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Nanotechnology in Hormone Replacement Therapy: Safe and Efficacy of Transdermal Estriol and Estradiol Nanoparticles after 5 Years Follow-Up Study

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***SUMMARY*. This study aimed to evaluate the safety and efficacy of a novel protocol of transdermal Hormone Replacement Therapy (HRT) based on a nanostructured formulation of Estriol (0.1 %) + Estradiol (0.25 %) restoring serum levels and relieving menopausal symptoms. We evaluated 122 women with mean age of 56.88 (± 6.27) as part a longitudinal prospective study on post-menopausal women with natural menopause, received in the right forearm a transdermal formulation of (EE) daily for 60 months. Clinical parameters including the degree of satisfaction with symptomatic relief, serum concentrations of estradiol, weight, blood pressure, and bilateral mammography BI-RADS were compared between the baseline and five years after treatment. New evidence regarding this HRT protocol was assessed. The transdermal nanoformulation estradiol improved clinical parameters. Satisfaction with treatment was 92 %. Serum concentrations of estradiol changed significantly after treatment ($p < 0.05$). Weight and systolic and diastolic blood pressures had no significant differences ($p > 0.05$) over the years. No vaginal bleeding was observed. Bilateral mammography assessment of the breasts following 60 months of HRT with bioidentical estradiol treatment found normal results in all women. This paper shows for the first time the effectiveness of a nanostructured transdermal formulation enhancer on the delivery of estradiol and estriol measured *in vivo* using Raman Confocal Spectroscopy. The Nanostructured formulation is safe and effective in reestablishing estradiol serum levels and relieving menopausal symptoms. The nanoformulation may serve as a good choice for hormone replacement therapy to protect against other post-menopausal symptoms.**

INTRODUCTION

The first-pass metabolism is related with a great number of side effects in many drugs, since the concentration of these bioactive compounds is reduced before it reaches the systemic circulation [1]. Nanotechnology comprises a new paradigm in science, in which new strategies with nanostructured drugs have been presenting interesting and unique properties such transdermal absorption [2]. The technology of controlled release of drugs represents one of the frontiers on medical science; it involves multidisciplinary aspects and may provide an important contribution to improve human health [3,4].

Hormones play an important role on human health controlling and regulating different pro-

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cesses. Recent studies have been reported that regular and stable levels have beneficial effects for human physiology [5]. During the menopausal transition, the ovarian production of estrogen and progesterone declines. This condition is related with physiologic changes such as loss of libido, vaginal dryness, insomnia, osteoporosis and cardiovascular disease [6].

Hormonal replacement therapy (HRT) has been used for the treatment of menopause symptomatology; however, little is known concerning the use of Transdermal Bioidentical Hormone Replacement Therapy (THRT) as an alternative approach for those who have impeditive reasons for other ways of drugs administration [7-9]. In this kind of treatment, side effects are highly dependent on dose and route of administration. Thus, strategies on hormonal replacement therapy are being developed in a perspective where benefits must always be advantageous when compared to the risks [10,11].

Researchers have been using confocal Raman spectroscopy, as an interesting method of measuring bioactive compounds, providing detailed information in real time after a local application 10. This technique requires no sample preparation and is prepared to provide new data on the administration process of the molecule through the skin. Compared with the currently available methodologies, confocal Raman spectroscopy becomes robust, accurate, and provides reliable data [11].

Recent studies have been conducted for a better understanding of some the active pharmaceutical permeation through the skin [12]. The confocal Raman spectroscopy demonstrated to be an interesting method of measuring bioactive compounds providing detailed information in real time [13].

The present study was designed to assess the long term efficacy and safety of a nanostructured formulation of Estriol+Estradiol for treating signs and symptoms related to the symptomatology in women with natural menopause and evaluate its effects on clinical laboratorial and radiographic parameters after 5 years follow-up.

MATERIALS AND METHODS

Ethics

Iinitially, a written informed consent was provided for individuals willing to participate in a protocol approved by the Institutional Review Board of the Paulista University, Brazil. Eligible patients were then assigned were then enrolled to the study. This was a long-term clinical trial study of female patients aged 45-72 years old treated for menopause-related hormone imbalances from January 1, 2003 to April 30, 2008. The Paulista University Health Science Center at Sao Paulo Institutional Review Board reviewed and approved this study; protocol #533/2009.

Study Design

One hundred and twenty-two patients (aged 45 to 72 years old) were enrolled into a pilot, single-center, prospective study. Inclusion criteria included patients with persistent amenorrhea (for ≥ 6 months), with typical climacteric complaints such as hot flushes, sweating, insomnia, depressive disturbance, genital itching and dyspareunia.

Exclusion criteria included patients unwilling to complete the treatment protocol, subjects with any disease that would impede the use of the substances used in the study (e.g, mental diseases) as well as subjects that had used any type of HRT within six months prior to the study.

Volunteers were recruited from a referenced Gynecology Medical Center in Fortaleza City, the Capital of Ceara state, located in Northeast Brazil where patient charts are maintained. Climacteric symptoms have a relevant prevalence in this part of the country.

Clinical Evaluation

At baseline, patients received a complete gynecological clinical exam where blood pressure, weight and others clinical parameters were evaluated. After the baseline visit, subjects were instructed to perform a blood exam including bilateral mammogram radiographic.

The THRT consultation consists of an extensive clinical evaluation followed by an educational lecture about THRT. The patients were questioned regarding medical history and menopausal symptoms classified as “absent = 0,” “mild = 1,” “moderate = 2” or “severe = 3”. During initial evaluation and follow-up visits, it was used a standardized form to monitor symptom resolution and adverse effects.

The patients also were educated on several components of this kind of therapy such as: hormonal changes in menopause, associated factors, symptoms, risks and benefits of THRT dosage forms and the importance of the follow-up visits.

Blood samples were collected from the subjects early in the morning after an overnight fast. To minimize variation, each subject sample was

	Baseline (n = 122)	After THRT Therapy (n = 100)	Significance
Age (years)	56.88 ± 6.27	61.42 ± 6.27	P = 0.7 [a]
Weight (kg)	62.33 ± 6.27	62.22 ± 6.27	P = 0.89 [b]
Serum estradiol	33.80 ± 48.5	53.48 ± 78.7	P = 0.02 [b]
Systolic blood pressure (mmHg)	12.2 ± 1.18	11.1 ± 1.01	P = 0.33 [b]
Diastolic blood pressure (mm Hg)	7.67 ± 7.9	7.67 ± 6.7	P = 0.9 [b]
Undefined Bi-rads	9	4	P = 0.15 [c]

Table 1. Characteristics of women on the Baseline and women treated with THRT after 60 month follow-up. [a] Fisher's exact test; [b] t-test; [c] Chi Square test.

analyzed at the same time of the day. Pre and post treatment data, such as age, weight, estradiol levels, Bi-rads classification and systolic and diastolic blood pressure are shown in Table 1.

After serum testing, the identification hormone deficiencies, was determined by the gynecologist, and then, additional bioidentical hormones such as estriol, estradiol, or a combination of both were recommended and the patients were evaluated each 6 months after THRT initiation. All the patients were instructed about how to use the pump for transdermal application (performed in the presence of an experienced gynecologist) who performed all clinical examinations to guarantee standardization and correct use of the THRT. Compliance was defined as completing seventy percent or more of the transdermal applications.

Transdermal Hormone Replacement Therapy

Patients received an transdermal dose on the right forearm a nanoparticulated formulation of Estriol (0.1 %) + Estradiol (0.25 %) (EE), (Estriol+Estradiol/ Biolipid B2®; Evidence, Fortaleza, Brazil) given daily for 60 months.

The severity of the menopausal symptoms was evaluated according to the answers in the form full filled each six months of the study. Satisfaction with the hormone therapy was also evaluated at those times. Compliance with the regimen was checked by personal interviews. Furthermore, serum concentrations of estradiol and blood pressure were measured.

During the 5 years of trial, Patients were instructed to complete evaluation forms each six months after the first consultation to monitor menopausal symptoms and side effects. The subjects continued to use their regular non-supervised, self-performed transdermal bioidentical hormone measures. The clinical exam with the same parameters was repeated at 3 and after six months during five years.

Nanostructured Bioidentical Hormone Emulsion Preparation

The Nanostructured Bioidentical Hormones formulation was prepared at the Department of Nanotechnology, Institute of Applied Biotechnology (Patent Pending PCT No.WO2012/009778.A2).

The Biolipid/B2 is a stable colloidal nanostructured system, biocompatible and developed to enhance the transdermal drug delivery of hormones.

Two hormone+BIOLIPID/B2® formulations (Transdermal Penetration Enhancer Vehicle) were prepared. The emulsions were prepared, and the following mass ratios were obtained: Nanoformulation 1: 0,1 % Estriol + Biolipid®B2; Nanoformulation 2: 0,25 % Estradiol + Biolipid®B2.

Estriol and Estradiol were obtained from Sigma Aldrich. The main composition of this emulsion is based on the nanoparticulated hormones (estriol/estradiol) and oleic acid, phospholipids, protein and bionutrients compatible with the dermal structure.

Serum levels Assay

Serum levels of estradiol were obtained by radio-immunoassay. The results of estradiol were expressed as picograms per milliliter (pg/mL). Blood was collected from each participant at the baseline visit and after 3 and each 6 months after baseline.

The patients had their baseline blood test before initiating the bioidentical transdermal hormone replacement therapy (THRT). Samples were analyzed on the same reference laboratory of clinical analyses (Clementino Fraga, Fortaleza-CE/Brazil). Laboratory personnel were blinded to treatment status.

BI-RADS Evaluation

The data of 100 women who participated in the study were evaluated. The mammograms

were independently read by two certified radiologists who must reach consensus about referral for further diagnostic assessment. Assessment and diagnosis were repeated and completed with ultrasound and biopsies were performed when necessary. The American College of Radiology (ACR) guidelines [14] define a negative screening examination as one that is negative or has benign findings (BIRADS categories 1 and 2) and a positive screening examination as one for which referral is initiated (BI-RADS categories 0, 4 or 5). BI-RADS 3 was initially included in this study. Although this category is negative, it suggests short interval follow-up [14]. Thus, this category was excluded and merged into BI-RADS 0.

Scanning electron microscope (SEM) Assay

The electron microscopy analyses of the size of nanoparticles, was obtained by an equipment TESCAN SEM (Model VEGA/XMU, Brno, Czech Republic) using accelerating voltage of the 30Kv. All samples analyzed for SEM were previously sputtered with a ~20nm gold layer in order to obtain the images of the hormone nanoparticles.

Particle Size and distribution assay

The analyses of the size and distribution of nanoparticles, was performed by the equipment Zetasizer Nano ZS90 from Malvern Instruments Ltd. (Worcestershire, UK, England) using a method for the measurement of the nanoparticles.

This technique is used to measure particle and molecule size. This technique measures the diffusion of particles moving under Brownian motion, and converts this to size and a size distribution using the Stokes-Einstein relationship, indicated for the types of nanoparticles commonly used in biological applications. The estriol and estradiol nanoparticles samples were analyzed in order to obtain the graphic distributions of the hormone particles.

Raman spectrometer assay

Raman Confocal spectroscopy measurements were performed using the model 3510 Skin Composition Analyzer (model 3510 SCA, River Diagnostics, Rotterdam, The Netherlands).

To confirm the data, the experiments were performed twice on the volar forearms of volunteers [12]. The arm of the volunteer was placed on a fused silica window mounted in the measurement stage. Laser light is focused into the skin with a microscope objective located under the window. An internal video camera allows for inspection of the skin surface and selection of the measurement spot [13].

The spectral resolution is 4 cm^{-1} throughout the entire spectral range. A detailed Raman depth profiles could be acquired across the stratum corneum, viable epidermis and dermis. All Raman spectra were calibrated and corrected for instrument response using built-in instrument control software of the model 3510 SCA (River Diagnostics).

The test area was a marked in a 4 x 4 cm in the arm of the volunteer and treated with 70 µL of the nanoformulation of both hormones and then was evaluated.

The Nanostructured hormones were applied on the skin and gently spread using the tip of the micropipette, without rubbing [12,13], 10 min after the application the measurements started. The volunteers place the volar forearm on a fused silica window for the measurement stage of the Raman spectrometer. Depth profiles were then collected in the period interval of 1, 3, 6, 21, and 24 h after transdermal hormones (EE) administration. Six depth profiles were collected within each hour time period, thus, were acquired across the skin layers a detailed Raman depth and percent profiles from the nanoparticles formulation hormones.

Statistical Analysis

All statistical analyses were performed using SPSS statistical package for Windows version 10.0 (SPSS Inc., Chicago, IL, USA). The data are presented as the mean ± SEM or as the medians. The data are presented as the mean ± SEM or as the medians. Differences between baseline and after treatment were evaluated by Student's T test to compare medians. Statistical differences were considered to be significant at $p < 0.05$.

Categorical variables were compared using chi-square and Fisher's exact tests. Continuous variables were tested for normality using the Shapiro Wilk-W Test. Normally distributed variables were reported as means (standard deviations). Paired data were compared using the Wilcoxon signed-rank test. All tests were two-sided.

RESULTS

Twenty two women were excluded from study with some of the events such as: absence to consultation through the study, urinary tract

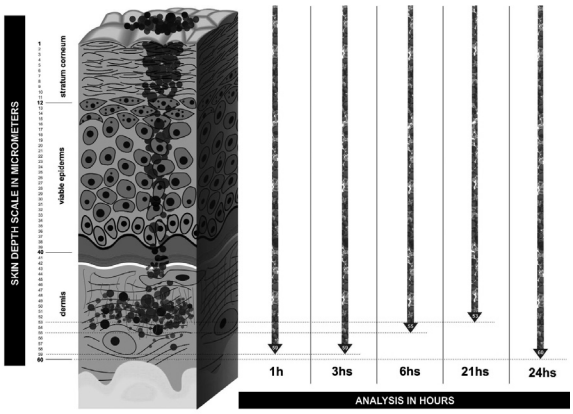

Figure 1. Skin Layer Analysis - Nanoparticles concentration of Estriol+Estradiol on the dermis. The skin depth concentration of hormones was measured at 1,3,6,21 and 24 hours after transdermal application. (Model 3510 SCA, River Diagnostics, Rotterdam, The Netherlands). ■ Estriol+Estradiol.

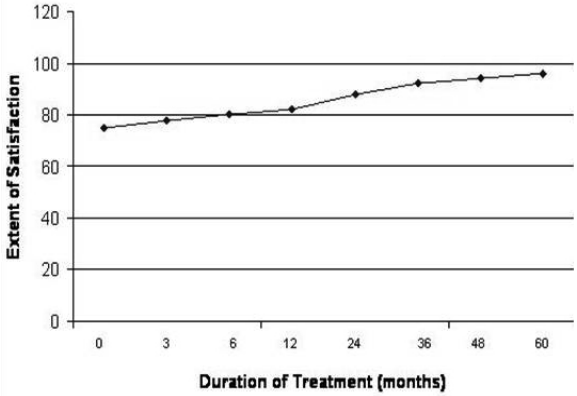

Figure 2. The satisfaction of the treatment in 100 women submitted to the transdermal hormone replacement therapy (THRT) during 60 months. Estriol + Estradiol emulsion was administered topically in women with menopause. The continuation of treatment seemed to induce further increases in the extent of satisfaction: 85.2 after one year of THRT and 92.5 ± 4.2 % at the end of treatment (P < 0.05).

infection or viral infection. The mean age of the population at baseline was 56.88 ± 6.27 years. Baseline and post-treatment data, such as weight, blood pressure and estradiol serum levels are shown in Table 1.

Figure 1 shows the Estriol+Estradiol (EE) nanoparticles through skin layers performed using the model 3510 Skin Composition Analyzer (model 3510 SCA, River Diagnostics, Rotterdam, The Netherlands).

Effect of Estradiol+Estriol Nanoparticles formulation the menopausal symptomatology

The menopausal symptomatology analysis of the volunteers subjected to THRT that received a combination of Estradiol (0.25 %) + Estriol (0.1 %) emulsion revealed a relevant decreasing of menopausal symptoms (Table 2), receiving median score 1 (range, 0-1), whereas a significant symptomatology was found in the charts of patients on the baseline, receiving a median score 3 (range, 2-3). These values were statistically significant (P < 0.05), when THRT treatment was compared to the baseline.

Effect of Estradiol+ Estriol Nanoparticles formulation on the therapy satisfaction

The comparison of the feeling of satisfaction with this hormone therapy between baseline and the end of treatment is shown in Figure 2. At the end of the first month, the extent of satisfaction with the hormone therapy was ~75 %. The continuation of treatment seemed to induce further increases in the extent of satisfaction: 85.2 after one year of THRT and 92.5 ± 4.2 % at the end of treatment (P < 0.05).

Effect of Estradiol+ Estriol Nanoparticles formulation on the estradiol serum concentration

The serum concentration of estradiol, over the 60-month study is shown in Figure 3. Statistical analysis of mean estradiol pretreatment values at baseline was 33.80 ± 48.5 pg/mL and after 60 months of THRT showed a significant increase by 53.48 ± 78.7 pg/mL. The data reached a statistical difference (p < 0.05) in serum after the treatment with the Transdermal Bioidentical Hormone. Final serum values were compared

Symptomatolgy	Baseline	After THRT	Significance
Scores	3 (2-3)	0 (0-1) *	P < 0.05 *

Table 2. Symptomatology analysis of the effect of the nanoparticles formulation of Estriol+Estradiol emulsion on menopausal complaints. * P<0.05 compared to baseline values (Kruskal-Wallis).

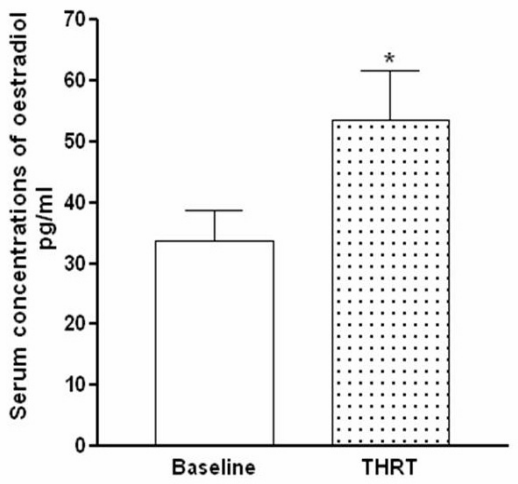

Figure 3. Effect of Nanoparticles formulation emulsion on Estradiol serum concentration (ESC) in 100 women submitted to the transdermal hormone replacement therapy (THRT) during 60 months. The emulsion was administered topically in women with menopause. Mean values are shown, SE is indicated by error bars. *P < 0.05 was considered significantly different compared to baseline values (Student's T test).

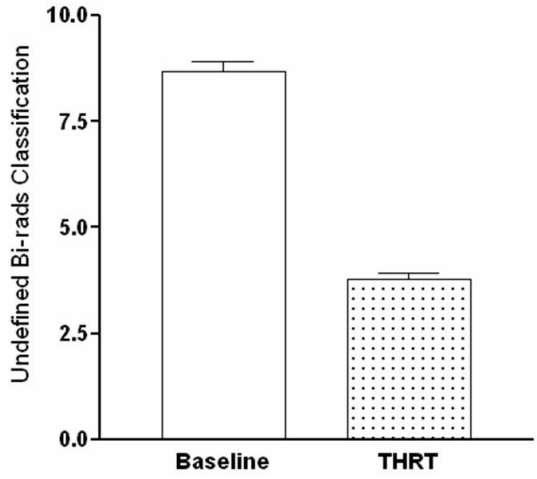

Figure 4. Effect of Nanoparticles formulation emulsion on Bi-RADS undefined classification in 100 women submitted to the transdermal hormone replacement therapy (THRT) during 60 months. Estriol+Estradiol emulsion was administered topically in women with menopause. *P < 0.05 was considered significantly different compared to baseline values (Student's T test).

prior to study entry (mean estradiol values prior to study entry and at the end of this study.

Effect of Estradiol+ Estriol Nanoparticles formulation the BI-RADS classification

Concerning the BI-RADS classification, during the 60 months of hormone replacement treatment, no statistically significant changes were observed, however it was performed a comparison on B-0 index that establishes the "undefined" diagnosis. On the Baseline there was 9 patients with B-0 diagnosis and after 60 months of THRT this number was cut by half with only 4 patients with B-0 classification at the end of treatment showed a relevant decrease, however it was not statistically significant (P >0.05) as compared with pretreatment values respectively (Fig. 4).

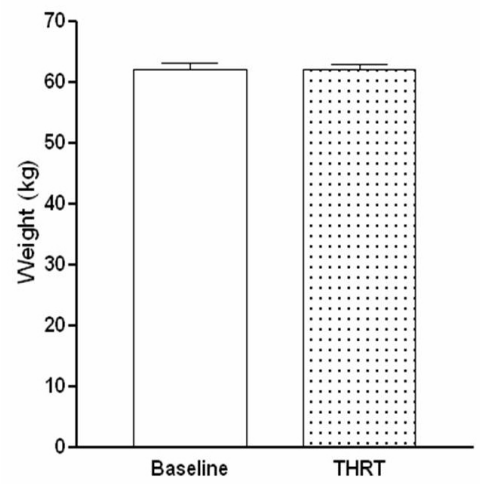

Figure 5. Effect of Nanoparticles formulation emulsion on weight in 100 women submitted to the transdermal hormone replacement therapy (THRT) during 60 months. Estriol+Estradiol emulsion was administered topically in women with menopause. Mean values are shown, SE is indicated by error bars. *P < 0.05 was considered significantly different compared to baseline values (Student's T test).

Effect of Estradiol+ Estriol Nanoparticles formulation on the weight and blood pressure

Changes in weight and blood pressure before and after treatment are shown in Figures 5 and 6. No statistical significant changes in these parameters were observed after 60 months of THRT treatment.

Ultrasound assessment of the breasts following 60 months of treatment found no tumor in all women evaluated. No one patient reported vaginal bleeding and no adverse effects were reported by the patients during the study.

Figure 7 shows the Estriol+Estradiol particles at Scan Electron Microscopy and the size and distribution of the nanoparticles through Zeta Sizer Nano ZS-90 (Malvern, WR, UK).

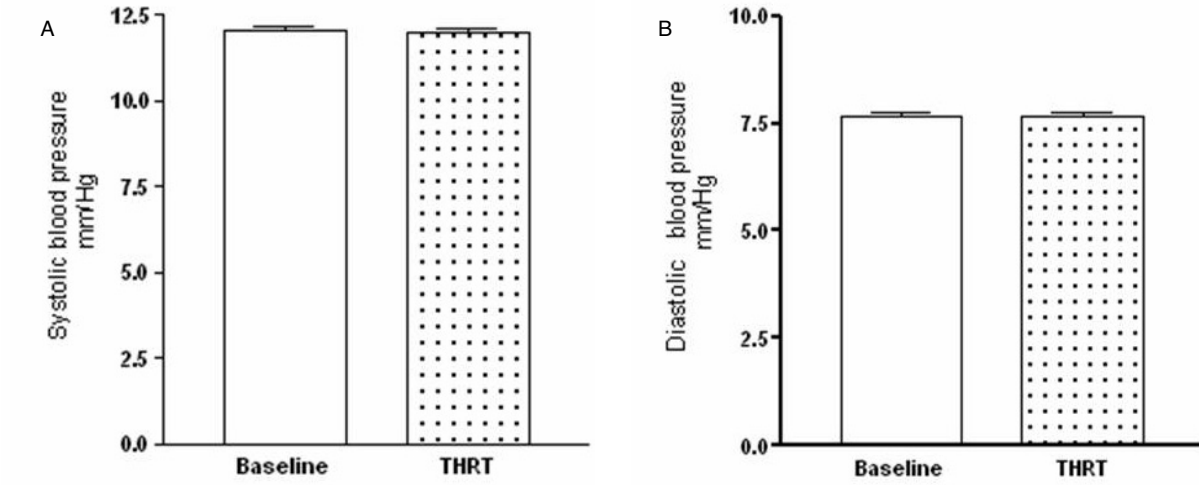

Figure 6. Effect of Nanoparticles formulation emulsion on Systolic (**A**) and Diastolic (**B**) Blood pressure in 100 women submitted to the transdermal hormone replacement therapy (THRT) during 60 months. Estriol+Estradiol emulsion was administered topically in women with menopause. Mean values are shown, SE is indicated by error bars. *P < 0.05 was considered significantly different compared to baseline values (Student's T test).

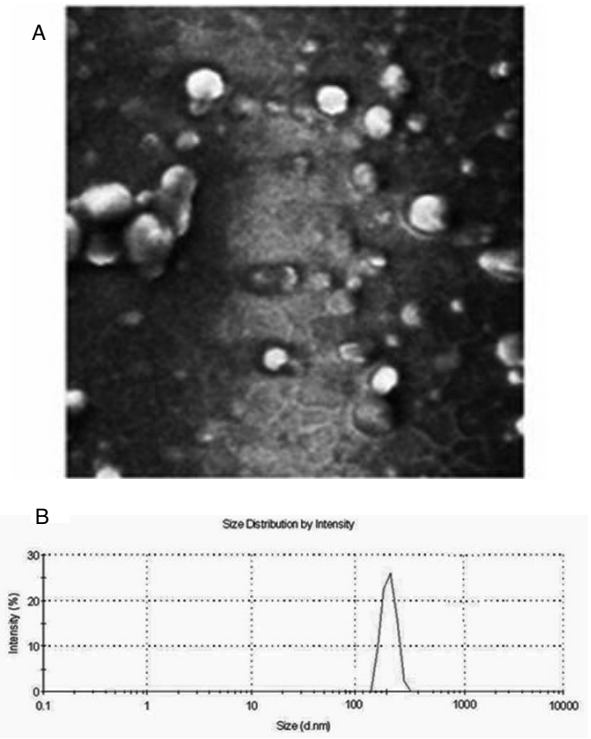

Figure 7. (**A**) Microscopic analyses of Estriol+Estradiol particles at Scanning electron microscope (SEM). (**B**) Size and distribution particles analysis (Estriol+Estradiol Nanoformulation). Zetasizer Nano ZS90 from Malvern Instruments Ltd. (Worcestershire,UK.England).

DISCUSSION

The transdermal estrogen therapy protocol is an effective treatment for relieving menopausal symptoms, particularly in those women for whom the adverse effects of the orally administered drugs are seen as an impeditive factor, since hormones are cleared in this stage when orally administered [15]). Thefirst-pass metabolism is a phenomenon related with the drug metabolism where the concentration of the drug is greatly reduced before it reaches the systemic circulation.

The nanoformulation of Estriol+Estradiol (EE) have shown an interesting controlled released system, delivering estriol and estradiol as early as one to 24 h after its application on the volar arm of the subjects. The skin concentration analysis can be seen by Raman confocal spectroscopy data (Table 3).

This transdermal protocol configure the basis for the development of an innovative proposal that was able to validate safety and effectiveness of the nanostructured formulation used. Many studies relates the use of transdermal hormone therapy to relieve menopausal symptoms and also used for prevention of osteoporosis and cardiovascular diseases [16]. Topical application of active substances offers an additional option in hormone therapy [17]. The Estriol+Estradiol emul-

	1 h	3 h	6 h	21 h	24 h
Stratum corneum	0.6 %	3 %	5 %	5 %	5 %
Viable Epidermis	5 %	12 %	9 %	33 %	12 %
Dermis	16 %	10 %	9 %	16 %	10 %

Table 3. Raman Confocal spectroscopy analysis on skin layers of estradiol nanoparticles concentration.

sion was studied in women showing expressive results on these patients [18].

In the present study, we have shown the safe and efficacy of a transdermal nanoparticulated controlled system, this data was evaluated following 122 women for 60 months.

As far as we concern, for first time Confocal Raman Spectroscopy was used to validate the transdermal absorption, thus, this methodology was able to explain the effect of penetration of hormones enhancer (BIOLIPID/B2®). This compound was able to show a very stable controlled released system delivering nanoparticles of estradiol and estriol, from one to 24 h evaluation. Through this technique it was possible to measure in vivo the depth and percentage of tested nanoparticulated hormones. This data can be seen clearly on Figures 1 and 3.

The transdermal effect of a nanoparticles formulation of Estriol+Estradiol emulsion plays a positive role on menopausal symptomatology; this effect was associated with reduction of the main complaints recorded at baseline (Table 2). Other interesting issue to be noticed over this 60 month study is that the transdermal therapy was not able to produce any kind of skin irritation or redness on the volunteers.

The weight gain is an important matter to be discussed, which is characteristically seen in the patients subjected to the use of oral hormones or contraceptives [19]) However after 60 month therapy, this phenomenon was not seen (Fig. 5) over the 60 months study follow-up. No statistical differences were observed between baseline and after THRT ($P > 0.05$).

The importance of this transdermal therapy, promotes a continuous stable systolic and diastolic blood pressure, in the present study, the systolic blood pressure showed a slight decrease 60 months after starting treatment as compared with mean baseline values (Fig. 6). The reason for these results is not known, and further investigation in a larger number of patients is indicated.

It is reasonable to speculate, the mechanism of action of transdermal hormone therapy. When applied to the stratum corneum (topical application) Estriol+Estradiol emulsion is absorbed into the underlying tissues, being able to deliver the hormones nanoparticles as a very stable drug released system, thus, it was possible to measure the increasing serum level of estradiol reducing menopausal complaints.

A previous study showed that the use of percutaneous 17 β-estradiol emulsion was indicated for the treatment of vasomotor symptoms in postmenopausal women. This clinical approach has been shown as an actual tendency towards to choose this kind of therapy to treat menopausal disorders [20]).

From the findings of this experiment we can assume that this nanoparticles formulation, have proven to be safe and efficacy treating menopausal symptomatology.

CONCLUSIONS

Within the limitation of this trial, it was demonstrated that Estriol+Estradiol nanoemulsion is effective in reducing menopausal symptoms and the tested emulsion was able to restore the serum levels of estradiol in all patients and no statistical variations were seen on weight or blood pressure in post-menopausal women. These activities could support the continued investigation of nanoparticulated hormones as a potential therapeutic agent in hormone replacement therapy. The results may have an important impact in order to create in a close future an effective agent for use in the government women health programs, thus further studies are warranted to clarify its usefulness in a large sample.

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