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EVALUATION OF SAFETY AND SKIN TOLERABILITY OF ORGANIC COTTON PADS IN CASE OF IRRITATIVE VULVITIS

Michela MORI, Antonella PRATICÒ, Roberta VILLA, Alice BUZZELLA, Riccardo VICINI, Silvia BUSOLI BADIALE, Ornella PASTORIS, Claudio ANGELINETTA, Maurizio BARBIERI CARONES

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ORIGINAL ARTICLE

Evaluation of safety and skin tolerability of organic cotton pads in case of irritative vulvitis

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ABSTRACT

BACKGROUND: The woman vaginal environment is a fragile and delicate ecosystem that is often impaired by physical and chemical agents. This condition tends to damage skin barrier causing allergic reactions that lead to chronic irritating conditions.

METHODS: Clinical and *in-vitro* studies were performed on organic cotton pads in order to assess if their use can prevent the onset of irritant conditions. During clinical studies, the panelists' skin and mucosae state were checked through a gynecological clinical examination in order to assess tissue dryness and alterations. Moreover, each panelist answered a sensorial questionnaire at the end of the test. Data were gathered and the product acceptability of use was registered in terms of itching, irritations and burning feelings. The panelist score was calculated based on VNS Scale (0-10, where 0 is the minimum value and 10 is the maximum).

RESULTS: From a careful analysis of the first part of the study, it is possible to state that the tested product (organic cotton pads) has proved to reduce the onset of irritative phenomena and slight undesired effects caused by the conventional use of synthetic pads. *In-vitro* tests were conducted to study possible biological processes involved during allergic and sensitizing events produced by vulvitis. In particular, a pro-sensitizing test, a skin irritation on RHE (adapted from OECD 439) and tests to assess the soothing activity were performed on cell substrates.

CONCLUSIONS: Results demonstrated that organic cotton pads, in each part, are safe and do not impair any physiological activities of the tissue substrates.

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KEY WORDS: Vulvitis - Menstrual hygiene products - Cotton fiber - Feminine hygiene products.

The woman vaginal environment is a fragile and delicate ecosystem, where millions of microorganisms live, constituting the physiological vaginal habitat.

Symptoms of vulvitis are itching, burning and pain at vulvo-vaginal site. Therefore, it has to be considered a real contact dermatitis.[1]

Vulvitis is caused by different irritating factors, which are mainly based on bad personal care and lifestyle. The skin outside the vagina become more sensitive to physical and chemical agents, that tend to damage the skin barrier causing allergic reactions, which could lead to chronic irritative conditions.[2, 3]

The external irritative agents are several: tight clothes, synthetic underwear (nylon, lycra), oiled or perfumed toilet paper, the use of synthetic or less transpired (with high content of cellulose) menstrual pads, application of many topic cosmetics (deodorant sprays, depilatory wax, aggressive detergents) and the use of barrier contraceptive.[4]

Further to external factors, there are also intrin-

sic personal/endogenous factors caused by high levels of endogenous hormone, which leads to a disequilibrium of the vaginal flora or blood stagnation in case of menstrual period, that contribute to the onset of contact dermatitis, like pregnancy and menstrual cycle.[5] Furthermore, there are situations that can worsen the irritating skin conditions and create a favorable environment for pathogens in a moment when vulvar mucosae are more vulnerable, such as: urinary incontinence, obesity, psychosomatic factors, concomitant pathologies (*e.g.* diabetes), antibiotic therapies and poor personal care.

Another etiology factor of an irritating vulvitis as a concomitant use of synthetic pads, that can alter the physiological environment of vulvo-vaginal habitat, because of cutaneous frictions and poor perspiration. This situation stimulates micro-organisms proliferation. Several studies underlined that this major problem is more recurrent in fertile women aged between 14 and 75 years old.[6, 7]

Consequences of above-mentioned conditions are several and most commonly due to a concomitant complication of pathogen infection (pyogenic or mycotic), such as *Candida Albicans*, that can cause cystic and urethritis pathologies.

Generally, vulvo-vaginal phlogosis is a burden state of tissue occurring in women during fertile period and postmenopause.[7]

Considering all these factors, safety and tolerability evaluations sanitary pads are important in order to assess if natural based products can be safe and the directions of use are correct.

This study aimed to evaluate, both clinically and *in vitro*, if organic cotton pads can prevent the onset of irritating phenomena that usually occur during and after the use of non-cotton pads. To this end, clinical evaluations of vaginal skin and mucosae alterations (erythema and edema) and dryness were carried out on 200 women, who declared to be used to wear conventional synthetic pads and suffer from irritating phenomena during and after menstrual period. The product's tolerability of use was evaluated.

In the meantime, *in-vitro* tests were performed to understand which biological processes were involved. A pro-sensitizing test was carried out on human monocyte cell line, since skin sensitization is defined as the toxicological endpoint associated with chemicals that have the intrinsic ability to cause skin allergy in humans. This process, also called allergic contact dermatitis (ACD), is a cell mediated hypersensitivity immune response.[8-12]

In-vitro models studying human skin are important tools in the field of research and development of pharmaceutical and cosmetic industries.[13, 14] Chemical-induced skin irritation, mainly manifested in erythema and edema, is the result of a cascade of events beginning with the penetration of chemicals through the stratum corneum, where they may damage the underlying layers of keratinocytes and other skin cells. The damaged cells may either release inflammatory mediators or induce an inflammatory cascade. In order to assess the potential skin irritation of the tested product and to consider the involved events, an OECD 439 adapted method was conducted on RHE.[15, 16]

Several skin irritants can trigger different inflammatory processes. The principal mechanisms used by epidermal cells to participate in the immune and inflammatory skin reaction are the production and responses to cytokines.[17, 18] The amount of interleukins produced in cell cultures of human keratinocytes treated with the tested sample was evaluated to verify if the product tested does not have irritating action. In particular, the synthesis of interleukin-1α (IL-1α) in human keratinocyte cell cultures was measured.

Materials and methods

Clinical trial

This clinical trial was carried out on 200 women, aged 20-40, in their fertility period, from October 2017 to February 2018. In particular, volunteers were recruit following specific inclusion and exclusion criteria: fertility period, users of conventional synthetic pads, presence of irritative phenomena during and after menstrual cycle, no amenorrhea, in a good general health, no dermatopathies, no pharmacological product process and no particular changes in their daily routine.

Panelists were then examined at three timings:

- step 1 basal value of the clinical study with an irritative condition in action: T0;
- step 2 use of the product conventional synthetic pads T1-synthetic pads during menstrual cycle;

TABLE I.—*Clinical scores of skin tolerability.*

Skin alterations (erythema and edema) Vaginal skin and mucosae alterations (erythema and edema)	
Erythema	Edema
No erythema	No edema
Slight erythema (hardly visible)	Very slight edema (hardly visible)
Clearly visible erythema	Slight edema
Moderate erythema	Moderate edema (about 1mm raised skin)
Serious erythema (dark red with possible formation of light eschars)	Strong edema (extended swelling even beyond the application area)

• step 3 use of the product organic cotton pad T2-cotton pads during the second menstrual cycle.

During this period, the skin and mucosae state of panelists were checked by a gynecologist. All the answers given by panelists in the sensorial test in accordance with the VNS Scale (0-10, where 0 is the minimum value and 10 is the maximum) were collected at the end (Table I).

During the clinical trial all the evaluations on the tested product were taken into account by gynecologists in order to have information on the vaginal skin and/or mucosal alterations, in terms of erythema and edema, mucosae and skin dryness.

Moreover, subjective evaluations made by volunteers, who had to underline cases of exacerbate itching, burning or irritations, were taking into consideration as well.

All the evaluations were taken at the beginning of the clinical trial, defined as basal value at time 0 (T0) and after the products use: first menstrual (T1) and second (T2) menstrual periods. The vaginal mucosae and skin state of the volunteers during the period of product use was evaluated as well.

At the end, personal evaluations were collected (self-assessment).

The statistical analysis of clinical data was performed using the Wilcoxon test by fixing the threshold of acceptability at 5%.

In-vitro tests

Pro-sensitizing test (OECD 442E)

The test was carried out on human monocytes THP-1, as surrogates for dendritic cells. It is known that THP-1 cells show enhanced CD86 (BD-PharMingen) and/or CD54 (Dako-Agilent) expression when rated with sensitizers. THP-1 cells were cultured in RPMI-1640 medium supplemented with fetal bovine serum (10%), 2-mercaptoethanol (0.05 mM) and antibiotics (1%) (all reagents were purchased from Euroclone spa). Cells were incubated at standard culture conditions (37 °C, 5% CO_2). Good cell culture practices were used.

The tested product was incubated overnight in complete culture, then cells were treated with the obtained liquid of maceration (filtered through a 0.22 μM) and subsequent dilutions 1:2 (TS). As positive control 2,4-dinitrochlorobenzene (DNCB from Sigma Aldrich srl) at the concentration of 4 μg/mL was used.

The product underwent a preliminary cytotoxicity evaluation through MTS (Promega Italia srl, Milan, Italy) test which allows to choose a non-cytotoxic concentration to be put in contact with THP-1 cells. The assay was performed after a 24-hours treatment by adding a small amount of an MTS solution directly to culture wells, incubated for 1-4 hours, and then by recording the absorbance (optical density, OD) at 450 nm with a 96-well plate reader. The quantity of formed formazan measured at 450 nm is directly proportional to the number of living cells in culture.

A suitable number of THP-1 cells was precultured for 48 hours, seeded into a 24 well flat-bottom plate and treated for 24 hours with test sample and positive control. Untreated cells in culture medium were used as negative control. The expression levels of CD86 and CD54 were analyzed through flow cytometry. Based on the geometric mean fluorescence intensity (MFI), the relative fluorescence intensity (RFI) of CD86 and CD54 for positive control (CTRL) cells and chemical-treated cells were calculated following the equation:

$$RFI = \frac{MFI\ (chemical\ treated\ cells) - MFI\ (IgG1\ treated\ cells)}{MFI\ (CTRL\ cells) - MFI\ (IgG1\ CTRL\ cells)} \times 100$$

For CD86/CD54 expression measurement, each test chemical is tested in at least two independent runs to derive a single prediction (positive or negative).

Skin irritation on RHE (OECD 439 modified protocol)

A skin irritation test on Reconstructed Human Epidermis (RHE by Episkin™), made up of normal human keratinocytes cultured on an inert polycarbonate filter at the air-liquid interface in a chemically defined medium, was performed on organic cotton pads. DPBS was used as negative control, whereas SDS was considered as positive control.

The following sample portions were tested: 1) whole sample; 2) inner part of the sanitary pad; 3) part of the sanitary pad in contact with the skin; 4) part of the sanitary pad containing glue.

Each portion was incubated for 72 h at 37 °C in the growth medium.

RHE tissues were treated for 18 h at 37 °C with the test substance following the scheme below:

- negative control: DPBS;
- positive control: 5% SDS;
- tested sample: TS (liquid of maceration).

Each experiment was carried out in triplicate. Cell viability was evaluated through MTT assay by reading the optical density (OD) at 540 nm.

In-vitro evaluation of soothing activity

This test was conducted to verify the absence of a possible irritating action of the organic cotton pads on human keratinocytes (Huker) cultured in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) and incubated at standard culture conditions (37 °C, 5% CO_2). Good cell culture practices were used.

The following sample portions were tested:

- TS1: whole sample;
- TS2: inner part of the pad;
- TS3: part of the pad in contact with the skin;
- TS4: part of the pad containing the glue.

Each portion was incubated for 24 hours at 37 °C in growth medium; cells were treated with the obtained liquid of maceration. As positive control lipopolysaccharides (LPS by Sigma Aldrich srl, Milan, Italy) were used.

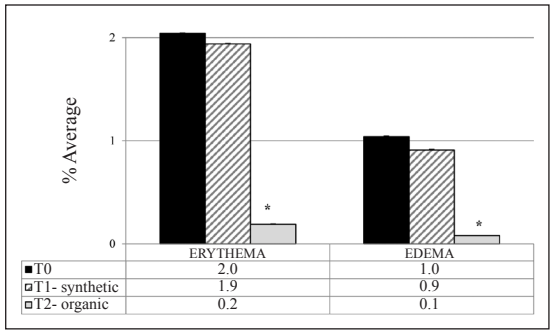
Cells were treated with non-toxic concentrations of the tested product and the positive control (PC). Untreated cells were used as negative control (NC). The amount of IL-1α in the culture medium was determined by ELISA (enzyme-linked immunosorbent assay by Peprotech EC LTD), which is used specifically for IL-1α detection. The detection was made through secondary biotinylated antibody which reacts with streptavidn-HRP. The colorimetric reaction is proportional to the amount of cytokine present in the medium and is read at 450 nm. The values were interpolated in a standard curve of IL-1α and results were expressed in pg/mL as follow: NC= negative control (untreated cells); PC= positive control (LPS); 1= whole sample; 2= inner part of the sanitary pad; 3= part of the sanitary pad in contact with the skin; 4= part of the sanitary pad containing glue.

The statistical analysis was performed according to *t*-test with a threshold of 95% and 99%.

Results

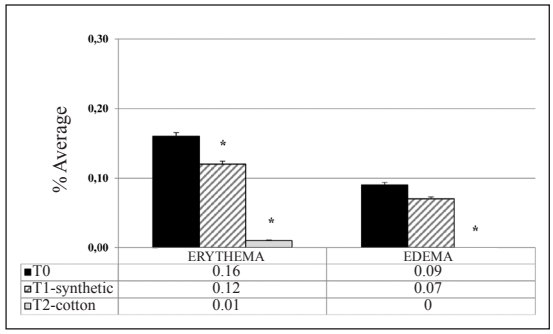
In Figure 1 skin alterations, in terms of erythema and edema, evaluated on a clinical score basis are reported. Data show a significant reduction in skin alterations, both for erythema and edema, after one menstrual cycle of organic cotton pads use (T2) compared to women that used conventional synthetic pads (T1). Moreover, it is evident that using organic cotton pads can significantly reduce skin alterations, as highlighted by the comparison with the initial condition (T0). Clinical evaluations show that vaginal skin swelling among participants wearing organic cotton pads was significantly lower than those wearing conventional synthetic pads, with an average rating of 0.9 (synthetic) *versus* 0.1 (cotton) (95% confidence level).

In Figure 2 mucosae alterations, in terms of erythema and edema evaluated on a clinical score basis are reported. Data show a significant reduction in mucosae alteration, both in terms of erythema and edema, after one menstrual cycle of organic



	ERYTHEMA	EDEMA
T0	2.0	1.0
T1- synthetic	1.9	0.9
T2- organic	0.2	0.1

Figure 1.—Evaluation of skin alterations by means of erythema and edema onset in accordance with the clinical scores reported in Table I at time 0 (T0 - skin situation at the beginning of the study), after the first menstrual cycle by using synthetic pads (T1) and after the second menstrual cycle by using organic cotton pads (T2).



	ERYTHEMA	EDEMA
T0	0.16	0.09
T1-synthetic	0.12	0.07
T2-cotton	0.01	0

Figure 2.—Evaluation of mucosae alterations by means of erythema and edema onset in accordance with the clinical scores reported in Table I at Time 0 (T0: skin situation at the beginning of the study), after the first menstrual cycle by using synthetic pads (T1) and after the second menstrual cycle by using organic cotton pads (T2).

cotton pads use compared the use of conventional synthetic pads during the first menstrual cycle (T1). Moreover, it is evident that using organic cotton pads can significantly reduce mucosae alteration compared to the initial conditions (T0).

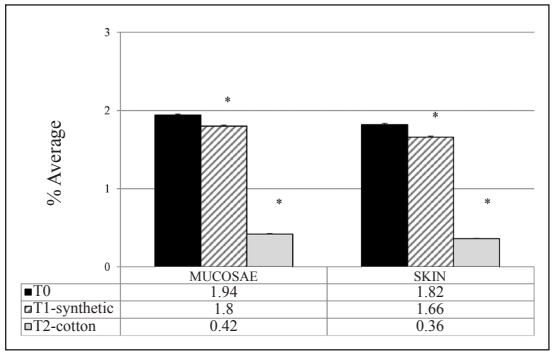
Evaluations of skin and mucosae dryness at T0, T1 after the use of conventional synthetic pads, and T2 after use of organic cotton pads are reported in Figure 3. As it is shown, mucosae and skin dryness among participants wearing organic cotton pads is significantly lower than those wearing conventional synthetic pads, with an average rating of 1.8 (synthetic) *versus* 0.4 (cotton) (95% confidence level).

Itching, burning and irritation phenomena after the use of pads, both synthetic (T1) and organic cotton (T2) are reported in Figure 4 based on the average of the clinical scores gathered among women enrolled.

Vaginal itching among participants wearing cotton pads (T2) was significantly lower than those wearing conventional synthetic pads (T1) with an average rating of 3.2 (synthetic) *versus* 0.6 (cotton) (95% confidence level).

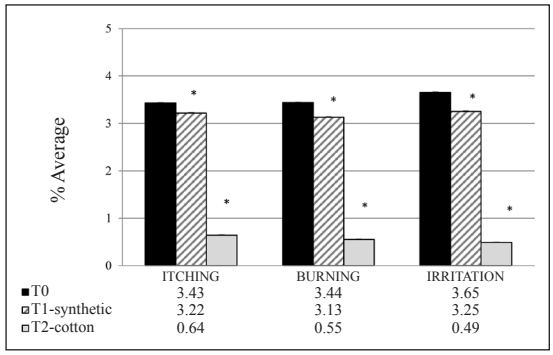
Vaginal irritation among participants wearing cotton pads (T2) was significantly lower than those wearing conventional synthetic pads (T1) with an average rating of 3.3 (synthetic) *versus* 0.5 (cotton) (95% confidence level).

After using cotton pads, most women reported a significantly lower skin irritation state after using organic cotton pads compared to convention-



	MUCOSAE	SKIN
T0	1.94	1.82
T1-synthetic	1.8	1.66
T2-cotton	0.42	0.36

Figure 3.—Evaluation of skin and mucosae dryness onset in accordance with the clinical scores reported in Table I at Time 0 (T0 - skin situation at the beginning of the study), after the first menstrual cycle by using synthetic pads (T1) and after the second menstrual cycle by using organic cotton pads (T2) are reported.



	ITCHING	BURNING	IRRITATION
T0	3.43	3.44	3.65
T1-synthetic	3.22	3.13	3.25
T2-cotton	0.64	0.55	0.49

Figure 4.—Evaluation of skin itching, burning and irritation are reported in accordance with the clinical scores reported in Table I at time 0 (skin situation at the beginning of the study), after the first menstrual cycle by using synthetic pads (T1) and after the second menstrual cycle by using organic cotton pads (T2) are reported.

al synthetic pads with an average rating of 6.17 (synthetic) *versus* 1.01 (cotton) (95% confidence level).

Pro-sensitizing test

The test was conducted on organic cotton pads. The product underwent a preliminary cytotoxicity screening on THP-1 cells where non-cytotoxic concentrations to be used were chosen through an MTT Test.

The expression of CD86 and CD54 was evaluated at least twice in independent runs.

The prediction is to be considered positive if at least one of the following conditions is met in 2 of 2 or in at least 2 of 3 independent runs, otherwise the prediction will be considered negative:

The relative fluorescence intensity (RFI) evaluated for CD86 was equal to or higher than 150% at any tested concentrations (with cell viability ≥50%), the RFI of CD54 is equal to or higher than 200% at any tested concentrations (with cell viability ≥50%).

The expression of RFI values calculated for CD86 and CD54 are reported in Table II. The table shows % values for positive control (DNCB) and the 8 concentrations of the tested product.

The calculated RFI values are within the limits of the prediction model. It can therefore be stated that there is no coexpression of CD86/CD54.

Skin irritation on RHE (OECD 439 modified protocol)

In Figure 5 percentage averages of tissue viability after incubation with tested product are reported. Test was performed according to OECD Test Guidelines 439 (*In vitro* Skin Irritation: Reconstructed Human Epidermis Test Method) and was carried out on the liquid of maceration obtained by incubating the product in tissue culture medium for 72 h. In particular, RHE tissue viability has been evaluated on negative control (NC), positive control (PC), Whole sample (TS1), Inner part of the sanitary pad (TS2), Part of the sanitary pad in contact with the skin (TS3), Part of the sanitary pad containing the glue TS4). The irritating potential of tested product has been predicted based on the viability of tissues exposed to the tested substance. The tested substance is considered to be irritant to skin (R38), if the average viability after 42 minutes of exposure and 42 hours post incubation is less or equal (≤) to 50% compared

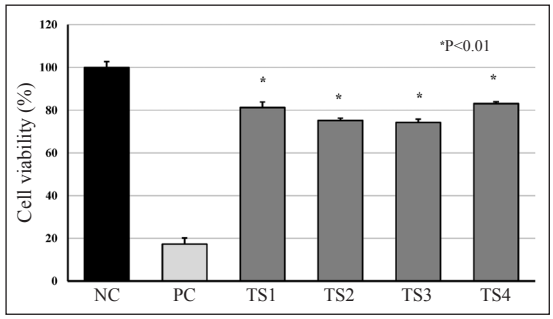


Figure 5.—RHE tissue viability after 72 h of contact with liquid of maceration of test product (organic cotton pads) are reported. The test was performed according to OECD Test Guidelines 439 (*in vitro* Skin Irritation: reconstructed human epidermis test method). Figure shows % average values of negative control (NC), positive control (PC), Whole sample (TS1); Inner part of the sanitary pad (TS2), part of the sanitary pad in contact with the skin (TS3), Part of the sanitary pad containing the glue (TS4) (6 replicates ±SD; *t*-test, P<0.01).

TABLE II.—*RFI values for positive control (DNCB) and the 8 concentrations of the tested (cotton pads) product are reported.*

		NC	DNCB	Sample concentrations %							
				100	50	25	12.5	6.25	3.125	1.5625	0.7813
CD86	Average	100.0	184.43	95.90	97.54	96.72	94.26	94.26	95.08	93.44	91.80
	SD	3.77	8.83	3.74	4.61	2.89	4.02	4.18	4.58	2.93	3.67
	t test			0.08689	0.26072	0.12119	0.05976	0.01675 *	0.04552 *	0.00805 **	0.00828 **
CD54	Average	100.0	206.29	94.41	90.21	88.81	87.41	84.62	81.82	83.22	86.01
	SD	6.52	7.52	4.57	3.00	3.16	4.00	2.62	2.07	2.12	3.54
	t test			0.04878 *	0.00342 **	0.00132 **	0.00038 ***	0.00087 ***	0.00113 **	0.00135 **	0.00452 **

3 values ±SD; *t*-test *P<0.05; **P<0.01; ***P<0.001 statistical analysis.

TABLE III.—*Criteria for* in vitro *interpretation of RHE tissue viability results in accordance with OECD 439, according to EU classification, the irritating potential of the tested substances is predicted for distinguishing between R38 skin irritating and no-label (non-skin irritating) test substances.*

	In vivo prediction
Mean tissue viability is ≤50%	R38, irritant (I) or GHS category 2
Mean tissue viability is >50%	No label, not irritant (NI)

to the negative control. In this case, the viability of RHE tissues treated with the tested product (liquid of maceration) was higher than 50% of the negative control, which means that the product does not have irritating behavior when in contact with RHE tissues, as reported in Table III.

In-vitro evaluation of soothing activity

Before studying interleukins production, the viability cell test is carried out in order to understand which range of concentrations are safe for cell cultures. Viability of cells in contact with liquids of maceration obtained from different components of the tested sample has been calculated. The tested product demonstrates to have non-cytotoxic effects on keratinocyte cell cultures, even when tested without any dilutions. Figure 6 shows the graphical evaluation of soothing activity of the tested product (organic cotton pad) in terms of IL-1α production, when a keratinocyte cell culture has been in contact for 24 hours with liquid of macerations of the different part of the pad. In Table IV, values of the IL-1a produced by keratinocytes are reported.

Absorbance values measured at 450 nm are directly proportional to the quantity of IL-1α produced by cells. The values are interpolated in a standard curve of IL-1α and the results are expressed as pg/mL.

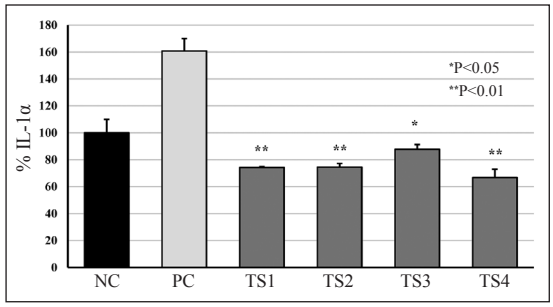


Figure 6.—Percentage of IL-1α production in keratinocyte cell culture respect to the negative control, when exposed for 24 hours to positive control lipopolysaccharides (PC) and to liquid of maceration of: whole sample (1); Inner part of the sanitary pad (2), part of the sanitary pad in contact with the skin (3), part of the sanitary pad containing the glue (4) (6 replicates ±SD; *t*-student test P<0.01)

So, it is possible to conclude that there is no production of IL-1α, meaning an absence of irritation activity of the product in terms of allergic reactions.

Discussion

The numerous and complexes factors involved during the genesis and progress of a vulvo-vaginal irritation make it difficult to adopt an effective pharmaceutical approach. Moreover, in case of chronic condition, an accurate prophylaxis mainly based on the elimination of the irritating or hyper-sensitizing agents is imperative. The abundant use of pads during fertile and post-menopausal periods makes it necessary to use organic pads composed by natural fibers, such as hydrophilic cotton, which helps the physiological conditions of vaginal environment with unalterable pH range and preserves the natural hydrolipidic barrier. The use of cotton materials prevents increased allergic risks and reduces frictions, which cause contact dermatitis.[19, 20]

Since it is a very sensitive contact site, the in-

TABLE IV.—*Values of IL-1α content in keratinocyte cell culture after 24 hours at 37 °C in growth medium are reported. Cells were treated with liquid of maceration obtained from sample portions: whole sample (1); inner part of pas (2); part of pad in contact with skin (3); part of pad contained glue (4).*

	NC	PC	Sample			
			1	2	3	4
OD average value	0.179	0.282	0.136	0.136	0.159	0.123
IL-1α mean value (pg/mL)	84.53	135.95	62.78	63.03	74.20	56.45
IL-1α (% of control)	100.0	160.8	74.3	74.6	87.8	66.8

timal equilibrium can be easily altered by several irritating, infective and allergic agents. Using a correct pad can prevent different allergic conditions. Pads, which are used for several reasons during different time of a woman life, are mainly made up of synthetic materials often containing absorbent powders used to increase the performance of the product, even if they can irritate the skin and mucosae in contact and cause vulvovaginal irritation. Moreover, synthetic pads impair the correct gas and humidity exchange, increasing the site temperature which stimulates the proliferation of microorganisms originating the onset of infections. On the contrary, organic natural based pads allow this natural exchange making the site breathable. They are therefore safer and more delicate for vaginal skin and mucosae.

In addition, cotton is an extremely absorbent natural material, in which synthetic absorbent powders ae not used, offering a softer and fresher condition, preventing odorless onset and maintaining a good state of humidity and temperature.

Data from the clinical study conducted on 200 women show that the use of organic cotton pads can reduce or even prevent any skin and mucosae symptoms related to vaginal irritation, such as erythema and edema, or phenomena of itching, burning and dryness.

These results are confirmed also by *in-vitro* tests results conducted on different part of organic cotton pads.

The skin sensitization test represents an important step for the evaluation of the safety of substances, especially if used in consumer products. Skin sensitization is the toxicological endpoint associated with chemicals that have the intrinsic ability to cause skin allergy in humans, resulting in a disease called ACD, a cell-mediated delayed-type hypersensitivity immune response induced by low-molecular-weight chemical compounds. The key biological events underlying the skin sensitization process are well established: the activation of dendritic cells (DC), typically assessed by the expression of cell surface markers, chemokines and cytokines, that is considered to be a key event. In the proposed pro-sensitizing test, the modulation of the CD86 and CD54 membrane markers in THP-1 cells, a human monocytic leukemia cell line used as a surrogate model for DC, is measured by flow cytometry following a 24 h exposure to the tested sample. In the cells treated with the tested sample a significative change in the expression of CD86 and CD54 compared to untreated cells has not been observed at any tested concentration. The lack of coexpression of the two markers means that the tested product does not have pro-sensitizing activity.

Chemical-induced skin irritation, mainly manifested by erythema and edema, is the result of a cascade of events beginning with penetration of the chemicals through the stratum corneum where they may damage the underlying layers of keratinocytes and other skin cells. The damaged cells may either release inflammatory mediators or induce an inflammatory cascade which also acts on the cells in the dermis, particularly the stromal and endothelial cells of the blood vessels. It is the dilation and the increased permeability of the endothelial cells that produce the observed erythema and edema.

The test on RHE, which was conducted to analyze the potential skin irritation of the organic cotton pads, demonstrates that the product can be considered safe for the skin.

Notably, the RHE-based test methods, in the absence of any vascularization in the *in-vitro* test system, measure the beginning of the events of the cascade, *e.g.* cell/tissue damage, by using cell viability as readout.

Skin is the body's largest organ with a unique architecture acting as a barrier against infection and other environmental hazards. Inflammation is considered a manifestation of an abnormal skin conditions. However, low levels of inflammation or what is considered to be the onset of inflammation can occur even in absence of clinical manifestations as erythema (skin redness).

Different skin irritants can trigger different inflammatory processes. The main mechanisms used by epidermal cells to participate in the immune and inflammatory skin reactions are the production of cytokines and the responses to cytokines. Interleukin-1α (IL-1α) is constitutively produced in large amounts by keratinocytes and has the primary role of maintaining the skin barrier function, preventing microorganisms from entering into the body. The results gathered from each part of the organic cotton pads showed that

the levels of IL-1α in cells treated with the tested product are comparable or lower than those of untreated cells. Therefore, the tested product does not stimulate the production of IL-1α *in vitro*.

Conclusions

In conclusion, organic cotton pads have demonstrated to be well tolerated and useful in preventing and improving irritative state of vulvo-vaginal skin and mucosae. These results are taken from a clinical evaluation on 200 women affected by an irritative condition of vaginal tissue as a consequence of utilization of conventional synthetic pad. The clinical study is supported by *in-vitro* tests, in which possible skin sensibilization, irritation and inflammation are evaluated on cell substrates, by analyzing organic cotton pads in each part.

The use of organic cotton pads, made by natural fibers both outside and inside, has to be considered a better choice during menstrual cycle in women that have already demonstrated irritative conditions when using conventional synthetic pads.

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