



EFFECTS OF A NEW TESTOSTERONE TRANSDERMAL DELIVERY SYSTEM, BIOLIPID B2®-TESTOSTERONE IN HEALTHY MIDDLE AGED MEN: A CONFOCAL RAMAN SPECTROSCOPY STUDY

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ABSTRACT

The Transdermal Delivery System (BIOLIPID B2®) is a nanoemulsion that can be applied directly to the skin by a metered pump to deliver hormones into the systemic circulation. The aims of this study were to assess the efficacy of BIOLIPID B2® to deliver testosterone systemically and evaluate the short-term effects on Thyroid-Stimulating Hormone (TSH), C-reactive protein (CRP) serum levels and blood pressure in middle aged men with testosterone deficiency. An open label randomized prospective study enrolled 14 Brazilian middle aged men with testosterone deficiency. The volunteers received in the forearm 50mg of transdermal BIOLIPID B2®-testosterone nanostructured formulation daily for 3 months. Confocal Raman Spectroscopy (CRS) was used to determine depth and concentration of testosterone on skin layers. Clinical parameters and serum concentrations were compared between the baseline and 3 months after treatment. Serum concentrations of testosterone, CPR and TSH changed significantly ($p < 0.05$) after treatment. There was, also a significant change in the waist circumference, weight and body mass index ($p > 0.05$) and no changes were observed in the blood pressure. No adverse events were attributed to this transdermal hormone replacement therapy protocol. The BIOLIPID B2® nanoemulsion formulation is safe and effective in reestablishing testosterone and Thyroid-Stimulating Hormone (TSH) serum levels. This transdermal protocol may serve as a strategy for hormone replacement therapy in middle aged men.

Keywords: BIOLIPID B2®, testosterone, transdermal delivery, andropause, C-reactive protein.

INTRODUCTION

Much attention has been drawn to the adverse effects related with drugs administered orally.^{1,2} Over the last decade, a great number of testosterone and other hormones preparations have been developed for pathologies treatment and replacement therapy purposes³, these include oral, intranasal, sublingual and subcutaneous implants.^{4,6}

It seems that the transdermal preparations are the better choice for hormone replacement therapy.^{4,7} Oral administration of testosterone leads to rapid metabolism by the liver that can bring relevant adverse effects for human physiology.^{8,9} Other occasional undesired events such as redness or itching are common with transdermal patches.⁴

Previous studies on confocal Raman spectroscopy, elected this methodology to validate the efficacy of controlled released drugs. This method can describe in real time relevant information about drugs profile such as depth and concentration of the investigated compound in each layer of the skin.⁴

Nanotechnology has been proving to become a part of medicine field bringing new strategies and perspectives in the health sciences.^{4,6} Recently new and strong evidences suggest that this kind of drugs have interesting and unique properties.⁷

The present study was designed to determine the short-term effects of transdermal HRT of a nanostructured formulation of BIOLIPID B2® Testosterone (5%) treating signs and symptoms related to early andropause and evaluate its effects on clinical, and laboratorial parameters after 3 months follow-up.

SUBJECTS AND METHODS

Ethics

Initially, a written informed consent was provided for individuals willing to participate in a protocol approved by the Ethical committee of University Paulista, UNIP, Sao Paulo/Brazil and written informed consent was obtained from every subject (UNIP #437/09). The study was registered at clinicaltrials.gov.

Study Protocol

This was a prospective short-term clinical trial study of male patients aged 36-68 years old treated for early andropause related hormone imbalances. Other results of this study are published elsewhere. Volunteers were recruited from referenced Medical Center in Teresina city, capital of Piaui State, where patient charts are maintained.

This study is a prospective short-term clinical trial study trial assessing the effects of Transdermal testosterone-BIOLIPID B2® on clinical symptoms and hormone serum levels in early andropause men.

The 14 men enrolled for this trial fulfilled the following inclusion criteria: 1) age between 35 and 70 years; 2) no use of any medication known to interfere with hormonal levels in the past 6 months. Patients presenting diabetes, history of cancer, thromboembolism or established cardio vascular disease were excluded.

Twelve patients dropped out in the three months of follow-up. Therefore, 14 patients completed the study. In the present analysis, the subjects received daily in the right forearm a transdermal nanostructured formulation of testosterone (5%) + Biolipid B2® (Evidence Pharmaceuticals, SP, Brazil) for

three months. The effects of transdermal hormone formulation were analyzed.

Clinical evaluation was performed before the treatment. Anthropometric measurements included body weight, body mass index, waist circumference and blood pressure were assessed before and during treatment. Blood pressure was measured using a digital sphygmomanometer (Omron HEM 742, Rio de Janeiro, Brazil). All exams were performed by experienced board certified physicians.

Nanostructured Emulsion Preparation

The nanoemulsion was developed at the Laboratory of Nanotechnology at University Potiguar in association with the Laboratory of Advanced Materials at Federal University of Ceara. The hormone+BIOLIPID/B2[®] formulation was prepared and the following mass ratio was obtained: Nanoformulation: Testosterone (5%) + Biolipid B2[®].

Testosterone was purchased from Sigma Aldrich. The main composition of this nanoemulsion is based on the nanoparticulated testosterone hormone and a transdermal penetration enhancer vehicle (Biolipid B2[®], Evidence Pharmaceuticals, SP, Brazil) based on oleic acid, phospholipids and nutrients compatible with the dermal structure developed to enhance the transdermal drug delivery of hormone through the skin layers.⁴

The hormones nanoparticles were prepared using a water-oil emulsion method with slight modifications (PCT Patent #WO 2012/009778 A2). The hormones nanoparticles were emulsified using a homogenizer (Art Labortechnik, Müllheim, Germany) at 20.000 rpm for 180 s. The resultant emulsions were stored for 3h at room temperature.⁴

Size and Zeta-potential Measurements

The size and zeta-potential of the testosterone particles were measured by a Malvern Zetasizer Nanoseries-ZS90 (England, UK). The size measurements were performed in disposable sizing cuvettes at a laser wavelength of 633 nm and a scattering angle of 90°, while the zeta-potential measurements were performed in disposable zeta-potential cells. Before the measurement, the testosterone particles were diluted 1:360 times with Milli-Q water. Each measurement was repeated for 3 runs per sample at 25°C.

Particle size measurements

Particle size analysis was performed by dynamic light scattering (DLS), also known as photon correlation spectroscopy, using a particle size analyzer (Malvern NanoZS90 Zetasizer England, UK). Prior to the measurements, all samples were diluted (1:360) using Milli-Q water to yield a suitable scattering intensity. DLS data were analyzed at 25°C and with a fixed light incidence angle of 90°. The mean hydrodynamic diameter (Z-average) and the polydispersity index (PDI) were determined as a measure of the width of the particle size distribution. The Z-average and PDI of the analyzed samples were obtained by calculating the average of 13 runs. The measurements were performed in triplicate.

Clinical Evaluation

The THRT consultation consisted on a brief lecture about risks and benefits of Transdermal HRT. During the whole treatment patients were asked to respond questions about any adverse effects using a standardized form to monitor symptoms resolution. During this evaluation it was used a

standardized form to monitor symptom resolution and adverse effects.

The Blood samples were collected from the subjects early in the morning after an overnight fast. After serum testing, the identification hormone deficiencies, was determined and then, if necessary, additional transdermal testosterone was prescribed. The patients were evaluated 3 months after THRT treatment protocol.

All the patients were instructed about how to use the testosterone pump for transdermal application, performed in the presence of an experienced physician, in order to guarantee standardization and correct use of the THRT. Compliance was defined as completing seventy percent or more of the transdermal applications.

Compliance and satisfaction with the treatment was also evaluated by personal interviews. Furthermore, serum concentrations of blood lipids, biochemical inflammatory markers were measured and are published elsewhere.

During the 3 months of trial, patients were instructed to complete evaluation forms each three months after the first consultation to monitor symptoms and side effects.

Transdermal Testosterone Therapy Assay (TTT)

Patients received a transdermal dose (1 pump=0.8g) on the right forearm of testosterone (5%) + Biolipid B2[®], this nanoformulation was administered daily for 3 months.

Satisfaction with the transdermal hormone therapy was also evaluated. Compliance with the regimen was checked by personal interviews. Furthermore, serum concentrations of Thyroid stimulating hormone (TSH) and Testosterone were measured. During the 3 months of trial, patients were instructed to complete evaluation forms after the first consultation, in order to monitor early andropause symptoms and side effects.

Serum levels Assay

The patients had their baseline blood test before initiating the transdermal testosterone therapy (TTT). Samples were analyzed on the same clinical analysis laboratory where the personnel were blinded to treatment status.

Serum levels of TSH were obtained by radio-immunoassay. The results of TSH are expressed as units per liter (IU/L). Blood was collected from each participant at the baseline visit and after 3 months. Serum levels of testosterone were obtained by radio-immunoassay. The results are expressed as picograms per milliliter (ng/mL).

Raman spectrometer assay

Raman Confocal spectroscopy measurements were performed using the model 3510 Skin Composition Analyzer (River Diagnostics, Rotterdam, The Netherlands). The test area was a marked in a 4×4 cm in the forearm of the volunteer and treated with 70 µL of the testosterone (5%) + BIOLIPID B2[®] and then was evaluated. The formulation was applied on the skin and gently spread using the tip of the micropipette, without rubbing, 10 minutes after the application the measurements started.

The forearm of the volunteer was placed on a fused silica window adapted 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. To confirm the data, the experiments were performed twice on the volar forearms of volunteers.⁴

A detailed Raman depth profiles was acquired through the stratum corneum, viable epidermis and dermis. All Raman spectra were calibrated and corrected for instrument response using built-in instrument control software (River Diagnostics).

Depth profiles were collected in the period interval of 1, 3, 6, 21 and 24 hours after transdermal testosterone application. Six depth profiles were collected within each hour time period.

Scanning electron microscope (SEM) Assay images

The electron microscopy analysis of the 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.

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. Differences between baseline and after treatment were evaluated by Student's T test to compare means. Friedman test was used for the analysis of Kupperman score, followed by the sign test. Categorical variables were compared using chi-square and Fisher's exact tests. Normally distributed variables were reported as means (standard deviations). Paired data were compared using the Wilcoxon signed-rank test. Statistical differences were considered to be significant at $p < 0.05$.

Table 1. Clinical and anthropometric variables and andropause symptoms of 14 middle-aged men submitted to TTT on Baseline and after 3 months of treatment

	Baseline (n=20)	After THRT (n=14)	Significance
Age (years)	50.8 ± 10.1	50.8 ± 10.1	$P=1^b$
Weight (kg)	77.37 ± 16.3	73.91 ± 13.1	$P=0.32^b$
Body Mass Index (BMI)	26.02 ± 3.3	24.93 ± 2.4	$P=0.005^b$
Waist Circumference	98.2 ± 12.09	94.6 ± 10.3	$P=0.001^b$
Thyroid-Stimulating Hormone (µIU/mL)	2.5 ± 1.8	1.97 ± 1.3	$P=0.02^b$
Systolic blood pressure (mmHg)	13.14 ± 1.834	12.21 ± 1.36	$P=0.45^b$
Diastolic blood pressure (mm Hg)	8.5 ± 1.22	7.6 ± 0.74	$P=0.18^b$
Treatment Satisfaction (%)	~58	~92	$P < 0.05^c$

Values expressed as mean ± SD.

P = difference between Baseline and 60 months after THRT.

* $P < 0.05$ compared to baseline values (Kruskal-Wallis).

Legend for statistical tests: ^a Fisher's exact test; ^b t-test; ^c Chi Square test.

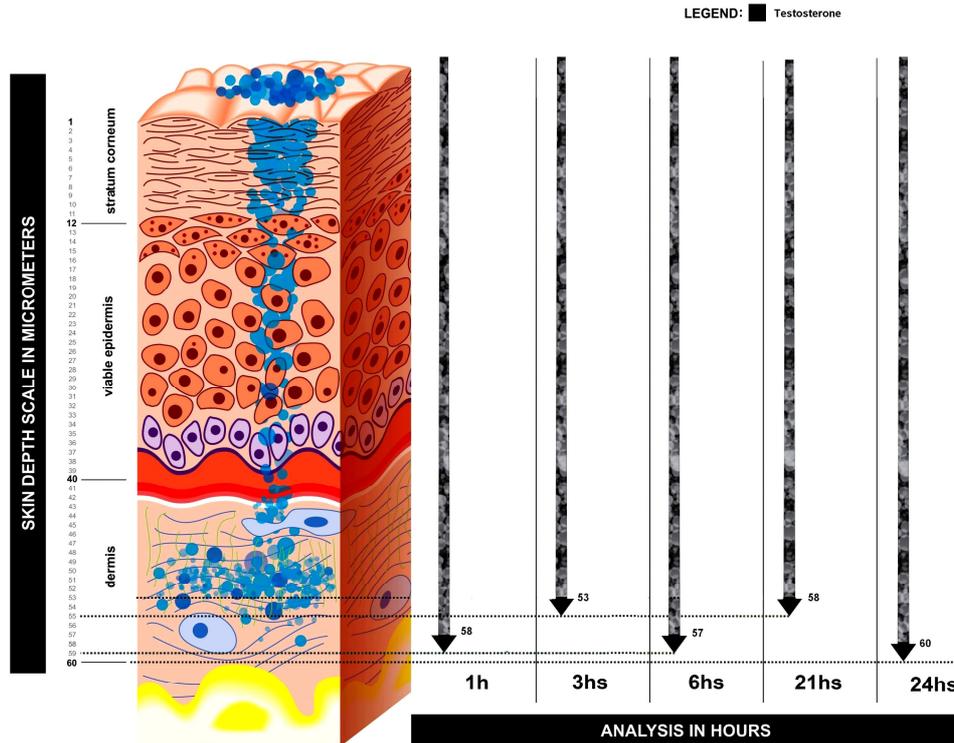


Figure 1: Skin layer Analysis - Nanoparticles concentration of testosterone particles on the dermis. The skin depth concentration was measured at 1,3,6,21 and 24 hours after transdermal application. (River Diagnostics, Model 3510 SCA, Rotterdam, The Netherlands).

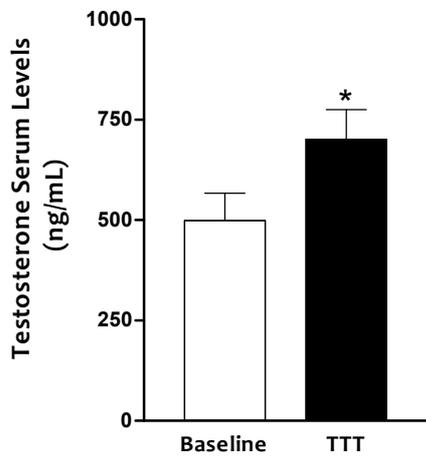


Figure 2: Effect of BIOLIPID B2®-Testosterone (5%) formulation administered topically in 14 middle aged men submitted to TTT during 3 months on testosterone serum levels. The Nanoemulsion was administered topically on the right forearm. Mean values are shown, SE is indicated by error bars. *P<0.05 was considered significantly different compared to baseline values (Student's T test).

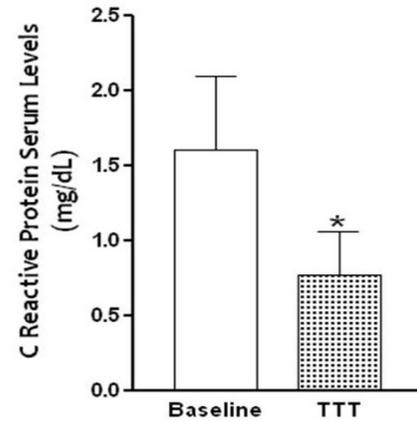


Figure 3: Effect of BIOLIPID B2®-Testosterone (5%) formulation administered topically in 14 middle aged men submitted to TTT during 3 months on C-reactive protein serum. The Nanoemulsion was administered topically on the right forearm. Mean values are shown, SE is indicated by error bars. *P<0.05 was considered significantly different compared to baseline values (Student's T test).

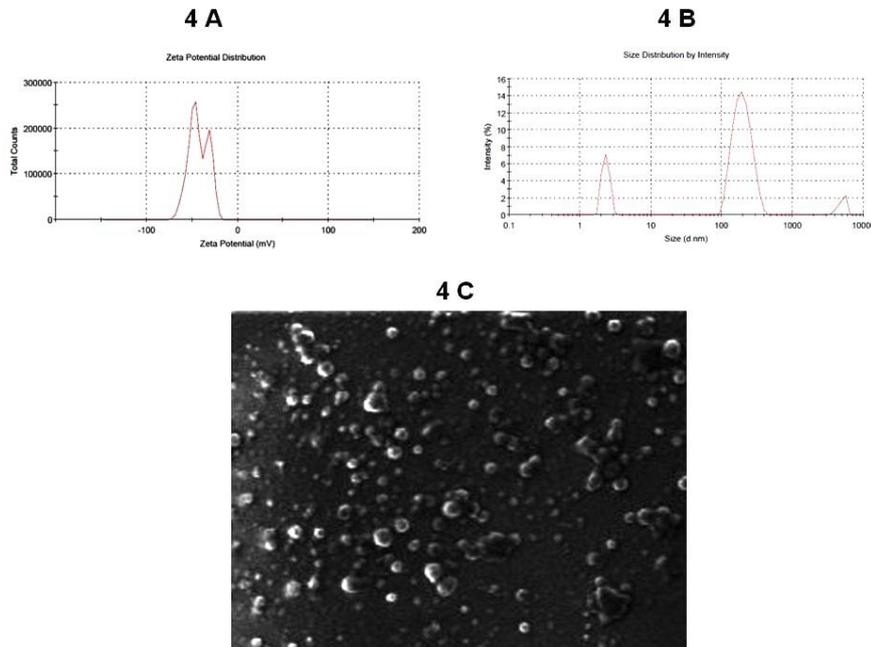


Figure 4A: Zeta potential analysis on BIOLIPID B2®-Testosterone (5%) formulation administered topically in 14 middle aged men submitted to TTT during 3 months. Zetasizer Nano ZS90 (Malvern Instruments Ltd. UK.England).

Figure 4B: Size distribution intensity analysis on BIOLIPID B2®-Testosterone (5%) formulation administered topically in 14 middle aged men submitted to TTT during 3 months. Zetasizer Nano ZS90 (Malvern Instruments Ltd. UK.England).

Figure 4C: Scanning Electron Microscopic Analyses of testosterone particles morphology administered topically in 66 postmenopausal women submitted to THRT during 60 months (TESCAN SEM -Model VEGA/XMU, Brno, Czech Republic).

RESULTS

Almost twenty-five percent of the initial population was excluded from study. Anthropometric measurements included body weight, waist circumference, body mass index and Blood pressure was checked twice in a visit.

The effects of transdermal emulsion was beneficial during 3 months follow-up Table 1 presents age, weight, blood pressure and body mass index and waist circumference for early andropause before and after 3 months of treatment in the group of 14 participants. The weight, systolic and

diastolic blood pressure did not reach statistical changes with TTT.

At baseline, all patients presented symptoms that improved significantly with treatment. The extent of satisfaction with the transdermal testosterone therapy at baseline was ~58%, with the continuation induced further increases in the extent of satisfaction: 78.2 after one month and $94 \pm 4.2\%$ at the end of study (Table 1). These values were statistically significant ($P < 0.05$), when the mean values from baseline were compared after 3 months of treatment.

Adverse events

No patient reported any adverse effects during the study and no other adverse effects were seen.

Treatment outcomes

Confocal Raman Spectroscopy of testosterone nanoparticles depth and concentration on skin layers.

The figure 1 shows in a graphical representation of Testosterone nanoparticles flow through the skin layers. This data were acquired through Confocal Raman Spectroscopy Analysis (CRSA) (River Diagnostics, Skin Composition Analyzer/3510, Rotterdam, The Netherlands). These main results represent the concentration and the depth of nanoparticles on each skin layer after 1, 3, 6, 21, 24 hours after transdermal HRT clinical procedure. The Confocal Raman spectroscopy was able to show important data analysis about the depth and concentration of hormones particles on skin layers over the hours after transdermal locally application. The skin depth concentration of testosterone was measured in Stratum Corneum (SC); Viable Epidermis (EPI); Dermis (D) at 1,3,6,21 and 24 hours (Figure 1).

Effect of Transdermal BIOLIPID B2®-Testosterone (5%) formulation on Testosterone serum levels

The serum level of testosterone, over the 3 months of TTT is shown in figure 2. Statistical analysis of mean testosterone pretreatment values at baseline was 498.59 ± 255.7 (ng/mL) and after 3 months of THRT showed a significant increase to 701.21 ± 276.8 . The data reached a statistical difference ($p < 0.05$).

Effect of Transdermal BIOLIPID B2®-Testosterone (5%) formulation on C-reactive protein (CRP) serum levels

The serum level of CRP, over the 3 months of TTT is shown in figure 3. Statistical analysis of mean CRP pretreatment values at baseline was 1.61 ± 1.4 mg/dL and after 3 months of THRT showed a significant decrease by 1.06 ± 1.2 . The data reached a statistical difference ($p < 0.05$) after the treatment with the transdermal formulation.

Zeta potential measurements

The nanoformulation of Testosterone presented a high negative average zeta potential of -42.8 mV as shown in figure 4A.

Hormone particle size measurements

The mean particle sizes of the testosterone nanoparticles measured by DLS are presented in figure 4B. The testosterone nanoparticles showed a homogenous size distribution with a mean diameter of $93.3 - 179.6$ nm.

The physical stability of the testosterone nanoparticles was also evaluated by examining changes of mean particle sizes during storage conditions for 2 months at room temperature. The testosterone nanoparticles did not show statistically significant changes in their mean diameter ($p > 0.05$) when stored at room temperature for 2 months. This short-term stability study indicates good physical stability, this observation ensure that the testosterone particles will remain stable in a long-term storage.

Testosterone nanoparticles morphology by SEM Assay

The Scanning Electron Microscopy (SEM) is a simple and practical method to obtain information about the mean size

and the surface morphology of particles. The morphology of the testosterone nanoparticles determined by SEM is shown in Figure 4C. The images reveal that the testosterone particles were almost spherical and uniform in shape with smooth surfaces. The mean diameter was in the range of 100-600 nm and there was no visible aggregation of particles.

DISCUSSION

The natural aging process submits men to lower testosterone levels. The production of sperm gradually becomes lower, physical and psychological symptoms become part of these low levels. According to the literature it is estimated that testosterone decreases about 10% every decade after the third decade.

Recent studies have shown that the restoration of testosterone level improves andropause symptoms, such as the osteopenia, increased fat mass, mood disturbance, loss of skeletal muscle and sexual dysfunction.^{8,9} In the other hand many articles have shown that injection of artificial testosterone can induce undesired side effects such as benign prostatic hyperplasia, cardiovascular events and in many cases prostate cancer.¹⁰⁻¹⁴ Therefore, identification of identical testosterone structure by Confocal Raman Spectroscopy can protect Leydig cells and support the continued the transdermal treatment protocol for minimizing the andropause symptomatology.

The Nanotechnology is an important agent in medical sciences. It can bring into the light, new and strong evidences that may represent important shift of old paradigms in the medical sciences.

As far as we concern, this is for first time that Confocal Raman Spectroscopy was used to validate the transdermal absorption of testosterone through the skin. This methodology can explain the effect of the enhancer BIOLIPID B2® used to deliver testosterone nanoparticles during 24 hours of the day. Through this strategy it was possible to measure *in vivo* the depth and percentage of testosterone (Figure 1).

The advanced knowledge in delivery systems is able to restore with safe and efficacy physiologic functions in different pathologies.⁴ The first-pass metabolism is related with many undesired effects, since the concentration of the drug is greatly reduced before it reaches systemic circulation.

A limitation of our study is that we were not able to adjust for drugs offer additional advantages in transdermal hormone therapy comparing with non-users. In the present study, we have shown the efficacy of a transdermal nanoparticulated hormone as a stable controlled release system. This data was evaluated following 14 men for 3 months with andropause symptoms.

There are strong evidences supporting that, transdermal hormone therapy has cardioprotective effect, probably due to its lack of effect on the coagulation cascade and resistance to activated protein C, reducing the cardiovascular events compared with non-users. Andropause men have a high risk of osteopenia and other cardiovascular diseases.

The BIOLIPID B2®-testosterone formulation was shown to deliver testosterone systemically to all humans evaluated in this study. The concentrations of hormone in the first 12h following BIOLIPID B2®-testosterone administration were found to be statistically different from baseline.

No serious or unexpected adverse events were reported or observed during the course of this three month trial. The drug nanoformulation and protocol administered were well tolerated by all subjects. The BIOLIPID B2®-testosterone

formulation provide a convenient delivery of testosterone with rapid drying, with no skin residue or irritation.

The transdermal testosterone therapy (TTT) did not cause any alterations on systolic and diastolic blood pressure (Table 1) in the treated subjects. During the study, any other antihypertensive was prescribed. These results are consistent with other studies, where transdermal testosterone did not have any impact on blood pressure. It is reasonable to understand that this positive effect might have been achieved by the transdermal route.

This paper shows for the first time the level changes in circulating testosterone, CRP and TSH. In this investigation, over the 3 months of treatment this transdermal HRT protocol did not have any harmful effect on blood pressure, moreover the mean serum fasting of TSH levels decreased significantly ($p < 0.05$). These outcomes are consistent with some previous studies, which used the transdermal route for HRT.⁴

In our perspective, the absence of side effects shows that transdermal route of TTT may be the best choice. Other interesting outcome was the absence of nausea during the treatment, maybe, related to the transdermal delivery. Future controlled investigations with different formulations are needed to unravel this question.

The weight gain is an important matter and after 3 month therapy, this phenomenon was not seen (Table 1). Concerning the systolic and diastolic blood pressure, in the present study, the systolic blood pressure showed a slight decrease after starting treatment as compared with mean baseline values (Table 1). The reason for these results is not known, and further investigation in a larger number of patients is indicated.

The transdermal testosterone protocol used in this study was an effective strategy relieving early andropause symptoms. The findings demonstrated that in 3 months treatment with this nanostructured transdermal formulation was able to reduce significantly andropause symptoms when compared to the baseline complaints (Table 1). The findings also suggest the safe and efficacy of this protocol restoring the testosterone levels (Figure 3).

The nanostructured testosterone formulation have shown an interesting controlled released system, delivering hormones as early as one to 24 hours after its application on the volar arm of the patients. A very consistent and stable controlled skin concentration analysis can be assessed by confocal Raman spectroscopy results (Figure 1). This transdermal protocol configures the basis for the development of an innovative proposal of the nanostructured formulation used previously by our research group.

The hypothesis that holds the present study on the efficacy of the transdermal HRT, address to the one of the components of the enhancer BIOLIPID B2[®], the Oleic acid and other long chain fatty acids, normally used as lipid fluidizers allowing faster transport through the skin layers, therefore current evidence suggests that this emulsion facilitates the penetration of testosterone.

The transdermal hormone enhancer, Biolipid B2[®], have been proving to be a vehicle able to deliver nanoparticles of testosterone over the three skin layers. In Brazil, this enhancer (BIOLIPID B2[®]) has been used in more than 20 states and has the approval by the Brazilian Drugs Regulation Agency (ANVISA). This vehicle demonstrated to be an excellent option for men with contraindications to orally administered hormones.

The particle sizes obtained by DLS are in agreement with the results obtained by previous studies that used the same technique.⁴ It should be noted that this methods is based on a new patent process. The size detection of nanoparticles by DLS is carried out in aqueous state meaning where the testosterone nanospheres are hydrated, so the diameters detected by this technique are usually larger than the non-hydrated diameters.

Regarding the Zeta potential the absolute value of -42.8 mV (Figure 4A), suggests that the nanoparticles developed in the present study are considered physically stable due to the electrostatic repulsion conferred by the chemical nature of the lipid matrix and possibly the adsorption of negatively charged ions onto the nanoparticles surface.

CONCLUSION

From the findings of this experiment we can assume that this nanostructured transdermal delivery system, have proven to be safe treating andropause symptomatology. Therefore, the present clinical data indicate that transdermal BIOLIPID B2[®]-testosterone (5%) could represent an interesting strategy for Hormone Replacement Therapy. Within the limitation of this trial, it was demonstrated that the nanoemulsion is effective in reducing symptoms and was able to restore the serum levels of testosterone and TSH with significant statistical variations.

These outcomes support the continued investigation of transdermal nanoparticulated hormones as a potential therapeutic agent in andropause therapy, thus, further studies are warranted to clarify its usefulness in a large sample.

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AUTHOR CONTRIBUTIONS

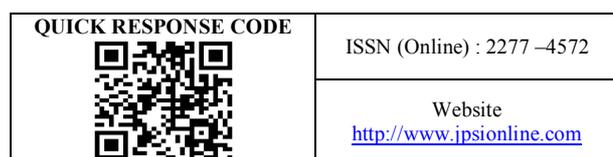
Barros GB, Umbelino S, Freitas A and Guerreiro S contributed to the data collection. Ruela R and Borges BC were responsible for the bibliographic review, data tabulation. Botelho MA was responsible for the statistical analysis. Queiroz DC was responsible for the SEM Images and Nanosizer analysis. Almeida JG, Quintans JR L and Botelho M.A were responsible for the project conception and manuscript writing.

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