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Review

ANIMAL MODELS IN BICOMPATIBILITY ASSESSMENTS OF IMPLANTS IN SOFT AND HARD TISSUES

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"We should never resort to experiments in cases where observation can provide the necessary information; no experiment should be performed without a clear and definite goal and without the conviction that the goal will be achieved and that the result will be real and simple; we should not unnecessarily repeat experiments (only) if we cause the least suffering, using the lowest ranks of animals and avoiding inflicting pain, we should try to provide adequate observation to avoid the need for repetition". Marshall Hall, 1847.

Abstract

The ethical dilemmas of using animals as *in vivo* models in preclinical and clinical examinations have been increasingly present in recent decades. Small laboratory animals

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(rats, rabbits) will continue to be used because they are cost-effective and permit the formation of statistically testable cohort groups; a task that, for financial, maintenance and care reasons, is almost prohibitive for larger animals. Technological advances in the production of new biomaterials for clinical use are enormous, but screening tests and methods used to assess biocompatibility lag behind these advances. The assessment of biological responses is slow and based on millennial recovery mechanisms in eukaryotic organisms. Therefore, the goal of researchers in this field is to re-evaluate old methods of biocompatibility assessment and introduce new methods of evaluation, especially for *in vivo* testing. In that sense, a revision of the ISO standards was planned and conducted in 2017, which insisted on cytotoxicity testing in cell lines and produced concrete proposals on how biocompatibility should be quantified. *In vivo* biocompatibility evaluation of biomaterials used for soft tissue recovery commonly utilises rats. Rabbits are recommended for implants used for hard tissues, because of the rabbit's size, the possibility of implanting the biomaterials on a larger bone surface, and because of the peculiarities of rabbit bone tissue that favours rapid recovery after bone defects and enables easy reading of the results.

Key words: biocompatibility, bone, rabbit, rat, subcutis

INTRODUCTION

Until recently, all chemicals intended for human consumption (e.g. hair dyes) were subject to animal testing, which is now prohibited in many cases (Beck et al., 1994). Many pharmaceuticals and cosmetics are now labelled as "not tested on animals". Experimentation on animals in order to test and research a new substance and monitor its effect in the body are depicted in ancient drawings and writings. There are traces of these procedures in ancient Egypt, China and India, countries that were the cradle of traditional medicine. The first recorded experiments date from the 5th century BC. Aristotle wrote about these kinds of experiments in the 4th century BC. Galen of Pergamon, in the 2nd century BC, was the first to record some standards in animal dissection. With the development of medicine and experimental sciences in the 17th century, William Harvey created an experimental protocol for working with animals by studying the bloodstream of the mammalian organism. In the 18th century, Voltaire wrote about various experiments performed on animals. The 19th century brought about an expansion of the natural sciences which set the official patterns for this type of research. Claude Bernard wrote: "An introduction to the study of Experimental Medicine", and Jeremy Bentham noted: "It is not a question of whether animals can reason or speak, but whether they can (physically) suffer". These actions, coupled with actions of other philosophers and scientists, highlighted the ethical problem of animal suffering that can be caused during research. In 1824, the first association for prevention of animal cruelty was made official. In 1835, under the patronage of Princess Victoria, an animal protection society was formed. In 1854, after the dramatic death of two dogs with experimentally induced epilepsy, there was a stormy public reaction (at a British Medical Association meeting), and in 1875, anti-vivisection associations were formed and a petition for preventing animal abuse for scientific purposes was issued.

Recently, significant technological advances have been achieved in the production of new biomaterials (Williams, 2004; Nešović et al., 2017; Surudžić et al. 2016a; Abudabbus et al., 2018; Nešović et al., 2020; Đošić et al., 2009). Chemical engineers, chemists and biomaterials experts are contributing to the development of novel biomaterials by introducing new methods (nanotechnology in recent years). They are an essential part of multidisciplinary teams dealing with research into biomaterials necessary for clinical use and substitution of damaged tissues in veterinary and human medicine and dentistry (Surudžić et al., 2016b; Abudabbus et al., 2016; Abudabbus et al., 2018; Nešović et al., 2018; Nešović et al., 2019a; Nešović et al., 2019b; Đošić et al., 2017; Stevanović et al., 2018; Stevanović et al., 2020a, Mohan et al., 2018).

Before experiments on animals are performed, alternative procedures, such as mathematical models, *in vitro* research, and species selection on a lower phylogenetic scale, must be ruled out. For those *in vivo* experiments that are deemed necessary, the 3R conditions must be implemented (Russell & Burch, 1959). 3R stands for: reduction - using the smallest possible number of animals; refinement - using the same animal for repeated or different experiments, using improved surgical techniques, improving care conditions or reducing the dose/volume of drugs and; replacement - using cell cultures, microorganisms, chemical, *in silico* models (computer and mathematical models and statistics) or other methods.

However, an ultimate condition for using biomaterials is to determine their safety in clinical testing *in vivo* on experimental animals before using them in clinical therapy or tissue replacement (Rand, 2008). This has caused the great increase in the number of disciplines, organizations and experts who deal with animal welfare issues, including purposeful selection of animal models for specific experimental protocols and analyses (Williams, 2006). The exploration of animal species characteristics, similarities and differences, in morphological and physiological terms (Monteiro-Riviere et al., 1990; Grada et al., 2018), is necessary in order to correctly interpret the underlying mechanisms of the foreign body response to new substances: drugs, cosmetics, medical devices, implants, artificial materials or tissue engineering organs intended for tissue replacement (Vecchia and Bunge, 2005). Before introducing artificial substances into a mammalian body, detailed preparation and verification of the reaction that will occur after a specific time is required (Van Ravenzwaay and Leibold, 2004; Grada et al., 2018).

The ultimate goal of all *in vivo* biomaterial research procedures performed on animals is to obtain beneficial results at the lowest possible cost. This can be accomplished by using a justified number of animals, by applying protocols that minimize animal suffering and provide them with quality of life at a humane level, and by providing adequate care during the experiment. The main idea is that the results obtained justify the experiments performed and contribute to mankind, other animals and the environment (Dorsett-Martin and Wysocki, 2008; Engel et al., 2015). Depending on the type of substance or biomaterial implanted in the body, as well as the tissues in which it is implanted (skin, bone, muscle, etc.), the selection of animal species

must correspond precisely with the material used and its intended purpose (Upman and Meunch, 2004). The countries of the European Union have developed national standards with the aim of establishing joint global projects, such as those promulgated by the Federal Drug Association (FDA) and International Standards Organization (ISO), to create a basis for common international standards that would establish strict *in vivo* protocols, and also enable efficient exchange of information.

EU LEGISLATIVE COMPLIANCE AND ETHICS COMMITTEE APPROVAL IS THE FIRST STEP IN CODUCTING *IN VIVO* BIOCOMPATIBILITY ASSESSMENT EXPERIMENTS

Animal experimentation requires a solid and rational moral foundation, and it is, therefore, necessary to continuously monitor and improve directives and regulations dealing with the conditions under which animals can be used in research. Researchers have to make decisions that are objective and emphatic and, together with ethics committees, have to keep re-evaluating protocols that remain a difficult and sensitive matter. In Serbia, experimental animals are governed by the Law of Animal Welfare (Official Gazette, 2009), which is in accordance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. According to Serbia's animal welfare law, animal testing can only be performed by individuals listed in the Register for Animal Testing, which is maintained by Ministry of Agriculture, if they meet the requirements regarding facilities, equipment and training in welfare of experimental animals (Article 33). Animal testing can be performed based on only approval brought by the Minister of Agriculture, which in turn is based on the expert opinion of the Ethics Commission for the protection of welfare of experimental animals (Article 34). Individuals working with experimental animals must appoint an expert in the protection of experimental animals' welfare to overview the research; the expert must fulfil the criteria: academic studies graduate - master in veterinary sciences (DVM), or basic studies in veterinary science lasting at least five years - and be trained in experimental animal welfare (Article 35). Surgical interventions on animals during the experiment are performed only by a veterinarian or an authorized scientist (Article 36). Animals must be provided with appropriate housing, care and attention before, during and after the experiment (Article 40). Animal testing cannot be performed if there are alternative methods of conducting the analysis that achieves the same goal. Animal tests must be performed on those animals that best suit the purpose of the test, and on the smallest number animals needed to achieve that goal. When conducting experiments on animals, methods that inflict the least pain, suffering, fear or stress must be used (Article 41). For the purpose of considering professional issues, providing expert opinion and participating in the realization of project tasks in the field of animal welfare, the Minister of Agriculture, in accordance with the regulations governing the state administration, establishes a special working group – the Ethical

Council for experimental animals' welfare (Article 48). Scientific research organizations and other legal entities conducting animal experiments form an Ethics Commission for the protection of experimental animals' welfare, either within their organization or together with other scientific research organizations or legal entities conducting animal experiments. The Ethics Commission consists of veterinary surgeons, veterinarians with experience in breeding experimental animals, experts with experience in applying statistics in research, representatives of associations and organizations with the goals of protecting animal welfare, and researchers from related scientific fields (Article 51). Scientific research organizations and other legal entities conducting animal experiments, besides forming their own Ethics Commission, must implement their own policy for the protection of experimental animal welfare, which must be in accordance with the Law on Animal Welfare. Based on the Law on Animal Welfare, various rulebooks have been established on: conditions for entry in the register for animal examinations; content and manner of maintaining the register; experimental animal welfare training program; application form for approval of conducting animal examinations; method of care; treatment and euthanasia of experimental animals; content and manner of keeping reproduction, traffic, care, management and experimental records (Official Gazette, 2010). The EU Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Directive 86/609/EEC, 2013) must be adhered to. Finally, there is an international organization of animal welfare experts, the Regional Animal Welfare Centre (RAWC), whose members are countries of the Balkan peninsula. Its primary goal is to build structures and processes to actively share knowledge and expertise related to implementing the EU legislation and to facilitate consistent implementation and enforcement of the EU legislation by identifying difficulties, bottlenecks and related risks for animal welfare (Regional Animal Welfare Centre, 2015).

BIOCOMPATIBILITY

Biocompatibility refers to the behaviour of biomaterial introduced into a living organism (Williams, 1990). According to the IUPAC definition, biocompatibility (biomedical therapy) is the ability of a material to act with an adequate host response in a given situation (Vert et al., 2012). Biocompatibility is the ability of a material to come into contact with a living system and not cause an adverse effect (IUPAC, 2012). Initial material safety tests are first assessed by a large number of *in vitro* tests that comply with ISO 10993 (or similar standards) (Upman and Meunch, 2004). These tests do not determine the biocompatibility of the material, but they are a very important step towards animal testing (preclinical and clinical trials) that will later determine the biocompatibility of the material (European Commission [2004]; OECD, 2004; USEPA, 2004).

To assess the biological safety of biomaterials, general and special toxicological tests are used, which give insight into their toxicity. Tests include assessment of: 1)

carcinogenicity, 2) mutagenicity, 3) teratogenicity, 4) antigenicity, 5) implantation reaction, 6) mucosal and skin irritation, 7) haemolysis, 8) exothermic reaction and 9) cytotoxicity (Ishikawa and Matsuya, 2003). In the implantation test, inflammation, necrosis and biomaterial encapsulation are examined histologically, i.e., all cellular and tissue parameters of the organism's response to the introduced foreign body are evaluated (Ishikawa and Matsuya, 2003). Histological evaluations can be analysed semiquantitatively, quantitatively, qualitatively, morphometrically, and by using immuhistochemical analyses of the tested tissue in contrast with intact tissue and with biomaterials that have already been tested and proven harmless when introduced in the living organism. Following the recommendations of the standard for test procedures, all changes are observed for specific time periods. In soft tissue, this is 7, 15, 30 and 60 days after implantation in the subcutis, but in bone tissue is from 7 days to 6 months and longer, given that the time for recovery and ossification of a bone defect is significantly longer than soft skin tissue regeneration (Marković et al., 2009, Lužajić Božinovski et al., 2018).

RAT, A COMMON ANIMAL MODEL FOR ASSESSING SOFT TISSUE BIOCOMPATIBILITY

Rodents are a very common animal model for evaluating reparation of tissue damage. (Monteiro-Riviere et al., 1990; Vechia and Binge, 2005; Dorset-Martin and Wysocky, 2008; Engel et al., 2015; Grada et al., 2018). The regeneration of rat skin tissue is faster and more effective than in many other animal species (Rand, 2008). The subcutis, according to screening test protocols, can be used to evaluate the biocompatibility of both hard and soft materials (Upman and Meunch, 2004).

Novel biomaterials must be tested, whether they are hydrogels intended for skin wound healing or hard materials such as various Ca-P hydroxyapatites which are mainly used for tissue regeneration. Both these types of materials are initially tested in the subcutis (Stojanović et al., 2008; Marković et al., 2009; Marković et al., 2012). Subcutis screening tests (measuring biocompatibility, assessed by evaluating the host's reaction to the foreign material) evaluate the body's defence response, which can be an indicator of the harmlessness of any substance that enters the living organism (Van Ravenzwaay and Leibold, 2004). Therefore, the subcutis is the first place to assess the biocompatibility of any material intended for future clinical use (Williams, 2006). The biocompatibility and harmlessness of different hard biomaterials intended for bone treatment are also initially tested in soft tissue (i.e. the subcutis). The idea behind this concept is as follows: if the material shows low levels of biocompatibility in the subcutis, it will not pass to the next stage (testing on bone tissue). This order of testing lessens the suffering of the experimental animals used and saves funds and time (Anderson, 2016; Ratner, 2016; Marković et al., 2009).

Dynamic multifunctional hydrogel scaffolds show great promise in treating skin repair disorders, which is seen through the stimulation of regenerated tissue in

different types of exposed wounds (Wu et al., 2007; Kamoun et al., 2017; Stojkovska et al., 2018). The scaffolds are particularly appropriate in situations with disturbed physiological function when there is a high risk of bacterial infections (Pencheva et al.,

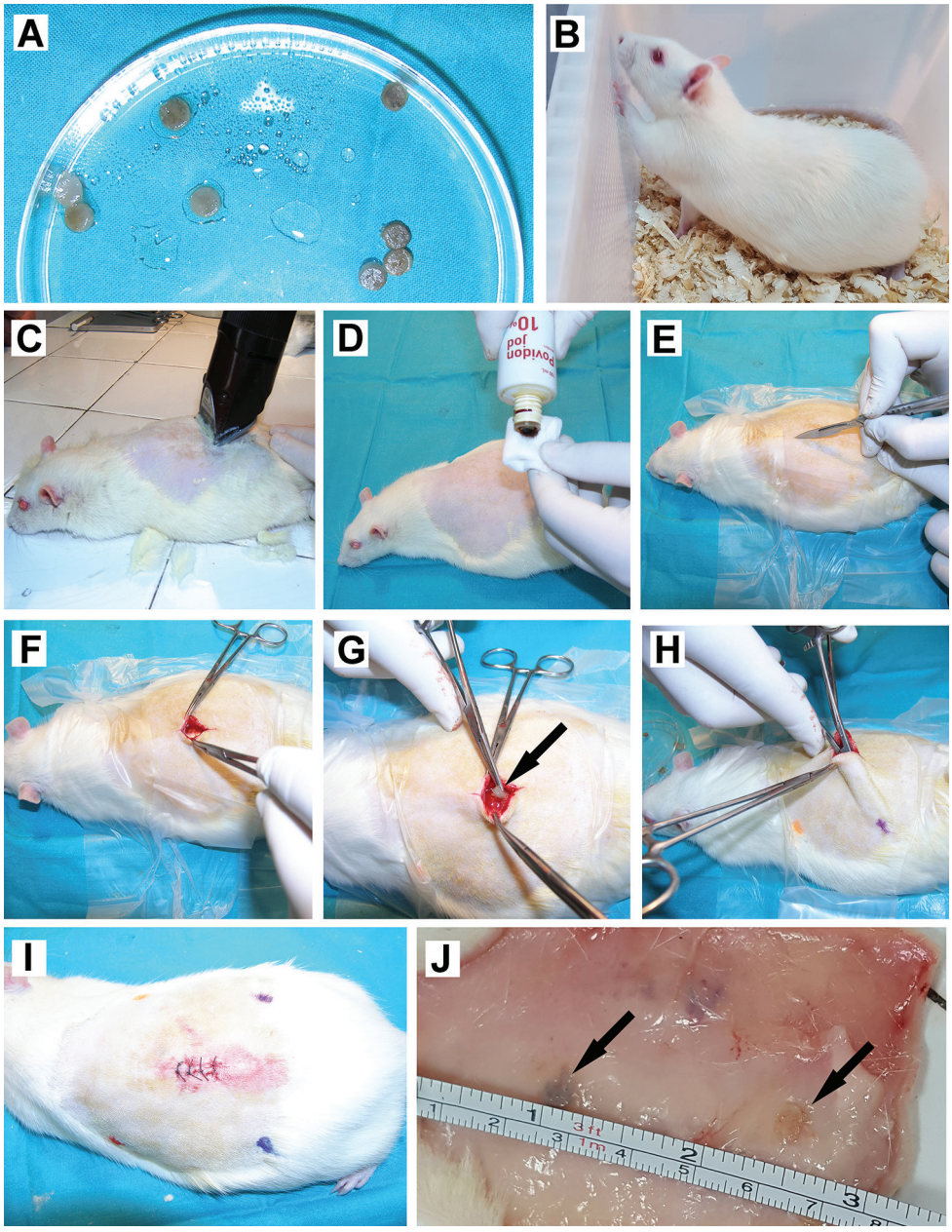

Figure 1. Surgical implantation of hydrogels performed in accordance with the permit issued by the Ethical Committees of the Faculty of Veterinary Medicine University of Belgrade (Certificate No. 01-488: 323-07-4087/2016-05/6).

2012; Fun, 2014). Materials that are nanotechnologically enhanced with silver particles or graphene, which have antibacterial properties and improve resilience and strength, have shown satisfactory levels of biocompatibility and promote skin regeneration (Lužajić Božinovski et al., 2018).

Animals and experimental protocol

Novel silver/polyvinyl alcohol (Ag/PVA) and silver/polyvinyl alcohol/graphene (Ag/PVA/Gr) hydrogels, potential wound dressing biomaterials, were electrochemically synthesized (Abudabbus et al., 2016; Abudabbus et al., 2018; Nešović et al., 2017) at the Faculty of Technology and Metallurgy, University of Belgrade. Sterilized nanocomposite hydrogel discs, 5 mm × 2 mm, and commercial Suprasorb®©, a calcium alginate dressing (Fig 1A), were implanted in the subcutaneous tissue of adult rats (Wistar/Albino), 3-4 months of age, 250-350 g average weight (Figure 1B). At the midline level of the of the thoracic spinal column of the rat, a small skin incision was made to create three pockets in the subcutis of the lateral sides of the back, which were distanced 3 cm from the back midline (Figure 1C, 1D, 1E, 1F). The implants were introduced in the pockets and the distance between each implant area was about 4.5 to 5 cm (Figure 1G, 1H). Surgical implantation of the hydrogels was performed according to the permit issued by the Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade (Certificate number No 01-488: 323-07-4087/2016-05/6). All animals survived the surgery and no wound healing complications were observed, either after the surgery, or during the whole experiment. The experimental materials were retrieved from the rats 7, 15, 30 and 60 days post implantation. The animals were sacrificed and the implants together with the surrounding tissue were dissected (Figure 1I, 1J). Tissue samples were processed for histological procedures and analysis.

RABBIT, A COMMON ANIMAL MODEL FOR ASSESSING HARD TISSUE BIOCOMPATIBILITY

Rabbits are commonly used in animal experiments given that they are easily handled, have a rapid bone turnover rate, and are fully mature within 6 months (Grada et al., 2018). Rabbit cranial defects are the first choice for basic verification of bone graft materials and evaluations of bone tissue regeneration (Pripatnanont, 2009; Xu, 2008; Micić, 2020).

It is crucial to correctly mark the anatomical regions for implantation in the rabbit animal model to enable proper evaluation of biomaterials (Sohn et al., 2010). Critical size defects (CSD) are based on the anatomy and histology of the rabbit tissue and are studied in the different bones used in implantology (Borie et al., 2017; Micić, 2020). Although there are only minor differences in bone composition between various animal models and humans, when choosing an appropriate model, these differences

must be taken into consideration because they can be essential to the outcome of the experiment (Shand 2002). No single species fulfils all the requirements of an ideal model, and therefore, an understanding of the differences in bone architecture and remodelling is essential when selecting a suitable model for testing (Frame, 1980). The most common bone defect models are located in the tibia, radius, mandible and the cranium (Kleinschmidt and Hollinger, 1992). Among them, the cranial model is best suited for implantation, given its biological inertness due to poor blood supply and limited bone marrow (Frame, 1980; Le Guehennec et al., 2005). Unlike some other tissues (e.g. cartilage), bone has great regenerative potential (Van't Hof et al., 2003; Pérez-Sánchez 2010). In many situations, when bone fractures or other injuries occur, the bone can heal without the formation of scar tissue (Sohn et al., 2010). Despite this ability, when more extensive fractures or more massive defects are present, regeneration will not be complete (Borie, 2017). Bone grafting involves implanting material that promotes bone regeneration alone, or in combination with other materials, through osteogenesis, osteoinduction and/or osteoconduction, utilising one or all of these processes. (Kleinschmidt and Hollinger, 1992). Choosing the ideal bone graft depends on several factors such as tissue viability, damage size, graft size, shape, graft volume, biocompatibility, biomechanical capabilities and cost (Guehennec, 2005; Micić, 2020). In order to determine whether a newly developed biomaterial intended for bone implantation conforms to the requirements of biocompatibility, mechanical stability and safety, it must undergo the recommended *in vivo* testing (Pérez-Sánchez, 2010; Borie, 2017; Stevanović et al., 2018; Stevanović et al., 2020a; Stevanović et al., 2020b; Đošić et al., 2017). Rabbits are one of the most commonly used animal models for exploring bone-implant interactions and for analysing the macrostructure and microstructure in bone composition and remodelling (Kleinschmidt and Hollinger, 1992; Guehennec, 2005).

Animals and experimental protocol

Novel silver/hydroxyapatite (Ag/HAP) and silver/hydroxyapatite/graphene (Ag/HAP/Gr) biocomposite coatings on titanium, potential bone implant materials, were electrophoretically deposited (Janković et al., 2015a; Janković et al., 2015b) at the Faculty of Technology and Metallurgy, University of Belgrade. Chinchilla rabbits, weighing 3.2 to 4.5 kg were selected for one experiment (Figure 2B, 2C). The animals were housed in separate cages under standard laboratory conditions and fed standard diet. Animal selection, management, surgical preparation and procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade (Certificate number No 01-488: 323-07-4087/2016-05/5), in compliance with the ARRIVE Guidelines on Animal Research (2016). Implantation of the materials (titanium coated with silver and hydroxyapatite nanoparticles) (Figure 2A) was under general anaesthesia (intramuscular injection of 35 mg/kg ketamine and 5 mg/kg xylazine in the same syringe). Analgesia was performed with an intramuscular injection of butorfanol (0.1 mg/kg). The surgical site (Figure 2D, 2E, 2F) was shaved

and the skin disinfected with 70% alcohol and povidone iodine. Local anaesthesia (2% lidocaine) was administered in the surgical area. Four defects of 5 mm diameter were made in each calvaria (Figure 2G), and calcium phosphate synthetic bone grafts were implanted. (Figure 2H, 2I). After implanting the tested materials, the corresponding tissue was sewn in layers with resorbable suture material (Vicryl 3-0, Ethicon, USA) (Figure 2J). Postoperative protocol included administration of an antibiotic

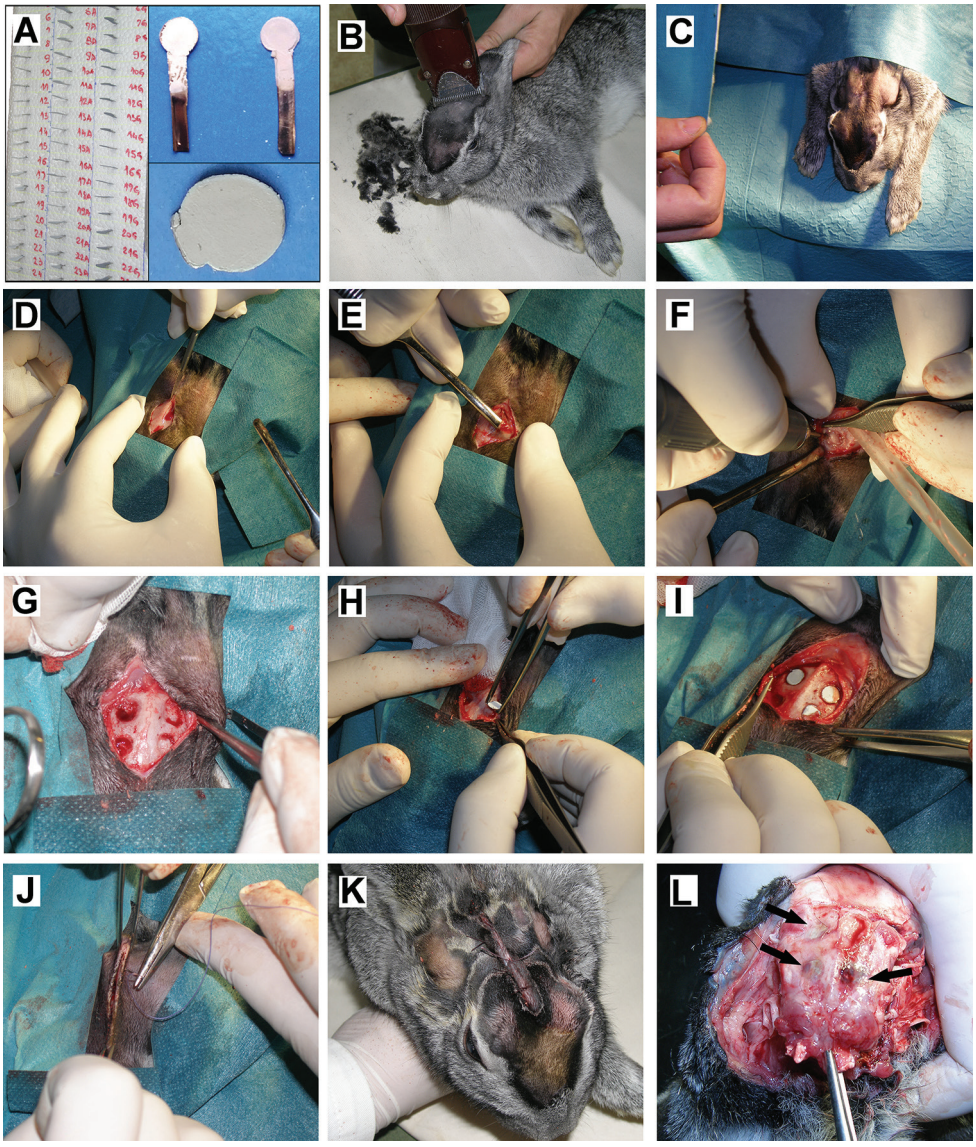

Figure 2. Animal selection, management, surgical preparation and protocol, approved by the Ethical Committees of the Faculty of Veterinary Medicine University of Belgrade (Certificate No. 01-488: 323-07-4087/2016-05/5)

(oxytetracycline 15 mg/kg) and an analgetic (buforphanol 0.1 mg/kg im). The tissue in the periimplant area was analysed 2, 4, 8 and 12 weeks postoperatively, after the animals were sacrificed by injecting a lethal dose of pentobarbital sodium, 100 mg/kg (Figure 2K, 2L). The implants' harmful properties were evaluated and compared to control graft samples and to intact bone materials. The foreign body biological response was histologically analysed in the bone tissue, and the results obtained showed the biomaterials used in this research have a satisfactory level of biocompatibility.

CONCLUSION

Any animal research that is necessary for the development of new drugs or biomaterials intended for clinical use in both veterinary and human medicine must comply with contemporary ethical standards and justify the purpose of any experimental work performed on animals. There is an on-going search for new conditions that provide a modern and more humane approach for performing animal experiments for medical purposes. In addition, laws, controls and inspections are becoming more rigorous, as is shown with the introduction of various regulations. Rats and rabbits are favoured for biocompatibility assessments of new biomaterials, rather than dogs, pigs or monkeys. In order to reduce additional and repeated experiments, the research results obtained must be clear and precise, thus reducing pain, suffering and stress in experimental animals to the necessary minimum, and provide valid data for further evaluation and use of the biomaterial for clinical purposes.

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Authors' contributions

M.D., MS. V. and T.V. conceived of the presented idea and supervised the writing of the manuscript. PB. B. processed data about the surgical procedures and biomaterial implantation. All authors provided critical feedback and helped shape the research, analysis and contributed to the final manuscript. Đ.M. researched and provided data about various biomaterials. M.I., LB. T. and G.V. worked out all of the technical details, processed data about animal experimentation regulations and designed the figures.

Competing interests

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PRIMENA ANIMALNIH MODELA U PROCENI BIOKOMPATIBILNOSTI IMPLANTATA ZA TVRDA I MEKA TKIVA

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Kratak sadržaj

Etičke dileme koje proizilaze iz korišćenja životinja kao in vivo modela u pretkliničkim i kliničkim ispitivanjima su sve prisutnije poslednjih decenija. Male laboratorijske životinje će se koristiti i u neposrednoj budućnosti zbog ekonomske isplatljivosti, kao i zbog toga što omogućavaju formiranje grupa iz kojih se mogu dobiti statistički obradivi podaci, što nije slučaj sa velikim životinjama. Tehnološki napredak u proizvodnji novih biomaterijala za kliničku primenu je ogroman, ali skrining testovi i metode koje se koriste za procenu biokompatibilnosti zaostaju za tim napretkom. Procena biološkog odgovora nakon implantacije biomaterijala je spora i zasniva se na milenijumskim mehanizmima oporavka prisutnim kod eukariota. Cilj istraživača iz ove oblasti je preispitivanje klasičnih metoda procene biokompatibilnosti i uvođenje novih, posebno za in vivo istraživanja. U cilju ostvarivanja ovog zadatka, u 2017. godini je sprovedena revizija odgovarajućih ISO standarda, pa se sada insistira na ispitivanju citotoksičnosti novih biomaterijala na ćelijskim linijama. Takođe, neki istraživači su izneli konkretne predloge o uspostvaljnju novih načina procenjivanja biokompatibilnosti. U proceni biokompatibilnosti biomaterijala in vivo koji se koriste za oporavak mekog tkiva, uobičajeno se koriste pacovi, dok se kunići preporučuju za implantate koji se koriste za tvrda tkiva zbog njihove veličine i mogućnosti implantacije biomaterijala na veću površinu kostiju, kao i zbog osobenosti koštanog tkiva kunića koje se brže oporavlja nakon oštećenja i omogućava lako očitavanje rezultata.

Ključne reči: biokompatibilnost, kost, kunić, pacov, potkožno tkivo