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A Preliminary Study on the Relationship between Micronucleus Formation and Cell Cycle

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ABSTRACT

Micronucleus is an abnormal structure in eukaryotic cells. It is the result of various physical and chemical factors, such as radiation and chemical drugs acting on dividing cells. It is generally believed that micronuclei are originated in backward chromosomes and fragments that have lost centromere and are formed at the end of mitosis. However, other experiments suggest that interphase cells can also form micronuclei. In this study, human peripheral blood lymphocytes were cultured in vitro and cyclophosphamide 20 ug/ml was injected at specific times of the cell cycle to observe the relationship between micronuclei formation and cell cycle. The experimental results confirmed that the cell cycle micronucleus formation.

KEYWORDS: cyclophosphamide; lymphocyte; cell cycle; micronucleus

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Preface

In this study, human peripheral blood lymphocyte micronucleus (MN) was induced by γ-ray, and cell cycle control, BrdU immunofluorescence staining, cytokine B (CB) block cytoplasmic splitting were used to study the micronuclei formation of human lymphocytes and the quantitative relationship between the cell cycle. Results: There was no significant change in micronucleus rate (MNF) and micronucleus cell rate (MNCF) between G0 and G1 lymphocyte irradiation and control group (P> 0.05). Compared with control group, MNCF increased significantly (P <0.01). (3) Compared with the control group, MNF and MNCF increased 90 times and 70 times higher than those of the control group. CONCLUSION: MN can form MN in S and G2 phases in human lymphocyte cycle, but M phase is the main stage of MN formation.

1. Topic background

1.1. Source of the project

Sanmenxia Vocational and Technical College Experimental Teaching Center Meng Quanke teacher.

1.2. Related research

1.2.1 Research status of micronuclei

Micronucleus is located in the cytoplasm independent of the main nucleus of the nucleosome, the staining with the main nucleus, but lighter than the main nucleus, its diameter is less than the main nucleus 1/3, mainly by external damage factors (biological, physical and chemical). The role of cells, resulting in cell chromosome loss or fracture, which in the cytoplasm to form one or several small nuclei [1]. Therefore, the micronucleus test in the foreign compounds (such as drugs, food additives, pesticides, cosmetics and environmental pollutants) genetic toxicity and occupational exposure to genetic damage monitoring and on-site ecological environment testing, diagnosis and prevention of liver cancer, esophageal cancer, lung cancer and other malignant tumors have been a lot of applications. The biggest advantage of the micronucleus test is the economic, simple, rapid, and a large number of comparative

studies at home and abroad, the more consistent view is that the method in the sensitivity, specificity and accuracy, and the classic chromosome aberration analysis method is basically the same [2]. Thus, it is particularly suitable as an experimental method for screening a large number of compounds and on-site populations.

Micronucleus test was founded in the mid-1970s (1973 - 1975) [3,4], many countries and international organizations, has its provisions as new drugs, food additives, pesticides, cosmetics and other toxicological safety assessment will do experiment [5 - 8]. In recent years, with the rapid development of molecular biology technology and penetration into the micronucleus research, greatly expanded the micronucleus test detection and application range, has developed into a simultaneous detection of chromosome breakage, loss, split delay, split imbalance, gene amplification, no separation, DNA damage repair disorder, Hprt gene mutation, apoptosis, cell division imbalance and other genetic endpoints detection, and thus in recent years, the international community has proposed a new micro-nuclear test concept [9], thus greatly expanding the scope of application of micronucleus test [10,11]. Of course, to achieve a test of multiple genetic damage to the end of the test, the need for more new technical means with, such as FISH technology and image analysis technology, which also on China's laboratory conditions and research level put higher requirements.

Due to the synthesis of a large number of new compounds, the application of atomic energy, the discharge of various industrial wastes, it is necessary to have a highly sensitive and technically simple test system to monitor changes in the environment. Only the eukaryotic test system can directly speculate the genetic hazards of mutagenic substances to humans or other higher organisms. In this respect, micronucleus test is an ideal method. At present, many departments at home and abroad have used micronucleus test for radiation damage, radiation protection, chemical mutagen, new drug test, chromosomal genetic disease and early diagnosis of cancer and other aspects. Micronucleus test has become an important method to detect the genetic toxicity of drugs, radiation and toxic substances, and to reflect its cytogenetic damage to human cells or in vitro. In recent years, with the analysis of DNA fragments, the study of apoptosis, the application of monoclonal antibodies, human genomics research and tumor genome anatomical plan to further enrich the micronucleus theory.

1.2.2 Mechanism of micronucleus formation

Micronucleus formation mechanism, most scholars believe that micronucleus mainly from chromosomal aberration without filamentous fragments and single or multiple chromosomes. Cao Jia and other technicians using electron microscopy, BrdU, DNA probe hybridization techniques [12 - 14] to study the micro-core structure and function in a more systematic way and found that some of the lymphocytes micronucleus has a certain structure and function with the ability of DNA replication, different mutagens induced micronuclei have different chromosomal composition, and there is a certain regularity. Such as the aneuploid fracture agent (chemical mutagen) induced micronucleus, mainly composed of the loss of the entire chromosome; chromosome cleavage agent (physical factors) induced micronucleus, mainly composed of chromosome fragments.

When micronuclei were used as an evaluation index for radiation injury, drug screening, and tumor prevention and control experiments to make it form a 'micronucleus assay' that has been affirmed, people are increasingly concerned with and focusing on cell micronuclei in various discussion on the forming mechanism of factors.

1.3. Purpose and meaning

The micronucleus experiment has been widely used since its establishment, because it is sensitive, stable, and can detect chromosomal integrity and chromosome separation to change the two genetic endpoints. With the development of molecular biology technology, it has become a multi-molecular toxicology. It has wide application prospect in the fields of aneuploid poison detection, mutagenic screening and biotoxic mechanism of foreign compounds. It is true that the current micronucleus experiment still exists: the specificity and sensitivity of the DNA probes used to detect micronuclei and telomeres are not very satisfactory and cannot fully analyze which chromosomes are derived from the micronuclei, the micronucleus automated analysis. However, with the continuous discovery of new DNA probes, the development of multicolor fluorescence in situ hybridization and computer-assisted laser confocal microscopy, the micronucleus chromosomal source can be detected more precisely and quickly by micronucleus experiment. To evaluate the toxic effects of foreign compounds.

Micronucleus test has become an important method to detect the genetic toxicity of drugs, radiation and toxic substances, and to reflect its cytogenetic damage to human cells or in vitro. In recent years, with the analysis of DNA fragments, the study of apoptosis, monoclonal antibody application, human genomics research and tumor genome anatomy plan to further enrich the micronucleus theory, but the study is still limited to young erythrocytes, mature lymphocytes and in vitro cell lines. With the further expansion of the scope of micronucleus test, the new micro-nuclear test is coming to us, our scholars should strengthen the research on this method, increase the intensity of new technology demonstration and promote to narrow the distance with the international researcher.

1.4. Design of the guiding ideology

The purpose of this trial is to grasp the basic principles and basic skills of micronucleus test, by studying cyclophosphamide induced human peripheral blood lymphoid micronucleus formation, to determine the micronucleus in the cell cycle which period or the formation of micronucleus rate (MNF)maximum.

2. Program Demonstration

2.1. Design principles of the program

In this study, human peripheral blood lymphocytes were selected as the test subjects to observe the effect of cyclophosphamide on the production of micronuclei and to determine when the micronucleus was formed during the period of the cell cycle or when is the biggest micronucleus rate (MNF).

2.2. Demonstration of the program

2.2.1 Selection of experimental objects

The micronucleus experiment has been extended from bone marrow erythrocytes to a variety of somatic cells and in vitro cultured cells. In the past that red blood cell maturation process can be discharged from the main nucleus. Micronucleus retention in the cell, easy to identify, and peripheral blood containing micronuclei and erythrocytes are often removed by the spleen, so the conventional micronucleus test to mouse bone marrow polychromatic red blood cells as the object of study. 80 years later found that the micronuclei erythrocytes in the mouse spleen cannot be removed effectively. The micronucleus test of peripheral blood erythrocytes in mice was consistent with the micronucleus test of bone marrow polychromatic erythrocytes. Obviously the former is easier. And can be the same animal for multiple sampling analysis. Recently, the method has been generally accepted by international organizations, Japanese scholars also directly isolated from the mouse micronuclei for micronuclei chromosome composition of the study [23]. It has also recently been found that red blood cells can also be micronucleus excreted. And the micronuclei to be discharged mainly originate from the whole chromosome [24].

With the development of technology, has been able to effectively identify micronuclei in nucleated cells. Human peripheral blood lymphocytes, solid tissue biopsy cells, tissue exfoliated cells (oral, urinary tract) micronucleus rate to a certain extent, reflecting the radiation and foreign compounds causes genetic damage to certain organs, tumor investigation showed, diagnosis has a greater reference value, many studies put them as a biological dose monitoring indicators. Germ cell (mainly sperm) micronucleus rate, due to its special genetic status, there are more studies reported.

Cultured cells micronucleus experiments avoid the influence of individual differences, easy to control the experimental conditions, easy to operate. In vitro culture of blood lymphocytes, a variety of mammalian cell lines, primary tumor cells, liver cells and Vicia faba root tips, purple grass and other plant cells have been widely used in micronucleus experimental research. In short, according to the experimental needs to select the appropriate cells for micronucleus experimental study.

2.2.2 Methods of experimental research

(1)Cell kinetic block micronuclei analysis: 1985, Feneeh et al established the method, with cytoplasmic blockers block cytoplasmic division, but does not affect the nuclear division, mitotic cells were specific morphology of the binuclear cells, not cells that undergo nuclear fragmentation maintain mononuclear cell morphology. The rate of bi-nucleated cells and the nucleation rate of nucleated nucleus pulposus were measured, and the genetic damage caused by mutagenic factors and the influence of cell cycle were obtained. This method is popular. Rapid and widely used, not only with the immunofluorescence and in-situ hybridization technology mentioned later, can accurately distinguish the source of micronucleus, to determine the sister chromatid monomer in the offspring of the sub-city. Moreover, by observing whether there is a chromatin bridge between the two subgenus nuclei of the bi-nucleated cells, chromosomal segregation disorder can be detected; inhibition of DNA resection with cytarabine can inhibit the change of micronucleus rate of binucleated cells and detect DNA double-strand damage and DNA excision repair defects; observed with 6-lysyl purine in the growth of the dual-core and multinucleated cell rate, can detect HPRT gene mutation [25].

(2) Immunofluorescence micronucleus analysis: scleroderma patients with serum containing anti-centromere (antikinetochore antibodies), can specifically with the human and some mammalian centromere protein binding, by immunofluorescence detection of micronuclei whether there is centromere protein, can be preliminary to determine whether the micronucleus from the whole chromosome or chromosome fragments. Because scleroderma patients often have significant CREST signs (ie, calcareous, Raynaud's phenomenon, esophageal and finger motility disorders

and scleroderma), this method is also known as CREST staining. This method was first reported in Vig [26] in 1986, and many of the studies were followed by this method. We also used this method to analyze micronuclei induced by Kunming Begonia and acrylamide [27, 28]. Since this method detects centromeric proteins, if the compound can inhibit the inactivation of the centromere or synthesis, it results in false negative results. Studies have shown that, mitomyein c, 5-azacyti-dine, diazepam, pyrimethamine and hydroq-none can be obtained by the above-mentioned pathways leading to a centromere positive micronucleus rate decreased. However, this method is simple, when the test compound has no effect on the centromere protein, it is quite accurate and reliable, so many scholars respected.

(3) Fluorescence in-situ hybridization micronucleus analysis: the use of centromeric DNA probe or the simultaneous application of centromere and telomere DNA probe, by fluorescence in-situ hybridization, detection of nuclei and telomere signal, can be accurate to determine whether the micronucleus is derived from the whole chromosome or chromosome fragment, the number of chromosomes and fragments contained in the micronucleus is estimated. Application of chromosomal specific DNA probe, can detect the sister chromatid monomer is the generation of cell distribution (including chromosome loss and chromosome is not separated), to determine whether the micronucleus contains a few chromosomes. So as to in-depth analysis of the induction of micronuclei formation of the physical and chemical factors of genetic toxicity and toxic targets and so on.

(4) In-situ initiation of DNA synthesis micronucleus analysis: with oligonucleotide primers, through a few rounds of PCR in-situ amplification of the target DNA sequence. And incorporation of digoxigenin-labeled dUTP, such as fluorescence in situ hybridization of the same micron nuclei within the centromere or telomeres show fluorescence signals. Russo et al. used this method to detect mitochondrial C and colchicine-induced mouse spleen cells and telomere composition, that this method is highly sensitive, highly reproducible and faster.

(5) Apoptosis detection of micronucleus cells: apoptosis is a kind of genetic regulation of cell physiological death mechanism, due to some oncogenes and tumor suppressor genes involved in the regulation of apoptosis, has aroused widespread concern. Especially the P53 gene, which is involved both in cell cycle arrest and apoptosis due to DNA damage, and also played important role in maintaining group stability. Studies have suggested that P53 is also associated with micronucleus formation. Cell genetic material damage may occur: still normal division, micronucleus and aneuploidy formation and apoptosis. It can be seen that apoptosis is also an important indicator of the follow-up effect of genotoxic injury. It is important to evaluate the apoptosis rate when the genetic function of the compound is evaluated.

3. Materials and methods

3.1. Materials and Instruments

3.1.1 Drugs and reagents

1 mg / ml Brdu, PHA powder (injection), 1 ug / ml colchicine, inactivated calf serum (1 mg / ml), saline, 0.5% KCl solution, distilled water, methanol, glacial acetic acid, Giemsa stock solution.

3.1.2 Instruments and equipment

80 ℃ incubator, ultra clean table, alcohol lamp, 10 ml centrifuge tube, tweezers, alcohol lamp, glove, mask, centrifuge, electronic Libra, optical microscope, 37 ℃ incubator, pipette, refrigerator, test tube rack, regular clock, degreasing slides, matches and so on.

3.2. Experimental steps

3.2.1 Solution configuration

(1). Conventional solution

1) 1/15 mol / L PBS (Phosphate Buffer Solution, PBS)

A solution: 1 / 15mol / L Na2HPO4 solution

Na2HPO4 9.465 g

Distilled water was added to 1000 ml

B solution: l / 15mol / L KH2PO4 solution

KH2P04 9.07g

Distilled water added to 1000 ml

Packed in a brown bottle, stored in the refrigerator at 4 ℃, with a, B two different liquid mixture in different proportions, you can get the required pH buffer, see the following table:

pH	A solution (ml)	B liquid (ml)	pH	A liquid (ml)	B liquid (ml)
5.29	2.5	97.5	6.81	50.0	50.0
5.59	5.0	95.0	6.98	60.0	40.0
5.91	10.0	90.0	7.17	70.0	30.0
6.24	20.0	80.0	7.38	80.0	20.0
6.47	30.0	70.0	7.73	90.0	10.0
6.64	40.0	60.0	8.04	95.0	5.0

2) 10 μg / ml colchicine

Colchicine 10 mg

Saline 100 ml

Pour into the tea bottle, for the stock solution, 4 ℃ refrigerator to save. Add 9 ml saline into the stock solution 1ml before using.

3) Giemsa Dye

① stock liquid

Giemsa powder 1g

Pure glycerol 66ml

Methanol 66ml

First, Giemsa powder placed in the mortar plus a small amount of glycerol, fully ground, was no particles of paste. And then add all the glycerin, into the 56 ℃ incubator for 2 hours, then add methanol, stored in a brown bottle. Generally used for two weeks as well.

② Working fluid

The stock solution was mixed with phosphate buffer at pH 6.8 at 1:20 before using

(2) Cell culture medium

1) 0.01 mol / LPBS (Phosphate Buffer Saline, PBS) pH 7.2.

0.2mol / L disodium hydrogen phosphate solution (a liquid):

$NaH_2PO_4 \cdot 12H_2O$ 35.814g

Double distilled water to 500ml

0.2mol / L sodium dihydrogen phosphate solution (B liquid):

$NaH_2PO_4 \cdot 12H_2O$ 15.601g

Double distilled water to 500ml

Take the liquid 36ml, B liquid 14ml and NaCl 8.2 grams, add double distilled water to 1000ml. Mixing completely then packing into different container, after autoclave storage in 4 ℃ refrigerator spare.

2) Hanks solution

1. Stock solution

NaCl 160 g

KCl 8g

$MgSO_4 \cdot 7H_2O$ 2g

$MgCI_2\ 6H_2O$ 2 g

Dissolved in 800ml of water.

CaCl2 (anhydrous) 2.8 g

Dissolved in 100 ml of distilled water.

After mixing the two liquids, add water to 1000ml with filter paper filter, plus 2ml chloroform preservative, set 4 ℃ refrigerator spare.

Raw liquid B

Na2HPO412H2O 3.04 g

KH2PO4 1.2g

Glucose 20.0g

Dissolved in 800ml distilled water, filtered with filter paper, and then add 0.5% phenol red 80ml, add water to 1000ml, and finally add 2ml chloroform preservative, set 4 ℃ refrigerator spare.

3) 1640 culture medium (containing 10% calf serum)

RPMI-1640 powder 10.39 g

Double distilled water to 1000ml

Through the appropriate amount of C02 gas, slowly stirring while adding CO2, so that it was completely transparent. Adjust the pH to 7.2 with NaHCO31.5 g.

Double resistance 100u / ml 10ml

Inactivated calf serum 110ml

Mix the above liquid, immediately with G6 filter sterilization equipment, set 4 ℃ refrigerator spare.

4) BrdU solution (200 ug / ml)

With sterile penicillin bottle, weighed at room temperature 1.0mg, sterilized under sterile conditions into the normal saline 9.0ml, dissolved, mix, wrapped with black paper, away from light and stored in the ice box. The best way is making the solution before using.

5) Cyclophosphamide solution

A sprayed cyclophosphamide powder (dose 0.02 g) was taken from the refrigerator and dissolved in 20 ml of purified water so that the concentration of cyclophosphamide was 10 mg / ml. As a result of the concentration of 1mg / ml, so when used to dilute 10 times that is prepared into 1mg / ml cyclophosphamide solution.

6) Tablet fixative

Methanol: glacial acetic acid (3: 1) mixture, is now with the first use.

7) Peripheral blood lymphocyte culture medium configuration

Will be two bottles of heparin 40ml, each bottle plus 10ml calf serum, configured into 20% calf blood bottle culture, sealed, into the 4 ℃ refrigerator for storage.

3.2.2 Blood and peripheral blood lymphocyte culture

Take 5ml of healthy men's venous blood and add into 3 flasks respectively (50 ml 20% calf blood bottle culture medium). After the blood was added to the culture medium, shake well (not too vigorous). So there are three groups in each period.

1. G0 period: 8ml plus the blood of the culture medium, without PHA, immediately after inoculation by adding 1mg / ml cyclophosphamide 0.2ml, 37 ℃ incubator in the cultivation of 18-24h harvest.

2. Adding a PHA into the remaining blood culture solution (first with 2ml was dissolved with distilled water).

① , G1 phase: 10ml plus blood culture medium, immediately add 1mg / ml cyclophosphamide 0.2ml into 37 ℃ incubator and harvesting after 22h.

② , S.G2 period: 20ml blood culture medium was inoculated and cultured in 37 ℃ incubator for 48h. After incubation for 20h-24h, add 1mg / ml cyclophosphamide 0.4ml; before harvesting 2.5-3h, add 1 ug / ml colchicine 1.05ml; 30min before harvesting, add 1mg / ml Brdu 0.33ml.

③ , M phase: 10ml plus blood culture medium inoculated in 37 ℃ incubator for 46h, add 1mg / ml cyclophosphamide 0.2ml, 2.5-3h before harvesting, add 1 ug / ml colchicine 0.53ml and 0.17 ml of 1mg / ml Brdu were added 30min before harvesting.

3.2.3 Peripheral blood lymphocytes

(1) Close the nucleus collection culture: remove the bottle when the culture time is full, gently remove the bottle cap, with a cap dropper to each bottle of supernatant (1.0ml), and then two bottles of parallel specimens into the 10ml centrifuge tube. If the culture is removed when mixed, then the two bottles after mixing into the 10ml centrifuge tube, with the control after the balance of 1500 r / min centrifugal 5 minutes, suction to the supernatant liquid.

(2) Low permeability: to have 2ml culture centrifuge tube by adding 0.5% KCl solution 8ml, mixed 37oC after holding and low permeability 10 minutes.

(3) Pre-fixed: low permeability after adding fixed solution 0.5ml, mix 1000 rpm after centrifugation for 5 minutes, remove the supernatant with dropper (try to clean the net, but cannot lose the sediment).

(4) Fixed: the sediment gently mix, along the centrifuge tube wall to join the fixed solution and quickly and gently mixed the sediment, breakdown all the clots formed, then add the liquid to 3 - 4ml, cover it , put 37 ℃ insulation fixed for 15 minutes. After fixing, centrifuge with 1000 rpm for 5 minutes, take out and remove the supernatant (clear the supernatant as much as possible, but cannot lose the sediment), repeat and fix 2 times.

(5) Producer: After the third fixed to the supernatant, leaving the sediment 0.2 - 0.4ml, mix then use a dropper drop respectively into the pre-cooling of the slide until one-third of the slide, blow gently and make it scattered, then fixed with burning, the unstained specimens was stored and spared.

3.2.4 Dyeing

The test was taken Giemsa staining, until the bone marrow tablets dry naturally after the preparation of fresh pH7.4 phosphate buffer 10% of the Giemsa dye stained 10 - 15min, stained with distilled water after washing and dry naturally.

3.2.5 Observations

Firstly, used low magnification, select the area with cell distribution evenly, moderate density, complete shape, well-dyed. Then, turned to the high-power microscope to take pictures of the results.

4. Conclusion and experience

4.1. Conclusion

Cell cycle can be formed at all times the micronucleus, the most of the period of micronucleus is G1 phase, followed by the G2 phase. The micronucleus rate of S phase cells was significantly lower than that of G1 phase, which indicated that most of the G1 phase micronucleus cells could not enter S phase, and the cell proliferation was discontinued, which had far-reaching significance for the prevention of cancer.

4.2. Experience

4.2.1 Summary

In the process of production, the slide should be placed in advance of the refrigerator, ready to be frozen. Production of the film to the way, the use of frozen slide is because the effect of frozen slides is better than ordinary slides.

Dyeing: In this experiment, Giemsa staining was used. When dyeing, the dyeing solution should be covered with the whole slide, making the dyeing process more complete. At the same moment, put paper below the slide to prevent Giemsa dye contaminate the experimental bench. Dyeing time should be not less than 15 minutes, with distilled water rinse the dye, you should let the distilled water with a small water flow evenly from the slide at one end of the red, and cannot let the water directly contact the stained cells to prevent wash the cells away.

4.2.2 Existing problems

After the discovery and summary of the data, the formulation and modification of the program, and the stages of the experiment, some experimental results were obtained, but many problems and a large number of areas to be improved were found during the experiment.

(1) The application of dyeing methods

At the time of micronucleus test, Giemsa staining, feulgen staining and acridine orange fluorescence staining were selected. This test takes a simple, easy-to-use Giemsa staining, although this method is particularly prominent advantages, there are also some shortcomings. In general, this staining does not distinguish between DNA, RNA and other basophilic particles in cells, and is therefore prone to false positives, overestimating the levels of micronuclei produced in erythrocytes. Due to the limitation of test time and laboratory conditions, only one dyeing method has been adopted in this experiment. It is hoped that in later experiments, several different dyeing methods can be taken at the same time. For example, Giemsa staining and acridine orange fluorescence staining can be applied to the same test, in order to more accurately observe the test results.

(2) To explore the mechanism of chromosome damage in micronucleus test

This test is a traditional micronucleus test, can only observe the chemical toxic substances for the genetic toxicity of lymphocytes, cannot identify toxic drugs are aneuploidy or chromosome breakage agent. If the introduction of immunofluorescence techniques into this traditional micronucleus test, highlighting the centromeric portion of the chromosome, it can be determined by identifying whether the micronucleus in the cell is a chromosome fragment or a chromosomal fragment containing a centromere or one or more composition of the chromosomes, to identify the types of chemical toxic substances.

Hope that fluorescence immunoassay will be introduced in future experiments to determine the types of genotoxic substances and the most important causes and mechanisms of chromosome damage.

5. Appendix

Attachment 1: Experimental drug

Drug Name	Nature	Chemical	Source
Methanol	Analysis pure	CH_3OH	Sinopharm Group Chemical Reagent Co., Ltd.
Glacial acetic acid	Analysis pure	CH_3COOH	Wuhan Hongshan Zhongnan Chemical Reagent Co., Ltd.
Cyclophosphamide	Chemistry pure	$C_3H_7O_2$	China Pharmaceutical (Group) Shanghai Chemical Reagent Company
Sodium chloride	Chemistry Pure	NaCl	China Pharmaceutical (Group) Shanghai Chemical Reagent Company
Potassium chloride	Chemistry Pure	KCl	China Pharmaceutical (Group) Shanghai Chemical Reagent Company

Attachment 2: Experimental apparatus

Instrument Name	Model	Source
Optical microscope	E-100	Nanjing Ji Fei Technology Co., Ltd
Electronic balance	KD-BML	Zhejiang Jinhua City Kedi Instrument Equipment Co., Ltd
High - speed centrifuge	M800	Jiangsu Jintan billion - Electronics Co., Ltd

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