PERMEABILITY AND BIOCOMPATIBILITY OF NOVEL MEDICATED HYDROGEL WOUND DRESSINGS

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□ *Hydrogel dressings are being popularized for wound care management because of their softness, tissue compatibility, and ability to enhance wound healing process. PVP-CMC and PVP-CMC-BA hydrogels were prepared using polyvinylpyrrolidone (PVP), sodium-carboxymethylcellulose (CMC), polyethyleneglycol (PEG), agar, glycerine and without/with boric acid (BA). Permeability: water vapor transmission and microbe penetration and biocompatibility: cytotoxicity, skin irritation, and skin sensitization tests of hydrogels were performed. Water vapor transmission ability of hydrogels shows positive response to gas permeability, whereas microbe penetration ability by Staphylococcus aureus and Escherichia coli shoius negative penetrability; thus these two hydrogel dressings could be considered as a good barrier against microbes having an excellent oxygen diffusion property. In vitro assay of cytotoxicity tuas carried out in presence of human immortalized non-tumorigenic keratinocyte cell line (HaCaT), which was further confirmed with balb/c 3T3 mouse fibroblasts cells. The skin irritation test was evaluated on albino rabbits and sensitization test was carried out on albino guinea-pigs under in vivo stale. Both PVP-CMC and PVP-CMC-BA represented mostly negative effect on cytotoxicity, dermal irritation, and/ or sensitization. All the results indicate that both PVP-CMC and PVP-CMC-BA hydrogels have great potential for biomedical applications, especially for burn skin treatment as well as wound dressings materials.*

Keywords Biocompatibility, Cytotoxicity, Hydrogels, Skin irritation, Wound dressing

INTRODUCTION

Hydrogels are well known for their intensive use for medical purposes, for instance contact lenses, coaling of catheters, artificial skin, controlled release drug delivery systems, and so on. Moreover, hydrogels are drawing more attention to be used for the healing of skin lesions in the form

of bandages, adhesives, and burn wound dressings (1-3). Wound dressing hydrogels are cross-linked three-dimensional networks of hydrophilic polymers, which can absorb substantial amounts of water (4). A hydrogel dressing prevents the wound from microbial contamination, inhibits the loss of body fluids, provides free flow of oxygen to the wound, and generally accelerates the healing process (5). Each biomaterial, which needs to be in contact with living organs, has to fulfil some basic requirements like non-toxicity, functionality, sterilizability, and biocompatibility (6, 7). The application of the biomaterials for medical purposes should not be responsible for any adverse reaction in the organism and should not cause any danger to the patient's life. Biocompatible materials must be non-toxic, non- allergenic, non-carcinogenic, non-mutagenic, and should not influence the fertility of the patient (2). Biocompatibility of the device is normally investigated using analytical chemistry, *in-vitro* tests and animal models. It depends on several factors, such as: the chemical and physical nature of its component materials, the type of patient tissue that will be exposed to the device and duration of that exposure. The primary purpose of biocompatibility assessment of a device is to protect the patient's safety as in some cases substances may leach off from the medical device into adjacent tissue while in use. These leachables or extractables are sometimes not biologically safe. Therefore, it is mandatory to perform a biocompatibility test to assure the safety use of this medical device or product (8, 9). Hydrogels are generally considered as biocompatible materials, because they are soft, porous, and their tissue-like consistency provides minimum frictional irritation upon implantation (i.e., they are tissue compatible). Moreover, hydrogels are non- or only slightly thrombogenic when they get in contact with blood (10).

The remarkable interest in the application of radiation techniques to obtain hydrogels for biomedical purposes began in the late sixties, as an evidence of papers and patents by Japanese and American scientists, led by Kaetsu in Japan and Hoffman in the United States demonstrates (6). Since that time until today, various kinds of hydrogels have been reported and research is still continuing on improvement and implementation of hydrogels in different ways including using of natural and synthetic polymers like polyvinylpyrrolidone (PVP) and sodium-carboxymethylcellulose (CMC) for the synthesis of wound dressing hydrogels (11).

In the present study, we have prepared PVP-CMC hydrogels without and with boric acid (BA) by moist heat treatment, a physical stimulation technique (12, 13) and designated the novel hydrogels as "PVP-CMC" and "PVP-CMC-BA," respectively. BA was incorporated to develop antimicrobial properties within the hydrogel. Use of BA for medical purposes, such as in creams and ointments has already been reported (14, 15) but incorporation of BA in hydrogel to improve its medicinal value is a novel approach

that was reported in our previous article (13). We also thoroughly studied the properties of PVP-CMC hydrogels (12).as well as PVP-CMC-BA hydrogels (13). which were prepared containing 0-4% of BA. The PVP-CMC-BA hydrogel containing 3% BA showed reasonably good performance in water absorption, elasticity, mechanical, and antibacterial properties point of view. Moreover, 3% BA maintains the permissible limit of dose of BA from the health care point of view (15). The development of hydrogel is intended for its use as wound dressing material for health care application. Thus, considering the limitation of BA in health care application, PVP-CMC-BA hydrogel was prepared with 3% BA and examined their permeability as well as biocompatibility: cytotoxicity (in vitro) and skin irritation and skin sensitization tests (in vivo). Water vapor transmission (16, 17) and microbe penetration tests (18, 19) of hydrogels were also performed as these tests are very essential before recommending to use hydrogel as a wound dressing material. *In-vitro* studies of the PVP-CMC and PVP-CMC-BA hydrogels were conducted on human immortalized non-tumorigenic keratinocyte cell line (HaCaT) (20, 21). Furthermore, the PVP-CMC-BA hydrogel was confirmed by *in-vitro* cytotoxicity study on balb/c 3T3 mouse fibroblasts cells (22, 23), as well as *in-vivo* skin irritation tests on rabbits and skin sensitization test on guinea-pigs.

EXPERIMENTAL

Permeability of hydrogel by means of water vapor transmission and microbe penetration assessment and biocompatibility of hydrogel by means of cytotoxicity *(in-vitro),* skin irritation and sensitization (*in-vivo)* studies on animals were carried out to evaluate its potential as medical device for health care application. The biocompatibility confirmation tests on balb/c 3T3 mouse fibroblasts cells and on animals were carried out at National Institute of Public Health (accredited laboratory No 1206.3) Prague, Czech Republic.

Materials

Hydrogel

Polyvinylpyrrolidone K30 (PVP: molecular weight 40,000), polyethylene glycol 3,000 (PEG: average molecular weight 3,015-3,685) and agar were obtained from Fluka, Switzerland; sodium carboxymethyl cellulose (CMC) was purchased from Sinopharm Chem. Reagent Co., Ltd., China; boric acid (BA) from Sigma-Aldrich, USA and glycerin obtained from Lachema Ltd., Czech Republic.

Cytotoxicity Assay

The cytotoxic effect of hydrogel was investigated in presence of two kinds of cells, that is (i) Human immortalized non-tumorigenic keratinocyte cell line (HaCaT) and (ii) Balb/c 3T3 mouse fibroblasts. The materials required to conduct these tests are mentioned below.

i. Extract of PVP-CMC and PVP-CMC-BA hydrogels, human immortalized non-tumorigenic keratinocyte cell line (HaCaT) supplied by Cell Lines Service, Germany. Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, USA), containing 4,500 mg/L D-glucose, L-glutamine, and 110 mg/L sodium pyruvate supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/ml -0.1 mg/ml) was used as the cell culture medium. As a positive control dodecyl sulfate sodium salt (SDS, Signia) having the final concentration of 1, 10, 20 µg/ml DMEM and as a negative control medical polyethylene were used.
ii. Extract of PVP-CMC-BA hydrogel, Balb/c 3T3 mouse fibroblasts. DMEM (Dulbecco's Minimum Essential Medium, Sevapharma, Czech Republic) supplemented with antibiotics (penicillin; 100 IU/ml, streptomycin; 100 \ig/ml) and 10% of inactivated calf serum (Vyroba ser a.s., Hustoopece na Hane), pH 7.2, was used as the culture medium. I)odecyisulphate sodium salt (SDS, Sigma) having the final concentration of 1, 10, 20 (1 g/ml DMEM without serum was used as positive control (PC). Hydron (poly [(2-hydroxyethyl) methacrylate] (Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague) was used for negative control (NC). The extraction vehicle without test material subjected to extract conditions and test procedures: culture medium without serum was designated as reagent control (RC). Cell control (C) was the culture medium without serum.

Skin Irritation

Healthy young adult albino rabbits (single outbred strain, females, average weight 2-3 kg) were considered as model animals. PVP-CMC-BA hydrogel was used as test material (TM), distilled water as negative control (NC), also ascorbic acid, gauze, semi-occlusive bandages were used to perform the test. The test was preformed according to ISO- 10993-10: 2003 (24).

Skin Sensitization

Healthy young adult guinea-pigs (single outbred strain (Dunkin Hartley), females, average weight 250-320 g, nulliparous, not pregnant), were used as model animals. PVP-CMC-BA hydrogel was used as test material (TM), and saline solution was used as extraction vehicle. Extraction vehicle

without PVP-CMC-BA hydrogel was considered as reagent control (RC). The test was preformed according to ISO- 10993-10:2003 (24).

Methods

Preparation of Hydrogels

The ingredients used for the preparation of hydrogel wound dressings were PVP, CMC, BA, PEG, agar, and glycerin. The polymer solutions were prepared in sealed bottles by dissolving weighed components in water. Then, moist heat treatment [15 lbs (107 kPa) pressure, 120°C, 20 minutes] was applied to the solutions followed by solution casting in petri dishes of 85 mm diameter. The polymer solutions were then allowed to cool at room temperature (20-22°C) under an aseptic environment to achieve the desired hydrogels (12, 13, 25). The hydrogel without BA was designated as "PVP- CMC" and the hydrogel with BA was designated as "PVP-CMC-BA." The composition of PVP-CMC and PVP-CMC-BA hydrogels has been shown in Table 1.

Water Vapor Transmission Test

The water vapor permeability (WVT) of the hydrogel films (PVP-CMC and PVP-CMC-BA) was determined gravimetrically (16, 17). Each aluminum foil dish (internal diameter of 5.5 cm and height 1 cm) was filled with 10 ml of distilled water (100% RH). A circular piece of dry hydrogel film was placed on the dish and sealed with glue. Another circular piece of the same film was fixed on another dish without water as a reference. Both samples and references were accurately weighed and then placed in a desiccator containing calcium chloride (CaClg) as a desiccant, and filled with silica gel (0% RH). At specific intervals (24, 48, 72, and 96 hours) the dishes were weighed and the profile of mass change was plotted versus time, for each film. For each hydrogel experiments were conducted in triplicates. The amounts of water vapor transmission through the films were measured using the following Eq. 1 (17).

$$WVT = \frac{Wx}{tAP_0 (RH_1 - RH_2)}$$

TABLE 1 Composition of Hydrogels (W/V %)

Sample index	PVP	CMC	PEG	Agar	Glycerin	BA	Water
PVP-CMC	0.2	0.8	1	2	1	0	95
PVP-CMC-BA	0.2	0.8	1	2	1	3	92

In this equation, WVT is the water vapor transmission. W/t is the mass change (flux, g/h) resulted from the slope of profile of the mass change versus time; x is die thickness of the film used in the scale of mm. A is the area of the film in the scale of m^2, which is equal to the surface of the dish. Po is the vapor pressure of pure water vapor transmission that is equal to 3.159 kpa at 25°C. (RH_1-RH_9) is the relative humidity gradient of the inside and outside moisture contact of the examined dish.

Microbe Penetration Test

The bacterial penetration ability in hydrogels (PVP-CMC and PVP-CMC- BA) was examined in presence of *Staphylococcus aureus and Escherichia coli.* The hydrogels were first made into rectangular pieces of size 30 x 30 mm^2 and ^2.5 mm thickness. The upper surface of hydrogel was contaminated with 0.02 ml of culture inoculums (10^7 CFU/ml). Contaminated hydrogels were then placed on the surface of sterile nutrient agar plate in the petri dish (diameter 85 mm) and then incubated at 37°C for 24 hours. After that, the hydrogels were removed and examined the formation of colony on the surface where the contaminated hydrogels were placed (18, 19).

Evaluation of Cytotoxicity (In-Vitro)

Extract preparation. The PVP-CMC and PVP-CMC-BA hydrogels were extracted according to ISO 10993-12 in the ratio of 0.1 g of the hydrogel per 1 ml of extraction vehicle (DMEM without serum) in chemically inert closed containers by using aseptic techniques. The individual hydrogel of PVP-CMC and PVP-CMC-BA was incubated in DMEM medium at 37°C with stirring for 24 hours. The hydrogel extract was then diluted in culture medium DMEM without serum to achieve the final concentration as 100, 50, 25, 10, 5, and 1%.

Test on HaCaT cells. Cells were preincubated in petridishes (9.2 cm^2) using 2.5 ml of DMEM medium having the concentration of cell 2 x 10°/pe tridishes (26, 27). The final concentration of extract 100, 50, and 10 were tested on HaCaT cells. Subsequently, the culture medium of HaCaT cell cultivation was replaced with the extraction fluids of hydrogels and as a control set, only extract medium (DMEM + FBS) was treated under same conditions. To assess the cytotoxic effect of PVP-CMC and PVP-CMC-BA hydrogels on *HaCaT* cell culture, the MTT assay (22, 28, 29) was performed. Two variants of test were performed. In fact, the effects of hydrogel extract on cell were tested either by short term or by long term. The “short term” was consisted from 1 day precultivation; 1 day cultivation in extracts and MTT test. The “long term” consisted from 1 day precultivation, 3 days cultivation in extract following by 3 days cultivation in medium and MTT test.

Cell viability of HaCaT. Tetrazolium (Vybrant, MTT cell proliferation assay kit) was used to determine cell viability. The cell viability of HaCaT cells was assessed after cell cultivation in hydrogel extracts (100, 50, 10, and 0%) by MTT assay [reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, which turns from yellow to a purple formazan product] (29). The absorbance value of the medium without cells was subtracted as background. The cell viability (%) of HaCaT cells was presented compared to cells cultivated in medium without hydrogel extracts.

Cell morphology of HaCaT. The morphology of HaCaT was assessed after cultivation of cells in hydrogel extracts (100, 50, 10, and 0%) from 24 hours until 120 hours. The morphology of HaCaT cells from each culture plates was observed using an inverted phase contrast microscope (Olympus) equipped with an objective of 40x magnification.

Test on Balb/ c 3T3 mouse fibroblasts cells. The Balb/c 3T3 mouse fibroblasts cells were seeded into individual wells of 96-well microtitre tissue culture plates. The cell suspension of 1 x 10^5 cells/ml DMEM was inoculated in the volume of 0.1 ml (1 x 10^4 cells) per well. After 24 hours of pre-incubation, the medium was removed and replaced by 0.2 ml of liquid extracts of hydrogel (test material, TM) and controls (positive controls (PC), negative control (NC), reagent control (RC) or culture medium (C)). At the end of 24 hours treatment (37°C, 7.5% CO_2), the medium was removed and the cells were stained by Neutral Red dye according to INVITTOX Protocol No. 46 (0.2 ml Neutral Red solution per well, 3 hours incubation, Neutral Red desorb solution-ethanol/acetic acid). The test was carried out in compliance with SOP 2/13 tests for *in- vitro* cytotoxicity and test on extract (ISO 10993-5) (30).

The cell viability was quantitatively determined as the Neutral Red uptake measured by fluorescence-luminescence reader FLX800TBI (Bio Tech). The evaluation of cytotoxicity by means of cold light fluorimetry is based on the incorporation of a vital dye (Neutral Red) into living cells (Neutral Red Uptake) and detection of fluorescence in the system of excitation (530 nm) and emission (590 rim) filters. The results (Fluorescence Units, FSU) obtained for wells treated with the PVP-CMC-BA were compared to untreated control wells (culture medium, 100% viability) and converted to a percentage value (31). The mean FSU value of eight blank wells (containing only Neutral red desorb solution) was subtracted from the mean FSU value of four treated wells (treated with the test material, PC, NC, RC, and C). The cytotoxic effect of hydrogel extract on Balb/c 3T3 mouse fibroblasts cells was evaluated on the basis of ISO 10993-5 (28). The cell viability was calculated following Eq. 2:

$$\text{Viability}(\%) = \frac{\text{Mean FSU of test wells} - \text{Mean FSU of blanks}}{\text{Mean FSU of cell control wells} - \text{Mean FSU of blanks}} \times 100 \quad (2)$$

All the test samples on HaCaT as well as Balb/c 3T3 and controls were run in quadruplicates.

Evaluation of Skin Irritation (In-Vivo)

The skin irritation test was carried out on albino rabbits (Fig. 8) following ISO standard (SOP 3/13 tests for irritation and delayed-type hypersensitivity) (24). Three healthy albino rabbits were employed for this test and they were kept in individual cages under 20°C =L 2°C, 55% dt 10% relative humidity, natural illumination, conventional diet and water, with sufficient quantity of ascorbic acid to adjust them to this environment. After being acclimatized for 5 days, *single exposure test* was carried out. The fur on the backs of the rabbits was clipped (area approx. 10 x 25 cm^2) smoothly in a sufficient distance on right side of the spine. The hydrogel was applied on one of the clipped part of skin, and a gauze moistened with 0.5 ml distilled water (negative control) was applied to another part. The application sites were covered by semi occlusive bandages for 4 hours, so that the applied hydrogel and gauze would not move from their places. After 4 hours, the patches were removed and the application sites were observed at 1 hour, 24 hours, 48 hours, and 72 hours. The skin reactions for erythema and oedema were described and evaluated according to the ISO norm of classification system for skin reactions. The irritation response of PVP-CMC-BA hydrogel categories follow the response category depicted in Table 4. The preliminary Irritation Index (Pn) was determined using the Primary Irritation Scores (Pis) for each animal at each time interval of observation (24, 48, and 72 hours after removal of the patches). The Pis for both erythema and oedema at each specified time were added arid divided by the total number of observations (24).

Evaluation of Sensitization (In-Vivo)

The test was carried out on healthy albino guinea-pigs (Fig. 9) following ISO standard (SOP 3/13 Tests for irritation and delayed-type hypersensitivity) (24). Fifteen healthy albino guinea-pigs were considered for sensitization test which were kept in individual cages for 14 days under 20°C d= 2°C, 55% =h 10% relative humidity, natural illumination, conventional diet and water, with sufficient quantity of ascorbic acid to adjust themselves to the said environment. The acclimatized albino guinea-pigs were divided into two groups: the test group (10) and the control group (5). The experiment was carried out in several phases (i.e., Intradermal induction phase, Topical induction phase, and Challenge phase). The initial day was considered as day-0, and termed as ‘‘Intradermal induction phase.” Three pairs of intradermal injections of 0.1 ml volume were injected in the shoulder region, that is,

intrascapular region of the test group animals, (a) A 50:50 (V/V) mixture of Freund's complete adjuvant mixed with the extraction vehicle (saline solution), (b) The extract at the concentration 100%. The extract was prepared using 6 cm^2 hydrogel per 1 ml of saline solution, incubated at 37°C for 72 hours, (c.) The extract at the concentration used in (b), emulsified in a 50:50 (V/V) mixture of Freund's complete adjuvant and the extraction vehicle. Similarly, three pairs of intradermal injections 0.1 ml volume were also given to the animals from control group in the same sites as in the test animals, containing (a) A 50:50 (V/V) mixture of Freund's complete adjuvant mixed with the extraction vehicle (saline solution), (b) The extraction vehicle (saline solution), (c) The extraction vehicle mixed/emulsified with the mixture prepared according to (a). Then, all the test and control sets of animals were incubated for 6 days in the same environment where they were acclimatized earlier.

On the 6th day, all the animals were massaged by 10% sodium lauryl sulfate mixed with petroleum, in the intrascapular region. After 24 hours, a piece of filter paper (20 x 40 mm^2) soaked in the 100% hydrogel extract was applied on the intrascapular region of the each tested animal. The control animals were also treated in the same way but except 100% hydrogel extract. The extraction vehicle (saline solution) was applied to control set of animals. Finally, all the patches of filter papers were held tightly with occlusive dressings until 48 hours and then removed. These 6-7 days are termed as "Topical induction phase." Subsequently, all the animals were then again incubated for 14 days at the former environmental condition. After 14 days, both test and control animals were treated with 100% of hydrogel extract. The filter papers were soaked with hydrogel extract and applied to one clipped flank of each animal. The patches were again covered with occlusive dressings and after 24 hours both dressings and patches were removed. Day 22-23 is called "Challenge phase." Afterward, the appearance of any erythema/oedema on the challenged skin was observed up to 72 hours in every 24-hour intervals.

RESULTS AMD DISCUSSIOW

Water Vapor Transmission

A wound dressing should be capable of free flow of oxygen to the wound. Oxygen is essential for cell growth, which can make the healing process faster. So, it is desirable that the wound dressing hydrogels should have oxygen/gas permeable property. Thus, the oxygen diffusion property of hydrogels was confirmed by measuring water vapor transmission (WVT) rate and loss of water in time under controlled environment and the results are presented in Fig. 1 and Table 2. It can be seen from the Fig. 1 that rate of

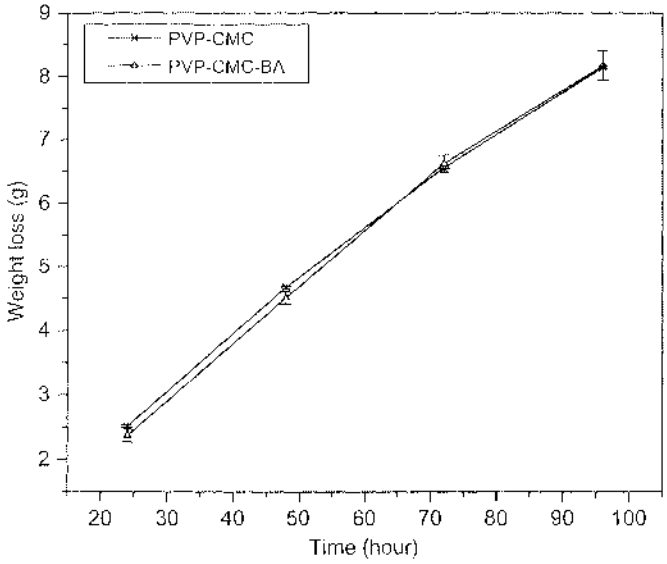

FIGURE 1 Profile of water vapor transmission th rough PVP-CMC and PVP-CMC-BA hydrogel films.

TABLE 2 Water Vapor Transmission of PVP-CMC and PVP-CMC-BA Hydrogels

Sample index	Film thickness (mean ± SD; n = 15) (μm)	W/t Mass change (mg/h)	WVT (mean ± SD; n = 3) (mg mm/m^2 h kpa)
PVP-CMC	132 ± 2	95.0	16.73 ± 0.23
PVP-CMC-BA	684 ± 28	93.5	79.2 ± 0.58

water vapor permeation was constant for both PVP-CMC and PVP-CMC-BA hydrogel films. It is well known that increasing the hydrophilic nature of a polymer membrane induces water vapor tendency and as a result increases water vapor permeation (16). Both PVP-CMC and PVP-CMC-BA contain a combination of hydrophilic polymers, that is, PVP, CMC, PEG, and agar, which are responsible for high rate of water vapor transmission through the hydrogels. On the other hand, due to the presence of moisture absorbing materials (CaCk, silica gel) in the desiccators, the water molecules come out of the other side of the film are absorbed by the water absorbing materials (17). This indicates that the hydrogels are porous as well as permeable for oxygen/gas.

Further, it can be observed from the Table 2 that WVT value of the PVP- CMC-BA is 5 times higher than the value for PVP-CMC. This occurs may be due to the following reasons: PVP-CMC hydrogel in dry state is smooth, transparent, and flexible film like but the surface of dry film of PVP-CMC- BA hydrogel is not smooth and thickness becomes about 4-5 times higher

than the dry PVP-CMC film due to the presence of boric acid which is recrystallized and appears as small white dots throughout the film. When water vapor transmits through the films the mass change (W/t) values are very close for both PVP-CMC and PVP-CMC-BA as shown in Fig. 1. But, as the thickness of PVP-CMC-BA film is about 5 times higher, and WVT is directly proportional to the thickness of the hydrogel film, thus the WVT value of the PVP-CMC-BA hydrogel becomes 5 times higher than the value for PVP-CMC hydrogel. It also signifies that incorporation of boric acid not only provides antimicrobial property in hydrogel but also enhances the water vapor transmission capacity of the hydrogel, which is very important for wound dressing purposes.

Microbe Penetration

One of the important characteristics of wound dressing material is that the dressing material must have adequate bacterial barrier property to protect the wound from bacterial infection. The goal of wound therapy is to keep the wound micro-organism content as low as possible in order to prevent infection and accordingly to stimulate the wound healing process faster (18). Gram positive *Staphylococcus aureus (S. aureus)* and gram negative *Escherichia coli (E. coli)* bacteria were used to conduct microbe penetration test of hydrogels as shown in Fig. 2. After removing the hydrogels from the surface of nutrient agar (NA) plates it was visible that there was no growth of

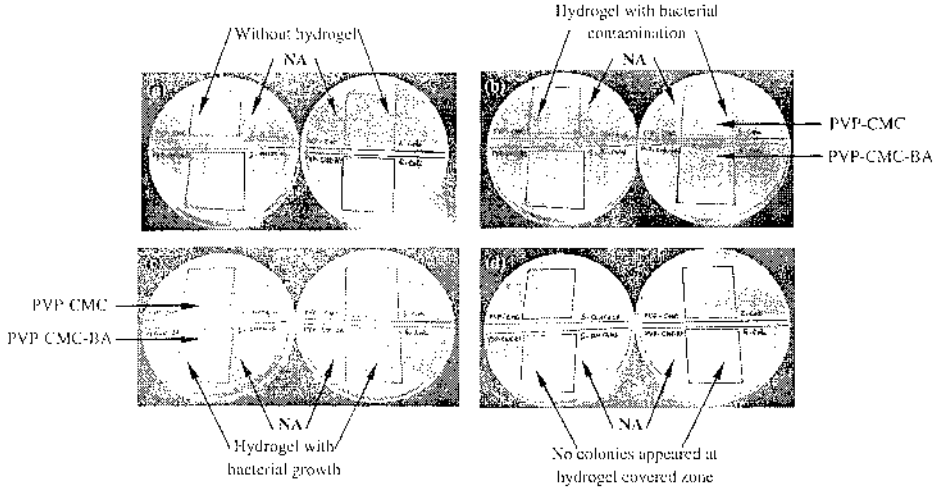

FIGURE 2 Visual image *of* microbe penetration test with Staphylococcus aureus ancl Escherichia coli (a) nutrient, agar (NA) plates without hydrogels (b) NA plates (initial) with hydrogcls, upper surface contaminated with bacterial strain (c) NA plates (after *24* hours) with hydrogcls, upper surface with bacterial growth (cl) NA plates after removal of hydrogels from the surface (after 24 hours).

bacteria on NA plates after 24-hour incubation in 37°C. This indicates that neither *S. aureus* nor *E. coli* passed through the hydrogels. Some bacterial growth *(S. aureus andE. coli*) was noticed on the surface of PVP-CMC hydrogel, but these were not able to penetrate through the hydrogel of PVP-CMC to grow on the surface of NA plates. It happened probably due to the composition of PVP-CMC hydrogel, which is free from any antimicrobial agents, thus maybe it was easy to consume nutrients from the said hydrogel by the *S. aureus* and *E. coli*, which was allowed to grow on it. But, in the case of PVP-CMC-BA hydrogel, BA is functioning as an antibacterial agent, which neither allows microbes to sustain on the surface of hydrogel nor to grow on it. Therefore, PVP-CMC and PVP-CMC-BA hydrogel dressings could be considered as a good barrier against the microbes.

Biocompatibility

The biocompatibility test of any medical product/devices is essential to assure the safety use of it. The primary purpose of a device biocompatibility assessment is to protect the patient's safety. Biocompatibility of the device is normally investigated using analytical chemistry, *in-vitro* test, and animal models. Here, the biocompatibility of hydrogels is evaluated by cytotoxicity (*in-vitro* cell culture model), skin irritation, and skin sensitization tests were performed on animal models.

The evaluation of cytotoxicity is very important for hydrogels that will be used for wound dressing applications (22). *In-vitro* methods of cytotoxicity can demonstrate the occurrence of changes within the cells, ranging from cell death to very subtle alterations of certain cellular functions. The assessment of cell death can be based in the integrity of cell membrane, ascertained by the uptake of foreign molecules (e.g., neutral red) into the cell (2). MTT assay is often used to evaluate the *in-vitro* cytotoxicity of polymeric components as it is a quick, effective method for testing mitochondrial impairment and correlates quite well with cell proliferation. It is based on the use of tétrazolium salt 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl tétrazolium bromide (MTT), which can be converted to an insoluble blue formazan product by mitochondrial enzymes in viable cells (22).

HaCaT cell line is often used to evaluate cytotoxicity of hydrogels as skin consists of different types of cells: keratinocytes, melanocytes, and fibroblasts (29, 32, 33). Further, it is well established that HaCaT cells play a crucial role in epidermal tissue regeneration. Hence, in our study, human skin ker- atinocites (HaCaT) cell line and Balb/c 3T3 mouse fibroblasts cells were exploited as an *in-vitro* model for cytotoxicity assay. The cytotoxic effect on cell viability and cell morphology was depicted in Figs. 3-7.

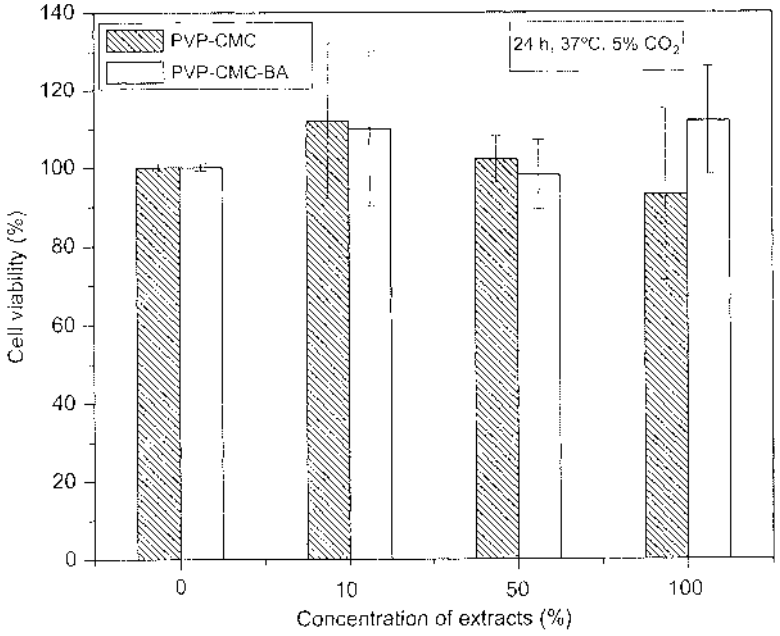

FIGURE 3 Cell viability of human skin keratinocitcs (HaCaT) in presence of hydrogel extracts (24 hour).

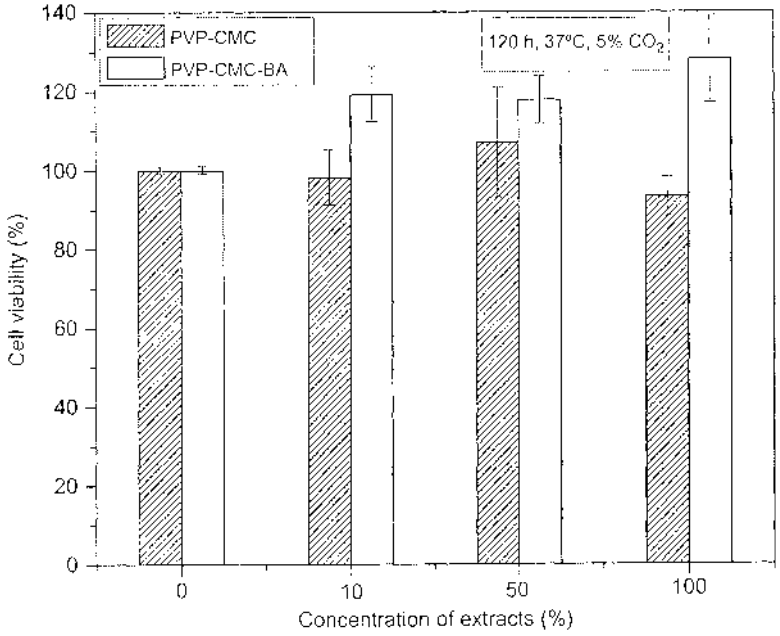

FIGURE 4 Cell viability of human skin keratinocites (HaCaT) in presence of hydrogcl extracts (120 hour).

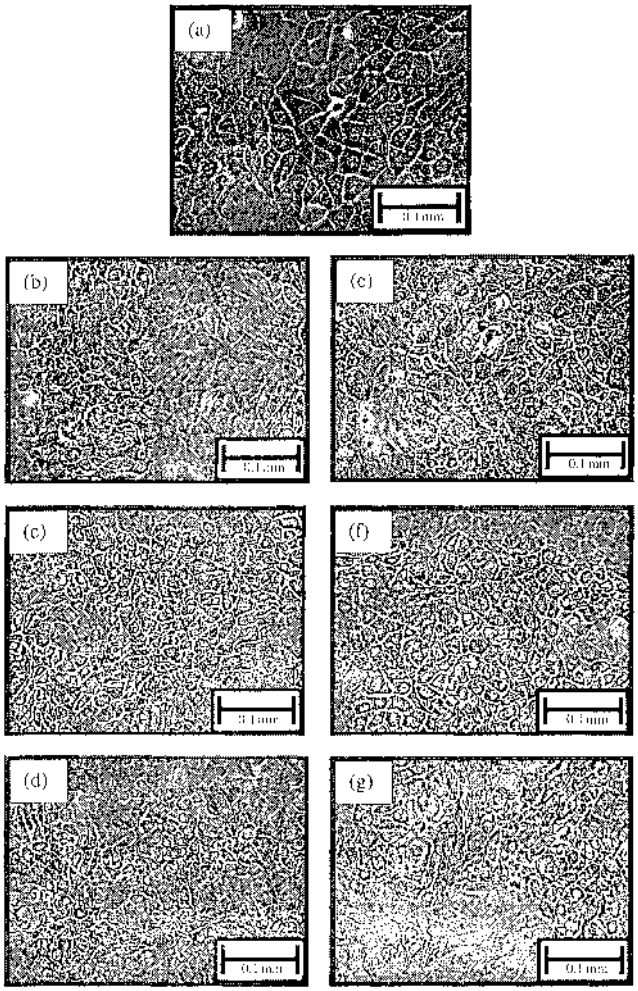

FIGURE 5 Optical micrograph of human skin (HaCaT) ccll growth after 24 hour cultivation in absence and presence of hydrogels extract: (a) control (b) PVP-CMC 10% (c) PVP-CMC 50% (d) PVP-CMC 100% (c) PVP-CMC-BA 10% (f) PVP-CMC-BA 50% (g) PVP-CMC-BA 100%.

It is clear from Figs. 3, 4, and 7 that both PVP-CMC and PVP-CMC-BA do not have much cytotoxic effect, as they show high cell viability in all extract concentrations. Furthermore, it is understandable from Figs. 5 and 6 that there was no significant difference in the morphology of the HaCaT cells after 24 hours cell culturing in DMEM medium with hydrogel extract of PVP-CMC and PVP-CMC-BA. Even after 120 hours not much significant difference occurred on cell morphology of HaCaT cells that were grown within the extract of PVP-CMC even though some notable differences in cell morphology and appearance of few vacuoles have been found in case of

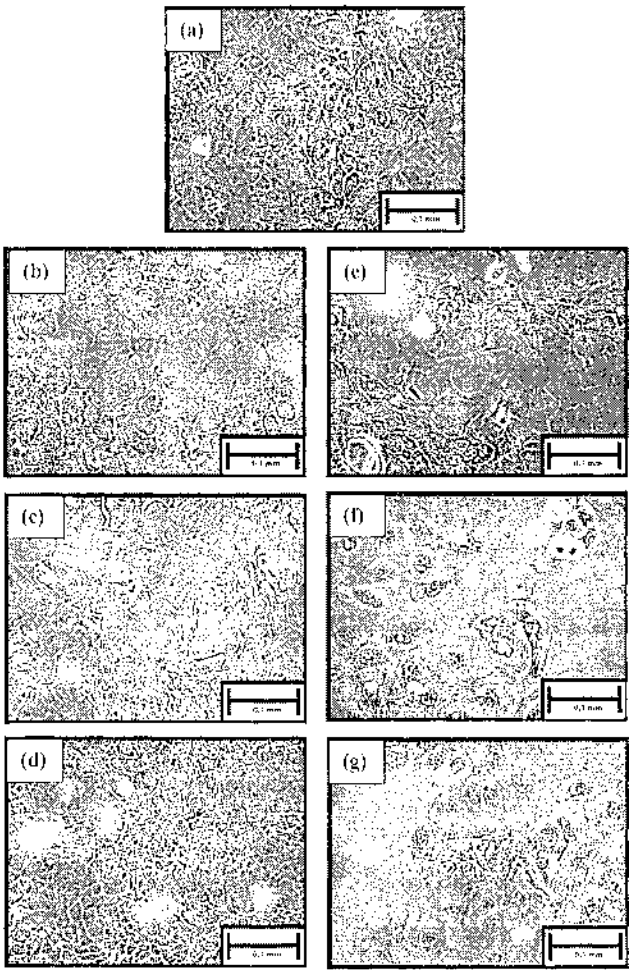

FIGURE 6 Optical micrograph of human skin (HaCaT) ccll growth after 120 hours cultivation in absence and presence of hydrogels extract: (a) control (b) PVP-CMC 10% (c) PVP-GMC 50% (d) PVP-CMC 100% (c) PVP-CMC-BA 10% (f) PVP-CMC-BA 50% (g) PVP-CMC-BA 100%.

PVP-CMC-BA (Fig. 6). The appearance of these vacuoles may be due to the fusion of multiple membranes of HaCaT cells.

The skin irritation and sensitization response of PVP-CMC-BA hydrogel was tested on the animal skin on albino rabbits and albino guinea-pigs, respectively, and observed data illustrated in Table 3 and Fig. 8 for skin irritation and in Table 4 and Fig. 9 for skin sensitization. Skin irritation is a locally arising inflammatory reaction, which appears shortly after stimulation. Its main characteristics are the development of transient inflammatory reactions as evident by clinical signs of irritation: erythema (redness), oedema (swelling), itching, and pain. Whereas, skin sensitization

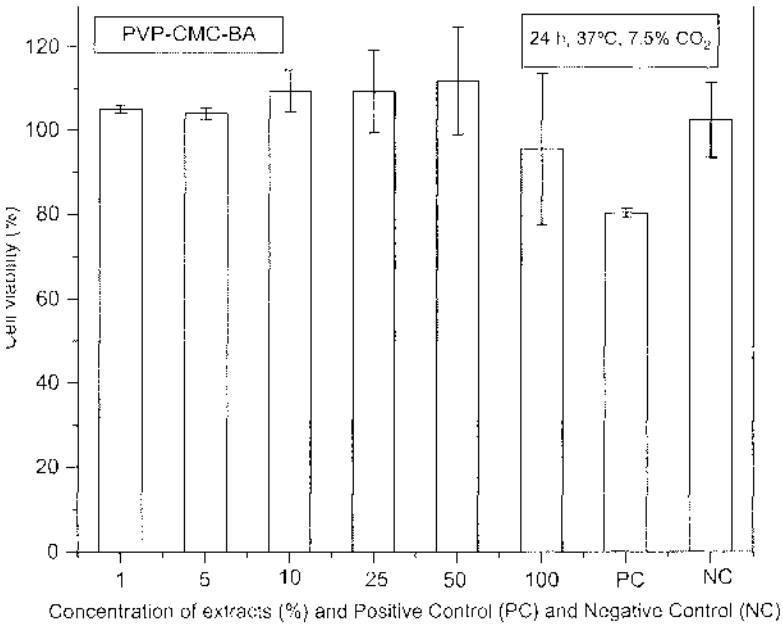

FIGURE 7 Cell viability (Balb/c 3TB mouse fibroblasts) in presence of hydrogel extracts (24 hour).

means an allergic reaction to a particular irritant that results in the development of skin inflammation and itchiness. Unlike skin irritation, skin becomes increasingly reactive to the substance as a result of subsequent exposures.

It can be made out from Tables 3 and 4 that until 72 hours, there was no appearance of erythema and oedema in presence of test material (i.e., either hydrogel or hydrogel extract) which was applied on the skin of albino rabbits (Fig. 8) and skin of albino guinea-pigs (Fig. 9). All the results indicated positive response concerning skin irritation and sensitization points of view. Thus, animal model tests confirmed that the medicated hydrogels (PVP-CMC and PVP-CMC-BA) do not have any adverse effect on the skin cells and they are biocompatible.

TABLE 3 Irritation Response

	Erythema/oedema (scores)	
Observation time	Distilled water (NC)	PVP-CMC-BA
1 h after patch removal	0/0	0/0
24 h after patch removal	0/0	0/0
48 h after patch removal	0/0	0/0
72 h after patch removal	0/0	0/0

$P_{II} = 0.00$.

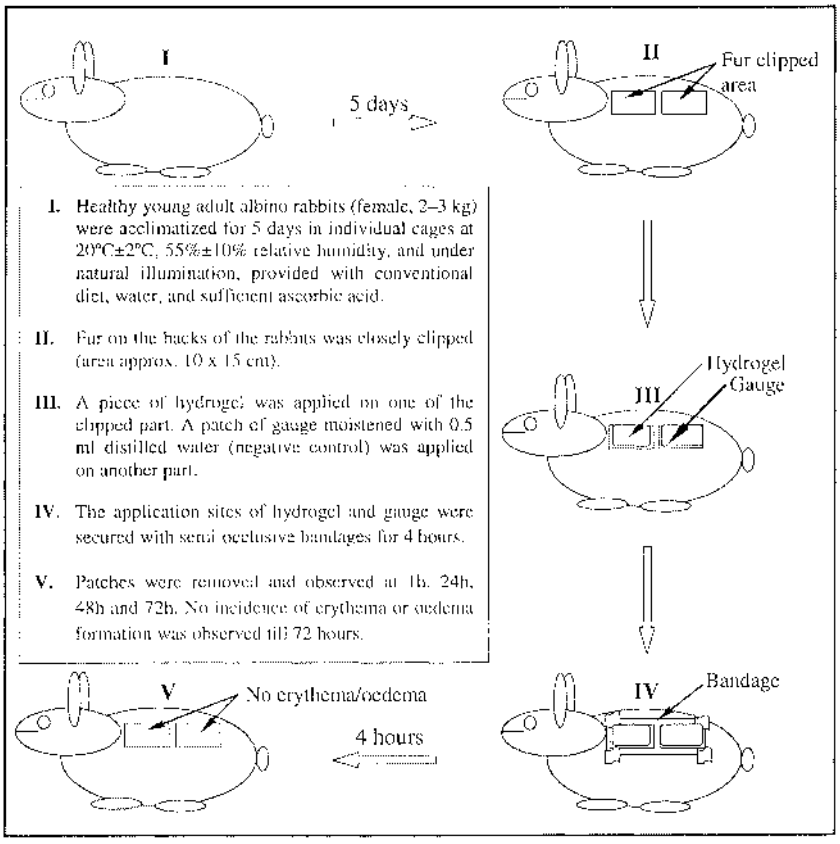

FIGURE 8 Scheme of skin irritation test on albino rabbits (Experiment, conducted at National Institute of Public Health, accredited laboratory No. 1206.3, Prague.

TABLE 4 Sensitization Response

	Reaction erythema/oedema (scores)					
	Observation time					
	24 h		48 h		72 h	
Animal no.	Saline solution (RC)	PVP-CMC-BA extract	Saline solution (RC)	PVP-CMC-BA	Saline solution (RC)	PVP-CMC-BA
1	0/0	0/0	0/0	0/0	0/0	0/0
2	0/0	0/0	0/0	0/0	0/0	0/0
3	0/0	0/0	0/0	0/0	0/0	0/0
4	0/0	0/0	0/0	0/0	0/0	0/0
5	0/0	0/0	0/0	0/0	0/0	0/0

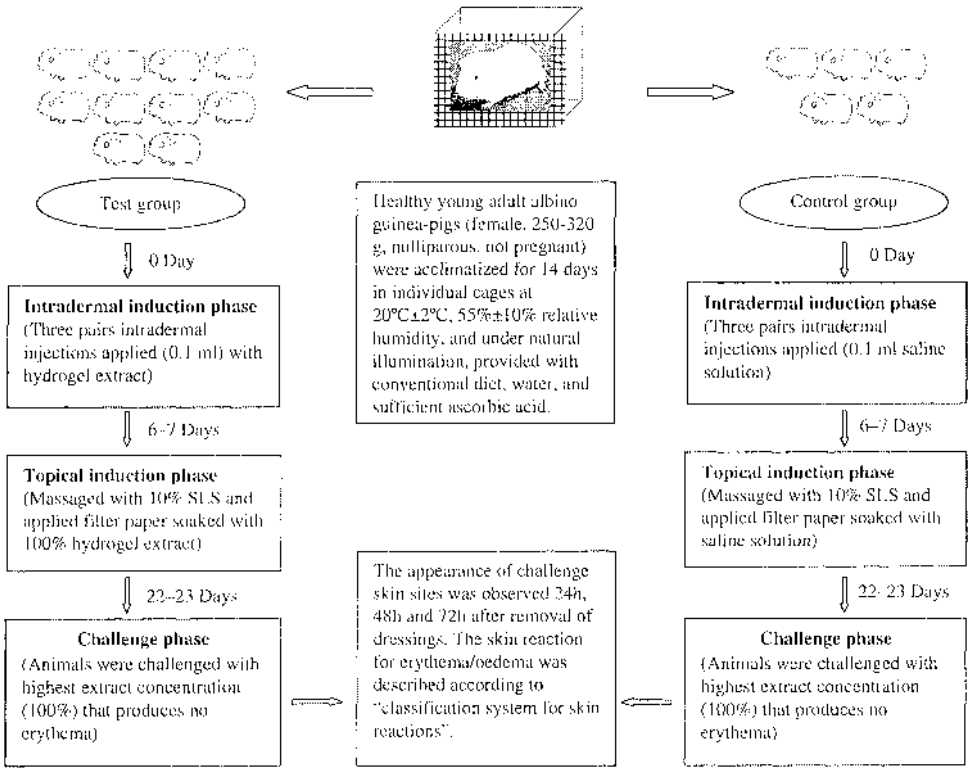

FIGURE 9 Schemc of sensitization test on albino guinea-pigs (Experiment conducted at National Institute of Public Health, accredited laboratory No. 1206.3, Prague).

CONCLUSION

PVP-CMC and PVP-CMC-BA, hydrogels showing a high rate of water vapor permeability also performed as a good barrier for bacterial penetration. Furthermore, these hydrogels exhibit mostly negative effect on cytotoxicity, dermal irritation, and/or sensitization during studies of *in-vitro* cell culture and *in-vivo* animal models. Therefore, it can be concluded that both PVP-CMC and PVP-CMC-BA hydrogels are human/animal friendly biomaterials, have great potential for biomedical applications, and could be recommended for burn skin treatment as well as wound dressings materials.

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