Research Article

Sensitization Potential of Medical Devices Detected by *In Vitro* and *In Vivo* Methods

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Abstract

Medical devices must be tested before marketing in accordance with ISO EN 10993-10 in order to avoid skin sensitization. This standard predominantly refers to the *in vivo* test but does not exclude the use of *in vitro* methods that have been sufficiently technically and scientifically validated for medical device testing. It is foreseen that, due to the complexity of the sensitization endpoint, a combination of several methods will be needed to address all key events occurring in the sensitization process. The objective of this pilot study was to evaluate the sensitization potential of selected medical devices using a combination of *in chemico* (DPRA, OECD TG 442C) and *in vitro* (LuSens, OECD TG 442D) methods in comparison with the *in vivo* (LLNA DA, OECD TG 442A) method and to suggest a possible testing strategy for the safety assessment of medical device extracts. Overall, one of the 42 tested samples exhibited positive results in all employed test methods, while 33 samples were predicted as non-sensitizing in all three performed methods. This study demonstrated good agreement between *in vitro* and *in vivo* results regarding non-sensitizing samples; however, some discrepancies in positive classification were recorded. A testing strategy is suggested in which negative results are accepted and any positive results in the *in chemico* or *in vitro* tests are followed up with a third *in vitro* test and evaluated in accordance with the "2 out of 3 approach". This strategy may reduce and replace animal use for testing the sensitization potential of medical devices.

1 Introduction

A medical device (MD) is defined as any instrument, apparatus, implant or material intended by the producer to be used, separately or in combination, for specific medical uses, e.g., diagnosis, monitoring, cure of disease or injury, adjustment or support of the anatomy or physiological process, assisting or maintaining life, or control of conception (WHO, 2020). The broad spectrum of medical devices, comprising catheters, gloves, blood bags, wound dressings, tissue engineering articles, etc., is globally increasing, and a high rate of research and progress in this area has been recently reported (Myers et al., 2017). Medical devices usually contain diverse materials such as plastics, cotton, rubber, latex, gels, metallic alloys or biological derivatives (Goud, 2017). They may be in contact with the human body for a short period (≤ 24 h), for a prolonged period (> 24 h to 30 days) or permanently (> 30 days).

With the aim to ensure safety for the end-users of MDs, all incoming marketed articles have to be tested according to "Biological evaluation of medical devices" (ISO 10993), which contains a collection of standards for evaluating MDs for the purpose of managing biological risks (ISO, 2009; Strickland et al., 2019). The range of endpoints for biocompatibility evaluation is defined by the nature of body contact (e.g., contact with healthy skin vs with damaged skin vs implanted device in bone or tissue) and time persistence (e.g., short term vs long term application/contact). Three fundamental items of information obligatory for all types of MDs comprise data on their cytotoxicity, sensitization and irritation/intracutaneous reactivity.

Skin sensitization is defined as a dermal reaction initiated by immunological responses to a chemical or material, which lead to a delayed-type hypersensitivity response after cutaneous contact and subsequent penetration into the epidermis, resulting in allergic contact dermatitis (OECD, 1992; Basketter et al., 2005). During

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their lifetime, humans are exposed to numerous non-immunogenic, low molecular weight chemicals called haptens that can elicit sensitizing reactions upon repeated exposure. In most cases, haptens elicit an immune response only when attached to a large carrier such as a protein (Kimber et al., 2002, 2011). Chemical sensitizers mostly exhibit electrophilic properties and may react with various nucleophiles. Lysine and cysteine are the nucleophiles most often reported to react with electrophilic allergens (Ahlfors et al., 2003), forming extremely stable covalent bonds and thus participating in skin sensitization responses (Gerberick et al., 2004).

Testing for sensitization induced by chemicals extractable from medical devices is a key element of ISO 10993 – Part 10 (ISO, 2010). The *in vivo* methods using guinea pigs, i.e., the Guinea Pig Maximisation Test (GPMT) and the Buehler Test (TG 406) (OECD, 1992), cover the whole skin sensitization process (Jowsey et al., 2006), evaluating both the induction and elicitation stages of skin sensitization (ISO, 2009, 2010; Basketter et al., 2012; Mertl et al., 2019). An alternative refinement method using mice, the local lymph node assay (LLNA: DA), was introduced into the standard in 2011. The LLNA: DA (TG 442A) (OECD, 2010), which assesses solely the induction response, has been broadly accepted as it has advantages over the guinea pig tests both in terms of animal welfare and because it provides an objective measurement of the induction stage of skin sensitization. However, the recent development of non-animal methods to classify the skin sensitization potential of chemicals and cosmetics ingredients (Bauch et al., 2012; Myers et al., 2017; Kirk, 2018) has suggested that *in vitro* testing may be more effective, both in terms of cost and time, than *in vivo* testing and may also be applicable to MD (Myers et al., 2017).

The current knowledge on the chemical and biological mechanisms related to skin sensitization has been utilized in the development of an adverse outcome pathway (AOP) with defined key events for which corresponding non-animal test methods have been developed (MacKay, 2013; OECD, 2014). A combination test methods has been implemented with regard to key events (KE) of the AOP, starting with the molecular initiating event, i.e., covalent binding of haptens to skin proteins (KE1), leading to activation at cellular level of keratinocytes (KE2) and dendritic cells (KE3) and dendritic cells migrating from the epidermis to the local lymph node, where they act as antigen-presenting cells and induce proliferation of T-cells (KE4), finally resulting in the adverse outcome allergic contact dermatitis (Jowsey et al., 2006; OECD, 2014).

Several *in chemico* and *in vitro* tests have been accepted as OECD test guidelines for skin sensitization testing, including the direct peptide reactivity assay (DPRA), a cell-free *in chemico* assay that uses lysine- and cysteine-containing test peptides for evaluation of the test compounds' reactivity, the amino acid derivative reactivity assay (ADRA) (OECD TG 442C; Gerberick et al., 2004; OECD, 2019); the KeratinoSens™ and LuSens assays, based on human keratinocyte cell lines and activation of the Nrf2/Keap1/ARE signaling pathway (OECD TG 442D; Natsch, 2010; Natsch et al., 2011; OECD, 2018a), the human cell line activation test (h-CLAT), which uses a human monocytic leukemia cell line (THP-1) and measures the expression of CD86/54, the U937 cell line activation test (USENS™), and the interleukin-8 reporter gene assay (IL-8 Luc assay) (OECD TG 442E; Sakaguchi et al., 2009; OECD, 2018b). Taking into consideration that each of these validated assays covers only one key event, a single alternative method may not be sufficient to achieve satisfactory accuracy due to the complexity of the skin sensitization mechanism (Coleman et al., 2015).

Although risk assessment for chemicals based on well-defined prediction models has already acquired broad regulatory acceptance, the procedures must still be adapted and evaluated for MDs, which can be performed with the MDs as such or with extracts obtained with polar or non-polar solvents. ISO 10993-10, which addresses the skin sensitization endpoint, predominantly refers to *in vivo* testing, however, it does not exclude the use of *in vitro* methods that have been sufficiently technically and scientifically validated.

Thus, the objective of this pilot study was to evaluate the sensitization potential of selected, commercially available medical devices using validated *in chemico* and *in vitro* methods in comparison to the *in vivo* method, and to suggest a possible testing strategy that could be applied in the process of safety assessment of MD and their extracts. We combined the DPRA and LuSens assays, addressing KE1 and KE2 respectively, and compared the outcome with the *in vivo* LLNA: DA.

2 Animals, materials and methods

The list of the 42 tested medical devices, including their description and composition, is given in Table 1.

2.1 Preparation of medical device extracts

For LLNA, the samples were extracted in appropriate polar and non-polar solvents for testing using a surface/volume ratio according to ISO EN 10993-12 (ISO, 2012) for 72 h at 37°C. The extracts were prepared in cottonseed oil (non-polar vehicle) and physiological saline solution (polar vehicle). Solid samples with a defined surface and a thickness of more than 0.5 mm were extracted with a surface/volume ratio of 3 cm^2/mL; samples thinner than 0.5 mm were extracted with a ratio of 6 cm^2/mL; and samples with an undefined surface were extracted with a ratio of 0.2 g/mL. The extraction conditions are based on a standardized approach, which represents an exaggerated use of the product.

The highly sensitive LuSens assay requires the exclusion of antibiotics from the cell culture as they may affect the induction of reporter gene expression. Sterility of the extracted samples had to

Abbreviations
AOP, adverse outcome pathway; ATP, adenosine triphosphate; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; DPRA, direct peptide reactivity assay; h-CLAT, human cell line activation test; KE, key event; LLNA, local lymph node assay; MD, medical device; OECD, Organisation for Economic Co-operation and Development; PBS, phosphate-buffered saline

Tab. 1: Commercially available medical devices classified according to material type

Sample	Description	Composition	Risk component
1	Silicone sealing ring	Silicone 5307	Not specified
2	Barrel	Polypropylene, Tatren RM 45 55	Not specified
3	Antimicrobial Sol SB15	Non-woven textile	Not specified
4	Hemostatic collagen sponge	Chicken collagen	Not specified
5	Hemostatic collagen sponge	Horse collagen	Not specified
6	Hemostatic absorbent material	Non-woven textile 100% cellulose	Not specified
7	Pad for acute and chronic wounds	Oxidized cellulose	Not specified
8	Filling of medical material	Plastic crumblings	Not specified
9	Textile	100% cotton	Not specified
10	Textile	50% cotton, 50% plastic	Not specified
11	Two-layer laminate	Laminate	Not specified
12	Gauze	Bleached gauze, 17 threads	Not specified
13	Filling of medical material	Plastic crumblings	Not specified
14	Wood applicator	Cotton wool	Not specified
15	Examination latex gloves	Natural rubber latex	Not specified
16	Maternity pads	Cotton	Not specified
17	Absorbent underpads	Cotton	Not specified
18	Foam for very dry skin	Aqua purificata, oils (primrose oil, apricot kernel oil and macadamia oil), propane-butane mixture	Apricot kernel oil, primrose oil, macadamia oil
19	Eardrops	Glycerol, lidocaine	Not specified
20	Nasal spray	Water, soybean oil, glycerol, xylitol, emulsifiers, kappa-carrageenan, fucoidan (algae + seaweed), tocopherol acetate, EDTA, Siberian fir (essential) oil	Siberian fir oil
21	Anesthesia needles	Stainless steel	Not specified
22	Epidural and block nervous catheters	Pebax® (polyether block amide)	Not specified
23	Ear spray	Aqua purificata, phenoxyethanol, ethylhexylglycerin, propylene glycol, polysorbate 80, tocopherol acetate, extract (*Chamomilla recutita* flower, *Malva sylvestris*, *Aloe barbadensis*, *Hippophae rhamnoides* fruit), parfum green tea	Extracts, parfum
24	Nasal spray	Aqua purificata, sodium chloride, benzalkonium chloride, citric acid, hydroxyethyl urea, disodium phosphate, sodium hyaluronate, D-panthenol, polysorbate 80, extract (*Hippophae rhamnoides* fruit, *Aloe ferox*), *Eucalyptus globulus* leaf oil	Extracts, essential oil
25	Aniball light pink	Silicone, KEG-2003	Not specified
26	Bioactive wound cover	Bioactive absorbent material (hydrogen calcium salt of oxidized cellulose)	Not specified
27	Aniball dark pink	Silicone, KEG-2003	Not specified
28	Aniball Inco salmon pink	Silicone, KEG-2003	Not specified
29	Dental composite (polymerized filling)	Silane treated glass, isopropylidenediphenol PEG-2 dimethacrylate, silane treated silica, bisphenol A-glycidyl methacrylate, urethane dimethacrylate,	Methacrylates

Sample	Description	Composition	Risk component
		triethylene glycol dimethacrylate, 2-hydroxyethyl methacrylate, camphorquinone, 2-(dimethylamino) ethyl methacrylate, 3,5-di-tert-4-butylhydroxytoluene	
30	Lubricant gel cherry	Aqua, hydroxyethylcellulose, glycerin, propanediol, panthenol, citric acid, *Stevia rebaudiana* extract, potassium sorbate, sorbic acid, cherry aroma, sodium hydroxide 50%, benzoic acid	Extract, cherry aroma
31	Hydrogel burn spray	Aqua, sodium hyaluronate, propylene glycol, extract (*Aloe barbadensis*, *Calendula officinalis*), phenoxyethanol, ethylhexylglycerin, sodium hydroxide	Extracts
32	Spray for safe removal of ticks	Cryogenic spray (tetrafluoropropane)	Not specified
33	Lubricant gel strawberry	Aqua, hydroxyethyl cellulose, glycerin, propanediol, panthenol, citric acid, *Stevia rebaudiana* extract, potassium sorbate, sorbic acid, strawberry aroma, sodium hydroxide 50%, benzoic acid	Extract, strawberry aroma
34	Lubricant gel natural	Aqua, hydroxyethyl cellulose, glycerine, propanediol, panthenol, citric acid, sodium hydroxide 50%, benzoic acid	Not specified
35	Rubber material for MD	Black rubber	Not specified
36	Bandage material with pad	Cotton wool	Not specified
37	Thigh-high stocking	Cotton, elastane LYCRA, PAD nylon	Not specified
38	Knitted knee sleeve with silicone-stabilized patella	50% Pad nylon, 25% natural rubber latex, 8% elastane LYCRA, 17% polyester	Latex
39	Hemostatic dressing	Oxidized regenerated cellulose	Not specified
40	Urinary catheter	Plastic (thermoplastic elastomer)	Not specified
41	Three-layer sandwich textile material	Saška purple sash, PU, terry leny cloth	Not specified
42	Three-layer sandwich textile material	Saška milky (PA), PU, terry leny cloth	Not specified

be ensured prior to extraction. Thus, sterilization of the MDs by EtOH or UV-light was performed. The sterilization by EtOH was done by rinsing for approximately 30 s, after which the sample was left at room temperature to dry. Sterilization by UV-light was done for approximately 20 min under a UVC germicidal lamp in a biological safety cabinet class II, EuroFlow type EF/S (Clean Air Techniek B. V., Netherlands), 10 min from each side of the sample. After sterilization, a visual check (to see if any dyes or other substances had been released) as well as a mechanical check (to see if the structure of the substance had been disturbed) was done. Only samples not apparently influenced by the sterilization process were subjected to further testing. Samples delivered to the lab as sterile were tested without additional sterilization treatment.

For LuSens, the same extraction ratio of the samples as for LLNA was used. However, a shorter extraction time of 24 h at 37°C was employed according to the recommendations for cytotoxicity testing in tissue cultures (ISO 10993-5). The samples were extracted in DMSO or in Dulbecco's Modified Eagle's Medium (DMEM) and passed through a 0.2 µm filter (Whatman™ 6753-2502) to minimize the risk of biological contamination prior to testing.

For the DPRA, the samples were extracted in physiological saline solution and passed through a 0.45 µm filter (Fisher Scientific 3511.0057) prior to testing.

2.2 Toxicological tests

Direct peptide reactivity assay (DPRA)

The DPRA was performed according to OECD TG 442C (OECD, 2019) with minor modifications. DPRA was performed in cysteine-only modification. Acetonitrile was used as negative control. Peptides containing cysteine were obtained from GenScript (Piscataway, NJ). Briefly, 50 µL test extract plus 200µL acetonitrile or 250 µL test extract was mixed with 750 µL peptide solution (0.5 mM in sodium phosphate buffer, pH 7.5) and incubated in the dark for 24 h at 25°C. The volume of 50 µL sample is in accordance with OECD TG 442C, while the volume of 250 µL extract was used in addition to account for the testing of an extract instead of a chemical. Following incubation, the relative peptide concentration was measured by reverse-phase high-performance liquid chromatography (HPLC, Ecom HPLC) on a Chromolith-C18 column (5.0 mm ∗ 100 mm) with gradient elution and UV detection at 220 nm (Ecom UV/VIS detector)

using an external standard linear calibration curve. Three samples were prepared for all tested extracts and each sample was measured in triplicate. Cysteine peptide percent depletion values were calculated and used to categorize each extract into one of four reactivity classes (< 13.89%, minimal; 13.89% - 23.09%, low; 23.09% - 98.24%, moderate; > 98.24%, high reactivity class), which distinguish sensitizers and non-sensitizers (OECD, 2019) according to the prediction model.

ARE-Nrf2 luciferase test method (LuSens)

The LuSens assay was performed according to OECD TG 442D (OECD, 2018a). The LuSens cell line was kindly provided by BASF SE (Germany) and was tested for mycoplasma contamination during routine use with negative results. Cells were maintained in T75 flasks with 20 mL medium (10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 0.005% puromycin dihydrochloride) at 37°C in a humid atmosphere containing 5% CO_2 to a confluence of 80-90%. Then cells were washed with 10 mL PBS containing 0.05% EDTA. PBS was aspirated and cells were trypsinized by adding 2 mL trypsin and incubating the cells at 37°C until cells had detached (5-10 minutes). After cells had detached, they were resuspended in 8 mL medium per T75 flask, cells were seeded at density $0.7 * 10^6$ per T75 culture flask and incubated until the next cell passage (twice a week). Cell suspensions from passage 4 to 16 were used in the experiments.

120 µL cell suspension (10^4 cells/mL) was seeded into 96-well plates and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. Then, the medium was replaced with DMEM with 1% inactivated FBS (Merck) containing the sample extract in a final volume of 200 µL per well. DMEM extracts were tested at concentrations of 100, 75, 50, 25, 10 and 1%; DMSO extracts were tested at concentrations of 1, 0.5, 0.25, 0.125, 0.063, 0.031%, as the final DMSO concentration should not exceed 1% in LuSens cell culture to avoid cytotoxic effects. After 48 h incubation, luciferase activity was measured using the Steady-Glo or One-Glo luciferase substrate (Promega), 1:1 in PBS, using a plate reader (GLOMAX Multi Reader, Promega). In parallel, cell viability was determined by MTT assay (Mosmann, 1983). The resulting formazan concentrations were measured with an Eon High Performance Microplate Spectrophotometer (BioTek Instruments) at 590 nm.

The extract is considered to have sensitizing potential if the luciferase activity equals or exceeds a 1.5-fold induction compared to the vehicle control at concentrations that do not reduce cell viability under 70% (Urbisch et al., 2015; Ramirez et al., 2017). Two independent experimental runs were carried out with each sample tested in triplicate. For acceptance of the assay, at least 3 tested concentrations must retain a viability above or equal to 70%. The positive control, 120 µM ethylene glycol dimethacrylate, which must induce luciferase expression above 2.5-fold in comparison to the vehicle controls, and the negative control, 5000 µM DL-lactic acid, were included in each test run. Further, the average standard deviation of the vehicle controls should not exceed 20% (OECD, 2018a). As vehicle controls, DMEM with 1% DMSO (for DMSO extracts) and DMEM (for DMEM extracts) were used.

Local lymph node assay: DA (LLNA: DA)

The LLNA: DA, in which the proliferation of cells in the lymph nodes is assessed by measuring intracellular ATP (adenosine triphosphate) using a bioluminescence method, was performed according to OECD TG 442A (OECD, 2010). The experimental procedure was conducted under SPF conditions in the accredited animal facility of the NIPH, Prague, Czech Republic (File number 16OZ23091/2017-17214; Ref. number 9806/2018-MZE-17214) in conformity with EU legislation related to protection of animals used for scientific purposes (Directive 2010/63/EU). The project was approved by the Ministry of Health of the Czech Republic (MZDR 6593/2019-4/OVZ).

Newborn mice were delivered from Charles River Laboratories (Germany), randomly selected and group-housed (4 individuals per cage) in cages on wooden bedding material especially suitable for SPF. Mice were housed in standard environmental conditions (22 ± 2 °C, 55 ± 10% relative humidity) with artificial lighting (12 h day/night light cycle) and fed *ad libitum* on a commercial ST1 diet (Velaz, Czech Republic) with an unlimited supply of drinking water.

Healthy, non-pregnant, female BALB/c mice, 8-12 weeks of age, acclimatized for 7 days before the start of the test, were used. Four animals were used per test group as stated in OECD 442A, i.e., non-polar extract (cottonseed oil), polar extract (physiological saline solution), or sample tested as such, negative control group treated with respective vehicle, and positive control group treated with 1-chloro-2,4-dinitrobenzene in acetone:cottonseed oil (4:1, v/v). The total number of animals used in one experiment was 28 individuals, in the case of testing 2 samples in both extract vehicles (polar and non-polar) plus negative control with saline, negative control with cottonseed oil, and positive control.

The experimental schedule of the assay was as follows: On Day 1, individual animals from each group were identified, their weight and any clinical observation were recorded. According to OECD TG 442A, which was developed for chemicals, ears are then pretreated with 1% SLS aqueous solution; however, we did not consider this step relevant for extracts and therefore omitted it. The samples, positive and negative controls were applied in a volume of 25 µL to the dorsum of each ear in the morning on Days 1, 2, 3 and 7. After application, each animal was returned to the home cage. On Day 8, the weight of each animal and any clinical observations were recorded (weight 17-21 g ± 0.5 g, without clinical symptoms in all experiments), and the animals were humanely killed by diethyl ether (Penta).

The auricular lymph nodes were removed, and cell suspensions were prepared by gentle grinding of the lymph nodes on a cell strainer (BD Falcon, 352360), rinsing with 2 mL of phosphate-buffered saline (PBS), followed by shaking. A volume of 100 µL cell suspension was added to 9.9 mL PBS. The diluted cell suspension was shaken, 100 µL was transferred into a microtiter plate and 100 µL Cell Titer-Glo (Promega) was added per well. The intensity of emitted light was measured using the luminescence reader GLOMAX Multi Reader (Promega). Each sample was measured in triplicate. The results are considered positive for sensitization potential when the stimulation index (SI) ≥ 1.8 in comparison to the respective negative control (ICCVAM,

Tab. 2: Results of testing of sample extracts by specific methods for skin sensitization
Results are indicated as -, negative result; +, positive result; +?, equivocal result; x, samples that could not be tested due to the lack of test material; *, samples that could not be tested as unsuitable for specific method or vehicle extraction. Samples were classified as positive in the *in vitro* evaluation if they tested positive at least once in both *in vitro* tests. Samples were classified as equivocal in the *in vitro* evaluation if they tested positive twice in either DPRA or LuSens. Otherwise, samples were classified as negative. The final evaluation as sensitizer by *in vivo* LLNA: DA was defined as a sample with at least one positive result in one of the tested solvents or used as such.

Sample	Description	DPRA		LuSens		*In vitro*	LLNA: DA			*In vivo*
		50 µL	250 µL	DMEM	DMSO	Final evaluation	Polar	Non-polar	As such	Final evaluation
1	Silicone sealing ring	-	-	-	-	-	-	-		-
2	Barrel	-	+	-	-	-	-	-		-
3	Antimicrobial Sol SB15	-	-	+	+	+?	-	-		-
4	Hemostatic collagen sponge	-	x	*	-	-	-	-		-
5	Hemostatic collagen sponge	-	x	*	-	-	-	-		-
6	Hemostatic absorbent material	-	+	*	-	-	-	-		-
7	Pad for acute and chronic wounds	-	+	*	-	-	-	-		-
8	Filling of medical material	-	-	-	+	-	+	-		+
9	Textile	-	-	-	-	-	-	-		-
10	Textile	-	-	-	-	-	-	-		-
11	Two-layer laminate	-	-	-	-	-	-	-		-
12	Gauze	-	-	-	-	-	-	-		-
13	Filling of medical material	-	-	-	-	-	-	-		-
14	Wood applicator	-	x	-	-	-	-	-		-
15	Examination latex gloves	+	+	+	+	+	-	-		-
16	Maternity pads	-	+	-	-	-	-	-		-
17	Absorbent underpads	-	-	-	-	-	-	-		-
18	Foam for very dry skin	-	-	*	-	-	-	-		-
19	Eardrops	-	-	*	-	-			-	-
20	Nasal spray	+	+	+	+	+			-	-
21	Anesthesia needles	-	x	x	-	-	-	-		-
22	Epidural and block nervous catheters	-	-	x	-	-	-	-		-
23	Ear spray	-	-	+	+	+?			+	+
24	Nasal spray	+	-	+	+	+			+	+
25	Aniball light pink	-	-	-	-	-	-	-		-
26	Bioactive wound cover	-	+	*	-	-	-	-		-
27	Aniball dark pink	-	-	-	-	-	-	-		-
28	Aniball Inco salmon pink	-	-	-	-	-	-	-		-
29	Dental composite	-	-	-	+	-	-	-		-
30	Lubricant gel cherry	-	-	-	-	-			+	+
31	Hydrogel burn spray	*	*	*	-	*			-	-
32	Spray for safe removal of ticks	*	*	*	*	*			-	-
33	Lubricant gel strawberry	-	-	-	-	-			-	-

Sample	Description	DPRA		LuSens		*In vitro*	LLNA: DA			*In vivo*
		50 µL	250 µL	DMEM	DMSO	Final evaluation	Polar	Non-polar	As such	Final evaluation
34	Lubricant gel natural	-	-	-	-	-			-	-
35	Rubber material for MD	+	+	+	+	+	-	-		-
36	Bandage material with pad	-	-	-	-	-	-	-		-
37	Thigh-high stocking	-	-	-	-	-	-	-		-
38	Knitted knee sleeve with silicone-stabilized patella	+	+	-	-	+?	-	-		-
39	Hemostatic dressing	-	-	-	-	-	-	-		-
40	Urinary catheter	-	-	-	-	-	-	-		-
41	Three-layer sandwich textile material	-	-	-	-	-	-	-		-
42	Three-layer sandwich textile material	-	-	*	-	-	-	-		-

2010). However, there are some situations where borderline results (SI value between 1.8 and 2.5) may be acceptable depending on the foreseen use of the medical device (OECD, 2010).

2.3 Statistical analyses

The results were expressed as concordance, discordance and accuracy and analyzed using three different statistical tests (p-value < 0.05): McNemar's test, analyzing the statistical significance of the differences in classifier performances (McNemar, 1947), Cohen's kappa, useful for either interrater or intrarater reliability testing (McHugh, 2012), and Kendall's Tau, a nonparametric measure of the degree of correlation (Puka, 2011). For data processing, the statistical software Stata was used.

3 Results

3.1 Testability of samples in the three assays

The results of the 42 samples tested in the study are shown in Table 2. 40 samples were tested in at least one test volume in the DPRA, while two samples of sprays were unsuitable for extraction (samples 31 and 32). Four samples were tested only in the 50 µL volume owing to lack of material (marked with x). The DPRA test method is considered not applicable to the testing of metal compounds, which are known to react with proteins via mechanisms other than covalent binding and may lead to false-positive results. However, sample 21 (anesthesia needles) probably did not release metal ions into the extract as the result was negative.

41 samples were tested in at least one vehicle in the LuSens assay, while one sample was unsuitable for extraction in both vehicles (sample 32), as it induced a strong cryogenic effect and could not be dosed properly. Nine samples were unsuitable for extraction in DMEM (*), one sample was cytotoxic in DMEM at all concentrations tested (sample 42), and the lack of material allowed testing of 2 samples in only one vehicle (samples 21 and 22). Of the samples that were unsuitable for extraction with DMEM, those containing collagen (samples 4, 5, 6, 7 and 26) formed a jelly-like mass after application of the DMEM extracts to the cells. This thick layer, which could not be removed from the cell monolayer, interfered with consequent analysis and evaluation. The other samples that were unsuitable for extraction with DMEM (samples 18, 19 and 31) were in the form of foam, which prohibited homogeneous extraction.

All animals were healthy, non-pregnant female BALB/c mice, 8-12 weeks old, with weight between 17-21 g ± 0.5 g, without clinical signs before and after treatment or any adverse effects. All data obtained in this study were included in the evaluation of results. Forty-two samples were tested in the LLNA: DA, of which 33 samples were tested in both vehicles and 9 samples were tested as such.

3.2 Classification of samples

In the DPRA, ten samples tested positive, four in both tested volumes (50 and 250 µL), one only in the lower volume, and five only in the higher volume. In the LuSens, eight samples tested positive, six of them in both used extractant vehicles (DMSO and DMEM) and two only in DMSO.

Samples were classified as positive in the *in vitro* evaluation if they tested positive at least once in both the *in chemico* and *in vitro* test, i.e., in DPRA 50 µL or DPRA 250 µL and LuSens DMEM or LuSens DMSO (samples 15, 20, 24 and 35), while samples with no or only one positive outcome were considered non-sensitizers. Samples with two positive results in either the DPRA or in the LuSens were deemed equivocal in the *in vitro* evaluation (samples 3, 23 and 38).

A sensitizer in the *in vivo* LLNA: DA was defined as a sample with at least one positive result in one of the tested solvents or used as such. All of the results were clearly positive or clearly negative; no borderline results were obtained. Four samples were classified as positive (samples 8, 23, 24 and 30). Three of the positive samples, i.e., samples 23, 24 and 30, were applied as

Tab. 3: Statistical comparison of *in vitro* and *in vivo* test method results
DPRA was performed with two different volumes (50 µL and 250 µL) and LuSens with two extraction vehicles (DMEM and DMSO). Analysis was done using McNemar's test (Symmetry), Cohen's kappa (Kappa) and Kendall's Tau (Tau-c). The table shows the concordance, discordance and accuracy between the tests based on the total number of results for both tests (n). *, $p < 0.05$.

Comparison	Symmetry	Kappa	Tau-c	n (total)	concordant (n)	discordant (n)	accuracy (%)
LLNA: DA vs DPRA 50 µL	0.7055	0.2128	0.455	40	33	7	82.5
LLNA: DA vs DPRA 250µL	0.1655	0.8897	0.240	36	23	13	63.9
LLNA: DA vs LuSens DMEM	0.4142	0.0536	0.124	30	24	6	80.0
LLNA: DA vs LuSens DMSO	0.1025	0.0016*	0.004*	41	35	6	85.4

Tab. 4: Statistical comparison of *in vitro* test method results
DPRA was performed with two different volumes (50 µL and 250 µL) and LuSens with two extraction vehicles (DMEM and DMSO). Analysis was done using McNemar's test (Symmetry), Cohen's kappa (Kappa) and Kendall's Tau (Tau-c). The table shows the concordance, discordance and accuracy between the tests based on the total number of results for both tests (n). *, $p < 0.05$.

Comparison	Symmetry	Kappa	Tau-c	n (total)	concordance	discrepancy	accuracy (%)
DPRA 50 vs DPRA 250	0.1025	0.0011*	0.003*	36	30	6	83.3
DPRA 50 vs LuSens DMSO	0.1797	0.0002*	< 0.001*	40	35	5	87.5
DPRA 50 vs LuSens DMEM	0.5637	0.0001*	< 0.001*	30	27	3	90.0
DPRA 250 vs LuSens DMSO	0.7630	0.1773	0.375	36	25	11	69.4
DPRA 250 vs LuSens DMEM	1.0000	0.0233*	0.055	29	23	6	79.3
LuSens DMEM vs LuSens DMSO	0.1573	< 0.001*	< 0.001*	30	28	2	93.3

such, suggesting the importance of physico-chemical properties and content of active substances for a positive response in experimental animals.

Only one of the 42 tested samples exhibited positive results in all employed test methods (sample 24); 33 samples were predicted as samples with non-sensitizing potential in all three performed methods. The samples that were equivocal or positive in the *in vitro* assays but negative in the LLNA: DA may be considered false-positive (samples 3, 15, 20, 35, 38). The samples that were equivocal or negative in the *in vitro* assays but positive in the LLNA:DA may be considered false-negative (samples 8, 23 and 30).

3.3 Correlation of test methods

The results of the statistical comparison of performance and correlation of the *in vitro* and *in vivo* methods for skin sensitization are summarized in Table 3. Very good concordance was achieved between the *in vivo* and *in vitro* methods (LLNA: DA and LuSens DMSO, accuracy 85% and LLNA: DA and DPRA 50, accuracy 82%). According to Cohen's kappa and Kendall's Tau-c test, the interrater reliability between LLNA: DA and LuSens DMSO methods is statistically significant ($p < 0.05$), and this comparison also achieves the highest accuracy.

The results of the statistical evaluation of performance and correlation of the two *in vitro* methods for skin sensitization are summarized in Table 4. According to the Cohen's kappa and Kendall's Tau-c test, the interrater reliability between DPRA 50 and LuSens DMSO, and DPRA 50 and LuSens DMEM is statistically significant. As expected, the reliability between the two extraction volumes or two extraction vehicles of the respective tests is also statistically significant. The highest accuracy (90%) between different *in vitro* tests was achieved by DPRA 50 vs LuSens DMEM.

McNemar's test (Symmetry) did not reach statistical significance between tests.

4 Discussion

The increasing regulatory and societal demand for alternative methods for safety testing is also reflected in the medical device industry. In January 2021, the ISO standards were updated to include the *in vitro* skin irritation test on reconstructed human tissues as predictor of intracutaneous irritation (De Jong et al., 2018; ISO, 2021), and ISO 10993-10 is currently under revision (ISO, 2010; Grundrtröm and Borrebaeck, 2019). However, multiple toxicity endpoints in medical device safety testing still demand animal experiments, particularly tests for skin sensitization, for which huge numbers of laboratory guinea pigs and mice are still used.

From the animal welfare perspective, the LLNA is undoubtedly a refinement method compared to the conventional toxicological *in vivo* methods, as the animals are subjected to less painful and stressful procedures than, e.g., in the guinea pig maximization test (Basketter et al., 2005; Hoffmann, 2015). However, the variability in the animal data is an indicator of uncertainty with regard to the LLNA endpoint, particularly in the case of substances that have been tested only in a single LLNA study (EPA, 2018).

Since the first publication of the adverse outcome pathway for skin sensitization in 2012 (OECD, 2012), efforts have been made to develop an integrated approach to testing and assessment (IATA) for *in vitro* skin sensitization testing by integrating various types of chemical and toxicological data into the decision-making process (OECD, 2014, 2017). Skin sensitization is a comprehensive immune response, therefore, alternative methods that assess a single key event are considered insufficient for evaluating the ability of a test substance to induce skin sensitization. Thus, focus has been increasingly laid on an assessment that combines at least two *in chemico* or *in vitro* methods (addressing different key events), resulting in the "2 out of 3 approach" (OECD, 2017; Bauch et al., 2012). Here, a single test may be performed for each of the key events 1, 2 and 3 (peptide/protein binding, keratinocyte activation, and dendritic cell activation), respectively. Where the first two tests are in agreement, decision-making is unequivocal. Where the tests are discordant, a third KE test should be performed.

All contemporary assays reflect different ways of assessing the reactivity of chemical compounds while exhibiting specific technical limitations. The relevance of prediction based on their combined use depends on how the different assays compensate for each other's disadvantages. It has been suggested that no more than two methods may be necessary (namely DPRA and h-CLAT), and this may render a considerably higher level of efficiency than the "2 out of 3 approach" (Roberts and Patlewicz, 2018). A sequential testing strategy combining 1 to 3 models to cover the main key events of the skin sensitization AOP has been suggested for a bottom-up approach, which would minimize the danger of a false-negative conclusion, essential from the perspective of medical device users. A study published by Otsubo et al. (2017) suggested that a binary test battery of KeratinoSens™ and h-CLAT could be useful as part of a bottom-up approach, where the testing strategy suggests using test methods that can reliably define non-sensitizers (Scott et al., 2010). An international endeavor has been made to construct a defined approach (DA) consisting of an established data interpretation procedure applied to results generated with a defined collection of information sources to derive a prediction of skin sensitization (OECD, 2017).

Medical devices commonly contain a mixture of materials in low concentrations and therefore pose specific testing challenges in comparison to pharmaceutical or chemical testing, where a solution of the test substance with a defined concentration can be prepared (Myers et al., 2017). Medical devices often need to be extracted prior to safety testing using polar and non-polar solvents according to ISO 10993-12. Culture media, water, physiological saline solution or oil are recommended as extraction vehicles (ISO, 2012). Thus, possible sensitizers extracted from a device are frequently extremely diluted, and their sensitizing potential may be affected by other components present in the extract. While alternative tests for skin sensitization have been validated and recommended for testing of chemical substances, their usefulness to predict the skin sensitization potential of mixtures is being investigated. Novel studies comprise optimizations for testing of botanical extracts and ingredients (Gan et al., 2013; Moreira et al., 2017; Nishijo et al., 2019), mixtures, and finished products (Settivari et al., 2015; de Ávila et al., 2019; Cottrez et al., 2020). For example, the applicability of KeratinoSens™ (OECD TG 442D) has been evaluated recently for assessment of plant extracts, which were negative in the test but positive when spiked with known sensitizers. The study provided a proof of principle for testing of mixtures in this assay and verified its ability to detect minor constituents with sensitizing potential (Andres et al., 2013). The *in chemico* test DPRA has been optimized with regard to the used volume (mDPRA) or process (photo-mDPRA) (de Ávila et al., 2017), e.g., to predict the skin sensitizing potential of botanical extracts (Moreira et al., 2017) or even metals, specifically platinum (Hemming et al., 2019).

The current study was designed to determine if the validated DPRA and LuSens assays can detect sensitizers at low concentrations, which would make them possible alternatives for skin sensitization testing of medical device extracts. The samples that were equivocal or positive in the *in vitro* assays but negative in the LLNA: DA may be considered false-positive (No. 3, 15, 20, 35, 38), but actually the LLNA: DA may be insufficiently sensitive to medical device extracts, as it has never been validated specifically for the purpose of testing medical devices and their extracts. Many sensitizers need to be present at 2-20% in the LLNA to cause a positive response (ICCVAM, 2011), although OECD even requires testing the highest dose that is soluble and does not produce systemic toxicity, for chemicals. The level of 2-20% may not be reached for real sensitizing impurities in an extract, but the application of that device in practice may potentially sensitize humans, i.e., the LLNA result might be false-negative.

The samples that were equivocal or negative in the *in vitro* assays but positive in the LLNA:DA may be considered as false-negative (samples 8, 23 and 30). Sample 8 consisted of plastic crumblings from recycled raw material. The technological process of manufacture included intense washing with detergents, which probably were not rinsed effectively, and unknown impurities from plastic recycling might have been present. The residual detergents and impurities probably caused a positive response in LLNA: DA and also LuSens (DMSO). A different batch of this material (sample 13), manufactured under modified conditions including thorough rinsing, yielded negative results in all the test methods. The suspected sensitizing agent in lubricant gel cherry (sample 30) is cherry aroma, which is a synthetic and not a natural food aroma. The other tested samples from the same manufacturer, lubricant gel strawberry and lubricant gel natural (samples 33 and 34), with a similar formulation except for the aroma, both provided negative results.

Samples 20, 23 and 24 might be positive in the *in vitro* tests due to the higher sensitivity of these methods to plant extracts, essential oils and perfumes, which are used as odorous ingredi-

ents. They represent complex mixtures, which may include naturally occurring contact sensitizers. The main components of essential oils are monoterpene and sesquiterpene hydrocarbons, monoterpene and sesquiterpene alcohols, esters, ethers, aldehydes, ketones and oxides. Commonly, every oil contains a few ingredients from each major chemical group. The spectrum of reported skin reactions to essential oils includes contact urticaria, irritant contact dermatitis, phototoxic reactions, and allergic contact dermatitis (Bleasel et al., 2002; Lalko and Api, 2006). These compounds may well cause problems when included in the composition of medical devices.

In vitro methods also may be more sensitive to materials such as latex, rubber or silicone as seen by positive results for samples 15, 35 and 38. The natural rubber latex is frequently reported to cause sensitization or allergy, leading to serious occupational health issues. The first reports of allergy to natural rubber latex included only contact urticaria, although anaphylactic reactions were later also described with increasing frequency (Valks et al., 2004). A novel form of natural rubber (guayule) is now commercially available for use in latex medical devices, e.g., medical gloves and catheters. These products do not cause reactions in humans who are allergic to latex. Guayule contains terpene resin comprising hundreds of isoprenoid compounds. A study by Cornish et al. (2010) reported that guayule derivatives (guayulins) are contact sensitizers in guinea pigs, although they appeared much less sensitizing in humans. However, in mice, no sensitization or irritation by guayulin A was observed, but guayule resin was irritating at concentrations of 10% and higher with no confirmed potential of sensitization (Cornish et al., 2010).

The combination of DPRA and LuSens assays helped to improve the overall prediction power compared with the single test method approach in case of non-sensitizing materials. This result supports the widely-held belief that integrated approaches to skin sensitization testing surpass single non-animal methods (Rovida et al., 2015). Nevertheless, the tests are not suitable for testing some types of medical devices (e.g., metal compounds and aerosol sprays). The modified version of DPRA, using a higher volume of the sample, did not improve the test performance. The two *in vitro* tests applied in combination in our study provided several cases of disagreement between the non-animal test methods and the LLNA: DA data, showing a higher sensitivity of the alternative tests to some materials as described above (latex, rubber, silicone, plant extracts, essential oils and perfumes). Therefore, it would be desirable to incorporate a third *in vitro* method covering KE3 (e.g., h-CLAT, OECD 442D) in case of positive or equivocal results in the KE1 and KE2 tests in a "2 of 3" approach.

According to medical device manufacturers, the majority of *in vivo* sensitization tests provide negative results (Myers et al., 2017), which was seen also in this study (only 4 positive results in the *in vitro* methods and 4 positive results in the *in vivo* method). Huge numbers of experimental animals are still employed to prove lack of sensitization potential, while numerous alternative methods, based on cells and tissues of human origin with higher relevance to clinical practice, have demonstrated their accuracy and reliability (Myers et al., 2017; Bergal et al., 2020). Skin sensitization is a complex immune response, therefore, all key events available *in vitro* to confirm the absence of sensitization potential should be tested to ensure the safe use of the medical device foreseen to come into contact with the human body.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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