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Potent Antibiotic Producing

Vitro Regeneration System

Discovering Thoughts, Inventing Future

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Isolation and Characterization of Potent Antibiotic Producing Marine Actinomycetes from Tiruchendur and Kulasekarapattinam, Tamilnadu

By Prasantha Kumari. Mantada, Girija Sankar. G, Prabhakar.T

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Abstract - A set of 97 actinomycete strains which were isolated from 24 marine samples such as sea water, sea sediments, sponges, and corals obtained from Tiruchendur and Kulasekarapattinam, Tamilnadu state, India. The actinomycetes isolated from these eco systems are capable of producing antibiotics that strongly inhibit the growth of gram positive and gram negative bacteria and yeast like fungi. A total of 27 isolates representing the range of morphological diversity observed from each sample, were obtained in pure culture. However of the 27, four were found to produce antibiotic substances. KSP-SS-06-1C/1 exhibited higher activity and was, therefore selected for further studies. The purification and characterization of the substances is now in progress.

Keywords : marine actinomycetes, isolation, biochemical characterization, kulasekarapattinam, antimicrobial activity.

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Isolation and Characterization of Potent Antibiotic Producing Marine Actinomycetes from Tiruchendur and Kulasekarapattinam, Tamilnadu

Prasantha Kumari. Mantada [α], Girija Sankar. G [σ], Prabhakar.T [ρ]

Abstract - **A set of 97 actinomycete strains which were isolated from 24 marine samples such as sea water, sea sediments, sponges, and corals obtained from Tiruchendur and Kulasekarapattinam, Tamilnadu state, India. The actinomycetes isolated from these eco systems are capable of producing antibiotics that strongly inhibit the growth of gram positive and gram negative bacteria and yeast like fungi. A total of 27 isolates representing the range of morphological diversity observed from each sample, were obtained in pure culture. However of the 27, four were found to produce antibiotic substances. KSP-SS-06-1C/1 exhibited higher activity and was, therefore selected for further studies. The purification and characterization of the substances is now in progress.**

Keywords : marine actinomycetes, isolation, biochemical characterization, kulasekarapattinam, antimicrobial activity.

I. Introduction

As a great promising for new natural products which have not been observed from terrestrial microorganisms, marine bacteria are being developed for the discovery of bioactive substances with new types of structure, with growing intensive interest. The achievements have been well reviewed (Bernan, V.S et al.,1997) ,where many novel antibiotics were obtained from actinomycetes (Okami, Y & K. Hotta,1998) .Thus actinomycetes associated with marine plants and animals are expected to be a potential source for new natural bioactive agents. Actinomycetes in marine environment are found in water, sediments, fishes, fauna, free swimming vertebrates, invertebrates, corals, coral reefs, sponges and marine snow (Alan C. Ward and Nagamani Bora, 2006).

II. Materials and Methods

a) Sites of Sampling

A total of 24 marine samples were collected from Tiruchendur and Kulasekarapattinam located in Thoothukudi district, Tamilnadu state, India.

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Tiruchendur: This area is located along the southern coast of Tamilnadu state. It extends over a distance of 28 km of the south eastern coast line border of Bay of Bengal. It is 37 km away from Thoothukudi and 85 km from Kanyakumari. Latitude - $8^0$28'60 N, Longitude-$78^0$7'0 E.

Kulasekarapattinam: It is about 51 km south of Thoothukudi and about 13 km south of Tiruchendur and about 116 km away from Kanyakumari. Latitude - $8^0$05'and $9^0$30' N, Longitude-77^0 05' and 78^0 25' E.

b) Collection and Processing of samples

The marine samples include sea water, sea sediments, sponges and corals which were collected in sterile containers containing sterile starch casein broth at depth of 5,10,15,25,30 m respectively at a distance of about 10-15 kms off the sea shore. All samples were labeled and were brought to the laboratory. They were stored at a temperature between 6 to 10°C for further use.

c) Isolation of Actinomycetes

The collected marine samples were serially diluted to ten folds with sterile sea water (1:10 w/v).To germinate actinomycetes spores, each diluted sample (10^{-6},10^{-7},10^{-8},10^{-9},10^{-10}) were heated to 35-45°C for 10 min in a shaking incubator (Grein, A. and Meyers, S.P , 1958). 1mL from each diluted sample was inoculated in 250mL Erlenmeyer flask containing 50 mL of sterile molten starch casein agar medium (g/L: Starch, 19; Casein, 0.3; KNO_3, 2; NaCl, 2; K_2HPO_4, 2; $MgSO_4.7H_20$, 0.05; $CaCO_3$, 0.02; $FeSO_4.7H_2O$, 0.01; Agar, 20) supplemented with Cyclohexamide (50 μg/mL) and Rifampicin (5μg/mL) to prevent fungal and bacterial growth respectively, (Haque et al.,1992) poured into sterile petriplates and incubated at 28°C for 14 days. After 14 days, colonies of unicellular bacteria were counted on agar plates (Dwight, R. and Fenical, W, 1991). The average colony count of each dilution was found to be 20-250 colonies.

d) Identification of Actinomycetes

Actinomycetes were recognized by their characteristic tough leathery colonies, branched vegetative mycelia, and, when present, aerial mycelia

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and spore formation. Because of these criteria, only colonies with well developed and branched hyphae were included in this study.

e) Determination of Antimicrobial Activity

i. *Test Micro organisms*

Anti microbial activity of the selected isolates were tested against following organisms such as *Bacillus subtilis* (NCIM-2063), *Staphylococcus aureus* (NCIM-2901), *Escherichia coli* (NCIM-2065), *Saccharomyces cerevisiae* (NCIM-3047), *Candida albicans* (NCIM-3100), *Aspergillus niger* (NCIM-787), *Pseudomonas aeruginosa* (NCIM-2037). All the test organisms employed in the present study were supplied by The National Collection of Industrial Microorganisms, National Chemistry Laboratory, Pune, India.

f) Screening of antibiotic producing strains

i. *Primary Screening Test*

The antimicrobial activity of isolates were tested by Cross Streak Plate Method (Elliah,P. et al.,1996), (Dhanasekaran, D. and Selvamani, S, 2009) . Each actinomycete isolate was transferred from starch casein agar plate to nutrient agar plate. This is done by streaking a straight line of the actinomycete inoculum across the surface of nutrient agar medium in the plate and incubated at 28°C for 7days. Inoculum of the test micro organisms were then streaked at right angles to the actinomycetes straight line colony (Zhomghui Zheng. and Wei Zeng, 2000).Control plates were also maintained to assess the normal growth of the pathogenic organisms.

ii. *Secondary Screening Test*

The active isolates resulted from primary screening, promising isolates were tested for their extra cellular antibiotic production capabilities under submerged fermentation conditions. The production medium containing (g/L: Sucrose, 1.0; Yeast extract, 2.0; KNO_3, 3.8) was used for antibiotic production test. Well sporulated, 7 to 10 days old isolates were taken and 5ml of sterile water was added to each slant and 5ml of spore suspension was added to 45mL of respective production media and incubated at 28°C for 6 days on a rotary shaker. Then samples were collected in to sterile centrifuge tubes and centrifuged at 4000 rpm for 15 minutes to separate the fermented broth and the mycelium. The clear supernatant was used for antibiotic assay using agar diffusion method (Barry AL, Thornsberry C, 1985), employing nutrient agar for bacteria and potato dextrose agar for fungi.

g) Characterization of Actinomycetes

i. *Determination of the color of the actinomycete isolates*

Morphological characters and color determination of selected isolate was studied on different media such as Yeast extract malt extract agar (ISP-2), Oat meal agar (ISP-3),Inorganic salts starch agar medium (ISP-4), Glycerol aspargine agar medium (ISP-5) (Rathna Kala, R. and Chandrika,V,1993) in accordance with the International *Streptomycetes* Project (ISP) (Shirling, E.B. and Gottlieb, D. 1966). Color was determined using the color names lists (Pridham TG, 1964).

ii. *Morphological Characterization*

Morphological characterization consisting of macromophology and micromorphology. Micromorphology was noted by the naked eye and by observation with magnifying lens. To study aerial mycelium and its spore characteristics was identified by inclined cover slip culture technique (Kawato, N. and Shinobu, R, 1959).

iii. *Biochemical Characterization*

Actinomycetes isolates are characterized using Melanin reaction, H_2S production, Tyrosinase reaction, Starch hydrolysis, Gelatin hydrolysis, Casein hydrolysis, Nitrate reduction, Milk coagulation and peptonization according to International *Streptomycetes* Project.

iv. *Physiological and Cultural Characterization*

The ability to grow at various Growth temperature range (10-70°C), range of pH (3-10) and in different concentrations of NaCl (2-16 g/L) on medium was also tested.

v. *Chemo taxonomical characterization*

Isomers of diaminopimelic acid (DAP) in cell wall hydrolysates and whole cell sugars of actinomycete were determined by thin layer chromatography following the standard methods of (Waksman SA, Henrici AT, 1943) and (Boone KE, Pine L, 1968).

III. Results and Discussion

The selective isolation process resulted in isolation of 97 actinomycetes strains from 24 marine samples. Out of which 10 samples collected from Kulase karapattinam and 14 from Tiruchendur resulted in isolation of selective and high number of actinomycetes. Among the collected samples, Kulase karapattinam samples yielded 41 strains of actinomycetes and the Tiruchendur samples yielded 56 strains as shown in (Table 1). Among 97 actinomycete colonies isolated, 27 isolates were morphologically distinct, among 27 isolates only four isolates exhibited the anti microbial activity in the primary screening test against bacterial and fungal pathogens. Whereas, in the secondary screening test out of four active isolates KSP-SS-06-1C/1 isolate showed the potent activity against both bacterial and fungal pathogens. Hence, this strain has been taken for further character analysis. Zone of inhibitions of all four isolates were shown in (Table 2). The morphological and cultural characteristics of most

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active isolate were studied on various media in accordance with International Streptomycetes Project. The growth characteristics, presence of mycelium and soluble pigments were observed (Table 3).Gram staining reveals that the active isolate was gram positive. Chemotaxonomy plays a major role in identification of actinomycetes to generic level. In this study, the active isolate contained LL-DA and absence of characteristic sugars, belongs to cell wall type-I. Different physiological characteristics are influencing the growth rate of actinomycetes. Assessment of physiological characteristics of the strain revealed that they could be grow well at 25°C and 40°C temperature, pH 7.0-10 respectively. However, the strain had maximum growth rate at a NaCl concentration of 2-7%. In this study various biochemical tests were performed and result were shown in (Table 4).

IV. Conclusion

A total of 27 isolates of actinomycetes from marine samples collected from coastal areas of Kulasekarapattinam and Tiruchendur of Tamilanadu state, India. Out of 27 isolates 4 isolates showed good antimicrobial activity against pathogenic bacteria and fungi. starch casein agar supplemented with Rifampicin and Cyclohexamide was found to be suitable for isolating actinomycetes from marine samples. Out of the 4 isolates one isolateshowed potent activity. After performing some biochemical tests, most active isolate belongs to the genus *Streptomyces*. The physiological characteristics of actinomycetes varied depending on the available nutrients in the medium and physical conditions. The present study is an attempt to identify and characterize versatile strains of Streptomyces. Further purification and characterization secondary metabolites can be carried out.

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Table 1 : Distribution of actinomycetes in various marine samples

Sample code	Type	Characters	Total no. of Isolates
KSP-06	Sponge	White, brain like mass, tiny spores are present	08
KSP-SS-06,1A,1B,1C	Sea Sediment	Green colored sediments	19
KSP-M1,M2,M3	Mixture	Pale Yellow colored Species	02
KSP-04	Sponge	Thick Green color *Ircinia* Species	09
KSP-SW-I,II	Sea water	Light Green color – Water	03
TCH-01,02,03	Sponge	Dark brown in color, Finger like Projections	18
TCH-04	Sponge	*Ircinia* Species	10
TCH-05,06,07	Sponge	Unknown species	08
TCH-08	Sponge	Light Grape colored *Hydella* Species	06
TCH-09,10	Corals	White Hard substance	02
TCH-11,12,14	Sponge	Unknown species, Dark Brown In color	07
TCH-13	Sponge	*Ircinia* Species, hard to cut, Tilden like smell	05

KSP-Kulasekarapattinam, TCH- Tiruchendur, Total no. of Samples-24,
Total no. of Isolates = 97

Table 2 : Antimicrobial activity of selected isolates

Test organisms	KSP-04/1	KSP-SS-06-1C/1	TCH-09/1	TCH-13/1
Bacillus subtilis	11	22.4	10.4	12.5
Staphylococcus aureus	14	21.9	11	17.5
Escherichia coli	12	20.4	10.9	13.2
Saccharomyces cerevisiae	13.7	19.5	11.5	12.0
Candida albicans	11.6	13.9	-	-
Aspergillus niger	9.8	11.7	10	9.8
Pseudomonas aeruginosa	11.8	12.8	10.5	10.3

- = No activity

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Table 3 : Cultural characteristics of isolate KSP-SS-06-1C/1

Name of the medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
Yeast extract malt extract medium(ISP-2)	Abundant, spreading, powdery	Abundant, powdery, grey	Pale yellow	None
Oat meal agar(ISP-3)	Moderate, spreading, powdery	Good, powdery, grey	Pale yellow	None
Inorganic salt starch agar(ISP-4)	Poor, spreading, powdery	Absent	None	None
Glycerol Aspargine agar (ISP-5)	Good, spreading, powdery	Abundant, powdery, grey	Yellow	None
Nutrient agar	Moderate, spreading, powdery	Moderate, powdery, grey	Pale yellow	None
Starch casein agar	Abundant, spreading, powdery	Abundant, powdery, grey	Pale yellow	None
Bennett's agar	Good, spreading, powdery	Moderate, powdery, grey	Yellow	None

Table 4 : Biochemical characteristics of isolate KSP-SS-06-1C/1

S.No	Reaction	Observation	Results
1.	Melanin Reaction on		
	ISP-1	No color change	Negative
	ISP-7	No color change	Negative
2.	H_2S production	No color change	Negative
3.	Tyrosinase reaction	No color change	Negative
4.	Starch Hydrolysis	Growth zone=9 mm Hydrolyzed zone=23 mm	Positive
5.	Casein Hydrolysis	No hydrolyzed zone	Negative
6.	Gelatin Hydrolysis	No hydrolyzed zone	Negative
7.	Milk coagulation and Peptonization	Coagulation followed by Peptonization	Positive Positive
8.	Nitrate Reduction	Pink color was observed	Positive
9.	Growth Temperature Range	25°C to 45°C	
10.	Chemical Tolerance(pH)	6.5 to 10	
11.	NaCl Tolerance	2% to 7%	
12.	Cell Wall Composition	LL-DAP, Glycine, Type-I	
13.	Gram staining	Stained to violet color	Positive
14.	Spore staining	No staining observed	Negative

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Cercetări Privind Utilizarea Anticorpilor Monoclonali Şi Policlonali În Evidenţierea Celulelor Stem Umane Neuronale În Creierul Fetal Ovin

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Introduction - Stem cells were first isolated from the internal cell mass of blastocyst and grown *in vitro*. Variants of these techniques for obtaining and cultivation are used today in laboratories around the world.

The most important publications related to stem cells have been centralized in the years 2001 and 2002 by R. Edwards.

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Early research on stem cells in vitro cultivation (Fig. 1) were made in 1963 by J. Paul and E. Edwards, who prepared for a group of cells growing rabbit embryo preimplantaţional in drops of NCTC 109 , 199, F10. (Theia, N.D., 2000).

A stem cell has two main properties: long-term capacity autoreînnoire without senescence and pluripotenţă and ability to differentiate into one or more specialized cell types. These cells can provide an inexhaustible source of cells for transplantation. Totipotente stem cells that have the ability to generate all types of tissue plays a critical role in human development, providing material for the development of all tissues and organs in the embryo and in all extra embryonic tissues.

GJSFR-G Classification : *FOR Code: 060410*



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At first it was thought that these adult stem cells regenerate only a very limited set of cell lines, but they demonstrated during a much greater plasticity.

II. Importance of Stem Cells

Neurodegenerative diseases are characterized by gradual and progressive damage or loss of nerve cells and neural tissue. Common are Alzheimer's disease, Parkinson's and multiple sclerosis. Neurodegenerative disorders affecting over 22 million people worldwide. Some symptomatic treatments have become possible in the past 15 years, but still not found complete treatment of these diseases.

Multiple sclerosis produce progressive brain destruction of myelin protective foil spinal neurons with progress towards irreversible disease. It is estimated that over 2.5 million people on the globe suffering from this disease. This number includes those 10,000 people in Romania. Cell replacement strategies are suitable for multiple sclerozelor because, unlike other tissues, the mammalian brain and spinal cord have a limited capacity to renew.

There is evidence that embryonic stem cells and those derived from fetal sources can generate neural cells that can be used to replace lost neurons. Mesenchymal stem cells make neurons able to migrate through the brain and spinal cord tissue seats where dysfunction is present sclerotic

a) Stem Cells - Characterization Overview

Stem cells are undifferentiated primary cells that have the ability so to differentiate into other cell types. This category includes both bone marrow cells and peripheral blood cells and embryonic cells, whose differentiation capacity up to 200 other different cell lines property which called evolutionary plasticity (Erghelegiu, Marina, 2005).

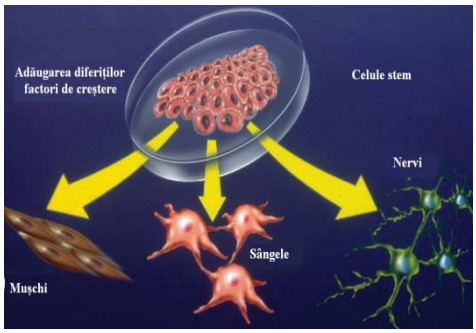

Figure 1 : Differentiation of embryonic stem cells (http://www.srsp.net/kassel/images/Pluripotent_Stem_Cells.jpg)

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Researchers believe that stem cell therapy, also called regenerative medicine, has the potential to change the face of common diseases in humans and animals by using them to replace specific tissues or organ development. Stem cell therapy experiments are currently in various medical centers around the world. There are at least 10-15 such trials in progress. The most important application of human stem cells is the generation of cells and tissues that can be used in so-called 'cell-based therapies".

b) Classification of Stem Cells

i. *Embryonic stem cells*

Embryonic stem cells from the blastocyst. Blastocyst stage of embryonic development is preimplantaţional, when the embryo consists of 150 Murin compacted blastomere arranged as spherical (Gottlieb, DI., 1999).

The blastocyst stage, embryonic cells are divided into cells forming embrioblastul (interior) and trophoblastic cells forming (from outside). Embrioblastului stem cells are cells pluripotente thus give rise to all tissues of the body except those forming the placenta and fetal envelopes. Trophoblastic cells can give rise only to the placenta and fetal envelopes (I Vintilă, 2005).

Embryonic stem cells [ES], similar to embryonic germ cells [EG], may come from the primordial germ cells which form the ova and sperm, can be isolated and grown in vitro where further proliferation and maintain their differentiation capacity (Soria , B., 2000).

The criteria defining embryonic stem cells have been developed by Weaver, CH (2006) whose studies have contributed significantly to the establishment of lists of essential characteristics of these cells.

ii. *Adult stem cells*

Adult stem cell is undifferentiated, but is in a differentiated tissue. It is able to renew the lifetime of the organism, but also generates precursors differentiated tissue. I pluripotente cellsthat can give rise to all tissues except the placenta and fetal bags.

Adult stem cells, although difficult to isolate the various organs and tissues and a small period of life, do not develop tumors, and the probability of rejecting the body is weak, especially in patients receiving organ and tissue cells harvested from their own.

c) Adult Somatic Pluripotent Stem Cells Sources And Differentiation Potency

Regarding the embryonic stem cells (Fig. 2), the main source is the embryo before the blastocyst stage. Can be obtained from the blastocyst (us) clone, is an important source of embryos resulting from insemination, in vitro "(Fraichard, A., 1995).

The 2nd source are cells derived from fertilization, in vitro"a ova with sperm are in the 'fertile banks". Researchers do not need to create embryos from sperm and ova, because it can obtain from fertilization clinics.

Sometimes couples have a child trying to create more embryos, but not all use. They can donate them for research, otherwise will be destroyed.

The 3rd source is embryonic stem cells created by SCNT (Somatic Cell Nuclear Transfer = somatic cell nuclear transfer).

This is the process by which genetic material of certain cells in the body is transferred to an egg enucleator, thus resulting in an 'embryo" produced without fertilization by a sperm. It is assumed that in fact this is a technique for cloning, which raises many questions around this subiect. Cloning is developing embryos for therapeutic use in treating diseases. The method assumes that the genetic information of a somatic cell to be transferred into an egg from which DNA was manipulated eliminat. Last source is coming from fetuses in first trimester abortions.

Adult stem cell harvesting is practiced in the medical world for over 30 years, at least in terms of their harvesting bone marrow cells (Fig. 11). 200 x 109 red blood cells are created every day in the body of haematopoietic stem cells (Eglitis, MA and Mezey, E., 1997)

For example, only 1 of 10.000-15.000 cells is bone marrow hematopoietic stem cells (blood forming).

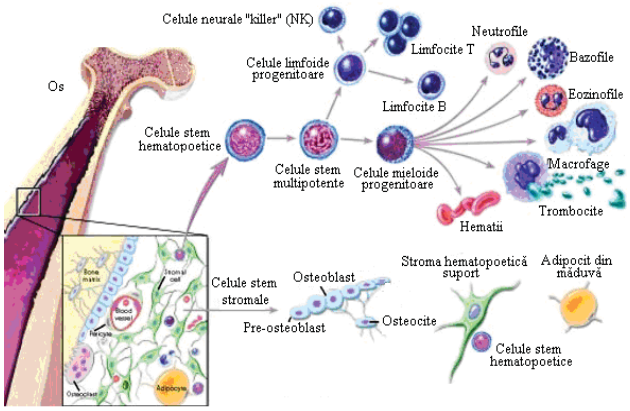

Figure 2 : differentiation of bone marrow stem cells
http://stemcells.nih.gov/info/basics/basics4.asp

The collection of the blood seems to have some advantages over harvesting of the bone marrow (Ferrari, G., 1998), meaning that you can harvest a sufficient number of stem cells, which can support more intensive meetings chemotherapy.

Basic material for the cultivation of cells in culture serve mesenchymal stem cells (CD34-), which were first identified by A. Fridenstein (et al., 1973) in the bone marrow. CD34-negative cells, which are predecessors of the bone marrow niche and leave the bloodstream. Got the blood in the appropriate

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microenvironment, they enter into interaction with signal-regulatory cells mature and form a stromal framework, which can differentiate them in glial tissue and fibroblasts. (Schuldiner, 2000)

It was also shown that in the event of localized damage, stem cells mezenhimale receive signals from damaged tissues, which trigger reparative mechanisms (Tavasolli et al., 1991). Mezenhimale and hematopoietic stem cells found in cord blood. In experimental studies it was shown that during long-term cultivation of mononuclear of cord blood, endothelial-cells predecessors under the action of certain cytokines in culture form colonies of fibroblasts. (M. Nied, 1997).

III. Human Stem Cells Evidentiation using Antibodies, Antigens, Markers and Flow Cytometry Technique

a) *Antibodies*

i. *General Characterization Of Antibodies*

Antibodies are a type of protein molecules, are also known as the immunoglobulins. There are 5 types of immunoglobulins: IgG, IgA, IGD, IgE and IgM. Antibodies are produced by B lymphocytes naturally in both forms of exocytosis: the plasma membrane and secretory full. They form the B cell antigen receptor specific antibodies found in plasma and paste specific receptors flat region of immunoglobulin. Antibodies are molecules in the shape of the letter Y (Fig. 3) is composed of 2 heavy chains and 2 light chains of polypeptides These chains are linked together by covalent and necovalente (www.sinauer.com).

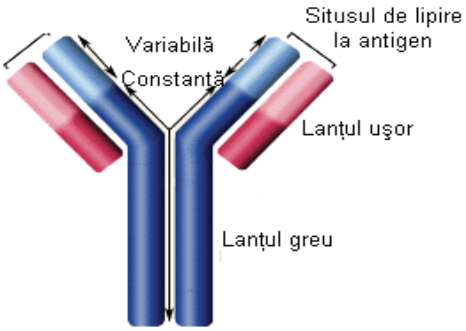

Figure 3 : The structure of an antibody (http://images.google.ro/imgres?imgurl=http://www.capemaster.net)

Primary antibodies are antibodies formed against antigens of interest (a protein, peptide, carbohydrate, or other small molecules) and are typically unconjugated. Primary antibodies that recognize and bind with high affinity and specificity of epitopii unique. A primary antibody can be very useful for the detection of biomarkers for diseases such as cancer, diabetes, Parkinson's and Alzheimer's disease and are used for the study of ADME and multi-drug resistance (MDR) of therapeutic agents (http://en.wikipedia. org / wiki / Primary _antibodies).

Specific primary antibodies used by me in my experiments were Synaptophisin, NPT II, DsRed Murin, CD Murin 31 and 20 murine Cytokeratina.

ii. *Secondary Antibodies*

A secondary antibody is an antibody that binds the primary antibody or antibody fragment. Usually they are labeled with probes that make them easy to use for detection, purification and cell sorting.

Second antibodies may be polyclonal or monoclonal and are available with specificity for whole Ig molecules or antibody fragments, such as FC (fragment crystallizable region - which is the terminal region of an antibody that interacts with receptors on the surface of a cell) or Fab regions (fragment antigen-binding - that is the region of an antibody that binds to the antigen).

Are selected by using primary antibodies, primary antibody class (eg, IgG or IgM), and the type of probe that is preferred. Identification of optimal secondary antibody is normally carried out by more tests (http://en.wikipedia.org/wiki/Secondary_antibody).

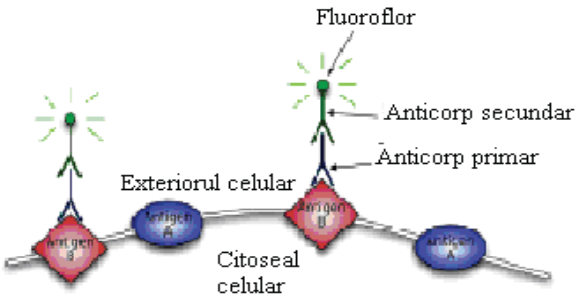

Figure 4 : Attaching the secondary antibody primary antibody http://upload.wikimedia. org/wikipedia/commons/thumb/f/fc/Primary-Secondaryantibody.svg/300px-Primary-Secondaryantibody.svg.png

Secondary antibodies specific to my use of my experiments are the Alexa 488 goat anti Flor Murin and goat anti rabbit Alexa Flor 633 Murin both goat and goat anti-rabbit anti-goat Alexa 594 anti Flor Murin.

b) *Antigens*

Antigen (Greek: anti = against and geano = to give birth, to generate) is the term that defines any substance endogenous or exogenous origin, which once reached the body, is recognized as its own and causes an immune response, aimed at neutralizing and elimination. (Http://ro.wikipedia.org/wiki/Antigen).

Due to the similarity of these stem cells have with other types of cells, the question of how to differentiate. The answer came through stem cell markers. These markers are represented such as protein receptors from the cell envelope. They have the ability to selectively bind or join with other molecules

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signals. Normally, cells use these receptors and the molecules attached to them for communication and smooth the body functions.

Currently researchers use to identify specific stem populations, a combination of chemical properties of fluorescence with a single model of surface receptors. Technique called 'florescence-activated cell sorting (FACS) (Shamblott, MJ et al., 2001). Identification of stem cells is also based on the presence of genes by PCR technique (polymerase chain reaction). (Shamblott, M.J., 2001).

c) *Flow Cytometry*

Continuous-flow cytometry is a technique of counting and examination of microscopic particles such as cells and chromosomes by their suspension in a stream of fluid and passing it through an electronic device detection. This allows simultaneous analysis of physical and chemical characteristics of thousands of particles per second.

Flow cytometry is used routinely in the diagnosis of health disorders, particularly in cases of blood cancer, but has many other applications, both in research and in clinical practice (Recktenwald DJ. 1993).

► *The principle of the flow cytometry*

A beam of light (laser light, usually) a single wavelength is directed by a hydrodynamic flow of liquid concentrate. A series of detectors are directed towards the point where the stream passes through beam of light: one beam of light according to the dispersion of the forward (Forward Scatter) and the dispersion of the light beam perpendicular (Side Scatter). Each particle size from 0.2 - 150 micrometer beam of light passing through chemical substances found in fluorescent particles or attached to particles can be excited to emit light at a wavelength greater than the source of light. FSC correlates with cell volume and SSC depends on the complexity inside the particle. (Givan A. 2001).

IV. Results of National and International Research Regarding the Culture of Stem Cells

a) *Current Status of the National Research*

► The first public bank of placental blood in our country who have free access to all Romanian citizens working in Cluj-Napoca, Romania using EUROCORD Foundation. Another private bank placental blood was opened in Bucharest, the CMU, in collaboration with the Stem-Health Greece (http://www.contraboli.ro/bank-of-cells-stem-to-Cluj).

► A team of doctors including Dr. Irinel Popescu, performed the first stem cell transplant for liver disease in Romania. Patient T.L. aged 46 years, initially suffered from a liver cirrhosis and liver cancer.After four years of operation ill T.L. was cured as cirrhosis and cancer. (http://www.evenimentul.ro/ article / first-transplant-to-cell-stem-in-romania.html).

► Other investigations were performed by Daria Maria Groza in his doctoral thesis titled experimental research on behavior in vitro and in vivo stem cells in human reproduction", 2009. The aim of this PhD thesis is the practical application of current knowledge on stem cells from fetal and Annexes umbilical cord blood and experimental research on the isolation, differentiation and in utero transplantation of human stem cells obtained from animal model, using cutting-edge biotechnologies.

b) *Current Status of the International Research*

► A new stage in the use of adult stem cells is one from 1989, the discovery and use as a source of adult stem cells, extracted from umbilical cord blood. Even in that year, was successful cord blood transfusion. This was the first clinical evidence that cord blood is indeed a source of adult stem cells. (http://www.produsenaturiste.net/pages/Stimularea-pe-cale-naturala-a-celulelor-stem-adulte.html).

► In America there was an experiment of this kind. In laboratory conditions the stem cells of a cow have obtained a functional kidneys that were transplanted into animals. The research and experiences of adult stem cells raises many moral and ethical questions. (http://dictionar.romedic.ro/transplant-de-celule-stem).

V. Purpose and Objectives of Research

a) *The Aim of the Research*

International studies conducted to date in the stem cells have demonstrated their characteristics, become nowadays an important tool of regenerative medicine. In Romania, there are few studies that follow this line of inquiry, which is focused mainly on elucidating the specifics of adult stem cells.

The present study was undertaken to test the ability of monoclonal antibodies following primary Synaptophisin, NPT II, DsRed Murin, CD Murin 31 and 20 murine Cytokeratina and the secondary antibodies Alexa Flor 488 both goat and goat anti Murin anti rabbit Alexa Flor 633 the goat anti Murin and goat anti rabbit and Alexa 594 goat anti Flor Murin. They are cited in the literature (JW Goding, 1984, AM Campbell, 1984) and are used in order to highlight the existing human stem cells in fetal sheep brain.

It should be stressed that although there are research findings involving stem cells, the results are far from satisfactory. This is why we chose to perform the study.

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b) *Objectives of the Research*

Research objective was to identify the most appropriate methods of recognizing human stem cells migrated into the fetal sheep brain. Methodological objectives are targeted testing several sets of monoclonal and polyclonal antibodies to identify possibilities for use in highlighting human stem cells in fetal sheep brain. To this end, the plan was compiled experimental research study taking into 8 sets of monoclonal and polyclonal antibodies, whose effectiveness has been tested on brain derived from fetal sheep. He practiced the same testing methodology, based on specific fluorescence emission principle, that red color when nerve cells and double sheep red - green for human neurons. Citometriei in continuous flow technique, we determined that the antibody is suitable to identify human stem cells.

VI. Researches Regarding Monoclonal and Policlonal Antibodies Utilization in Stem Cells Evidentiation

a) *Materials and Methods*

i. *Materials*

a. *Biological Materials*

To test monoclonal antibodies has been studied a lot of sheep and their fetuses belonging merino breed of livestock of the farm in Reno, Nevada

As organic materials were used:

-Fetal sheep brain, stem cells from existing cell lines in the laboratory cell bank in Reno, Nevada, antibodies, antigens, markers.

b. *Chemical Materials*

- Solutions of PBS (Phosphate Buffered Saline), NGS, blocking solution (buffer bloking), Ethanol, Methanol, Alcohol different concentrations (100, 95 and 75%), antigen recovery solution (antigen retrival), DAPI (4 ',6-diamidino-2-phenylindole)-fluorescent dye that binds to DNA, Hoecst, Media Dako, Dulbeco, DMEM (Dulbeco odified Eagle Medium), sterile water, alcohol medicinal and disinfection solutions.

c. *Laboratory Equipment*

Equipment used to carry out experiments was: microtome, electron microscope, confocal microscope, Olympus Bx 60 microscope (Epifluorescence microscope), PCR devices, UV lights, centrifuges, stirrer ultraturax (Helidolph), Apparatus for ice product (Bremen), autoclave (Raypa), Bath Marine (Bioblock Fisher Scientific polystat), analytical balance (320m AW Schimadzu) Biohazard waste containers (Bremen), deionizator water (SMEG WP 3000), oven (Memmet), refrigerators, freezers, fume for chemicals (Kottermann) PCR hood (sterile), microcentrifuge (Sigma 1-15), pH - meter (InoLab), UV-VISNano Drop ND-1000, Vortex (Schimadzu).

d. *Matriale Laboratory Supplies*

Glassware, blades, blades, tubes, Bisturie, gloves, pipettes (Eppendorf) with adjustable volume of 0,2-5 ml, 5-10 ml, 50-100 ml and 100-1000 ml, tubes (Eppendorf) for 1.5 ml pipette tips of different sizes (large, medium and small), the volume of 0,2-5 ml, 5-10 ml, 50-100 ml and 100-1000 ml.

ii. *Staining methods*

Specific antibody testing methods are used to reach the stage and coloring other steps must be taken first, as follows:

a. *Sampling of biological material (tissue from fetal sheep brain)*

→ *Collection*

Harvesting is the operation of taking small pieces of tissue or organ, or in the living body or from cadavers. Although often considered a trivial operation, it is actually one of the most important steps in making permanent preparation.

→ *The sampling of the brain*

Adult animals in 58-60 days pregnant were subjected to surgery, which were removed through the incision made uterine horns, fetuses intraperitoneally injecting is a certain amount of human stem cells. After a week of the operation of adult animals are slaughtered and their fetuses are collected organs of interest. They are treated and kept.

Parts of the tissues of these organs are preserved in paraffin blocks or ice. In this way can be stored for long periods of time.

b. *The inclusion of biological material in blocks of paraffin*

→ *Standing preparation*

Make a permanent preparation requires a more complicated procedure in comparison with fresh preparation. To be completed execution of a number of stages or times of work, each having a decisive influence on the quality of the preparation.

In most cases these phases are: harvesting, fixing, washing, including, cutting, coloring and installation. How to do these steps is often tailored to the needs of parts processed. Thus, these working time can be shortened or extended, simplified or complicated depending on the situation. The fact is that only one who knows how to work this time Drinking from the sample needs to obtain the final preparation of histological quality.

→ *Fixing*

For histological study is not enough evidence to be examined to be transparent and have adequate optical contrast.

Cells and tissues (especially those taken from animals) are unstable physically and chemically. Cells

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and extracellular materials must be "preserved" so that structural changes and chemical composition of parts processed histologically to be minimal. This "conservation" is the subject of fixing. Simply put, fix is to stop the vital phenomena of cells and tissues as faithful preservation of their structure.

Formaldehyde is a gas, but the histology is used as a solution from 37 to 40% formaldehyde in water solution called formalin. If Formalin is used as a fixative in simple aqueous solutions, it should be diluted with a few days before, and when using the formalin neutralized can be used immediately.

→ *Money*

After setting Catchers action must be stopped by removal of parts. Otherwise, continue acting, Catchers will gradually distort parts of biological parts. The only set in which parts can be stored for longer without the major changes is Formalin. Catchers washing of the end of fixing parts water or alcohol use, depending on composition Catchers used.

Money should be made compulsory in water after fixation with dichromate, chromic acid or dinitrogen osmium. May be used for this purpose special containers with perforated walls and cork than cork.

→ *Inclusion in paraffin*

After washing, water is extracted components (dehydration) and replaced with solvents of paraffin (clarification), and the final piece is infiltrated with molten paraffin (parafinare). Standard procedure is valid for pieces with a thickness of 3-5 mm. Times for dehydration, clarification, infiltration with paraffin can be shortened or extended, as the pieces are larger or smaller.

If using fixatorii alcoholics early stages of dehydration are omitted. For the entire procedure volume of liquid must be 10 to 20 times the volume of the piece.

c. *Slicing*

After solidification of paraffin blocks containing biological parts, it can move to cutting them. The operation is executed most frequently with a rotary microtome, although it may be used microtoame tilt or sliding. Sections are obtained as thin slices, with an average thickness of 6 to 7 ìm. With a special skill can obtain ribbons consisting of a sequence of sections that may have a thickness of only 4 ìm. Sections obtained will be glued to strips of glass with a thin layer of albumin Mayer.

d. *Deparaffinization*

Paraffin is removed using Xylene. Allow slides in this solution for 10 minutes at room temperature. If necessary, repeat until the total removal of paraffin.

e. *Staining*

Histology using numerous coloring solutions, typically used at room temperature.

This extraction process is called differentiation or color fading. The success of both methods of staining depends on the colors that generally have a greater affinity to certain structures than to others. Staining is called simple when using a single color (eg simple methylene blue color), dual two solutions are used coloring (eg with hematoxylin-eozină dual color) and three solutions are used tricromică coloring (eg tricrom Masson). The most used method of staining of histological sections with hematoxylin is dual color (hemalaun) and eozin.

f. *Mounting*

After staining, sections are mounted in a substance that protects and does not affect section contrasts. The mounting medium is used Canada balsam. Canada balsam is naturally a yellow viscous liquid, which is softened by heating. By drying it becomes solid and the installation must be done properly mixed with xylene.

b) Staining Techniques

The application of immunohistochemical methods are-established some general principles concerning the purpose of methods, choice of primary antibodies, the binding agent system view.

- The purpose of primary processing methods for possible adaptation involves making immunohistochemical methods. In most cases, these techniques are performed for malignant tumors and rarely imunofenotipizarea with other injuries. This should be considered especially in harvesting, fixing and including in paraffin..
- Inhibition of endogenous peroxidase is always done with hydrogen peroxide 3%, 5 minute is usually sufficient.
- Choice of buffer solution is optional only in part, because some antibodies require saline phosphate buffer, pH 7.2 to 7.6 and other antibodies require the use of Tris buffer.
- Choice of primary antibody should be adequate for the purposes resulting from examination of preparations stained with routine methods.

Viewing will be done preferably with diaminbenzidin (DAB). DAB solution is activated with hydrogen peroxide before and in this form is active only 12 o'clock. Aminoethyl carbazole (AEC) prevent dehydration in alcohols and clarification so that installation will be done in aqueous medium and preparations are not stable.

i. *In vivo staining*

This technique is used to stain living tissues. Causing certain cells or structures to get a contrasting color, they can more easily study the morphology and position within a tissue. The main purpose of staining is to discover certain cytological details could be omitted.

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By this technique can show where there are chemicals or specific chemical reactions which take place within cells or tissues.

ii. *In vitro staining*

Staining in vitro cell or tissue staining means it is no longer alive. Certain colors are often combined to have more staining power than a single dye to reveal the detail and quality than a single color. Used with restraint protocols and sample preparation, this technique can be used in making the diagnosis.

iii. *Counterstain Technique*

This is the technique that is used when using the simple technique of color are not results, making the cells or cellular structures more visible. For example, "crystal violet" color only gram-positive bacteria. Counterstain technique is used with safrarin all cells, permiting the identification of Gram-negative bacteria.

Often these techniques are called 'critical techniques", because colors are placed in living organisms. However, some dyes are toxic to organisms.

To achieve the desired results, colors are used in dilutions from 1:5000 to 1:500000. (Howey, 2000).

c) Interpretation of the Immunohistochemistry Stained Tissue

Although positive imunoreacţiile eliminates much of the examiner's subjectivity, it is necessary first of all a strict evaluation of the quality of preparation. To this effect must be taken into account a number of parameters:

- external positive control - will include with the blade to study in technical work (eg investigation of anti-CD3, external positive control section of thymus; enolază investigate the neuronal specific external positive control section of cerebral cortex).
- External Negative Control - is a section of the biopsy to study that instead of antibody solution is applied "negative control" the result will compare with that obtained on section treated with antibody. Most antibodies are supplied with the negative control solution.
- internal positive control - turns on section substrates antibody positive potential (eg anti-von Willebrand stains the cytoplasm of endothelial cells, anti-smooth muscle Actin stains miocitele smooth mioepiteliale cells and miofibroblastele).
- internal negative control - no color antibody substrates that are potentially negative (eg epithelialsquamous citokeratina 8 no color, no color desmina epithelial cells or connective).

i. *Immunohistochemistry protocol*

a. *From paraffin tissues*

1. remove paraffin, using Xylene. Allow slides in this solution for 10 minutes at room temperature. If necessary, repeat until the total removal of paraffin.
2. Are place of the blade tissue rehydration, repeated transfer of ethanol solutions of concentrations 100%, 95% and 75% for one minute at each concentration. Finally leave the water a minute.
3. Se incubated in solution of antigen retrival (antigen recovery solution) at a temperature of 93 ° C for 5 minutes, then left to cool at room temperature.
4. Leave in PBS solution.

After these steps, slides with fixed tissue are prepared for staining with primary and secondary antibodies, in order to highlight the cells of interest.

b. *From paraffin and ice tissues*

1. Rinse slides in PBS .Se of 3 x 5 minute
2. Incubate in NGS solution, which is a buffer - the buffer solution (consisting of PBS + 10% normal goat serum) for 1 hours at 4 ° C, or 15 to 30 minutes at room temperature
3. Rinse slides with PBS solution + 2% NGS, 2 x 5 minute
4. Incubated with primary antibody diluted with PBS + 2% NGS for 3 hours at room temperature or overnight at 4 ° C in a room humidifier. Primary antibody is on all slides, except the negative control strip, which is available only secondary antibody.
5. Rinse slides with PBS solution + 2% NGS, 3 x 5 minute
6. Incubated with secondary antibody, diluted in PBS + 2% NGS for 1 hours at room temperature.
7. Secondary antibody is on all slides, the amount of 2.5 ml / blade.
8. Rinse slides with PBS solution + 2% NGS, 3 x 5 minutes.
9. Rinse slides with PBS solution
10. Add a few drops of each blade DAPI dye and leave 5 minute. If there is DAPI, HOECHST solution can be used in dilution of 1 ml / 5 ml PBS
11. Rinse with PBS solution
12. Dry slides at room temperature and sealed with the strip, using Cytoseal 60 for sealing blades.

d) Importance of Staining Technique

Staining technique is the procedure by which a histological specimen is viewed in full resolution by setting it on a blade placed on the support slot located on the light microscope. Stainingul is an ancillary technique used in microscopy to enhance the contrast of the image seen in electron microscope.

The biochemistry of this technique is used for substrate and DNA specific dyes, proteins, fats and carbohydrates, to qualify and quantify the presence of specific components. This technique is widely used in biology and medicine to highlight structures in biological tissue. Staining can be used to define and examine muscle or connective tissue, organic cells or cell populations.

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e) *Antibodies Test Sets*

i. *Testing of the first set of antibodies by performing a dual staining on the tissues in paraffin*

a. *Results and discussion*

In test 1, in which primary antibodies were used Synaptophisina NPT II murine and rabbit, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 633 goat anti-rabbit, although they were strictly observed the reaction conditions, concentration and reaction sequence of recommended protocols, have not achieved expected results.

In figure 5 it can be seen that there has been coloring, but failed to identify human stem cells, so could not determine their existence in the individual brain with number 2246.

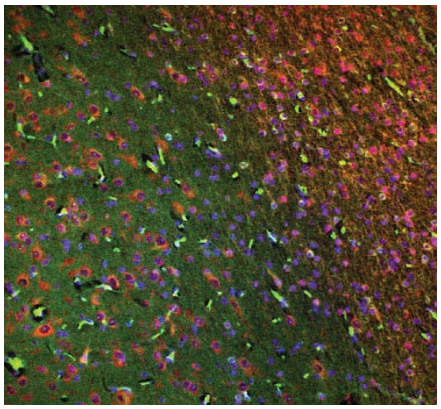

Figure 5 : Double staining with antibodies Synaptophisină primary murine and rabbit NPT II, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 633 goat anti-rabbit (original)

The literature data were found to refer to test these antibodies in highlighting human neural stem cells exist in fetal sheep brain.

ii. *Testing of the II set of antibodies by making a single color, the tissues in paraffin*

To conduct this test in order to reflect existing human stem cells in fetal sheep brain, we used the individual with the number 2269.

There is an easy color to be able to easily distinguish between human and sheep cells, using as primary antibody Murin DsRed and the secondary antibody Alexa 488 goat anti Flor Murin. 10 blades were used.

b. *Results and discussion*

For test 2, the primary antibody were used DsRed Murin, and the secondary antibody Alexa 488 goat anti Flor Murin. In Figure 6 you can see the dye occurred, but failed to identify human stem cells, so could not determine their existence in the individual brain with number 2246.

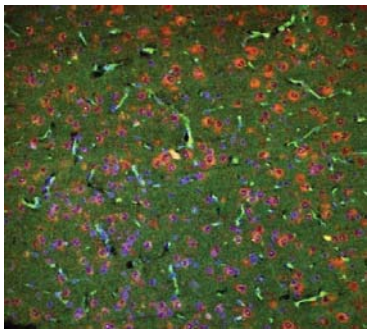

Figure 6 : Staining with primary antibody DsRed Murin and secondary antibody Alexa 488 goat anti Flor murine (original)

Neither this set of antibodies were not found in the literature data refer to test these antibodies in highlighting human neural stem cells exist in fetal sheep brain.

iii. *Testing of the III antibodies by performing a set of double color, the tissues in paraffin*

To conduct this test in order to reflect existing human stem cells in fetal sheep brain, we used the individual with the number 2267. Double staining was performed in order to lighten the difference between human and sheep cells, using as primary antibodies and CD 31 Murin Murin Cytokeratina 20 and the secondary antibodies Alexa 488 goat anti-Murin Flor and Flor Alexa 633 goat Anti Murin. 20 blades were used.

c. *Results and discussion*

Neither test has been conducted with 3 primary antibodies and CD 31 mouse and Cytokeratin 20 and the secondary antibodies Alexa 488 goat anti-mouse Flor and Alexa flor 633 goat anti mouse, did not lead to expected results. Note that in this case have been strictly observed the reaction conditions, concentration and reaction sequence of recommended protocols. In Figure 7 it can be seen that there has been coloring, but failed to identify human stem cells, so could not determine their existence in the individual brain with number 2267.

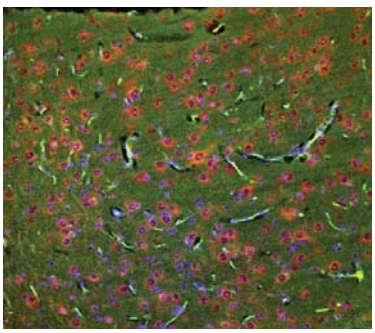

Figure 7 : Double staining with primary antibodies CD 31 and Cytokeratina Murin Murin and 20 antibodies Alexa 488 goat anti-Murin Flor and Flor Alexa 633 goat anti-murine (Original)

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Neither this set of antibodies were not found in the literature data refer to test these antibodies in highlighting human neural stem cells exist in fetal sheep brain.

iv. *Testing of the IV set of antibodies, by making a double color, the tissues in paraffin*

To conduct this test in order to reflect existing human stem cells in fetal sheep brain, we used the individual with the number 2248. Double staining was performed, with 30 slides available. In order to lighten the difference between human and sheep cells were used as primary antibodies UCHL1 polyclonal, rabbit monoclonal DsRed mouse, and the secondary antibodies Alexa Flor 633 goat anti rabbit and Alexa 488 goat anti Flor mouse.

d. *Results and discussion*

In test 4, where I used UCHL1 polyclonal primary antibodies, rabbit monoclonal DsRed mouse, and the secondary antibodies Alexa Flor 633 goat anti rabbit and Alexa 488 goat anti Flor mouse. In Figure 8 you can see the dye occurred, but failed to identify human stem cells, so could not determine their existence in the individual brain with number 2248.

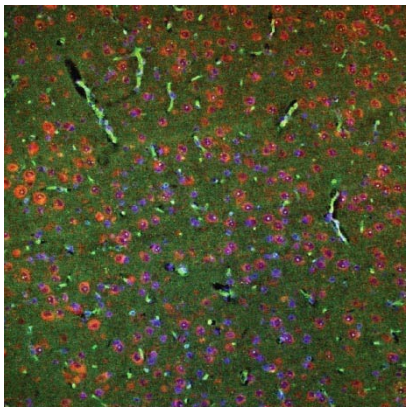

Figure 8 : Double staining with UCHL1 polyclonal primary antibodies, rabbit monoclonal DsRed Murin, and the secondary antibodies Alexa 633 goat anti rabbit Flor and Flor Alexa 488 goat anti-mouse (original)

The literature data were found to refer to test these antibodies in highlighting human neural stem cells exist in fetal sheep brain.

v. *Testing of the V set of antibodies, by making a double color, the tissues in paraffin*

To conduct this test in order to reflect existing human stem cells in fetal sheep brain, we used the individual with the number 2270. Double staining was performed, with 30 slides available. In order to lighten the difference between human and sheep cells were used as primary antibodies monoclonal DsRed Murin and Chromatogranină A Rabbit, and the secondary antibodies.

e. *Results and discussion*

For test 5, DsRed was used as primary monoclonal antibody and Chromatogranină Murin A Rabbit, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 633 goat anti-rabbit.

Again, although they were strictly observed the reaction conditions, concentration and reaction sequence of recommended protocols, have not achieved expected results. In Figure 9 you can see the dye occurred, but failed to identify human stem cells, so could not determine their existence in the individual brain with number 2270.

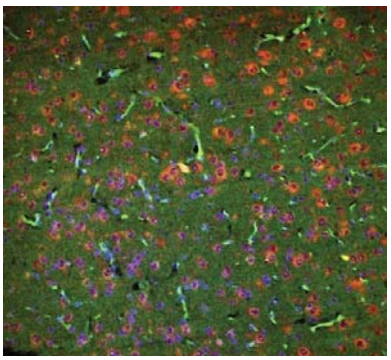

Figure 9 : Double staining with primary antibodies and DsRed monoclonal Murin Chromatogranină A Rabbit, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 633 goat anti-rabbit (original)

Neither this set of antibodies were not found in the literature data refer to test these antibodies in highlighting human neural stem cells exist in fetal sheep brain.

vi. *Testing of the VI set of antibodies by performing a double color, the tissues in paraffin*

To conduct this test in order to reflect existing human stem cells in fetal sheep brain, we used the individual with the number 2280. Double staining was performed, with 30 slides available. In order to lighten the difference between human and sheep cells were used as primary antibodies and Oligodendrocyte Murin Chromatogranină A Rabbit, and the secondary antibodies Alexa 594 goat anti Murin Flor and Flor Alexa 633 goat anti-rabbit.

f. *Results and discussion*

For test 6, primary antibodies were used Oligodendrocyte Murin and Chromatogranină A Rabbit, and the secondary antibodies Alexa 594 goat anti Murin Flor and Flor Alexa 633 goat anti-rabbit.

Again, although they were strictly observed the reaction conditions, concentration and reaction sequence of recommended protocols, have not achieved expected results. In figure 10 it can be seen that there has been coloring, but failed to identify human stem cells, so could not determine their existence in the individual brain with number 2280.

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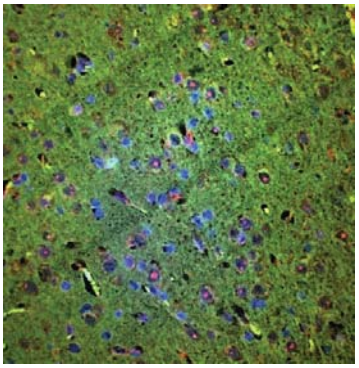

Figure 10 : Double staining with primary antibodies and Oligodenrdocyte Murin Chromatogranină A Rabbit, and the secondary antibodies Alexa 594 goat anti Murin Flor and Flor Alexa 633 goat anti-rabbit (original)

Neither this set of antibodies were not found in the literature data refer to test these antibodies in highlighting human neural stem cells exist in fetal sheep brain.

vii. *Testing of the VII antibodies by performing a set of double color, the tissues in paraffin*

To conduct this test in order to reflect existing human stem cells in fetal sheep brain, we used the individual with the number 2281. Double staining was performed in order to lighten the difference between human and sheep cells, using as primary antibodies and DsRed Murin Chromatogranina A Rabbit, and the secondary antibodies Alexa 488 goat anti Flor Murin and Alexa Flor 594 goat anti-rabbit. 20 slides were used.

g. Results and discussion

For test 7, primary antibodies were used DsRed Murin and Chromatogranină A Rabbit, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 594 goat anti-rabbit. In figure 11 it can be seen that there has been coloring, but failed to identify human stem cells, so could not determine their existence in the individual brain with number 2281.

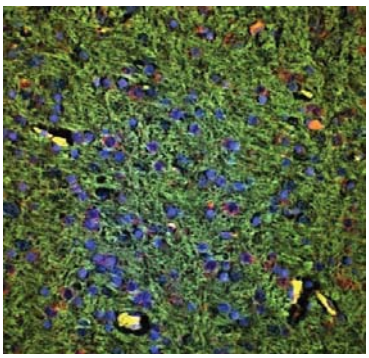

Figure 11 : Double staining with primary antibodies and DsRed Murin Chromatogranină A Rabbit, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 594 goat anti-rabbit (original)

Neither this set of antibodies were not found in the literature data refer to test these antibodies in highlighting human neural stem cells exist in fetal sheep brain.

viii. *Testing of the VIII antibodies by performing a set of double color, the tissues in paraffin*

To conduct this test in order to reflect existing human stem cells in fetal sheep brain were used individuals in 2270, 2271, 2272, 2273, 2274. Double staining was performed, with each 4 blades available from each individual, 20 slides.

In order to lighten the difference between human and sheep cells were used as primary antibodies monoclonal DsRed Murin and Chromatogranină A Rabbit, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 594 goat anti-rabbit.

h. Results and discussion

The test 8 was conducted with DsRed monoclonal primary antibodies and Chromatogranin A Rabbit, and the secondary antibodies Alexa 488 goat anti mouse and Alexa 594 goat anti-rabbit. The only test that led to expected results. Note that in this case have been strictly observed the reaction conditions, concentration and reaction sequence of recommended protocols. In figure 12 it can be seen that there has been coloring and stem cells could be identified uname, so it could establish their existence in the brain of all individuals analyzed, which had a positive DsRed mouse primary antibody and a rabbit with Chromatogranin A secondary antibodies Alexa 488 goat anti mouse Flor and Flor Alexa 594 goat anti-rabbit.

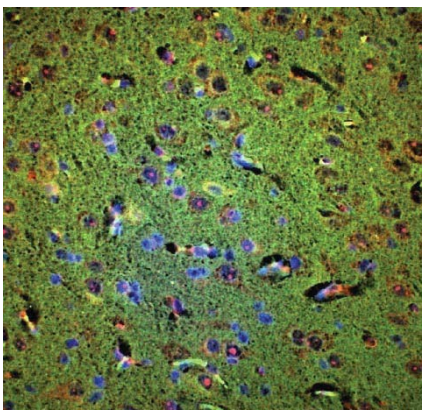

Figure 12 : Double staining with primary antibodies and DsRed monoclonal Murin Chromatogranină A Rabbit, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 594 goat anti-rabbit (original)

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V. Characterization of the Antibodies using the Flow Cytometry Technnique

Flow cytometry is a technology that allows a single cell to be measured for a variety of features, the measurements are determined only by observation of cells passing through a liquid. The instruments used for this can gather information about cells by measuring the fluorescent emission of visible light, allowing a cell sorting based on physical characteristics, biochemical and antigenic. (http://en.wikipedia.org/wiki/Flow_cytometry)

a) Materials and Methods

i. *Materials*

a. Biological materials

As organic materials were used: fetal sheep brain, stem cells from existing cell lines in the laboratory cell bank in Reno, Nevada, blood fetal sheep

b. Chemical materials

-dyes fluorescent-FITC (fluoresceinisothio-cyanate), PE (ficoeritrină), PCA (aloficocianină) antibodies: CD4, CD8, CD25, Mouse IgG 1

c. Laboratory equipment

-flow-cytometric apparatus continue

d. Matriale Laboratory supplies

-Glassware, tubes, gloves, pipettes (Eppendorf) with adjustable volume of 0,2-5 ml, 5-10 ml, 50-100 ml and 100-1000 ml, tubes (Eppendorf) of 1.5 ml pipette tips of different sizes (large, medium and small), the volume of 0,2-5 ml, 5-10 ml, 50-100 ml and 100-1000 ml.

b) Results and Discussion

Following application of flow cytometric technique to identify human stem cells from fetal sheep brain, some of the foundings are:

1. Is observed on CD4-positive population ov marked with P1 representing 9 events, respectively, and 0.41% of total CD 25 FITC-positive population ov marked P2, representing 77 events, ie 3.50% of total cells (Fig. 13). Starting from the premise that the antibodies used were created to recognize human stem cells, we can say that 3.91% of analyzed cells are human stem cells from individuals in 2164 (Annex. 1).

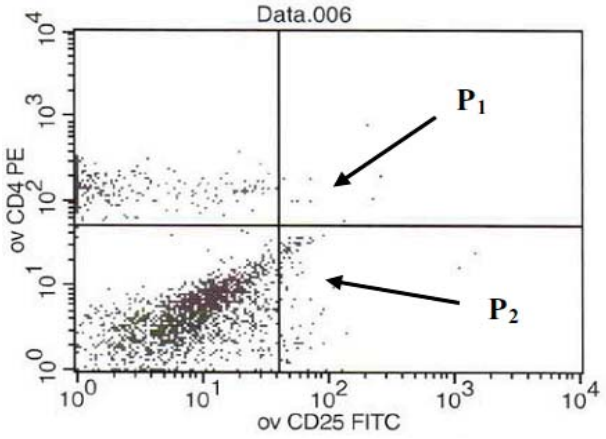

Figure 13 : Results of analysis of CD4 and ov ov CD25 antibody (original)

2. Positive population is observed on ovCD8 marked P1 representing 40 events, respectively, and 1.07% of total CD4-positive population ov FITC marked P2, 767 events representing respectively 20.49% of total cells (Fig. 14). Starting from the premise that the antibodies used were created to recognize human stem cells, we can say that 21.56% of analyzed cells are human stem cells from individuals in 2165 (Annex 1).

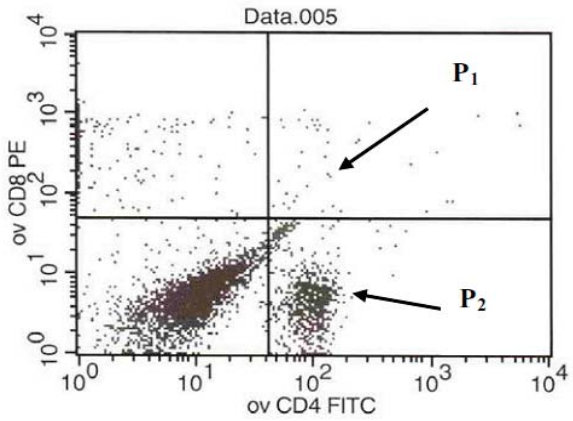

Figure 14 : Results of analysis of antibodies ov ov CD8 and CD4 (original)

3. Positive population is observed on ovCD8 marked P1 representing 84 events, respectively, and 1.31% of total CD4-positive population ov FITC marked P2, representing the 2083 events, respectively 32.38% of total cells (Fig. 15). Starting from the premise that the antibodies used were created to recognize human stem cells, we can say that 33.69% of analyzed cells are human stem cells from individuals in 2166 (Annex 2)

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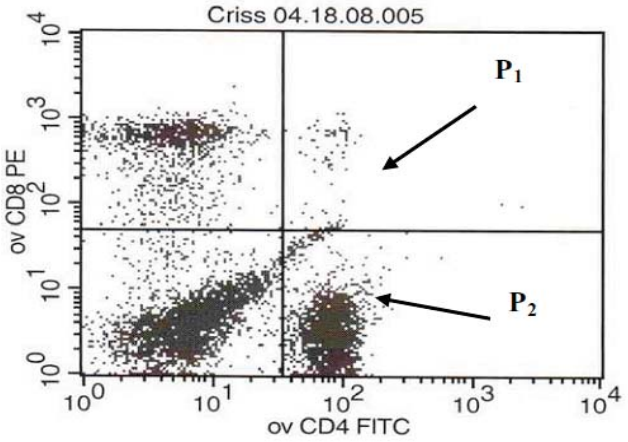

Figure 15 : Results of analysis of antibodies ov ov CD8 and CD4 (original)

4. Positive population is observed on ovCD4 marked P1 representing 45 events, respectively, and 0.68% of total positive population ov CD 25 FITC marked P2, representing 202 events, ie 3.06% of total cells (Figure 16) Starting from the premise that the antibodies used were created to recognize human stem cells, we can say that 3.74% of analyzed cells are human stem cells from individuals in 2169 (Annex 2).

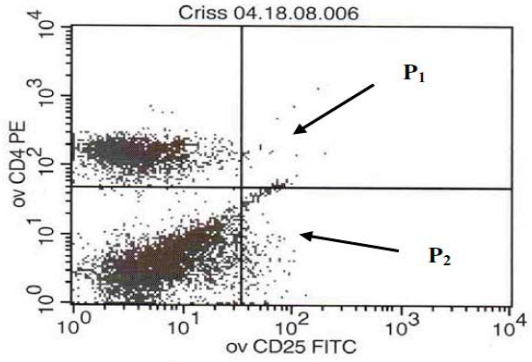

Figure 16 : Results of analysis of CD4 and ov ov CD25 antibody (original)

VI. Statistical Intepretation of the Data Regarding the Total Amount of the Cells, Sheep Neurons and Human Stem cells Identiffied by the used Antibodies

Average number of cells shown in fetal sheep brain recorded at between 761.70 when disclosure was made with the set of antibodies VII and 610.50 when highlighting kit antibody was performed with V. There was variability between 30.68% for set VI antibody and 15.74% for set II antibodies (Table 3)

Table 3 : Averages and dispersion parameters for all cells highlighted in the 8 sets of antibodies used

Issue	n	$\overline{X}$	±	$s_{\overline{X}}$	V%
Set I of antibodies	20	674,95	±	1,79	1,19
Set II of antibodies	10	691,50	±	1,72	0,79
Set III of antibodies	20	757,10	±	0,79	0,47
Set IV of antibodies	30	675,03	±	1,67	1,36
Set Vof antibodies	30	614,33	±	1,28	1,14
Set VI of antibodies	30	680,60	±	2,08	1,67
Set VII of antibodies	20	744,05	±	1,67	1,01
Set VIII of antibodies	20	764,05	±	1,21	0,71

The average number of neurons in the brain of fetal sheep sheep highlight recorded values between 614.33 when evidence was made with the set of antibody V and shown as 764.05 when performed with a set of antibodies VIII. There was variability between 0.47% for set III antibody and 1.67% for set VI antibody (Table 4)

Table 4 : Averages and dispersion parameters for sheep neurons highlighted in the 8 sets antibody

Issue	n	$\overline{X}$	±	$s_{\overline{X}}$	V%
Set I of antibodies	20	225,90	±	1,74	39,43
Set II of antibodies	10	192,40	±	1,59	56,97
Set III of antibodies	20	123,90	±	1,65	35,39
Set IV of antibodies	30	160,60	±	0,93	27,30
Set Vof antibodies	30	167,23	±	1,48	32,72
Set VI of antibodies	30	213,47	±	0,98	34,99
Set VII of antibodies	20	175,50	±	1,31	39,66
Set VIII of antibodies	20	158,90	±	1,19	22,87

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Human stem cells were identified only when testing was performed with the set VIII antibody. He obtained an average of 4.50 cells and a very high coefficient of variability, 48.29% respectively. If we refer to the total number of cells in the brain evidenced by Kit antibody VIII (Table 1), found that the number of identified human stem cells is only 5.91% of them.

*Table 5 :*The averages and dispersion parameters for the human stem cells emphasized with VIIIth set of antibodies

Specificare Issue	n	$\overline{X}$	±	$s_{\overline{X}}$	V%
Setul VIII de anticorpi	20	4,80	±	0,39	36,04

VII. Conclusions

1. After conducting the experiment to test the possible use of staining techniques (staining) of neural stem cells in sheep tissue with a series of monoclonal antibodies, the result the following conclusion: Set 1 of antibodies, consisting of primary antibodies Synaptophisin and NPT II, and the secondary antibodies Alexa 488 and Alexa 633, not suitable for use to reflect neural stem cells in sheep tissue by staining technique developed and perfected in the laboratory of the Department of Biotechnology University Reno, Nevada.

2. Neither set of antibodies 2, consisting of DsRed Murin primary antibody and secondary antibody Alexa 488 goat antimurin, not suitable for use to reflect neural stem cells in sheep tissue by staining technique developed and perfected in the laboratory of the Department of Biotechnology, University Reno , Nevada, USA.

Again assume that their structure does not lend itself to the formation of a COMPEX stem cells producing fluorescence, visible confocal microscope.

3. The results were also negative for antibodies set 3, consisting of primary antibodies CD 31 murine and 20 Murin Cytokeratina and secondary antibody Alexa 488 goat anti-Murin, not suitable for use to reflect neural stem cells from sheep tissue by staining technique developed and perfected in the laboratory of the Department of Biotechnology, University Reno, Nevada.

Our results show that neither CD 31 murine primary antibody structure and Cytokeratina 20 Murin and secondary antibody Alexa 488 goat anti-Murin is not compatible with stem cells to form a COMPEX producing fluorescence, visible confocal microscope.

4. Set 4 antibody, UCHL1 up of primary polyclonal antibodies, monoclonal rabbit DsRed Murin, and the secondary antibodies Alexa 633 goat anti-rabbit and Alexa 488 goat anti Murin not suitable for use to reflect neural stem cells in sheep tissue The staining technique developed and perfected in the laboratory of the Department of Biotechnology, University Reno, Nevada.

Suppose that the structure does not lend itself to the formation of a COMPEX stem cells producing fluorescence, visible confocal microscope.

5. Neither set of antibodies 5, 6 and 7 were positiv for what I have searche for.

8. The results were positive for antibodies set 8, consisting of primary DsRed monoclonal antibodies and Chromatogranină Murin A, and the secondary antibodies Alexa 488 goat anti-rabbit and Alexa 594 goat anti Murin, which do not lend themselves to use stem cells to highlight neural tissue of sheep by the dye technique developed and perfected in the laboratory of the Department of Biotechnology, University Reno, Nevada.

The results show that the structure of DsRed rabbit primary antibody with secondary antibody Alexa 488 goat anti rabbit is compatible with stem cells to form a COMPEX that produces visible fluorescence confocal microscope.

9. Mention that for the first time that this type of testing was efectuatpe neural stem cells.

10. Este should continue and expand research to identify other antibodies whose structure have availability for evidence of human neural stem cells.

11. Citometriei technique in continuous flow were determined antibodies that can identify existing human stem cells in fetal sheep brain, and the quantity they are found in each individual analysis.

12. The overall analysis of data obtained by flow fluorocitometrie may find the following: the highest percentage of grafting human stem cells in fetal sheep brain in the amount of 33.69% was obtained from the individual with the number 2166 and the lowest percentage of engrafting of human stem cells in fetal sheep brain was the amount of 1,74% , was obtained from the inndivids with numbers 2270, 2271, 2272, 2273, 2274.

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Is it Possible Artificial Sex Regulation in Mammals?

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Abstract - In spite of, impressive breakthrough in modern genetics and molecular biology the problem of artificial sex regulation of mammals still remains unsolved. Moreover, the very problem of sex origin of eukaryotes in the process of evolution still has not settled. Existing theories and hypothesizes mainly concern the maintenance and biological reasonability of sexual mode of replication. Their theoretic foundation is based on Darwin's and Mendel's ideas that sex was originated due to natural selection and genes. On the basis of other model of genesis and sex evolution of eukaryote, it was suggested the idea of artificial sex regulation of mammals. Seemingly, the sex differentiation (SD) in animals and human is determined by the amount of constitutive heterochromatin region (cHR) in the Y chromosomes of the undifferentiated embryonic gonads (UEG) via cell thermoregulation. It is assumed the medulla and cortex tissue cells in the UEG differ in vulnerability to the increase of the intracellular temperature because of their anatomical position in genital ridges. If the amount of the cHR on Y chromosome is enough for efficient elimination of redundant metabolic heat from rapidly growing UEG cells the medulla tissue survives. Otherwise it doomed to degeneration and a cortex tissue will remain in the UEG. For artificial regulation of the SD it is proposed to remove a layer of cortex in the UEG.

Keywords : sex differentiation; sex regulation; cell thermoregulation; constitutive heterochromatin; Y chromosome; genital ridges; non coding DNAs.

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Is it Possible Artificial Sex Regulation in Mammals?

A.I. Ibraimov

Abstract - In spite of, impressive breakthrough in modern genetics and molecular biology the problem of artificial sex regulation of mammals still remains unsolved. Moreover, the very problem of sex origin of eukaryotes in the process of evolution still has not settled. Existing theories and hypothesizes mainly concern the maintenance and biological reasonability of sexual mode of replication. Their theoretic foundation is based on Darwin's and Mendel's ideas that sex was originated due to natural selection and genes. On the basis of other model of genesis and sex evolution of eukaryote, it was suggested the idea of artificial sex regulation of mammals. Seemingly, the sex differentiation (SD) in animals and human is determined by the amount of constitutive heterochromatin region (cHR) in the Y chromosomes of the undifferentiated embryonic gonads (UEG) via cell thermoregulation. It is assumed the medulla and cortex tissue cells in the UEG differ in vulnerability to the increase of the intracellular temperature because of their anatomical position in genital ridges. If the amount of the cHR on Y chromosome is enough for efficient elimination of redundant metabolic heat from rapidly growing UEG cells the medulla tissue survives. Otherwise it doomed to degeneration and a cortex tissue will remain in the UEG. For artificial regulation of the SD it is proposed to remove a layer of cortex in the UEG.

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I. Introduction

In many mammals, including us, the genetic sex is determined at fertilization by sex chromosomes carried by the father's sperm, X in the case of female and Y in the case of male. At the early stages of embryonic development a pair of undifferentiated embryonic gonads (UEG) and both rudimentary female and male reproductive system develops in the embryo. As result of this all embryos are potentially bisexual.

For the time being the mechanisms of the sex differentiation (SD) are not known. At present the balance hypotheses, worked out by Bridges (1939) and Goldschmidt (1955) are generally accepted. According to these hypotheses, the interaction of genes, located in the sex chromosomes and autosomes, underlie the SD. Their theoretic foundation is based on Darwin's and Mendel's ideas that sex was originated due to natural selection and genes.

However, almost nothing is known about concrete mechanisms and types of gene interaction at the SD. The problem also becomes complicated especially as: a) the number, localization, products, and types of these gene interactions are not determined; b) the role of the sex chromosomes in the embryo SD remains not completely clear; c) there are no ideas as regards the possible role of a great amount of constitutive heterochromatin region (cHR) of the Y chromosome in the SD.

Indeed it is hard to believe that having impressive breakthrough in modern genetics and molecular biology the reasons and mechanisms of sex origin are still unknown. This probably has to do with the fact that in the basis of all hypothesizes and theories on sex biology lies idea on all-powered role of natural selection and genes in eukaryotic organisms' evolution. Although they help to explain reasonably and justify such widespread propagation of sexual reproduction in the world of eukaryotes; nevertheless, these approaches had little help in the development of theories and hypothesizes explaining of sex origin (Ibraimov 2012). Moreover they were not able to show ways of their experimental check. As Ridley (2010) begrudgingly admitted: 'Sex is not used simply for want of an alternative. Nothing, in an evolutionary sense, forces organisms to reproduce sexually'.

We offer the idea that probably sex and sexual reproduction of eukaryotic organisms are the result of the long evolution of non coding DNAs (ncDNAs), which led to the origin of mitotic chromosome, mitosis, meiosis, sex determination and differentiation mechanism. It is supposed that sex and sexual reproduction were a direct consequence of the origin of unicellular eukaryote, but not are result of emergence of some specific structural genes determining the sex development. Genes pertaining to sex appeared later, but again not for determination and differentiation of sex, but for development of the secondary sexual characters with the help of hormones (Ibraimov 2008; 2009; 2010; 2011; 2012).

The overwhelming majority of modern biologists associate development and life evolution with structural, functional and frequency changes of genes. If the role of non-informative, i.e. ncDNAs in

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these processes is admitted, then only as a secondary one. The possible role of ncDNAs in the development and the evolution of eukaryotes, except for maybe satellite DNAs and chromosomal heterochromatic regions (HRs), is not considered seriously. Mostly, they are considered as "surplus" DNAs.

However, recently there has appeared data about the possible role of ncDNAs in individual development and evolution (Ibraimov 2003, 2004, 2008, 2009, 2011, 2012; Ibraimov and Tabaldiev 2007), including the sex differentiation of higher eukaryotes (Ibraimov 2008). In particular, on the basis of the cell thermoregulation concept there was proposed a hypothesis of a possible sex differentiation mechanism. Seemingly, the SD in animals and human is determined by the amount of constitutive heterochromatin region (cHR) in the sex chromosomes of the undifferentiated embryonic gonads (UEG) via cell thermoregulation. If the amount of the cHR on Y chromosome is enough for efficient elimination of heat difference between the nucleus and cytoplasm in rapidly growing UEG cells the medulla tissue survives. Otherwise it doomed to degeneration and a cortex tissue will remain in the UEG (in more detail see Ibraimov 2008). Thus, the sex differentiation is an immediate consequence of cell thermoregulation, but not a result of some specific structural gene emergences determining sex development. In other words, the basis of SD at the stage of indifferent primordial gonads is mainly physical processes rather than chemical.

II. What are the Approaches for Artificial Regulation of Sex?

Currently the following approaches are practiced: chemical (hormonal), physical and cellular methods of artificial regulation of sex. The disadvantage of the first approach, as we know, is the reversibility of the sex after the termination of the sex hormones. The second approach starts from the classical embryo-genetic studies by Jost (1972) on removing of the future gonad (genital ridges) in early embryos of rabbits: removal of genital ridges before the formation of the gonads led to the development of embryos as females.

Scientists tried to separate the sperm by size, weight, mobility in an electric field and by other methods. Despite the large number of experiments carried out, the problem of regulating sex in a mammal by physical separation of sperm has still not been solved.

It is possible to receive organisms with desired sex from animals using cell engineering such as transplantation and cloning. Currently, a method of dividing embryos has been developed. Embryos of pets can be divided into 4, 8 or more parts. At the same time one part of the embryo is planted into the uterus of the female, the remaining parts are frozen and stored for a long time in liquid nitrogen. After the birth of the offspring transplants of other parts of the divided embryo can be done. In this case, the sex will be the same as in the birth of the animal.

The essence of our idea is to transform the embryo with XX karyotype in the male fetus, which will be able to produce gametes of only one type (X sperm) in the future, and it is based on the following assumptions:

1. Sex-determining ability of Y chromosome is not due to the presence there in *SRY* / *Sry* and other not yet identified genes, but due to the fact that it has a large block of cHR.
2. It is the presence of cHR in Y chromosome that ensures the survival of medulla at the stage of indifferent primordial gonads from heat degeneration through the mechanisms of cell thermoregulation.
3. A two-layer structure of the genital ridges –medulla inside and cortex outside - has profound biological significance: the emergence of a male depends on the survival of medulla, and not on the presence of *SRY* and others genes that has to do with sex determination.
4. Location of the medulla in the depth of genital ridges puts her in a difficult position in terms of the effective removal of metabolic heat, as it is surrounded by a layer of adipose tissue (mesentery) and cortex. Rise in the temperature of the medulla tissue above the physiologically acceptable levels may cause it to heat death, with all the ensuing consequences. Medulla survival depends on timely removal of excess heat through the layers of the mesentery and the cortex outside the genital ridges.
5. There are mammals that determine male sex without the *SRY* gene.
6. Along with genetic factors (the presence of the sex chromosomes of a particular type, *SRY* and other hypothetic genes) on the formation of sex in some vertebrates and invertebrates environmental factor, such as temperature has a significant impact as well.
7. Production of gametes does not depend on the type of gonads, if testis is formed, germinal cells develop into sperm cells, and if the ovary is formed, then the same cells form eggs.
8. For the normal functioning of mammalian organisms, as well as for other homoiotherms, body temperature needs to be maintained within very narrow limits (temperature homeostasis), which is provided by organ-based mechanisms of physiological thermoregulation. Obviously, the ultimate goal of any thermoregulation is to maintain a constant temperature inside the cells. However, while maintaining the equal temperature between

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nucleus and cytoplasm, the role of organ-based physiological thermoregulation is limited and mechanisms of intracellular thermoregulation must be turned on through a layer of condensed chromatin, which is located inside the nuclear membrane (nuclear envelope).

9. As known, complex systems function poorly. It is hard to imagine that such an important process as sex determination in mammals has been assigned to *SRY* and other, more than a hundred, genes. Perhaps Nature invented the simplest way, to determine sex through the physical survival of the medulla, while giving each species of mammals a defined window of time (f.e.: on 10.5-12th day of embryonic development in the mouse).

Thus, our idea of artificial regulation of sex is based on the lately discovered cell thermoregulation (CT) phenomenon, the essence of which is in the following: the peripheral layer of condensed chromatin (CC) of nucleus, being the densest domains in a cell, apparently conducts heat between the cytoplasm and nucleus when there is a difference in temperature between them. The assumed heat conductivity effect of CC, consisting of highly repetitious DNA (the main component of cHR), is stipulated by its principal features: condensed state during the interphase, association with the lamina and the inner nuclear membrane, replication at the end of the S period of a cell cycle, formation of the chromocenter, genetic inertness, and wide variability in the quantitative contents both within and between species (Ibraimov2003; 2004). The reality of the CT existence is shown at the organism level. In particular, it turned out that the individuals in the population differ from one another by the heat conductivity of their bodies (BHC). At that the BHC value depends on amount of the constitutive heterochromatin in their genomes (Ibraimov and Tabaldiev2007; Ibraimov et al., 2010 a,b).

As known, the sexual development in the mammals is a process consisting of at least three stages: the 1ststage is the chromosome determination of sex (XX or XY); the 2nd stage is SD (the development of testicles or ovaries); the 3st stage is the development of the secondary sexual characteristics. In case the testicles are formed, they excrete the testosterone hormone, circulating over embryo, and causing the development of the somatic cells by the male type.

On the contrary, the lack of testosterone results in invariable development of the somatic cells by the female type.

Now let's try to ground our assumption. (1). The heat conductive effect of the CC especially strongly increases in conditions of mulicellularity (Ibraimov 2003, 2004); (2). By the 3rd week of the embryo development in human, the cHRs is completely formed (Prokofyeva-Belgovskaya 1988), and they are able to exert their heat conductive effects in the cells. (3). In mammals it has been shown that at equivalent gestational ages, males are developmentally more advanced than females. Medulla, being located in the very middle of the genital ridges closed to aorta and surrounded with cortex and mesentery probably experiences the greatest problems with removal of the excessive metabolic heat in comparison with cortex. Obviously, the cortex having a relative advantage in supporting the intracellular temperature homeostasis than the medulla, other things being equal, has more chances to preserve and further develop in to the female tissue.

It is possible that the medulla of the UEG is more vulnerable to the temperature increase than the cortex tissue. The following data testify to this: a) the clinical consequences of cryptorchidism in boys; b) location of scrotum outside the body in the mammals. In other words, the direction of development of the genital ridges towards the male or female side is not something strictly fixed, it will depend on the environment in which it is being implemented. In our opinion, most likely this is the temperature influence on the cells of the genital ridges, and seemingly on which it depends, whether the medulla tissue will remain or not. If the medulla tissue is not provided with the timely and efficient removal of excessive metabolic heat, it will be doomed to degeneration having making for the cell from the cortex. Just in this meaning we understand the role of cHR in SD, as efficient means of medullar tissue protection against "heat death".

It could be possible to test our hypothesis experimentally (Fig. 1). At genital ridges with the karyotype XX to remove its cortical layer preserving the medulla tissue. If our hypothesis is true then a male with a female genotype (XX) will be developed, which at usual crossing results only in females. Such experiments could give an answer to two interrelated question: 1) what does the SD depend on, either on the gene balance or on the "dose" of the cHR on Y chromosome?; 2) why does at genotype XX the medulla tissue preliminarily degenerate?, either from the "heat death" or from the impact of the gene products, produced by the cortex cells, on the medulla tissue?

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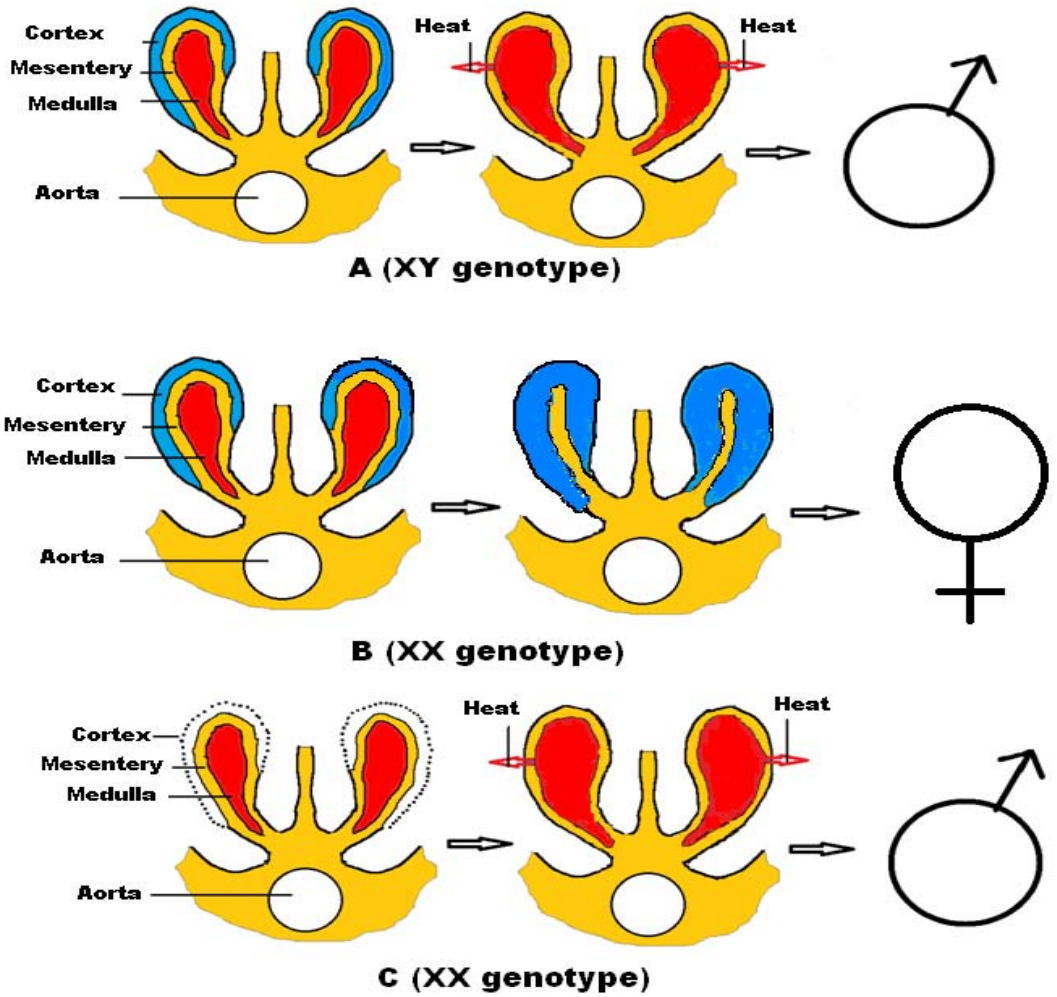

Figure 1

A. Genital ridges of the embryo with the genotype XY. Due to the presence of a Y chromosome with large block of constitutive heterochromatin (cHR) in medulla cells metabolic heat effectively deduces into the surrounding genital ridges space through the mechanisms of cell thermoregulation that allows medulla and subsequent development of the testis to survive.

B. Genital ridges of the embryo with the genotype XX. Due to lack of Y chromosome with large cHR in fetal cells excess metabolic heat is not deduced in the surrounding genital ridges space, which will lead to thermal degeneration of medulla and preservation of the tissue of cortex only with subsequent development of the ovary.

C. Genital ridges of the embryo with the genotype XX with remote cortex. After surgical removal of the cortex from the genital ridges medulla cells can survive despite the absence of Y chromosome in its nuclei. Excess metabolic heat of medulla cells from such embryos will be deduced in the surrounding genital ridges space due to lack of cortex layer, which, along with the mesentery, prevented the retraction of heat energy from the medulla tissue that, ultimately, allow it to survive and helps the development of the testis.

Theoretically, the problem in realizing our ideas may be associated mainly with *Sry* gene. In mammals, it has been known for 20 years that the Y chromosomal gene *Sry* is necessary for male sex differentiation, and many others genes have since been implicated in testis development. Thus, Y chromosome analysis revealed that a hypothetical factor responsible for the formation of the sex, in fact exists – it is gene *SRY* / *Sry* (sex region of Y-chromosome) and is localized on the short arm of Y chromosome. *SRY* – a gene that controls male sexual development; produces testis-determining factor (*TDF*), a product that triggers the formation of testes from undifferentiated embryonic gonad tissue. Its protein - a transcription factor, contains *HMG*-domain and belongs to the *SOX*-family proteins. *SRY* is not present on the X-chromosome gene. For the determination of the male

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phenotype also protein to another gene *Sox*-family - *SOX9*, localized to an autosome is need. *SRY* and *SOX9* genes are expressed together in the gonad ridges of males. Expression screens such as microarray analyses have resulted in hundreds of candidate genes that show sex-specific expression patterns. However, it has been difficult to place these genes into a network of gene regulation and function. Even for *Sry*, it is still not known how its expression is regulated, what proteins might interact with it, and which genes it regulates (for more details see Wilhelm et al., 2007).

However, while *Sry* is found only in mammals, *Sox9* is present in all vertebrates. Thus, *Sox9* is more ancient gene and apparently plays a more general role in sex determination than *Sry*. In mammals, *Sox9* activates related gene *Sry*. Thus, *Sry* may act primarily as a "switch" that activates the gene *Sox9*, and protein *Sox9* initiates an evolutionarily conserved pathway for the formation of the testes. But in any case, the final answer to the question whether you can do without the *Sry* gene during artificial regulation of sex in mammals comes after the relevant experiments.

Returning to the classical experiments by Jost (1972), it should be noted that the author did not conduct experiments on the removal of the cortex to the genital ridges, so it seems to us highly likely to change the sexual differentiation of the fetus. From available sources of information, we could not find information as well as conducted experiments there any one presented on all to removal the cortex or medulla of the genital ridges.

III. The Hypothetic Mechanism of Sex Regulation in Mammals

We believe that the impact of cHR Y chromosome on sex regulation starts with the formation of genital ridges and continue until the end of ontogeny, through the protection of *SRY* and other genes localized on this chromosome, from the harmful effects of excess heat through mechanisms of cell thermoregulation. Role of cHR on the Y chromosome at the stage of differentiation of sex is, apparently, in the preservation of medulla cells from heat degeneration due to their location in the back of the depth of the body of genital ridges (within the mesentery and the cortex). As it is well known, medulla cells of mammals begin operating earlier than the cortex cells. If medulla maintains its viability (for Nature allocated for this type short time in the early stages of embryogenesis), then it will continue to exist, and cortical tissue subjected to thermal degeneration.

Survival of the medulla in the genital ridges, where the embryo has XY karyotype, is explained by the fact that, due to the presence of cHR on the Y chromosome cells efficiently derive excess heat into the surrounding space. The thing is that removals of excess metabolic heat outside the cell are fraught with difficulties, particularly from the nucleus. Latter possesses two ways to remove the excess heat: increase its size, it is not possible because of the numerous organelles in the cytoplasm or increase the thermal conductivity of the layer of condensed chromatin (CC) around the nucleus. Next, through a dense layer of CC thermal energy is deduced in the extracellular space through the cell skeleton (microfilaments, intermediate filaments, microtubules). We believe that, in general, the second option is implemented (see more Ibraimov, 2003, 2004).

Apparently, the heat diverting property of cHR on the Y chromosomes in the cells has its own characteristics. It seems very likely that the *SRY* and other genes, potentially affecting the production of testosterone, are not localized on the Y chromosome by chance. Indeed, when the medulla cells begin functioning, as a result of the accelerated metabolism heat might build up above physiologically acceptable level, which is fraught with negative consequences ("heat death" of the cells). If the layer around the nucleus of the CC is sufficiently dense, then the cell can avoid the heat death, through the timely removal of excess heat in the extracellular space. Such cells have the possibility of having cHR on the Y chromosome, and perhaps for this reason medulla tissue of embryo with XY genotype survive. It is known that transgenic XX mice carrying a genomic fragment containing the *Sry* gene develop as males, which are sterile in spermatogenesis (Koopman et al., 1991). Therefore, we argue that the presence of that *SRY* and other genes in the genome are not sufficient for the survival and development of the medulla. The availability of cHR on the Y chromosome is necessary, as it provides effective removal of excess metabolic heat from the medulla through mechanisms of cell thermoregulation.

Perhaps, following analogy is appropriate here. As it is well known, high pressure mercury lamps are used as a source of ultraviolet light for fluorescence microscopes. For normal operation of such lamps timely removal of excess heat outside the glass bulb is very important. Different heat diverting devices are invented for this aim. Recently, manufacturers of fluorescent microscopes began to supply its products with special cooling fins for these lamps to keep the temperature below 230° C. We believe that the survival of the medulla in the body of genital ridges depends on the timely removal of excess metabolic heat from the nucleus of cells. Perhaps, *SRY* and other genes located on Y chromosome are more sensitive to increased intracellular temperatures than genes located on autosomes. Apparently, Nature solved this engineering task by making cHR on Y chromosome as the heat removal, which in contact with the *SRY* and other genes outputs thermal energy outside the cell nucleus. It is no

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accident that *SRY* produced protein binding to the minor groove of the DNA induces a sharp bend of 60–85°. Biochemical analysis of *SRY* protein expressed in human XY sex-reversed patients revealed that DNA binding and bending are integral parts of *SRY function* (Harley et al., 1992; Pontigga et al., 1994).

IV. Conclusion

According to Lyon (1992)"...the X-chromosome is clearly involved at some point in the sex-determination pathway". The point that I am trying to convey is that: a) the SD is one of the most important examples of how the physical state of the DNA molecular (in this case the level of compacting of the peripheral layer of CC around the cell nucleus) influences to the cell differentiation; b) X-inactivation is not involved in the SD; c) X-chromosome is not being inactivated, but it is heterochromatinized in order to compensate the lacking in the female karyotype the largest block of the cHR in the interest of the cell thermoregulation. Thus it would be more correct to speak about compensation of the heterochromatin dosage, and not about the dosage (double) of genes (Ibraimov 2003, 2004, 2008, 2010, 2011, 2012).

The reasons why the cortex tissue in the genital ridges is not exposed to the heat degeneration as the medullar tissue because of the problems connected with the CT in addition to the above mentioned, are also explained by the fact that: a) it has been shown that at equivalent gestational ages, males are developmentally more advanced than females, even before the gonads form. So, as for example a detailed analysis of XX v. XY developmental differences in mice has shown that XY fetuses are indeed larger than XX fetuses prior to gonad SD (Burgoyneetal.1995). The human testicles are formed earlier (6th–12^{th}week) than the ovaries (14th–16^{th}week); b) by the time of the ovaries formation, the problems with heat conductivity out from the deep parts of the organs and embryo tissues become considerably less complicated, as by this time the circulation system of fetus begins to function, i.e. a distant transfer of heat energy in a body (Ibraimov and Tabaldiev 2007), that eliminates the danger of the cell heat degeneration including the cortex tissues; c) with mammals and human the development towards the male sex is determined by availability of a testicle, to be more precise by a hormone secreted by it – testosterone, and the development towards the female sex is not induced, i.e. it is not the consequence of availability of the embryo ovary. It rather should be considered as the consequence of the testicle absence (Ohno et al.,1971); d) random X-inactivation of either the maternally - or paternally – inherited X chromosome most likely justifies to the participation of the facultative heterochromatin in the CT, and not to the dosage compensation of genes; and last c) homoiotherms keep the constant temperature in a body not so much they possess a 4-chamber heart and lung respiration (as a result the tissues obtain more oxygen) for acceleration of heat production, as their ability to efficiently remove the excessive heat from the organism. This is promoted by high conductivity of their bodies which are provided, in addition with the cHR, with the availability of G+ and Q+ bands in the chromosomes which intensify the compactization of the peripheral CC layer in the interphase nuclei, thus accelerating the liquidation of the appeared temperature difference between the nucleus and cytoplasm in the cell (Ibraimov 2003, 2004; Ibraimov and Tabaldiev 2007). So, the cause of the differential survivability of the medulla and cortex cells is the CT (preservation of the intracellular temperature homeostasis), which is determined by the cHRs on Y chromosome.

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An Efficient *in vitro* Regeneration System for Tori (*Brassica campestris*)-7

By Sabrina Shameen Alam, Laila Khaleda & Mohammad Al-Forkan

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Abstract - An efficient *in vitro* regeneration system for Tori (*Brassica campestris*) -7 was developed from calli of hypocotyls and cotyledonary leaves. The optimum medium for callus induction was found with 0.5 mgl^{-1} 2, 4- dichloro-phenoxyacetic acid (2,4-D). The best shooting medium contained 3.0 mgl-1 6, benzyl amino purine (BAP), 0.1 mgl^{-1} naphtalene acetic acid (NAA), and 5.0 mgl^{-1} $AgNO_3$. Maximum number of shoots were produced when 0.5 mgl^{-1} kinetin was used, whereas the combined effect of 2.0 mgl^{-1} BAP, 0.1 mgl^{-1} NAA, and 5.0 mgl^{-1} $AgNO_3$ regenerated calli the most. The effect of $AgNO_3$ was found for callogenesis at 0.5 mgl^{-1} and for regeneration at 5.0 mgl^{-1}. *In vitro* regenerated shoots of Tori-7 developed roots on medium with 1.0 mgl^{-1} indole 3- butyric acid (IBA). The developed efficient *in vitro* regeneration protocol can be used as a baseline for Agrobacterium-mediated genetic transformation of the studied plants.

Keywords : *agrobacterium, in vitro regeneration, and tori-7.*

GJSFR-G Classification : *FOR Code: 270805*



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An Efficient *in vitro* Regeneration System for Tori (*Brassica campestris*)-7

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Abstract - An efficient *in vitro* regeneration system for Tori (*Brassica campestris*) -7 was developed from calli of hypocotyls and cotyledonary leaves. The optimum medium for callus induction was found with 0.5 mgl^{-1} 2, 4- dichlorophenoxyacetic acid (2,4-D). The best shooting medium contained 3.0 mgl^{-1} 6, benzyl amino purine (BAP), 0.1 mgl^{-1} naphtalene acetic acid (NAA), and 5.0 mgl^{-1} $AgNO_3$. Maximum number of shoots were produced when 0.5 mgl^{-1} kinetin was used, whereas the combined effect of 2.0 mgl^{-1} BAP, 0.1 mgl^{-1} NAA, and 5.0 mgl^{-1} $AgNO_3$ regenerated calli the most. The effect of $AgNO_3$ was found for callogenesis at 0.5 mgl^{-1} and for regeneration at 5.0 mgl^{-1}. *In vitro* regenerated shoots of Tori-7 developed roots on medium with 1.0 mgl^{-1} indole 3-butyric acid (IBA). The developed efficient *in vitro* regeneration protocol can be used as a baseline for *Agrobacterium*-mediated genetic transformation of the studied plants.

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I. Introduction

Mustard (including rape) is one the most important crops and accounts 3.08% of the total production of major crops in Bangladesh. The production of oil seed in 1994-95 was 307,602 metric tons in the country, from which 179,524 metric tons of oil was produced that can supply only 27% of the daily per head oil need. Hence, we need to increase annual *Brassica* production. Improvement of *Brassica* production rate can be achieved through *in vitro* tissue culture of mustard. Tissue culture or *in vitro* micropropagation technique has been applied for *Brassicas* for a long time observed by Ali *et al* (2007) and John *et al* (1991). The combination of 2.4-D, BAP, NAA and $AgNO_3$ was found to be the best medium for callus initiation and growth for *Brassica napus* by Ali *et al,* (2007). The use of $AgNO_3$ was reported by Khan et al (2003) in *Brassica napus* and Ali *et al* (2007) for plant regeneration. However, the best condition for Bangladeshi *Brassicca* local variety (Tori-7) regeneration system is not known. The present study was designed to screen out the varietal response of Tori-7 regarding its response to specific cultural conditions in comparison with and finally, to establish an efficient genotype-independent *in vitro* cultural system for the initiation and development of embryogenic calli with an ultimate goal of plant regeneration that can be used in future as a baseline for *Agrobacterium*–mediated genetic transformation for quality improvement.

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II. Materials and Methods

Tori-7 (*Brassica campestris)* seeds were collected from Bangladesh Agricultural Research Institute (BARI), Hathazari Substation, Chittagong. The explants collected from *in vitro* grown seedlings used for the experiments were: hypocotyls and cotyledons with petiole. Hypocotyl segments of 0.5-1.0 cm and cotyledonary leaves with petioles were used for the experiments. MS (Murashige and Skoog, 1962) medium was prepared with 3% (w/v) sucrose and solidified with 0.4% (w/v) agar. Only half strength MS medium was used for seed germination whereas, MS media supplemented with different PGRs (Plant Growth Hormones) such as 2, 4-D, BAP and additives such as proline and casein hydrolysate (CH) were used for callus induction. Shoots were developed by using media with BAP and the effect of hormones and additives were checked. Initiation of roots were tried on media supplemented with NAA and IBA. The plantlets with sufficient rooting system were taken out of the culture vessels and the roots were washed under tap water. The *in vitro* grown rooted plants were then transferred into small pots. Hardening was carried out by periodical exposure of the plants to natural environment.

III. Results and Discussions

Cotyledons and hypocotyls, both formed large calli on MS medium supplemented with 0.5 mg l^{-1} 2, 4-D. The increasing concentration of 2, 4-D greatly reduced the percentage of callus formation as well as size of the callus (Fig.1), though this effective concentration of 2,4-D does not agree with many previous reports such as Cardoza and Stewart (2003), who found the best callus induction using 1.0 mg l^{-1} 2, 4-D. Quain and Zhang (2004) and Khan *et al.* (2002) reported best callus induction medium using 2,4-D at 1.5 mg l^{-1} and 2.0 mg l^{-1}, respectively. These differences with the optimal concentration of 2, 4-D of current investigation can be assumed as a result of varietal differences and variations in the exo- and endogenous environments. The use of BAP on callogenesis was found to increase the size and frequency of callus formation. Withdrawal of BAP from the medium reduced

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the percentage of hypocotyl callus formation even when 2, 4-D concentration was increased to 1.0 mg l^{-1}. The explants became brown that did not produce any significant callus (Fig. 2). On the other hand, withdrawal of $AgNO_3$ from the medium caused the explants become brown and dead (data not shown). The use of $AgNO_3$ was described to improve the growth of the callus by Noman *et al.* (2008) that supports the result. About 12% of the hypocotyl calli and 1-2% of the cotyledonary explants produced roots within 20 days of culture on medium containing 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} IAA (Fig. 3). Hypocotyls produced callus of embryogenic nature on proline containing medium though cotyledons responded less in proline and CH contacting media (Fig. 3). The increasing BAP reduced shooting (Fig.4). When NAA at 0.1 mg l^{-1} was used in combination with BAP, the percentage of shoot formation raised to 80% from 47.8% for hypocotyl explants. George and Rao (1980) observed maximum regeneration from cotyledon explants in *Brassica juncea* on medium supplemented with BAP and NAA rather than BAP only, which supports the present findings. About 20% cotyledonary calli started shooting within 10-12 days. The medium without $AgNO_3$ but with NAA gave shoots at a percentage of 65-80% (data not shown). Presence of CH in media reduced shooting frequency.

Spontaneous root generation occurs sometimes on MS medium with hormonal supplements for the induction of shoots (Fig.5). Tori-7 responded only on media containing IBA (Fig.5). This finding again suggests the genotypic and environmental difference may cause the variety to respond.

On the basis of observation and results taken, Tori-7 was better in terms of producing more shoots but the subsequent growth and establishment was difficult that is supported by Dunwell (1981), Dietert *et al.* (1982) and Glimelius (1984) who concluded the difficulties of regeneration of *Brassica campestris* than other *Brassicas*.

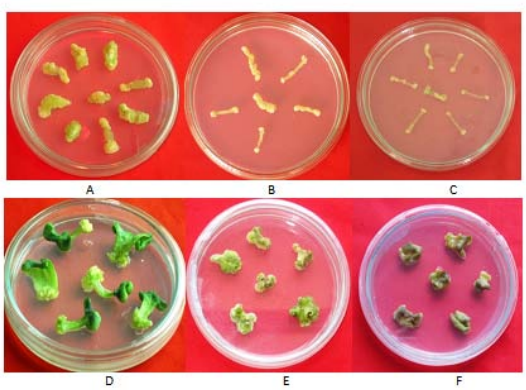

Fig. 1 : Decreasing level of callus formation in hypocotyls (A-C) and cotyledons (D-F) with increasing concentrations of 2,4-D from 0.5 mg l^{-1} to 2.0 mg l^{-1}

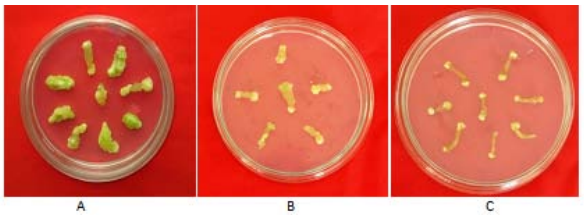

Fig. 2 : Combined action of BAP and $AgNO_3$ increases callus formation, (A) media with both BAP and $AgNO_3$, (B) media with BAP but no $AgNO_3$ and (C) media with $AgNO_3$ but no BAP

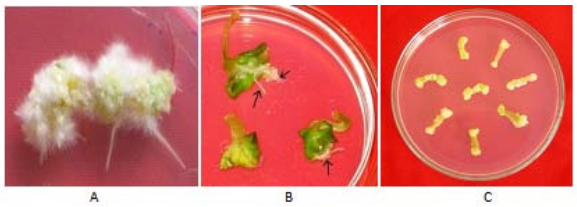

Fig. 3 : Effect of hormones; initiation of root from hypocotyl (A) and cotyledon (B) explants on callus induction medium containing both IBA and IAA, and proline: callus appearing at the edges of hypocotyls (C)

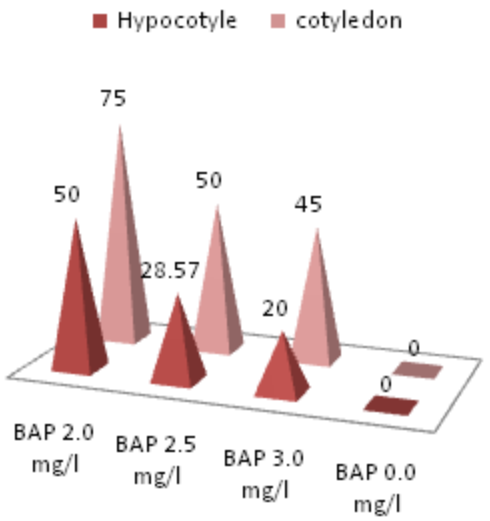

Fig. 4 : Change in the percentage of shoot formation from Tori-7 calli on different media; changing BAP concentration changes shooting frequency

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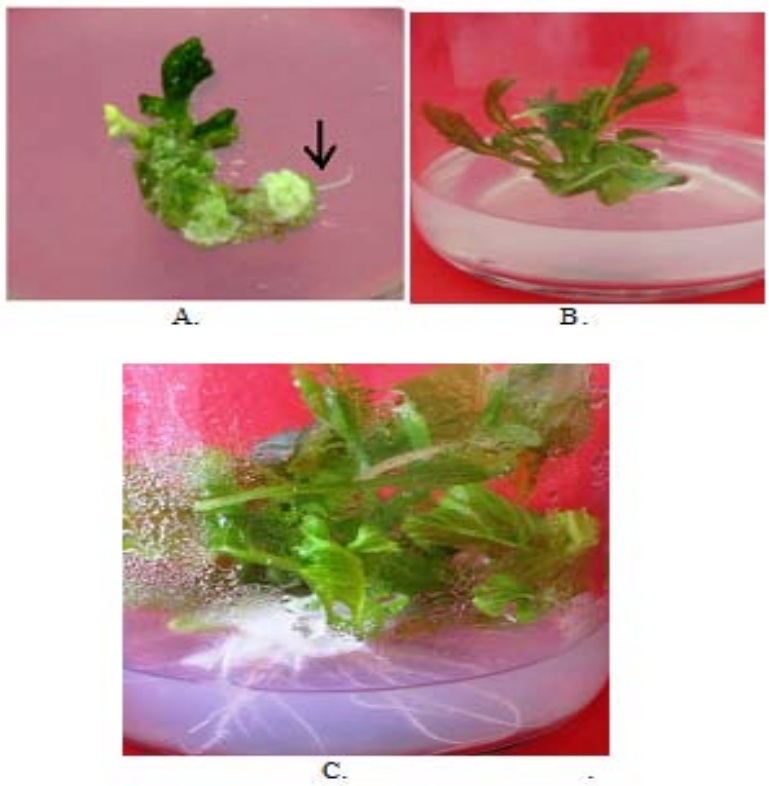

Fig. 5 : Different stages of regeneration; spontaneous root formation on shooting medium unattached to shoots (A), shoot elongation from Tori-7 hypocotyls on medium containing kinetin (B) and formation of rooting system of Tori-7 on IBA containing medium (C)

IV. Conclusion

The present study has described an efficient in vitro regeneration system for a local *Brassicas* variety namely Tori-7. The optimum medium for callus induction was found with 0 .5 mgl^{-1} 2, 4-D, where as the best shooting medium was 3.0 mgl^{-1} BAP, 0.1 mgl^{-1} NAA, and 5.0 mgl^{-1} $AgNO_3$. Maximum number of shoots were produced when 0.5 mgl^{-1} kinetin was used, whereas the combined effect of 2.0 mgl^{-1} BAP, 0.1 mgl^{-1} NAA, and 5.0 mgl^{-1} $AgNO_3$ regenerated calli the most. *In vitro* regenerated shoots of Tori-7 developed roots on medium with 1.0 mgl^{-1} indole 3-butyric acid (IBA). The developed efficient *in vitro* regeneration protocol will be supportive for increasing the local productivity.

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Serum Homocysteine and Paraoxonase1 Levels in Women with Polycystic Ovary Syndrome Treated with Metformin Versus Metformin and Folic Acid

By Dr. Tarek H. Abed Tawfik Al-Khyat, Dr. Melal Mohammed Al-Jeborry & Qusay Mohsin Kadium

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Abstract - Background: Polycystic ovary syndrome (PCOS) is the most common reproductive endocrinopathy and currently considered to be a part of the metabolic syndrome. Women with PCOS cluster risk factors associated with risk of atherosclerosis. These risk factors include insulin resistance, dyslipidemia, hyperandrogenemia, obesity, endothelial dysfunction and oxidative stress.

Aim of the study: Study of the relationship between homocysteine and paraoxonase 1 in PCOS in two different regimes of treatment with Metformin and Folic acid in order to elucidate some risk factors that may lead to atherosclerosis and vascular diseases in the corresponding patients.

Patients and Methods: Fifty patients with PCOS were enrolled in this study that subdivided into two groups according to type of treatment received i.e. G1 (comprising 25 patients) treated with Metformin only, G2 (comprisin25 patients) with Metformin and Folic acid. Serum Homocysteine (Hcy), Paraoxonase 1(PON1) Apolipoprrptein A-1(Apo A-1) and Apolipoprotein B(Apo B) were determind by using enzyme-linked immunosorbent assay (ELISA). Reduced Glutathion (GSH) and Malondialdehyde (MDA) were determined by using colorimetric method.

GJSFR-G Classification : FOR Code: 320307p, 100499



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Results: The results indicated a significant increase in the serum levels of Hcy($p<0.01$), PON1 and GSH($p<0.05$) anda significantdecreasein thelevels of MDA and Apo B/Apo A-1 ratio($p<0.01$)in PCOS group after Metformin only(G1-A).But, after Metformin with Folic acid treatment (G2-A)Hcy, Apo B/Apo A-1 ratio and MDA levels were significantly lower ($p<0.01$) and PON1 as well as GSH levels were significantly higher ($p<0.01$) in comparison with those before treatment. Whereas, a significant decreasein level ofHcy, MDA ($p<0.01$) and Apo B/Apo A-1 ratio($p<0.05$) anda significantincrease in PON1($p<0.01$) and GSH levels ($p<0.05$) in (G2-A)as compared with (G1-A). The results revealed a significant positive correlation between Hcywith MDA and Apo B/Apo A-1 ratio as well as between PON1 and GSH. And a significant negative correlation between HcywithGSH, PON1and also, between PON1 with MDA and Apo B/Apo A-1 ratio in patients groups.

Conclusion: Folic acid administration to PCOS patients tends to lower homocysteine levels in the serumof the corresponding patients and alleviate the oxidative stress in those patients. Besides, increase of PON1 prevents atherogenic effects by changing Apo B/Apo A-1 ratio.

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I. Introduction

Polycystic ovarian syndrome (PCOS) is one of the most common female endocrine disorders and is a complex, heterogeneous disorder of uncertain aetiology, but it can be classified as a genetic disease [1]. Itis one of the most common causes of anovulatory infertility with a prevalence between 6% and 10% based on the U.S. National Institute of Health (NIH) criteria and as high as 15% when the broader Rotterdam criteria are applied [2]. Typically, PCOS is first identified during the early reproductive years. The clinical expression varies but commonly includes oligo-ovulation or anovulation, hyperandrogenism (either clinical or biochemical) and the presence of polycystic ovaries [3]. Theaetiology ofPCOS is not fully known, but many environmental and genetic factors may cause this disease, such as diet, pollution and sedentary lifestyle. Stress may contribute to its development [4].

Homocysteine (Hcy) is an intermediate product formed during the breakdown of the amino acid methionine, and may undergo remethylation to methionine (folate and cobalamin is involved in Hcyremethylation) or trans-sulphuration to cystathionine then to cysteine (vitamin B6 is involved in Hcy trans-sulphuration) [5]. Excess Hcy in the blood stream may cause injures to arterial vessels due to its irritant nature, and result in inflammation and plaque formation by impaired endothelial function, increased oxidative stress, alterations of lipid metabolism, increasing platelet adhesiveness, activation of the coagulation system and stimulating vascular smooth muscle cell proliferation [6].

PON1 is a member of family of proteins that also includes PON2 and PON3. that share considerable structural homology and are located adjacently on chromosome 7 in humans. All the three proteins prevent oxidative stress and fight inflammation [7]. PON1 has different anti-atherogenic features such as protection from free radical induced oxidation of cholesterol in arterial wall and protection against harmful effects of oxidized LDL. PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is

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associated with apoA-I incorporation in the high-density lipoprotein (HDL) particles [8].

The ApoB/ApoA-I ratio represents the balance of pro-atherogenic and anti-atherogenic lipoproteins, which is the better parameter than routine lipid measurements in predicting risk of cardiovascular diseases(CVD), might also be potential biomarkers in predicting risk of CVD in patients with PCOS[9]. Various studies had demonstrated that this ratio was associated with the incidence metabolic syndrome (MS), insulin resistance (IR) and increased free androgen index and visceral adiposity [10].

Oxidative stress, defined as the rate of Reactive Oxygen Species (ROS) (and/or Reactive Nitrogen Species (RNS) production) and the rate of their removal by cellular defense mechanisms [11].In humans, oxidative stress is thought to be involved in the development of an environment unsuitable for normal female physiological reactions [12]. This, in turn, can lead to a number of reproductive diseases including endometriosis, PCOS, and unexplained infertility. It can also cause complications during pregnancy, such spontaneous abortion, recurrent pregnancy loss, preeclampsia, and intrauterine growth restriction [13].

Metformin is an insulin-sensitizing drug from the biguanide class. It is orally administered drug used to lower blood glucose concentrations in patients with non-insulin dependent diabetes mellitus (NIDDM), and is now also used in the treatment of polycystic ovary syndrome [14]. Metformin had many benefits such as, decrease in body mass index (BMI), androgens in both lean and obese women activity for around 20% andimprovement of menstrual cycles (ovulation rates). Normal menstrual cycles achieved within 3 months of starting treatment in some groups of patients, reduce early pregnancy losses and reduce risk of miscarriage,a 10-fold reduction in gestational diabetes (31% to 3%) and prevention or delay of onset of diabetes, lowering the blood insulin levels, increasing insulin sensitivity and lowering glucose level[15]. However, serum vitamin B12 and Folic acid levels are known to decrease during Metformin therapy probably due to malabsorption. Hence, Hcy levels might increase [16]. Folic acid may help reduce certain complications of PCOS as Folic acid supplements are "likely effective" for the treatment of high Hcy levels. Folate is involved in Hcy remethylation to methionine.

Women with PCOS may be able to improve their chances of getting pregnant by taking Folic acid every day [17] and besides possibly reducing infertility associated with PCOS, Folic acid may also improve pregnancy outcomes with PCOS. As with all pregnant women, pregnant women with PCOS need to get sufficient Folic acid to prevent neural tube defects. Both low Folic acid levels and PCOS are linked to miscarriage. Pregnant women with PCOS may thus help reduce their risk of miscarriage by taking Folic acid [18].

In the present study, the relationship between homocysteine, paraoxonase 1 and oxidative stress levels was elucidated in different regimes of treatment with Metformin and Folic acid in Iraqi patients inflicted with polycystic ovarian syndrome and the effect of those treatments regime on ApoB/Apo A-1 ratios in the corresponding patients.

II. Patients and Methods

This study was conducted in Babylon Maternity and Pediatric Teaching Hospital and in the laboratory of Biochemistry Department, College of Medicine, University of Babylon in the period starting from November 2012 to June 2013. Fifty patients with PCOS were enrolled in this stady. The patients were divided into two groups according to type of treatments received. The first group (G1) included 25 patients with PCOS received Metformin only, their age ranged between (18 - 30) years. The second group (G2) included 30 patients who received Metformin with Folic acid, their age ranged between (19 - 30) years. Full history was taken for all the patients which includes: age, address, length, weight, past history of diseases, obstetrical history, smoking, family history of disease, medical history. No drugs were prescribed to those patients that may interfere with the measured parameters (fertility drugs, oral anti-diabetic agents and oral contraceptive pills).

Seven to ten milliliters of blood were obtained from those patients. Blood samples were collected in tubes without anticoagulants and were left for 15 minutes at room temperature to clot. After that, the blood samples were centrifuged at 1500 xg for approximately 10 minutes. The serum was isolated and divided into five aliquots using eppendrof tubes and stored at (-20°C) until time of use.

Serum Hcy and PON1, were determined using ELISA kit provided by CUSABIO, China. Whereas, Apolipoprotein A-1 and B were determined using ELISA kit provided byEAGLE Biosciences, USA. The determination of Serum Hcy, PON1, Apo A-1 and Apo B is based on sandwich principle., using ELISA technique .Serum GSH concentration was determined by using a modified procedure utilizing Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNBwhen Sulfhydryl group of (GSH) reduces disulfide chromogen DTNB and change it to an intensely yellow compound which is proportional to total GSH and measured spectro-photometrically at 412 nm. The method of determination of serum MDA was based on the reaction with thiobarbituric acid (TBA) at 90-100°C and pH 2-3 for 15 minutes to form pink color product; forming an MDA-TBA_2 adduct that absorbs strongly at 532 nm.

All statistical analysis was performed by using SPSS version 18 for windows. Data were expressed as Mean ± SD. The normality of the distribution of all

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variables was assessed by the ANOVA, LSD and Pearson correlation analysisthat have been used to determine the significant difference between PCOS patients groups. P values less than (0.05) is considered significant while valuesless than (0.01) is considered highlysignificant.

III. Results and Discussion

The results in table (1) show non-significant differences in the age among different groups. This is due to the selection of subjects who are nearly within the same age. In fact, this is an important aspect for the comparison of other parameters especially those parameters which vary with age.

The body mass index (BMI) is a measure for human body shape based on an individual's mass and height. This anthropometric parameter is an important measure of obesity. Elevated BMI was associated with insulin resistance [19]. In our study, neither Metformin therapy nor Metformin and Folic acid therapy changed the BMI significantly. Our results were in agreement with at least two studies that failed to demonstrate the non-significant BMI reduction during the treatment with Metformin only [15,20] and also, during the treatment with Metformin plus Folic acid [15,21]. The small number of patients in the studies and differences in the treatment periods may explain these discrepancies.

In this study, A significant increase in Hcy ($p<0.01$), a significantly low in Apo B/Apo A-1 ratio and MDA levels ($p<0.01$) were observed in PCOS patients treated with Metformin only while there were significant increase in PON1 and GSH ($p<0.05$) in the corresponding patients. The results revealed a high significant decrease in Hcy, Apo B/Apo A-1 ratio and MDA levels ($p<0.01$)in patients on Metformin plus Folic acid but, there weresignificant increase in PON1 and GSH($p<0.01$)in the corresponding patients. Table (1).

Some investigators found that Metformin tends to decrease Folic acid which in turn leads to increase of Hcy in the PCOS patients [22, 23]. in spite of the benefits of such treatment in PCOS patients to improve fertility, normalization of menstrual cycles and reduction of thecal androgen production, there were some harms in using this drug alone[15]. For this reason Folic acid was administrated for PCOS patients with Metformin to improve antioxidant status and to lower Hcy levels. Previous studies that are done by Palomba [21] and Kilicdag [20] revealed a significant decrease in Hcy levels after adding Folic acid to Metformin in PCOS patients.

Elevated Hcy levels can damage endothelial cells, impairing the release of nitric oxide (NO) and leading to a net increase in the production of superoxide ($O_2^{\bullet}$-) which lead the formation of atherosclerosis plagues and myocardial infarction (MI). It also leads to structural changes in lipoproteins especially LDL molecules [6]. This may give the idea about the correlation between Hcy, oxidative stress and Apo B/ApoA-I ratio as shown in table (2).

The significant increase in PON1 associated with Folic acid administration to Metformin indicate the essential role of this vitamin in the improving lipid status since the latter is involved in the incorporating of Apo A-1 in HDL-particles[8]. This was confirmed by the negative correlation between PON1 and Apo B/ApoA-I ratio in PCOS patients after treatment with Metformin and Folic acid in the present study.

PON1 may acts synergistically with Folic acid to lower Hcy levels since PON1 can hydrolyze Hcy-thiolactone back to Hcy and Hcy may be then converted either back to methionine (this reaction which needs folate and vitamin B12 as co-factors), or condensed with serine to form cystathionine in a reaction that is dependent on vitamin B6 as reported by Yilmaz [24]. This conclusion can be attributed to negative correlation between PON1 and Hcy in PCOS patients treated with Metformin and Folic acid as reported in table (2).

The results revealed a significant decrease inApoB/ApoA-I ratio after treatment with of Metformin plus Folic acid in PCOS patients because Metformin therapy improves glycemic control and may decrease oxidative stress related oxidation of LDL [25] as well as the protective effects of Folic acid supplementation on endothelial dysfunction by direct effect on free-radical oxidation of LDL lipids and prevent the structural and functional modification of Apo A-1in HDL due to nitration [9,26]. In fact, the decrease in Apo B/ApoA-I ratio levels in this study is more important because it is a potential biomarkers in predicting risk of CVD in patients with PCOS [9].

In the present study, the addition Folic acid to Metformin had further effect on oxidative stress parameters (decrease MDA and increase GSH) due to the fact that Metformin was able to regulate ovarian oxidative stress by decrease androgen level via reducing pituitary gonadotropin secretion [23, 27]. And Folic acidhad direct antioxidant role and free radical scavenging activity[28].

The unique idea in our work is the relationship among Hcy, PON1, oxidative stress and Apo B/Apo A-1 ratio since previous studies lack the correlations among those parameters and the results in table 2 confirmed such relations among different parameters.

Finally administration of a combined treatment of Metformin and Folic acid is quite essential for the lowering of serum levels of Hcy, oxidative stress and improving apolipoprotein status in PCOS patients to exclude further complications associated with this disease.

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Table 1 : The anthropometric and clinical characteristicsof PCOS groups before and after treatment

Variable	Group	N	Mean ± SD	Range	*P* Value
Age (year)	G1-B	25	22.84 ± 3.85	18 – 30	
	G2-B	25	23.92 ± 3.54	19 – 30	P_1 >0.05
BMI (kg/m^2)	G1-B	25	29.25 ± 5.99	21.77– 44.96	P_1> 0.05
	G1-A		29.01 ± 6.01	21.91 –45.54	P_2> 0.05
	G2-B	25	30.56 ± 5.83	22.14– 47.38	P_3> 0.05
	G2-A		28.73 ± 5.67	19.56– 46.09	P_4> 0.05
Hcy (nmol/ml)	G1-B	25	13.96 ± 0.68	12.69 – 15.50	P_1>0.05
	G1-A		16.12 ± 1.03	14.69 -19.93	P_2<0.01
	G2-B	25	14.30±0.79	12.78 – 16.03	P_3<0.01
	G2-A		12.75±0.77	11.50 – 14.21	P_4<0.01
PON1 (mlU/ml)	G1-B	25	163.44±16.54	125.42 – 191.44	P_1>0.05
	G1-A		170.51±7.71	155.49- 183.13	P_2<0.05
	G2-B	25	165.12± 10.62	143.36- 182.06	P_3<0.01
	G2-A		188.15±12.2	167.00 – 211.92	P_4<0.01
GSH (μM)	G1-B	25	22.23±5.18	11.99 – 31.26	P_1>0.05
	G1-A		25.22±5.25	16.12 – 34.01	P_2<0.05
	G2-B	25	20.95±4.91	12.26 – 30.15	P_3<0.01
	G2-A		28.05±4.60	18.97 – 36.86	P_4<0.05
MDA (μM)	G1-B	25	5.89±0.91	4.23 – 8.09	P_1>0.05
	G1-A		5.03±0.85	3.87 – 6.79	P_2<0.01
	G2-B	25	6.10±0.57	5.15 – 7.73	P_3<0.01
	G2-A		4.39±0.69	3.65 – 6.87	P_4<0.01
Apo B/Apo A-1 ratio	G1-B	25	11.16±2.72	5.59– 16.81	P_1>0.05
	G1-A		9.25±1.52	5.47 – 12.20	P_2<0.01
	G2-B	25	10.31±1.96	6.39 – 14.06	P_3<0.01
	G2-A		7.94±1.61	5.19 – 10.67	P_4<0.05

G1-B= patients group before treated with Metformin only, G1-A= patients group after treated with Metformin only, G2-B= patients group before treated with Metformin and Folic acid, G2-A= patients group after treated with Metformin and Folic acid, SD= standard deviation, N= number of patient, BMI= body mass index, HCY= Homocysteine, PON1= Paraoxonase1, CAT= Catalase, GSH= Glutathione, MDA= Malondialdehyde, Apo B/Apo A-1 ratio=Apolipoprotein B/ Apolipoprotein A-1 ratio, P_1= between G1-B and G2-B, P_2= between G1-B and G1-A, P_3= between G2-B and G2-A, P_4= between G1-A and G2-A.

Table 2 : The Pearson correlations between different parameters in PCOS groups before and after treatment

		G1-B		G1-A		G2-B		G2-A	
		Hcy nmol/l	PON1 mlU/ml	Hcy nmol/l	PON1 mlU/ml	Hcy nmol/l	PON1 mlU/ml	Hcy nmol/l	PON1 mlU/ml
Age (year)	r	- 0.106	- 0.040			0.275	- 0.249		
	P-value	0.615	0.850			0.182	0.230		
BMI	r	0.156	- 0.388	0.141	- 0.315	0.0.157	- 0.140	0.371	- 0.184
(kg/m^2)	P-value	0.457	0.055	0.501	0.125	0.454	0.504	0.068	0.378

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MDA (μM)	r	0.517	- 0.751	0.424	- 0.703	0.788	- 0.697	0.506	- 0.611
	P-value	0.008	0.000	0.034	0.000	0.000	0.000	0.010	0.001
GSH (μM)	r	- 0.682	0.751	- 0.529	0.688	- 0.701	0.765	- 0.850	0.890
	P-value	0.000	0.000	0.044	0.000	0.000	0.000	0.000	0.000
Apo B/ Apo A-1 ratio	r	0.553	- 0.554	0.413	- 0.554	0.634	- 0.752	0.752	- 0.680
	P-value	0.004	0.004	0.040	0.004	0.001	0.000	0.000	0.000
Hcy nmol/l	r		- 0.463		- 0.418		- 0.537		- 0.843
	P-value		0.020		0.038		0.006		0.000

G1-B= patients group before treated with Metformin only, G1-A= patients group after treated with Metformin only, G2-B= patients group before treated with Metformin and Folic acid, G2-A= patients group after treated with Metformin and Folic acid, BMI= body mass index, HCY= Homocysteine, PON1= Paraoxonase1, GSH= Glutathione, MDA= Malondialdehyde, Apo B/Apo A-1 ratio=Apolipoprotein B/ Apolipoprotein A-1 ratio and r= pearson correlations coefficient

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3. Submission of Manuscripts,

4. Manuscript's Category,

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- Consequences, including definite statistics - if the consequences are quantitative in nature, account quantitative data; results of any numerical analysis should be reported
- Significant conclusions or questions that track from the research(es)

Approach:

- Single section, and succinct
- As a outline of job done, it is always written in past tense
- A conceptual should situate on its own, and not submit to any other part of the paper such as a form or table
- Center on shortening results - bound background information to a verdict or two, if completely necessary
- What you account in an conceptual must be regular with what you reported in the manuscript
- Exact spelling, clearness of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else

Introduction:

The **Introduction** should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable to comprehend and calculate the purpose of your study without having to submit to other works. The basis for the study should be offered. Give most important references but shun difficult to make a comprehensive appraisal of the topic. In the introduction, describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will have no attention in your result. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here. Following approach can create a valuable beginning:

- Explain the value (significance) of the study
- Shield the model - why did you employ this particular system or method? What is its compensation? You strength remark on its appropriateness from a abstract point of vision as well as point out sensible reasons for using it.
- Present a justification. Status your particular theory (es) or aim(s), and describe the logic that led you to choose them.
- Very for a short time explain the tentative propose and how it skilled the declared objectives.

Approach:

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- Sort out your thoughts; manufacture one key point with every section. If you make the four points listed above, you will need a least of four paragraphs.

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- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

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- Report the method (not particulars of each process that engaged the same methodology)
- Describe the method entirely
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures
- Simplify - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in every other part of the paper - avoid familiar lists, and use full sentences.

What to keep away from

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- Leave out information that is immaterial to a third party.

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Content

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- Present a background, such as by describing the question that was addressed by creation an exacting study.
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- Never confuse figures with tables - there is a difference.

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Approach:

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References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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