoses after 25, 40 and 48 h, post incubation respectively. Sub culturing of growing lymphocytes was performed from 3 to 45 days to obtain a lymphoblastoid cells. The characterization of their differentiation is required Establishment of goat blood lymphocyte culture will help in gene marker's detection in their somatic cells.

المستخلص

يهدف هذا البحث الى حساب دورة انقسام خلايا الماعز الجسمية خارج الجسم الحي.تمت تنمية خلايا دم الماعز اللمفاوية في الوسط الزرعي (RPMI-1640) الحاوي على مادة البروموديوكسي يوريدين 15 مايكروغرام/ مل المعلّمة لجزيئة الـ DNA في الخلية لمدة 72 ساعة. احتسبت دالة الاورمة (Blastogenic index) (BI) و دالة الانقسام (Mitotic index) (MI) و دورة الانقسام التعاقبية (Cell Cycle Progression) (CCP) و معدل التبادل الكروماتيدي الشقيقي (Sister chromatid exchanges SCE).استخدمت طريقتين في زرع خلايا الدم اللمفية و هي: الاولى زرع من الدم مباشرة، و الثانية من البلازما بعد تلازن كريات الدم الحمراء. لم تلاحظ فروق معنوية في دالة الاورمة و الانقسام الخلوي لكن ظهرت فروق معنوية في دورة الخلية الانقسامية بين الطريقين.قورنت نتائج التحليلات الخلوية الوراثية للخلايا اللمفاوية المعزولة من ماعز تمثل عدة ظروف اجنبية متأقلمة في القطر. كما قورنت تلك النتائج مع خلايا دم لمفاوية معزولة من الاغنام و الانسان. كانت خلايا الماعز اقلها استجابة للمحفز PHA على الانقسام و اقلها معدلاً في تردد التبادل الكروماتيدي لكنها كانت سريعة في دورتها الانقسامية.ظهر الانقسام الاول و الثاني و الثالث لدورة الخلايا اللمفاوية بعد 25 و 40 و 48 ساعة بعد

الحضن، على التوالي . تم استزراع الخلايا اللمفاوية كل اسبوع ابتداءً من اليوم الثالث من تنميتها في الوسط الزرعي و لمدة 45 يوماً للحصول على خلايا لمفاوية مولّدة (Lymphoblastoid). ان ايجاد هذا النظام الزرعي لخلايا الماعز سيسهل مستقبلاً دراسة الدوال الوراثية Genetic markers.

Introduction

The developments of techniques for differentially staining the two chromatids of a chromosome greatly improve the study of chromosomal alterations and functions. It was shown that if bromodeoxyuridine (BrdU) was incorporated into chromosomes for two consecutive S-phases, the sister chromatids exhibited a differential pattern of folding depending upon whether they are singly or doubly substituted with BrdU [1].

A complete set of studies by [2, 3] demonstrated that sister chromatid differentiation could be observed by fluorescence microscopy following growth in BrdU and staining with Hoechst 33258, or DAPI. It was possible to quantitate sister chromatid exchanges (SCE) production and use the specific staining pattern of metaphases that had undergone one, two, or three replications in the presence of BrdU to determine cell cycle durations.

Successful application of this system on mouse bone marrow cells, human blood lymphocytes, sheep blood lymphocytes and calves blood lymphocytes were achieved [4] [5 , 6]. In this investigation an attempt to apply this system on goat blood lymphocytes; to determine there non-specific transformation (Blastogenesis) and cycling progression was implemented.

Materials and Methods

Animals: fifteen local breed goats (5 males + 10 females Arabic local breed) from the animal unit at the center of biotechnology research in Nahran University were included in this investigation. All animals were healthy at the time of testing.

i. Cytogenetic analysis:

Blood lymphocytes were cultured in RPMI-1640 medium for blaastogenesis cell division and replicative index (RI) under optimum conditions [7, 8]. Two cultures were initiated from each blood samples. Briefly 0.5 ml of heparinized blood was inoculated in 4.5 ml of the medium containing 10% heat-inactivated fetal calf serum and PHA (IAEC) in 20 µg/ml and BrdU in 10 µg/ml. The cultures were kept in the dark at 37°C for 68 hrs. Colchicine (Houde France) was added at a concentration of 5 µg/ml 3h before harvest. The cells were treated after harvesting with 0.075 M KCL, fixed with methanol: glacial acetic acid (3:1 v/v) and pellets were spread on slides. Air-dried slides were then stained with a 2% Giemsa solution. The blood lymphocyte transformation that expressed blastogenesis was determined as percentage of blast in 1000 inoculated lymphocytes (BI). The mitotic index (MI) was counted as a ratio of mitoses to interphase nuclei in at least 1000 cells. For each animal, 10-15 good metaphases were examined for chromosome counting. Identical slides from same animal culture, were stained in 4, 6-diamidino-2-phenyl indol (DAPI) solution [3], and the sister chromatid exchange (SCEs) were counted in 50 well spread second metaphases. The replicative metaphase cells as percentage of first, second and third cell divisions in the presence of BrdU was determined [9].

ii. Whole Blood (WB) versus leukocytes-rich plasma (LRP)

Blood sample from same animal was divided into two parts; one was treated as whole blood, while 0.1 ml of PHA was added to the 2nd part and incubated at 37°C for 3h. Leukocytes-rich plasma was then prepared by centrifugate at 500 rpm. 0.5 ml from WB or from LRP was inoculated in complete medium as described above (I) BI, MI, RI and SCEs were then examined.

iii. Incubation Time: Cell Cycle Progression

Blood taken from the same animals in the above experiments was tested. 5 ml of blood was inoculated in RPMI-1640 complete medium at37C, on successive times (12-72 hrs.). Colchicine were added for the last 3 hrs. of each period. Slides were then prepared and examined for scoring BI, MI and RI.

iv.Culturing of blood lymphocytes from different breed

Blood cells isolated from Angora, Jumnabgri and Nobey goats that where adapted in Iraq (Dept. of Animal Health, IAEC) were cultured under similar conditions (Exp. 1).

v. Culturing of lymphocytes from different species

Goat, sheep and human blood lymphocytes were grown *in vitro* under identical conditions.

vi. Lymphoblast Subculturing

From cultures of the same animals in step II (before adding the colchicines) inoculation of 1 ml into 4 ml of fresh medium, for further growth was manipulated every week for 9 weeks. Successive inoculation was performed till day 45th for obtaining lymphoblast-cells. BI, MI and RI were scored after each subculture.

Statistical analysis

Data were analyzed by student "t" test.

Results and discussion

Peripheral blood lymphocytes from 15 local breed goats, and other breeds had grown successfully in RPMI-1640 medium. Their growth was similar to those of different breeds of sheep that was previously investigated [6].

Cell Cycle Progression

As a function of incubation time,

a) Blast-forming blood lymphocytes had started to appear with increased percentage after 16 hrs. of incubation till 72 hrs.after that they showed a decline Table (1).

b) First appearance to goat lymphocytes at mitoses was observed after first 5h of incubation where 100% of this mitosis was at first division (M1). The ratio of dividing cells was increased as a function of time; at 48 hrs. the second (M2) and the very low rate of third cell division (M3) were started to appear. These rates were increased after 72 hrs. However, using leukocyte rich plasma, and at equal number of lymphocytes second cell division started to appear after 40 h versus 48 hrs. in WB-culture techniques. These results may suggest that cellular component of erythrocytes in WB play an important role(s) for the proliferation of the lymphocytes [1,10]. Thus the duration of goat

lymphocyte cycling in vitro is ranging as 25 h for the first cell cycle (post PHA-Stimulation), 15-20 h for the second and third cell division, post first cell division, although cultural conditions are playing a racial roles in the duration of cell cycling, such as PHA, type of medium, serum concentration, blood storage, abeling technique as well as WB versus of LRP-techniques, [4,5,10].

Table (1): Goats blood lymphocytes cycle progression under different culture conditions

Incubation time (hr)	Whole blood culture					Leukocyte rich plasma				
	BI (%)	MI (%)	Cell cycle progression			BI (%)	MI (%)	Cell cycle progression		
			M1 (%)	M2 (%)	M3 (%)			M1 (%)	M2 (%)	M3 (%)
13+3h	4.6	0.0	-	-	-	2.5	0.0	-	-	-
18+3h	5.2	0.0	-	-	-	6.8	0.0	-	-	-
22+3h	18.0	0.1	100	-	-	4.7	0.15	100	-	-
37+3h	19.0	0.5	100	-	-	8.7	0.75	58.0	42.0	0
45+3h	28.0	0.8	30.0	69.0	1.0	15.0	0.80	38.0	32.0	30.0
69+3h	17.5	1.3	23.8	28.5	47.6	17.5	1.90	28.0	36.0	35.0

+3h for colchicines treatment.

Sex Difference

The incidence of sister chromatid exchanges in blood lymphocyte for both sexes presented in the Table (2). The mean frequency of SCEs for the males was 5.6±1.7 and for the females 4.7±0.3. Although the number of females was twice more than the number of males, there were no significant differences between them in BI, MI and RI values. Similar observations were seen in sheep lymphocytes [11].

Biodiversity could be seen clearly in cellular response to non-specific (and replicative) indexes were higher in local bread (Arabic) than in Angora goat, Indian breed Jamunapari or African breed Nobe, Table (3). The causes of these variations are unknown. They may due to genetic characterization [12] or due to adaptive characterization [13].

Table (2): Cytogenetic analysis of blood lymphocytes isolated from male and female goats

Parameters	Male (n=5)	Female n=12
1. Blastogenic index (%)	7.5-27.5	8.2-30.5
2. Mitotic index	1.5-8.5	5.0-7.3
3. Cell Cycle progression		
M1 (%)	30.0	23.0
M2(%)	31.0	29.0
M3(%)	39.0	48.0
RI	2.09	2.40
4. Sister Chromatid Exchanges (SCE) per cell	5.6±1.7	4.7±0.8

Table (3): Peripheral blood lymphocytes proliferative activity *in vitro* after stimulation with PHA for 72 h: compression between different breed:

Breed	Blastogenic index (BI)	Mitotic index (MI)	Cell Cycle progression			
			M1 (%)	M2(%)	M3(%)	RI
Arabic (local) [male]	21.0	1.7	33	35	32	1.99
Arabic (local) [female]	21.0	2.0	36	28	35	1.97
Angora: adapted in Iraq [male]	13.0	1.0	80	20	0	1.20
Jumnabari adapted in Iraq [male]	16.3	1.2	66	31	3	1.37
Nobei: adapted in Iraq [female]	12.0	3.0	90	10	0	1.10

Cytogenetic analysis of goat, sheep and human peripherals blood lymphocytes:

Under similar conditions peripheral blood lymphocytes that were isolated from goat, sheep and human were cultured in vitro for 72 h. the results are presented in Table (4). Goat lymphocytes presented a lowest rate of cellular division than sheep and human cells, i. e MI for goat was 2.1±0.8 significantly lower than that of sheep (5.9±0.3) or human cells (5.7±3.2). for 72 h incubation, goat cells exhibited a highest (P<=0.01) rate of cellular replication (RI = 2.22) which were 47.5% of these cells are in M3, comparing to sheep M3= 26% and human cells M3= 37.2%. it is interested to note that goat somatic cells have 60 chromosomes which is higher than those of human cells 46 chromosomes or sheep cells 56 chromosomes [14] although they have a lowest rate of SCE/cell than the other species. These variations in the cellular division, replication and SCEs might result from the species differences (P<=0.01) in response to PHA, colchicines [15, 5] or in response to BrdU[9].

Table (4): Cytogenetic analysis of peripheral blood lymphocytes isolated from different species and grown in vitro under similar conditions:

Parameters	Goat (Caprin)	Sheep (ovin)	Human
Mitotic index	1.3-2.9	5.7-6.2	2.9-8.3
Cell Cycle progression			
M1 (%)	23.0	27.0	32.0
M2(%)	28.5	47.0	30.8
M3(%)	47.5	26.0	37.2
RI	2.22	1.99	1.62
Sister Chromatid Exchanges (SCE) per cell	5.1±0.7	7.3±0.7	6.5±0.2

Long-term culture (3-45 days) of peripheral blood lymphocytes

Several attempts to maintain consistently healthy goat peripheral blood lymphocyte culture 45 days were succeeded. Subculturing of blast cells in fresh medium at constant period 7 days retained excellent cellularity with mitoses evident at 15th day of culture

(Table 5). Additional administration of medium with mitogen every 7th day had no deleterious effect on the cells; it rethread promoted their growth. More efforts are required to characterize these cells, whether they are plasma cells or lymphoblast cells by running immunological characterization.

Table (5): Cytogenetic analysis of (Subcultured) peripheral blood lymphocytes isolated from goats to get on lymphoblast:

Sample	Blasto index		Mitotic index		Replicative index	
	0	P1*	0	P1*	0	P1*
	21.75	30.2	2.5	1.01	1.48	1.90
	33.80	29.5	1.0	0.47	1.94	2.10
	No mitoses	-	-	-	-	-
	15.30	23.0	0.80	0.55	2.10	0.00
	16.50	23.3	1.20	0.90	1.63	2.00
	26.10	37.7	1.26	0.99	2.06	2.3
	29.20	30.6	1.35	0.00	2.15	1.95
	No mitoses	-	-	-	-	-
	23.2	38	0.94	0.9	2.10	1.79
	25.0	36.3	1.55	0.78	2.12	1.89

***cells subcultured from the primary blood culture (grown for 72h) and analyzed after one month of growth in RPMI-1640 complete medium.**

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