ΑΝΑΣΚΟΠΗΣΗ

Βιοηθική και κλινικές μελέτες σε ζώα

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ΠΕΡΙΛΗΨΗ

Εισαγωγή: Ο αριθμός των ζώων που χρησιμοποιούνται στην έρευνα έχει αυξηθεί με την πρόοδο και την ανάπτυξη της ιατρικής τεχνολογίας. Κάθε χρόνο, εκατομμύρια πειραματόζωα χρησιμοποιούνται σε όλο τον κόσμο. Ο πόνος, η αγωνία και ο θάνατος που βιώνουν τα ζώα κατά τη διάρκεια επιστημονικών πειραμάτων αποτελούν εδώ και πολλά χρόνια θέμα συζήτησης. Εκτός από το έντονο θέμα της ηθικής που προκύπτει, υπάρχουν κι επιπλέον μειονεκτήματα αυτών των πειραμάτων όπως η ανάγκη για εξειδικευμένο ανθρώπινο δυναμικό, τα χρονοβόρα πρωτόκολλα και το υψηλό κόστος. Πλέον, αρκετές εναλλακτικές λύσεις για τις δοκιμές σε ζώα έχουν αναπτυχθεί με σκοπό να ξεπερα-στούν τα μειονεκτήματα που σχετίζονται με αυτά αλλά και να αποφευχθούν παράλληλα οι ανήθικες διαδικασίες.

Σκοπός: Σκοπός της παρούσας εργασίας είναι να αποδειχθεί ότι το συγκεκριμένο πεδίο της Ιστομηχανικής μπορεί να αποτελέσει μελλοντικά ένα σημαντικό εργαλείο που να οδηγήσει στην μείωση χρήσης ζώων και στην εφαρμογή των 3RS.

Υλικό – Μέθοδος: Χρησιμοποιήσαμε τη βάση δεδομένων PUBMED για να επιλέξουμε τις μελέτες που περιγράφουν αυτά τα προϊόντα της ιστομηχανικής τα οποία αντικαθιστούν τα ζώα κατά τα πειράματα. Η έρευνα έλαβε χώρα μεταξύ Ιουνίου Αυγούστου 2018 και επαναλήφθηκε το Μάρτιο του 2021.

Αποτελέσματα: Εντοπίστηκαν 5 μελέτες: οι 3 αφορούν τα δέρματα πλήρους πάχους και πειραματικά αποδεικνύουν ότι υπάρχει η δυνατότητα κατασκευής υλικών που μπορούν να χρησιμοποιηθούν για in vitro έρευνες και οι άλλες 2 αναφέρονται στην ανακατασκευασμένη ανθρώπινη επιδερμίδα όπου πειραματικά με τη χρήση διαφόρων χημικών ουσιών ελέγχεται η αντίδρασή τους σε αυτά.

Συμπεράσματα: Οι νέες τεχνολογίες που αναπτύχθηκαν στον τομέα της μηχανικής των ιστών μας δίνουν τη δυνατότητα κατασκευής λειτουργικών ιστών που μπορούν να χρησιμοποιηθούν ως μοντέλα για την αξιολόγηση του κινδύνου για τον άνθρωπο που πιθανόν να κρύβουν κάποιες χημικές ουσίες που χρησιμοποιούνται στην παραγωγή προϊόντων.

Λέξεις Κλειδιά: Ιστομηχανική, εναλλακτικές λύσεις, πειράματα σε ζώα, βιοηθική.

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REVIEW

Bioethics and clinical trials on animals

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ABSTRACT
Introduction: The number of animals used in research has increased following the progress and development of medical technology. Every year, millions of animals are used around the world. The pain, distress and death experienced by animals during scientific experiments have been a matter of debate for many years. In addition to the strong ethical issue, there are further drawbacks of these experiments, such as the need for skilled human resources, time consuming protocols and high costs. Now, several alternatives to animal testing have been developed to overcome the disadvantages associated with them but also to avoid unethical procedures.
Aim: The aim of this review was to designate the specific field of tissue engineering that can be an essential tool in the future and lead to the reduction of animal use and the application of the 3RS.
Material - Methods: We used the online data base PUBMED to choose the studies that describe the products of tissue engineering that are being used instead of living animals in experiments. The research took place the months June- August 2018 and again in March of 2021.
Results: Five studies are analyzed: 3 refer to full thickness skins and experimentally demonstrate that it is possible to manufacture materials that can be used for in vitro investigations and the other 2 refer to rebuilt human epidermis where experimentally using different chemicals controls their reaction to that's all.
Conclusion: The new technologies developed in the field of tissue engineering allow us to build functional tissues that can be used as models for risk assessment for humans that may conceal some chemical substances used in the production.

Keywords: Tissue engineering, alternative solutions, experiments on animals, bioethics.

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INTRODUCTION

Experiments on animals are a crucial issue in today's society. It is believed that more than 100 million vertebrate animals are being used in research worldwide. Vertebrates are animals with a spine, such as humans. Also, there being used many invertebrates, such as worms and flies. Unfortunately, most of these animals are submitted to euthanasia, or die after the experiments. The animal species in use are usually mice and rats, fishes, rabbits, cats, dogs, monkeys, and many more, even though the vast majority of them are mice and rodents.[1] The research is equally multivarious, and it includes the following fields: medicine, aesthetics, defense, genetics, and research on behavior. These researches are carried out by government agencies, universities, pharmaceutical companies, and other commercial agencies.

It is acceptable as it is defined in the law 2015 of 21.2/27.2.92 of the Republic of Greece, " Ratification of the European Agreement for the protection of the vertebrate animals who are being used for experimental or other scientific purposes"[2] that humans have the moral obligation to respect all animals and to take under serious consideration the fact that they suffer and have a memory of it, as well as that an animal may continue experiencing pain or agony even after the general restoration of its health. Being aware of the pain and the agony that is being provoked to the animals, Willian Russell and Rex Burch (1959)[3] suggested that if it is necessary to use animals in experiments, it has to be put every possible effort into their

replacement with alternative solutions, non - sensible, to reduce the minimum number of animals that are going to be used and refining the experiments in which animals have been used and suffered in agony, installing the strategy of 3Rs (replacement, reduction, refinement). A science that offers the 3R principle development in tissue engineering. Tissue-engineering, or the engineering of tissues, is the combination of cell use, engineering, methods of materials, and relevant biochemical and physicochemical factors. It aims to improve or replace biological functions. If and when it will be characterized as a field of biomaterials, today it has evolved in importance and the action field, and it is considered autonomous. An essential part of tissue engineering is the development of 3D's equal to skin made from tissues and capable of mimicking basic anatomical, metabolical, cellular, and functional aspects of the natural human skin. As a result, it can experimentally be used in vitro systems and replace the animals in toxicological experiments. The field of this specific application is vast because it includes medicines, cosmetics, cleaning products, etc.[4] The replacement of animal testing with alternative methods that apply through tissue engineering does not hide dangers for us humans.

Furthermore, that does not mean that we stop the medical progress. On the contrary, replacing animal testing will improve the quality and the "decency" of science. Different alternative methods could replace the animals in various experiments. Though there are still moral issues in the biggest part of those alternative methods. For what reason an experiment on flies or zebra-fishes to be considered moral, but on rodents, is it immoral? Are the experiments carried out according to the same scientific and animal welfare standards?[5] Is there a scale of moral evaluation? Can we grade the moral in an experiment on flies with a lower number and with a higher one in an experiment on rodents or other animals? Using the above methods, ensure the aim of the 3Rs is fulfilled to a certain degree. Ideally, no animal should be an object of experimentation without that fact to influence the experiments that are necessary to be done. Tissue engineering could help with that.

PURPOSE

The purpose of this study is to designate the specific field of tissue engineering that can be an essential tool in the future and lead to the reduction of animal use and the application of the 3RS.

MATERIAL AND METHODS

For the selection of the studies that describe the products of tissue engineering that is being used instead of living animals in experiments, has been used the online database PUBMED.

The research has been held between June – August 2018 and again in March of 2021. The results were inferior, so they were tested many keywords. Eventually, they use the following keywords: tissue engineering, alternative, animal experiments, bioethics. Based on the following research questions, the research and the studies election were carried out.

- Is there the possibility of constructing a skin's equivalent with vessels that could be used for in vitro experiments?
- How important is the bioreactor in the production process?
- Is it necessary for a vessel net to exist in the final product?
- Can the skin's equivalence replace the Draize Test (test of the skin and eye's irritation from chemical substances)?
- Can the results of the irritation tests be announced so we can compare them with the OECD standards?

The criteria of the studies' introductions were the following:

- References that the material in the question can be used as an alternative solution instead of living animals in experiments.
- Description of the products of tissue engineering and particularly of skin's equivalences.
- Answers to the questions of the research

The keywords gave a result of 86 studies. With the completion of the selection process, as it appears analytically in the Table 1, 5 was the number of valid studies for the particular research. The main object of those studies is the proof that the skin's equivalents that are a product of tissue engineering can be excellent alternative solutions instead to animal testing. From table 2, we figure out that the number of studies is particularly small, and the years of extractions data are recent. That means that the field needs more analysis and studying, so the skin's equivalents can be improved and better results from their use. Nevertheless, in all the studies, the results are encouraging since there are references in every single one that there is success in the side experiments and that it can be used in many different fields (medicine, food, cosmetics, etc.). Essential is the reference in all of the studies of the alternative use instead of animals in relevant experiments. From the studies that we selected, we gather the above indications, as they appear in table 2. Table 3 as describes the method that was followed in each of the studies above.

Following references to the results of each study

Study 1[6]

This particular study describes the development of a model that uses a biological scaffold, human epidermal small-vascular endothelial cells, and human fibroblasts.

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Combining the above with the suitable bioreactor could lead to the development of a functioning model of the human skin with a vascular net that uses the tech BioVaSc. That model mimics the natural conditions of a fully human skin organ to be used as a developed substitutefor animal models in medical research. The results of the experiments showed that with the mentioning method, we take a material that:

1) The culture in the bioreactor eases the formation of a layered epidermis, including the vascular dermal layer.
2) The human keratinocytes from a properly differentiated skin with the dermal-epidermal joint.
3) The structure of the vessels is formed from human endothelial cells, and they show a bigger diameter than the vessels of the human skin.
4) The observation confirms the epidermal block and the vascular perfusion.

In conclusion, the skin's equivalents with a vascular net represent a developed organ model for scientific research. The vascular network allows the interaction between cellular and non-cellular parts of the blood flow with different layers of tissue. That can be particularly interesting considering the immunological research and relevant studies on transferring lymphoceles. For clinical applications, this particular skin's equivalent can be used as a vascular skin transplant to cure wounds of the deep epidermis and eventually replace the respectively animal ones.

Study 2[7]

This particular study refers (as the one above) to the construction of a skin's equivalent from biological materials using a scaffold with a vascular network. It is a product of tissue engineering, it improves the effectiveness of the equivalent using instead of primary microvascular endothelial cells (mvEC), a new development technology (upcyte mvEC) endothelial cells with the ability to multiply that preserve through the differentiated function of theirs. As a result, creating the skin's equivalents with vessels that can represent better deferent organs such as the liver.

From these experiments, it has been proved that it is possible to create a promising system that will have the ability to be used in vitro experiments. Furthermore, new skin's equivalent with the Upcyte endothelial cells' technology allows us to create a human tissue model that would be useful as an alternative solution to animal testing for the measurement of drug's infiltration, distribution, and metabolism.

Study 3[8]

As it was mentioned before, to ensure the functionality of the skin's equivalents, we should create cultural conditions that resemble the natural environment. As a result,

constant feeding of the cells with nutrients, co-culture of different cell types, proper structures and bioreactors of advanced technology are required. The bioreactors preserve the conditions of the natural tissue at the wanted levels. Furthermore, to ensure the efficient provision of nutrients it has been developed the vascular skeleton BioVasc. These human tissue models consist of a new technology that can be used as an alternative solution to animal testing for pharmacokinetics (drug's infiltration, distribution, and metabolism) and pharmacodynamic studies.

These new technologies create in the field of tissue engineering allow us to have long-term functional tissues and use them as in vitro models. It has been proved that the non-cellular biological scaffold with a vascular network (BioVasc) can be used for the mentioning purpose. A necessary condition to construct it is a suitable bioreactor that can preserve the conditions at the ideal levels. The bioreactor that has been used provides the proper biochemical and physical ordinance. The provision of nutrients through the vascular network is possible to achieve the differentiation process of the cells and influence their function.

Study 4[9]

This particular study used the evaluation of the skin's irritation by a chemical substance, was used the reconstructed human epidermis, 12 irritating and13 non-irritating substances. These 25 chemical substances refer to the performance prototypes of the European Center for the Confirmation of Alternative Methods (ECVAM). The reconstructed tissues were exposed to the chemical substances for 15 minutes, and after, they stayed for 42 hours in a new cultural environment. The analysis on the used environment showed high reliability and accuracy in discriminating the irritating and non-irritating chemical substances.

1. Reliability: the ratio of the intra-laboratory reproducibility is 93,5%, and the interlaboratory reproducibility is 96,5%. Those numbers prove that this test has a high e producibility rate and the model's reliability is also high.

2. Predictability: the average sensitivity, specialization, and accuracy of the particular experimental model are 83,3%, 73,1%, and 78% in order. According to ESAC (the database of ESAC-Net provides European report's data concerning the consumption of the anti-germ substance in the community as well as in the hospital field), the acceptable experimental model has to have: a sensitivity of 80%, specialization of 70% and accuracy 75% (109). The results of the experiments satisfy these qualifications.

In conclusion, the experimental procedure that was followed is suitable for use as an

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autonomous method to separate the irritating and non-irritating substances for the skin.

Explanations

Reliability: is the degree of coherence of a measure. A test will be reliable when it gives the same repeated result under the same circumstances.

Reproducibility: is the degree of spreading among the results taken with the same method on the same sample, under different circumstances, like different analysts, different devices, reactor cartels, different times.

In vitro reproductivity: in the same lab.

Interlaboratory reproductivity: different labs.

Sensibility: it expresses the signal to the analyst's concentration change.

Specialization: the ability of an analysis method to separate the analyst from other chemical substances of the sample.

Accuracy: it refers to the difference of the average of a series of measurements and the acceptable value as true.

Study 5 [10]

In this particular method for evaluating skin from a chemical substance, it has been used a reconstructed human epidermis, and 20 referring substances. For these tests, the blind method was used. The results show high reliability in the separation of the irritating and the non-irritating substances. Furthermore, the reconstructed human epidermis can be used experimentally to test the irritability of the epidermis.

In the following table (4) we see that the reliability, predictivity and the reasons for the reproductivity satisfy the standards of OECD, so the experimental procedure can be used as a method to separate the irritating and the non-irritating substances in the skin. (In 1982, the OECD was the first international organization to agree on harmonized guidelines for the testing of chemicals.)[4]

Comparing the results

Studies 1,2, and 3 belong to the same use of the biological scaffolding with vascular net (BioVaSc) that demands a bioreactor where the growth of the tissues will occur. The 3d skin's equivalents manufactured from tissue can mimic the primary anatomical, metabolical, cellular, and functional sides of the natural human skin. So, they can be used as a wound coverer for significant dermal flaws or wounds or as in vitro systems instead of animal models in basic research. Two types of tissue-engineering models are available. The first one refers to the "reconstructed human epidermis" which represents the level of the epidermis. On the other side, the second one refers to "a skin's equivalent of "full-depth", and represents the dermal and the epidermal levels. Despite the constant progress, the use of today's skin's equivalents for medical purposes and as a test system remains

restricted due to the lack of a functional vascular system.

In skintransplant, an existing vascular system supports a quick anastomosis of the donor's skin to the host, but the skin's implants that are manufactured from tissues ha have to form new vessels with angiogenesis, which delays the embodiment of the implant. As a result, biotechnological skin's implants are more likely to get rejected. Furthermore, the vascular system of the skin is necessary for several natural functions, including the growth of skin conditions, wound healing etc. The skin's models with a vascular system have restricted abilities, and cannot represent the natural conditions of a full organ. Because there is no such model to represent the natural conditions of a full organ, it remains a scientific and medical need for animal models. To overcome those restrictions and create a tissue with a vascular network aw, it has been referred above, it can be used as a biological scaffold with a vascular net (BioVaSc) that demands a bioreactor in which the growth of the tissue will take place. (studies 1,2,3).

We use the Draize test for the past 60 years to test the skin's and eye's irritability to chemicals.[11] This procedure includes the application of 0,5 ml or 0,5 g or a controlled substance on the eye or the skin of a conscious animal, leaving it there for a specific amount of time before it gets washed out, and record the data of their results. The animals are observed for at least 14 days for red marks and swelling during the testing procedure and for redness, swelling, bleeding, extraction, turbidity, or even blindness on the tested eye. In addition, in studies 4 and 5 it has been tested if the reconstructed human epidermis can be used in experiments of skins irritation control to reduce animal use in testing.

DISCUSSION

At first, it was not easy to find studies. Accept the PUBMED there were tries on other data dases relevant to the alternative solutions to bring any positive outcome. Still, the research on PUBMED had many difficulties as the results were usually irrelevant. We tested a lot of different keywords without some essential results, and only the studies referring above had some relevant results. Perhaps there is an issue using similar verbal terms among the researchers and the 3RS organizers, something that should be solved and develop a standard international terminology for issues relevant to alternative solutions. That would particularly help in research and its results. Comparing the results of the studies, we noticed that creating a tissue with a vascular net it can be used a biological scaffold with a vascular network (BioVaSc) which demands a bioreactor in which the growth of the tissues will take place (studies 1,2,3) and in studies 4 and 5 it has been checked if the reconstructed human epidermis can be used in experiments

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of skin's irritation testing to reduce the animal use in testing.

As an alternative solution to animal testing, human tissue models can be viable and used to develop new products, including pharmaceutical products, chemicals, cosmetics, and food. Such alternative methods contribute to the 3RS principle are classified as "superior" and possibly more reliable. The 3RS principle provides the motive and the frame for discussions that aim to better treat the animals used in danger evaluations and product development. The new technologies developed in tissue engineering allow us to construct functional tissues that can be used as models to evaluate the possible danger that some substances may hide. To create such advanced human tissue models, it is necessary for suitable culture conditions to exist, where the microenvironment is simulated. At the studies' experiments, it has been proven that the biological scaffold with a vascular system ensures the attachment pf different cellular types, leading to the tissues' differential process, making the long-term function of these 3d biological tissues possible. All the necessary biochemical and natural settling orders are provided with a suitable bioreactor to achieve the pre-referring events. In this model of co-culturing tissues, we can reach angiogenesis,an important condition to create complex tissues, functional equivalents to human tissue.

Another field where tissue engineering could play an important role is the experiments of irritation trials on the skin or the eye of different products. For example, the irritation of the skin test is possible using the alternative approach using in vitro trials with the reconstructed human epidermis made by epidermal keratin cells. Those models can accurately mimic the morphology, structure, and metabolism of the human epidermis with accuracy. Currently, several models are available for sale from trading companies or academic institutes, and they have been used for various research topics such as irritation, corrosion, blocking, structure, and absorption of the skin.

If the alternative solutions of tissue engineering actually have such abilities, why aren't they been used wider or why don't we have more relevant results or why don't they develop faster? Sure, some important technical issues and barriers should be overpassed, such as the development of more effective scaffolds and the creation of bigger vascular structures. Also, the tissue engineers perhaps don't want to change the existing practices for years and be informed or trained for new techniques. So, the problems are many as well as obstacles. Sure, though, it is worth every effort to stop the animals from being tortured by the human species.

CONCLUSIONS

The confirmation of the skin's equivalents as an alternative solution has shown that using the reconstructed tissues leads to provisions that are at least as reliable as the ones being used on living animals. Furthermore, understanding the physiology and the pathology of the tissues, the skin's equivalents of tissues engineering could be a more reliable tool than the living animals with their uncontrollable complexity. It has been suggested that the engineers who are occupied with the subject think and verify in cases of using their products as alternative solutions that those have a relevant biological function. It is not easy for all the research to be replaced by alternative tissue engineering solutions because the issue engineering cannot develop complex organs.

Despite all that, the field of tissue engineering can contribute to the development of alternative solutions for use in experiments instead of living animals. It is a fact that this contribution raises steadily year after year, and it has not been finalized yet. The exciting parts and different functions of the 3Rs could have a big part in the best development of alternative solutions. An international standard terminology should be developed for issues relevant to alternative solutions to ease the research. Tissues engineers and 3RS organizations could cooperate and organize relevant conventions or actions. Scientific magazines can invite relevant issues and raise the published studies. Universities could organize relevant classes or allow postgraduate studies on the subject of connecting tissue engineering to alternative experimental solutions. Suppose the development of such alternative solutions to tissue engineering is promoted correctly. In that case, this particular science not only helps the development of human health but also contributes to the reduction of the develop human health and contributes to the reduction of animal use and their suffering in the labs. In the best interest of science to continue the studies in the field of alternative solutions, so in some time in the near future the progressive replacement of animal use in the labs to happen.

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TABLE 1 selection studies process, from PUBMED.

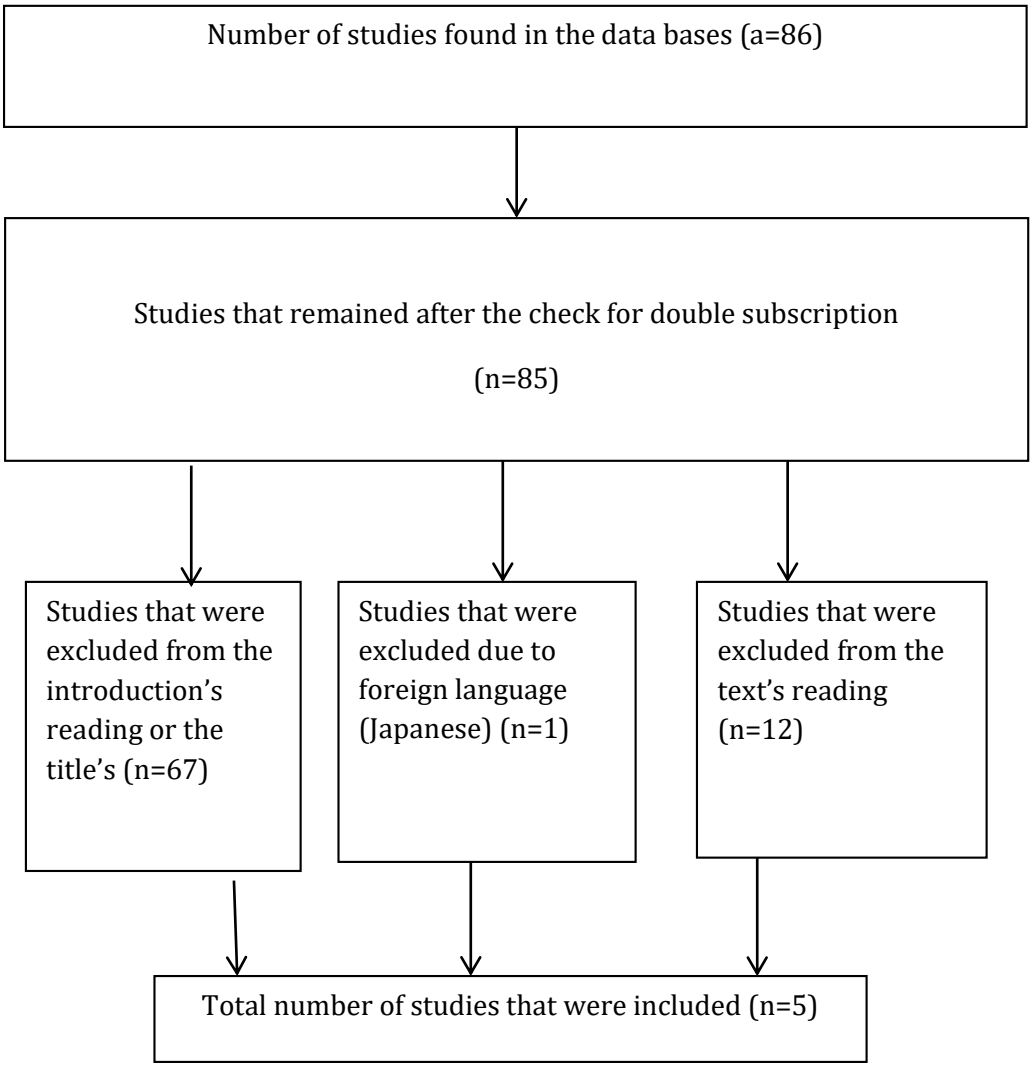

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TABLE 2. Data of selected studies.

Writer	Country	Year	R/F	Title	Kind of study	Keywords	Purpose	Field	Result
Groeber et al	Germany	2016	1	A First Vascularized Skin Equivalent as an Alternative to Animal Experimentation	Article	skin equivalents, alternative to animal testing, vascularization, tissue engineering	Skin's equivalents, products of tissue engineering as an alternative solution to the animal testing	Scientific research	The skin's equivalents with a vascular network represent an improved model of tools for scientific research.
Scheller et al	Germany	2013	2	Upcyte_ Microvascular Endothelial Cells Repopulate Decellularized Scaffold	Article	None	BioVasc – a tissue engineering product that proves to be a successful alternative solution to animal testing for PHORMACOCYNETICS.	PHARMACOCYNATICS AND PHARMACODYNAMIC studies	The multicellular models of human tissue would be useful as alternative solutions to animal testing for the measurement of drug infiltration, distribution, and metabolism.
Schanza et al	Germany	2010	3	Vascularised human tissue models: A new approach for the refinement of biomedical research	Article	Tissue engineering Vascularised scaffold Bioreactor technology Human liver test system Human intestine tissue model	BioVasc – a tissue-engineering product that proves to be a successful alternative solution to animal testing for pharmacokinetics	PHARMACOCYNATICS AND PHARMACODYNAMIC studies	As an alternative solution, human tissue models can be used to develop new products, including pharmaceutical products, chemicals, beauty products, and food.

						Alternative to animal models			
Kojima et al	Japan	2012	4	Validation Study of the In Vitro Skin Irritation Test with the LabCyte EPI-MODEL24	Confirmation study	in vitro, interleukin-1 alpha (IL-1α), MTT, reconstructed human epidermis, skin irritation, validation	The reconstructive epidermidis (RhE) LabCyte EPI-MODEL24 as an alternative solution for animal testing for skin irritation tests of products.	Skin's irritation	The analysis showed excellent results and reliability (100%) at the separation of irritating and non-irritating substances.
Groeber et al	Germany	2016	5	Catch-up validation study of an in vitro skin irritation test method based on an open-source reconstructed epidermis	Confirmation study	In vitro skin irritation testing, Open-source reconstructed epidermis, Validation, Alternative test methods	A reconstructive epidermidis can be used as an alternative model for skin irritation testing	Skin's irritation	A reconstructive epidermidis could be used from the labs for irritation testing.

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TABLE 3. Methods that have been used in each study.

R/F	Title	Method
1	A First Vascularized Skin Equivalent as an Alternative to Animal Experimentation	Skin's equivalents, a product of tissue engineering, vaccinated with human fibroblasts, keratin cells, and small-vascular endothelial cells, were tested experimentally for their effective use as an in vitro model for skin research.
2	Upcyte_ Microvascular Endothelial Cells Repopulate Decellularized Scaffold	Experimental testing of the skin's equivalents is used as an alternative tool for studies of pharmacokinetics (infiltration, distribution, metabolism) and pharmacodynamics.
3	Vascularised human tissue models: A new approach for the refinement of biomedical research	Experimental testing of the skin's equivalents is an alternative tool for studies of pharmacokinetics (infiltration, distribution, metabolism) and pharmacodynamics.
4	Validation Study of the In Vitro Skin Irritation Test with the LabCyte EPI-MODEL24	Experimental testing of 25 chemical solutions on skin's equivalents to verify their use as an alternative to the human epidermis on skin irritation tests.
5	Catch-up validation study of an in vitro skin irritation test method based on an open-source reconstructed epidermis.	Experimental testing with 20 chemical solutions to verify the use of the skin's equivalents as an alternative to skin irritation tests.

TABLE 4. Comparative value table, concerning the performance standards of OECD.

Parameter (%)	**Acceptable limits**	**Average**
Sensibility	80	90
Specialization	70	70
Accuracy	75	80
Fraction of in vitro reproductivity	90	87
Fraction of interlaboratory reproductivity	80	85

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