IJAMB

Vol. 6, n.1 2024

INTERNATIONAL JOURNAL OF ADVANCES IN MEDICAL BIOTECHNOLOGY

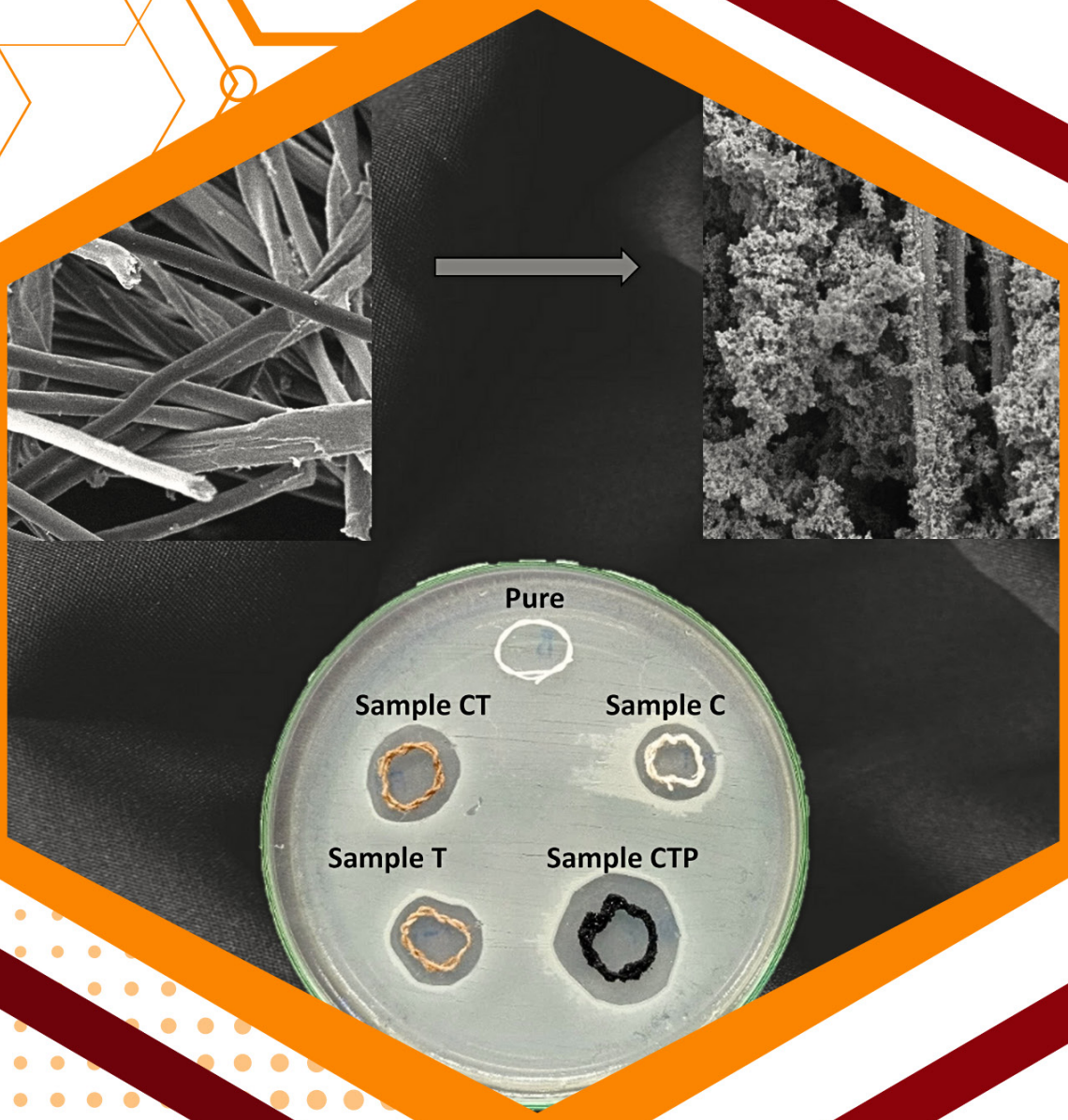


Figure credit: H. Pequeno de Oliveira
Cover design: T. Mariano

ORIGINAL ARTICLE

IJAMB
International Journal of Advances in Medical Biotechnology

Synthesis, characterization and antibacterial evaluation of cotton fiber coated with chitosan-agar/tannin derivative/polypyrrole composites

C. A. Nascimento Filho[1]; F. A. G. Silva Junior[1]; M. Matiuzzi da Costa[1]; H. Pequeno de Oliveira[1*]

*Corresponding author: E-mail address: helinando.oliveira@univasf.edu.br

Received: 01 March 2024; May 15 April 2024.

Abstract: The development of chemically modified fibers for biomedical applications can be conveniently addressed through the production of new materials for sutures. Herein, it is proposed the mutual coating of chitosan (sample C) and a tannin-derivative (Tanfloc) – sample T for the following polymerization of polypyrrole – sample P in a complex structure that combines the outstanding performance of components as antibacterial and antibiofilm agents. The characterization of modified fibers was provided by SEM images, FTIR spectrum, and mechanical assays that were conducted and confirmed the adequate deposition of coating layers on fibers. The best antibacterial activity of the modified fiber was observed for the system prepared in the presence of three components (sample CTP) that returned a complete elimination of *Staphylococcus aureus* after 60 min of contact and a reduction in the biofilm formation in order of 99.38%.

Keywords: Antibacterial. Antibiofilm. Polypyrrole. Chitosan. Wearables. Cotton.

Introduction

Suture threads are essential components for clinical treatments (prevention of the spread of infections)[1] making the adequate choice of materials for use in wound treatment[2]. Materials based on cotton and silk have excellent properties such as good knot security[2], requiring additional treatment to acquire antibacterial properties (against *Staphylococcus aureus* and *Escherichia coli)*[3]. The typical procedure is based on the coating of the fibers, which can adversely affect the intrinsic physical and handling (mechanical properties) of the resulting material[4]. As a standard procedure to avoid these drawbacks and to reduce the risk of surgical site infections it is explored the incorporation of antibacterial agents such as triclosan, which has been used as an antiseptic for *in vitro* and *in vivo* applications with direct reduction in the adherence of selective clinical strains of Gram positive, Gram negative, antibiotic-resistant and biofilm-forming bacteria to the surface of surgical sutures[5]. Polyglactin is another essential compound that has been applied in association with triclosan as a coating layer for the prevention of *in vivo* bacterial colonization[6].

Despite the extensive use of triclosan, it has been reported the limited action of this compound against antibiotic-resistant bacteria[7]. On the other hand, the use of metallic nanoparticles (MNPs) has been progressively considered in related applications due to their non-toxic behavior for mammalian cells[8]. Based on these aspects, combinations of silver nanoparticles, antimicrobial peptides, and chitosan have attracted interest in the medical field due to their characteristic biodegradability, biocompatibility, bioadhesiveness, and non-toxicity behavior[9]. Alternatively, materials such as chitosan[10], tannin derivatives[11] and conducting polymers such as polypyrrole[12] can be associated with cotton fiber to improve its physical, handling, and antibacterial properties.

Chitosan (a derivative of chitin) is an edible, biodegradable, and biocompatible compound that has been widely used for several medical purposes, taking advantage of its antibacterial activity[13] against *Staphylococcus aureus*[14] which can be controlled by deacetylation degree, molecular weight, and pH[15]. Different mechanisms have been proposed to explain the antibacterial properties of chitosan: the change in the cell permeability due to the interaction between polycationic chitosan with electronegative charges on the bacterial cell surface[16] and the chelating activity that selectively binds essential metals and nutrients, inhibiting bacterial growth[17].

On the other hand, tannin derivatives possess remarkable properties such as antimutagenic and antitumor agents[18]. In addition, antiviral and antibacterial responses have been reported for tannins applied as additives in biopolymers, collagen and chitosan, agarose, and starch as a result of their

[1] Federal University of São Francisco Valley, Juazeiro, Bahia, Brazil.

https://doi.org/10.52466/ijamb.v6i1.120.

unique characteristics[13], involving the inhibition in the proliferation of contaminants, tissue regeneration, and wound healing[19]. In addition, tannin-derivative compounds have been successfully applied in the green synthesis of metal nanoparticles and as antibacterial agents, as reported in Ref. [20].

Another important building block applied in the improvement of the antibacterial activity of compounds is the conducting polymer polypyrrole, which has been considered one of the most studied polymers, synthesized by electrochemical or chemical methods[21], and characterized by excellent antibacterial properties for application in wearable devices[22–25], odor control, textile-based medical devices such as bandages and wet wipes[21] being indicated for disease and infection control such as in multiple infections from resistant bacteria - methicillin-resistant *S. aureus* (MRSA) as well as in textile products for domestic use[21].

In particular, it is critical to the production of suture threads that can be able to inhibit bacterial growth at the surgical site without inducing resistance to antibiotics. Herein, a simple strategy to chemically modify cotton fibers by successive coating with chitosan, tannin-derivatives (Tanfloc), and polypyrrole is proposed to provide a material that not only preserves the intrinsic properties of the pristine fibers but also introduces the multifunctional activity with high antibacterial activity, fast kinetics for kill time assays and antibiofilm activity.

Materials and Methods

Materials

Bacteriological granulated agar (Merck, Darmstadt, Germany), pyrrole (Sigma-Aldrich, Saint Louis, USA), anhydrous ferric chloride (Exodus, Sumaré, Brazil), chitosan (Exodus, Sumaré, Brazil), hydrochloric acid (Sigma-Aldrich, Saint Louis, United States), bacteriological tryptone soy broth (TSB) and broth plate count agar (Merck, Darmstadt, Germany) and acetic acid (Vetec Química, Rio de Janeiro, Brazil) were used as received. Tanfloc® is a cationic compound from the tannin extract of *Acacia mearnsii* that is produced from the Mannich method that is based on the incorporation of imine cation in structure (responsible by cationic behavior of tannin derivative). Tanfloc® is produced by Tanac S.A. Brazil and more details about structure and applications are available in Refs. [26,27]. Pyrrole was distilled under reduced pressure before each experiment. The saline solution was prepared by dissolving 0.85 g of sodium chloride in 100 mL of an aqueous solution. All solutions were made using Milli-Q water with a resistivity of 18.2 MΩcm.

Methods

Cotton threads (Linhas Extra Forte, São Paulo, Brazil) with 0.5 mm in diameter and 10 cm in length were immersed in a 1% acetic acid solution for 12 h to activate the surface of fibers to be coated by additives[28]. Following this step, the successive coating of fiber by different additives was conducted to evaluate the influence of fillers on the overall response of fiber coated with chitosan (sample C), fiber coated with Tanfloc (sample T), and combined activity of fibers coated with chitosan, Tanfloc and polypyrrole (sample CTP) in comparison with negative control – pristine cotton fiber.

Characterization techniques

The morphology of fibers and the aggregation level induced by the progressive incorporation of fillers were evaluated from scanning electron microscopy carried out using an SEM Vega 3XM - Tescan (Brno – Kohoutovice, Czech Republic) at an acceleration voltage of 10 kV. The chemical composition of coating layers was scrutinized by the Fourier transform infrared spectrum (FTIR) - KBr method in an IR Prestige-21 Fourier transform infrared spectrometer Shimadzu (Japan). Mechanical evaluation of the changes in the response of the fibers under chemical treatment was analyzed from an electromechanical universal machine (EMIC model DL 10000, Instron, São José dos Pinhais, PR, Brazil) with output processed from TESC software.

D. Preparation of sample C

The solution was prepared by dissolving 500 mg of chitosan in 49 mL of water and 1 mL of acetic acid (10 mg mL^{-1}). After the complete dispersion of chitosan, 500 mg of agar was added. After that, the cotton fibers were immersed for 30 minutes in the resulting solution, washed with Milli-Q water, and dried in an oven at 90°C for 5 minutes. This procedure was repeated three times to ensure uniformity of the chitosan coating layer on cotton fiber[28].

Preparation of sample T

The sample T was prepared by dissolving 500 mg of Tanfloc in 50 mL of water (10 mg mL^{-1}). After complete dispersion of the Tanfloc in the solution, the cotton fibers were immersed in this solution for 30 minutes, washed with Milli-Q water, and dried in an oven at 90 °C for 5 minutes. This procedure was repeated three times to ensure uniformity of the coated cotton fiber.

Preparation of sample CT

The steps described in sections D and E were conducted in sequence from which the chitosan was firstly deposited on cotton fibers for the following deposition of the Tanfloc resulting in the sequence of

deposition of layers of chitosan and Tanfloc – sample CT.

Preparation of sample CTP

Cotton fibers modified with chitosan and Tanfloc were immersed in a solution of pyrrole (0.36 M) and HCl (0.1 M) and left under stirring for 30 minutes [28]. After this step, the fibers were polymerized by adding a solution of ferric chloride (0.36 M) at 4°C followed by agitation for 12 hours.

Agar diffusion test

Agar diffusion assays were performed according to Ref.[27]. The Gram-positive *S. aureus* bacterial inoculum (ATCC 25923) was prepared from a culture kept in agar at 4°C. An aliquot of bacterial inoculum was inserted into a saline solution with a concentration of 0.85%, leaving the bacterial suspension with a turbidity of 0.5 on the McFarland scale (10^8 CFU mL^{-1}). With the help of a swab, this bacterial suspension was dispersed in a Petri dish containing plate count agar (PCA) culture medium. Different samples (C, T, CT, and CTP) were incorporated into the plates to evaluate their antibacterial activity. After this step, the plate was incubated at 37 °C for 24h[27].

Kill time assays

The shortest time required for modified fibers to actuate in the elimination of the bacteria was determined from kill time assays, conducted as follows: 5 mL of 10^8 CFU mL^{-1} bacterial solution was placed in a test tube, which was later diluted to 10^6 CFU mL^{-1}. After homogenizing the bacterial solution, the different fiber-based samples (C, T, CT, and CTP) were placed in four tubes. The positive control and the blank were performed to compare with the response of four different systems. Then, 100 µL aliquots were removed from the tubes at fixed intervals of time (1 h, 2 h, 3 h, and 4 h) and placed in Petri dishes containing plate count agar (PCA). The plates were kept at 37 °C for 24 h[29] for the following counting of the viable cells.

Biofilm assays

Using a bacteriological loop, the inoculum of *S. aureus* (ATCC 25923) was dispersed in 10 mL of tryptic soy broth (TSB) in different tubes. After preparing the bacterial solutions, different samples (C, T, CT, and CTP) were added to four tubes. The positive and negative controls were performed to compare with the results of the modified fibers. Then, the tubes were incubated at 37 °C for 24h.

After this process, the fibers were transferred to new tubes containing 10 mL of saline solution. The resulting solutions (with samples) were sonicated in a bath (f = 40 kHz) for 15 min to remove species trapped in the wall of the tubes (adhered biofilms). Then, 100 µL aliquots were removed in triplicate from each system and seeded in a PCA medium. After that step, the plates were incubated at 37°C for 24h, and the colonies were counted to determine the number of viable cells in the biofilms [27,30,31].

Toxicity assays

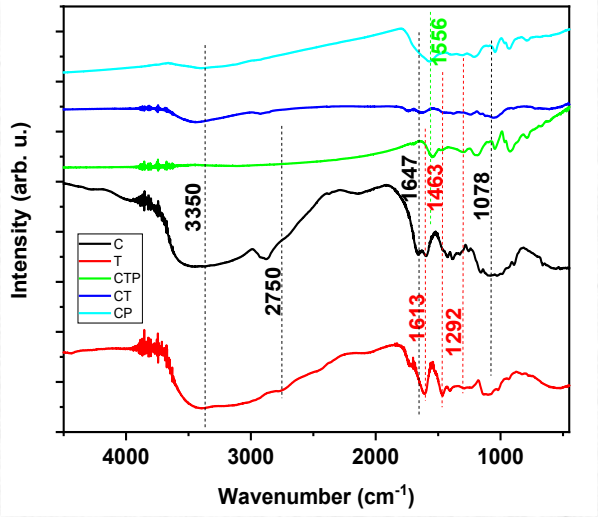
The toxic potential of cotton fibers associated with different materials (chitosan, Tanfloc, chitosan, and Tanfloc, chitosan, Tanfloc, and polypyrrole) was evaluated against *Artemia Salina*. For this, the fibers with different compositions were placed in test tubes vigorously shaken, and subsequently sonicated for 30 minutes to improve the dispersion of impregnated compounds in the solution.

Results and Discussion

Structure and morphology

The characteristic groups of additives incorporated into cotton fibers were scrutinized by the FTIR spectrum, summarized in Fig. 1. As can be seen, the FTIR spectrum of the fiber/chitosan (sample C) shows peaks attributed to the chitosan structure in the range of 500 cm^{-1} to 1084 cm^{-1} with bands attributed to the amine group at 1374 cm^{-1}. A wide absorption band between 3500 cm^{-1} and 2750 cm^{-1} is observed for chitosan components, due to the stretching of the primary amine (NH_2) and hydroxyl groups (OH^-) of chitosan. The characteristic bands at 1647 cm^{-1} and 1078 cm^{-1} are attributed to the twisting of the —NH group and the stretching of C—O, respectively. The characteristic band around 1660 cm^{-1} corresponds to the C=O stretching vibration of the amide group of chitosan[32].

Figure 1 - FTIR spectrum for samples C, T, CT, CP and CTP.



Source: Own authorship, 2023.

These shifts in the characteristic bands can be

attributed to the response of the amine groups ($-NH_2$) from chitosan at protonated cationic form ($-NH_3^+$) in an acidic solution, that remains in contact with the nucleophilic surface of the cotton fiber by hydrogen bonds and/or dipole-dipole interactions[33].

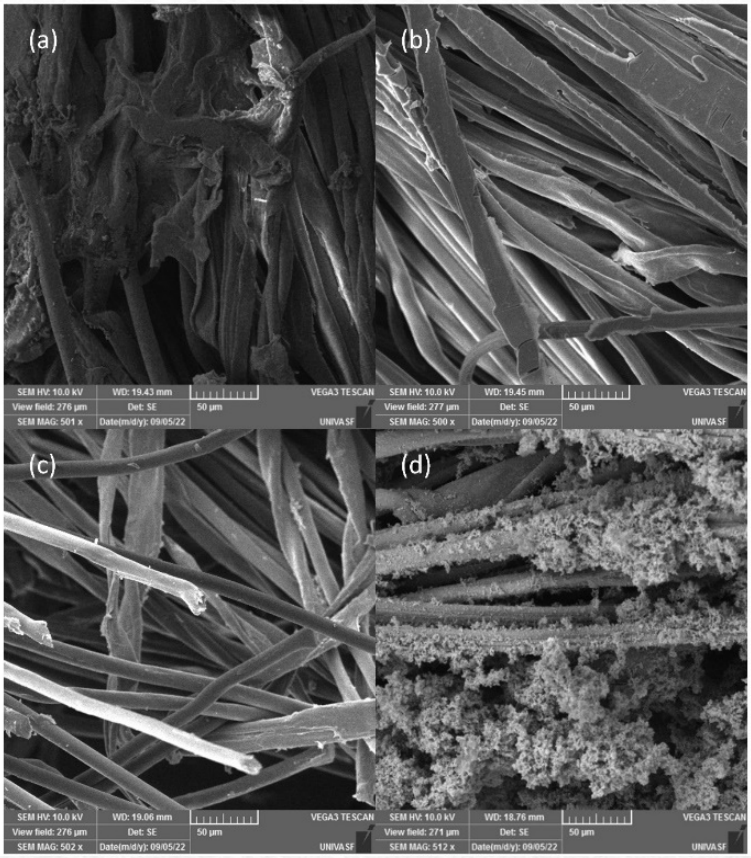
For sample T, the peak at 3350 cm^{-1} is attributed to the –OH elongation of the phenolic tannin. The small peak near 2943 cm^{-1} has been associated with the bond stretching vibration between aromatic C–H groups[34]. The peak at 1795 cm^{-1} belonged to the carboxyl-carbonyl group[35] while the peak at 1613 cm^{-1} corresponds to the –C=C– aromatic ring[34] and the band at 1463 cm^{-1} is assigned to methylene groups. The peak at 1292 and 1039 cm^{-1} was mainly due to the plane strain of the phenolic –OH group [36,37]. A band attributed to the –OH in the plane strain of the carboxylic acid group can be seen at 1141 cm^{-1} [38]. The off-plane vibrations from the deformation of the C-H bond in benzene rings were represented by small absorption bands at 936, 841, and 771 cm^{-1} [39].

The response of samples prepared with the mutual incorporation of additives into the fibers (sample CTP) is characterized by the presence of bands at 1600 cm^{-1} (attributed to the double bond between carbons (C=C) of the stretching of the pyrrole ring) and the peak at 1033 cm^{-1} which is assigned to the in the –C–H plane deformation of the pyrrole unit [32] while bands at 1463 and 1556 cm^{-1} are assigned to the C-C and C=C stretching vibrations of the polypyrrole ring confirming the incorporation of polypyrrole on fibers after their chemical polymerization.

SEM images were performed to evaluate the changes in the morphology of cotton fibers under successive steps of coating by antibacterial additives. As shown in Fig. 2a, the incorporation of chitosan into fibers results in a thick film that results in the aggregation of fibers due to the adhesive properties of chitosan. On the other hand, the impregnation of Tanfloc on cotton fibers (see SEM image in Fig. 2b) preserves the integrity of the pristine material and produces a thin layer on fibers, maximizing the surface area of material with the minimum aggregation of fibers by agglomerates of additives. An interesting finding is observed for samples prepared in the mutual presence of Tanfloc and chitosan. The typical behavior of the Tanfloc coating prevails and a coating on individual fibers is established (see Fig. 2c) preserving the intrinsic morphology of the fibers (minimal aggregation of fibers in agglomerates).

The polymerization of polypyrrole was provided on the CT samples (see Fig. 2d) in which is possible

Figure 2 - SEM images at the same magnification (500 x) for (a) cotton fibers coated with chitosan (sample C), (b) Tanfloc (sample T), (c) mutual deposition of chitosan and Tanfloc (sample CT), and (d) after polymerization of polypyrrole on chitosan/Tanfloc layer (sample CTP).

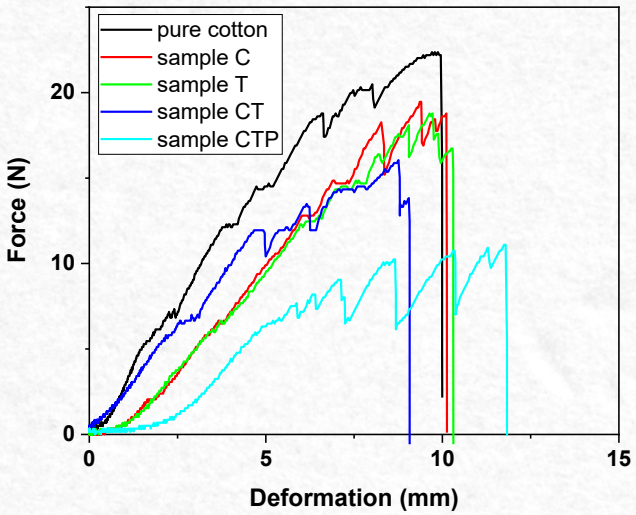


Source: Own authorship, 2023.

to identify the presence of grains of the conducting polymer dispersed on the surface of modified fibers.

Another important characterization technique evaluated to provide information about the modification in the structure of fibers is the mechanical characterization of pure and modified fibers from the force-deformation assays. As shown in Fig. 3, in comparison with the cotton thread, it is observed a general trend of reduction in the maximum force applied in the fibers applied before the rupture, with a more pronounced effect observed for the sample modified with polypyrrole, following the order sample CTP> sample CT> sample T> sample C> pure cotton.

Figure 3 - Force-deformation curves for samples prepared with different coating layers (sample C, sample T, sample CT, sample CTP, and pure cotton – negative control).



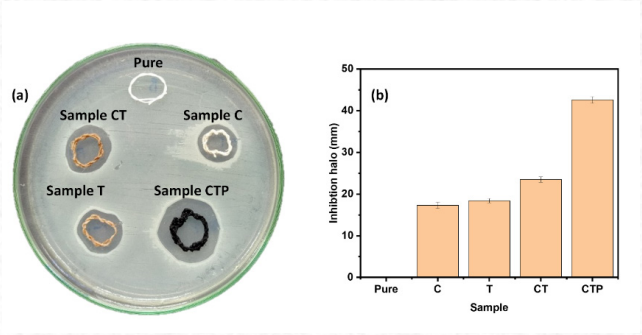
Source: Own authorship, 2023.

This process can be explained by the disruption in the interactions of modified fibers induced by the successive incorporation of chitosan, Tanfloc, and the polymerization of polypyrrole. However, it is worth mentioning that this reduction in resistance does not affect the functionality or the main purpose of the fibers for specific applications in sutures, since the skin is considered a non-linear elastic material with low sensitivity to the rate of deformation [40] and that the forces applied in the force-deformation tests are higher than the required forces involved in the skin/muscle set that is under treatment.

Antibacterial assays (agar diffusion assays)

The agar diffusion assays were conducted for pure (negative control experiment) and modified fibers that were separated in a length of 5 cm and disposed of as circles to make possible the identification of the corresponding inhibition haloes for each modified fiber. All of the modified fibers were disposed of as circles on plates that were inoculated with S. aureus for 24 h at 37 oC. Fig. 4a summarizes the inhibition zones obtained for negative control (pure cotton fiber) and samples C, T, TP, and CTP. As can be seen (and as expected) the pure fiber returned negligible activity while the isolated and combined incorporation of additives returned different antibacterial activity. The quantitative evaluation of the inhibition haloes is shown in Fig. 4b (as a result of experiments in triplicate) indicating that overall performance follows the order: sample CTP> sample CT> sample T > sample C. The antibacterial activity observed for chitosan-modified fiber is associated with the amino group, which binds to the surface components of bacteria and inhibits their growth[41].

Figure 4 - Inhibition halo assays (a) image of the plate containing circles of pure and modified fibers (sample C, sample T, sample CT, sample CTP) and (b) measured values for inhibition haloes.



Source: Own authorship, 2023.

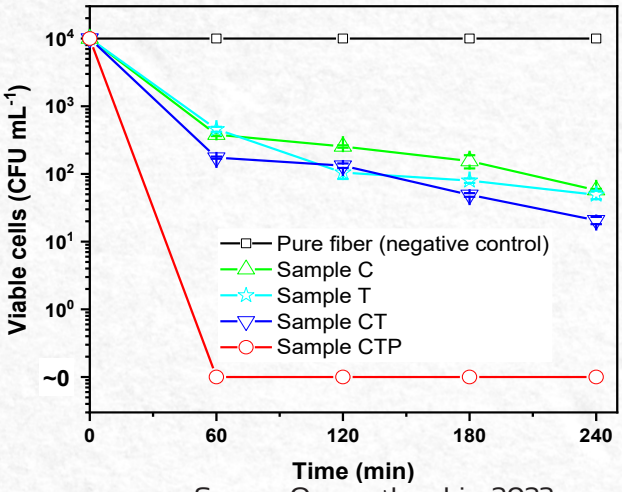
On the other hand, the inhibition zones observed as a consequence of the incorporation of the Tanfloc can be explained by its characteristic ability to form complexes with macromolecules, in addition to its polyphenolic nature of material[42]. In terms of the outstanding performance of the polypyrrole, this process is attributed to the formation of positive charges along the PPy chain, resulting in a strong electrostatic interaction between oppositely charged species (cationic surface of polypyrrole and negatively charged surface of bacteria) as a main source of antibacterial activity of the material.

Kill time assays

The kinetics of death of *S. aureus* provided by different experimental systems (coating layers) were determined from the direct measurement of the number of viable cells after a fixed interval of time of interaction of bacteria and antibacterial agent. As can be seen in Fig. 5, for samples C, T, CT, and CTP, the general trend is observed with the progressive reduction in the number of viable cells with the increasing time of treatment, while the negative control (as expected) returned negligible variation in the number of viable cells of *S. aureus*.

The higher value in the slope of the curves preserves a direct relationship with the effectiveness of the antibacterial agent. By direct comparison of the responses, it can be seen that fiber CTP eliminates the viable cells after 60 min of interaction. Intermediate performance is observed for samples C, T, and CT, an indication that polypyrrole is extremely important to provide outstanding performance in terms of bacterial inhibition in association with chitosan and Tanfloc layers.

Figure 5 - Results of the kill-time assays with the number of viable cells as a function of the time of interaction with the antibacterial agent for sample C, sample T, sample CT, sample CTP, and negative control.



Source: Own authorship, 2023.

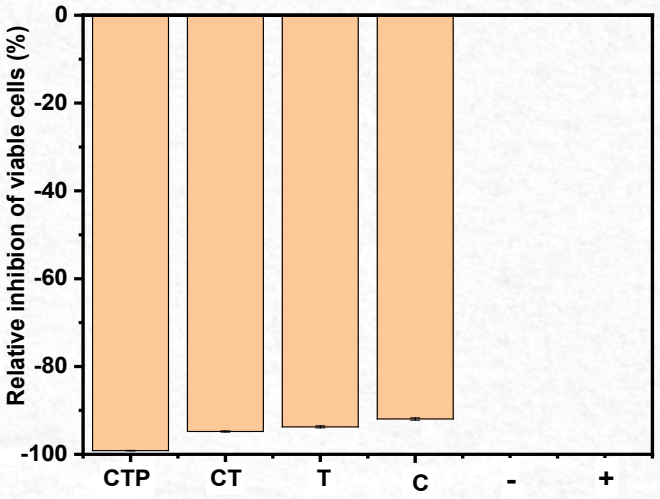
The general mechanism for the fast inhibitory activity of sample CTP can be attributed to the cationic behavior of the synthesized compound, which electrostatically interacts with negatively charged cell walls of bacteria attracting the material to the modified cotton surfaces. This process is combined with the release of reactive species from all of the additives that inhibit bacterial development, as reported in previous studies[30,43,44] that considered the isolated activity of additives.

Antibiofilm activity

The antibiofilm activity of compounds was evaluated from the direct counting of viable cells adhered in biofilm structures on cotton fibers (pure and modified). Results summarized in Fig. 6 indicated that all of the modified fibers are characterized by strong antibiofilm activity for all of the compositions that are compared with negative and positive controls (in which negligible activity is observed for cotton fibers – no growth of bacteria in negative control and complete growth of biofilm on cotton fibers – positive control). For all of the coating treatments, the antibiofilm activity of materials is higher than 91%, following the order sample CTP (99.38% of inhibition)>sample CT (95.32% of inhibition)>sample T(93.98% of inhibition)>sample C (91.18% of inhibition).

As can be seen, Fig. 6 shows a reduction in biofilm formation with the unmodified fiber applied as the control group. The fiber treated with chitosan, Tanfloc, and polypyrrole showed the greatest reduction (96.9%) in biofilm formation compared to the group of modified fibers. The electrostatic interaction of bacteria (as planktonic form and as biofilm) is followed by the migration of reactive species along with the cell wall that inhibits vital processes in bacteria[45].

Figure 6 - Results for the relative biofilm inhibition against *S. aureus* for sample C, sample T, sample CT, and sample CTP in comparison with the positive control.



Source: Own authorship, 2023

These results confirm the relevance of the polypyrrole in the overall antibacterial activity of the coating, due to the combined diffusive activity of reactive species in polypyrrole and the intrinsic antibacterial activity of the material, which provides outstanding performance in terms of the planktonic and biofilm inhibition of modified cotton fibers. The successive coating of additives (chitosan and Tanfloc) before the chemical polymerization of polypyrrole improves the activity of the resulting material.

Toxicity test with Artemia Salina

Artemia Salina nauplii have been explored in the evaluation of the toxicity of a large number of different compounds, including nanoparticles as a biological model in toxicology given its small body size, short life cycle, and relatively simple cultivation setup[46].

The toxicity test using the microcrustacean *Artemia salina* is one of the most common methods that

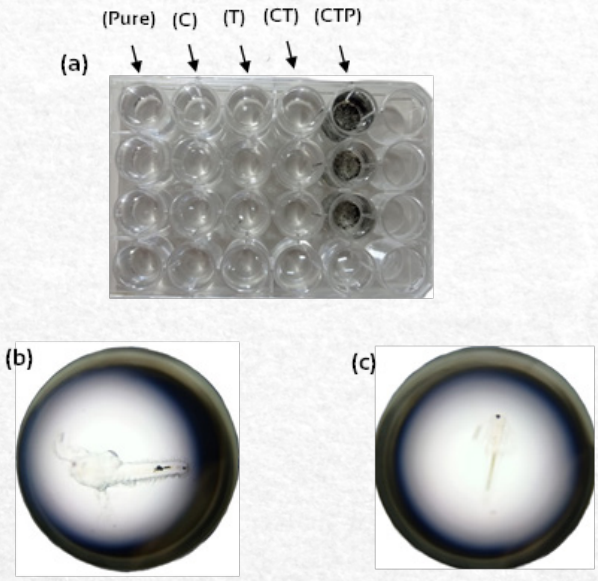
are associated with the use of animals to evaluate the toxicity of substances, acting as a bioindicator due to its reduced degree of tolerance and clear response to small environmental variations [47].

The results of the assays for the mortality of *Artemia Salina nauplii* returned a percentage of zero immobilization, and there was no death for the samples analyzed in the triplicates. All *Artemia Salina nauplii* were alive after 48 h of the cytotoxicity test, showing that there is no toxicity of the material under study.

Accumulation of material in the guts of *Artemia Salina nauplii* was observed in each triplicate and for all materials using a stereomicroscope equipped with a camera. Compared to controls, the exposed *Artemia Salina* entrails were full of particles (which were detached from the fibers after strong agitation and sonication).

No nauplii of *Artemia Salina* from a set of 120 (10 in each triplicate and 30 for each type of fiber, four different associations tested) that interacted with the different fibers died within 48 h of the toxicity test, as shown in Figs. 7a to 7c. These results suggest that the different fibers tested are not toxic to *Artemia Salina*. The ingested particles appeared as a long dark streak within the bowels. This can be explained by the lack of food intake and absorption and the filling of the intestine with particles.

Figure 7 - Toxicity assay in (a) pure fiber, fiber with chitosan, fiber with Tanfloc, fiber with chitosan and Tanfloc, fiber with chitosan, Tanfloc, and polypyrrole using *Artemia Salina*. In (b) a nauplius without contact with the materials in (c), in contact with the materials showing the animal's abdomen with the material.



Source: Own authorship, 2023.

Conclusions

The mutual incorporation of chitosan, Tanfloc, and polypyrrole as coating layers on cotton fibers preserves the mechanical properties of the pure material (due to the plastic properties of the Tanfloc coating layer) enriched by cationic behavior of chitosan and the outstanding antibacterial properties of polypyrrole that renders a quasi-complete biofilm removal for modified cotton fibers, a characteristic time for complete bacterial elimination of 60 minutes and a good degree of diffusion of reactive species that improves the antibacterial properties of the compound with negligible toxicity to *Arthemia Salina*. An important aspect to be considered is the prevention of critical changes in the mechanical resistance of fibers due to the self-aggregation of polypyrrole grains. To avoid these critical effects, the previous incorporation of chitosan and Tanfloc as coating layers protects the fibers against aggressive conditions of polymerization, maintaining the mechanical resistance of the resulting fiber at an acceptable limit for biomedical applications. These results confer to this multilayer coating procedure a promising application in antibacterial-modified fiber-based surfaces.

Acknowledgment

This work was supported by FACEPE, FAPESB, FINEP, and CNPq. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

Declarations

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] Dhom J, Bloes DA, Peschel A, Hofmann UK. Bacterial adhesion to suture material in a contaminated wound model: Comparison of monofilament, braided, and barbed sutures. Journal of Orthopaedic Research 2017;35(4):925–933.

[2] Chen X, Hou D, Wang L, Zhang Q, Zou J, Sun G. Antibacterial Surgical Silk Sutures Using a High-Performance Slow-Release Carrier Coating System. ACS Appl Mater Interfaces 2015;7(40):22394–22403.

[3] Thi Tran QM, Thi Nguyen HA, Doan V-D, Tran Q-H, Nguyen VC. Biosynthesis of Zinc Oxide Nanoparticles Using Aqueous *Piper betle* Leaf Extract and Its Application in Surgical Sutures. J Nanomater 2021;2021:8833864.

[4] Guambo MP, Spencer L, Vispo NS, et al. Natural Cellulose Fibers for Surgical Suture Applications. Polymers (Basel) 2020;12(12):3042.

[5] Leaper D, Assadian O, Hubner N-O, et al. Antimicrobial sutures and prevention of surgical site infection: assessment of the safety of the antiseptic triclosan. Int Wound J 2011;8(6):556–566.

[6] Matz D, Teuteberg S, Wiencierz A, Soysal SD, Heizmann O. Do antibacterial skin sutures reduce surgical site infections after elective open abdominal surgery? - Study protocol of a prospective, randomized controlled single center trial. Trials 2019;20(1):390.

[7] Sajid MS, Craciunas L, Sains P, Singh KK, Baig MK. Use of antibacterial sutures for skin closure in controlling surgical site infections: a systematic review of published randomized, controlled trials. Gastroenterol Rep (Oxf) 2013;1(1):42–50.

[8] Vieira D, Angel SN, Honjol Y, et al. Engineering surgical stitches to prevent bacterial infection. Sci Rep 2022;12(1):834.

[9] Umair MM, Jiang Z, Ullah N, Safdar W, Xie Z, Ren X. Development and characterisation of antibacterial suture functionalised with N-halamines. Journal of Industrial Textiles 2015;46(1):59–74.

[10] Sanchez Ramirez DO, Varesano A, Carletto RA, et al. Antibacterial properties of polypyrrole-treated fabrics by ultrasound deposition. Materials Science and Engineering C 2019;102:164–170.

[11] Chaudhari M, Mengi S. Evaluation of phytoconstituents of Terminalia arjuna for wound healing activity in rats. Phytotherapy Research 2006;20(9):799–805.

[12] Xie J, Pan W, Guo Z, Jiao SS, Ping Yang L. In situ polymerization of polypyrrole on cotton fabrics as flexible electrothermal materials. J Eng Fiber Fabr 2019;14:1558925019827447.

[13]Flinčec Grgac S, Tarbuk A, Dekanić T, Sujka W, Draczyński Z. The Chitosan Implementation into Cotton and Polyester/Cotton Blend Fabrics. Materials 2020;13(7):1616.

[14]Rabea EI, Badawy MET, Stevens C V., Smagghe G, Steurbaut W. Chitosan as Antimicrobial Agent: Applications and Mode of Action. Biomacromolecules [homepage on the Internet] 2003;4(6):1457–1465.

[15]Ardean C, Davidescu CM, Nemeş NS, et al. Factors Influencing the Antibacterial Activity of Chitosan and Chitosan Modified by Functionalization. Int J Mol Sci 2021;22(14):7449.

[16]Martínez-Camacho AP, Cortez-Rocha MO, Ezquerra-Brauer JM, et al. Chitosan composite films: Thermal, structural, mechanical and antifungal properties. Carbohydr Polym 2010;82(2):305–315.

[17] Remya S, Mohan CO, Bindu J, Sivaraman GK, Venkateshwarlu G, Ravishankar CN. Effect of chitosan based active packaging film on the keeping quality of chilled stored barracuda fish. J Food Sci Technol [homepage on the Internet] 2016 [cited 2024 Feb 7];53(1):685–693.

[18] Hussein RA, El-Anssary AA. Plants Secondary Metabolites: The Key Drivers of the Pharmacological Actions of Medicinal Plants. In: Builders PF, editor. . Rijeka: IntechOpen, 2018; p. Ch. 2.

[19]Krafts KP. Tissue repair. Organogenesis 2010;6(4):225–233.

[20] Lima Oliveira D, Silva D, Costa D, et al. Synergistic Antibacterial Activity of Green Gold Nanoparticles and Tannin-Based Derivatives. BioChem 2022, Vol 2, Pages 269-279 [homepage on the Internet] 2022;2(4):269–279.

[21] Varesano A, Vineis C, Tonetti C, Mazzuchetti G, Bobba V. Antibacterial property on Gram-positive bacteria of polypyrrole-coated fabrics. J Appl Polym Sci 2015;132(12):41670.

[22]Lima RMAP, Oliveira MCA de, Oliveira HP de. Wearable supercapacitors based on graphene nanoplatelets/carbon nanotubes/polypyrrole composites on cotton yarns electrodes. SN Appl Sci 2019;1(4):325.

[23] Lima RMAP, Alcaraz-Espinoza JJ, Silva FAG Da, Oliveira HP De. Multifunctional Wearable Electronic Textiles Using Cotton Fibers with Polypyrrole and Carbon Nanotubes. ACS Appl Mater Interfaces 2018;10(16):13783–13795.

[24] Candido ICM, Oliveira GDS, Viana GG, Silva FAG Da, Costa MM Da, Oliveira HP De. Wearable Triboelectric Nanogenerators Based on Chemical Modification of Conventional Textiles for Application in Electrically Driven Antibacterial Devices. ACS Appl Electron Mater 2022;4(1):334–344.

[25]Alcaraz-Espinoza JJ, Melo CP De, Oliveira HP De. Fabrication of Highly Flexible Hierarchical Polypyrrole/Carbon Nanotube on Eggshell Membranes for Supercapacitors. ACS Omega 2017;2(6):2866–2877.

[26] Mangrich AS, Doumer ME, Mallmannn AS, Wolf CR. Química Verde no Tratamento de Águas: Uso de Coagulante Derivado de Tanino de Acacia mearnsii. Revista Virtual de Química 2014;6(1):1–14.

[27] Lima Oliveira EG De, Vieira SA, Silva FA Da, Costa MM Da, Gomes ASL, Oliveira HP De. Synergistic Antibacterial Activity of Green Gold Nanoparticles and Tannin-Based Derivatives. BioChem 2022;2(4):269–279.

[28]Zhang Q, Qiao Y, Zhu J, et al. Electroactive and antibacterial surgical sutures based on chitosan-gelatin/tannic acid/polypyrrole composite coating. Compos B Eng 2021;223:109140.

[29] Guimarães ML, Silva FAG da, Souza AM de, Costa MM da, Oliveira HP de. All-green wound dressing prototype based on Nile tilapia skin impregnated with silver nanoparticles reduced by essential oil. Applied Nanoscience 2021 12:2 2022;12(2):129–138.

[30] Silva FAG da, Alcaraz-Espinoza JJ, Costa MM da, Oliveira HP de. Low intensity electric field inactivation of Gram-positive and Gram-negative bacteria via metal-free polymeric composite. Materials Science and Engineering C 2019;99:827–837.

[31] Santos MR dos, Silva FAG da, Ferrais PP, Lima RS de, Costa MM da, Oliveira HP de. Polyaniline-coated Calotropis procera L. hollow tubular fibers with remarkable antibacterial activity. SN Appl Sci 2020;2(9):1550.

[32] Huang H, Wu J, Lin X, et al. Self-assembly of polypyrrole/chitosan composite hydrogels. Carbohydr Polym 2013;95(1):72–76.

[33] Sharma NK, Vishwakarma J, Rai S, Alomar TS, AlMasoud N, Bhattarai A. Green Route Synthesis and Characterization Techniques of Silver Nanoparticles and Their Biological Adeptness. ACS Omega 2022;7(31):27004–27020.

[34] YURTSEVER M, ŞENGL A. Adsorption and desorption behavior of silver ions onto valonia tannin resin. Transactions of Nonferrous Metals Society of China 2012;22(11):2846–2854.

[35] Badawi MA, Negm NA, Abou Kana MTH, Hefni HH, Abdel Moneem MM. Adsorption of aluminum and lead from wastewater by chitosan-tannic acid modified biopolymers: Isotherms, kinetics, thermodynamics and process mechanism. Int J Biol Macromol 2017;99:465–476.

[36] Zhou Z, Liu F, Huang Y, Wang Z, Li G. Biosorption of palladium(II) from aqueous solution by grafting chitosan on persimmon tannin extract. Int J Biol Macromol 2015;77:336–343.

[37] Rubentheren V, Ward TA, Chee CY, Nair P, Salami E, Fearday C. Effects of heat treatment on chitosan nanocomposite film reinforced with nanocrystalline cellulose and tannic acid. Carbohydr Polym 2016;140:202–208.

[38]Azadikhah F, Karimi AR, Yousefi GH, Hadizadeh M. Dual antioxidant-photosensitizing hydrogel system: Cross-linking of chitosan with tannic acid for enhanced photodynamic efficacy. Int J Biol Macromol 2021;188:114–125.

[39] Agarwal S, Rajoria P, Rani A. Adsorption of tannic acid from aqueous solution onto chitosan/NaOH/fly ash composites: Equilibrium, kinetics, thermodynamics and modeling. J Environ Chem Eng 2018;6(1):1486–1499.

[40] Yang W, Sherman VR, Gludovatz B, et al. On the tear resistance of skin. Nat Commun 2015;6:6649.

[41]Siralertmukul K, Watcharamul S, Wicheanpaisan N, Nuisin R. Potential Antibacterial Activity of Polystyrene Nanoparticles/Chitosan Coated on Cotton Fabrics. Macromol Symp 2015;354(1):324–333.

[42] Bruyne T De, Pieters L, Witvrouw M, Clercq E De, Berghe D Vanden, Vlietinck AJ. Biological evaluation of proanthocyanidin dimers and related polyphenols. J Nat Prod 1999;62(7):954–958.

[43] Liu L, Shi H, Yu H, Zhou R, Yin J, Luan S. One-step hydrophobization of tannic acid for antibacterial coating on catheters to prevent catheter-associated infections. Biomater Sci 2019;7(12):5035–5043.

[44] Hossain F, Follett P, Salmieri S, Vu KD, Harich M, Lacroix M. Synergistic Effects of Nanocomposite Films Containing Essential Oil Nanoemulsions in Combination with Ionizing Radiation for Control of Rice Weevil Sitophilus oryzae in Stored Grains. J Food Sci 2019;84(6):1439–1446.

[45] Silva Jr . FAG da, Queiroz JC, Macedo ER, et al. Antibacterial behavior of polypyrrole: The influence of morphology and additives incorporation. Materials Science and Engineering: C 2016;62:317–322.

[46] Libralato G. The case of Artemia spp. in nanoecotoxicology. Mar Environ Res 2014;101(1):38–43.

[47] Arcanjo DDR, Albuquerque ACM, Melo-Neto B, Santana LCLR, Medeiros MGF, Citó AMGL. Bioactivity evaluation against Artemia salina Leach of medicinal plants used in Brazilian Northeastern folk medicine. Brazilian Journal of Biology 2012 ;72(3):505–509.

ORIGINAL ARTICLE

IJAMB
International Journal of Advances in Medical Biotechnology

Managing Knowledge for pharma and biotech innovation

L. M. B. Ferreira[1]; S. A. Cavicchioli[2]; C. Pinheiro[1]; H. S. Barud[2]; C. Coury[3]

*Corresponding author: E-mail address: lmb.ferreira@unesp.br

Received: 01 March 2024; May 15 April 2024.

Abstract: Knowledge Management (KM) involves a deliberate and systematic organization of people, processes, structure, and technology with the main objective of creating value for innovation from the reuse of data and information. Although there are several models for KM in various types of organizations, there is nothing concrete to integrate the knowledge generated in collaborative University-Industry projects in the pharmaceutical and biotechnology areas. This work aimed to gather elements for the creation of a sustainable model of effective articulation in this scenario. It is a strategic action that can bring benefits of intellectual, economic, and social impact. This research used different instruments: systematic mapping, questionnaires, and experience reports. The mapping highlighted the need to consider the following aspects for the development of KM models: collaborative/competitive arrangements, tacit/explicit knowledge managers and change screening. The questionnaire and report demonstrated that the challenges go beyond aspects such as data organization. They must prioritize the social aspect of knowledge sharing, using safe coordination to prevent misconduct.

Keywords: Innovation. University-Industry Interaction. Data Management. Information Management. Information Technology.

Introduction

Innovation is not clearly defined but it is mainly understood as the involvement of new ideas to generate new techniques, products, and processes. Herein, it is important to emphasize that innovation is not limited to creating something completely new, but rather seeks to facilitate everyday life, reduce costs, and make things more accessible. In Brazil, scientific innovation is understood as the last link in the chain of Science-Technology-Innovation (ST&I), and it is crucial to translate the intellectual and economic impacts of research made in universities to society. Using this linear relationship, innovative products and services are thought to be the outcome of scientific advances, but the reality is always like this? Further, can innovative actions stimulate discoveries in basic science? These intriguing questions are pressing in the current situation in Brazil, where it is possible to observe a certain polarization between basic and applied research.

The influence of pure science in advancing innovation was criticized by Matt Ridley during his provocative point called "The Myth of Basic Science", which stimulated thoughtful responses on social media about the role and benefits of science and technology. The divergences about the role of basic science and technology in innovation raised pointed to bidirectional influences[1]. However, the current understanding of innovation points to a context-dependent factor with great influences on local or regional vocation and development. Herein, the partnership between the University-Government-Industry was also another influential point, thus giving rise to the Triple Helix Theory. However, changes in the global scenario have expanded the form of relationships between these actors. This traditional triad has been strengthened with new models of collaboration for knowledge generation, including society (Quadruple Helix) and the environment (Quintuple Helix) with important helices in the dynamics of innovation" [2].

The University-Pharmaceutical or Biotech Industry interface is a specific example of the quintuple helix operation and plays a fundamental role in meeting the demands of innovation in the health area through scientific entrepreneurship. In this context, the contribution of private capital to universities through public-private partnerships has the potential to create favorable conditions for the better functioning of the whole gear. This scenario may seem like the solution

[1]UNESP- State University of São Paulo, Faculty of Pharmaceutical Sciences, Rodovia Araraquara-Jaú, Araraquara-Brazil.
[2] UNIARA – University of Araraquara, Voluntários da Pátria Street, n°1309 – Center zip code: 14801-320. Araraquara - SP.
[3]Federal University of São Carlos. Doctoral Student – Production Engineering Department. Washington Luís Highway km 235 – SP-310, São Carlos, SP, Brazil.

https://doi.org/10.52466/ijamb.v6i1.121

to the problems being faced by the country, however, it could generate even greater discrepancies when we consider that Industry and Academia have essentially different objectives. Because they have different focuses, Industry and Academia walk at different paces. While the industrial sector aligns its efforts towards the agile development of products and obtaining profit in the short term, the academic environment is concerned with the generation of publications that guide knowledge as well as the training and qualification of personnel, which usually occurs in the long term [3].

Qualitative research carried out focusing on the University-Pharmaceutical Industry interface in Brazil showed that many challenges need to be overcome so that the knowledge generated in universities can be effectively transformed into solutions for the population. Among the various points raised by the respondents and interviewees, the processes that involve Knowledge Management (KM) were highlighted. Despite the existence of Technological Innovation Centers, issues related to sharing information with the agility and organization necessary to support decision-making in emergencies such as the one we are experiencing during the COVID-19 pandemic are still far from expected. Some processes that involve KM that were mentioned are: streamlining the flow of information, increasing the efficiency of continuous training of employees, and improving data analysis and management[4].

KM involves a deliberate and systematic organization of people, processes, and technology as well as the organizational structure itself with the main objective of creating value for innovation from the reuse of data and information. This coordination is achieved through the creation, sharing, and application of lessons learned and best practices in the "organization memory"[5]. KM also involves data and information management flows in two main dimensions: tacit and explicit [6]. The implementation of systematic KM practices generates several benefits, such as improving the financial performance of global startups, executing external knowledge search strategies complemented by internal innovation management, and facilitating digital collaborations[7-9].

Although there are several models to manage knowledge within various types of organizations, there is nothing concrete to integrate the fundamental knowledge generated in universities with those that are more applied and of a more technological nature, commonly generated and used by Industry. In this way, the main objective of this work was to identify some of the main elements that should be considered for the creation of a sustainable KM model for collaborative projects. The establishment of this articulation is a strategic action to catalyze scientific entrepreneurship in the context of the Quintuple Helix. Because innovation is a long-term, complex, and risky endeavor, a sustainable KM model would be a valuable contributor to Brazilian initiatives in closing some important gaps in collaborative projects.

Material and methods

In this work, an exploratory study was carried out with a view to a future creation of a model for KM of scientific and technological innovation projects, preferably those situated within University-Industry collaborations in the context of the quintuple helix. The research was conducted using three different instruments to meet the breadth and complexity of this topic: i. Systematic mapping of the Scientific Knowledge Management topic following two generically defined guiding questions: (Q1) What are the main elements that involve Knowledge Management? and (Q2) What ICT-based tools currently exist to support the Knowledge Management process? ii. The questionnaire aimed at parties involved in scientific entrepreneurship that takes place at the University-Pharmaceutical Industry interface. The questionnaire was disseminated via social networks. The questionnaire was created based on two models of KM: the North American model (Davenport & Prusak) and the Japanese model (Nonaka & Takeuchi).; iii. Field Diary containing the researchers' perceptions and experience report around the theme. The data from the systematic mapping, questionnaire, and field diary were analyzed using qualitative analysis software (ATLAS.ti, following content analysis processes as recommended by Bardin[10]. The Excel software was also used in the process of generating the graphs.

Shane (2005) states that technology is "the incorporation of knowledge in different ways, making it possible to create new products, explore new markets, use new ways of organizing, incorporate new raw materials or use new processes to meet customer needs"11. In this context, entrepreneurs are the driving force that catalyzes the process of transforming knowledge into inventions and innovations with potential economic and social impacts. Commonly, all this dynamic is dictated by technological entrepreneurship, that is, one that directly seeks the practical utility of the invention without worrying too much about understanding the scientific aspects that underlie its applicability[12]. However, the crisis triggered by COVID-19 has exposed the need for technological solutions, especially those that focus on human health (e.g., the production of medicines and vaccines), which must necessarily include

aspects related to fundamental knowledge so that well-informed decisions are made. In this context, scientific entrepreneurship emerges as a necessary development of the articulation between Science and Technological Innovation, aiming at minimizing risks and maximizing gains with the creation of new products and/or services. The results presented in this work highlight some of the main elements that should be considered in the creation of KM models for scientific entrepreneurship.

Systematic Mapping - Identifying Knowledge Management Model Elements

The initial total of 50 articles was refined according to the research questions, closing in 19 articles from primary studies and 9 secondary studies. The results of the systematic mapping allowed a better structuring of the research topic around different topics related to KM of innovative projects, preferably those that happen collaboratively between the University and Industry. The survey on the main topics used by the authors considered not only the keywords explicitly defined in the articles but also the text codes. Such codes allowed a panoramic mapping of the main elements that involve KM practices. The emerging themes were then listed based on the codes defined and allocated within the two guiding questions (Table I).

The KM process is complex and dynamic and involves different actors in the collaborative interface. Despite multiple approaches and some prominent models (e.g., the SECI model of Nonaka-Takeuchi and the model of Davenport-Prusak), some main elements could be identified and can be scaled both within the human and technological dimensions. The use of information and communication technologies as digital platforms is considered necessary, but not sufficient to deal with all the dynamics of KM practices. In addition, many of these technologies perform simple system analysis operations such as structuring and archiving data and information but lack some of the key elements shown to be important in all KM practices (e.g., disambiguation, data analysis, knowledge reliability).

Some of the main elements related to KM that go beyond the domain of Information and Communication Technologies (ICTs) refer to the human element or "social character" of knowledge. Although digital social networks are common, currently they do not seem to satisfy the practical needs of KM in terms of traceability and definition of information impact in contexts of interest to a project or organization. It is worth mentioning that this social element is one of the basic engines of the Nonaka-Takeuchi model, especially when talking about exchanges of tacit knowledge[13]. Some emerging themes refer to the possibility of uniting people around the shared construction of academic and business experiences. This type of functionality within platforms is in line with the results obtained via the questionnaire – open questions, which were evaluated in the second stage of this work.

The need to transpose concepts linked to social theories to digital platforms should be considered when planning the types of functionalities that should be created. In this context, the so-called "crowdsolving" platforms stood out among the results, in which individuals are brought together to provide collective solutions to problems previously defined by an organization and/or community. In addition to typical data and information processing operations, such types of platforms can encompass different types of typologies in how human relationships are managed. For example, the possibility of creating competitive and collaborative arrangements is something that should be considered depending on the urgency of solving the challenge.

Table I - Main emerging themes defined in the systematic mapping.

Articles (Author/Date)	Keywords defined by the author	(Q1) What are the main elements that involve Knowledge Management?	(Q2) What ICT-based tools currently exist to support the Knowledge Management process?
(Angelidou; Mount; Pandza, 2022)[8]	Collaboration, complementarity, search for external knowledge, managerial innovation	●Significant learning Tacit knowledge manager ●Explicit Knowledge Manager (Technical) ●Knowledge reliability ●Disambiguation resolution ●Agility ●Academic experience ●Business Experience ●Communications Manager ●Change screening ●Data Manager ●Metrics Manager ●Competitive arrangements ●Collaborative arrangements	●Crowdsolving Platforms ●Platforms for digital documentation
(Battisti *et al.*, 2022)[9]	KM practices, financial performance, global startups		
(Bozic; Bachkirov; Cerne, 2021) [14]	Science-practice gap, rigor-relevance debate, knowledge creation, collaborative challenges, grounded theory		
(Chen *et al.*, 2022)[15]	knowledge management, data-driven decisions, dynamic capabilities, hidden knowledge		
(Marijan; Gotlieb, 2021)[16]	Software engineering, collaborative research, knowledge co-creation, collaborative model, technology transfer, knowledge transfer, research-based innovation		
(Spanellis; Macbryde; Dorfler, 2021)[17]	Knowledge-based systems, causal mapping, knowledge sharing		
(Zhao; Oberoi, 2022)[18]	Crowdsolving, crowdsourcing, SECI model		
(Zhong *et al.*, 2022)[19]	knowledge mapping,		
(Naprawski, 2021)[20]	Agile knowledge management, online reorganization		
(Wohlin; Runeson, 2021)[21]	Technology transfer model, university -Industry collaborative model		
(Abbas *et al.*, 2022)[7]	Lessons learned, collaborative platform, integration		
(Albats; Alexander; Cunningham, 2022)[22]	Academic entrepreneurship, digital platform		
(Myneni *et al.*, 2016)[23]	Cognition, information management		
(Nakayama; Hustad; Sutcliffe, 2021)[24]	Documentation system, tacit knowledge, explicit knowledge, knowledge sharing		
(Alsulami; Hashim; Abduljabbar, 2022)[25]	knowledge sharing		
(Pudjiarti; Lisdiyono; Werdiningsih, 2022)[26]	Regulatory implementation, innovation performance		
(Saunders; Radicic, 2022)[27]	Open innovation, cooperation for innovation		
(Muscio; Shibayama; Ramaciotti, 2022)[28]	Student entrepreneurship, entrepreneurial universe, academic training		
(Chopra *et al.*, 2021)[29]	Sustainability		
(Edwards, 2022)[30]	Information management		
(Gomez-Marin *et al.*, 2022)[31]	Sustainable indicators, collaborative mapping, organizational memory		
(Hadi; Liu; Li, 2022)[32]	knowledge brokers		
(Stemberkova *et al.*, 2021)[33]	Transferência de tecnologia		
(Di Vaio *et al.*, 2021)[34]	Digital transformation, sustainable performance		
(Benitez-Hidalgo *et al.*, 2021)[35]	Knowledge extraction, semantics		
(Allen *et al.*, 2021)[36]	data visualization		
(Ketikidis; Solomon, 2018)[37]	entrepreneurial education		
(Schaefer; Makatsaria, 2021)[38]	Market intelligence, data analysis		

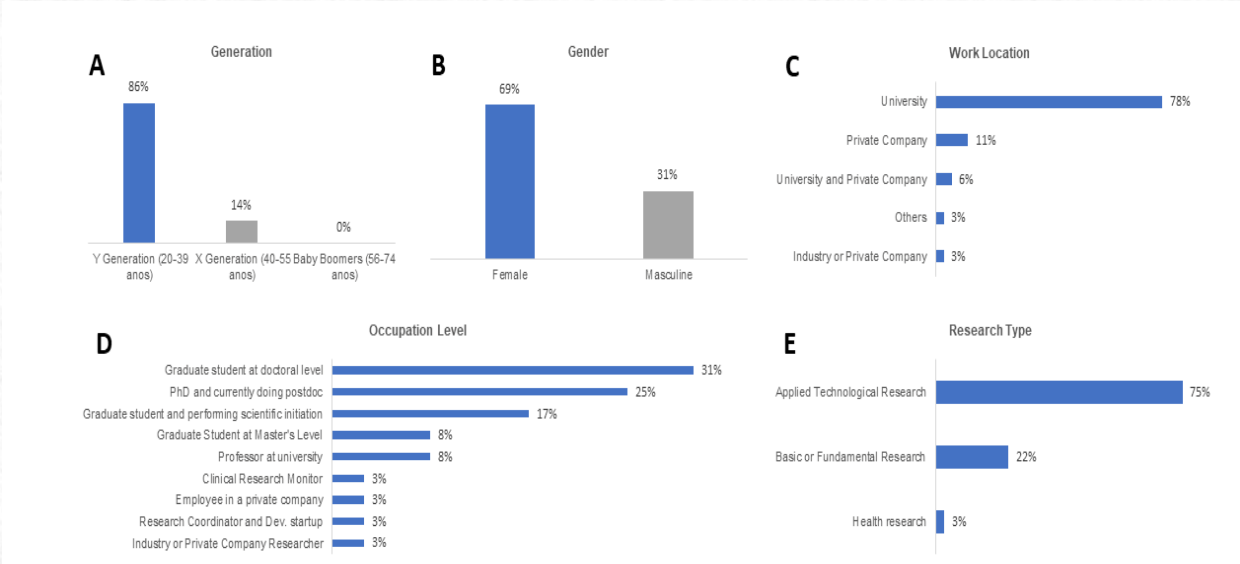
Source: Original search results.

Questionnaire - Characterization of the respondent population

The socio-demographic characterization of the respondents aimed to identify some possible elements (e.g., sex, age) that may influence perceptions about the KM processes of science-based projects. The characterization of the personal profile of the respondents, shown in Figure 1A-B, showed most female participants (69%), while male respondents totaled 31%. Regarding age group, most respondents belong to Generation Y (20-39 years old, 86%) and a minority belong to Generation X (40-55 years old, 14%). No respondents belonging to Generation Z (under 20 years old) or Baby Boomers (56-74 years old) were counted. This classification based on a generational approach is an important aspect to be considered as it interferes both with the perception of the values associated with KM processes and the difficulties, especially with processes that involve the application of ICT-based tools[39] .

The characterization of the professional profile is shown in Figure 1C-E. It was identified that 78% of the respondents are inserted only in the University, 11% are inserted in a private company and 6% are part of both the University and a private company. A minority (3%) is inserted elsewhere. Regarding the level of training, most respondents are doctoral students (31%), post-doctoral students (25%), and scientific initiation (17%). A minority is composed of master's students (8%), professors (8%), coordinators in a private company (3%), startup coordinators (3%), and researchers from Industry or private companies (3%). Regarding the type of research they develop, 75% work with technological research, 22% with fundamental research, and 3% with research in Health. This last result is particularly interesting because it may constitute evidence that in Brazil, we have not yet managed to create the culture of an entrepreneurial Science, that is, one that seeks to align the fundamental knowledge with technological innovation.

Figure 1 - Characterization of the personal and professional profile of the respondents.



Source: Original search results.

Questionnaire - Assessment of the perception of the parties involved about the workflows in the KM of science-based projects

KM workflows permeate dimensions related to organizational culture and learning, social capital, and technological tools. More specifically, the American model of KM focuses mainly on applications of ICT resources to facilitate the flow of data and information within the organization. A series of questions were formulated to understand some operational aspects to assess how respondents interact with such flows. The results are shown in Figures 2 and 3 and focus on understanding how the Collect → Transfer → Storage → Processing of data and information dynamics takes place. Most respondents work with quantitative data (92%) and a minority work with qualitative data (8%). Regarding data transfer processes, 44% use flash drives and external hard drives immediately after data collection, and 31% also use flash drives, but do so periodically. Another 14% leave the data stored on the computer connected to the equipment. Minorities use cloud storage (3%), physical storage + cloud (3%), and notebook notes (3%).

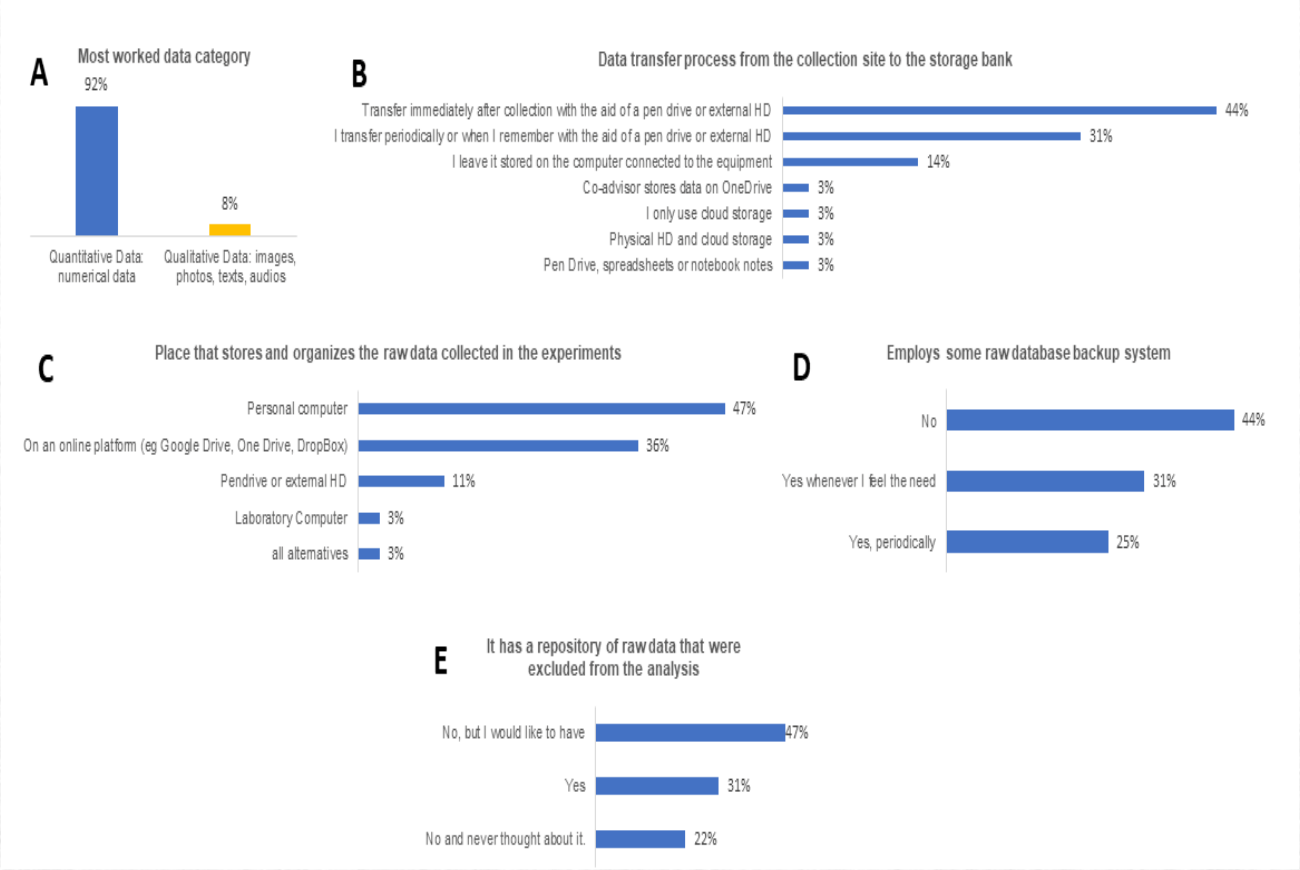
The storage of data/information from the projects is a highly relevant element in interactive University-Industry contexts as it is directly related to aspects related to security and their sharing.

Regarding the storage location of the raw data collected: 47% use a personal computer, 36% use some online platform such as Google Drive, One Drive and Dropbox, and PenDrive/external HD (11%). A minority storage on the laboratory computer (3%) or use all alternatives (3%). Most respondents do not use any backup system for the data collected (44%), and others use backup whenever they feel the need (31%). The minority (25%) said they use backup. About a repository of raw data that was excluded from analysis: most do not have it but would like to (47%), 31% have the repository and 22% do not have it and had never thought about it.

Still considering data and information management processes, Figure 3 presents the results related to metadata storage management and analysis processes. Regarding the repository for metadata, 42% of respondents said they have and organize the metadata manually, another 42% do not have it but would like to have it, and 14% do not have it and had never thought about it. Regarding the processes that involve Information Management: 53% said that some data is analyzed manually and others are analyzed automatically; 38% perform data analysis only manually following a previously established protocol. Only 8% do a fully automated analysis; About the process of generating meaning from the data: 36% compare the results with support material, 31% generate multiple graphs to be able to better visualize the information, 8% compare the results with the support material (reference values and articles of the area).

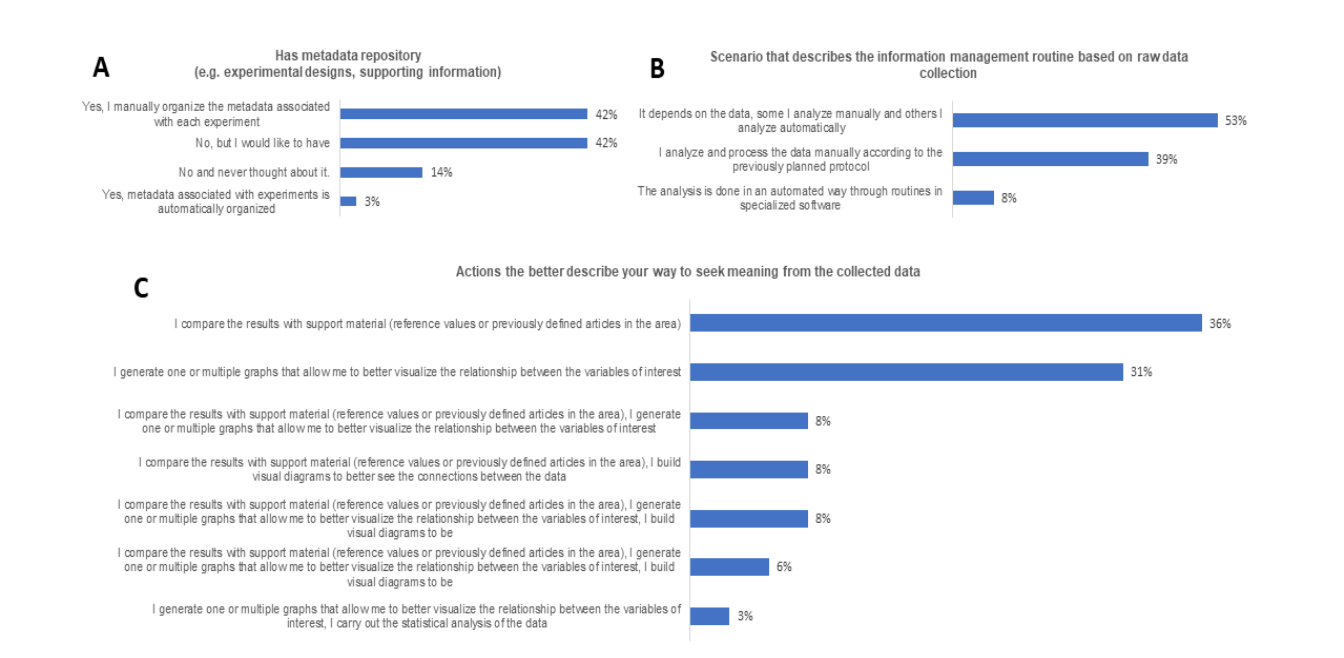
When analyzing together the results shown in Figures 2 and 3, it is evident that several respondents still use manual and unsafe means to manage data/information from science-based projects. Some important aspects related to the use of backup systems and metadata management are still far from best practices and may incur KM problems. Such problems can range from the lack of systematization of databases, which impairs analysis and decision-making processes, to the leakage of sensitive and/or confidential information. It is also worth mentioning that these results are in line with the main parameters considered within the context of knowledge/information management established by some authors, such as people, processes, culture, technology, and structure[30].

Figure 2 - Knowledge Management processes and routines - aspects involving data and information management (Part 1).



Source: Original search results.

Figure 3 - Knowledge Management processes and routines - aspects involving data and information management (Part 2).



Source: Original search results.

Questionnaire - Assessment of potential challenges and elements of value in processes involving the KM of science-based projects

The challenges and perceived value of some KM elements were also evaluated (Figure 4). More specifically, we sought to understand the perception of value that some respondents have on the processes of socialization, externalization, internalization, and combination, which are established in Nonaka and Takeuchi's KM model (Figure 4 A-D). It was observed that all these model processes have a lot of value to respondents for acquiring knowledge. Most of the respondents (81%) attributed a lot of value (grade 5) to the "externalization" process, defined in terms of writing, recording, drawing, and making the information visual (Figure 5-B). A total of 72% of respondents also gave a high value (grade 5) to the "internalization" process, defined as studying, reading, listening, and watching. While the first refers to the articulation of tacit knowledge into explicit, the second uses explicit knowledge, that is, rationalized, into something tacitly internalized by the individual.

The components "socialization" and "combination" were considered valuable (grade 5) by 67% and 56% of the respondents, respectively. Socialization was defined in terms of interacting with people, hands-on observation, and group discussions. It is an extremely important process in sharing experiences, creating tacit knowledge in multiple ways (e.g., mental models and technical skills)[13]. In turn, the combination component seeks to systematize different sets of explicit knowledge using schemas, records, etc. Despite not opposing the common sense that socialization is an important component in knowledge-sharing processes, the results presented emphasize the importance of internalization and externalization processes. This may not necessarily be related to the sharing of information, but to actions that aim to deepen the understanding of knowledge on a given topic.

The other questions regarding the value of KM processes focused on topics related to University--Industry collaborative projects (Figure 4 E-L). The results showed that 92% of respondents are interested in this type of project, with 72% and 78% of respondents giving high value (grade 5) to the following interaction products: obtaining financial resources for research (e.g., equipment and various materials) and the possibility of having the collaboration well evaluated by the platform for future collaborations, respectively (Figure 4 – H and I). In comparison, fewer respondents, 50 and 56%, think that the receipt of salaries and the dissemination of research in scientific journals is valuable (Figure 4 – F and G).

Questionnaire - Answers to open questions

The open questions at the end of the online questionnaire aimed to raise the main topics and more subjective elements related to the KM processes of research projects with innovative potential. A series of codes emerged from the thematic analysis of the responses and were based on hypotheses for the construction of digital platforms for KM (Table II).

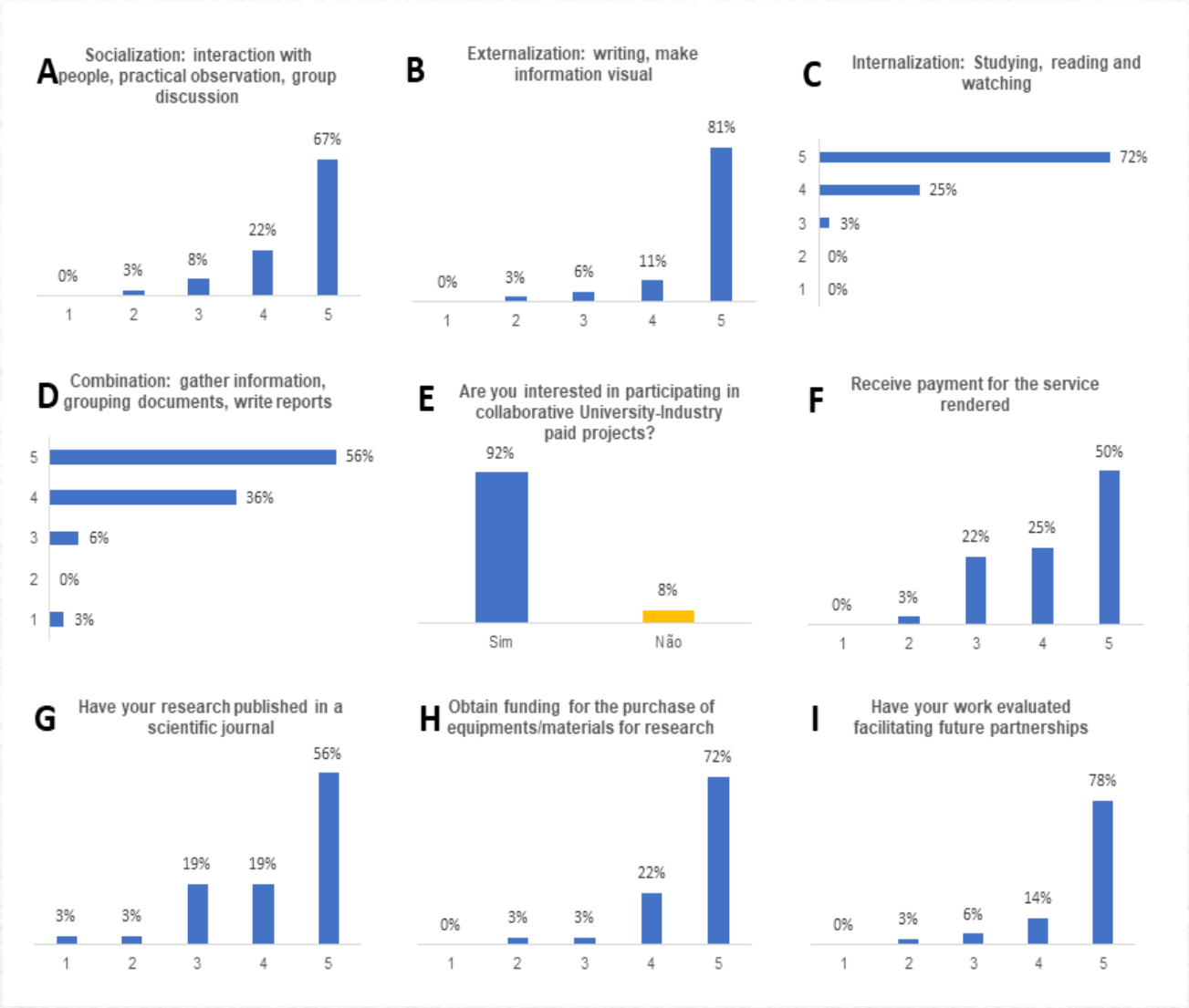
Tacit knowledge management is the most

challenging form of management due to the influence of adjacent subjectivity in interactions. The perception is that currently there are no efficient ways to manage this type of knowledge, but there are hypotheses of interesting solutions that could be investigated. Here, respondents cited the creation of study groups, information records, and means of sharing experiences as effective ways to build KM for tacit knowledge.

The KM of University-Industry collaborative projects is something that needs to be guided by members who make up both sides of the coin. In this work, we mainly explore the academic side, but according to the respondents, different views can be integrated, aiming at a management that favors both the development of new products and the generation of more knowledge around themes of mutual interest. In this case, academics can provide basic (and even frontier) knowledge to be assimilated by members of the industry in the development of innovation projects.

Another aspect that became clear is that the generation of scientific publications should not be the only indicator to guide University-Industry interactions. The fruits of these interactions go far beyond that and can even bring a return to society as it promotes the generation of new innovative products and services. Thus, depending on how KM is practiced, the benefits of the interface can effectively benefit the triple helix parties: government, university, industry. Establishing the main conditions of success for each of these parts is research that needs to be addressed in future works.

Figure 4 - Challenges and value elements of the main processes involved in Knowledge Management. The statements were rated on a five-point scale: (1) Not valuable – (5) Very valuable.



Source: Original search results.

Table II - Coding of emerging themes in the open questions of the online questionnaire on Knowledge Management in University-Industry collaborative projects.

Questions	Parts	Thematic Codes	Hypotheses
It is known that tacit knowledge (that inherent to the person and from experiences) is difficult to share. What is the importance of this type of knowledge in innovative projects? Do you believe that there are currently efficient ways to manage this type of knowledge?	"... tacit knowledge is what differentiates people and is fundamental for collaborative projects, as it is what makes it possible to generate empathy and real applicability of techniques and theories. My suggestion is to analyze it in a systematic and even psychoanalytic way, try to understand the scenario, the problem, possible solutions. This ends up making the situation more translucent and measurable." "... of great importance and currently I don't see efficient ways of sharing this knowledge. Years of work are often lost with the departure of the person who owns this knowledge..." "Collaborations are essential so that tacit knowledge is disseminated to other groups, reducing steps and accelerating the process of research and development of new products. There are a few ways to manage this knowledge on researcher platforms, such as Research Gate and/or discussion forums. But still nothing very efficient. "	Study group Keep Information Records share experiences	Currently, there are no efficient ways to manage tacit knowledge, but this can be done through the creation of study groups and spaces for sharing experiences.
Do you believe that a Knowledge Management of innovative projects that take place in collaboration between the University-Industry can bring a competitive advantage to the Industry? And for the University? Because?	"Yes, because the vision of the academic environment is limited. The industry has a different vision and applicability than academia, and one can benefit from the other. " "I believe so, since the knowledge of academia combined with technology and industrial speed can generate great results. Since the academic scientist may have a different view from the person who works only in the industry."	Integration of different views Provides fundamental knowledge Acquire industrial vision	The KM of collaborative projects can add to both the University and the Industry, that is, the integration of the two mental models is possible and can be used to achieve greater goals and with social impact.
What is for you a successful scenario of the Knowledge Management process in University-Industry collaborative projects?	"It is an idea that is created and developed together, with a specific purpose and goals that both fields (academic and industrial) benefit and progress with the development of a product/idea." "Transfer of knowledge and technology, training, alignment of interests of both parties."	Product generation Generating more knowledge (publications)	The success of collaborative scenarios occurs when they are able to produce both products and more knowledge for the population.
Are you interested in participating in UNpaid University-Industry collaborative projects? If yes, what would encourage you to participate? What would you consider viable to receive in return besides financial gratification?	"Yes, the experience of professional practice, proof for services and time spent." "Yes, the recognition and confidence in my work, as well as the possibility of expanding my knowledge in different areas.	Recognition	The differential of unpaid collaborative projects goes beyond financial gratification. It can unfold into recognition for the participants.

Source: Original search results.

Experience Report

The consolidation of science-based entrepreneurship still represents a challenge for the country since fundamental research is far from technological research. This becomes even more complex when it comes to research for innovation, that is, research in close connection with the economic market. Articulating these three types of research represents an action for the consolidation of science-based entrepreneurship and, therefore, must contemplate the specificities of each one of them. Experiences from both academia and industry clearly show that researchers have different mental models and focus of action. Thus, the creation of a sustainable KM model that can promote this articulation will possibly require a strong investment in leadership training capable of understanding the different interests of the parties involved.

One of the most likely consequences of the increase in private investment and decrease in public investment is that the University is forced to fully conform to the molds of the business world. In a period of crisis and pressure like the one we are experiencing; this could possibly be reflected in the polarization between basic and applied research. As applied research manages to produce tangible results in a shorter period, it is quite possible that it would receive greater investment from the private sector, thus standing out from basic research. However, this dichotomy between basic research and applied research does not exist. The two types of approach are not mutually exclusive. On the contrary, they are closely intertwined, and one catalyzes the development of the other. In this way, the current Brazilian scenario requires the parties involved take these points into account for the creation of solutions focused on KM model of scientific entrepreneurship. A good guide for this problem is the Stokes diagram, which proposes a research classification system so that scientists can guide their activities according to the need to produce results to expand the knowledge and have practical utility [40].

Ethical issues regarding data and information security, especially information of a confidential nature, must also be considered. Most universities still do not have an adequate structure to deal with these issues. It will certainly be necessary to implement information security mechanisms that are not yet in the public domain. Another point also refers to misconduct actions such as plagiarism, predatory competition, improper manipulation of data, etc. These issues need to be thought through and regulatory mechanisms need to be created so that people within the model can feel safe and motivated in relation to knowledge sharing.

Final considerations

This work showed that the KM of University-Industry collaborative projects aimed at fostering science-based entrepreneurship is complex and composed of several elements related not only to operational issues of digital technologies, but also to social aspects that encompass human relations in the knowledge construction process. This aspect was evident both by the systematic mapping of articles in the area and by the questionnaires disseminated over the internet. Among the results of the closed questions, it is worth mentioning the lack of automated processes for organizing backups and metadata as well as processes related to data sharing security. Open questions highlighted the difficulties in managing tacit knowledge as well as the importance of integrating industry and university mental models in innovation processes. The hypotheses built from the answers to open questions provide a direction for future research and can be assimilated in future initiatives of developing digital solutions for KM in collaborative projects.

Something that should be considered in KM, in addition to the way of obtaining and collecting data, is the discussion of these data and their interpretation by the research and development team, to establish an understanding of its meaning and then produce an applicable knowledge. This process, perhaps, represents a more time-consuming step, as it depends on the elaboration of logical thinking about the data obtained, which can make the management model more time-consuming and the development of a product/technology slower, thus being a limiting step. Coordinating this discussion of data with the fast pace of production in an industry, for example, is challenging, given the constant demand for creation and innovation in this sector.

References

[1] Witze A. Does innovation always come from science? Nature, 2015;527

[2] Mineiro, A. A. DA C. et al. Da Hélice Tríplice a Quíntupla: Uma Revisão Sistemática. Revista Economia & Gestão, v. 18, n. 51, p. 77–93, 2019.

[3] Birnbaum MJ. Pharma and Academia: What We Have Here Is a Failure to Communicate. Cell Metab. 2016;24(3):365–7.

[4] Ferreira LMB, Oliveira AB de F. Projetos de inovação na interface Universidade-Indústria Farmacêutica: desafios e oportunidades. Universidade de São Paulo; 2021.

[5] Dalkir K. Knowledge management in theory and practice. Knowledge Management in Theory and Practice. 2013. 1–356 p.

[6] Bandera C, Keshtkar F, Bartolacci MR, Neerudu S, Passerini K. Knowledge management and the entrepreneur: Insights from Ikujiro Nonaka's Dynamic Knowledge Creation model (SECI). Int J Innov Stud. 2017;1(3):163–74.

[7] Abbas Y, Martinetti A, Rajabalinejad M, Schuberth F, van Dongen LAM. Facilitating digital collaboration through knowledge management: a case study. Knowl Manag Res Pract. 2022;00(00):1–17.

[8] Angelidou S, Mount M, Pandza K. Exploring the asymmetric complementarity between external knowledge search and management innovation. Technovation. 2022;115(December 2021):102472.

[9] Battisti E, Alfiero S, Quaglia R, Yahiaoui D. Financial performance and global start-ups: the impact of knowledge management practices. J Int Manag. 2022;28(4):100938.

[10]Bardin L. Análise de conteúdo. 1st ed. EDIÇÕES 70 - Grupo Almedina; 2011.

[11] Shane SA. Sobre Solo Fértil. Como identificar grandes oportunidades para empreendimentos em alta tecnologia. 1st ed. Porto Alegre: Bookman; 2005.

[12]Bailetti T. Technology Entrepreneurship: Overview, Definition, and Distinctive Aspects. Technol Innov Manag Rev. 2012;2(2):5–12.

[13]Farnese ML, Barbieri B, Chirumbolo A, Patriotta G. Managing knowledge in organizations: A nonaka's SECI model operationalization. Front Psychol. 2019;10(December):1–15.

[14]Božič K, Bachkirov AA, Černe M. Towards better understanding and narrowing of the science-practice gap: A practitioner-centered approach to management knowledge creation. Eur Manag J. 2021;(March 2020).

[15]Chen Y, Luo H, Chen J, Guo Y. Building data-driven dynamic capabilities to arrest knowledge hiding: A knowledge management perspective. J Bus Res. 2022;139(January 2021):1138–54.

[16]Marijan D, Gotlieb A. Industry-Academia research collaboration in software engineering: The Certus model. Inf Softw Technol. 2021;132(November 2019):106473.

[17]Spanellis A, MacBryde J, Dörfler V. A dynamic model of knowledge management in innovative technology companies: A case from the energy sector. Eur J Oper Res. 2021;292(2):784–97.

[18]Zhao Z, Oberoi P. Designing crowdsolving Ba: A closer look at the features of crowdsolving platforms to manage organizational knowledge. Inf Manag. 2022;59(4):103641.

[19]Zhong D, Fan J, Yang G, Tian B, Zhang Y. Knowledge management of product design: A requirements-oriented knowledge management framework based on Kansei engineering and knowledge map. Adv Eng Informatics. 2022;52 (December 2021):101541.

[20]Naprawski T. Towards agile knowledge management in an online organization. Procedia Comput Sci. 2021;192:4406–15.

[21]Wohlin C, Runeson P. Guiding the selection of research methodology in industry–academia collaboration in software engineering. Inf Softw Technol. 2021;140(June):106678.

[22]Albats E, Alexander AT, Cunningham JA. Traditional, virtual, and digital intermediaries in university-industry collaboration: exploring institutional logics and bounded rationality. Technol Forecast Soc Change. 2022;177(December 2021):121470.

[23]Myneni S, Patel VL, Bova GS, Wang J, Ackerman CF, Berlinicke CA, et al. Resolving complex research data management issues in biomedical laboratories: Qualitative study of an industry-academia collaboration. Comput Methods Programs Biomed. 2016;126:160–70.

[24]Nakayama M, Hustad E, Sutcliffe N, Hustad E. ScienceDirect ScienceDirect Agility and system documentation in large-scale enterprise system Agility and system documentation in large-scale perspective enterprise system projects : a knowledge management projects : a knowledge management perspective. Procedia Comput Sci. 2021;181(2019):386–93.

[25]Alsulami ZA, Hashim HS, Abduljabbar ZA. Model to enhance knowledge sharing process in academia during COVID-19. Bull Electr Eng Informatics. 2022;11(1):558–66.

[26]Pudjiarti ES, Lisdiyono E, Werdiningsih R. Knowledge management to develop comprehensive networking of university-industry collaboration in technology and innovation performance. Int J Data Netw Sci. 2022;6(2):461–8.

[27]Saunders K, Radicic D. Managing the knowledge for innovation in Eastern European firms: open or closed innovation? J Sci Technol Policy Manag. 2022;

[28]Muscio A, Shibayama S, Ramaciotti L. Universities and start-up creation by Ph.D. graduates: the role of scientific and social capital of academic laboratories. J Technol Transf. 2022;47(1):147–75.

[29]Chopra M, Saini N, Kumar S, Varma A, Mangla SK, Lim WM. Past, present, and future of knowledge management for business sustainability. J Clean Prod. 2021;328(October):129592.

[30]Edwards JS. Where knowledge management and information management meet: Research directions. Int J Inf Manage. 2022;63(November 2021):102458.

[31]Gómez-Marín N, Cara-Jiménez J, Bernardo-Sánchez

A, Álvarez-de-Prado L, Ortega-Fernández F. Sustainable knowledge management in academia and research organizations in the innovation context. Int J Manag Educ. 2022;20(1).

[32]Hadi A, Liu Y, Li S. Transcending the silos through project management office: Knowledge transactions, brokerage roles, and enabling factors. Int J Proj Manag. 2022;40(2):142–54.

[33]Stemberkova R, Maresova P, David OO, Adeoye F. Knowledge management model for effective technology transfer at universities. Ind High Educ. 2021;35(6):638–49.

[34]Di Vaio A, Palladino R, Pezzi A, Kalisz DE. The role of digital innovation in knowledge management systems: A systematic literature review. J Bus Res. 2021;123(May 2020):220–31.

[35]Benítez-Hidalgo A, Barba-González C, García-Nieto J, Gutiérrez-Moncayo P, Paneque M, Nebro AJ, et al. TITAN: A knowledge-based platform for Big Data workflow management. Knowledge-Based Syst. 2021;232:107489.

[36]Allen L, Atkinson J, Jayasundara D, Cordiner J, Moghadam PZ. Data visualization for Industry 4.0: A stepping-stone toward a digital future, bridging the gap between academia and industry. Patterns. 2021;2(5):100266.

[37]Ketikidis P, Solomon A. Special issue: Co-producing knowledge, innovation and growth through university–industry collaboration – Lessons from experience. Ind High Educ. 2018;32(4):211–2.

[38]Schaefer C, Makatsaria A. International Journal of Intelligent Networks Framework of Data Analytics and Integrating Knowledge Management. Int J Intell Networks. 2021;2(July):156–65.

[39]Silva RC da. A abordagem geracional como proposta à gestão de pessoas. 2013.

[40]Stokes DE. Pasteur's Quadrant_ Basic Science and Technological Innovation. 1st ed. Brookings Institution Press; 1997. 1–150 p.

ORIGINAL ARTICLE

IJAMB
International Journal of Advances in Medical Biotechnology

Endocrine activities modulated by adipose-mesenchymal stem cell in an animal model induced to polycystic ovary syndrome

E. D. Alves[1]; L. G. D. Benevenuto[1]; J. A. Anselmo-Franci[2]; E. Ervolino[3]; B. P. Morais[4]; M. Andrade de Barros[4]; J. A. Achcar[1]; L. H. Montrezor[1]*

*Corresponding author: E-mail address: lhmontrezor@uniara.edu.br

Received: 15 January 2024; 18 March 2024.

Abstract: Purpose - Animal models offer a useful way to study the reproductive and metabolic abnormalities, including polycystic ovary syndrome (PCOS). Mesenchymal stem cells (MSCs) have received increasing attention as a potential cell-based therapy and regenerative medicine, due to their effects in modulation of different molecular and biological pathways. The aims of the present work were to investigate modulation of the ovarian microenvironment by adipose-mesenchymal stem cells (AdMSCs) in an animal model induced to PCOS. Methods - Female rats were divided into control, polycystic ovary, and mesenchymal stem cell groups, evaluated at two different times after PCOS induction and injection of AdMSCs. Results - The polycystic ovary group showed changes in ovarian cycles, the presence of cysts in the ovaries, and hyperandrogenemia. In addition, changes in plasma insulin, glucose, leptin, and osteocalcin were observed in the polycystic ovary group. These metabolic changes were modulated by the injection of AdMSCs into the ovary. Data are presented for female rats in an animal model integrating PCOS with AdMSCs, together with the relationships among ovaries, bones, and adipocytes. Conclusion - The results suggested the existence of endocrine-metabolic-reproductive microenvironment relationships modulated by AdMSCs, which should help in guiding further investigations to clarify pathophysiological mechanisms that have not yet been fully elucidated.

Keywords: Ovary microenvironment. Mesenchymal stem cell. Biotechnology. Polycystic Ovary Syndrome. Animal model.

Introduction

The mechanisms underlying ovarian dysfunction in PCOS have not been definitively established. Hyperandrogenism is a major PCOS characteristic, with evidence indicating that it plays a key role in PCOS pathogenesis [1]. Genetic, physiological, social, and environmental causes appear to contribute to the condition. Among fertility disorders, PCOS is the most common endocrine disorder[2], affecting 5.6 to 21.3% of women of reproductive age worldwide[3], depending on the diagnostic criteria applied.

Stem cell paracrine signaling has been highlighted as an important mechanism underlying stem cell mediated tissue regeneration and immunomodulation, and as a potential cell-based therapy[4-7]. Mesenchymal stem cells derived from bone marrow, cord blood, and adipose tissue have been found to secrete a wide spectrum of growth factors, cytokines, and extracellular vesicles that enhance angiogenesis, neurogenesis, and wound healing [8-11]. The production of paracrine signals from transplanted MSCs can improve tissue regeneration and induce functional recovery of defective tissue by activation of endogenous cells in host tissue[9], including female reproductive organ defects leading to infertility, such as endometriosis, premature ovary failure, and PCOS [5].

Understanding the etiology of PCOS is important for identifying biomarkers and developing possible therapies. Although studies are being carried out in humans, there are important ethical restrictions. Therefore, studies with animal models are indispensable for understanding the pathophysiological mechanisms of this syndrome[12-19]. Animal models have been proposed that use steroid hormones and steroidogenic enzyme inhibitors for the development of a phenotype associated with PCOS[13, 20-21]. However, there is no animal model that is totally effective for studying PCOS, and the choice of a model must be guided by the objectives of the study.

A suitable animal model could provide a useful way to study the physiopathology of the characteristic reproductive and metabolic abnormalities associated with PCOS. So far, there is no consensus on the best animal model, which should ideally reproduce

[1]Department of Biological Science and Health - University of Araraquara - UNIARA, Araraquara, São Paulo, Brazil.
[2]Department of Morphology, Physiology and Basic Pathology – USP, Ribeirão Preto, SP, Brazil.
[3]Department of Basic Science – Odontology - UNESP, Araçatuba, SP, Brazil.
[4]Regenera Stem Cells - Campinas, São Paulo, Brazil.

https://doi.org/10.52466/ijamb.v6i1.115

the key features associated with human PCOS [18,22]. Neonatal, peripubertal, and adult models demonstrate that activational action postnatal manipulations can generate sufficient POCS-like traits [23].

The aims of the present work were to investigate modulation of the ovarian microenvironment by AdMSCs in an animal model induced to PCOS. The results revealed reproductive and metabolic changes that were possibly mediated by mesenchymal stem cells.

Material and methods

Animals

Adult female Wistar Hannover rats (6 months old at the end of the experiments, *n* = 30) were obtained from the Ribeirão Preto Medical School (FMRP-USP, Ribeirão Preto, SP, Brazil). The animals were kept in the laboratory at the University of Araraquara (UNIARA, Araraquara, SP, Brazil), under controlled conditions of 22 ± 2 °C and 12-h light / dark cycles (lights on at 7:00 a.m.). Water and commercial chow diet were offered *ad libitum*. All the experimental procedures were approved by the Committee of Ethics in Animal Use (CEUA/UNIARA, n° 030/2016), following the norms of the National Council for Control of Animal Experimentation (CONCEA/MCTI, Brazil).

Polycystic ovary syndrome induction

The PCOS induction was performed with a single dose of estradiol valerate (EV) (Sigma-Aldrich, MO, USA) dissolved in mineral oil (2.0 mg/0.2 mL / rat, intramuscular) [22]. Control animals received intramuscular injection of 0.2 mL of mineral oil. In addition to practicality and low cost, this PCOS model induced by EV showed important signs related to the syndrome, including hyperandrogenemia, irregular estrous cycles, and polycystic ovarian morphology.

Adipose-Mesenchymal stem cell injection

Female dog AdMSCs were used (Regenera Stem Cells®, Campinas, SP, Brazil). The cells were thawed in a water bath (37 °C), resuspended in 3 mL of defrosting medium (Regenera Stem Cells®, Campinas, SP, Brazil), centrifuged at 1000 rpm for 5 min, washed twice with 3 mL of washing solution (Regenera Stem Cells®, Campinas, SP, Brazil), centrifuged again, and resuspended in 1 mL of saline. The animals were anesthetized using intraperitoneal ketamine (75 mg/kg, Agener, SP, Brazil), and xylazine (10 mg/kg, Coopers, SP, Brazil), and laparotomy was performed to access the ovaries. The ovaries were exposed, and the AdMSCs were injected (1×10^6 cells / 0.2 mL of saline / ovary), using 1 mL syringes. The control group animals underwent the same surgical procedures, and 0.2 mL of saline was injected into each ovary. After surgery, the animals were treated with subcutaneous enrofloxacin (10 mg/kg, Bayer, SP, Brazil). The AdMSCs application was performed on the same day as the PCO induction.

Experimental protocol

After PCO induction and AdMSCs injection, the rats were divided into 6 groups (*n* = 5 / group), according to the time after induction (30 and 60 days), and the treatment (C, PCO, or MSC). The evaluation times were based on the time required for PCOS to appear[24, 25] and for observations of possible responses modulated by biologically factors secreted by the MSCs [26]. The groups were labeled as follows: C 30 and C 60 (control, C groups); PCO 30 and PCO 60 (polycystic ovary, PCO groups); MSC 30 and MSC 60 (adipose-mesenchymal stem cell, MSC groups).

Estrous cycles and body mass

Estrous cycle analysis was performed daily for four weeks, prior to induction of PCO, to confirm the occurrence of normal and consecutive cycles. The animals used in the experiment had at least four consecutive regular 4-day cycles[12, 24]. The estrous cycles checks were continued daily until the end of the experimental period. Body mass analysis of the rats was performed on the day of arrival at the University Animal Facilities, on the day of induction of PCO and AdMSCs injection, weekly after the day of induction and injection, and on the day of euthanasia.

Gonadosomatic index and ovarian morphology

The animals were weighed before euthanasia. Subsequently, the ovaries were removed, cleaned, and weighed. The values obtained were used to determine the gonadosomatic index (GSI): (ovarian mass / body mass) x 100. The ovaries were removed, properly cleaned, placed in histological cassettes, and kept for 24 hours in 4% formaldehyde (prepared from paraformaldehyde), in 0.1 M sodium phosphate buffer (pH 7.2), at room temperature, and subsequently in running water for another 2 hours to remove excess formaldehyde. The ovaries were then dehydrated in increasing concentrations of alcohol (70°, 80°, 90°, and 100° GL), diaphanized in xylol, infiltrated, and included in paraffin. Serial longitudinal cuts of 5 µm were made. After every 10 cuts, histological glass slides were mounted, stained with hematoxylin-eosin (HE), and analyzed by optical microscopy (Eclipse TS100, Nykon, Tokyo, Japan). The images were analyzed using ISCapture IS500 v.4.3.1 software (TUCSEN Photonics, Fujian, China). Cystic follicles were defined as those devoid

of oocyte and displaying a large antral cavity, a thin granulosa cell layer, and a thickened theca interna cell layer[12, 24].

Hormones and glucose assays

At the end of the experimental periods, the rats were euthanized using pentobarbital sodium (Merck, NJ, USA) at 3% (0.6 mL / 100 g, intramuscular), and a 5 mL blood sample was obtained from the heart into heparinized syringes. Plasma was separated by centrifugation at 3000 rpm for 20 min, at 4°C, and was stored at -20 °C for subsequent determination of progesterone (P4), testosterone (T), luteinizing hormone (LH), and follicle-stimulating hormone (FSH), by radioimmunoassay (RIA). Plasma P4 and T concentrations were determined by double-antibody RIA, using kits provided by Biochem Immunosystem (Bologna, Italy). The lower limits for detection of progesterone and testosterone were 0.03 and 4.8 ng/mL, respectively. The intra-assay coefficients of variation were 6.5% for P4 and 4.5% for T. Plasma LH and FSH were assayed using a kit provided by the National Hormone and Peptide Program (Harbor-UCLA, USA). The antiserum for LH was LH-S10, using RP3 as reference. The FSH primary anti-body was anti-rat FSH-S11, with FSH-RP2 as standard. The lower limits of detection for LH and FSH were 0.04 and 0.2 ng/mL, while the intra-assay coefficients of variation were 3.4% and 6.3%, respectively.

Plasma levels of leptin, osteocalcin (OCN), and insulin were determined using commercially available ELISA kits, following the instructions of the manufacturer (EMD Millipore Corporation, MA, USA). Glucose was determined using a drop of blood obtained by cardiac puncture. The blood was added to a test strip that was inserted into the measurement device (Accu-Chek® Performa, Roche, SP, Brazil), according to the manufacturer's instructions.

Statistical analysis

The results are reported as means ± SD. The data were analyzed using ANOVA (analysis of variance) to compare the means, while the Fisher test was used for multiple comparisons. Statistical analyses were performed using a software program (Sigma Stat, Systat Software, CA, USA). Significant statistical differences among the means of the treatment groups were considered for *P*-values < 0.05.

Results

Morphometric data

The morphometric data of body weight, ovarian weight, and gonadosomatic index are presented in Table 1. *30 days*: Higher body weight was observed for the C group, compared to the PCO and MSC groups. The GSI was higher for the PCO and MSC groups, compared to the C group. *60 days*: Higher body weights were obtained for the C and PCO groups, compared to the MSC group. Comparison of the values obtained at 30 and 60 days showed a reduction of body weight for the C group. For the C and MSC groups, the ovarian weight and GSI were lower at 60 days of the experiment, compared to 30 days.

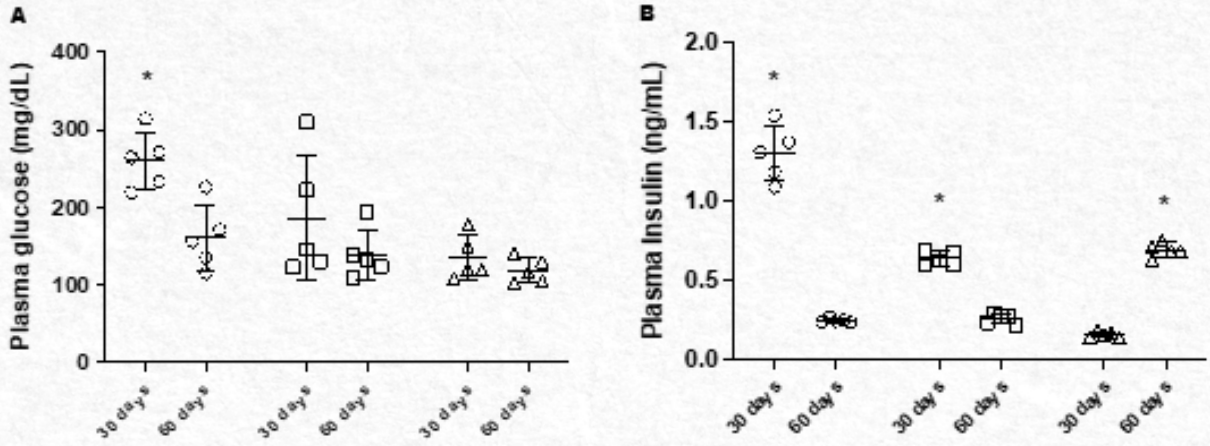
Table 1 - Body weight, ovary weight, and gonadosomatic index (GSI) for the control (C), polycystic ovary (PCO), and adipose-mesenchymal stem cell (MSC) groups, after 30 and 60 days of the *in vivo* experiments. The results are presented as mean ± SD (*n*=5). * Indicates statistically significant differences between means for different times in the same group. Different letters indicate statistically significant differences between groups for the same time (*P* < 0.05).

	BODY WEIGHT (g)		OVARY WEIGHT (g)		GSI (%)	
GROUPS	30 days	60 days	30 days	60 days	30 days	60 days
C	514.4 ± 14.1 ^{a*}	477.6 ± 14.7 ^{a}	0.065 ± 0.008 *	0.041 ± 0.01	0.049 ± 0.001 ^{a*}	0.008 ± 0.003
PCO	460.4 ± 30.7 ^{b}	446 ± 22.7 ^{a}	0.073 ± 0.03	0.041 ± 0.01	0.015 ± 0.007 ^{b}	0.009 ± 0.003
MSC	391.4 ± 26.8 ^{c}	404.8 ± 25.4 ^{b}	0.06 ± 0.01 *	0.038 ± 0.006	0.015 ± 0.002 ^{b*}	0.009 ± 0.001

Plasma glucose and insulin

The variations of plasma glucose and insulin are shown in Figure 1. *30 days*: There were decreases of blood glucose in the PCO and MSC groups, compared to the C group (Fig. 1A). In addition, there were reductions of plasma insulin in the PCO and MSC groups, compared to the C group (Fig. 1B). *60 days*: Plasma insulin was higher in the MSC group, compared to the PCO and C groups (Fig. 1B). The C group showed a reduction of blood glucose at 60 days of the experiment, compared to 30 days. The C and PCO groups showed decreased of plasma insulin at 60 days, compared to 30 days. Finally, the MSC group showed an increase of plasma insulin at 60 days, compared to 30 days.

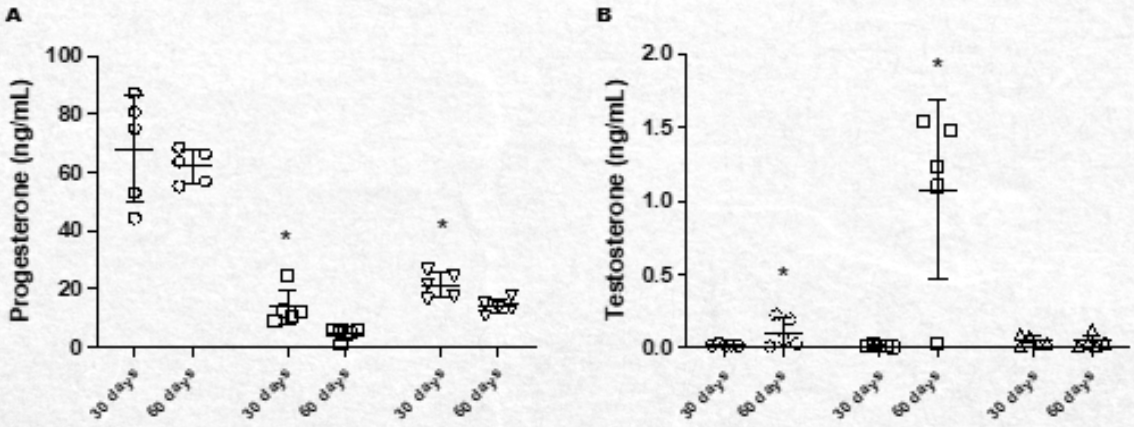
Figure 1 - Plasma glucose (A) and insulin (B) for the control (C - circles), polycystic ovary (PCO - squares), and adipose-mesenchymal stem cell (MSC - triangles) groups, after 30 and 60 days of the in vivo experiments. The results are presented as mean ± SD (n=5). * Indicates statistically significant differences (P < 0.05).



Plasma progesterone and testosterone

Plasma progesterone and testosterone are shown in Figure 2. *30 days*: There were reductions of plasma P4 in the MSC and PCO groups, compared to the C group (Fig. 2A). *60 days*: Plasma P4 showed a similar pattern to that at 30 days., with reductions in the MSC and PCO groups, compared to the C group (Fig. 2A). Higher plasma T was observed for the PCO group, compared to the C and MSC groups (Fig. 2B). The comparison between 30 and 60 days showed a reduction of plasma P4 in the PCO and MSC groups at 60 days, compared to 30 days. There was an increase of plasma T in the PCO and C groups at 60 days, compared to 30 days.

Figure 2 - Plasma progesterone (A) and testosterone (B) for the control (C - circles), polycystic ovary (PCO - squares), and adipose-mesenchymal stem cell (MSC - triangles) groups, after 30 and 60 days of the *in vivo* experiments. The results are presented as mean ± SD (*n*=5). * Indicates statistically significant differences (*P* < 0.05).



Plasma gonadotropins

The plasma variations of pituitary gonadotropins and the LH/FSH ratio are shown in Table 2. *30 days*: The LH concentration was higher for the C group, compared to the MSC group. The FSH concentration was higher for the C group, compared to the PCO and MSC groups. The LH/FSH ratio was higher for the MSC group, compared to the PCO and C groups. *60 days*: The LH concentration was higher for the MSC and PCO groups, compared to the C group. The FSH concentration was higher for the MSC group, compared to the PCO and C groups. The LH/FSH ratio was higher for the C group, compared to the PCO and the MSC groups. For the PCO and MSC groups, LH was higher at 60 days than at 30 days. For the C group, FSH was higher at 30 days than at 60 days, while for the MSC group, FSH was higher at 60 days than at 30 days. Finally, for the C and PCO groups, the LH/FSH ratio was higher at 60 days than at 30 days, while for the MSC group, it was higher at 30 days than at 60 days.

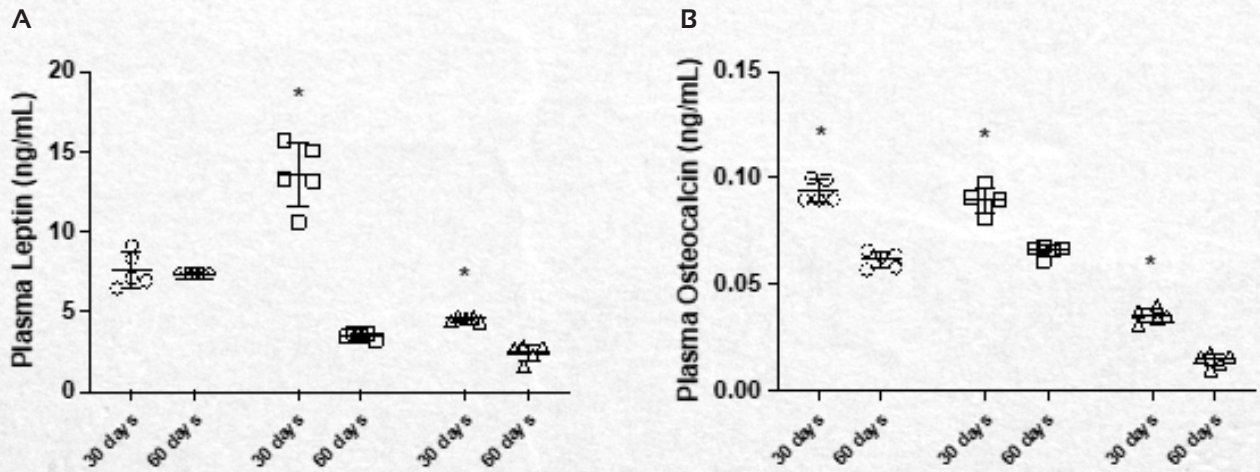
Table 2 - Luteinizing hormone (LH), follicle-stimulating hormone (FSH), and LH/FSH ratio values for the control (C), polycystic ovary (PCO), and adipose-mesenchymal stem cell (MSC) groups, after 30 and 60 days of the *in vivo* experiments. The results are presented as mean ± SD (*n*=5). *Indicates statistically significant differences between the times for the same group. Different letters indicate statistically significant differences between the groups at the same time ($P < 0.05$).

	LH (ng/mL)		FSH (ng/mL)		LH/FSH ratio	
GROUPS	**30 days**	**60 days**	**30 days**	**60 days**	**30 days**	**60 days**
C	0.334 ± 0.06 ^{a}	0.366 ± 0.05 ^{b}	4.078 ± 0.63 ^{a*}	0.293 ± 0.58 ^{c}	0.082 ± 0.018 ^{b}	2.33 ± 0.88 ^{a*}
PCO	0.231 ± 0.06 ^{a,b}	1.017 ± 0.28 ^{a*}	2.994 ± 0.92 ^{b}	2.35 ± 0.29 ^{b}	0.09 ± 0.052 ^{b}	0.43 ± 0.092 ^{b*}
MSC	0.179 ± 0.03 ^{b}	1.062 ± 0.13 ^{a*}	0.612 ± 0.44 ^{c}	4.716 ± 0.4 ^{a*}	0.422 ± 0.085 ^{a*}	0.228 ± 0.046 ^{c}

Plasma leptin and osteocalcin

Figure 3 presents the results for plasma leptin and osteocalcin. *30 days*: Plasma leptin was higher for the PCO group, compared to the C and MSC groups (Fig 3A). There was no significant difference in plasma osteocalcin between the C and PCO groups, but both values were higher than for the MSC group (Fig. 3B). *60 days*: There were reductions of plasma leptin in the PCO and MSC groups, compared to the C group (Fig. 3A). There was no significant difference in plasma osteocalcin between the C and PCO groups, but both values were higher than for the MSC group (Fig. 3B). In the PCO and MSC groups, leptin was lower at 60 days, compared to 30 days. In all the groups, osteocalcin was reduced at 60 days, compared to the 30 days.

Figure 3 - Plasma leptin (A) and osteocalcin (B) for the control (C - circles), polycystic ovary (PCO - squares), and adipose-mesenchymal stem cell (MSC - triangles) groups, after 30 and 60 days of the in vivo experiments. The results are presented as mean ± SD (n=5). *Indicates statistically significant differences ($P < 0.05$).



Ovarian morphology

Figure 4 shows images of the ovaries from all the groups for experimental times of both 30 and 60 day. At 30 days, the presence of follicles and corpus luteum could be seen for all the groups. Currently, there was no presence of ovarian cysts (Fig. 4A-F). After 60 days, there were ovarian cysts in the PCO group (Fig. 4I-J), while these cysts were absent in groups C (Fig. 4 G-H) and MSC (Fig. 4 K-L). In the latter groups, there were healthy follicles and corpus luteum. At 60 days, the numbers of healthy follicles were higher in the C and MSC groups, compared to the PCO group. On the other hand, the number of follicular cysts was higher in the PCO group, compared to groups C and MSC (Table 3).

Figure 4 - Histological photomicrographs of sections of the rat ovaries after 30 days (A-B: control group (C); C-D: polycystic ovary group (PCO); E-F: adipose-mesenchymal stem cell group (MSC)) and 60 days (G-H: control group (C); I-J: polycystic ovary group (PCO); K-L: adipose-mesenchymal stem cell group (MSC)) of the *in vivo* experiments. Red arrows indicate follicles. CL: corpus luteum; C: follicular cysts. Sections stained using hematoxylin-eosin; 100 x magnification; bars = 20 µm.

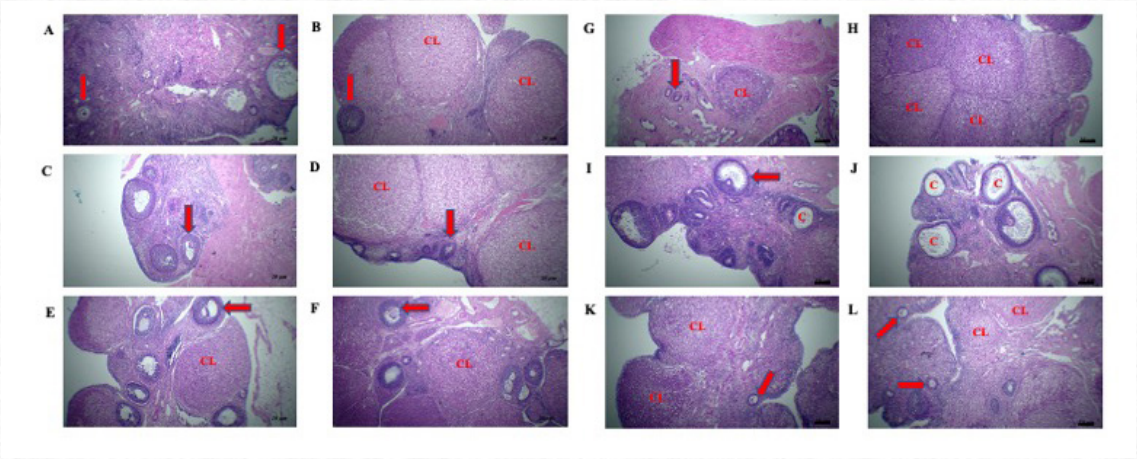


Table 3 - Number of follicles, corpus luteum, and follicular cysts from control (C 30 and C 60 days), polycystic ovary (PCO 30 and PCO 60 days), and adipose-mesenchymal stem cell (MSC 30 days, and MSC 60 days) rats. Data are shown as the mean ± SEM (*n*=5). *Indicates statistically significant differences between means for different times in the same group. Different letters indicate statistically significant differences between groups for the same time ($P < 0.05$).

	FOLLICLE		CORPUS LUTEUM		FOLLICULAR CYST	
GROUPS	**30 days**	**60 days**	**30 days**	**60 days**	**30 days**	**60 days**
C	10 ± 1 ^{a}	9 ± 0.7 ^{a}	6.1 ± 0.8 ^{a}	5.8 ± 0.6 ^{a}	0.28 ± 0.3 ^{b}	0.28 ± 0.3 ^{c}
PCO	3.1 ± 1.2 ^{b*}	2.3 ± 0.6 ^{b}	3.9 ± 0.7 ^{b*}	2.1 ± 0.8 ^{b}	4 ± 0.9 ^{a}	7 ± 1.1 ^{a*}
MSC	6.4 ± 0.8 ^{b}	8 ± 0.9 ^{a}	6 ± 0.6 ^{a}	4.8 ± 0.9 ^{a}	0.3 ± 0.2 ^{b}	0.4 ± 0.1 ^{b}

Discussion

Rodents and sheep induced to PCOS like women with PCOS have increased ovarian volume and multifollicular ovarian morphology. The body weight of animals induced to PCOS can increase or decrease, depending on the animal model and the PCOS induction method[12, 22, 27-29]. In the present study, the PCO and MSC groups had lower body mass and higher GSI, compared to the control group. Body weight analyses were performed considering weight gain between the two experimental times, with the greatest weight gain 60 days after PCOS induction being observed for the PCO group. In addition, the plasma levels of insulin, leptin, osteocalcin, and testosterone may have caused important metabolic changes that resulted in body weight and GSI modifications. Women with high T values exhibit increased body mass index, central adiposity, and insulin resistance[30]. Abnormal metabolic conditions could result from a sustained positive energy balance, when the energy intake is higher than the energy expenditure, and exacerbated body weight gain is associated with irregular fat accumulation, which leads to obesity[30-31] in women with PCOS[30, 32-33], and in animals induced to PCOS [13]. Overweight/obese women with PCOS are at increased risk of developing lipotoxicity due the excess fat free acid into non-adipose cells, including the muscle, liver,

pancreas, and ovaries[30].

Women with PCOS seem to have a level of peripheral insulin resistance that is much like that of women with type 2 diabetes, which is characterized by a 35-40% decrease in insulin-mediated glucose uptake, and insulin resistance might contribute to hyperandrogenism and gonadotropin abnormalities[30, 33]. Insulin resistance and glucose intolerance have also been described in animal models induced by PCOS[2, 13, 20]. The present results showed that up to 30 days after PCOS induction, plasma glucose and insulin levels were normal, with lower concentrations for the MSC group. However, 60 days after PCOS induction, all the groups showed high blood glucose, with only the MSC group showing functional relationships between insulin and glucose levels. MSCs transplantation has been found to reduce glucose levels in diabetic rats[34]. In human, it has been found to attenuate ß cell dysfunction by reversing ß cell differentiation in an IL-1Ra-mediated manner, in response to the elevated expression of proinflammatory cytokines[35]. Soluble factors, extracellular vesicles, and miRNAs secreted by mesenchymal stroma cells can reach many organs, modulating their functions[8]. Therefore, the results suggested that the injection of AdMSCs into the ovaries could have restored follicular steroidogenic activity, especially T synthesis, which modulated plasma glucose and insulin levels. In addition, chemical signals from the AdMSCs injected into the ovaries could have reached the pancreas, modulating insulin secretion.

Animals induced to PCOS develop anovulatory cycles[2, 36-37]. However, the causes for such cyclical changes are variable and depend on the treatment used[38]. In the present study, the PCO group showed changes in ovarian cycles, with the cyclicity returning to normal about 45 days after AdMSCs injection. The observation of ovarian changes, such as the presence of cysts in the ovaries of the PCO group and changes in plasma levels of P4, T, and pituitary gonadotropins, suggested that such cycle changes were probably mainly due to ovarian steroidogenic changes that altered the secretion of pituitary gonadotropins.

The plasma P4 concentrations were lower for the PCO group, compared to the control group, indicative of lower ovarian synthesis due to reduced follicular activity, fewer ovulations, and a reduced quantity of corpus luteum. The plasma P4 concentration in the MSC group was higher than in the PCO group, but lower than in the control group. It is interesting to note that in the MSC group, the ovary morphology did not show cysts, only follicles and corpus luteum. The results suggested that the higher P4 levels in the MSC group were related to the return of ovarian activities such as folliculogenesis, ovulation, and corpus luteum activity. MSC can both reducing nitric oxide synthase reducing fibrose and can also exert their healing effects by donating mitochondria to target cells, as an important mechanism in apoptosis prevention and metabolic damage[7]. In this way, since follicular steroidogenesis occurs in the mitochondrial matrix, of theca and granulosa cells, it appeared that paracrine effects on ovarian steroidogenesis could be driven by AdMSCs.

Rats and mice induced to PCOS present hyperandrogenism, with endocrine, reproductive, and metabolic characteristics like those found in women with PCOS, including adipocyte hypertrophy, insulin intolerance, increase in the size of the ovaries, and anovulatory cycles[28, 39]. In the present work, the MSC group presented plasma T levels like those of the control group. The plasma T levels, ovarian morphology with the presence of cysts, and absence of cyclicity indicated that this animal model was suitable for the intended purpose, especially at 60 days after induction of PCOS, providing a model for reproductive studies related to this syndrome. It is interesting to note that after PCOS induction and AdMSCs injection, plasma T levels did not change in the MSC group, compared to the control group. In addition, the ovary morphology of the MCS group showed a reduction in the number of follicular cysts, together with increases of the number of follicles and corpus luteum, compared to the PCO group, suggesting that the AdMSCs injection strategy would be appropriate for use in studies employing animal models to investigate ovarian dynamics. This potential is based on ovarian actions that are modulated by the factors secreted by the MCSs, including transforming growth factor (TGF), insulin growth factor (IGF-1), hepatocyte growth factor (HGF), interleukin (IL), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF)[7-8,40], and as previously mentioned, MSC can donate mitochondria to target cell, and they can reduce ovary fibrosis and apoptosis activities. However, future studies will be needed for molecular investigations.

Hypergonadism due to LH excess, observed in women with PCOS, is also a characteristic in monkeys[30, 37, 41], sheep[42], and rats[43] induced to PCOS. Such an endocrine change may reduce the sensitivity of the neuroendocrine system to negative feedback from ovarian steroids, especially lower sensitivity to testosterone[2]. Follicular granulosa cells from women with PCOS demonstrated abnormal responses in E2 secretion to gonadotropin stimuli[44]. It has been reported that MSCs improve ovarian function and assist ovarian functional recovery, but it remains unclear whether this effect is achieved

by the differentiation of MSCs into oocytes or by supporting steroidogenesis of the follicular and stromal cells[5, 36, 40, 45]. The results showed that the control group present normal feedback between plasma T levels and pituitary gonadotropins. However, the PCO group showed an increase of plasma LH at 60 days, despite high plasma T concentrations, suggesting lower sensitivity of the pituitary to T. This corroborated the vicious cycle of hyperandrogenemia, ovarian cysts, follicular hyperthecosis, increased LH, greater stimulus for T synthesis by theca cells, increased plasma T, and more cysts. On the other hand, there was an apparent restoration of feedback mechanisms between plasma T and LH in the MSC group, again suggesting paracrine modulation of the ovarian steroidogenesis microenvironment by AdMSCs.

Circulating leptin levels have been positively correlated with body fat, independent of PCOS, according to some studies[46-47], but not others[48-49]. Leptin directly affects steroidogenesis of thecal cells, and normally fluctuating concentrations of leptin in blood may be important in communicating the metabolic status of the animal to the reproductive system[50]. A low dose of leptin was found to increase P4 accumulation by luteinized porcine granulosa cells *in vitro*, whereas a high dose was inhibitory[51]. Leptin deficiency in female mice was associated with impaired folliculogenesis and increased follicular atresia[52]. The results showed that the highest leptin level occurred 30 days after PCOS induction, which could have inhibited ovarian steroidogenesis, since plasma levels of P4 and T were very low. In addition, folliculogenesis could also have been affected by leptin, because the ovarian morphology analyses showed the highest number of ovarian cysts in the PCO group. However, the MSC group showed a reduction in leptin levels, compared to the PCO group. As mentioned earlier, the reduction in leptin levels, the increase in P4 levels, and reduction in the number of ovarian cysts suggested that the injection of AdMSCs into the ovaries could have modulated ovarian activities such as steroidogenesis and folliculogenesis.

Osteocalcin, a polypeptide secreted by osteoblasts, is found at high concentrations in the bone extracellular matrix, and it also possesses several characteristics of a hormone[53]. Studies indicate that osteocalcin can regulate fat mass, insulin secretion, male fertility, and energy expenditure, according to multiple mechanisms, although fasting indices are not always well correlate with insulin resistance[52-53]. The results of the present study revealed metabolic changes that could be associated with OCN, especially in the MSC group. In this group, the lowest OCN concentration could be related to reductions of body weight, ovary weight, and GSI. In other work, osteocalcin decreased fat mass and serum triglycerides levels, with the effect of osteocalcin on fat mass possibly being indirect and secondary to another physiological action[52]. This was corroborated by the present results, with such changes probably being a consequence of the integrated effects of OCN, leptin, and insulin, especially for the 60-day time. However, future studies are needed to clarify these relationships. Osteocalcin can control glucose metabolism by directly affecting pancreatic islet biology, as well as insulin synthesis and secretion. Different to the present results, Hinoi et al.[54], found that insulin secretion was increased by OCN, due to its ability to increase cytosol calcium levels. In the animal model used in the present work, the injection of AdMSCs induced decreases of plasma OCN and leptin, increase of insulin, and decreases of body weight, ovary weight, and GSI, compared to the control and PCO groups. Soluble factors secreted by MSCs include TGF, IGF-1, HGF, IL, and PDGF [8], which can modulate hepatic, adipocyte, pancreas, skeletal muscle, and bone metabolism, affecting insulin synthesis and secretion, glucose metabolism, and body weight. In addition, as PCOS patients and rodent models are characterized by hyperandrogenism, it may not be surprising that OCN levels may change in PCOS[53], and results present here showed the relationship among OCN and testosterone were modulated by AdMSCs, compared to the PCO group.

In conclusion, the results revealed endocrine-metabolic-reproductive functions modulated by AdMSCs in the ovaries. However, despite the results showing changes among the P4, T, leptin, insulin, and OCN levels, and the LH:FSH ratio, it was not possible to determine whether the AdMSCs modulated ovarian activities directly or indirectly. The action of the AdMSCs injected into the ovaries could be direct, with modulation of the ovarian functions involving the hypothalamic-pituitary axis, or indirect, by the effects of leptin, osteocalcin, and insulin on the ovarian dynamics. The findings described here are interesting, because they present data from female rats, in an animal model integrating PCOS with AdMSCs, considering their effects and the relationships among ovaries, bones, and adipocytes. The results suggested the existence of endocrine-metabolic-reproductive microenvironment relationships modulated by AdMSCs, which could assist in guiding further investigations to clarify pathophysiological mechanisms that have not yet been fully elucidated.

Declarations

Conflict of Interest

The authors declare that there are no competing interests. The authors have read the journal's policy on disclosure of potential conflicts of interest and agreed with the journal's authorship statement.

Funding

This work was supported by the São Paulo State Research Foundation (FAPESP), SP, Brazil (Grant #2016/02811-4).

Ethical Approval

All the experimental procedures were approved by the Committee of Ethics in Animal Use (CEUA/UNIARA, n° 030/2016), following the norms of the National Council for Control of Animal Experimentation (CONCEA/MCTI, Brazil).

Author's contributions

Luís Henrique Montrezor: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. Eduardo Donato Alves: Methodology, Investigation, Writing - review & editing. Luíz Guilherme Dércore Benevenuto: Methodology, Investigation. Janete Aparecida Anselmo-Franci: Methodology, Writing - review & editing. Edilson Ervolino: Methodology. Bruna Pereira de Morais: Methodology, Writing – review & editing. Michele Andrade de Barros: Methodology. Jorge Alberto Achcar: Formal analysis, Writing - review & editing.

Acknowledgments

We thank Aline C. Bertato and Ruiter O. G. Carolino for technical support.

References

[1]. Aflatounian A, Eduwards MC, Paris VR, Bertoldo MJ, Desai R, Glichrist RB. Ledger WL, Handelsman DJ, Walters KA. Androgen signaling pathways driving reproductive and metabolic phenotypes in a PCOS mouse model. J Endocrinol. 2020;245(3):381-395.

[2].Padmanabhan V, Veiga-Lopez A. Sheep models of polycystic ovary syndrome phenotype. Mol Cell Endocrinol. 2013;373:8-20.

[3].Doretto L, Mari FC, Chaves AC. Polycystic ovary syndrome and psychotic disorders. Front Psychiatry. 2020;11:543.

[4]Spallanzani RG. Visceral adipose tissue mesenchymal stroma cells in the intersection of immunology and metabolism. Am J Physiol Endocrinol Metab. 2021;320:E512-E519.

[5]Esfandyari S, Chugh RM, Park H, Hobeika E, Ulin M, Al-Hendy A. Mesenchymal stem cells as a Bio-organ for treatment of female infertility. Cells. 2020;9:2253.

[6]Song N, Scholtemeijer M, Shah K. Mesenchymal stem cell immunomodulation: mechanisms and therapeutic potential. Trends Pharmacol Sci. 2020;41(9):653-664.

[7]Gomez-Salazar M, Gonzales-Galofre ZN, Casamitijana J, Crisan M, James AW, Péault B. 2020 Five decades later, are mesenchymal stem cells still relevant? Front Bioeng Biotechnol. 2003;8:148.

[8]Maacha S, Sidahmed H, Jacob S, Gentilcore G, Calzone R, Grivel JC, Cugno C. Paracrine mechanisms of mesenchymal stromal cells in angiogenesis. Stem Cell Inter. 2020;9:4356359.

[9]Yang K, Oark HJ, Han S, Lee J, Ko E, Kim J, Lee JS, Yu JH, Song KY, Cheong E, Cho SR, Chung S, Cho SW. Recapitulation of in vivo-like paracrine signals of human mesenchymal stem cells for functional neuronal differentiation of human neural stem cells in a 3D microfluidic system. Biomaterials. 2015;63:177-188.

[10]Segers VF, Lee RT. Stem-cell therapy for cardiac disease. Nature. 2008;451:937-942.

[11]Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. Plos One. 2008;3:e1886.

[12]Alves ED, Bonfá ALO, Pigatto GR, Anselmo-Franci JA, Achcar JA, Parizotto NA, Montrezor LH. Photobiomodulation can improve ovarian activity in polycystic ovary syndrome-induced rats. J Photochem Photobiol B Biol. 2019;194:6-13.

[13]Ryu Y, Kim SW, Kim YY, Ku SY. Animal Models for Human Polycystic Ovary Syndrome (PCOS) Focused on the Use of Indirect Hormonal Perturbations: A Review of the Literature. Int J Mol Sci. 2019;20(11):1-20.

[14]Kuang HY, Li Y, Zhang HP, Legro RS, Stener-Victorin E. Data do not support effectiveness of acupuncture for improving live birth rate in women with polycystic ovary syndrome. Chinese J Integ Med. 2018;24(5):399-400.

[15]Caldwell AS, Caldwell ASL, Edwards MC, Desai R, Jimenez H, Gilchrist RB, Handelsman DJ, Walters KA. Neuroendocrine androgen action is a key extraovarian mediator in the development of polycystic ovary syndrome. Proc Natl Acad Sci USA. 2017;114:3334–3343.

[16]Krishnan A, Muthusami S. Hormonal alterations in PCOS and its influence on bone metabolism. J Endocrinol. 2017;232(2):99-113.

[17]Marcondes RR, Maliqueo M, Fornes R, Benrick A, Hu M, Ivarsson N, Carlström M, Cushman SW, Stenkula KG, Maciel GAR, Stener-Victorin E. Exercise differentially affects metabolic functions and white adipose tissue in female letrozole- and dihydrotestosterone-induced mouse models of polycystic ovary syndrome. Mol Cel Endocrinol. 2017;448:66-76.

[18]Walters KA, Allan CM, Handelsman DJ. Rodent models for human polycystic ovary syndrome. Biol Reprod. 2012;86(5):1-12.

[19]Shi D, Vine DF. Animal model of polycystic ovary syndrome: a focused review of rodent models in relationship to clinical phenotypes and cardiometabolic risk. Fert Steril. 2012;98(1):185-193.

[20]Walters KA, Rodriguez PV, Aflatounain A, Handelsman DJ. Androgens and ovarian function: translation from basic discovery research of clinical impact. J Endocrinol. 2019;242(2):R23-R50.

[21]Wickenheisser JK, Nelson-DeGrave VL, McAllister JM. Dysregulation of cytochrome P450 17alpha-hydroxylase messenger ribonucleic acid stability in theca cells isolated from women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2005;90(3):1720-1727.

[22]Paixão L, Ramos RB, Lavarda A, Morsh DM, Spritzer PM. Animal model of hyperandrogenism and ovarian morphology changes as features of polycystic ovary syndrome: a systematic review. Reprod Biol Endocrinol. 2017;15:12.

[23]Stener-Victorin E, Padmanabhan V, Walters KA, Campbell RE, Bernick A, Giacobini P, Dumesic DA, Abbott DH. Animal models to understand the etiology and pathophysiology of polycystic ovary syndrome. Endocr Rev. 2020;41:538-576.

[24]Brawer JR, Munoz M, Farookhi R. Development of the polycystic ovarian condition (PCO) in the estradiol valerate-treated rat. Biol Reprod. 1986;35(3):647-655.

[25]Montrezor LH, Carvalho D, Dias MB, Anselmo-Franci JA, Bícego KC, Gargaglioni LH. Hypoxic and hypercapnic ventilatory responses in rats with polycystic ovaries. Resp Physiol Neurobiol. 2015;217:17–24.

[26]Li C, Li G, Liu M, Zhou T, Zhou H. Paracrine effect of inflammatory cytokine-activated bone marrow mesenchymal stem cell and its role in osteoblast function. J Biosci Bioeng. 2016;121(2):213-219.

[27]Ma Y, Andrisse S, Chen Y, Childress S, Xue P, Wang Z, Jones D, Ko CM, Wu S. Androgen receptor in the ovary theca cells plays a critical role in androgen-induced reproductive dysfunction. Endocrinol. 2017;158(1):98–108.

[28]Caldwell AS, Middleton LJ, Jimenez H, Desai R, Mcmahon AC, Allan CM, Handelsman CJ, Walters KA. Characterization of reproductive, metabolic, and endocrine features of polycystic ovary syndrome in female hyperandrogenic mouse models. Endocrinol. 2014;155:3146-3159.

[29]Van Houten EI, Kramer P, Mcluskey A, Karels B, Themmen AP, Visser JA. Reproductive and metabolic phenotype of a mouse model of PCOS. Endocrinol. 2012;153(6):2861–2869.

[30]Dumesic DA, Padmanabhan V, Chazenbalk GD, Abbott DH. Polycystic ovary syndrome as a plausible evolutionary outcome of metabolic adaptation. Reprod Biol Endocrinol. 2022;20:12.

[31]Oliveira M, Mathias LS, Rodrigues BM, Mariani BG, Graceli JB, De Sibio MT, Olimpio RMC, Moretto FCF, Deprá IC, Nogueira CR. The roles of triiodothyronine and irisin in improving the lipid profile and directing the browning of human adipose subcutaneous cells. Mol Cell Endocrinol. 2020;506:110744.

[32]Amirkhanloo F, Esmaeilzadeh S, Mirabi P, Abedini A, Amiri M, Saghebi R, Golsorkhtabaramiri M. Comparison of Foeniculum Vulgare versus metformin on insulin resistance and anthropometric indices of women with polycystic ovary, an open-label controlled trial study. Obesity Medicine. 2022;31:100401.

[33]Norman RJ, Dewailly D, Legro RS, Hickey TE. Polycystic ovary syndrome. The Lancet. 2007;370:685-597.

[34]Domouky AM, Hegab AS, Al-Shahat A, Raafat N. Mesenchymal stem cells and differentiated insulin producing cell are new horizons for pancreatic regeneration in type I diabetes mellitus. Int J Biochem Cell Biol. 2017;87:77-85.

[35]Wang L, Liu T, Liang R, Wang G, Liu Y, Zou J, Liu N, Zhang B, Liu Y, Ding X, Cai S, Wang Z, Xu X, Ricordi C, Wang S, Shen Z. Mesenchymal stem cells ameliorate ß cell dysfunction of human type 2 diabetic islet by reversing ß cell dedifferentiation. EBioMedicine. 2020;51:102615.

[36]Xie Q, Xiong XL, Xiao N, He K, Chen M, Peng J, Su X, Mei H, Dai Y, Wei D, Lin, G, Cheng L. Mesenchymal stem cells alleviate DHEA-induced polycystic ovary syndrome (PCOS) by inhibiting inflammation in mice. Stem Cells Inter. 2019;9782373.

[37]Abbott DH, Barnett DK, Bruns CM, Dumesic SA. Androgen excess fetal programming of female reproduction: a developmental aetiology for polycystic ovary syndrome? Hum Reprod Update. 2005;11:357-374.

[38]Sullivan SD, Moenter SM. Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: implications for a common fertility disorder. Proc Natl Acad Sci USA. 2004;101:7129-7134.

[39]Kawa IA, Masood A, Ganie MA, Fatima Q, Jeelani H, Manzoor S, Rizvi SM, Muzamil M, Rashid F. Bisphenol A (BPA) acts as an endocrine disruptor in women with polycystic ovary syndrome: hormonal and metabolic evaluation. Obesity Medicine. 2019;14:100090.

[40]Malard PF, Peixer MAS, Grazia JG, Brunel HSS, Feres LF, Villarroel CL, Siqueira LGB, Dode MAN, Pogue R, Viana JHM, Carvalho JL. Intraovarian injection of mesenchymal stem cells improve oocyte yield and in vitro embryo production in a bovine model of fertility

loss. Scientific Reports. 2020;10:8018.

[41]Dumesic DA, Abbott DH, Eisner JR, Goy RW. Prenatal exposure of female rhesus monkey to testosterone propionate increases serum luteinizing hormone levels in adulthood. Fertil Steril. 1997;67:155-163.

[42]Sharma TP, Herkimer C, West C, Ye W, Birch R, Robinson JE, Foster DL, Padmanabhan V. Fetal programming: prenatal androgen disrupts positive feedback actions of estradiol but does not affect timing of puberty in female sheep. Biol Reprod. 2020;66:924-933.

[43]Wu XY, Li ZL, Wu CY, Liu Y-M, Lin H, Wang S-H, Xiao W-F. Endocrine traits of polycystic ovary syndrome in prenatally androgenized female Sprague-Dawley rats. Endocr J. 2010;57:201-209.

[44]Harrison TNH, Chang RJ. Ovarian response to follicle-stimulating hormone in women with polycystic ovary syndrome is diminished compared to ovulatory controls. Clin Endocrinol. 2020;97:310-318.

[45]Zhao YX, Chen SR, Su PP, Huang FH, Shi YC, Shi QY, Lin S. Using mesenchymal stem cells to treat female infertility: an update on female reproductive diseases. Stem Cells Int. 2019;9071720.

[46]Tan BK, Chen J, Hu J, Amar O, Mattu HS, Adya R, Patel VH, Ramanjaneya M, Lehnert H, Randeva HS. Metformin increases the novel adipokine caronectin/CTRP3 in women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2013;98:E1891-900.

[47]Plati E, Kouskouni E, Malamitsi-Puchner A, Boutsikou M, Kaparos G, Baka S. Visfatin and leptina levels in women with polycystic ovaries undergoing ovarian stimulation. Fertil Steril. 2010;94,:451-1456.

[48]Barber TM, Franks S. Adipocyte biology in polycystic ovary syndrome. Mol Cell Endocrinol. 2013;373:68-76.

[49]Svendsen PF, Christiansen M, Hedley PL, Nilas L, Pedersen SB, Madsbad S. Adipose expression of adipocytokines in women with polycystic ovary syndrome. Fertil Steril. 2012;98:235-41.

[50]Swain JE, Dunn RL, McConnell D, Gonzales-Martinez J, Smith GD. Direct effects of leptin on mouse reproductive function: regulation of follicular, oocyte, and embryo development. Biol Reprod. 2004;71(5):1446-1452.

[51]Ruiz-Cortés ZT, Martel-Kennes Y, Gévry NY, Downey BR, Palin M-F, Murphy BD. Biphasic effects of leptin in porcine granulosa cells. Biol Reprod. 2003;68(3):789-796.

[52]Ferron M, Lacombe J. Regulation of energy metabolism by the skeleton: osteocalcin and beyond. Arch Biochem Bioph. 2014;561:137-146.

[53]Shan C, Yue J, Liu W. Broadening the role of osteocalcin in the hypothalamic-pituitary-gonadal axis. J Edocrinol. 2021;249(2):R43-R51.

[54]Hinoi E, Gao N, Jung DY, Yadav V, Yoshizawa T, Myers Jr MG, Chua Jr SC, Kim JK, Kaestner KH, Karsenty G. The sympathetic tone mediates leptin's inhibition of insulin secretion by modulating osteocalcin bioactivity. J Cell Biol. 2008;183:1235-1242.

ORIGINAL ARTICLE

IJAMB
International Journal of Advances in Medical Biotechnology

Induction of mineralized matrix production by recombinant human BMP-2 Immobilized in TEMPO-Oxidized Cellulose Hydrogel: a novel target for tissue repair

B. Domingos Neto[1]*; V.M. Hebling[1]; G.G.C. Gastaldi[1]; H.S.S. Araújo[2]; E. Trovatti[1]; M.R.C. Iemma[1];

*Corresponding author: E-mail address: bdneto@uniara.edu.br

Received: 10 January 2024; 05 March 2024.

Abstract: Bone morphogenetic proteins (BMPs) are potent promoters of osteogenesis, especially BMP-2, which has been highlighted for acting as a growth and differentiation factor that promotes new bone formation. There are several biomaterials that can be used to release bioactive substances, such as natural polymers. Cellulose has stood out for the possibility of its chemical modification using the reagent 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) to obtain a cellulose derivative (TEMPO oxidized cellulose nanofibers - ToCNF), which is shown to be a promising material for biological application. The objective of this work was to evaluate TEMPO cellulose immobilized with rhBMP-2 against the activity of inducing bone cell proliferation and differentiation in vitro, evaluating the ability to form bone matrix in pre-osteoblastic cell lineage of rats - MC3T3. Cell viability assays using resazurin were performed and for detection of mineralized matrix, Alizarin Red solution was used. The results reveal the good capacity of TEMPO cellulose functionalized with rhBM-2 in inducing the synthesis of mineralized bone matrix.

Keywords: Bone Morphogenetic Protein 2. Bone regeneration. TEMPO cellulose. Tissue engineering. Biocompatible materials.

Introduction

Bone morphogenetic proteins are a group of proteins belonging to the Transforming Growth Factor Beta (TGF-β) superfamily. BMPs are the most studied and most promising group of growth factors because they have a potent ability to promote bone and cartilage formation. The ability of BMPs to induce bone formation is called osteoinduction. Osteoinduction is due to the competence of this group of proteins to induce the undifferentiated mesenchymal cell, present in the receptor area, to transform into a bone-forming cell[1].

After the discovery of BMPs, several studies emerged on the osteoinduction capacity of these proteins, as they have become of great interest to several areas of medicine, especially regenerative medicine [2]. Some BMPs, such as BMP-2 (bone morphogenetic protein 2) have been highlighted for playing an important role in osteogenesis, and can be used as a powerful osteoinductive component in several tissue engineering products in the areas of orthopedics and dentistry[1,3].

BMP-2 has stood out for acting as a growth and differentiation factor in the body, which promotes new bone formation, acting extensively throughout the osteogenesis phase, both in vitro and *in vivo*[4,5]. Years of studies have been carried out on the application of different types of growth factors in bone regeneration. It is proven that, among all members of the BMP subgroup, BMP-2 is the most potent growth factor with positive effects on undifferentiated mesenchymal cell differentiation and osteogenesis[6]

The results of several animal experiments suggest a bright future for BMPs for bone reconstruction, bringing therapeutic benefits[7]. However, like other factors, BMPs require a transport system that releases them slowly and gradually, allowing an adequate condition for cell migration, proliferation and differentiation[6].

The use of BMPs in bone repair and regeneration is ushering in a new era for orthopedic and craniofacial reconstruction[8], as animal and human studies have already proven the ability of BMPs to promote bone formation[9]. Therefore, since their discovery, BMPs promise a promising future in the field of tissue engineering[10]. However, one of the biggest problems for researchers is to develop ways to deliver these osteoinductive factors and therefore achieve clinical success in humans[8,9].

Tissue engineering and regenerative medicine are constantly growing and have positive contributions to

[1]University of Araraquara - UNIARA, Araraquara, São Paulo, Brasil.

https://doi.org/10.52466/ijamb.v6i1.124

the advancement of various pathologies, especially on the world stage due to the low number of organs and tissues available for transplantation[11]. Tissue engineering is a multidisciplinary field that involves several areas of knowledge, mainly the medical, biological and engineering areas. It aims to improve new techniques and develop new biomaterials that restore, maintain or improve the function of different organs and tissues[12,13].

The choice of a biomaterial depends on the analysis of several conditions, such as biocompatibility, toxicity, biodegradability, as well as the material's degradation rate and biofunctionality. A biomaterial must also be able to retain the growth factor at the repair site for a period compatible with the time of tissue re-formation[9]. There are several biomaterials available, highlighting the natural polymers that can be of protein origin, such as collagen, and polysaccharide, such as cellulose, among others.

Hydrogels are three-dimensional polymeric networks formed by hydrophilic structure capable of absorbing and releasing water or biological fluid in response to environmental conditions[14]. The retention capacity of hydrogels is associated with the hydrophilic groups present in their composition (-OH-, -CONH-, -COO- and -SO2H-)[15] and other factors such as pH and temperature. While the expansion capacity is linked to the osmotic phenomenon[14]. There are several hydrogels with application in regenerative medicine, among them are cellulose-based hydrogels.

Cellulose is one of the most abundant polymers in nature, it is a polymer formed from the bonding of thousands of glucose units. This polymer can also be classified as a polysaccharide and is present in most plants, making up their cell walls. In the broad field of tissue engineering and biomedical applications, cellulose is recognized for having distinctive characteristics[16]. Although cellulose *in natura* does not present a degradation rate in time compatible with the repair time of most tissues, being a limitation for more complex biomedical applications[2]. However, cellulose has, among other characteristics, water retention capacity, hydrophilicity and biocompatibility[16]. In addition, it displays functional groups available for chemical modification. The oxidation of the glucose carbon-6 hydroxyl group with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) radical,generates the cellulose derivative (ToCNF or also called TEMPO cellulose) rich in carboxylic groups, highly hydrophilic, with the physical appearance of a viscous transparent hydrogel, formed by individualized nanofibrils[17].

Recent advances in bone regeneration have explored innovative biomaterials based on TEMPO-oxidized cellulose nanofibrils, as they have been shown to be resorbable in humans at physiological pH (~7.4). Approximately 90% by weight of the oxidized cellulose is solubilized within 21 days and converted into the sodium salt of polyglucuronic acid, which is easily eliminated by the body[18].

The study by Abouzeid et al.[19] evaluated the in situ mineralization of calcium phosphates (dicalcium phosphate dihydrate and hydroxyapatite) in hybrid hydrogels combining alginate, PVA, and TEMPO-oxidized cellulose nanofibrils (T-CNFs) under different pH levels, aiming at applications in bone regeneration and wound healing. The results showed that the hydrogels containing hydroxyapatite are more stable and promising for supporting the healing of both hard and soft tissues, demonstrating the potential of these materials in tissue engineering.

In line with this approach, the study by Gorgieva et al.[20] investigated gelatin scaffolds reinforced with TEMPO-oxidized cellulose nanofibrils modified with phosphonate groups to promote calcium deposition by mesenchymal stem cells. The modified scaffolds showed greater hydroxyapatite deposition and supported cell growth compared to unmodified scaffolds, demonstrating superior osteoinductive potential and standing out as a promising alternative for bone regeneration.

Salama et al.[21] then, assessed via calcium phosphates (CaP also developed a new biomaterial for bone regeneration composed of TEMPO-oxidized cellulose nanofibers (T-CNF) grafted with soy protein hydrolysate (SPH) and mineralized with calcium phosphate (CaP). This material demonstrated high biocompatibility, promoting the proliferation and differentiation of human mesenchymal stem cells and forming a mineralized matrix similar to bone apatite. These results indicate that the biomaterial is promising for bone tissue repair and regeneration, mimicking the composition and biological properties of natural bone.

Complementarily, the study by Yao et al.[22] developed biomimetic materials for bone regeneration using cellulose nanofibrils (CNFs) modified by TEMPO oxidation and chitin (ChNFs), which were enzymatically mineralized with alkaline phosphatase (ALP) to form calcium phosphate (CaP) deposits. The resulting materials demonstrated high stiffness and strength while maintaining flexibility and adjustable mechanical properties, showing great potential for applications in bone replacement and tissue regeneration with structural gradients.

Ingole et al.[23] developed and characterized hydroxyapatite (HA) nanocomposites reinforced with TEMPO-oxidized cellulose nanofibrils (TCNF) and cellulose nanocrystals (CNC) for bone regeneration applications. The composites containing TCNF exhibited superior mechanical properties, such as

compressive strength, elastic modulus, and fracture toughness, comparable to cortical bone. All HA composites, regardless of the type of nanocellulose, were biocompatible and promoted the viability of human osteoblasts, highlighting their potential for bone regeneration in load-bearing applications.

In the area of 3D printing, Im et al.[24] developed an osteogenic bioink composed of alginate, TEMPO-oxidized cellulose nanofibrils (TOCNFs), and polydopamine nanoparticles (PDANPs) for the creation of scaffolds intended for bone tissue engineering. The incorporation of TOCNFs and PDANPs increased printability, improved the mechanical properties and bioactivity of the bioink, and promoted the proliferation and osteogenic differentiation of osteoblastic cells.

Given the current scenario concerning studies on cellulose nanofibrils for bone repair, this study presents a novel approach by combining TEMPO-oxidized cellulose (ToCNF) with recombinant human bone morphogenetic protein BMP-2 (rhBMP-2).Therefore the objective of this work was to evaluate the ability of TEMPO cellulose hydrogel immobilized with human recombinant bone morphogenetic protein (rhBMP-2) to induce bone cell proliferation and differentiation *in vitro,* as well as the ability to form bone matrix in mouse pre-osteoblastic cell line - MC3T3.

Materials and Methods

Preparation and characterization of TEMPO-oxidized cellulose nanofibrils (ToCNF)

ToCNF was prepared in two steps, firstly, the bleaching, followed by chemical modification (oxidation). For bleaching, 10 g of milled sugarcane bagasse (~1 mm length) was extracted in a Soxhlet system using toluene/ethanol 2:1 (v/v) for 8 h and then dried overnight at room temperature. The dried fibers were dispersed in 1.3 % sodium chlorite solution (400 mL), the pH was adjusted to approximately 4 (± 0.5) with diluted acetic acid, the mixture was heated at 75 °C and stirred for 1 h, using a reflux condenser system. Then, the fibers were washed with deionized water up to neutral pH. The fibers were then dispersed in 2 wt% KOH aqueous solution (400 mL) and stirred at 85 °C for 2 h. Then, the fibers were again washed with deionized water up to neutral pH. The treatment with sodium chlorite solution (first step) was repeated. This was followed by treatment with 5 wt% KOH aqueous solution (400 mL) at 85 °C for 2 h, followed by washing with deionized water. For chemical oxidation, the bleached sugarcane bagasse suspension (10 g, 1 wt%), sodium bromide (1 mmol/g cellulose), and TEMPO reagent (0.1 mmol/g) were stirred together, and the sodium hypochlorite solution (5 mmol/g cellulose) was slowly dropped into the suspension. The pH of the suspension was maintained at ~10 by slowly dropping 0.1 M NaOH. After the pH was stable at ~10, the pulp was filtered and extensively washed with deionized water to neutral pH. The oxidized fibers were sonicated in an ultrasonicator (Hielscher, UP 400S, 400 W, 24 kHz) using an ice bath, until the formation of a transparent gel (1 min sonication, and 2 min interval, for about 10 min).

The ToCNF content in the hydrogel was determined by gravimetric analysis at 105 °C for 6 h. The carboxylic acid content in the structure of ToCNF was determined by conductometric titration[25]. The chemical modification was accessed by FTIR using a Perkin-Elmer Spectrum 100 FT-IR Spectrometer equipped with an ATR module with a selenite diamond crystal, the resolution being 4 cm^{-1} after 16 scans.

Cell culture

Human fibroblasts (GM07492) and osteoblast-like rat cells (OSTEO-1) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS). The osteoblast precursor cell line derived from mouse calvaria (MC3T3) was cultured in α-MEM medium supplemented with 10% FBS. All cell lines were incubated at 37 ± 2 °C in an atmosphere of 5% CO_2 until reaching 80-90% confluence to be used in the experiments.

Cellularization and ToCNF cell viability assay

ToCNF membranes were inserted into the wells of a 96-well plate and then with $1x10^4$/well and kept in incubation. Growth was monitored for 72 hours by the resazurin viability method.

Resazurin is a blue, weakly fluorescent dye, reducible to resorufin, a pink dye with high fluorescence. The reduction of resazurin takes place by the dehydrogenase enzymes of the mitochondria of living cells, therefore the fluorescence generated by resorufin indicates cellular viability as it is a metabolite generated only in the presence of living cells.

Every 24 hours, 200 µL of the resazurin solution was removed and transferred to a 96-well plate, and the fluorescence measured in a plate reader (SpectraMax i3, Molecular Devices, USA) at 570 nm excitation and 590 nm emission length.

Detection of rhBMP-2 induced mineralization by Alizarin Red staining assay

The alizarin red staining was used to detect calcium deposits of mineralized extracellular matrix in Osteo-1 cell culture. Osteo-1 cells treated or not treated with rhBMP-2 were seed in a 24 wells plate ($1x10^5$ cells/well) and incubated in DMEM culture medium supplemented with FBS 10% at 37 ± 2 °C in 5

% CO_2 atmosphere for 7 days. The samples were fixed using ethanol (70 % v/v) and stained with alizarin red 1% (1 % w/v) for 2 min at room temperature followed by washing with distilled water until excess dyed is removed. The stained wells were photographed under light Zeiss microscope. After imaging the hydroxyapatite crystals were destained in 0.1% acetic acid (v/v) and a 100 µl aliquot was transferred to a 96-well plate for absorbance (540 nm) reading in Spectra Max i3 plate reader (Molecular Devices). The values obtained were plotted in a bar graph using the Graph Pad Prism program.

Immobilization by adsorption of rhBMP-2 on TEMPO cellulose

In a 24-well plate, two wells were filled approximately with a volume of 0.3mL of ToCNF and 1 mL of PBS solution containing the purified rhBMP-2 at variable concentrations (mg/mL). aiming its immobilization on the surface of TEMPO cellulose by electrostatic attraction of oppositely charged , namely, the negative charges from TEMPO cellulose with the positive charges from the protein side chains. The plate was incubated at room temperature for 4 hours. Then, the solution from each well was removed using a micropipette for absorbance reading in Nano Drop (Thermo Fisher) - small volume spectrophotometer (1-2 µL).

Proliferation and Mineralization detection at the surface of ToCNF-rhBMP2

To evaluate the proliferation of cells with or without TEMPO cellulose, MC3T3 cells were seeded in triplicate in a 24-well plate previously filled or not with TEMPO cellulose, at a density of $5x10^3$ cells/well. The plate remained in an oven at 37°C in a humidified atmosphere containing 5% CO_2 for 48 hours. Every 24 hours the medium was removed and a volume of 1mL of resazurin solution (10% in culture medium) was added to each well followed by 4 hours of incubation. After the incubation period, 200 µL of the resazurin solution was removed and transferred to a 96-well plate, and the fluorescence measured in a plate reader (SpectraMax i3, Molecular Devices, USA) at 570 nm of excitation and 590 nm of emission length.

After chemical bonding of rhBMP-2 on TEMPO cellulose MC3T3 cells were seeded in a 24-well plate ($2x10^4$ cells/well) whose wells were pre-filled with rhBMP-2 treated or untreated TEMPO cellulose. After 24 hours the medium was removed, the wells were washed with PBS Buffer and the differentiation medium (α-MEM supplemented with 10%FBS, 10 mM β-glycophosphate, 0.1 mM dexamethasone and 50 mM ascorbic acid) was added to the corresponding wells of cellulose untreated with rhBMP-2. The medium was changed every 3 days and the plate remained in an incubator at 37° C in a humidified atmosphere with 5% CO_2. After 14 days, staining with Alizarin Red was performed to verify the presence of mineralized bone matrix by t mineralization nodules detection. For staining the cells were fixed in 4% paraformaldehyde solution (m/v) at room temperature for 10 minutes followed by washing with 1X PBS. After washing, 1mL of Alizarin Red solution was added to each well and the plate was incubated for 30 minutes at room temperature. Then the dye was removed, the wells washed with distilled water and images were captured using a Nikon Eclipse T5100 inverted microscope.

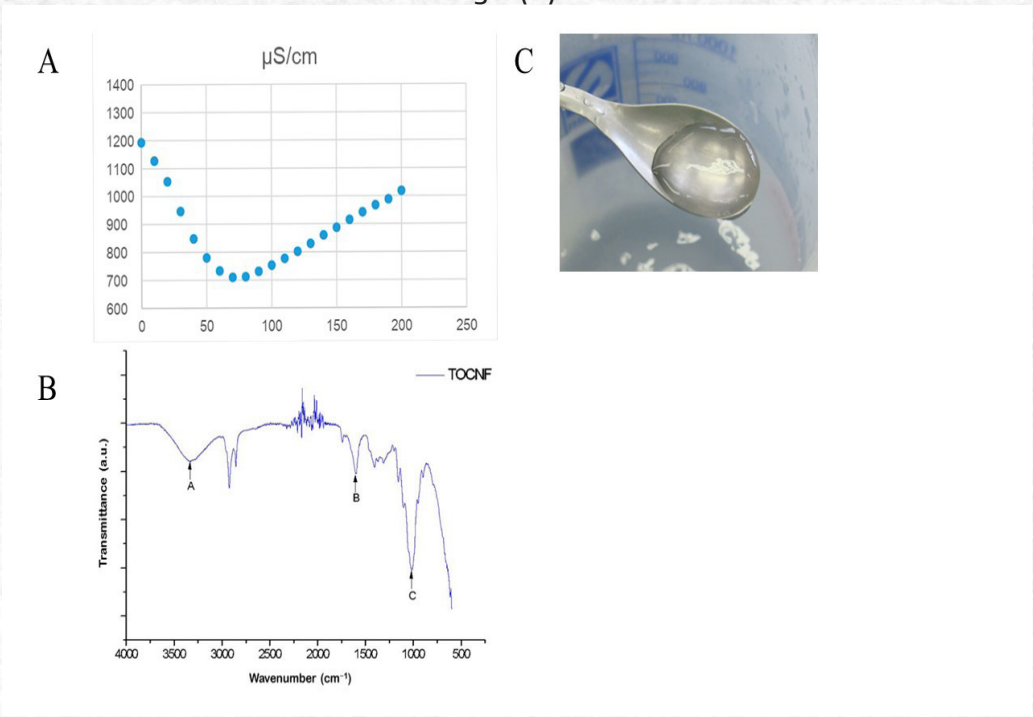
Results and Discussion

The curve of the conductometric titration of ToCNF is shown in Figure 1. The results indicated, by simple titration, that the number of free carboxylic acid in ToCNF was 0.25 mmol/g. These chemical modification was confirmed by FTIR, which results are shown in Fig. 1 B. ToCNF FTIR spectra bands at about 3300, 2900, and 1027 cm^{-1}, correspond to the vibrations of the -OH, C-H, and C-O-C groups, respectively, of the glucose structure, typical of cellulosic substrates. The strong peak at 1599 cm^{-1} is assigned to the asymmetric stretching vibration of carboxylate (C=O) formed after the oxidation of the C-6 primary hydroxyl groups of cellulose (26,27) with 2,2,6,6-tetramethylpiperidine-1-oxyl reagent (TEMPO. These results confirm the oxidation reaction of cellulose.

ToCNF membranes were inserted into the wells of a 96-well plate and then cellularized with 1x104/well and kept in incubation. Growth was monitored for 72 hours as shown in Photographs A, B, C, D and E in Figure 2.

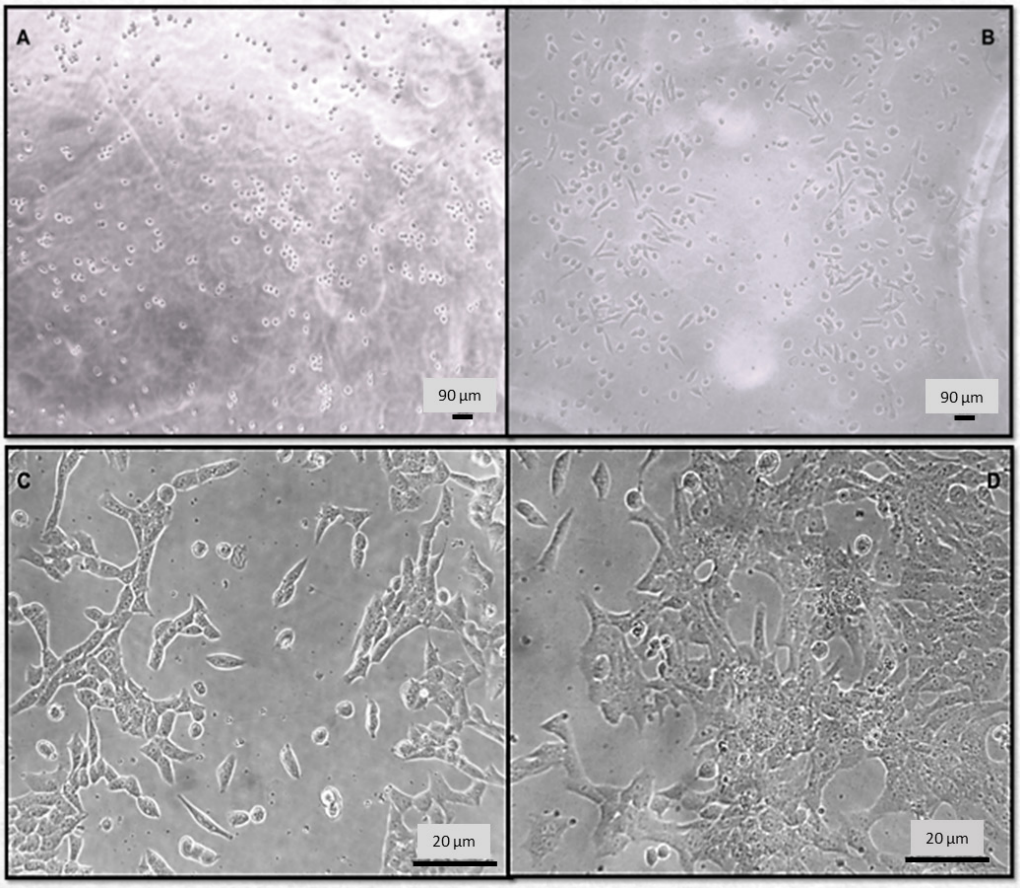
After cellularization of the ToCNF membranes, the resazurin method was adopted to verify the viability of the cells that were adhered to the cellulose membranes. Figure 3 shows the average of the fluorescence points measured at pre-established times. From these results it is possible to see that the ToCNF membranes had a lower cell viability compared to the control cells. However, there was a stabilization of cell growth demonstrating that ToCNF membranes were not cytotoxic (Figure 3).

Figure 1 - Conductometric titration curve (A) and FTIR-ATR spectra of ToCNF (B) and the TEMPO cellulose gel (C).



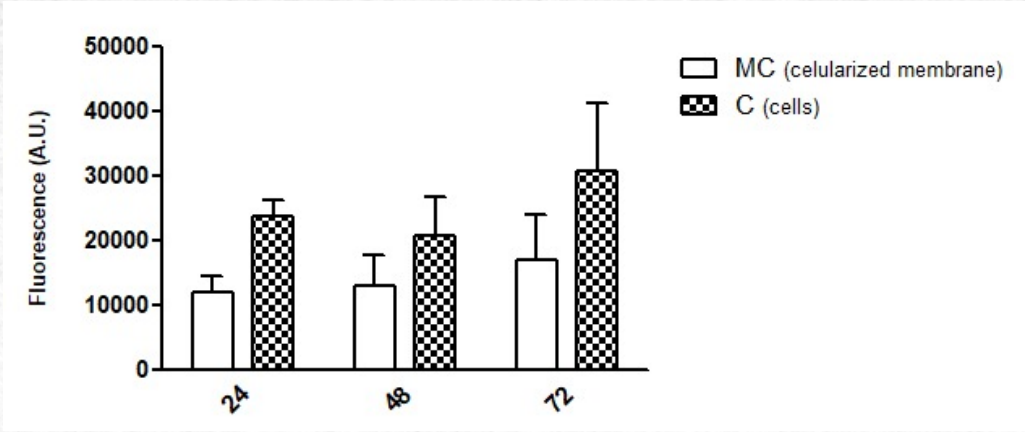
Source: Own authorship, 2024.

Figure 2 - Cellularization of the membranes (A – Cellulose membrane plus cells at time 0; B – Cellulose membrane after 4 hours; C – Cellulose membrane plus cells after 24 hours; D – Cellulose membrane plus cells after 48 hours).



Source: Own authorship, 2024.

Figure 3 - Cellular Proliferation of ToCNF membranes by Resazuirn Method.

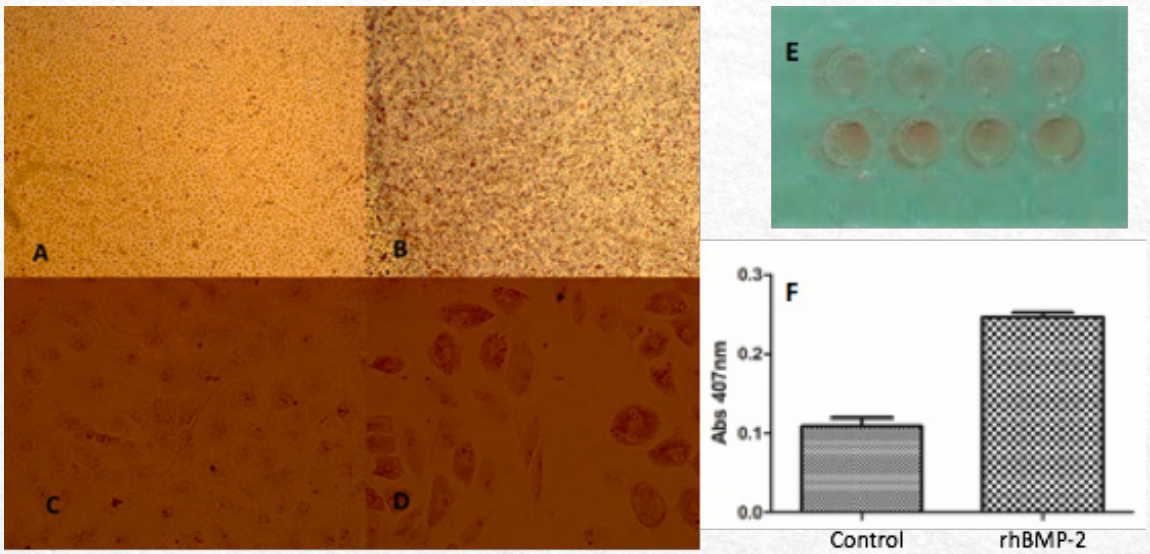


Source: Own authorship, 2024.

The results of the use of rhBMP2 into the mineralization culture medium are shown in Figure 4. The images of optical microscopy at two magnifications (A and B, and C and D) for the mineralized matrix free of rhBMP2 and in presence of rhBMP2 indicate that both the conditions led to the mineralization, however, with visual evidence of increased mineralization in the presence of the rhBMP2, evidenced by the intensity of the red color in this sample. These results showed the osteoinduction of rhBMP2, as it is one of its most important biological activities. Figure E shows the picture of the wells of the culture plate, with the mineralized culture replicates at the top and the mineralized culture replicates in presence of the rhBMP2 at the bottom. Figure 4 F shows the values of the absorbance of the mineralization tests carried out using the wells of Fig. 4 E, and the total agreement of the visual color intensity with the spectrophotometric reading.

In the experiments carried out to immobilize rhBMP-2 on TEMPO cellulose, it was possible to make an estimate in percentage of the concentration of protein adsorbed on the cellulose. In each experiment, a volume of 1 mL of solution was used, containing a certain concentration of rhBMP-2. After the incubation time applied in this work, the final reading of the protein concentration in each experiment was performed. Results were expressed as a percentage (Table 1).

Figure 4 - A and C shows the microscopy of the mineralized cells matrix formed in the medium free of rhBMP2, at two different magnification (20X and 40X); B and D shows the microscopy of the mineralized cells matrix formed in the media with rhBMP2, at two different magnification (20X and 40X); E shows the visual aspect of the plate wells with samples A and B; and F shows the spectrophotometric data reading of the test of mineralization for samples A and B.



Source: Own authorship, 2024.

Table 1 - Percentage of adsorption of rhBMP-2 on TEMPO cellulose.

Experiment	mg/mL (initial)	mg/mL (final)	%
1	0.225	0.110	48.8
2	0.244	0.120	49.1
Average	0.234	0.115	48.9

Source: Own authorship, 2024.

In the short incubation time adopted in this work, the results obtained in the two experiments gave a percentage of approximately 49% of adsorption of rhBMP-2 on TEMPO cellulose. In addition to being a low and affordable production cost strategy.

The cell proliferation assay was performed using resazurin. The blue, non-fluorescent resazurin is reduced to the pink, fluorescent resazurin, and its formation is directly proportional to mitochondrial metabolic activity and cell viability. For this work, readings of the times of 24 and 48 hours were made. The results were presented with the fluorescence values by arbitrary unit (AU), as shown in Figure 5.

The results revealed that the biomaterial used could promote viability and cellular proliferation by the increase in fluorescence.

The images in Figure 6 show TEMPO cellulose treated or not with rhBMP-2 before the induction of mineralized bone matrix synthesis, as well as revealing the synthesis of mineralized bone matrix stained with Alizarin Red. For TEMPO cellulose not immobilized with rhBPM-2, a culture medium containing osteoinductors (differentiation medium) was used. While for the TEMPO cellulose treated with the protein, there were no osteoinductive factors in the medium used.

Qualitatively, it is possible to observe that TEMPO cellulose - rhBMP-2 was able to induce the synthesis of mineralized bone matrix, as we can visualize calcium-alizarin crystals.

Figure 5 - Resazurin reduction at 24 and 48 hours, expressed as percentage reduction.

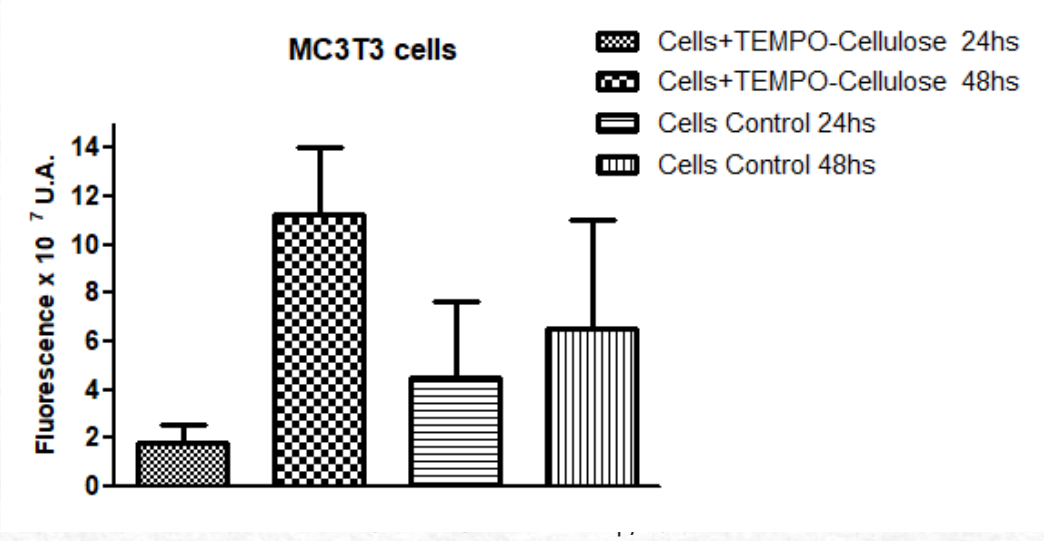
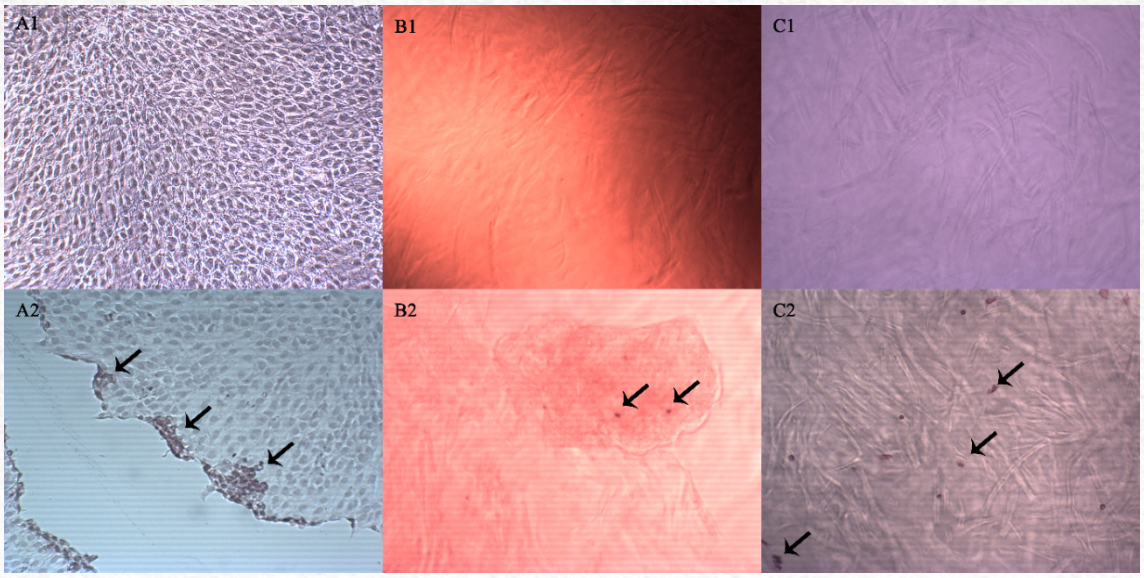


Figure 6 - TEMPO cellulose treated or not with rhBMP-2– Images captured at 10x magnification by optical microscopy. Arrows indicate the presence of mineralized bone matrix. Images A1, B1 and C1 show, respectively, the cell culture (MC3T3) without TEMPO cellulose, the cellularized TEMPO cellulose and treated with rhBMP-2 and the cellularized TEMPO cellulose and not treated with rh-BMP-2 before the induction of synthesis. of mineralized bone matrix. A2, B2 and C2 reveal the synthesis of mineralized bone matrix stained with Alizarin Red.



Source: Own authorship, 2024.

Conclusion

The cell viability results of cellularized ToCNF membranes indicate that the modified cellulose provides a stable environment for cell maintenance, corroborating the results of detection of proliferation and mineralization on the surface of ToCNF-rhBMP2. Overall, this study demonstrates that TEMPO cellulose functionalized with rhBM-2 has significant potential for use as a biomaterial in bone tissue repair. New studies should be encouraged to confirm the experiences found.

Acknowledgments

The authors would like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/O Programa de Suporte à Pós-Graduação de Instituições de Ensino Particulares (CAPES/PROSUP) foundations for their support.

References

[1] Bessa PC, Pedro AJ, Klösch B, Nobre A, van Griensven M, Reis RL, et al. Osteoinduction in human fat-derived stem cells by recombinant human bone morphogenetic protein-2 produced in Escherichia coli. Biotechnol Lett. 2008 Jan;30(1):15–21.

[2] Colange AL. Produção da BMP-2 recombinante de humano (rhBMP-2) para funcionalização de biopolímeros em reparo ósseo. 2017; Available from: https://uniara.com.br/arquivos/file/ppg/biotecnologia-medicina-regenerativa-quimica-medicinal/repositorio-cientifico/dissertacoes/2017/ana-lucia-colange.pdf

[3] Lee JH, Kim CS, Choi KH, Jung UW, Yun JH, Choi SH, et al. The induction of bone formation in rat calvarial defects and subcutaneous tissues by recombinant human BMP-2, produced in Escherichia coli. Biomaterials. 2010 May;31(13):3512–9.

[4] Kim HS, Park JC, Yun PY, Kim YK. Evaluation of bone healing using rhBMP-2 soaked hydroxyapatite in ridge augmentation: a prospective observational study. Maxillofac Plast Reconstr Surg. 2017 Dec;39(1):40.

[5] Hrubi E, Imre L, Robaszkiewicz A, Virág L, Kerényi F, Nagy K, et al. Diverse effect of BMP-2 homodimer on mesenchymal progenitors of different origin. Hum Cell. 2018 Apr;31(2):139–48.

[6] Khojasteh A, Behnia H, Naghdi N, Esmaeelinejad M, Alikhassy Z, Stevens M. Effects of different growth factors and carriers on bone regeneration: a systematic review. Oral Surg Oral Med Oral Pathol Oral Radiol. 2013 Dec;116(6):e405-423.

[7] Ihm HJ, Yang SJ, Huh JW, Choi SY, Cho SW. Soluble expression and purification of synthetic human bone morphogenetic protein-2 in Escherichia coli. BMB Rep. 2008 May 31;41(5):404–7.

[8]Haidar ZS, Hamdy RC, Tabrizian M. Delivery of recombinant bone morphogenetic proteins for bone regeneration and repair. Part A: Current challenges in BMP delivery. Biotechnol Lett. 2009 Dec;31(12):1817–24.

[9] Groeneveld EH, Burger EH. Bone morphogenetic proteins in human bone regeneration. Eur J Endocrinol. 2000 Jan;142(1):9–21.

[10]Bessa PC, Casal M, Reis RL. Bone morphogenetic proteins in tissue engineering: the road from the laboratory to the clinic, part I (basic concepts). Journal of Tissue Engineering and Regenerative Medicine. 2008;2(1):1–13.

[11]Leite MDR, Marinho TMA, Fook MVL. Obtenção e caracterização de scaffolds de policaprolactona produzidos a partir do sistema bioextruder. Revista Eletrônica de Materiais e Processos. 2016;11.

[12]Oliveira LS de AF, Oliveira CS, Machado APL, Rosa FP. Biomateriais com aplicação na regeneração óssea – método de análise e perspectivas futuras. Revista de Ciências Médicas e Biológicas. 2010 Nov 18;9:37–44.

[13]Tabata Y. Biomaterial technology for tissue engineering applications. J R Soc Interface. 2009 Jun 6;6 Suppl 3(Suppl 3):S311-324.

[14]Celestino V, Maestri G, Bierhalz A, Immich A. Produção e caracterização de hidrogel de carboximetilcelulose para aplicação na área de curativos biomédicos. In São Paulo; 2017.

[15]Yonezawa UG, Moura MR de, Aouada FA. Estado da arte: um estudo sobre polímeros biodegradáveis na germinação e desenvolvimento de plantas. Caderno de Ciências Agrárias. 2017 Aug 31;9(2):69–78.

[16]Aljohani W, Ullah MW, Zhang X, Yang G. Bioprinting and its applications in tissue engineering and regenerative medicine. Int J Biol Macromol. 2018 Feb;107(Pt A):261–75.

[17]Saito T, Kimura S, Nishiyama Y, Isogai A. Cellulose nanofibers prepared by TEMPO-mediated oxidation of native cellulose. Biomacromolecules. 2007 Aug;8(8):2485–91.

[18]Singh M, Ray AR, Vasudevan P. Biodegradation studies on periodate oxidized cellulose. Biomaterials. 1982 Jan;3(1):16–20.

[19]Abouzeid RE, Salama A, El-Fakharany EM, Guarino V. Mineralized Polyvinyl Alcohol/ Sodium Alginate Hydrogels Incorporating Cellulose Nanofibrils for Bone and Wound Healing. Molecules. 2022 Jan;27(3):697.

[20]Gorgieva S, Girandon L, Kokol V. Mineralization potential of cellulose-nanofibrils reinforced gelatine scaffolds for promoted calcium deposition by mesenchymal stem cells. Materials Science and Engineering: C. 2017 Apr 1;73:478–89.

[21]Salama A, Abou-Zeid RE, Cruz-Maya I, Guarino V. Soy protein hydrolysate grafted cellulose nanofibrils with bioactive signals for bone repair and regeneration. Carbohydrate Polymers. 2020 Feb 1;229:115472.

[22]Yao S, Xu Y, Zhou Y, Shao C, Liu Z, Jin B, et al. Calcium Phosphate Nanocluster-Loaded Injectable Hydrogel for Bone Regeneration. ACS Appl Bio Mater. 2019 Oct 21;2(10):4408–17.

[23]Ingole VH, Vuherer T, Maver U, Vinchurkar A, Ghule AV, Kokol V. Mechanical Properties and Cytotoxicity of Differently Structured Nanocellulose-hydroxyapatite Based Composites for Bone Regeneration Application. Nanomaterials. 2020 Jan;10(1):25.

[24]Im S, Choe G, Seok JM, Yeo SJ, Lee JH, Kim WD, et al. An osteogenic bioink composed of alginate, cellulose nanofibrils, and polydopamine nanoparticles for 3D bioprinting and bone tissue engineering. International Journal of Biological Macromolecules. 2022 Apr 30;205:520–9.

[25]Da Silva Perez D, Montanari S, Vignon MR. TEMPO-mediated oxidation of cellulose III. Biomacromolecules. 2003;4(5):1417–25.

[26]De Lima Pizi Cândido A, Fregonezi NF, Carvalho AJF, Trovatti E, Resende FA. TEMPO-Oxidized Cellulose Nanofibers In Vitro Cyto-genotoxicity Studies. BioNanoSci. 2020 Sep 1;10(3):766–72.

[27]Trovatti E, Tang H, Hajian A, Meng Q, Gandini A, Berglund LA, et al. Enhancing strength and toughness of cellulose nanofibril network structures with an adhesive peptide. Carbohydrate Polymers. 2018 Feb 1;181:256–63.

REVIEW ARTICLE

IJAMB
International Journal of Advances in Medical Biotechnology

The pharmaceutical patent process: National Health Surveillance Agency and National Institute of Industrial Property act differently in the process

L. H. De Souza[1]; C. S. T. Amaral[1]

*Corresponding author: E-mail address: leiraudhdsouza@uniara.edu.br

Received: 10 dezember 2024; 10 february 2024.

Abstract: The National Institute of Industrial Property (INPI) is the government body responsible for granting patents in the national territory; in the case of medicines, they need to be registered with the National Health Surveillance Agency (ANVISA) in order to be marketed. There was a divergence in legal interpretation (of Art. 229-C of the IPL) which caused damage to entrepreneurs, laboratories and the community in general, as there was no express provision as to whether ANVISA's opinion would be binding on the INPI's decision on patent issues. The aim of this research was to analyze this problem, raising its main points and demonstrating how dangerous and damaging bureaucracy and inefficient and obscure normative acts that give rise to dubious interpretation can be, based on the application of hermeneutic and dialectical methods. In 2021, the Superior Court of Justice (STJ), in Special Appeal n. 1543826, held that ANVISA's opinion would be a valid prerequisite for granting patents for pharmaceutical products or processes. It was found that the STJ decision increased ANVISA's "powers", but with the repeal of Art. 229-C of the IPL, the dilemma was extinguished and the competencies of each body re-established. It is therefore of the utmost importance to fill legal gaps and issue clear and specific laws so as not to leave room for harmful interpretations, guaranteeing original competences and legal certainty.

Keywords: Medicines. ANVISA. INPI. Patents.

Introduction

Health is one of the areas in which biotechnology operates and, specifically for this study, the pharmaceutical area, which uses biotechnological techniques to produce medicines such as antibiotics and biopharmaceuticals, e.g. using genetically modified cells to produce therapeutic proteins such as recombinant insulin for diabetic patients and monoclonal antibodies to treat cancer, for example [25].

The Institute of Science, Technology and Industrial Quality (ICTQ), based on research carried out, has verified the growth trend of the global biopharmaceuticals market, which is worth around US$160 billion a year. In Brazil, there are pharmaceutical companies with biopharmaceutical development projects, such as Libbs, Cristália, Recepta and BioNovis (a joint venture created by Aché, EMS, Hypermarcas and União Química laboratories)[25].

Due to the importance of medicines for the protection of human life, materializing the right to health and, as well as national economic development through the production and marketing of drugs, this research addresses the problem involving the two responsible bodies: the National Institute of Industrial Property (INPI) which protects property rights and the National Health Surveillance Agency (ANVISA) which grants registration for the marketing of drugs.

It should be noted that as part of the state's obligations, it is the government's role to establish policies and laws aimed at protecting human health, and there is a particular concern involving the process of registering drugs by the ANVISA and the analysis of patent applications by the INPI in the area of drugs.

ANVISA is the public body responsible for analyzing drug registration applications and defining the criteria and stages necessary for the release of a new drug in the national territory; only after the registration has been granted will the drug be released for sale[9]. The INPI is a federal authority responsible for granting patents and registering other intellectual properties, as well as supporting the technology transfer process[10].

What is the relationship between these two bodies when it comes to medicines? As a result of the amendment to the IPL and the insertion of Article

[1] University of Araraquara - UNIARA, Araraquara, São Paulo, Brazil.

https://doi.org/110.52466/ijamb.v6i1.116

229-C, a problem arose, involving the question of competence, as to whether or not ANVISA's opinion was binding on patent applications for medicines filed with the INPI, leading to a clash of competences between them. The Superior Court of Justice (STJ) was asked to give its opinion, taking the view that the opinion was binding. However, in the same year (2021), Law 14.195 was enacted, resolving the impasse and separating the competences, in the sense that the INPI is not bound by ANVISA's opinion when granting patents for pharmaceuticals.

The aim of this research is precisely to discuss the current rules for granting patents and registering medicines in Brazil, so that they can contribute to innovation and entrepreneurship in the area of pharmaceuticals. This is a literature review, carried out on the basis of journals, the government, the STJ and its decision on the matter, presented from the perspective of the hermeneutic and dialectical methods.

Materials and methods

A bibliographical survey was carried out, a literature review, with a documentary approach and analysis of data contained in literary, scientific and technical works, on the official websites of the Ministries of State, the INPI, ANVISA, the STJ and current legislation related to the subject.

The methods used were hermeneutic and dialectic. Hermeneutics, or the interpretative method, aims to understand a text or discourse, verifying its sense (its meaning). The dialectical method considers the permanent transformation of society. Through discourse based on reasoned arguments, with a view to transforming and improving antagonistic relations, criteria are established for the practice of conduct and the adoption of mechanisms aimed at the evolutionary process[20]. Thus, the dialectical method, following the Hegelian conception with the formulation of a thesis (initial statement), antithesis (refutation of this statement by contrary aspects) and synthesis (a new thesis based on logical convergence or this dialectical logic) was applied to organize the arguments and draw up the conclusions.

Relationship between drug registration and patents

According to Igor Simões[27], the government is concerned about the pharmaceutical sector, both in terms of registering drugs and granting patents. However, the sector is faced with the constant concern of the government compulsorily licensing patents (promoting patent infringement). In addition, Simões says that the problem involving the sector (registration, granting of patents for medicines and possible compulsory licensing) lies much more in the lack of investment and serious and effective public policies in the area of health, much more than in the granting of patents, and the population has the most to lose in this process of divergence.

In order to give the population greater access to medicines, the government is promoting incentives for the generic drug industry. The world market for generic drugs has grown by an average of 11% a year and consumption of these drugs in Brazil has also grown by approximately 220% since they were first made available in pharmacies in 2000. This is due to lower research and marketing costs, which is reflected in the value of the product to the consumer, representing savings of up to 40%[27]. However, the generic drug can only be manufactured and marketed if there is no patent in force and other rights that guarantee exclusivity rights.

Patent protection gives the holder a monopoly on the exploitation of the product for a certain period of time. It is worth noting that the LPI considers an invention patent to be a product or process that meets the following requirements: inventive step, novelty and industrial application. The patent granted is valid for 20 (twenty) years from the date of filing. Utility model patents are for objects of practical use, or part thereof, susceptible to industrial application, which present a new form or arrangement, involving the inventive act, which results in a functional improvement in its use or manufacture; it is valid for 15 (fifteen) years. And finally, the certificate of addition of invention translates an improvement or development introduced into the object of the invention, even without inventive step, but still within the same inventive concept; it is considered an accessory to the patent, which is why it has the same expiry date[8].

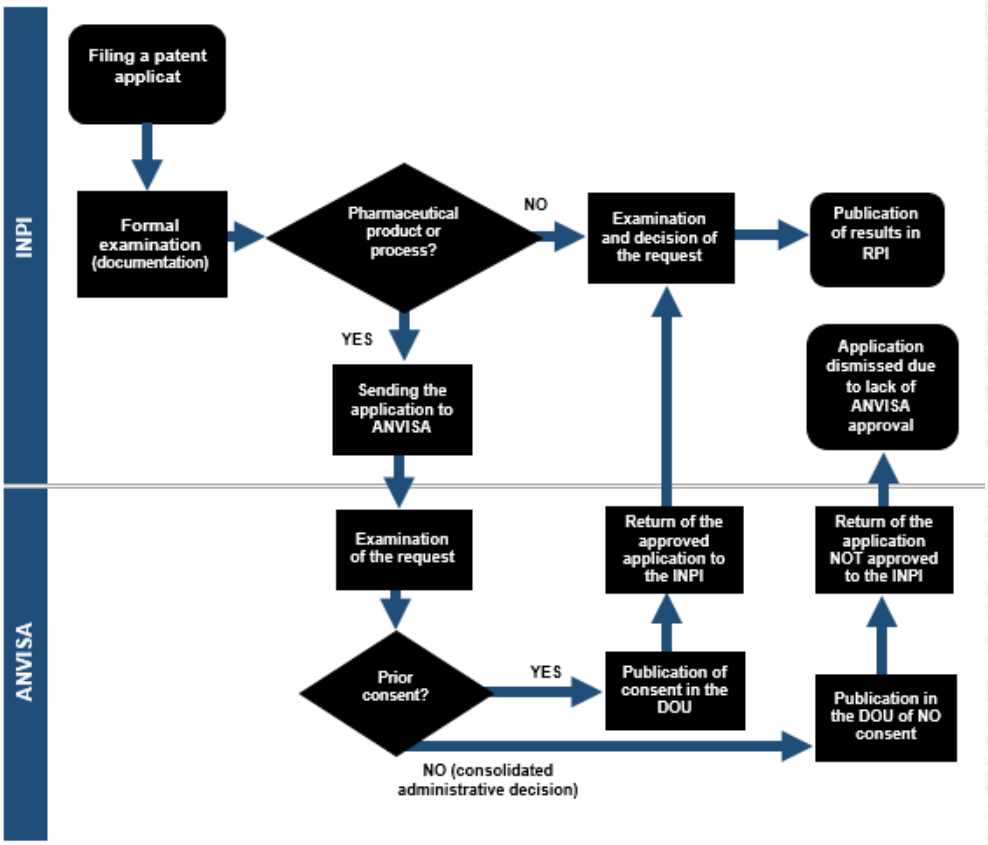
But in the case of medicines, the granting of the patent would be linked to the prior consent of ANVISA. The process follows the stages described in Figure 1.

Until 2001, the INPI examined drug patents without interference from the ANVISA. With the advent of Law n. 10.196, which came into force on February 14, 2001, Art. 229-C was included in Law n. 9.279/1996 (Industrial Property Law - LPI), determining that the granting of patents for pharmaceutical products and processes would henceforth depend on ANVISA's prior consent.

Thus, the granting of a patent does not authorize its holder to commercialize the medicine, it only protects and guarantees the property rights over the product, and may enter into contracts for the assignment of rights, for example. However, the drug cannot be made available on the consumer market, as this requires registration with ANVISA.

The legislative change has sparked discussions,

Figure 1 - Analysis of patent applications for pharmaceutical products/processes.



Source: Adapted from the Report of the Interministerial Working Group on Public Administration[apud 24].

especially regarding the constitutional right to property, which includes the right to patent protection. Therefore, if the requirements are present and the legal requirements for granting the patent by the INPI have been met, it would not be possible to include a new restriction, in this case the prior consent of ANVISA, which would be reflected in the consent of the Federal Government, thus hindering the exercise of this right.

For Denis Borges Barbosa[1], it is not possible to interpret Art. 229-C as ANVISA having the power to deny or admit patents, based on the judgment of convenience and opportunity inherent in the discretionary power of the Public Administration, because in this case, it would be totally incompatible with Art. 5, inc. XXIX of the Federal Constitution of 1988, which establishes that the legal requirements for granting a patent, in a binding procedure, can only be created by ordinary law. The INPI has a duty to listen to ANVISA, which will make a technical analysis aimed at protecting life and health, but it cannot bind the INPI's decision.

However, ANVISA's understanding with the legislative change was that it had the power to analyze the technical requirements for patentability and not only the issues inherent to efficacy and risks to life and health [24]. This discussion was taken to the Federal Attorney General's Office (AGU), which confirmed the Agency's role of analyzing only product/process issues associated with health risks[21].

In a patent application denied by ANVISA, the Federal Regional Court of the 2nd Region (TRF2) ruled that the Agency's legal powers had been overstepped, as they were restricted to examining potential health risks. ANVISA appealed the decision to the STJ, which held that ANVISA's favorable opinion was a prerequisite for the granting of patents for pharmaceutical products or processes, a discussion that will be revisited below.

In practice, from 2001 to 2017, the INPI only analyzed drug patent applications after ANVISA's analysis and approval. As long as this consent was not forthcoming, the processes remained stalled. This procedure meant that at the time, more than 21,733 patent applications for medicines remained paralyzed at the INPI for this reason[22].

In an attempt to resolve this issue and speed up the analysis of processes involving pharmaceuticals, the INPI and ANVISA formalized an agreement in March 2017. The rule included prior analysis by ANVISA, with the aim of ensuring that the drug was effective and did not pose any health risks, but did not bind the INPI in its analysis of the merits of the patent, and it could grant it even if ANVISA issued an unfavorable opinion, which was defined in Joint Ordinance n. 01, of April 12, 2017 (regulating the

procedures for applying Art. 229-C of the LPI).

In summary, Joint Ordinance n. 01/2017 determines the processing of patent applications for pharmaceutical products and processes:

►Art. 2: once the patent has been formally examined by the INPI, the procedure for granting ANVISA's prior consent will take place after the request for examination has been made (in accordance with Art. 33 of the LPI);

►Art. 2, §1°: the INPI will publish the notification of the forwarding of patent applications to ANVISA in the Electronic Industrial Property Magazine (RPI) and, when necessary, the decisions on examination priority;

►Art. 2, §2°: the INPI will make the updated full contents of patent applications available, together with the publication of the referral;

►Art. 3: The INPI will provide ANVISA with access to the information contained in its database;

►Art. 4: After receiving the patent applications forwarded to the INPI, ANVISA will analyze them taking into account aspects inherent to public health, issuing a technical opinion;

►Art. 6: When the INPI disagrees with ANVISA's opinion, it must state the reasons for its disagreement in a reasoned technical opinion;

►Art. 7: At the end of the INPI's examination of patent applications with ANVISA's consent, an official list of patent applications granted and published in the RPI will be sent to the Agency by the INPI;

►Art. 9: An Interinstitutional Articulation Group will be set up, with members from the INPI and ANVISA, with the aim of exchanging technical information and harmonizing understandings.

►Art. 11: the ordinance came into force sixty days after its publication (04/13/2017).

In 2017, the Interinstitutional Articulation Group (GAI) was created with the aim of analyzing and suggesting instruments, mechanisms and procedures for coordinated action between the INPI and ANVISA in the analysis of patents for pharmaceutical products and processes, under the terms of Joint Ordinance no. 2, of October 20, 2017. On March 26, 2018, the first technical meeting of the GAI was held by videoconference, the main purpose of which was to establish the Group's working methodology. On May 24, 2018, the Group's second meeting was held at the INPI's headquarters in Rio de Janeiro. At this meeting, the main results obtained over the period were presented, among them: the optimization of the flow of patent applications, the current methodology for forwarding letters and opinions, the creation of pages on the portals of both the INPI and ANVISA for greater transparency of the GAI's work[7].

In 2021, the 4th Panel of the Superior Court of Justice (STJ) ruled that ANVISA's opinion is a prerequisite for the validity of patents for pharmaceutical products or processes. According to Justice Luís Felipe Salomão, rapporteur of the case (REsp. No. 1543826), the best interpretation of Article 229-C of the IPL is to understand it as a prerequisite for the validity of pharmaceutical patents granted by the INPI. With this understanding, the justices annulled the decision of the Federal Regional Court of the 2nd Region (TRF2), which considered, in a patent application denied by ANVISA, that it had exceeded its legal powers, which were restricted to examining potential health risks[16].

ANVISA's negative opinion in cases where it is shown to be contrary to public health policies is binding and does not support the INPI's decision. ANVISA's attributions are restricted to examining potential health risks, and it is ANVISA's responsibility to determine before the INPI whether the granting of exclusivity rights (production, use, commercialization, importation or licensing) could lead to a situation that is harmful to public health[16].

However, Law n. 14.195, of August 27, 2021 (Conversion of Provisional Measure n. 1.040/2021) revoked Art. 229-C of Law 9.279/1996, establishing the end of ANVISA's prior consent for patent applications for pharmaceutical products and processes (Chart 1).

In a note, available on the official website of the Ministry of Development (2022), the INPI announced the procedures to be adopted:

a) the extinction of the flow of patent applications between the INPI and ANVISA since August 27, 2021;

b) applications that are returned by ANVISA will be processed normally at the INPI;

c) the applications concluded by ANVISA and forwarded to the INPI before the revocation of Art. 229-C were published in the Industrial Property Magazine (RPI) n. 2763;

d) the applications that were at ANVISA were returned to the INPI on August 30, 2021, a total of 1,284 applications, of which 54 already had the consent published by ANVISA before the revocation of the article, so they would be published in the RPI;

e) the INPI is awaiting the return of 19 patent applications that were under requirement or had already been denied;

f) the applications filed by December 31, 2016, included in the Backlog Combat Plan, have been forwarded for examination[10].

Chart 1 - INPI and ANVISA: chronology of the dilemma.

Law n. 10.196/2001 inserted Art. 229-C into the LPI: "The granting of patents for pharmaceutical products and processes will depend on the prior approval of the National Health Surveillance Agency - ANVISA."	
From 2001 to April/20	The INPI only analyzed patent applications after ANVISA's opinion and consent.
From April/2017 to July/2021	Agreement signed between the INPI and ANVISA - Joint Ordinance No. 01, of April 12, 2017 (regulates the procedures for applying Art. 229-C of the IPL).
On August 5th, 2021	The 4th Panel of the Superior Court of Justice (STJ) has ruled that ANVISA's opinion is a prerequisite for the validity of patents for pharmaceutical products or processes. According to Justice Luís Felipe Salomão, rapporteur of the case (REsp. no. 1543826/RJ).
Law n. 14.195/2021 repealed Art. 229-C of the LPI: extinguished the dilemma - re-established the competence of the INPI in granting patents.	

Source: Own authorship, 2023.

The issue of ANVISA's approval, the granting of patents by the INPI and compulsory licensing of medicines

Blocking the analysis of the patent application because of the wait for ANVISA's favorable opinion created insecurity; for the company it makes no sense to invest in a product/process without guarantees of commercial exclusivity. With the application distributed, the principle of prior art guarantees the right from the moment it is filed, but while the criterion was to wait for ANVISA's prior approval before adding it to the application, the risk was greater: in addition to the delay, the «secret» could fall into the public domain (leak out in some way) and be used by generic manufacturers.

The government can compulsorily license the patent in cases of lack of exploitation in the national territory, and how can you exploit it economically (market the drug) without ANVISA's approval? Since medicines cannot be marketed without authorization through the relevant registration with the Agency.

One term used by the informal media is that the government will authorize the «breaking of the patent» of a certain drug, which is nothing more than authorizing the compulsory licensing of the drug, i.e. the government authorizes another company to exploit the drug, implying the loss of exclusivity of economic exploitation by the patent holder.

The compulsory license is provided for in Art. 68 of Law 9.279/1996 (Industrial Property Law - LPI), and will be applied to the patent holder in the event of abusive exercise, abuse of economic power (proven by administrative or judicial decision), in the absence or insufficiency of manufacture of the product and in the absence of full use of the patented process, except in the case of economic infeasibility. This is because patent protection, as a protective reflex and guarantor of industrial property rights, must fulfill a social function and if it does not, i.e. if it is used outside the legal limits, the patent may be compulsorily licensed at the request of the government or an interested third party.

The legal hypotheses that give rise to compulsory licensing of patents are summarized below:

The exercise of a right in an abusive manner (abuse of rights): this is the exercise of a right by its holder that goes beyond the limitations imposed by its economic or social purpose, by good faith or by good customs (Art. 187, CC/2002), characterizing an unlawful act subject to reparation.

Abuse of economic power: unlawful conduct by an economic agent who has market power or who assumes a dominant position in the market exorbitates this power with a view to dominating the market, eliminating competition and arbitrarily increasing its profits, which are prohibited by Brazilian legislation (Art. 173, §4 of the Federal Constitution/88 cc. Art. 36 of the Antitrust Law - Law n. 12.529/2011). It is worth mentioning that the abuse of economic power must be recognized by the Administrative Council for Economic Defense (CADE), the competent administrative body, or by the Judiciary, in a sentence handed down.

Failing to exploit the object of the patent in Brazilian territory, due to the lack or insufficiency of manufacture of the product or lack of full use of the patented process: reveals the «misuse of property». It will be necessary to manufacture the product or use the patented process in Brazil, and importation will only be authorized in cases of economic infeasibility, which is highly subjective - there is no objective concept of what «economic infeasibility» means, making it seriously difficult to grant a license for this reason[19].

When marketing does not meet the needs of the market: when production is insufficient to meet the needs of the market.

In cases of emergency or public interest, declared by the Federal Executive Branch, if the patent holder or its licensee does not meet this need: through collective interest, usually occurs in patents for products and processes involving health, for example in the pharmaceutical industry in 2007 with Efavirenz, a drug for the treatment of HIV[26].

The compulsory license must be requested by a third party with a legitimate interest and the technical and economic capacity to exploit the patent effectively, with the aim of supplying the domestic market. It will be granted ex officio by the Federal Executive Power, exceptionally in cases of national emergency or public interest and, even in these cases, only if the patent holder is not meeting the needs of the market; the grant is exclusive and sub-licensing is not allowed[19].

The request for a compulsory license may be rejected by the INPI. It is the right of the patent holder to be notified of the request and to have a period (60 days) in which to respond, proving with documents the reasons for the disuse (the reasons must be legitimate) or that they are taking steps to start production and/or increase it, justifying the problems they are facing, or that they are not producing due to force majeure, explaining them. In this sense, if the arguments are proven and accepted by the INPI, the compulsory license will not be granted, and exclusivity will remain with the holder of the Patent Letter. In the event of inertia on the part of the owner, once the deadline for manifestation has passed, the request for a license will be granted under the conditions under which it was filed.

If the owner contests the license request, the INPI may carry out due diligence, appoint a commission (including external experts) to support the arbitration of the remuneration due, since there was no consensus between the parties. The arbitration will take into account the circumstances and peculiarities of the specific case, without forgetting to consider the economic value of the license granted[28].

Bill 12/2021 was converted into Law 14.200/2021, amending the LPI to enable the compulsory licensing of products to combat Covid-19, a necessary measure to deal with public health emergencies. It is now possible to compulsorily license patents or patent applications in cases of national or international emergency or in the public interest and in the face of a state of national public calamity (new wording of Art. 71 of the LPI). The compulsory license may be granted ex officio, on a temporary and non-exclusive basis, guaranteeing the rights of the holder to compensation, with the holder's remuneration being set at 1.5% of the net sales price of the product until its value is effectively established (Art. 71 and its §13 of the LPI).

Compulsory license versus patent revocation

A compulsory license should not be confused with patent revocation. The section above defined compulsory licensing and the legal hypotheses for requesting it. In this section we will summarize the hypotheses of patent termination, which is different from compulsory license,

According to Fabio Ulhoa Coelho[21], the industrial right protected by a patent will be extinguished by virtue of the expiry of its term, forfeiture, non-payment of the amounts due to the INPI, the resignation of its owner, the absence of a legal representative in Brazil, if the owner is domiciled or has its headquarters abroad.

The doubts that can arise regarding the termination of a patent are recurrent in the case of forfeiture. Forfeiture arises from abuse or disuse in the exercise of the right, which can be declared by the INPI ex officio or if requested by an interested third party, after the compulsory license has been granted (3 years from the grant), after two years of the license, the patent will fall into the public domain, if the abuse or disuse of the product or process is still verified. The defense and the adversarial process are guaranteed in the forfeiture claim, an administrative process with the INPI (LPI, Arts 80 to 83).

It is important to note that the law protects the rights of third parties in the event of the owner renouncing a patent (a unilateral act). Franchisees and/or licensees, for example, must decline to accept the act, and the INPI must prove that there is no damage to the others involved (interested contractors).

Art. 217 of the IPL states that «The person domiciled abroad must appoint and maintain a duly qualified attorney domiciled in the country, with powers to represent them administratively and judicially, including to receive summonses», failing which the patent will be extinguished.

As you can see, compulsory licensing is an institute for fulfilling the social function of property and protecting the market and the consumer, while termination is another instrument that will operate in hypotheses other than those, the main effect of which is to definitively end the right of the holder of the Letters Patent to the exclusive exploitation of industrial property.

Conclusion

Despite the «tug-of-war» between ANVISA and the INPI, the joint ordinance eliminated (until 2021) the contradictions and speeded up the registration procedure in each body, establishing objective

criteria, which culminated in a greater benefit to health. In 2021, the STJ ruled that ANVISA's opinion is binding and must be observed by the INPI when granting patents on medicines in the event that it contradicts public health policies.

It is clear that the STJ's decision had given ANVISA greater power, conditioning its favorable opinion on the granting of a patent by the INPI, especially given the lack of regulations on what «contradiction to public health policies» would mean, which is subjective.

Then, also in 2021, Law 14.195 repealed Art. 229-C of the LPI and extinguished the dilemma and legal uncertainty, re-establishing the competence of the INPI to grant patents and speeding up the process, which had been too slow.

References

[1] Barbosa, D. B. ANVISA's role in granting patents. In: BARBOSA, D. B. From technology to culture: essays and studies on intellectual property. Rio de Janeiro: Lumen Juris, 2011.

[2] Brazil. Constitution of the Federative Republic of Brazil of 1988. Available at: http://www.planalto.gov.br/ccivil_03/constituicao/constituicao.htm

[3] Brazil. Law n. 9.279, of May 14, 1996. Regulates rights and obligations relating to industrial property. Available at: http://www.planalto.gov.br/ccivil_03/Leis/L9279.htm

[4] Brazil. Law n. 9.610, of February 19, 1998. Amends, updates and consolidates copyright legislation and makes other provisions. Available at: http://www.planalto.gov.br/ccivil_03/leis/L9610.htm#art115

[5] Brazil. Law n. 14.195, of August 26, 2021. Provides for facilitating the opening of companies, [...]; amends Laws No. [...] 9.279, of May 14, 1996, [...] and makes other provisions. Available at: https://www.planalto.gov.br/ccivil_03/_Ato2019-2022/2021/Lei/L14200.htm

[6] Brazil. Law n. 14.200, of September 2, 2021. Amends Law No. 9.279, of May 14, 1996 (Industrial Property Law), to provide for the compulsory licensing of patents or patent applications in cases of declaration of national or international emergency or public interest, or recognition of a state of public calamity of national scope. Available at: https://www.planalto.gov.br/ccivil_03/_Ato2019-2022/2021/Lei/L14200.htm

[7] Brazil. Ministry of Economy. National Institute of Industrial Property (INPI). INPI and Anvisa agree on pharmaceutical patents. Brasília, May 25, 2018. Available at: https://www.doisamaisfarma.com.br/noticias/inpi-e-anvisa-convergem-entendimentos-sobre-patentes-farmaceuticas/

[8] Brazil. Ministry of Economy. National Institute of Industrial Property (INPI). Frequently asked questions - Patents. Brasília, May 13, 2020, current. July 14, 2021. Available at: https://www.gov.br/inpi/pt-br/servicos/perguntas-frequentes

[9] Brazil. Ministry of Health. National Health Surveillance Agency - ANVISA. Published: July 4, 2022, current. 3 nov. 2022. Available at: https://www.gov.br/anvisa/pt-br/assuntos/noticias-anvisa/2018/registro-de-novos-medicamentos-saiba-o-que-e-preciso#:~:text=A%20Anvisa%20%C3%A9%20o%20%C3%B3rg%C3%A3o,um%20novo%20medicamento%20%C3%A0%20popula%C3%A7%C3%A3o. &text=No%20Brasil%2C%20a%20Ag%C3%AAncia%20Nacional,em%20todo%20o%20territ%C3%B3rio%20nacional

[10]Brazil. Ministry of Development. National Institute of Industrial Property. INPI announces procedures after termination of prior approval for pharmaceutical patents. Published: Aug. 31, 2021, current. Nov. 3, 2022. Available at: https://www.gov.br/inpi/pt-br/central-de-conteudo/noticias/inpi-divulga-procedimentos-apos-extincao-da-anuencia-previa-de-patentes-farmaceuticas#:~:text=A%20Lei%2014.195%2C%20publicada%20em,C%20da%20Lei%2-09.279%2F1996

[11] Brazil. General Secretariat of the Presidency of the Republic. Official Press. Joint Ordinance no. 1, of April 12, 2017. Regulates the procedures for the application of article 229-C of Law No. 9,279, of May 14, 1996, added by Law n. 10,196, of February 14, 2001, and makes other provisions. Disponível em: http://www.in.gov.br/materia/-/asset_publisher/KujrwOTZC2Mb/content/id/20163436/do1-2017-04-13-portaria-conjunta-n-1-de-12-de-abril-de-2017-20163370

[12] Brazil. Services and Information from Brazil. National Institute of Industrial Property - INPI. Published on October 14, 2020, current. July 12, 2021. Available at: https://www.gov.br/pt-br/orgaos/instituto-nacional-da-propriedade-industrial

[13] Brazil. Superior Court of Justice. Doctrine: commemorative edition, 30 years of the STJ. Brasília: Superior Court of Justice, 2019.

[14] Brazil. Superior Court of Justice. Jurisprudence. Writ of Mandamus n. 3365/DF. JusBrasil. Available at: https://stj.jusbrasil.com.br/jurisprudencia/557578/mandado-de-seguranca-ms-3365?ref=feed

[15] Brazil. Federal Supreme Court. Jurisprudence. Writ of Mandamus No. 9562/SP. JusBrasil. Available at: https://stf.jusbrasil.com.br/jurisprudencia/687022/mandado-de-seguranca-ms-9562-sp

[16] Brazil. Superior Court of Justice. Anvisa's opinion

on pharmaceutical product patents is binding. Published: Aug. 18, 2021. Available at: https://www.stj.jus.br/sites/portalp/Paginas/Comunicacao/Noticias/18082021-Parecer-da-Anvisa-sobre-patente-de-produtos-farmaceuticos-e-vinculativo.aspx

[17] Brazil. Superior Court of Justice (3rd Panel). Special Appeal n. 1.191.612. Rapporteur: Minister Paulo de Tarso Sanseverino. Date of judgment: October 28, 2013.

[18] Brazil. Superior Court of Justice (3rd Panel). Special Appeal n. 1.315.621. Rapporteur: Minister Nancy Andrighi. Date of judgment: June 4, 2013.

[19] Campos, E. M. Patent infringement: compulsory license. Migalhas, Aug. 15, 2006. Available at:https://www.migalhas.com.br/dePeso/16,-MI28718,91041-Quebra+de+patentes+licenca+compulsoria

[20]Chagas, E. F. Marx's dialectical method: investigation and critical exposition of the object. Síntese [Journal of Philosophy at the Jesuit College]. 2011; 38 (120): 55-70. Available at: http://faje.edu.br/periodicos/index.php/Sintese/article/view/1036

[21]Coelho, F. U. Commercial law course: company law. São Paulo: Saraiva, 2010.

[22]National Association of Private Hospitals. Agreement between Anvisa and INPI will speed up patents for medicines. Published: March 24, 2017. Available at: https://www.anahp.com.br/noticias/noticias-do-mercado/acordo-entre-anvisa-e-inpi-vai-acelerar-patentes-de-remedios/

[23] National Institute of Industrial Property. Available at: http://www.inpi.gov.br/sobre/estrutura.

[24] Mendes, D. R. F.; Oliveira, M. A. C.; Pinheiro, A. A. Anvisa's prior consent: the evolution of the regulation of a State policy. Revista Brasileira de Políticas Públicas. 2014; 4(2): 156-172.

[25] Müller, M. Biotechnology: the future-present in the pharmaceutical industry. Institute of Science Technology and Industrial Quality - ICTQ, 2016. Available at: https://ictq.com.br/industria-farmaceutica/769-biotecnologia-o-futuro-presente-na-industria-farmaceutica

[26] Rodrigues, W. C. V.; Soler, O. Compulsory licensing of Efavirenz in Brazil in 2007: contextualization. Revista Panamericana de Salud Publica. 2009; 26(6):553-559. Available at: http://iris.paho.org/xmlui/bitstream/handle/123456789/9729/12.pdf?sequence=1

[27]Simões, I. What is the relationship between drug registration and patents? Migalhas, April 18, 2006. Available at: https://www.migalhas.com.br/dePeso/16,MI23436,31047-Qual+a+relação+entre+registro+de+medicamentos+e+patentes

[28] Storer, A.; Machado, E. D. Industrial property and the principle of the social function of property. In: National Congress of the National Council for Research and Postgraduate Studies in Law. 2007: 2320-2332.

RESEARCH PAPER

IJAMB
International Journal of Advances in Medical Biotechnology

The importance of the fourth dimensions of fundamental rights in biotechnology and its constitutional effectiveness

A. Eli Alves[1]; R. A. Bonotto Barbosa[1]

*Corresponding author: E-mail address: alexandre_elialves@hotmail.com

Received: 10 January 2024; 20 february 2024.

Abstract: This article analyzes the classification of biotechnology as part of the fourth dimension of fundamental rights, focusing on its essential content and effectiveness as a constitutional norm. The study explores how biotechnology is incorporated into international treaties and how this recognition impacts the enforcement of fundamental rights. A bibliographic and literature review was conducted, drawing on studies, scientific articles, and doctrines from respected researchers and specialists in the fields of biotechnology and fundamental rights. Sources were selected based on their relevance and contemporaneity, focusing on materials from the last ten years. The research examined key international treaties, such as the Convention on Biological Diversity, to assess their role in shaping biotechnology as a fundamental right.The findings reveal significant gaps in Brazilian legislation concerning biotechnology, which hinder the effective implementation of related fundamental rights, particularly in terms of equitable access and sustainable development. While international efforts to regulate biotechnology are advancing, national implementation remains inadequate. The study highlights the need for a more comprehensive normative framework and the development of public policies that ensure the responsible and safe advancement of biotechnology. It concludes that clearer legal interpretation and stronger policy measures are required to fully integrate biotechnology into the fourth dimension of fundamental rights, thereby promoting scientific and technological progress that benefits society effectively and safely.

Keywords: Biotechnology. Fundamental rights. Fourth dimension. Essential content. International treaties.

Introduction

Biotechnology, as a multidisciplinary science that encompasses various methodological techniques for the genetic manipulation of living organisms, has emerged as a powerful tool for developing products and services that benefit society across various aspects of contemporary life. The continuous evolution of this field has sparked debates regarding its classification as part of the fourth dimension of fundamental rights, representing a profound shift in current ethical, scientific, and legal discussions.

This scientific article aims to analyze the implications of classifying Biotechnology within the fourth dimension of fundamental rights, its essential content, and its effectiveness as a constitutional norm. To achieve this, the historical development of Biotechnology was examined to assess its impact on the effectiveness of fundamental rights. The relevance of this topic lies in the need to understand the challenges Biotechnology poses to contemporary society, especially in terms of its scope and implications for human rights. The classification of Biotechnology as a fundamental right, anchored in international treaties and agreements, holds the potential to ensure its applicability within national legislations, protecting its essential content and preventing restrictions that could hinder its safe and responsible development.

The primary objective of this article is to analyze Biotechnology as part of the fourth dimension of fundamental rights, its inclusion in international treaties, the recognition of its essential content, and the influence this classification has on the effectiveness of fundamental rights. The methodology utilized was based on bibliographic research and a comprehensive literature review, exploring the studies and doctrines of renowned researchers and experts in the field.

The following sections present the key findings and conclusions drawn from this analysis, as well as the ethical, scientific, legal, and social implications of classifying Biotechnology within the fourth dimension of fundamental rights. Finally, this article highlights the importance of appropriate regulation and the application of international treaties to ensure the responsible development of Biotechnology and its positive contributions to society.

[1] University of Araraquara - UNIARA, Araraquara, São Paulo, Brazil.

https://doi.org/10.52466/ijamb.v6i1.123

The importance of the dimensions of the fundamental rights

From the outset, it is important to note that, according to Lenza[1], the dimensions of fundamental rights represent a classification distinct from the older «generations» approach. This shift in perspective aims to avoid the notion of succession or replacement between dimensions, which could mislead the understanding of rights. There is no linear transition but rather a continuous addition of fundamental rights. In this research, we have therefore opted to use the term "dimensions" instead of "generations" when referring to this concept.

Sarlet[2] asserts that when analyzing fundamental rights, one must necessarily consider the historical evolution that stems from humanity's own transformations and progressions. These changes affect the content, ownership, effectiveness, and implementation of fundamental rights, resulting in what can be seen as a genuine mutation.

The importance of classifying fundamental rights lies in their connection to history and, consequently, the evolution of both rights and society. As Tavares[3] points out, human society's needs are infinite and inexhaustible, constantly being redefined and recreated, thus generating a cycle of new needs and advancements.

Therefore, discussing the various dimensions of human rights protections supports the argument that there is no eternal and immutable list of rights inherent to human beings. Instead, there is a continuous and persistent reconsideration of rights.[3]

According to Moraes[4],the Brazilian Supreme Federal Court, in its jurisprudence, has recognized only the triad of dimensions or stages, which will be discussed in the following section.

First dimension

The first dimension comprises what are known as rights of resistance, defense, and negative rights. Agra[5] notes that the origin of these rights coincides with that of the democratic rule of law, as they serve to limit the actions of an oppressive State—a legacy of absolutism—through the establishment of laws. At the same time, they guarantee civil and political rights, as well as freedom and private autonomy, without any state interference.

Thus, they are termed the first dimension, according to Wolkmer[6], because of their significance in the tradition of the political-legal institutions of Western modernity, which emerged during the 18th and 19th centuries. These rights reflect a historical context shaped by the ideologies of secularized natural law, Enlightenment rationalism, social contract theory, individualistic liberalism, and competitive capitalism.

Mendes[7] emphasizes that these rights began to be enshrined in national constitutions following the advances of the American and French revolutions. These revolutions prompted a shift towards the non-interference of rulers in citizens' lives, marking these rights as universal.

According to Moraes[4], the first generation represents a duty of omission, «in that rights to freedom are fulfilled through non-interference, respecting the individual's personal sphere and restraining the actions of the Liberal State.» Furthermore, «this generation includes individual rights that define the sphere of protection for individuals against State power, as well as political rights, which express the rights to nationality and political participation, synthesized in the right to vote and be elected[8]

By way of example, these rights include «freedom of expression, press, assembly, association, property, formal equality before the law, political participation, due process of law, habeas corpus, and the right to petition[5]. In summary, this is the first dimension.

Second dimension

The second dimension of rights emerged alongside the advancements of industrialization, which led to significant social and economic problems. At that time, socialist doctrine recognized that merely guaranteeing first-dimension rights was insufficient to ensure the effective enjoyment of those rights. Therefore, it advocated for a more active role from the State to ensure social justice through committed action.[2]

Barroso[8] further argues that the second dimension of rights is characterized by the consolidation of the social State, which arose in response to industrialization, struggles against inequality, and the spread of socialism. This dimension encompasses rights related to social freedoms.

According to Wolkmer, these rights correspond to «social, economic, and cultural rights» and are based on the principles of equality, with a positive scope. Instead of opposing the State, these rights require the State to guarantee and provide for the well-being of all individuals through public authorities.

This new dimension focuses not on protecting the individual from the State but on creating a list of claims that can be demanded from the State, which must act to satisfy these rights. Among the second-dimension rights are the right to work, protection against unemployment, a minimum wage, limits on

working hours, paid rest, and access to all levels of education.[3]

Moraes and Barroso[4],[8] classify these social rights as belonging to the second dimension, which requires the State to take action to ensure equality by addressing social, economic, and cultural needs. This aims to promote the full development of life in all its capacities, including labor rights and the provision of public services such as education, health, and social security, to enhance the general well-being of society.

In conclusion, the second generation of fundamental rights goes beyond simply ensuring provisions. What distinguishes this phase in the evolution of fundamental rights is its "positive" nature, implying that the State must assume the duty to act in order to meet social, economic, and cultural demands. This approach represents a significant advancement in the pursuit of equality and social well-being.

Third dimension

The fundamental rights of the third dimension are composed of rights of fraternity or solidarity, characterized by their transindividual, collective, and diffuse nature. This means that these rights are directed towards human beings as members of the human species, approached collectively. As a result, the responsibility for realizing these rights is also collective, not limited solely to the actions of the State but involving all members of society to ensure that these rights are upheld for everyone.[5]

Within this classification, according to Wolkmer[6], there are two doctrinal categories. The first offers a broad interpretation of solidarity or fraternity[9-12] and includes rights related to development, peace, self-determination of peoples, a healthy environment, quality of life, communication, and more.

The second provides a specific interpretation of transindividual rights. According to Oliveira Jr.[12], collective and diffuse rights fall into this category, gaining increasing significance in environmental law and consumer protection law.

In this realm, the third dimension includes the right to peace, development, environmental quality, and the preservation of historical and cultural heritage[7].

The third generation or dimension, still inspired by the motto of the French Revolution, centers on fraternity (or solidarity), encompassing rights that are not enjoyed individually but rather by society as a whole, directed towards the human race collectively. These rights have a global reach and require cooperation and collective responsibility for their realization.

The transindividual nature of these rights highlights their importance today, reflecting progress in the development and recognition of human rights. While first-dimension rights emphasize freedom and second-dimension rights highlight equality, third-dimension rights enshrine the principle of solidarity, establishing fundamental and enduring values within social formations. However, as can be observed, these generations of rights are cumulative and not mutually exclusive[8].

Fourth dimension

The fourth dimension, according to Wolkmer[6], arises from "new" rights, including biotechnology, bioethics, and the regulation of genetic engineering. These rights are directly connected to human life and encompass issues such as assisted human reproduction (artificial insemination), abortion, euthanasia, intrauterine surgeries, organ transplants, genetic engineering (cloning), contraception, and more[6]

This dimension will be explored in greater detail in the next section, where an in-depth analysis of biotechnology will be conducted.

Fifth dimension

Some scholars argue that the evolution of fundamental rights has reached a fifth generation, although opinions diverge on this matter. The most widely accepted classification refers to the rights of cybernetics and peace.

According to Oliveira Jr. and Wolkmer[6,12], this dimension addresses significant challenges arising from information technology, cyberspace, the internet, and virtual reality in general. As Wolkmer[6] emphasizes, the impact of developments in cybernetics, computer networks, electronic commerce, artificial intelligence, and the rapid dissemination of the internet has been extraordinary, both in the legal field and in global society at large.

Among dissenting voices, Sampaio[13] argues that this classification should instead focus on the duty of love and respect for all forms of life, advocating for the defense against all forms of prejudice.

Since the early 21st century, Bonavides[9] has supported the view that peace, as the opposite of war, must necessarily be recognized as a fundamental right and form a new dimension.

Peace, an aspiration held collectively over many centuries, is the culmination of all the reasons upon which human logic, under the guidance of law and justice, bases the act of governing society. It aims to punish terrorists, judge war criminals, imprison torturers, uphold the foundations of the social pact, and establish and maintain, as inviolable, the rules, principles, and clauses of the political community[9].

With this in mind, the concepts that doctrine

classifies as the fifth dimension are concluded, and the analysis moves toward the latest dimension.

Sixth dimension

The recent evolution of human rights in contemporary society has paved the way for the expansion of legal interests subject to judicial protection. Beyond material goods, the growing emphasis on ethical principles and new societal needs has brought additional concerns to the forefront, such as animal rights.

According to Agra[5], domestic animals were once considered mere objects, subordinated to property rights and governed by the provisions of the Civil Code. As a result of this conception, animals have not been recognized as holders of rights and, therefore, lack the legal standing to appear as parties within the legal system. Historically, they have been treated as property, tied to one of the parties involved in a dispute.

"To enable the legal recognition of non-human animals, it is necessary to attribute legal personality to them, detaching the concept of personhood from that of human beings—separating animals from the species Homo sapiens. In this regard, the 2002 Civil Code took a significant step by replacing the word 'man' with 'person' when addressing personality and capacity, highlighting that personhood and being human are independent concepts[5].

Thus, the discussion progresses toward a proposed sixth dimension of human rights, advocating for the recognition of the fundamental right to access potable water, which has gained prominence in international human rights law and comparative constitutional law. This right has become increasingly relevant due to its critical importance for life, health, and human development.[2]

In this context, Agra and Sarlet[5,2] argue that the evolution of human rights necessitates the consideration of new values and interests, encompassing the expansion of animal rights and the possible inclusion of the right to access potable water as a new dimension of fundamental rights.

The impacts of the biotechnology classification as fourth dimension

According to Diniz and Burillo,[14,15] biotechnology encompasses a set of methodological techniques that allow for the isolation of cells, animals, plants, or microorganisms to obtain products and catalyze chemical reactions that meet various human needs. This science of genetic engineering also enables the manipulation of living organisms, including the creation of transgenic or genetically modified organisms, with applications in the medicinal, scientific, industrial, agricultural, and environmental fields. As such, biotechnology represents a powerful tool for driving the production of goods and services that benefit society in multiple spheres of life. Furthermore, biotechnology, being multidisciplinary, is linked to a wide range of fields such as biology, microbiology, molecular biology, genetic engineering, cellular processes, organic and analytical chemistry, biochemistry, and biochemical engineering (bioprocesses).[16]

Biotechnology innovations have had a significant impact on the modern world, utilizing biological systems, living organisms, and their derivatives to manufacture or modify products and processes, thus driving development.[16]

Although the origins of biotechnology date back to the earliest stages of human history, its development, as cataloged by scholars, is more closely associated with recent history. In the 19th century, key figures such as Pasteur made advances in microbial fermentation processes. Between the 1940s and 1950s, efforts focused on antibiotic production, particularly the work of Chain and Florey in advancing Fleming's discovery of penicillin. In the 1950s, advances in biochemistry led to a better understanding of intermediary metabolism, while the 1960s saw significant progress in molecular genetics. The 1970s marked a turning point with the discovery of restriction enzymes by Arber, Smith, and Nathans, as well as ligases to join DNA fragments.[17]

The discovery of molecular DNA recombination sparked a revolution in biotechnology, with applications in various fields, generating numerous debates about its use in transgenic animals and plants, stem cell therapies, gene therapy, biological drugs, and vaccines. These innovations led to unprecedented impacts and, at the same time, raised ethical and moral questions about human rights.

Within this context, some scholars argue for the emergence of a fourth generation of rights to address challenges related to the increasingly complex effects of biological research, which allow for manipulation of an individual's genetic heritage. What are the limits of this potential (and increasingly likely) manipulation?[18]

Although there is no consensus on the subject, Ramos[19] notes that even critics acknowledge that the inexhaustibility of human rights transcends didactic classifications, requiring a broad understanding of these essential rights for a dignified human life.

As a result, great discussions and challenges of the new millennium arise, confronting the limits of science and the difficulties of legislating and regulating biotechnology on an international level. Sauwen[20] emphasizes that solutions to issues

such as procreation techniques, embryo and organ trafficking, the production of biochemical weapons, cloning, and other developments in genetic engineering must find their effectiveness in international agreements. For this reason, Mazzuoli and Bonavides[19] point out that this dimension reflects the globalization of fundamental rights, expanding beyond borders. In addition to biotechnology, this dimension includes participatory democracy, the right to truthful, non-manipulated information, and a universally dignified society.

The challenges continue to grow, as biotechnology's multidisciplinary nature confronts jurists, biologists, philosophers, theologians, psychologists, sociologists, and various humanists and health professionals, each with differing cultures and beliefs. This divergence makes it difficult for society to support biotechnology and to communicate effectively with the scientific community and the public.

Given these challenges, both biotechnology and bioengineering must consider scientific and ethical issues. Maluf[22] argues that the State must legislate, regulate, and ensure the dissemination of knowledge and safety standards, while also allowing for a broad ethical interpretation that extends beyond state competence and enters the delicate realm of individual rights.

This chapter is critical to this study's analysis, as it is directly connected to the essential content and, consequently, the effectiveness of fundamental rights, which will be explored further in the following sections.

The essential content of biotechnology and the effectiveness of the fundamental right

The importance of recognizing biotechnology as a fourth-generation right, for us, is not limited to mere didactic classification; it involves an evolution of human rights, which, being established in international treaties and agreements, ensure their applicability in domestic law. Being established in international treaties and agreements, ensure their applicability in domestic law. As a consequence, this right can be considered a fundamental right which, according to Agra,[5] is divided into two parts: the first being its core essence, and the second, its peripheral zone.

The core essence or essential content is configured as the limit that must be respected by the Supreme Federal Court when determining the density of a right, which, in no way, can be disregarded by judicial decisions, prohibiting its emptying or transformation into an exception. This core essence is defined as the very essence of the right, which must be realized regardless of factual circumstances.[5]

Silva,[23] in analysis from a strictly objective dimension, found that the essential content must be interpreted and applied as a fundamental right in the entirety of social life. Consequently, this right also means prohibiting restrictions to the point of making it inapplicable to all individuals or part of them.

According to Agra[5] understanding, only the peripheral zone will depend on factual circumstances but with applicability aimed at the principle of maximum effectiveness of fundamental rights.

The recognition of the fourth dimension of fundamental rights and the necessity of including their essential content to be protected is essential; otherwise, the application of balancing and proportionality in the interpretation of the norm will necessarily imply the possibility of restriction to the point of compromising the evolution of biotechnology in the country.

The current domestic legislations in our country are insufficient to regulate the issue, as they are limited to only a few laws,[24,25,26] which are absolutely insufficient to regulate the matter in our country.

Precisely because of this deficient domestic legislation, we understand that it is imperative to apply the international treaties and agreements that already have advanced precedents on the subject, justifying their applicability.

Piovesan's doctrine[27] argues that, for a large portion of contemporary internationalists, international law supersedes the State and highlights its supremacy over domestic law because it derives from a principle that is above to the will of the States. It is not to say that the power of the State is a delegation of international law; but it seems indisputable that international law constitutes a legal limit to said power.[27]

In this context, it is evident that there are already major international treaties and agreements, as observed in Table 1.

Given the existing gap in our legal system regarding biotechnology, it is imperative to apply the Federal Constitution of 1988, which explicitly enshrines, in its article 5, paragraph 2, an open clause for the inclusion of new fundamental rights. This provision states that the rights and guarantees expressed in the Constitution do not exclude others arising from the regime and principles it adopts or from international treaties to which the Federative Republic of Brazil is a party.[28]

Thus, when analyzing article 5, paragraph 2, of the Federal Constitution of the Republic of Brazil, it is noted that these rights are organized into distinct groups: one is the rights expressly stated in the Constitution (for example, the rights listed in subsections I to LXXIX of article 5 and in other

provisions scattered throughout the text of the Magna Carta); another is the rights expressed in international treaties to which Brazil is a party; and finally, a third group is the implicit rights (those implied in the guarantee rules, as well as those arising from the regime and principles adopted by the Constitution)[27].

Thus, there is support not only for the recognition of biotechnology as a right provided for in international treaties, but also, considering the premises of this right in our legal system, it is possible to recognize the fourth dimension of fundamental rights and, as a result, the essential content of biotechnology. This comes along with new principles already admitted internationally, which will allow an analysis without implying restrictions on development.

Table 1 – Main international treaties related to biotechnology, biolaw and genetic engineering.

International treaty/convention	Synthesised description
Convention on Biological Diversity (CBD)	Seeks to conserve biological diversity, use its components in a sustainable manner, and ensure the fair and equitable sharing of the benefits arising from the use of genetic resources.
Cartagena's Protocol on Biosecurity	Addresses the safety in handling, transporting, using, and transferring genetically modified organisms (GMOs) to protect biodiversity and human health.
Internacional Convention for the Protection of Plants Varities	Establishes international standards for the protection of intellectual property rights related to plant varieties developed through biotechnology.
Plants Varieties (UPOV)	Promotes the protection of intellectual property rights of breeders of new plant varieties, encouraging innovation in agriculture and the development of cultivars with beneficial traits.
Convention on the elimination of all forms of Discrimination against Women	Aims to eliminate discrimination against women, including health and reproduction issues that may have implications in biotechnology and biolaw.
Convention on People with Disabilites	Seeks to guarantee the rights of people with disabilities, including access to health services and technologies that may involve genetic engineering and other biotechnological applications.
Paris Agreement	Sets goals and actions to limit global warming to 1.5°C above pre-industrial levels, with impacts on bioenergy technologies and genetic engineering.

Source: produced by the authors (2023).

For the judge, instead of seeking to analyze the matter solely with constitutional principles, it will be possible to interpret it with the recognition of the essential content of biotechnology, avoiding restrictions on legal knowledge.

Therefore, the incidence of systematic and teleological interpretation is guaranteed with constitutional principles and with the applicability of the principle of maximum effectiveness of fundamental rights, emphasizing the dignity of the human person, based on a balance with human rights parameters, analyzing constitutionality.

The recognition of the fourth generation of fundamental rights and the inclusion of biotechnology as essential content, stemming from international treaties, give new contours to the effectiveness of fundamental rights, thus ensuring the absence of restrictions on technological advances that may be compromising the nation's development.[27]

Final considerations

This article is based on the premise of the importance of classifying biotechnology as a fourth-generation right, exploring its essential content and effectiveness as a fundamental right. Through a literature review and analysis of international treaties and agreements, the study aimed to understand the relevance of this multidisciplinary field, which encompasses various areas and methodological techniques for innovating processes and products to meet the demands of contemporary society.

After examining the dimensions of fundamental rights, the study aligns with the doctrine that advocates recognizing biotechnology as part of the fourth dimension of fundamental rights, addressing various societal concerns. To this end, a historical analysis of biotechnology from the 19th to the 20th century was conducted, highlighting significant mi-

lestones that enabled advances in the field. The major turning point came with the discovery of DNA molecular recombination, one of the key events that paved the way for previously unimaginable technological developments, such as transgenic organisms and stem cell-based therapies.

Having reached this stage, attention is directed to the essential content of biotechnology and its effectiveness as a fundamental right. Once its essential content is identified, this right must be respected and implemented regardless of factual circumstances. Additionally, the importance of protecting and regulating biotechnology was emphasized, with a focus on the need to use international treaties as references, given the insufficiency of domestic legislation.

In light of legislative deficiencies and significant gaps in the legal system, the relevance of the 1988 Federal Constitution is underscored as a source of support for recognizing biotechnology as a fundamental right. Article 5, paragraph 2 of the Constitution allows for the inclusion of new rights arising from international treaties, thereby strengthening the legal framework surrounding this complex issue.

The final considerations reaffirm the importance of biotechnology as a powerful tool for societal advancement, while emphasizing the need for a careful approach that accounts for ethical and moral issues as well as implications for human rights. Technological development in this field must be accompanied by appropriate legislation and regulation to ensure the safe dissemination of knowledge and the responsible use of advancements.

In this context, a systematic and teleological analysis, combined with the principle of maximum effectiveness of fundamental rights, allows for a coherent interpretation of the subject without undue restrictions from the subjectivity of judges or legislators. Recognizing the fourth dimension of fundamental rights and including biotechnology as essential content is crucial to ensuring the evolution of this field while respecting human dignity and advancing national goals of economic and social development.

Finally, the study highlights the importance of future research to deepen understanding of biotechnology, addressing its limitations and expanding knowledge. Science, law, and society must work together, drawing appropriate support from the scientific community and broader society, and developing clear and transparent communication about the challenges and possibilities of biotechnology.

In conclusion, the analysis of the implications of classifying biotechnology as part of the fourth dimension and recognizing its essential content as a fundamental right underscores the significance of this field in today's world. This study contributes to the advancement of scientific knowledge and ensures dignified coexistence by respecting the ethical, moral, and legal dimensions that encompass biotechnology in its various applications.

References

[1]. Lenza P. Constitutional Law schematized v. 3. São Paulo: Saraiva; 2021.

[2]. Sarlet IW, Marinoni LG, Mitidiero D. Course in Constitutional Law 10. São Paulo: Saraiva; 2021.

[3]. Tavares AR. Course in Constitutional Law. 21. São Paulo: Saraiva; 2023.

[4]. Moraes GP. Course in Constitutional Law. São Paulo: Atlas; 2018.

[5]. Agra WM. Course in Constitutional Law. 9. Belo Horizonte: Fórum; 2018.

[6]. Wolkmer AC. Introduction to the fundamentals of a general theory on "new" rights. Rev Juríd [Internet]. 2013 Ago [mentioned in 15 jul. 2023];2(31):121-148. Available at: http://revista.unicuritiba.edu.br/index.php/RevJur/article/view/593/454

[7]. Mendes GF, Branco PG. Course on Constitutional Law. 15. São Paulo: Saraiva; 2020.

[8]. Barroso LR. Course on Contemporary Constitutional: the fundamental concepts and the construction of a new model. 9. São Paulo: Saraiva Education; 2020.

[9]. Bonavides P. Course on Constitutional Law. São Paulo: Malheiros; 2011.

[10]. Lafter C. The reconstruction of Human Rights. São Paulo: Companhia das Letras; 1998.

[11]. Bedin GA. The rights of man and neoliberalism. 2. Publlisher Ijuí: Unijuí; 1998.

[12]. Oliveira Jr JA. Juridical Theory and new rights. Rio de Janeiro: Lumen Juris; 2000.

[13]. Sampaio JAL. Fundamental Rights: rethoric and historicity. Belo Horizonte: Del Rey; 2004.

[14]. Diniz MH. The present state of biolaw. São Paulo: Saraiva; 2001.

[15]. Burillo IZ. In: Casabona CMR, org. Biotechnology, law and bioethics: prospects in comparative law. Genetic Biotechnology in agriculture and livestock. Belo Horizonte: Del Rey and PUC Minas; 2002.

[16]. Alves AE, Bonotto RA, Claudino MC. Vesting' s contract at biostartups: risks, challenges and considerations. Rev Bras Direito Empres [Internet].

2022 [quoted in 22 jul. 2023];8(2):25-46. Available at: https://www.indexlaw.org/index.php/direitoempresarial/article/view/9237/pdf.

[17]. Grisolía S. In: Casabona CMR, org. Biotechnology in the third millenium. Biotechnology, law and bioethics. Belo Horizonte: Del Rey; 2002.

[18]. Bobbio N. The age of rights. Rio de Janeiro: Elsevier; 2004.

[19]. Ramos AC. Course on human rights. 7. São Paulo: Saraiva; 2020.

[20]. Sauwen RF, Hryniewicz S. "In vitro" law: from bioethics to biolaw. Rio de Janeiro: Lumen Juris; 1997.

[21]. Mazzuoli VO. Course on human rights. 6. Publisher São Paulo: Método; 2019.

[22]. Maluf E. Genetic manipulation and criminal law. São Paulo: Juarez de Oliveira; 2002.

[23]. Silva VA. Fundamental laws: essential content, restrictions and effectiveness. 2. Publisher São Paulo: Malheiros; 2009.

[24]. Brasil. Decree n. 6.041, February 7th, 2007. Instituted the Biotechnology Development Policy, the National Biotechnology Committee is created and offered other measures. [Internet]. Brasília, DF: Republic Residency; 2007 [quoted in 24th July 2023]. Available at: https://www.planalto.gov.br/ccivil_03/_ato2007-2010/2007/decreto/d6041.htm

[25]. Brazil. Law n. 10.332, December 19th, 2001. Instituted financing mechanism to the Science and Technology for Agriculture Business Program, to the Promotion of Health Research Program, to the Biotechnology and Genetic Resources Program – Genoma, to the Science and Technology Program for the Aviation Sector and to the Competitiveness Innovation Program, and offered other measures. [Internet]. Brasília, DF: Republic Presidency; 2001 [quoted in 24th July, 2023]. Available at: https://www.planalto.gov.br/ccivil_03/leis/leis_2001/l10332.htm

[26]. Brazil. Law n. 11.105, March 24th, 2005. Regulates the Subsections II, IV, and V of § 1 of art. 225 of the Federal Constitution establish safety standards and oversight mechanisms for activities involving genetically modified organisms (GMOs) and their derivatives, create the National Biosafety Council (CNBS), restructure the National Technical Biosafety Commission (CTNBio), provide for the National Biosafety Policy (PNB), revoke Law No. 8,974 of January 5, 1995, Provisional Measure No. 2,191-9 of August 23, 2001, and articles 5, 6, 7, 8, 9, 10, and 16 of Law No. 10,814 of December 15, 2003, and make other provisions. [Internet]. Brasília, DF: Republic Presidency; 2005 [quoted in 24th July, 2023]. Available at: https://www.planalto.gov.br/ccivil_03/_ato2004-2006/2005/lei/l11105.htm

[27]. Piovesan F. Human Righrs and the International Constitutional Law. 7. Publisher. São Paulo: Saraiva; 2006.

[28]. Brazil. [Constitution (1988)]. Constitution of the Federative Republic of Brazil [Internet]. Brasília, DF: Republic Presidency; 1988 [quoted in 24th July, 2023]. Available at: https://www.planalto.gov.br/ccivil_03/constituicao/constituicao.htm

[29]. Moreira E, Wanghon MO, Costa CR, Mileo BAP, Pereira PAR, Pinheiro VS. Biotechnological patents: an essay on the impacts of the biotechnology effects in the Brazilian Systems of Patents [Internet]. Belém, PA: CESUPA; [Unknown date] [quoted in 29th Jan 2024]. Available at: https://www.cesupa.br/saibamais/nupi/doc/PRODUCAONUPI/Patentes%20Biotecnol%C3%B3gicas.pdf.