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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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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.

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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).

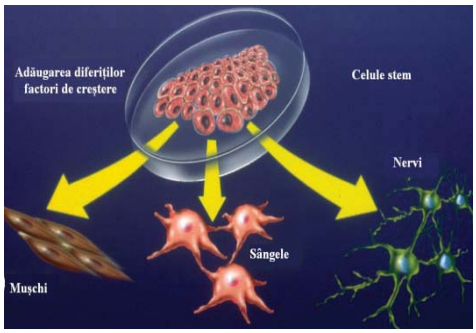

Figure 1 : Differentiation of embryonic stem cells (http://www.srsp.net/kassel/images/Pluripotent_Stem_Cells.jpg)

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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).

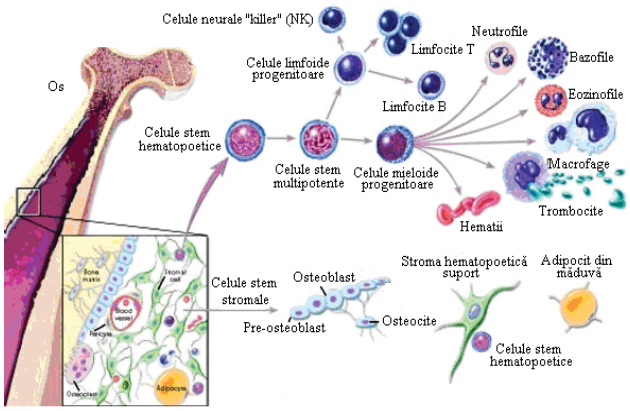

Figure 2 : differentiation of bone marrow stem cells
http://stemcells.nih.gov/info/basics/basics4.asp

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

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).

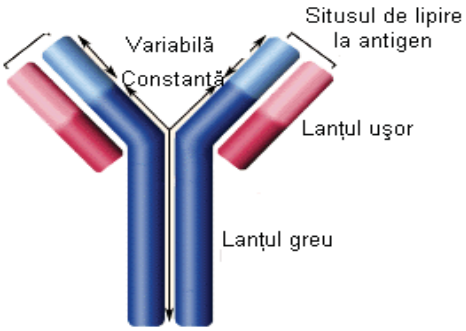

Figure 3 : The structure of an antibody (http://images.google.ro/imgres?imgurl=http://www.capemaster.net)

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).

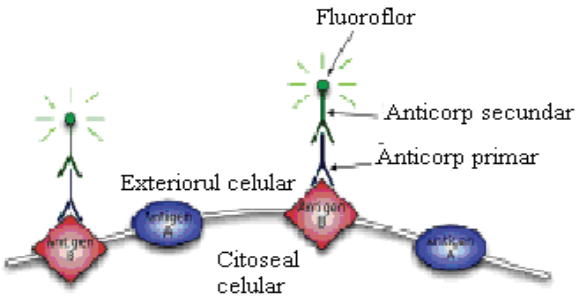

Figure 4 : Attaching the secondary antibody primary antibody http://upload.wikimedia. org/wikipedia/commons/thumb/f/fc/Primary-Secondaryantibody.svg/300px-Primary-Secondaryantibody.svg.png

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

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.

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

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.

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.

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.

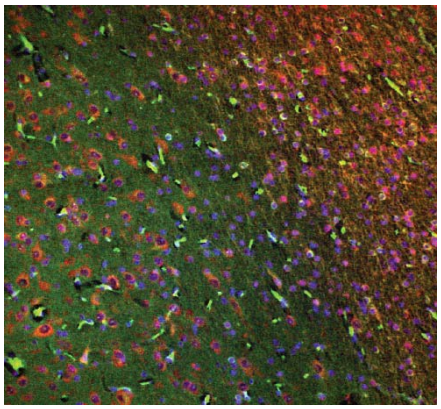

Figure 5 : Double staining with antibodies Synaptophisină primary murine and rabbit NPT II, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 633 goat anti-rabbit (original)

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.

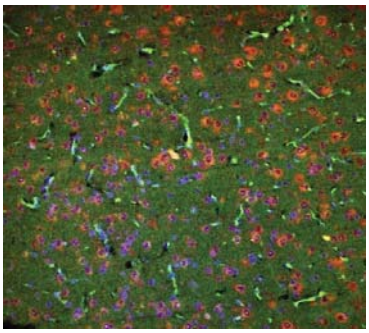

Figure 6 : Staining with primary antibody DsRed Murin and secondary antibody Alexa 488 goat anti Flor murine (original)

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.

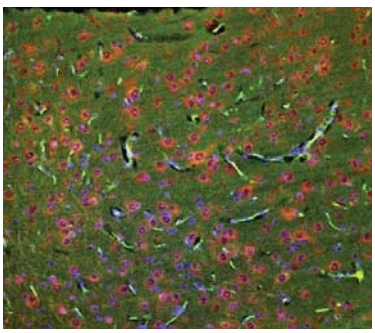

Figure 7 : Double staining with primary antibodies CD 31 and Cytokeratina Murin Murin and 20 antibodies Alexa 488 goat anti-Murin Flor and Flor Alexa 633 goat anti-murine (Original)

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.

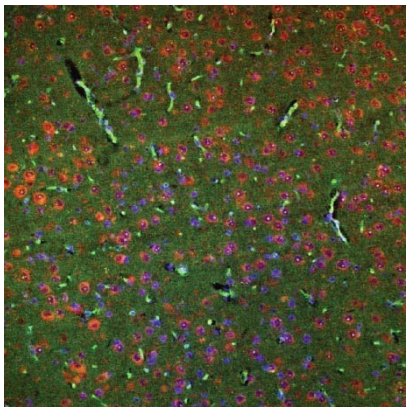

Figure 8 : Double staining with UCHL1 polyclonal primary antibodies, rabbit monoclonal DsRed Murin, and the secondary antibodies Alexa 633 goat anti rabbit Flor and Flor Alexa 488 goat anti-mouse (original)

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.

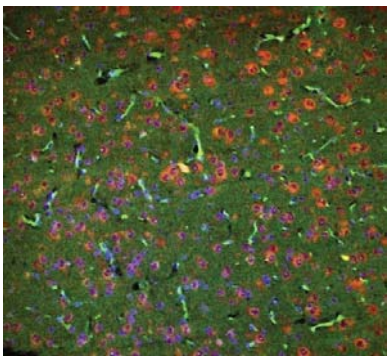

Figure 9 : Double staining with primary antibodies and DsRed monoclonal Murin Chromatogranină A Rabbit, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 633 goat anti-rabbit (original)

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.

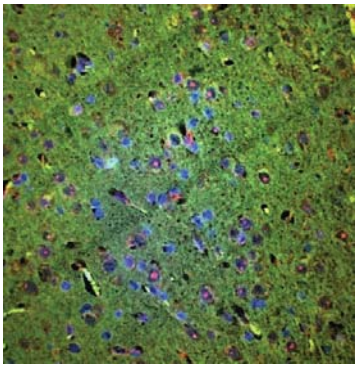

Figure 10 : Double staining with primary antibodies and Oligodenrdocyte Murin Chromatogranină A Rabbit, and the secondary antibodies Alexa 594 goat anti Murin Flor and Flor Alexa 633 goat anti-rabbit (original)

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.

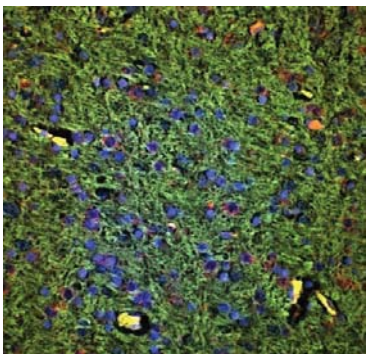

Figure 11 : Double staining with primary antibodies and DsRed Murin Chromatogranină A Rabbit, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 594 goat anti-rabbit (original)

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.

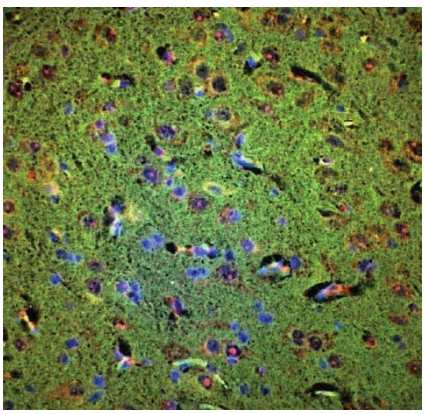

Figure 12 : Double staining with primary antibodies and DsRed monoclonal Murin Chromatogranină A Rabbit, and the secondary antibodies Alexa 488 goat anti Murin Flor and Flor Alexa 594 goat anti-rabbit (original)

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).

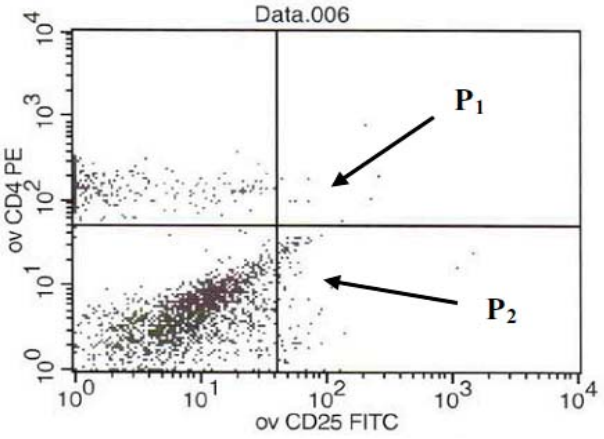

Figure 13 : Results of analysis of CD4 and ov ov CD25 antibody (original)

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).

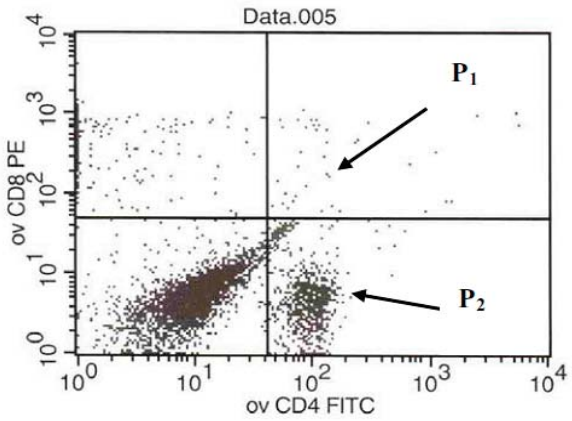

Figure 14 : Results of analysis of antibodies ov ov CD8 and CD4 (original)

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)

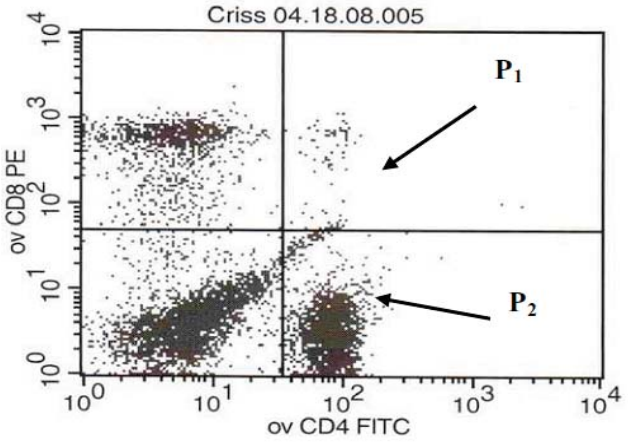

Figure 15 : Results of analysis of antibodies ov ov CD8 and CD4 (original)

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).

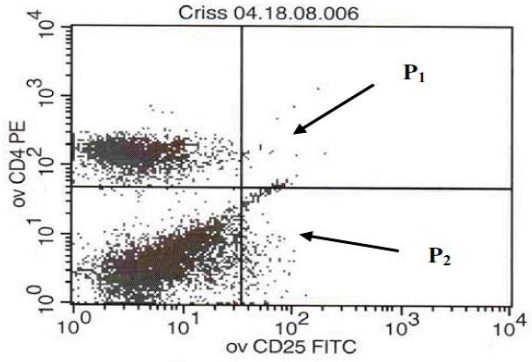

Figure 16 : Results of analysis of CD4 and ov ov CD25 antibody (original)

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

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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