ANTICANCER RESEARCH *37*: 6791-6797 (2017)
doi:10.21873/anticanres.12139

Transdermal Delivery of Luteinizing Hormone-releasing Hormone with Chitosan Microneedles: A Promising Tool for Androgen Deprivation Therapy

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Abstract. *Long-term administration of luteinizing hormone-releasing hormone analogs (LHRHa) is the main type of androgen-deprivation therapy (ADT) for lethal prostate cancer. A fully insertable microneedle system, composed of embeddable chitosan microneedles and a dissolvable polyvinyl alcohol/polyvinyl pyrrolidone supporting array, was developed for sustained delivery of LHRHa to the skin. A porcine cadaver skin test showed that chitosan microneedles can be fully embedded within the skin and microneedle-created micropores reseal within 7 days. The measured LHRHa loading amount was 73.3±2.8 μg per microneedle patch. After applying goserelin-containing microneedles to mice, serum LH levels increased initially and then declined below baseline at day 7. In contrast, serum testosterone levels increased to reach a peak at day 14 and then declined to a castration level at day 21. Additionally, such a castration level was maintained for 2 weeks. Therefore, transdermal delivery of goserelin with embeddable chitosan microneedles can produce a castrated state in mice. Such a system is a promising, feasible means of delivering ADT.*

Prostate cancer is the first leading malignancy and second cause of cancer death among males in Western countries and after lung cancer has been the second common cause of cancer death in men for two decades (1, 2). Since Huggins *et al.* introduced orchiectomy or estrogen for treating advanced prostate cancer (3), androgen deprivation therapy (ADT) has been applied not only in patients with metastatic prostate cancer, but also in those with localized prostate cancer (except in those with very low or low risk) and locally advanced disease as short- or long-term ADT before, during or after definitive radiotherapy (4, 5). Metastatic prostate cancer inevitably develops into castration-resistant disease; therefore, the current consensus guideline still recommended the importance of the castrated state by continuing ADT regardless of the use of several subsequent effective agents (6). Since Schally *et al.* determined and reported the sequence of luteinizing-hormone releasing hormone (LHRH) in 1972 (7), several LHRH analogs have been developed and used in medical castration, with the consensus that it is effectively the same as surgical orchiectomy (8). Although LHRH agonist therapy is associated with higher probabilities of adverse effects compared with orchiectomy (9), it has become the mainstay of ADT. Besides prostate cancer, LHRH analogs have been used for other medical conditions, including Silver–Russell syndrome (10), endometriosis (11), and breast cancer (12). Despite this, many efforts still focus on how to improve the use of LHRH analogs as an effective and convenient treatment for prostate cancer.

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Key Words: Prostate cancer, testosterone, microneedle, chitosan.

Compared to oral pills, intravenous or subcutaneous injection, transdermal drug delivery systems have several advantages, including no first-pass effect from gastrointestinal absorption, no local pain or discomfort from direct injection, slow release and avoidance of bolus administration, and easy manipulation. It is not easy for a large molecule, such as a protein (hydrophilic), to directly penetrate the hydrophobic *stratus corneum* of the skin without degradation (13, 14). Thus, the microneedle patch was developed to serve as a transdermal delivery system for large molecules, since it exhibits several characteristics, including less pain or discomfort, easy self-manipulation, convenience, and precise delivery and release of drug. For this reason, the aim of the study was to develop a transdermal delivery system for LHRH analogs with chitosan microneedles as a tool for ADT.

Materials and Methods

Reagents. Chitosan (91.2% deacetylated, viscosity=22 mPa·s)and fluorescein 5(6)-isothiocyanate (FITC; MW=389 Da) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Polydimethylsiloxane (PDMS; Sylgard 184), and Optimum Cutting Temperature compound were purchased from Dow Corning (Midland, MI, USA), Invitrogen (Eugene, OR, USA), and Tissue-Tek (Sakura Finetek, Torrance, CA, USA), respectively. All chemicals were used as received without additional treatment.

LHRH with 5-carboxytetramethylrhodamine (5-TAMRA) or without were purchased from Kelowna International Scientific Inc. (Taipei, Taiwan, ROC) for *in vitro* experiments. LHRH agonist goserelin was purchased from Bachem (Bubendorf, Switzerland) for *in vivo* efficacy experiments. Micro BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA) was used for *in vitro* LHRH measurement. Testosterone enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, New York, NY, USA) and LH ELISA kit (Cloud-Clone Corp, Katy, TX, USA) were used for quantitation of serum testosterone and LH concentrations.

Fabrication of chitosan microneedles and loading with FITC or TAMRA-LHRH. For visualization in experiments, FITC was incorporated into the chitosan, as well as TAMRA-LHRH. The fabrication of chitosan microneedles was modified from our previous published method (15). In brief, three pyramidal microneedle master structures were created using an electro-discharge machining process (Micropoint Technologies Pte, Ltd., Singapore). Each master structure consisted of 81 (9×9) pyramidal needles and the tip-to-tip distance of all samples was 600 μm. Microneedle molds were made from PDMS to inverse-replicate these master structures precisely. The cured PDMS molds were subsequently peeled from the master structures and repeatedly used to make chitosan microneedles (Figure 1A and B).

Chitosan powder was prepared to produce concentrated chitosan hydrogel, which was used for the casting process and served as the microneedle matrix as previously (15).For encapsulating within the microneedles, 16 g of chitosan powder was dissolved in 1% acetic acid at 20˚C overnight and then mixed with FITC at a ratio of 80 ml chitosan solution: 6 mg FITC (2 mg/ml in 99% ethanol) at 4˚C for 3 h with continuous stirring. Thereafter, the chitosan-FITC solution was dialyzed to form a diluted hydrogel (pH 5.5-6, 1.6 wt%). The FITC-chitosan hydrogel was then mixed with trehalose (10% dry weight of chitosan) and TAMRA-LHRH (TAMRA-LHRH/ 1.6wt%FITC-chitosan ratio at 1:1,000) at 50˚C with stirred and concentrated to 6 wt%. A similar fabrication process was also used to encapsulate goserelin in the microneedles at 1.5 mg goserelin per patch (about 5% drug-loading rate) and was concentrated at 40˚C with stirring to 6 wt%.

A double casting process was used to mold microneedles. The drug-loaded hydrogel was applied onto the PDMS mold as the first layer only, and the centrifugation process was used to fill the mold cavities as in the previous study (15). In brief, the PDMS mold was placed in a centrifuge tube and centrifuged at 3880 × *g* and 30"C for 4 h. When this first layer had dried, the second layer of polyvinyl alcohol/polyvinyl pyrrolidone(PVA/PVP) solution was subsequently placed onto it and this was followed by further centrifugation at 3,880 × *g* at 30˚C for 30 min. The filled mold was air dried at room temperature overnight and then put in an oven at 37˚C for 1 day. Finally, the drug-loaded microneedle patch was gently peeled from the mold and examined using a stereomicroscope (Olympus, Olympus Corporation, Tokyo, Japan).

In vitro LHRH-loading measurement. To measure the amount of LHRH loaded per microneedle, we dissolved the produced microneedle in double-distilled water maintained at 4˚C overnight to achieve the maximal release of loaded agent into the solution. Thereafter, the protein content was measured using a BCA kit according to the manufacturer's instructions by measuring the optical density and intrapolating values from the standard curve.

In vitro pig cadaver and in vivo mouse skin insertion. To avoid unnecessary animal testing, skin insertion into pig cadavers *in vitro* was firstly carried out. In order to evaluate the penetration potential of chitosan microneedles by aid of a homemade applicator, microneedle patches were fixed on the applicator first and then pressed manually against the cadaver skin for 5 min with an application force of 9.5 N per patch. The insertion site on the surface of the pig cadaver skin was then covered with blue tissue-marking dye (Shandon, Richard-Allan Scientific, Kalamazoo, MI, USA) for 1 min to identify the sites of *stratum corneum* penetration. After washing the skin and wiping residual dye from the skin surface, skin sections were processed for histological evaluation. The sites were excised from bulk skin using a scalpel. Each isolated skin section was embedded and frozen in liquid nitrogen, then prepared into 5-μm thick sections. The skin sections were finally viewed using an inverted fluorescence microscope (IX-71; Olympus, Tokyo, Japan).

In order to visualize the holes made by the microneedles in living animal skin, microneedles were inserted into the skin of a male 4-week-old ICR mice (National Cheng Kung University Animal Center, Tainan, Taiwan) by a homemade applicator under *i.p.* anesthesia with 35 mg/kg Zoletil 50 (Virbac, Carros, France), and the recovery of the skin wound after microneedle insertion was recorded daily until day 7.

In vivo transdermal delivery of goserelin to ICR mice using chitosan microneedles. The Institutional Animal Care and Use Committee of National Cheng Kung University approved all animal protocols, and experiments were conducted according to the guidelines of the Laboratory Animal Center of National Cheng Kung University (IACUC Approval No., 102072). Four-week-old male ICR mice weighting 20±5 g were used. In one experiment, 3 non-tumor-bearing ICR mice were treated with goserelin-carrying chitosan microneedle

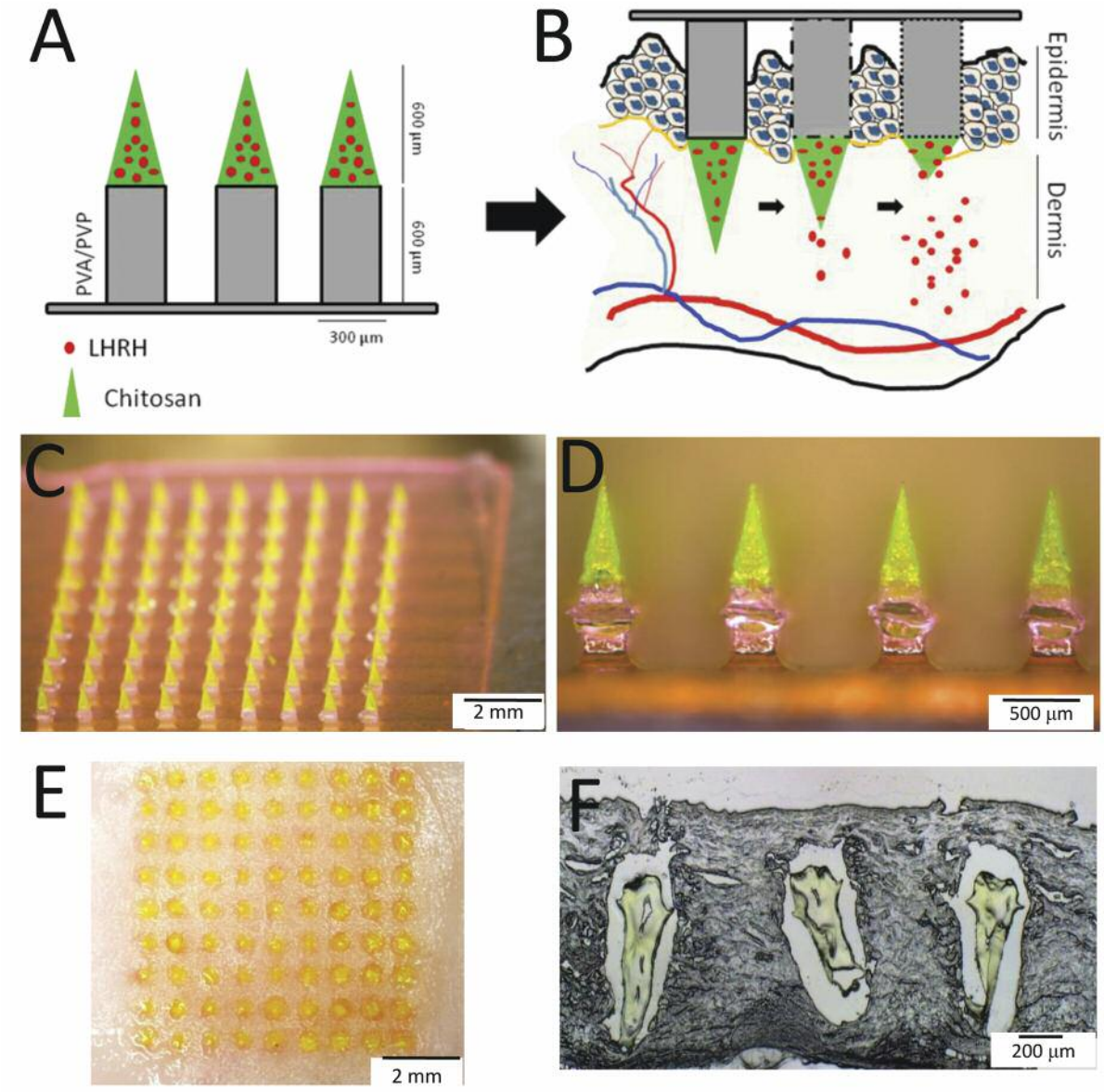

Figure 1. *Schematic illustration and in vitro skin penetration of the transdermal delivery of luteinizing hormone-releasing hormone L (HRH) with an embeddable microneedle system. A: The system is composed of LHRH-loaded chitosan microneedles on polyvinyl alcohol/polyvinyl pyrrolidone (PVA/PVP) supporting structures. B: The supporting array can provide additional length for counteracting skin deformation during penetration and offers mechanical strength for the complete insertion of the microneedles into the skin. After insertion, the supporting array is dissolved by skin interstitial fluid and the encapsulated LHRH is gradually released from the embedded microneedles. C and D: Bright-field micrographs of fluorescein 5(6)-isothiocyanate (FITC)-loaded microneedle array. E: The PVA/PPVP supporting array separates from the microneedles and porcine cadaver skin after skin insertion of the FTIC-loaded microneedles. F: Histological section of porcine skin pierced by the FITC-loaded microneedles.*

(one patch per mouse) and there was no control group. Immediately before applying the microneedle patch, each mouse was anesthetized with an intramuscular injection of Zoletil 50 (35 mg/kg; Virbac) and Rompun xylazine (2 mg/kg; Bayer, Leverkusen, Germany), and the hair on their backs was removed using depilatory cream (Domo Ointment Aid; c.G.M.P; New Taipei City, Taiwan, ROC). The blood sample was collected from the retro-orbital fossa and then centrifuged at 1,000 × *g* for 5 min to immediately separate the plasma, as well as the indicated time. Plasma testosterone and LH levels were determined using testosterone and LH ELISA kits, and the initial levels at 0 h were considered to be 100%. The percentage change in plasma testosterone and LH levels at each time interval after goserelin-containing microneedle therapy was calculated based on these initial values. This animal

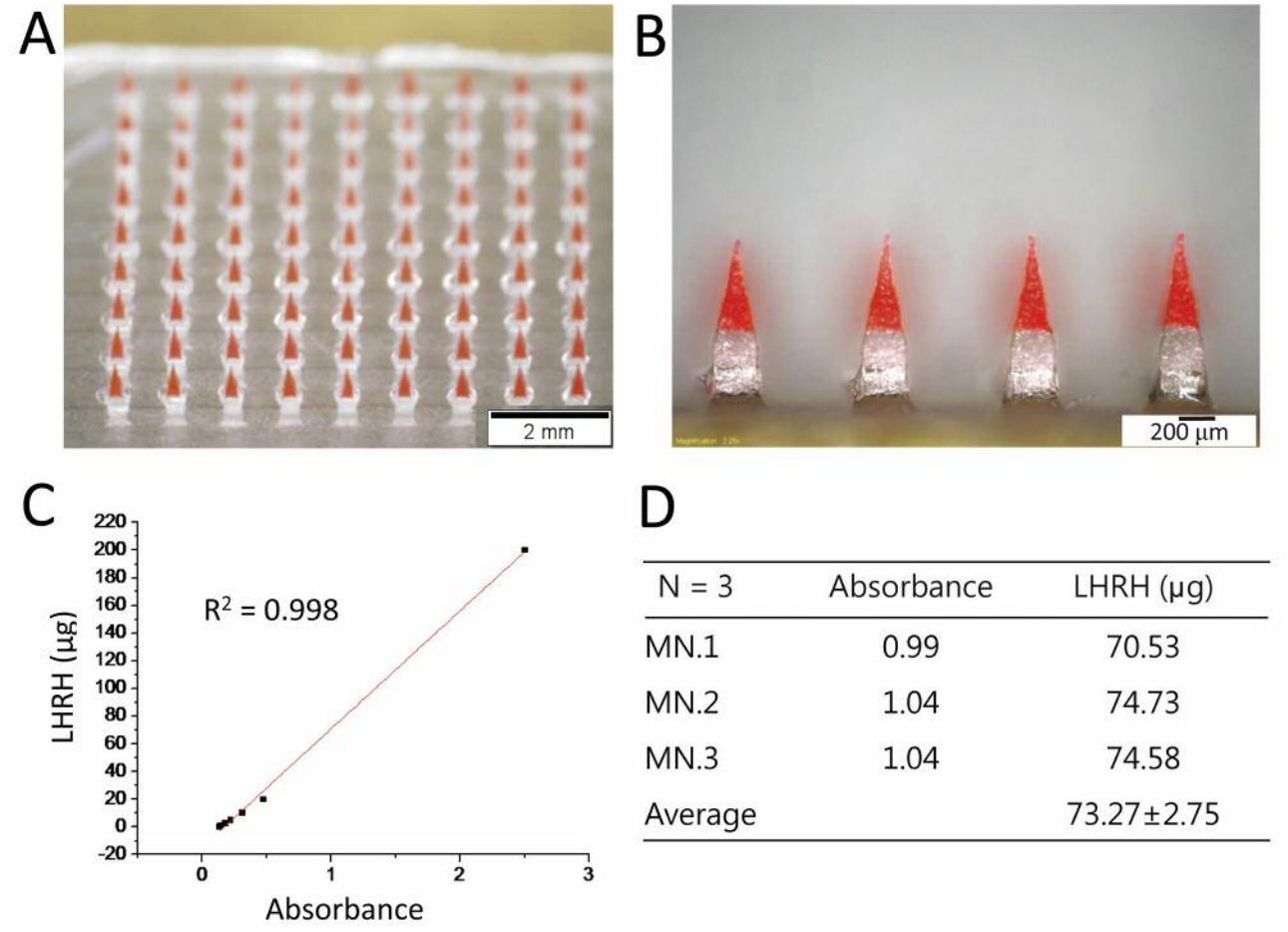

Figure 2. *Bright-field micrograph of chitosan microneedle (MN) patch containing luteinizing hormone-releasing hormone LHRH (A) and a magnified view (B). C: Standard curve for LHRH amount according to BCA kit. D: The amount of incorporated LHRH loaded into microneedles.*

experiment was repeated for three times. The data were similar among these three experiments and one of the data is shown here.

Statistical analysis. This study presents data as the mean±SD. A difference of $p<0.05$ was considered statistically significant compared to the baseline values using unpaired *t*-test.

Results

Production of a transdermal delivery system for LHRH with microneedles. This microneedle system is composed of chitosan and PVA/PVP supporting structures. Chitosan gel blends with either FITC, LHRH-TAMRA or gosrelin for subsequent studies. For research purposes, the microneedle size was reduced to fit the studied mouse model (Figure 1A and B). The produced microneedle was examined with aid of a stereomicroscope. The result showed good structural integrity of the produced microneedle (Figure 1C-1E and Figure 2A and B).

Chitosan microneedle loading with LHRH. To measure the amount of LHRH loaded by chitosan microneedles, the microneedles produced were dissolved in double-distilled water. The results showed that each microneedle patch can carry approximately 73.3±2.8 µg of LHRH (Figure 2C and D).

In vitro skin penetration test and in vivo wound healing. Histological section showed the produced microneedles are able to penetrate the epidermal and dermal layers of porcine cadaveric skin (Figure 1F). The skin recovery after microneedle insertion into ICR mice was almost complete within 7 days (Figure 3).

Biological effect of goserelin-containing chitosan microneedlesin ICR mice. For exploring the efficacy of such chitosan microneedle, we measured the changes of serum testosterone and LH levels. Before and after applying the goserelin-containing chitosan microneedles to ICR mice,

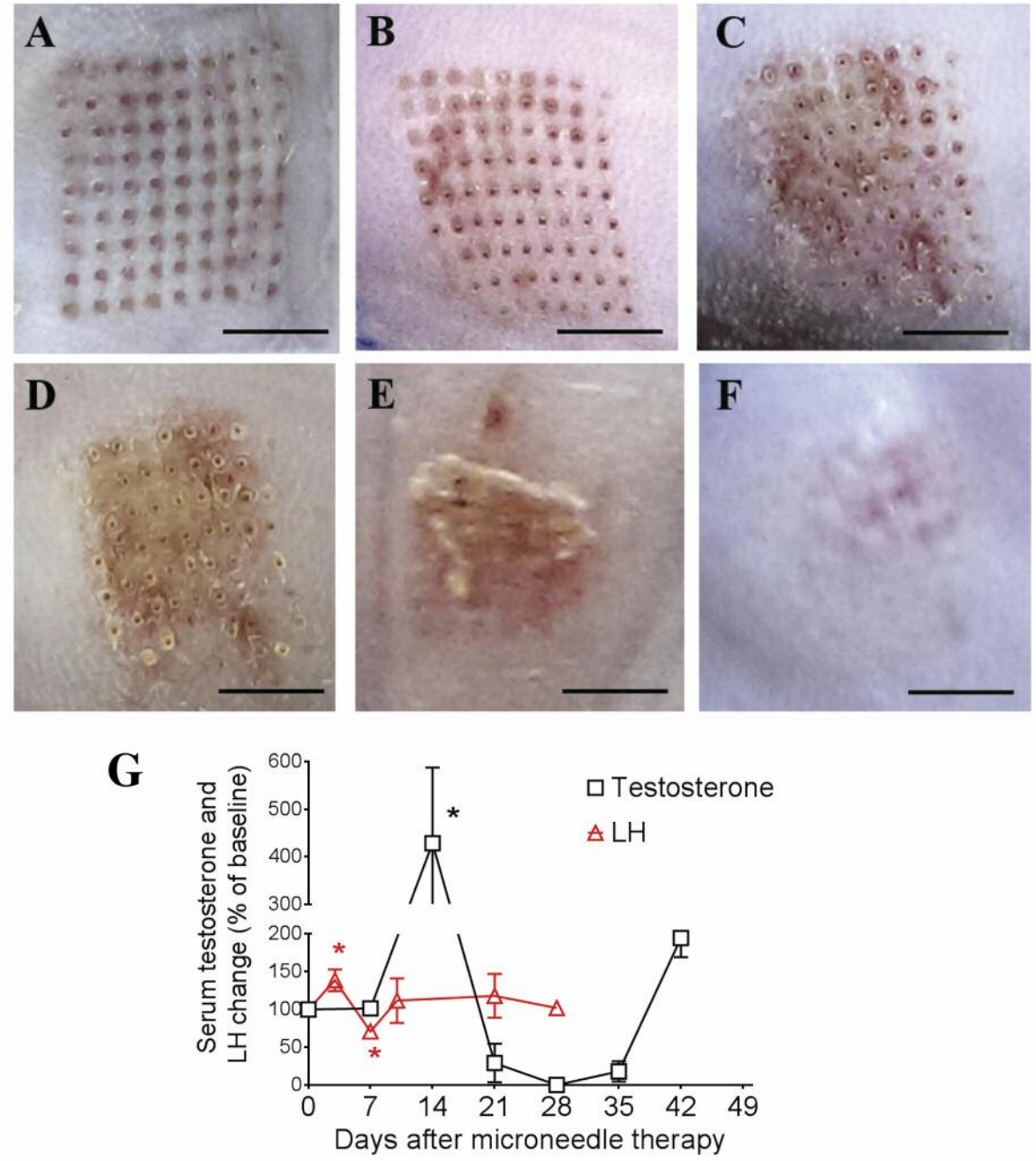

Figure 3. *A-F: Skin appearance on day 0 (A), 1 (B), 2 (C), 3 (D), 5 (E), and 7 (F), and changes of serum testosterone and luteinizing hormone (LH) after microneedle therapy in mice. G: Changes of serum testosterone and LH after goserelin-containing microneedle therapy in ICR mice (n=3). *Significantly different from baseline at p<0.05. The scale bar represents 4000 μm and the array area is 1×1 cm^2.*

serum LH level increased and declined within the first 7 days. In contrast, serum testosterone levels increased, reached a peak at day 14 and declined to a castration level at day 21. Such a castration level was maintained for 2 weeks (Figure 3G).

Discussion

In the current study, we developed a convenient tool for LHRH delivery, which is effective and safe for those requiring LHRH treatment. Such a chitosan microneedle

system successfully delivered bioactive LHRH subcutaneously in a mouse model. In the past, several efforts were made and the efficacy of LHRH therapy successfully increased. These improvements include modification of the salt, the 6th amino acid and formulation of synthetic LHRH (7, 16). Most significantly, LHRH can be made as a depot formulation and one injection of LHRH depot can produce a continuous delivery of LHRH and lead to a state of superagonist, rather than intermittent bolus administration. In a previous study, such superagonist regulatory peptides were shown to initially up-regulate LH, with maximum LH level at day 4, then down-regulation by day 8, and full suppression by day 21. Serum testosterone increased and reached a maximum at day 4, and then was down-regulated to basal values by day 8. Subcastrate values of testosterone were achieved by day 15 (17). Our data demonstrate that this chitosan microneedle array loaded with LHRH agonist is effective and feasible, with the potential for use in castration therapy for advanced and metastatic prostate cancer.

Although considered safe, injection site injury or vascular injury due to treatment with LHRH injection has been reported. These injection site injuries included mild burning pain, and the development of subcutaneous granuloma (18). Moreover, in rare cases, injection error may produce subcutaneous hematoma with hemorrhagic shock (19). Our data demonstrate that the wound from use of the array recovered quickly, and there was no obvious induration in the skin.

Several dissolving macromolecules were recently utilized to blend with bioactive protein for the production of drug-loaded microneedles. The loaded drug or protein can be released from the microneedles within several minutes after penetrating into the skin. Since the whole microneedle is composed of macromolecules, the amount loaded can be enhanced. Moreover the whole production procedure requires neither high temperature nor any organic solvents, so that the loaded protein remains bioactive. In addition, there is no need to deal with needle waste as with conventional injection because the macromolecule microneedles spontaneously dissolve within the penetration site. These macromolecules include carboxymethyl cellulose (20, 21), amylopectin (20), dextrin (22), haluronic acid (23), polyvinylpyrrolidone (24, 25) and polyvinyl alcohol (24). Chitosan is a polysaccharide macromolecule composed of both acetylated and deacetylated units of D-glucosamine, which is produced by treating chitin with alkali during various degrees of deacetylation (26). It exhibits several characteristics of biodegradability, biocompatibility, less toxicity and easy availability and has been approved by the American Food and Drug Administration as a wound dressing material (27). Since cationic chitosan can incorporate bioactive proteins at neutral pH, room temperature and in hydrophilic solution, other studies explored the potential of chitosan as a drug carrier, particularly for peptides, or proteins (28, 29). The loaded peptide or protein is slowly released *via* the imbibition and gradual degradation of the chitosan (30). Therefore, chitosan microneedles have great potential as an ideal drug delivery system.

There are some limitations to the current study. FirstIy, the number of animals studied was small. Further studies are required to determine the optimal therapy though this system. Secondly, the amount of incorporated drug may be limited. It is difficult to maintain the castrated state in humans. Thirdly, although wound healing was practically achieved within 7 days in the mouse model, this system may not be suitable for patients with bleeding tendency or immunocompromised status. Therefore, for practical application in humans, the incorporation of LHRH formulation should be investigated for the maximal loading amount in order to produce a longer castrated state. Currently, this system remains in a state of infancy and is difficult to compare with current means of ADT.

Conclusion

In the current study, we developed a transdermal delivery system for LHRH using the chitosan microneedle technique. Such a system can produce a castrated state in mice after LH surge. It represents a promising, feasible and convenient means of ADT in future.

Conflicts of Interest and Ethical Standards

None of the Authors has any conflict of interest in regard to this study. The Institutional Animal Care and Use Committee of National Cheng Kung University approved all animal protocols, and experiments were conducted according to the guidelines of the Laboratory Animal Center of National Cheng Kung University.

Acknowledgements

This study was supported by grants from Madou Sin-Lau Hospital, the Presbyterian Church in Taiwan and MOST (Grants103-2314-B-006-075- and 105-2314-B-039-048-).

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Received September 7, 2017
Revised October 10, 2017
Accepted October 12, 2017