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ANGIOGENIC POTENTIAL OF THE HYDROXYAPATITE-BASED COMPOSITES LOADED WITH PLANT EXTRACT AND NANOPARTICLES

Viktoriia Holubnycha
https://orcid.org/0000-0002-1241-2550
Public Health Department, Sumy State University, Sumy, Ukraine

Oleksandr Govorun
https://orcid.org/0000-0002-6626-1241
Department of Biology and Biology Teaching Methodology, Sumy State Pedagogical University named after A.S. Makarenko, Sumy, Ukraine

Veronika Pron
Academic and Research Medical Institute, Sumy State University, Sumy, Ukraine

Marharyta Holubnycha
https://orcid.org/0009-0008-7909-8755
Lviv Polytechnic National University, Lviv, Ukraine

Ganna Yanovska
https://orcid.org/0000-0001-8040-7457
Institute of Agrophysics, Polish Academy of Sciences, Lublin, Poland

ABSTRACT

Introduction. The growing ageing population and increasing incidence of bone-related disorders have intensified the demand for advanced regenerative materials. Hydroxyapatite-based composites enriched with metal nanoparticles and plant extracts show great promise due to their biocompatibility, antimicrobial activity, and ability to promote tissue regeneration. This study aimed to evaluate how such functionalized composites influence vessel formation using the chick chorioallantoic membrane (CAM) model.

Methods. Hydroxyapatite-based composites were prepared by incorporating silver nanoparticles (10 µg/g) and pomegranate peel extract (30 mg/g) into hydroxyapatite, followed by stirring and drying. Supernatants of the obtained composites were applied onto the chick chorioallantoic membrane (CAM) of fertilized eggs on embryonic day 10 to assess angiogenic activity. After 24–48 h incubation, CAM images were taken and analyzed morphometrically using ImageJ to quantify vessel density and bifurcation points. Statistical significance was evaluated with GraphPad Prism ($p < 0.05$). The study was approved by the Local Ethical Committee (Decision No. 3/10, 16 October 2025).

Results. Treatment with AgNPs loaded hydroxyapatite caused vessel dilation and tortuosity, whereas composites containing pomegranate extract—alone or combined with AgNPs—showed minimal vascular alterations. By day 12, a significant rise ($p < 0.05$) in vessel number was detected only in the group treated with composite containing pomegranate, highlighting its angiogenic activity, while the proportion of bifurcated vessels remained unchanged across all groups.

Discussion. This study evaluated hydroxyapatite-based composites functionalized with silver nanoparticles (AgNPs) and pomegranate peel extract. Overall, the study demonstrates that the angiogenic response of hydroxyapatite-based composites depends on their functional components: silver nanoparticles suppress vessel formation, whereas pomegranate peel extract enhances it, highlighting the importance of composition in designing biomaterials for regenerative applications.

Key words: bone regeneration, neovascularization, pomegranate, silver, health.

Corresponding author: *Viktoriia Holubnycha**,** Public Health Department, Sumy State University, Sumy, Ukraine, email:* *v.golubnichaya@med.sumdu.edu.ua*

РЕЗЮМЕ

Вікторія Голубнича
https://orcid.org/0000-0002-1241-2550
Кафедра громадського здоров'я, Навчально науковий медичний інституту, Сумський державний університет, Суми, Україна

Олександр Говорун
https://orcid.org/0000-0002-6626-1241
Кафедра біології та методики навчання біології, Сумський державний педагогічний університет, Суми, Україна

Вероніка Пронь
Навчально науковий медичний інституту, Сумський державний університет, Суми, Україна

Маргарита Голубнича
https://orcid.org/0009-0008-7909-8755
Львівський політехнічний національний університет, Львів, Україна

Ганна Яновська
https://orcid.org/0000-0001-8040-7457
Інституту Агрофізики, Польська академія наук, Люблін, Польща

АНГІОГЕННИЙ ПОТЕНЦІАЛ КОМПОЗИТІВ НА ОСНОВІ ГІДРОКСІАПАТИТУ, ЗБАГАЧЕНИХ РОСЛИННИМ ЕКСТРАКТОМ ТА НАНОЧАСТИНКАМИ

Вступ. Зростання кількості літніх людей та збільшення поширеності захворювань, пов'язаних із патологією кісткової тканини, збільшили попит на розробку інноваційних регенеративних матеріалів. Композити на основі гідроксиапатиту, збагачені металевими наночастинками та рослинними екстрактами, є дуже перспективними завдяки своїй біосумісності, антимікробній активності та здатності сприяти регенерації тканин. Це дослідження мало на меті оцінити, як композити функціоналізовані наночастинками та рослинною сировиною впливають на формування судин, використовуючи модель хоріоалантоїсної мембрани (САМ) курячих ембріонів.

Методи. Композити на основі гідроксиапатиту були виготовлені шляхом включення наночастинок срібла (10 мкг/г) та екстракту шкірки граната (30 мг/г) у гідроксиапатит, а потім перемішування та сушіння. Супернатанти отриманих композитів наносили на хоріоалантоїсну мембрану (САМ) курячих ембріонів на 10-й день ембріонального розвитку для оцінки ангіогенної активності. Після 24–48 годин інкубації були зроблені зображення САМ та проаналізовані морфометрично за допомогою ImageJ для кількісної оцінки щільності судин та точок біфуркації. Статистичну значущість оцінювали за допомогою GraphPad Prism ($p < 0,05$). Дослідження було схвалено комісією з біоетики СумДУ (Рішення № 3/10 від 16 жовтня 2025 року).

Результати. Лікування гідроксиапатитом, навантаженим AgNPs, викликало розширення та звивистість судин, тоді як композити, що містять екстракт граната — окремо, або в поєднанні з AgNPs — призвели до мінімальних змін судинного малюнку. Через дві доби інкубації значне збільшення ($p < 0,05$) кількості судин було виявлено лише в групі, яка підлягала впливу композиту в складі якого був екстракт гранату, що свідчить про його ангіогенну активність. Використання композиту із додаванням срібла та чистого гідроксиапатиту не призводило до помітного збільшення кількості судинних розгалужень.

Обговорення. У цьому дослідженні оцінювалися композити на основі гідроксиапатиту, функціоналізовані наночастинками срібла (AgNPs) та екстрактом шкірки граната. Загалом, дослідження демонструє, що ангіогенна реакція композитів на основі гідроксиапатиту залежить від їхніх функціональних компонентів: наночастинки срібла пригнічують утворення судин, тоді як екстракт шкірки граната посилює його, що підкреслює важливість складу в розробці біоматеріалів для регенеративних застосувань.

Ключові слова: регенерація кісток, неоваскуляризація, гранат, срібло, здоров'я.

Автор, відповідальний за листування: *Вікторія Голубнича, кафедра громадського здоров’я, Навчально-науковий медичний інституту, Сумський державний університет, Суми, Україна*
email: v.golubnichaya@med.sumdu.edu.ua

INTRODUCTION

The ageing of the global population, along with the rising incidence of traumatic injuries and oncogenic bone disorders, has substantially increased the demand for regenerative medicine and advanced approaches to the restoration of damaged tissues, including skin, cartilage, and bone. In response to this growing need, numerous natural and synthetic biomaterials have been developed, emerging as essential platforms for supporting and enhancing bone tissue regeneration [1]. Among the various biomaterials investigated, hydroxyapatite-based composites are considered particularly promising due to their chemical similarity to the mineral phase of bone and their favorable biocompatibility [2]. Nevertheless, these materials still exhibit several limitations, including insufficient mechanical strength and variability in biological performance [3]. Consequently, the optimization of their composition and the development of advanced formulations remain active areas of research in regenerative medicine.

Over the last decade, considerable attention has been given to metal nanoparticles and plant extracts as key supplements in composites, owing to their numerous outstanding features [4, 5]. Several studies have demonstrated their effectiveness against multidrug-resistant microorganisms, as well as the apparent absence of resistance development to them [6]. Another important property of nanoparticles and plant extracts is their ability to promote tissue regeneration. [7]. These findings highlight the potential of incorporating such bioactive components into regenerative medicine strategies, providing an avenue for enhancing the safety and efficacy of biomaterials.

Preclinical evaluation of the effectiveness and safety of such materials represents a critical step in their investigation. Historically, animal models have been used as the standard approach to address key issues in the preclinical evaluation of biomaterials. However, ethical concerns associated with the use of animals in scientific research have given rise to the principles of the 3Rs (reduction, refinement, and replacement) encouraging widely use of alternative methodologies [8]. The chick chorioallantois membrane (CAM) assay represents a versatile and ethically favorable *in vivo* model for the early-stage evaluation of candidate biomaterials in tissue engineering. Owing to its rapid assessment time, methodological straightforwardness, and cost-efficiency, this system is recognized as a valuable alternative to traditional preclinical models. In addition, the model provides an opportunity to study vessel formation, a critical prerequisite for the integration of biomaterials with the host tissue environment.

The objective of this work was to investigate the extent to which hydroxyapatite-based composites functionalized with plant extracts and nanoparticles modulate vessel formation within the CAM model.

MATERIALS AND METHODS

Media (Mueller-Hinton broth, Mueller-Hinton agar) were purchased at Hi Media (India). $CaCl_2$ anhydrous (CAS:10035-04-8), Na_2HPO_4 (CAS:7558-79-4), and NaOH (CAS:1310-73-2) were provided by Chempur (Poland), Sigma Aldrich (USA). Nano Pure Co. (Warsaw, Poland) provided AgNPs. Preparation of the pomegranate peels (*Punica granatum*) extract was prepared as described in our previous work [9].

Previously prepared hydroxyapatite (HA) was loaded with silver nanoparticles (AgNPs) and plant extract of pomegranate (PE). The target concentrations of the incorporated components were adjusted to 10 µg/g for silver, and 30 mg/g for the pomegranate peel extract within the composite. The suspension was subjected to continuous stirring for 12 h, followed by drying. The biological effects were evaluated using supernatants obtained after incubation of the HA-based composites, reflecting the activity of released soluble components. Prior examination of the antigenic potential obtained composites were poured with saline solution at WV rate 1 to 10, hold 24 hours at permanent shaking. Then, sterile distilled water was added to the composites in a ratio of 1:10 and incubated for 24 hours, and the resulting supernatant was used for CAM analysis.

For CAM assay, White Leghorn fertilized chicken eggs were incubated at 37˚C and 55-60% humidity. Start of incubation was determined as embryonic development day (EDD) 1. At EDD 3 the small hole (3 mm in diameter) was made in the shell with fallowing removal of 2-3 ml of albumen to detach the CAM from the shell. Then the eggshell window was sealed with silk tape. Then eggs were incubated at the same conditions before treatment. At EDD 10, all embryos were divided into groups:

I group – treated with HA+AgNPs 10 µg/g;

II group - treated with HA+PG 30 mg/g;

III group - treated with HA+ AgNPs 10μg/g +PG 30 mg/g;

IV group - treated with HA;

V group – non-treated.

Each group consists of six eggs. Fifty μl of the supernatant from tested sample was inoculated into the CAM. The hole was sealed, and eggs were further incubated for 24-48 h. At EDD11-12, eggshell was trimmed away to nearly the edge of the CAM, allowing a wide field of view, and the resulting concave space formed by the CAM was filled with a light grade mineral oil to destroy the opacity of the CAM surface. A ZOOM SM-6620 MICROmed stereomicroscope with 20x magnification, connected to a Coolsnap digital camera (Roper) and a computer with image management software (ImageJ), was used to obtain CAM images. At least five images of different areas of each animal were analyzed. The vessels area density and number of bifurcation points were quantified by morphometric evaluation of CAM images. Statistical significance was determined using one-way analysis of variance (ANOVA) performed with GraphPad Prism 9 software A p value < 0.05 was considered statistically significant. Figure 1 shows the general scheme of the experiment. The Local Ethical Committee approved experiment on chicken eggs (decision number 3/10 Date: 16 October 2025).

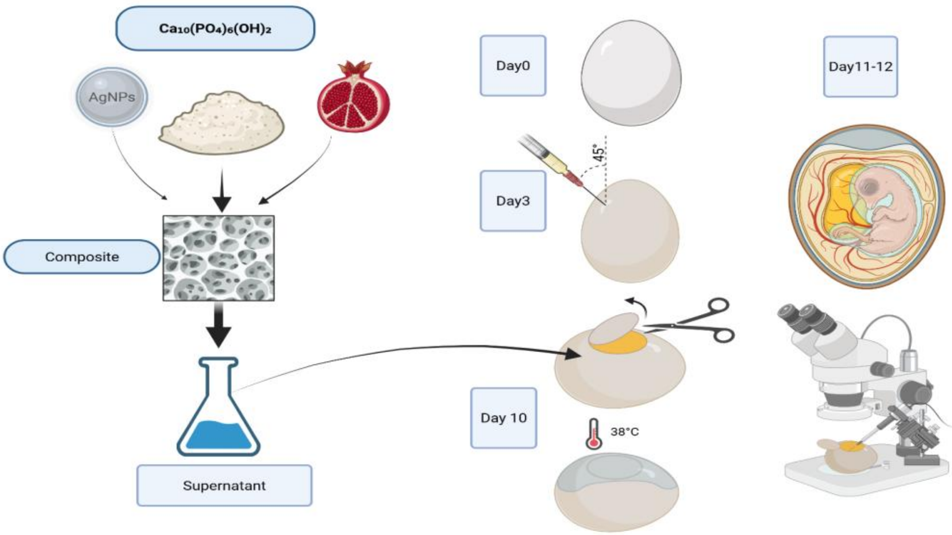

Fig. 1. Experimental procedure in schematic overview

Following the opening of the eggshell on day 3 and the placement of the matrices on the CAM (Day 10), the analysis of neovascularization was conducted using stereomicroscope on days 11, and 12. Abbreviations: CAM=chorion allantois membrane. Created with BioRender.com

RESULTS

Hydroxyapatite-based composites loaded with silver nanoparticles (AgNPs) and pomegranate peel extract at different concentrations were evaluated for their angiogenic potential using *in ovo* CAM assays. Figure 2 presents the vascular changes observed on day 11 between the control and experimental groups following composite treatment.

Application of the supernatant derived from hydroxyapatite loaded with AgNPs resulted in blood vessels appearing more tortuous, dilated, with indistinct contours. This observation contrasts with the groups treated with the hydroxyapatite-based composites containing pomegranate peel extract or both AgNPs and pomegranate peel extract, where essentially no visible vascular alterations were detected. In contrast, treatment with the supernatant obtained from pure hydroxyapatite resulted in marked blood vessel dilation and enhanced branching formation. All results were compared to the untreated control group, confirming that the presence of pomegranate extract may mitigate the angiogenic response induced by AgNPs alone.

The density of the vessel area on days 11 and 12 in treated and untreated CAMs is presented in Figure 3. On day 11, the number of blood vessels was significantly higher ($p < 0.05$) in the groups treated with supernatants derived from pure hydroxyapatite and hydroxyapatite loaded with Ag NPs or pomegranate peel extract. In contrast, the number of blood vessels was significantly lower in the groups treated with supernatants derived from hydroxyapatite loaded with both Ag NPs and pomegranate peel extract compared to the control group on the same day of embryo development.

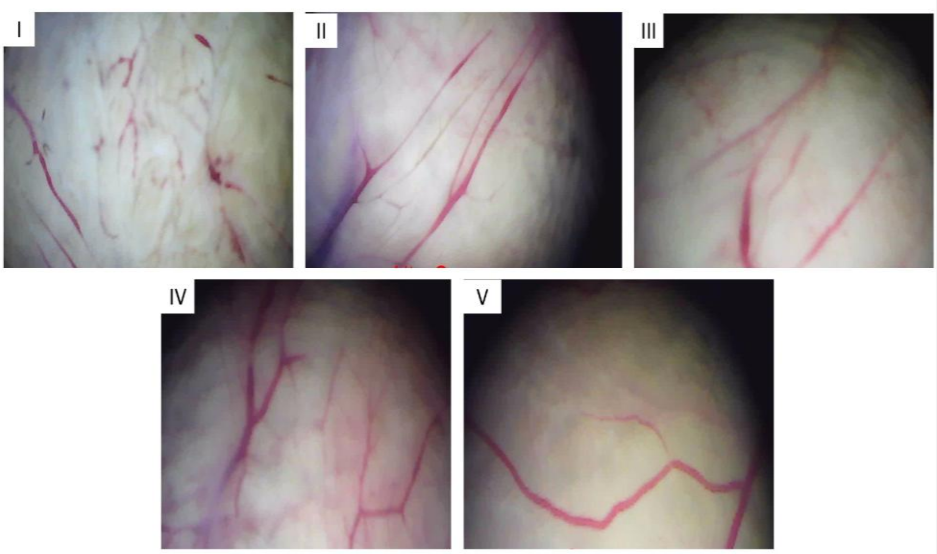

Fig. 2. Original color images taken at EDD11 of CAM development, at x20 magnification. I-V – experimental groups

On day 12, however, a significant increase ($p < 0.05$) in the number of blood vessels was observed only in the groups treated with supernatants derived from hydroxyapatite loaded with pomegranate peel extract, indicating the angiogenic potential of this agent.

No statistically significant differences were observed in the percentage of bifurcated vessels among the groups on each experimental day (Figure 4). In contrast, the number of secondary and tertiary vessels increased by more than 1.5-fold on EDD 12 compared to EDD 11 in groups III, IV, and V.

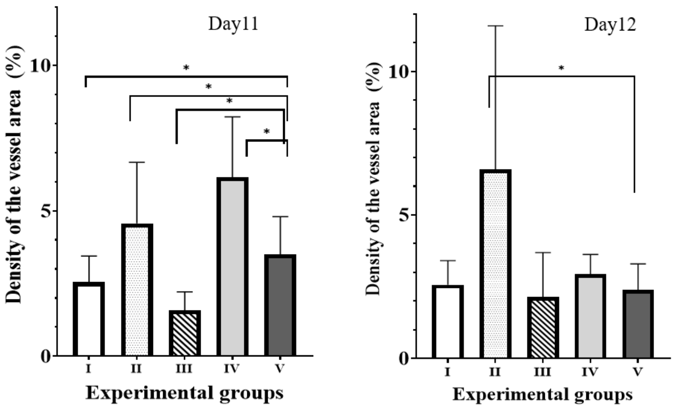

Fig. 3 Density of the vessel area on day 11 and day 12 in treated and non-treated CAM

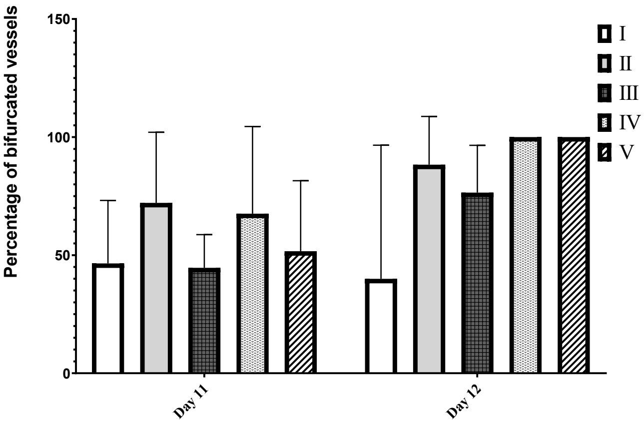

Fig. 4 Percentage of the bifurcated vessels on day 11 and day 12 in treated and non-treated CAM

DISCUSSION

Various biomaterials are currently being investigated as potential solutions in regenerative medicine. Angiogenesis is a fundamental process in tissue regeneration and must be thoroughly evaluated to ensure the successful translation of newly developed materials into clinical practice. The chorioallantois membrane (CAM) assay is widely used to study angiogenesis due to its cost-effectiveness, feasibility, and reproducibility [10].

Previous studies have reported on the antimicrobial activity of pomegranate peel extracts and silver nanoparticles (AgNPs) [11, 12]. In addition, our earlier *in vitro* investigations demonstrated a synergistic antimicrobial effect of these components. Therefore, biomaterials incorporating pomegranate peel extracts and AgNPs may be considered promising candidates for bioengineering and regenerative applications.

In the present study, the angiogenic response to bioactive molecules released from the hydroxyapatite-based composites functionalized with pomegranate peel extracts and AgNPs on angiogenesis were evaluated using the CAM model. Pure hydroxyapatite promoted neovascularization, whereas the incorporation of AgNPs inhibited angiogenesis and reduced vessel branching. In contrast, hydroxyapatite loaded with pomegranate peel extracts stimulated the formation of new blood vessels, which continued to progress throughout the observation period.

These results are consistent with previous reports demonstrating the anti-angiogenic activity of AgNPs [13]. Conversely, the findings of the present study contradict earlier research [14] that reported anti-angiogenic effects of pomegranate peel extracts tested in tumor-bearing animal models. This discrepancy may be attributed to differences in experimental models, extract composition, or concentration-dependent effects of bioactive compounds. Further studies addressing these variables are warranted to clarify the angiogenic potential of pomegranate peel extracts in different biological contexts.

CONCLUSIONS

In summary, hydroxyapatite-based composites functionalized with pomegranate peel extracts and silver nanoparticles exhibited distinct effects on angiogenesis in the CAM model. Pure hydroxyapatite promoted new vessel formation, while the incorporation of AgNPs inhibited angiogenesis and vessel branching. In contrast, composites containing pomegranate peel extracts enhanced neovascularization, indicating their potential to stimulate vascular growth and tissue regeneration. These findings highlight the importance of the compositional balance between bioactive agents and nanoparticles in designing multifunctional biomaterials for regenerative medicine.

PROSPECTS FOR FUTURE RESEARCH

In the future, it will be important to explore the underlying molecular mechanisms and to evaluate the angiogenic effects of these composites *in vitro* on cell lines, as well as the *in vivo* biocompatibility and regenerative potential.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. V.H., O.H., V.P., M.H., and A.Y. performed material preparation, data collection and analysis. The first draft of the manuscript was written by V.H. All authors read and approved of the final manuscript.

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CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

ARTIFICIAL INTELLIGENCE DISCLOSURE

During the preparation of this manuscript, the author utilized artificial intelligence tools exclusively to assist with language editing and grammar correction. No AI-generated content or analysis was used in the research itself.

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INFORMATION ABOUT THE AUTHORS

Viktoriia Holubnycha – PhD, MD, Associate Professor, Public Health Department, Sumy State University, Sumy, Ukraine
contact details (e-mail, work phone) v.golubnichaya@med.sumdu.edu.ua

Oleksandr Govorun – Candidate of Biological Science, Associate Professor, Department of Biology and Biology Teaching Methodology, Sumy State Pedagogical University named after A.S. Makarenko, Sumy, Ukraine
contact details (e-mail, work phone) a.govorun76@gmail.com

Veronika Pron – Student of Academic and Research Medical Institute, Sumy State University, Sumy, Ukraine
contact details (e-mail, work phone) tobulalanika@gmail.com

Marharyta Holubnycha – Student, Lviv Polytechnic National University, Lviv, Ukraine

Ganna Yanovska – Associate Professor, Institute of Agrophysics, Polish Academy of Sciences, Lublin, Poland