Morphological and electrophoretic data of primary pulmonary fibroblasts cultures obtained from normal and Ovalbumin-Challenged "Asthmatic" Wistar rats treated by speleotherapy in Cacica and Dej Romanian Salt Mines

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Abstract

Objective: To investigate the influence of salt mine medium from the Romanian Cacica and Dej Salt Mines upon the cell morphology and electrophoretic expression of pulmonary fibroblasts *in vitro* obtained from Wistar rats' lung, in normal and Ovalbumin challenged "asthmatic" conditions.

Materials and methods: Pulmonary fibroblasts cultures were prepared from Wistar rat lung. Cultures derived from lung rat develop with a monolayer of fibroblasts attached to the culture dish. Before cultures initiation, Wistar rats of 75-100 g weight were divided in two lots: control and ovalbumin challenged animals. Ten animals of each lot were send to Cacica and Dej Salt Mine for 14 days and maintained in the salt mine medium, as in speleotherapy treatment.

Results: Speleotherapy of Wistar rats had induced significant differences in cell morphology and electrophoretic expression of primary pulmonary fibroblasts cultures. The data obtained support the protective effects of speleotherapy by comparing with ovalbumin sensibilised animals.

Conclusions: The results of this study indicate the fact that speleotherapy induces changes on the morphology and protein expression of pulmonary fibroblasts *in vitro*, and these changes support the beneficial effects of speleotherapy.

Key words: speleotherapy, fibroblasts, salt mine

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INTRODUCTON

Asthma is a disorder characterized by chronic inflammation of the airways, airways hyper-responsiveness, and changes in airway architecture, termed remodeling. The cells responsible for maintenance of lung structure are the parenchymal cells of the lung, including epithelial cells, mesenchymal cells, and endothelial cells. Recent studies have suggested that the function of epithelial cells, smooth muscle cells, and fibroblasts cultured from lungs of individuals with asthma differs from the function of cells similarly cultured from individuals without asthma. These functional differences, particularly as they relate to repair and remodeling, could contribute airway structural alterations (Sugiura *et al.*, 2007).

The current study was designed to investigate the influence of salt mine medium from Cacica and Dej Salt Mines upon the cell morphology and electrophoretic expression of pulmonary fibroblasts *in vitro* obtained from Wistar rats' lung, in normal and Ovalbumin challenged "asthmatic" conditions.

Fibroblasts were cultured from lung parenchyma of control, ovalbumin-sensitized, and speleotherapy treated rats after ovalbumin-sensitization. Fibroblasts shape in culture can vary in accordance with the substrate, which on they is growing, and the space they have for movement.

Using pulmonary fibroblasts cultures to verify the therapeutic properties of saline mines medium, described as speleotherapy, represents an innovative and scientific new way to establish the medical methodology of preventing, treating and recovery of patients with various pulmonary problems.

MATERIALS AND METHODS

Materials

Phosphate Buffer Solution (PBS: NaCl 0,13M + KCl 2,6mM + Na_2HPO_4 x12 H_2O 8mM + KH_2PO_4 1,4mM); HAM-F12 culture medium (Sigma); penicillin 100 U/ml, streptomycin 100µg/ml; neomycin 50µg/ml (Sigma); fetal bovine serum (Sigma).

Rat Wistar Model of Allergic Asthma

Wistar rats of 75-100g weights were sensitized to Ovalbumin by i.m. injections.

Primary fibroblasts culture

After anaesthesia with chloroform, rats were killed. The thorax was opened and then the lungs were removed en bloc in a laminar flow hood using sterile technique and put into ice-cold sterile Phosphate Buffer Solution (PBS: NaCl 0,13M + KCl 2,6mM + Na_2HPO_4 x12 H_2O 8mM + KH_2PO_4 1,4mM). 1mm tissue pieces were suspended in 0.125% trypsin and 0.001% DNase and repeatedly stirred for 6 minutes and centrifuged at 1000g. The pellet was resuspended in **HAM-F12** medium with 4500mg/l glucose, 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml neomycin and 10% fetal bovine serum (Sugiura *et al*, 2007; Foster *et al*, 1990; Nunez *et al*, 1995).

Phase Contrast Microscopy

Phase contrast microscopy, first described in 1934 by Dutch physicist Frits Zernike, is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens, such as living cells (usually in culture), microorganisms, thin tissue slices, lithographic patterns, fibers, latex dispersions, glass fragments, and subcellular particles (including nuclei and other organelles).

SDS-PAGE Electrophoresis

The proteins electrophoresis from the total homogenate has as the purpose to establish the changes, which are revealed at the proteic level of fibroblasts cultures obtained from rats held on saline mine medium for the speleotherapy.

The proteins electrophoresis in gel of polyacrylamide was done in the denaturated conditions in the conformity with the techniques described by Laemmli (1979). The cultures have been washed with PBS, curetted from the culture plate and lyzed in buffer containing 0,5M Tris-HCl, pH 6,8 + 0,05% BPB + 10% glycerol + SDS 10%.

RESULTS

Control pulmonary fibroblasts culture of 9 days has a homogenic aspect with a high pre-confluence level. The cell division is to a high level and the cell morphology shows a typical microscopic view, described in the specific literature.

Pulmonary fibroblasts cultures of 9 days obtained from Ovalbumin sensitized rats presents many morphological changes from the control pulmonary fibroblasts culture, being observed an sensible number reducing of pulmonary fibroblasts in culture, the diminished cellular dividing frequency and an accentuated cellular morphopathology of the cells in culture.

After 9 days of culturing, the pre-confluence level is much lower than in the control case.

Pulmonary fibroblasts cultures of 9 days obtained from Ovalbumin sensitized rats and treated by speleotherapy in Cacica Salt Mine shows an improvement of the morphological parameters of the cells comparative with the cultures obtained from Ovalbumin-challenged asthmatic rats. By phase contrast microscopy, it is possible to observe a rising of the cells number.

Pulmonary fibroblasts cultures of 9 days obtained from Ovalbumin sensitized and treated by speleotherapy in Dej Salt Mine shows also an improvement of the morphological parameters of the cells comparative with the cultures obtained from Ovalbumin-challenged asthmatic rats. It is observed the rising of the cell population density and that of cell viability.

Pulmonary fibroblasts were homogenized with Laemmli buffer pH 6,8, and the proteins of the obtained homogenate were separated by 10 % SDS polyacrylamide gel electrophoresis that maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure.

Samples of 10µl were loaded into *wells* in the gel. One lane was reserved for Sigma molecular markers mixture of 205; 116; 97; 66; 55; 45; 36; 29; 24; 20; 14,2 and 6,5 KDa

Following electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250, that allowed visualization of the separated proteins. After staining, different proteins appeared as distinct bands within the gel (Towbin *et al.*, 1979).

Analysis with GeneTools version 4 software from SynGene of each track of the electrophoresis, allowed us to compare the profiles of the total proteins expression.

DISCUSSION

The present study evaluated morphological phenotypes related to repair and remodeling in fibroblasts obtained from control Wistar rats and from Ovalbumin-sensitized and -challenged rats, a model of asthma that results in airway hyperresponsiveness and chronic airway remodeling, as other authors had presented.

Compared with control fibroblasts, fibroblasts obtained from lung parenchyma of the "asthmatic" rats and Ovalbumin-sensitized rats treated in Cacica and Dej Salt Mines demonstrated the positive role of the saline medium for the “asthmatic” rats.

The current study focused on fibroblasts, which are believed to be cells that play a major role in the maintenance and remodeling of interstitial connective tissue. In this context, fibroblasts are believed to play a key role in maintaining and altering tissue structure. The ability of fibroblasts to migrate in response to chemotactic stimuli and to proliferate in response to specific growth factors is believed to control their accumulation at sites undergoing tissue repair. The ability of fibroblasts to produce and remodel extracellular matrix is thought to contribute to tissue structural changes. Remodeling of tissues likely involves fibroblast contractile activity.

In summary, the present study supports the concept that phenotypically altered fibroblasts can contribute to airway remodeling in asthma. Fibroblasts cultured from the lungs of chronically OVA-sensitized and -challenged animals demonstrated consistently augmented repair responses for a number of functional assays (Sugiura *et al.*, 2007).

CONCLUSIONS

- Phase contrast microscopy analyses of primary fibroblasts cultures reveals an cellular regeneration after animal exposure to saline medium in Cacica and Dej Salt Mines, comparative with the cells morphology of cultures from Ovalbumin sensitized rats.
- The morphological observations are confirmed by the electrophoretic analyses, which demonstrate through rising of the expression of many proteins and of total protein amount that the exposure of Ovalbumin-sensitized animals to the saline medium from Cacica and Dej Salt Mines is reversing the cells morphopathology of pulmonary fibroblasts in cultures;
- Wistar rats sensitized with Ovalbumin have a low number pulmonary fibroblasts output cultures, with a more sensitive morphopatologic level.

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TABLE 2 Protein expression analysis of the pulmonary fibroblasts cultures

Peak Nr.	Peak weights molecular limits (KDa)	CONTROL Quantity (µg/10µl)	OVALBUMIN Quantity (µg/10µl)	CACICA Quantity (µg/10µl)	DEJ Quantity (µg/10µl)
1	225 – 240	5,47	5,18	2,98	6,33
2	220 – 225	3,37	2,35	0,99	2,24
3	210 – 220	2,81	3,08	1,48	1,54
4	200 – 210	1,25	0,56	2,68	3,18
5	190 – 200	1,54	1,23	1,35	1,17
6	160 – 190	0,66	0,65	2,38	0,36
7	140 – 160	0,94	0,90	0,94	2,06
8	120– 140	0,90	2,81	0,70	0,53
9	105 – 120	3,01	1,07	1,00	0,58
10	100 –105	1,58	0,58	4,42	0,98
11	90 – 100	0,59	0,60	1,30	1,34
12	63 – 90	0,94	16,21	8,10	3,38
13	55 – 63	8,77	2,70	10,20	1,96
14	42 –55	0,80	0,34	10,34	0,80
15	40 – 42	2,78	0,39	0,70	0,75
16	37 – 40	2,88	1,38	0,61	14,47
17	35 – 37	0,36	3,11	3,29	6,29
18	34 – 35	2,16	2,16	1,19	0,53
19	32 – 34	8,48	0,44	1,64	7,62
20	30 – 32	3,79	0,55	2,17	2,39
21	23 – 30	4,78	1,86	2,05	1,35
22	19 – 23	4,16	6,64	4,64	12,93
23	6 – 19	18,64	12,62	16,80	15,94
TOTAL amount of proteins in 10 µl of sample:		80,66	67,41	81,95	88,72

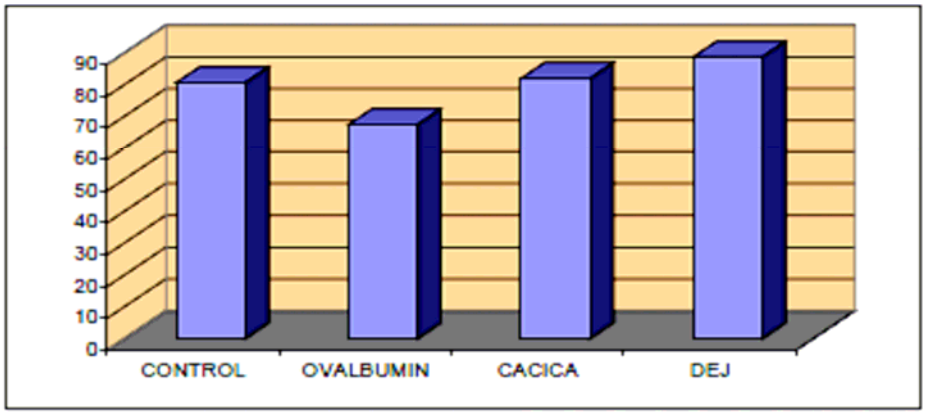
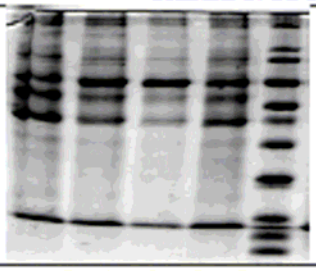


Fig.10 **TOTAL amount of proteins in 10 µl of sample**

TABLE 1: SDS polyacrylamide gel electrophoresis of the pulmonary fibroblasts cultures



Samples
5- 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats exposed to the saline medium of Dej Salt Mine
4- 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats exposed to the saline medium of Cacica Salt Mine
3- 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats
2- 9 days Control pulmonary fibroblasts culture
1- **Sigma** molecular markers

Fig. 1 – Electrophoretic profile of pulmonary fibroblasts cultures

Track 2 9 days Control pulmonary fibroblasts culture

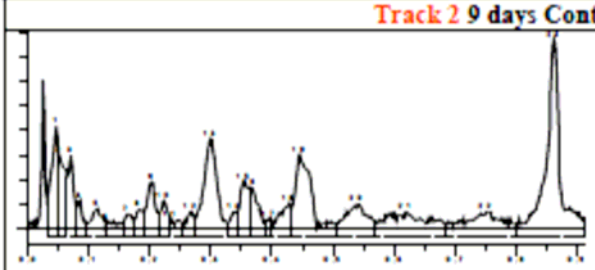


Fig. 2 Densitogram of 9 days Control pulmonary fibroblasts culture

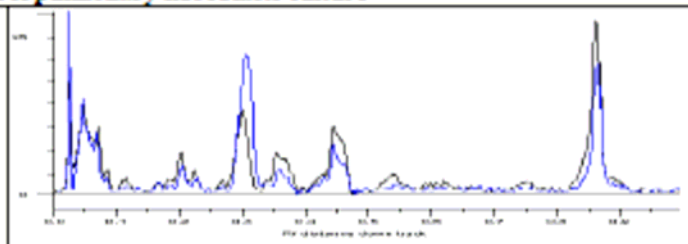


Fig. 6 Profile matching for
CONTROL (▬) – OVALBIMN (▬)

Track 3 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats

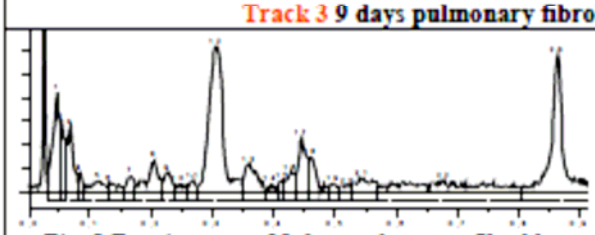


Fig. 3 Densitogram of 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats

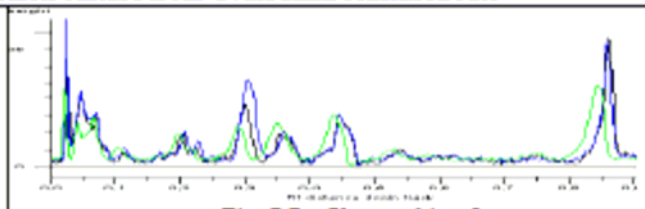


Fig. 7 Profile matching for
CONTROL (▬) - DEJ (▬) - CACICA(▬)

Track 4 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats exposed to the saline medium of Cacica Salt Mine

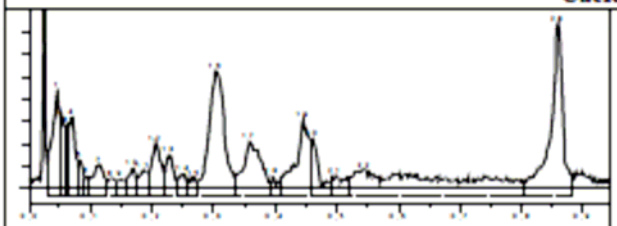


Fig. 4 Densitogram of 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats exposed to the saline medium of Cacica Salt Mine

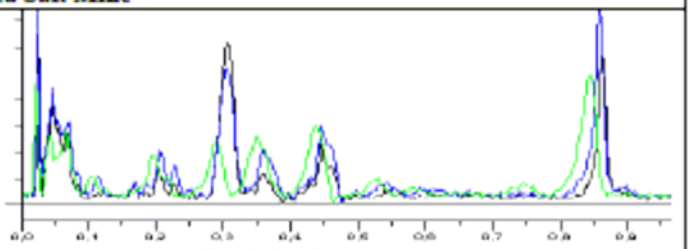


Fig. 8 Profile matching for
OVALBUMIN (▬) - DEJ (▬) - CACICA(▬)

Track 5 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats exposed to the saline medium of Dej Salt Mine

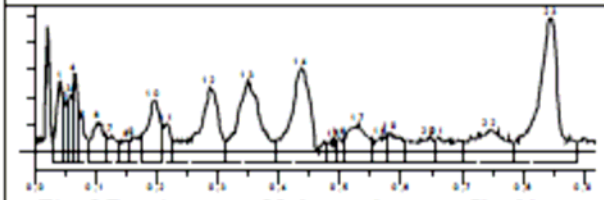


Fig. 5 Densitogram of 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats exposed to the saline medium of Dej Salt Mine

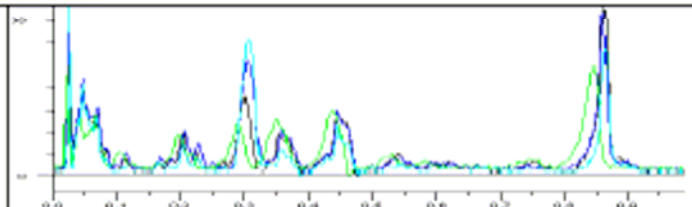


Fig. 9 Profile matching for **CONTROL (▬)**
OVALBUMIN (▬) - DEJ (▬) - CACICA(▬)

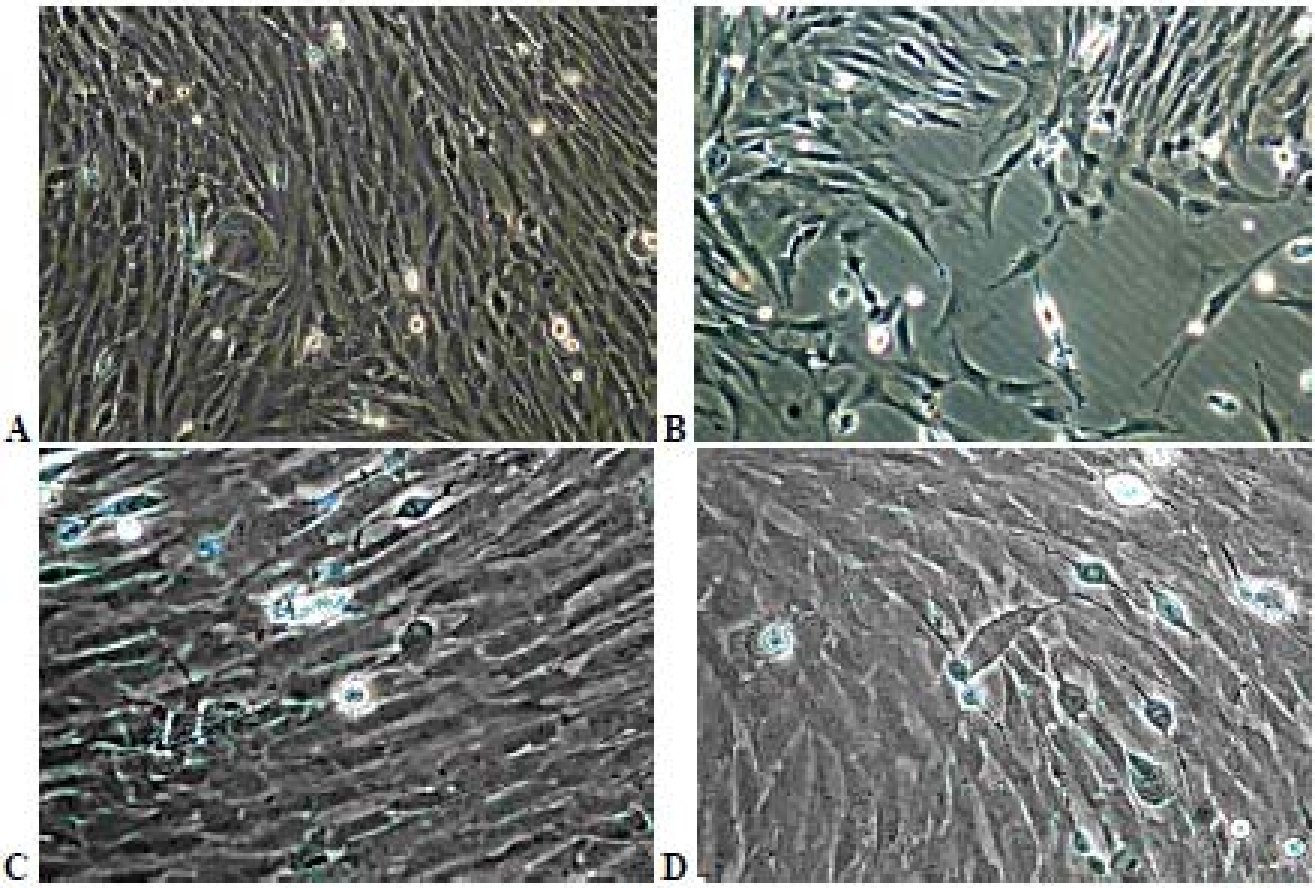


Fig. 11 Control pulmonary fibroblasts cultures of 9 days, A-B X 150, C-D X 300

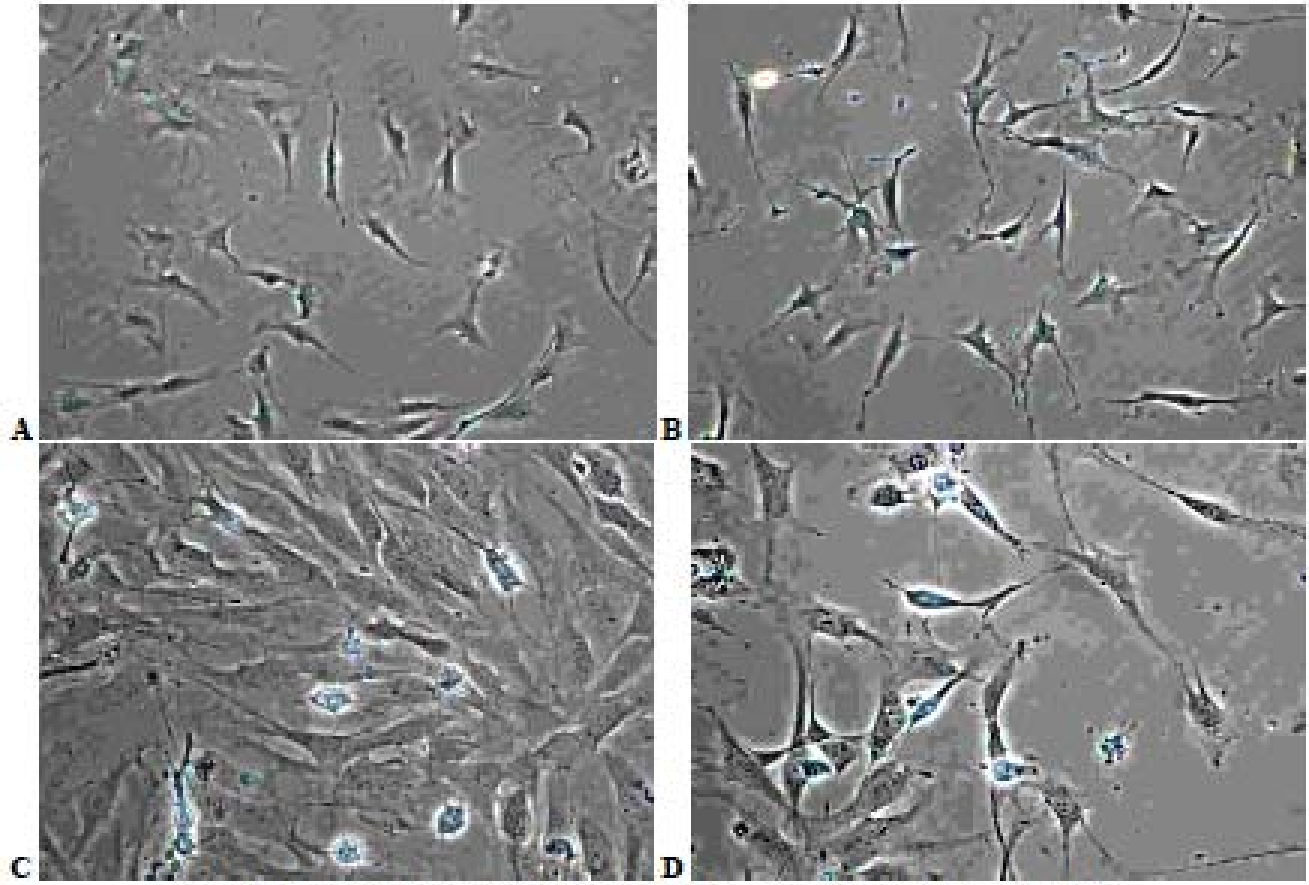


Fig. 12 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats, A-B X 150, C-D X 300

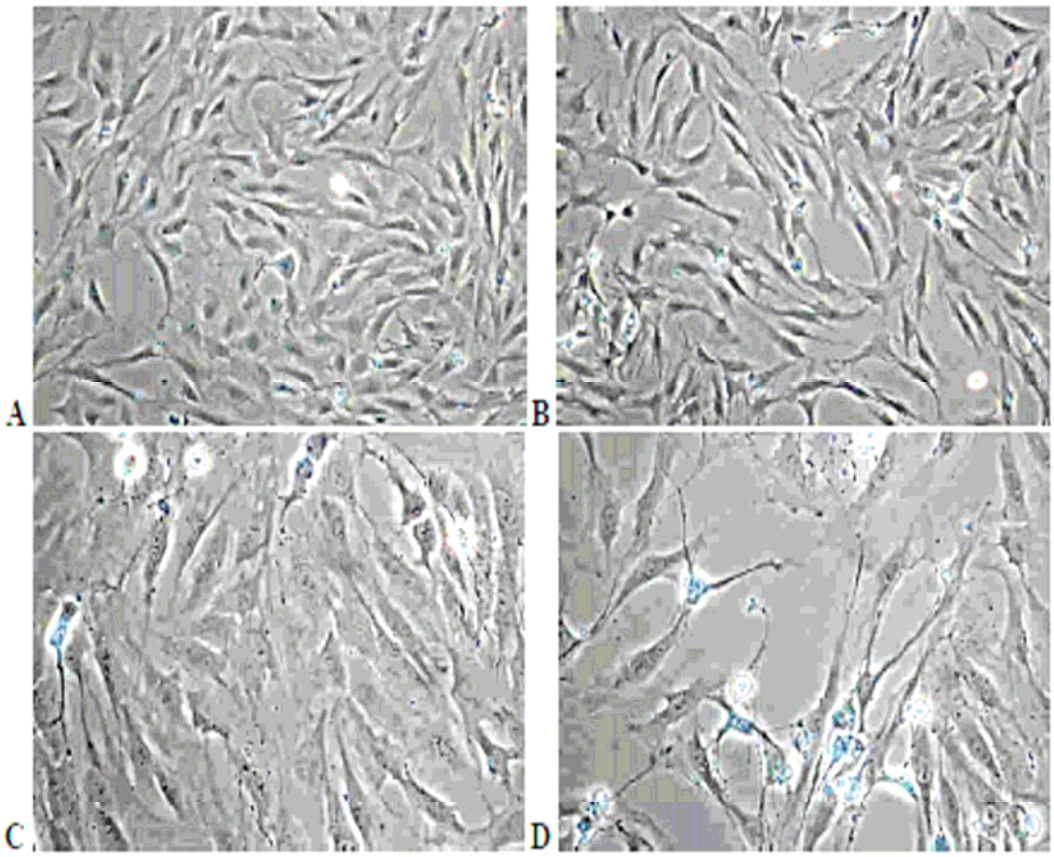


Fig. 13 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats exposed to the saline medium of Cacica Salt Mine, A-B X 150, C-D X 300

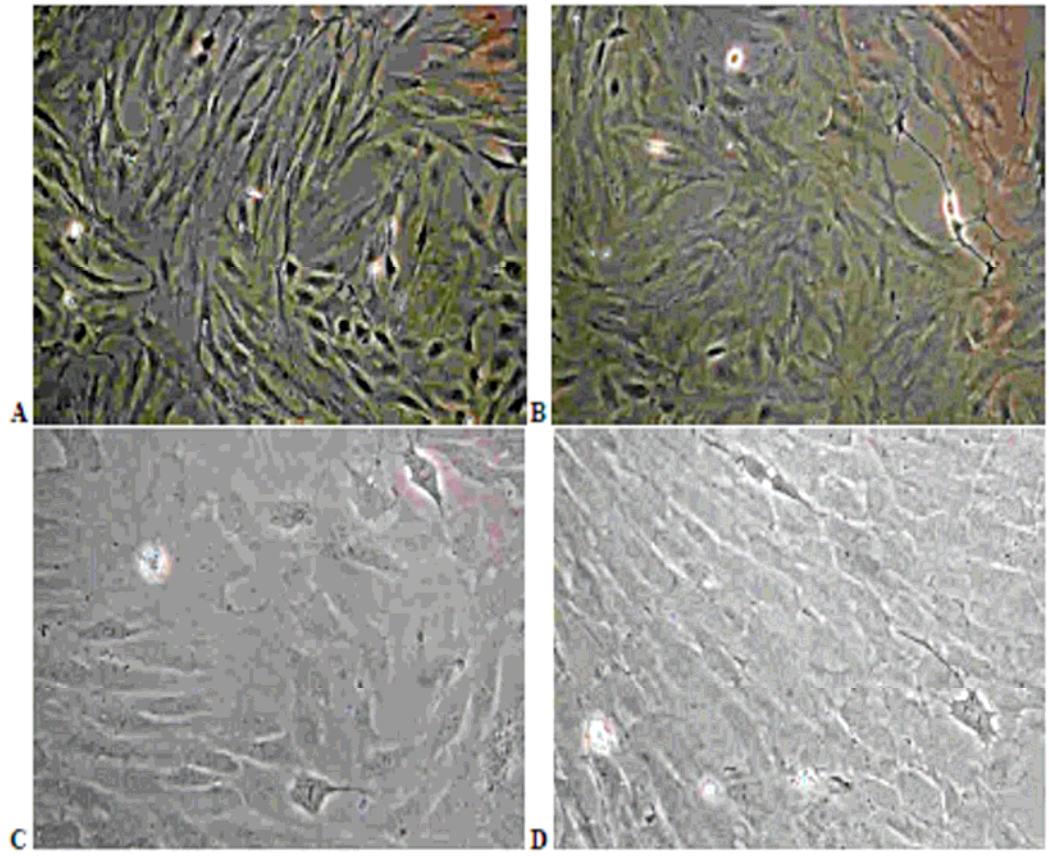


Fig. 14 9 days pulmonary fibroblasts culture from Ovalbumin-sensitised rats exposed to the saline medium of Dej Salt Mine, A-B X 150, C-D X 300