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# ECOLOGICALLY SAFE METHOD TO CONTROL THE EPIDEMIC SITUATION ON ANIMAL TUBERCULOSIS IN UKRAINE

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For adaptation, selection and accumulation of production strains bacterial mass, Sotona KF and Sotona-KhB growth media were developed, where cultures of *M. bovis* begin to grow earlier by (4.2 ± 1.1) days and give more bacterial mass accumulated. New technological procedures of manufacturing PPD-tuberculin by means of microfiltration and ultracentrifugation methods have been developed, permitting to obtain an active and specific allergen. A method for sensitization of experimental animals by inactivated *M. bovis* culture has been developed to perform the control study of the drug. The method is specific, safe for the health of animals and humans, it prevents the spread of living mycobacteria in nature.

**Key words:** tuberculosis, mycobacteria, PPD-tuberculin, membrane microfiltration, ultracentrifugation.

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Documents issued by the Ministry of Ecology and Natural Resources (MENR) of Ukraine, its Department of Ecological Security and EU, recognize the delayed-type hypersensitivity (DTHS) test - a tuberculin test - as the main method for detecting tuberculosis-infected animals [10-12]. In Ukraine, to perform the test, the State Enterprise "Sumy Biological Factory" manufactures tuberculin purified (*Purified Protein Derivative -PPD*) for mammals, developed by specialists of the Tuberculosis Laboratory at the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" (NSC IECVM) NAAS of Ukraine, which is manufactured using the *M. bovis* IEKVM-1 production strain [2, 5, 7].

Animal trade between the EU countries is performed in compliance with the EU Council Directive 97/12 of 17.03.1997, which implies tuberculinisation of animals to be carried out using *PPD* or *HCSM* tuberculins, which must be made of *M. bovis* "AN5" or "Valle" strains [6-8, 12, 13, 14]. Therefore, the current topical field of research is the harmonization of domestic *PPD*-tuberculins in compliance with the EU requirements.

When manufacturing *PPD*-tuberculin, the most vulnerable link in the technological process is deep sterilizing filtration through asbestos filters. Their application does not permit to receive a reliable sterile preparation. In addition, the use of deep filters causes significant protein loss, which reduces the yield of the drug. The membrane methods implementation into the PPD-tuberculin manufacture technology permits to improve the ecological safety of production by eliminating due to exclusion of asbestos filters from the technology, to reduce the use of water and the loss of tuberculoprotein [1, 2, 5].

According to the current veterinary legislation, safe farms of Ukraine perform compulsory scheduled allergic studies (tuberculinisation) of all livestock on epizootic (epidemiological) indices and before the sale of animals for breeding or production purposes [4, 7, 9]. At all state breeding enterprises, state and private breeding farms, regardless of the animal welfare terms, the breeding stock of cattle is studied for tuberculosis twice a year, and all growing animals are studied starting from the age of 40 days once a year. At farms supplying milk to children's and medical institutions, sanatoriums, health resorts or for manufacturing exported products, all the animal stock is examined by an allergic method twice a year, starting from 40 days of age. According to the veterinary report, in 2016, 3.4 million allergic studies for tuberculosis were performed [3-5].

The above facts convincingly testify that animal breeding of Ukraine with a stock of cattle about 4 million heads requires the annual production and using of a considerable quantity (about 8 million doses) of highly active and specific PPD-tuberculin for mammals that meets the requirements of the European Community and provides high-quality and efficient allergic test for tuberculosis. The technology for manufacturing and control of PPD-tuberculin by eliminating the use of live mycobacteria

to improve the environmental safety of the drug needs to be improved, which is particularly important for further maintenance of the epizootic well-being of the country on animal tuberculosis.

**The purpose** of the article was develop and implement into production an environmentally safe drug for allergic diagnosing of mammalian tuberculosis, meeting the specified EU requirements.

**Materials and methods.** The principle of obtaining the purified derivative of tuberculoprotein (*Purified Protein Derivative - PPD*) using the culture filtrate of mycobacteria, cultured on a synthetic growth medium by the trichloroacetic acid precipitation method, has remained unchanged since the development of the PPD-tuberculin manufacture technology by F.B. Seibert (1934, 1949). The disadvantage of this method is low yield and limited specificity of the final product - PPD-tuberculin. The most vulnerable link in the technological process is deep sterilizing filtration through asbestos filters, which does not permit to obtain a reliable sterile drug and results in the protein loss in significant amount with the above filters.

To avoid the said disadvantages, a technological scheme for obtaining PPD-tuberculin was suggested, which included the treatment of a culture fluid (after inactivation by autoclaving at 110$^{o}$ C, separating and utilizing the bacterial mass of the production strain mycobacteria) by the membrane microfiltration method using Sartoclean®CA capsules with a pore diameter of 0.45 μm and tuberculin protein fractions separation after precipitation with trichloroacetic acid by means of ultracentrifugation at 14,000 rpm followed by sterilizing microfiltration of the permeates obtained through Sartoclean®CA capsules with a pore diameter of 0.2 μm.

Sartoclean®CA single-layer filters made of cellulose acetate with a heterogeneous double layer, due to the built-in pre-filtration system necessary for cost-effective system layout, provide the highest rates of the total filter capacity and the highest protein yield while microbial retention filtration. After microfiltration with Sartoclean ® CS ultrafiltration membrane filters with a pore diameter of 0.20-0.45 μm (NBSP) and HOMM 150-1000 kDa, the drug contains protein fractions of mycobacteria with a molecular weight of 150-1000 kDa, which display the highest rates of diagnostic activity and specificity.

In general, during the project performing, the following methods were used: bacteriological (microscopic, cultural, biological), electron microscopic, radiological, immunological, molecular genetic, chemical (determining the mass percentage of sodium chloride, phenol, glycerol, etc.), biochemical (determining the mass percentage of protein by Kjeldahl method, etc.) and biotechnological methods (cultivation of mycobacteria, their inactivation, protein separation, purification by dialysis, methods of membrane microfiltration and ultracentrifugation) in compliance with generally accepted procedures [1, 2, 3, 13, 15].

Table 1

**Stages of performing experimental works**

| Stage | Works performed |
|---|---|
| First | Adaptation, selection, study of morphological, cultural and biological properties and deposition of *M. bovis* Vallee KMEEV-9 and *M. bovis* Vallee KMIEV-9KM production strains |
| Second | Development of the technology for manufacturing mammalian PPD-tuberculin purified using the *M. bovis* Vallee KMEI-9KM production strain, methods of membrane microfiltration and ultracentrifugation and their implementation into production |
| Third | Development of a method for determining the activity of purified (PPD) tuberculin for mammals in animals sensitized with avirulent cultures of *M. bovis* |
| Fourth | Manufacturing of experimental and production series of the drug using the developed and implemented technological methods and production strains. Performance of the control studies and determining the level of activity of the drug experimental series |

**Results of the study and their discussion.** The first stage of the environmentally safe tuberculin development is selection of the tuberculosis pathogen production strain. For this purpose, the adaptation, study of the morphological, cultural and biological properties of *M. bovis* Valle strain was carried out, since this strain, in combination with the AN-5 strain, was recommended by the EU Council Directive 97/12 of 17.03.1997 for manufacturing mammalian PPD-tuberculin. We obtained, within the framework of creative exchange with RUP “SN Vysheleskyi IEV” (Minsk), a sublimated strain of a bovine tuberculosis pathogen, which according to the “Strain Passport” was called “*M. bovis* Valle KMIEV-9“,we depreserved and adapted it to the growing media by Pavlovskyi, Levenshtein-Jensen, IECVM, Sotona KF and Sotona KhB.

In order to obtain a production strain with accelerated growth and increased accumulation of bacterial mass *M. bovis* Valle KMIEV-9 was subjected to gamma irradiation. After irradiation with the doses of 0.00645-206.40 C/kg by means of “Rocus” γ-emitter (source of radiation $^{60}$Co, 0.0138 Gy/s), the culture was sown on an elective growth medium. In this case, statistically significant acceleration of the

culture growth rate was not recorded. Changes in the microorganisms' ultrastructure were not observed either during electron microscopic studies. However, during this strain cultivation on the "Sotona KF growth medium for accelerated bacterial mass accumulation", accelerated growth of mycobacteria and increased accumulation of bacterial mass was recorded compared to the Sotona medium in the classical version. The initial growth onset of the *M. bovis* Valle KMIEV-9 production strain culture was observed by 3.0 ± 0.1 days earlier, formation of confluent growth (microbial film) - by 6.0 ± 0.3 days earlier. The yield of bacterial mass was by 7.4 ± 0.3 mg higher. That is, Sotona KF medium had some stimulating and selective properties, that permitted to obtain a strain with accelerated growth of mycobacteria. Sampled by means of selection mycobacteria isolate with accelerated growth after careful study of its cultural, morphological and biological properties was named "Bovine tuberculosis pathogen *M. bovis* Valle KMIEV-9KM production strain". After being transferred to Sotona KhB synthetic production medium, the phenomenon of growth acceleration persists for 2-3 generations and is further lost. Thus, the acquired properties of accelerated growth were the result of phenotypic modification, but not of the mutational changes at the genome level (not inherited). The obtained strain is a phenotypic modifier of the bovine tuberculosis pathogen *M. bovis* Valle KMIEV-9 strain.

During the study of electron microscopic specimens of *M. bovis* Valle KMIEV-9KM production strain cultures it was observed that mycobacteria have the appearance of short or moderately long sticks, located in the form of assemblies and conglomerations. Polymorphism of microorganisms is observed (fig. 1).

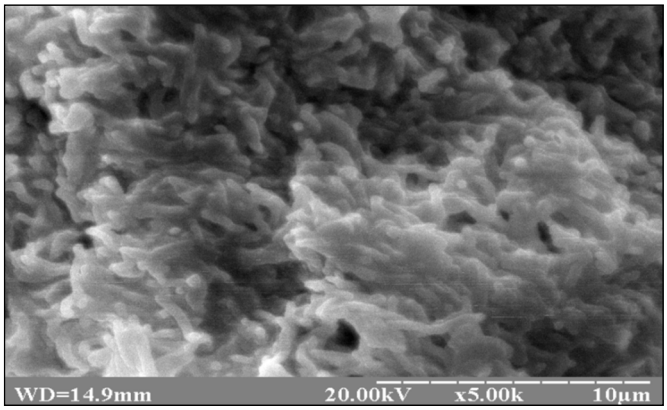


Fig. 1. Mycobacteria of the *M. bovis* Valle KMIEV-9KM production strain on the Pavlovskyi growth medium (conglomeration of rod-shaped forms)

Pure cultures of the first and subsequent generations of the M. bovis Valle KMIEV-9KM strain in the Sotona KF and Sotona KhB medium in smears stained by Ziehl-Neelsen have the appearance of red-colored sticks. At the beaf-extract agar (BEA) beaf-extract broth (BEB) the cultures do not grow; they only grow on egg and potato growth media at 37° C. Colonies of cultures are dry, small, ivory-colored. The growth rate in the subculture is 10-20 days. On the surface of liquid growth media a fragile film is formed without the medium turbidity.

During bacteriological studies, cultivation and bacterial mass accumulation of *M. bovis* Valle KMIEV-9 and *M. bovis* Valle KMIEV-9KM strains on Sotona KhB medium were performed. The terms of the initial confluent growth and production strains' accumulated bacterial mass were determined (table 2). *M. bovis* Valle KMIEV-9KM culture began to grow by two days earlier, giving accumulation of bacterial mass by 6 mg more. Microbial film was formed by 4.5 days earlier compared to the *M. bovis* Valle KMIEV-9 stock strain. Thus, the use of the *M. bovis* Valle KMIEV-9KM production strain permits to accelerate growth, to raise accumulation of mycobacteria bacterial mass; it permits to accelerate the technological process and increase the output of tuberculin.

Table 2

**Terms of the initial confluent growth onset (formation of a microbial film) and the weight of the accumulated bacterial mass of production strains**

| Study parameters | *M. bovis* Valle KMIEV-9 | *M. bovis* Valle KMIEV-9KM |
|---|---|---|
| Terms of the initial growth onset, days | 10.0 ± 1.0 | 8.0 ± 1.0 |
| Term of the microbial film formation, days | 19.5 ± 3.0 | 15.0 ± 0.9 |
| Accumulated bacterial mass, mg | 59.5 ± 0.3 | 65.5 ± 0.2 |

Using the suggested method, the suspension protein was separated from the liquid by means of ultracentrifugation at 14,000 rpm using an industrial high-speed separator of small volume (table 3).

Table 3

**Output of tuberculoprotein and mass percentage of protein in PPD-tuberculin manufactured according to different technological schemes**

| Features of the study | Output of tuberculoprotein after TCA precipitation, g | Output of tuberculoprotein per 1 l of medium, g | Mass percentage of protein, mg/cm$^3$ |
|---|---|---|---|
| Classic technology of tuberculin manufacture | 19.8 ± 0.2 | 1.4 ± 0.3 | 0.8 ± 0.1 |

| | | | |
|---|---|---|---|
| Technology using membrane microfiltration and ultracentrifugation | 28.4 ± 0.3 | 2.1 ± 0.4 | 1.4 ± 0.2 |

The materials of the table indicate that when using the membrane microfiltration and ultracentrifugation methods for tuberculin manufacture, the yield of tuberculoprotein in the drug is significantly increased (by 8.6 ± 0.1 g) after trichloroacetic acid precipitation, the yield of tuberculoprotein per 1 liter of medium - by 0.7 ± 0.1 g and the mass percentage of protein - by 0.6 ±0.1 mg / $cm^3$. Due to the use of ultrafiltration membranes with the filter capacity of 150-1000 kDa and capsules of Sartoclean®CA with a pore diameter of 0.20-0.45 μm, the drug is sterile, highly purified, containing protein fractions with the highest rates of diagnostic activity and specificity (150-1000 kDa ), while deep sterilization filtration through asbestos filters does not permit to obtain a reliably sterile drug causing a significant amount of protein and water losses with the said filters.

The next stage of work is the development of an environmentally safe method for determining the activity of purified PPD-tuberculin for mammals in animals sensitized with avirulent cultures of *M. bovis*. During manufacture of an allergen for diagnosing mammalian tuberculosis, the State Enterprise "Sumy Biological Factory" uses the "Method for monitoring the determination of the mammalian PPD-tuberculin purified activity" (Kassich et al., 1999), which provides for the use of both bovine tuberculosis pathogen and attenuated (live, weakened) *M. bovis* BCG vaccine strain for sensitization of animals. However, live cultures of *M bovis* are potentially dangerous in terms of epidemiology and epizootiology, since there is a possibility of reversion, i.e., reproduction of the attenuated strain's pathogenicity.

Our work is based on the task of developing a specific and safe for animals and humans technique for determining the activity of mammalian PPD-tuberculin purified in experiments on guinea pigs and large cattle using inactivated by autoclaving bacterial mass of *M. bovis* Valle. As a control, sensitized culture of animal BCG vaccine strain was used. Sensitization of large cattle and guinea pigs, determination of the tested series activity compared to that of the control, determination of the microbial contamination absence, other drug control stages were carried out in compliance with DSTU 4664: 2006: "Tuberculin purified for mammals in standard solution". Experimental pilot production series 1 (E) and 2 (E), PPD-tuberculin purified for mammals were manufactured in 2015 and 2016 using the developed and implemented technological methods and production strains.

Based on experiments performed on guinea pigs and healthy cattle vaccinated with inactivated by autoclaving *M. bovis* Valle culture (experimental animal groups) and the culture of the *M. bovis* BCG vaccines strain (control groups) it was determined that production series of "PPD-tuberculin for mammals purified", produced by the State Enterprise "Kherson biological factory" and tested for activity, specificity, absence of bacterial contamination and other indices provided by DSTU 4664: 2006 and the EU Council's Directive 97/12 of 17.03.1997, are active, specific and suitable for allergy study of animals with tuberculosis.

The activity of the tuberculin 1 (E) and 2 (E) series in international units (IU) was 49000 and 47000 IU/mg, respectively, which complies with DSTU 4664: 2006. Thus, methods for determining tuberculin activity in large cattle, guinea pigs sensitized with inactivated *M. bovis* Valle culture and in vaccinated with BCG vaccines animals are equally efficient. A method for controlling the tuberculin activity in animals, vaccinated with inactivated bacterial mass of *M. bovis* Valle, is efficient and specific, and besides, it prevents the living mycobacteria spread in nature.

The principle of obtaining purified derivative of tuberculoprotein (Purified Protein Derivative) out of a culture filtrate of mycobacteria cultured on a synthetic growth medium lies in precipitation of protein fractions with trichloroacetic acid or ammonium sulfate [1, 3, 5, 6].

A significant drawback of this method is the low yield and limited specificity of the final product. Tuberculin, manufactured using membrane fractionation techniques with a molecular weight of 3-300 kDa, has the highest specific activity. The authors [5, 6] showed that removal of the macromolecular fraction from the drug raises the specific protein content and the diagnostic specificity and, simultaneously, reduces the allergen reactogenicity. According to [7, 9], protein fractions with a molecular mass of 90 kDa or more have the highest activity, but small specificity (100% and 20%, respectively). Protein fractions with a molecular weight of 150-1000 kDa have the highest indices of diagnostic activity and specificity.

Therefore, we suggest the culture fluid treatment by the membrane microfiltration method after the bacterial mass separation using Sartoclean®CA capsules with a pore diameter of 0.45 μm NBSP and HOMM 150-1000 kDa and separation of tuberculin protein fractions after their trichloroacetic acid precipuitation by means of ultracentrifugation at 14,000 rpm followed by sterilizing microfiltration of the obtained permeates through Sartoclean®CA capsules with a pore diameter of 0.20 μm.

The use of membrane microfiltration and ultracentrifugation technology in the drug significantly raises the yield of protein by 8.6 ± 0.5 g, the yield of tuberculin per 1 liter of medium – by 0.7 ± 0.1 g and the protein fraction - by 0.6 ± 0.1 mg / cm3. After microfiltration with ultrafiltration membrane filters Sartoclean®CA with pore diameter 0.45-0.20 μm (NBSP) and HOMM 150-1000 kDa the drug contains protein fractions of mycobacteria with a molecular weight of 150-1000 kDa having the highest indices of diagnostic activity and specificity.

## Conclusions

1. Using the *M. bovis* Vallee KMIEV-9KM production strain in cultivation on Sotona KhB synthetic growth medium permits to accelerate growth and accumulation and raise the bacterial mass yield of mycobacteria by 6.0-7.9 mg per one bottle, gives an opportunity to accelerate the technological process and to raise tuberculin yield up to 1.20 ± 0.1 mg/cm$^3$.

2. In the drug, the protein output is significantly increased after TCA precipitation by 8.6 ± 0.5 g, tuberculin output per 1 liter of medium – by 0.7 ± 0.1 g, protein content – by 0.6 ± 0.1 mg/cm$^3$. The drug turns sterile and highly purified.

3. After microfiltration on ultrafiltration membrane filters Sartoclean®CA with pore diameter of 0.20-0.45 μm and HOMM 150-1000 kDa, the drug contains protein fractions of mycobacteria with a molecular weight of 150-1000 kDa having the highest indices of diagnostic activity and specificity.

4. The developed method to determine the activity of purified PPD tuberculin for mammals is specific and safe for the health of animals and humans, prevents the spread of living mycobacteria in nature. Determination of the tuberculin activity on vaccinated with inactivated *M. bovis* Vallee culture in cattle, guinea pigs and animals vaccinated with BCG vaccine strains is equally effective.

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## Реферати

**ЕКОЛОГІЧНО БЕЗПЕЧНИЙ ЗАСІБ КОНТРОЛЮ ЕПІДЕМІЧНОЇ СИТУАЦІЇ З ТУБЕРКУЛЬОЗУ ТВАРИН В УКРАЇНІ**

**Кассіч В.Ю., Уховський В.В., Сосницький О.І., Бібен І.А., Зажарський В.В., Кассіч О.В.**

Для адаптації, селекції та накопичення бактеріальної маси виробничих штамів розроблено живильні середовища Сотона КФ і Сотона-ХБ, на яких

**ЭКОЛОГИЧЕСКИ БЕЗОПАСНЫЙ СПОСОБ КОНТРОЛЯ ЭПИДЕМИЧЕСКОЙ СИТУАЦИИ ПО ТУБЕРКУЛЕЗУ ЖИВОТНЫХ В УКРАИНЕ**

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Для адаптации, селекции и накопления бактериальной массы производственных штаммов разработаны питательные среды Сотона КФ и Сотона-ХБ, на которых культуры

культури *M. bovis* починають рости раніше на (4,2 ± 1,1) діб і дають більше накопичення бактеріальної маси. Розроблено нові технологічні прийоми виготовлення ППД-туберкуліну з використанням методів мікрофільтрації та ультрацентрифугування, що дозволило отримати активний і специфічний алерген. Для проведення контрольних досліджень препарату розроблено спосіб сенсибілізації дослідних тварин інактивованою культурою *M. bovis.* Спосіб є специфічним, безпечним для здоров'я тварин і людини, він запобігає поширенню живих мікобактерій у природі.

**Ключові слова**: туберкульоз, мікобактерії, ППД-туберкулін, мембранна мікрофільтрація, ультрацентрифугування.

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*M. bovis* начинают расти раньше на (4,2±1,1) суток и дают большее накопление бактериальной массы. Разработаны новые технологические приемы изготовления ППД-туберкулина с использованием методов микрофильтрации и ультрацентрифугирования, что позволило получить активный и специфический аллерген. Для проведения контрольных исследований препарата разработан способ сенсибилизации опытных животных инактивированной культурой *M. bovis.* Способ является специфичным, безопасным для здоровья животных и людей и препятствует распространениюю живых микобактерий в природе.

**Ключевые слова**: туберкулез, микобактерии, ППД-туберкулин, мембранная микрофильтрация, ультрацентрифугирование.

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# МІЄЛОАРХІТЕКТОНІКА КАПСУЛИ КОЛІННОГО СУГЛОБА КОТІВ

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У статті показані кількісні співвідношення нервових елементів суглобової капсули котів в анатомічних частинах-антагоністах колінного суглоба (латеральна, медіальна, дорсальна, плантарна). Нервові структури виявляли нейроморфологічним методом – імпрегнацією азотнокислим сріблом за методами Більшовського-Грос в модифікації Лаврентьєва та Кампоса, які ми об'єднали. Статистично продемонстровано, що дорсальна частина суглобової капсули є найменш насиченою нервовими структурами. Бідніші рефлексогенні зони знаходяться в медіальній, латеральній та дорсальній частинах капсули через меншу кількість інкапсульованих закінчень. Рецептори представлені тільцями Фатер-Пачині, Руфіні, Краузе та вільними. У капсулі колінного суглоба котів, як представників фалангоходячих тварин, переважна більшість інкапсульованих нервових закінчень, ніж вільних. Ці дані можливо використати для вияснення закономірностей утворення больових відчуттів у колінному суглобі, для вияснення акупунктурних та акупресурних зон, мінімізування пошкоджень за доступів до органокомплексу синовіального середовища, а також у порівняльному аспекті для оцінки експериментального матеріалу.

**Ключові слова:** колінний суглоб, капсула, рефлексогенна зона, вільні рецептори, інкапсульовані нервові закінчення.

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Колінний суглоб має широкий діапазон рухів, і належна нейроанатомічна організація є критичною для стабільності коліна [6]. Синовіальна оболонка суглобу виконує ряд функцій, які забезпечують нормальну життєдіяльність суглобу у звичайних умовах, різноманітних навантаженнях та при виникненні патологічних змін локального характеру та організму в цілому [2]. Розподіл, розташування та просторова локалізація механорецепторів важливі для короткості та точності нейронного сигналу в пропріорецептивній інформації, яка необхідна для підтримання функціональної стабільності [14]. Більшість дослідників визнають потенційну роль механорецепторів у пропріоцептивній функції суглобів. Зовсім нещодавно було показано, що пошкодження суглобових механорецепторів може призвести до пропріорецептивних дефіцитів, які можуть призвести до періодичної нестабільності [14]. Це може бути обумовлене проблемами з механорецепторами, оброблюю та ретранслюванням соматосенсорної інформації у вищі центри або специфічним втручанням суглобів в конгінитивні процеси з боку болі [11]. Під час тотальної артропластики видаляється більша частина суглобової капсули, в тому числі більшість механорецепторів, важливих для пропріорецепції, що потенційно обмежує післяопераційне функціональне відновлення та має подовжений дефіцит пропріорецепції [12]. Ноцицептивні подразники сприймаються С-волокнами, що містяться в усіх структурах суглоба, за винятком суглобового хряща [5]. Основний механізм виникнення хронічного больового синдрому при захворюваннях опорно-рухового апарату – ноцицептивний [3]. Проблема регенерації нервових волокон привертає до себе увагу багатьох дослідників, так як ушкодження периферійних нервів викликає інвалідність, особливо в молодому віці [1]. Карта локалізації суглобових гілок