**Proceedings of the 28th IEEE**
**EMBS Annual International Conference**
**New York City, USA, Aug 30-Sept 3, 2006**



# Delivery of Active Collagenase to Skin Using a Lorentz-Force Actuated Needle-Free Injector

N. Catherine Hogan, Brian D. Hemond, Dawn M. Wendell, Andrew J. Taberner, and Ian W. Hunter

***Abstract*—The development of new therapeutics and the desire to reduce the frequency of needle stick injuries, overcome the improper reuse of needles, improve compliance, and reduce the prohibitive cost of disposal have fueled resurgent efforts to develop alternative drug delivery methods. This report discusses the use of a controllable Lorentz-force actuated needle-free injector to deliver a blend of bacterial collagenases to the skin. Drug ejected/injected using a pre-programmed pressure vs time profile through a 100 or 200 µm orifice showed activity comparable to that observed following ejection/injection using a conventional syringe as determined by a solution based fluorescence assay and zymography. In a live animal trial, necrosis, hair loss, and minimal bruising were observed at drug injected sites regardless of the delivery mode as were changes in tissue morphology.**

## I. Introduction

SEVERAL technologies have been developed to penetrate or by pass the skin barrier [1][2]. Needle-free injectors (NFI) are of interest because they combine the advantages of both transdermal and parenteral drug delivery methods. NFIs create a high pressure jet of fluid or powder that penetrates the skin with the amount of pressure required to do so being dependent on the size of the orifice, the desired depth of delivery, the viscosity of the drug, and individual skin variation. This technology not only obviates the need for a needle to inject medications through the skin but is relatively painless, psychologically more acceptable (increasing the likelihood of compliance), fast, and carries less risk of inadvertent infection since contaminated sharps or blood do not accumulate [3].

NFIs are currently being used to deliver therapies that cannot be administered orally such as anaesthetic agents [4], insulin [5]-[8], heparin [9], growth hormone [5][6], and a variety of vaccines with emphasis on the latter being powder formulation [10]-[12]. The continued development of protein-based therapeutics, which are anticipated to represent 30% of pharmaceutical sales by 2007 [13], requires the concomitant development of more innovative delivery technologies (routes of administration together with drug formulations) and/or more controllable delivery devices. Drug delivery technologies represent a valuable cost effective life cycle management resource [14][15].

Many of the new generation of biotech- and human genome- derived pharmaceuticals are large molecules such as proteins or carbohydrates subject to exacting conditions for dissolution and efficacy [16]. While it has been suggested that the high pressure required for their delivery using NFIs could potentially damage/shear many of these molecules [13][17][18], a more recent study by Benedek et al [19] showed that 18 commercially available proteins (6 groups of proteins including small peptides, hormones, and proteolytic enzymes) could be delivered using the Iject® from Bioject with no signs of degradation or aggregation. Careful control/modulation of the pressures used for delivery, the nozzle geometry, and the formulation and solvent conditions appear critical to successful delivery of these drugs [13][19].

We have developed a highly controllable Lorentz-force actuated NFI capable of reproducibly delivering drug to a desired tissue depth [20][21]. Using a pre-programmed pressure vs time profile, we used this NFI to deliver a blend of high molecular mass bacterial collagenases to the dermis. Effects of shearing due to propulsion through a small orifice under high pressure and penetration of skin are discussed in addition to the pharmacodynamics of the drug when administered using the NFI.

## II. Materials and Methods

### A. Experimental Protocol

A single castrated modern Suffolk lamb was used to determine drug efficacy following delivery using the NFI. The experimental protocols were approved by the IACUC at MIT and were conducted in accordance with the NIH Guide for the Use and Care of Laboratory Animals.

On the day of the experiment, the 10 week old animal was weighed, and then anaesthetized using a 1:1 mixture of ketamine (5.5 mg/kg):diazepam (0.28 mg/kg) given intravenously. The animal was intubated with a cuffed endotracheal tube, repositioned on its right side, and a

Manuscript received April 3, 2006. This work was supported in part by Norwood Abbey Limited, Victoria, Australia

N.C. Hogan is a Research Associate in the BioInstrumentation Laboratory at Massachusetts Institute of Technology, Cambridge, MA 02139 (phone: 617 324-6107, e-mail: hog@mit.edu).

B.D. Hemond is a doctoral student in the Mechanical Engineering Department, Massachusetts Institute of Technology, Cambridge, MA 02139 (e-mail: bhemond@mit.edu).

D.M. Wendell is a doctoral candidate in the Mechanical Engineering Department, Massachusetts Institute of Technology, Cambridge, MA 02139 (e-mail: dawn@mit.edu).

A.J. Taberner is a Research Scientist in the BioInstrumentation Laboratory at Massachusetts Institute of Technology, Cambridge, MA 02139 (e-mail: taberner@mit.edu).

I.W. Hunter is the Hatsopoulos Professor of Mechanical Engineering and Director of the BioInstrumentation Laboratory at Massachusetts Institute of Technology, Cambridge, MA 02139 (e-mail: ihunter@mit.edu).

stomach tube passed into the rumen. During the procedure the wether was given isoflurane (1-4% in balance oxygen).

While anaesthetized, the NFI was used to deliver 100 uL of a 10.5 U/mL solution of collagenase/0.1 mg/mL epinephrine (Cat.#'s C7926 and E4375, Sigma-Aldrich, St. Louis, MO) to the dermis of the skin following a 20 mm x 20 mm grid with each injection spaced at 10 mm for a total of nine injections. Nine 10 mm spaced injections of buffer (1x RB; 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.2 mM sodium azide, pH7.6) were delivered using the NFI to a similar grid (control) spaced approximately 35 mm distant from the protein grid. In addition, two sets of three sites with each site spaced 10 mm distant from the other were used to deliver buffer (negative control) or collagenase (positive control) using a conventional syringe (27.5 gauge needle).

Post injection, the animal was placed in sternal recumbancy and allowed to recover from anaesthesia. The oral gastric tube and endotracheal tube were removed and the wether was monitored for 64 days to determine if the treatment had any effect on health, disposition, or resulted in changes in tissue morphology at injection sites.

This procedure was repeated on the right mid-side of the animal at 25 weeks. Monitoring, biopsy acquisition, biochemical and histological analyses were as described for the left mid-side.

### *B. Tissue extraction*

Tissue plugs were obtained from post mortem lamb skin or from skin biopsies. In the latter case, nine to fourteen days post injection, biopsies were obtained at two sites within each injection grid. The animal was anaesthetized, the hair at the injection sites carefully clipped to avoid disruption of scabbing, and biopsies acquired using a 6 mm dermal biopsy punch (Miltex Inc., York, PA) under aseptic conditions. The biopsied tissue was immediately frozen in liquid $N_2$. Biopsied sites were stapled and cleansed with betadine.

For biochemical analyses, tissue plugs were allowed to thaw on ice in 2 mL of homogenization buffer [22]. The tissue was homogenized using 15 – 10s bursts of a PT1200C polytron (Kinematica AG, Lucern , SZ) at maximum power. The homogenate was transferred to a clean tube and tissue remaining in the original tube re-homogenized. The combined homogenates were each briefly centrifuged and the supernatants used for assays of soluble protein concentration, enzyme activity, and zymography.

### *C. EnzCheck solution-based fluorescent assay*

Collagenase activity was assayed using the EnzCheck Assay (Invitrogen Corp) with minor modifications. A small volume (10.0 uL) of tissue extract (concentrate or serially diluted in 1x RB) was transferred to replicate wells of a 384 well plate. 8.0 uL of 1x RB was added to each well followed by 2.0 uL of 100 µg/mL gelatin (or collagen I or IV) fluorescein conjugate. Fluorescence was determined over time using a SPECTRA MAX GeminiXS spectrofluorometer. Extract from post mortem plugs or biopsies obtained from skin injected with 1x RB served as negative controls for each delivery mode.

To demonstrate that increased fluorescence was due to cleavage of the substrate by exogenous collagenase, 1x RB was replaced with serial dilutions of the metal chelator 1, 10 phenanthroline. Chelator was incubated for 10 min with the tissue extract or serial dilutions of collagenase prior to the addition of substrate. Proteolytic activity was measured as a change in fluorescence at 535 nm.

A relative measure of collagenase activity was interpolated from a standard curve (U/mL enzyme detected/U/mg enzyme = mg of protein/mL) generated using serial dilutions of the collagenase used for injections, a fixed substrate concentration, and incubation time.

### *D. Zymography*

SDS polyacrylamide gels (10%) containing 0.1% gelatin (BioRad Laboratories, Inc., Richmond, CA) were loaded with stock collagenase or collagenase extracted from tissue biopsies post injection. Samples were mixed 1:2 with sample loading buffer (62.5 mM Tris-HCl, pH6.8, 4% SDS, 25% glycerol, 0.01% bromophenol blue) in the absence of reducing agent or heat. Each well was loaded with 30 uL of sample and gels were run at 100 V for 1 h. Following electrophoresis, gels were incubated in two changes of 2.5% Triton X-100 with gentle shaking for 30 min to displace the SDS and allow proteins to renature. The gels were then rinsed and incubated overnight at 37°C in substrate buffer (50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, pH7.5) with or without phenanthroline. Post incubation, the gels were stained for a minimum of 1 h with 0.5% Coomassie Blue R-250, 40% Me0H, and 10% acetic acid followed by destaining in 40% Me0H, 10% acetic acid until clear bands were visualized against a blue background.

### *E. Histochemistry*

Biopsies were fixed overnight in 10% neutral buffered formalin. The tissue was trimmed, placed in a cassette, and dehydrated after which it was infiltrated with paraffin for 6 h. Infiltrated tissue was positioned in an embedding tray and embedded with paraffin. Microtomed 5 µm sections were floated onto microscope slides, dried overnight at 60°C, deparaffinized, re-hydrated, and then stained with Masson's trichrome or hematoxylin-eosin.

## III. Results and Discussion

The concept of therapeutic enzymes is not new and many enzyme drugs have been developed for a wide range of disorders [23]. More specifically, collagenase has been used as a therapeutic drug in wound/burn debridement [24][25] and is under investigation in the US for the treatment of Dupuytren's and Peyronie's Disease [23].

### *A. Stability and activity of drug ejected using the NFI*

The collagenase used in these studies has been shown to

be relatively stable for prolonged periods (16 h) at room temperature in the presence of $Ca^{2+}$ and after several rounds (100x) of re-pressurization (<8% loss). In order to determine whether the molecular integrity of the drug was affected by ejection through a narrow orifice, the NFI was used to eject drug through a 100 or 200 µm orifice into a test tube. Post ejection drug activity was assessed using the EnzCheck assay and zymography and compared to the activity associated with the pre-ejected drug (Fig. 1).

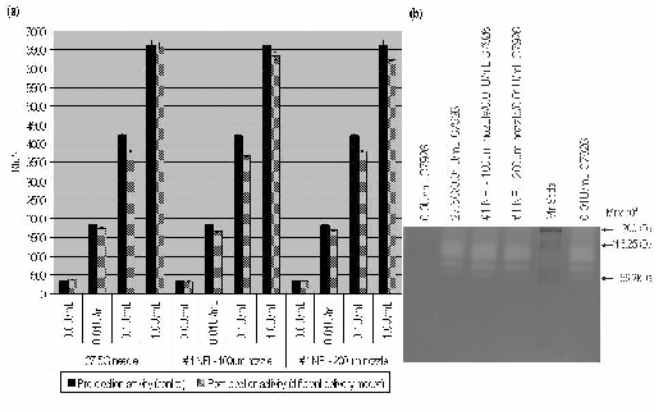

Fig. 1. Drug activity detected in post-ejected samples. (a) Dilutions of drug ejected from the NFI were aliquoted into duplicate wells of a 384 well plate and assayed using 100 µg/mL collagen type IV fluorescein conjugate. Drug ejected using a 27.5G needle served as a positive control while ejection of 1x RB using either delivery mode served as a negative control. Pre-ejected concentrations were used as references. (b) Gelatin zymogram comparing enzyme activity in the ejected samples to that in non-ejected samples. Molecular weight standards provided markers for $M_r$ determination.

Drug ejected through a 100 or 200 µm nozzle had activity comparable to that of drug ejected using a 27.5 gauge needle (Fig. 1a). Regardless of delivery mode, maximal loss in activity (<14%) when compared to the pre-ejected sample, was observed at a dilution of 0.1 U/mL which may reflect the steepest point in the standard curve.

The proteolytic activity observed in the zymogram agreed with the assay results. The number of bands, their relative molecular masses (~68 to 115 kDa) and intensities were comparable in samples containing enzyme alone or enzyme ejected from a 27.5 gauge needle or the NFI (Fig. 1b).

Having demonstrated that forcing drug through a narrow orifice under high pressure had marginal effect on activity, we then assessed the effect of tissue penetration on drug activity. Hemond et al [14] demonstrated that the depth and volume of drug delivered to the tissue can be determined by a pre-programmed waveform or pressure profile. By modifying the input waveform file, Wendall et al [15] created a variety of pressure profiles with a 2 to 3 ms "peak" pressure of 20 to 60 MPa followed by a 20 to 30 ms "follow-through" pressure of 10 to 30 MPa. A single waveform shown to reproducibly deliver dye to the dermis was used in each of these studies.

Collagenase activity detected in protein extracted from post mortem tissue injected with drug using the NFI or a 27.5 gauge needle was compared to that detected in tissue injected with 1x RB. Drug or buffer ejected into test tubes served as the device control (Fig. 2).

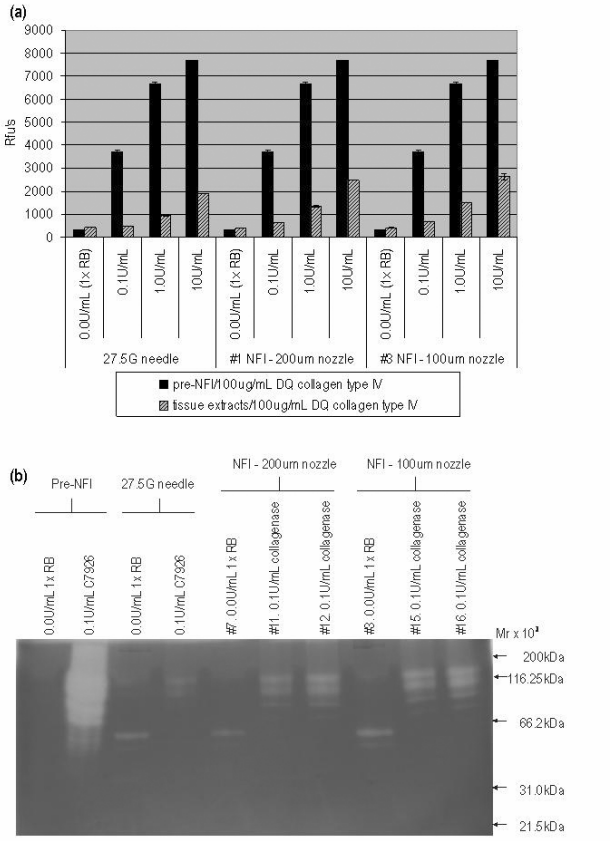


Fig. 2. Drug activity detected in 6 mm plugs obtained from post mortem lamb skin. (a) 10 uL of concentrated or serially diluted crude protein extracts from tissue plugs obtained from skin samples injected with C7926 were each aliquoted into four wells of a 384 well plate and activity assayed using 100 µg/mL collagen type IV fluorescein conjugate. Crude extract from plugs taken from tissue injected with drug using a 27.5G needle served as a positive control. Crude extract from skin plugs injected with 1x RB served as negative controls for each delivery mode. B. Gelatin zymograms showing enzyme activity in crude extracts used in (a). 1x RB (0.0 U/mL) or 0.1 U/mL C7926 used as injectate (Pre-NFI) served as negative and positive controls respectively.

The significant difference in activity, as determined by the assay, between non-injected but ejected drug (Pre-NFI) and drug observed in tissue extracts probably reflects the inherent difficulties associated with extracting proteins from skin and not degradation of the enzyme as bacterial collagenases ranging in Mr from 68-115 kDa were observed on zymograms (Fig. 2b) and well resolved high Mr proteins were observed on Coomassie Blue stained gels (not shown). Activity associated with extracts from NFI injected tissue was comparable to, if not better than, that observed in extracts from tissue injected using a conventional syringe. Device controls corroborated results shown in Fig. 1a.

Analysis of these samples using zymography confirmed the enzyme activity observed in the fluorescence based assay. It also suggested the presence of endogenous enzyme

activity in the negative control samples. That this activity is metalloproteinase in nature is indicated by its inhibition in the presence of 4 mM 1, 10 phenanthroline (not shown). It is of interest to note that this activity was not observed in extracts from skin injected with collagenase possibly inferring that it is not a result of degradation of a larger protein common to all extracts. However, without further analyses, it could also be argued that the protein concentration is below the detection limit of the assay. That it is not a contaminant in the 1x RB or collagenase itself is suggested by its absence in the pre-NFI controls.

*B. Pharmacodynamic Effect*

To verify drug efficacy, the NFI was used to deliver collagenase to the mid-side of a wether.

*B1. Physical evidence for drug activity post-injection*

The age of the animal at the time of the experiment appeared to influence drug efficacy independent of the delivery mode. Irritation resulting from injection using the NFI was observed in only two of 18 sites in the 10 week old animal. No wet injections, bleeding, or bruising were associated with delivery. Within nine days of the injections, significant hair loss and scabbing were observed in the areas injected with drug regardless of the delivery mode. No scabbing or hair loss was observed in either of the negative control grids. At this time, biopsies of the injection sites were acquired (two biopsies/grid).

At 21 days, sites injected with collagenase, whether using a 27.5 gauge needle or the NFI, showed signs of additional hair loss/bruising and were taking longer to heal than the negative controls. That the hair loss/bruising was not due to the acquisition of biopsies was evident from its absence in the negative controls. By day 43, negative control grids were difficult to distinguish from areas that had not been treated while test grids were characterized by significant hair loss regardless of delivery mode. By day 64, both the NFI and 27.5 gauge needle test grids, while healed, showed minimal to no hair re-growth.

Replication of this experiment on the same animal at 25 weeks resulted in a more muted response. Fourteen days post injection hair loss and minimal scabbing were observed in areas injected with drug using either the NFI or 27.5 gauge needle. Skin eruptions and scabbing at the sites of drug injection became evident approximately three weeks post injection. The general delay in response may reflect the age of the animal and/or slight differences in the amount of drug delivered. The use of a lower peak pressure (20 MPa) may have resulted in shallower penetration and a corresponding increase in wet injections. As with the first trial, injection of buffer using either delivery mode did not result in hair loss and/or skin eruptions and bruising.

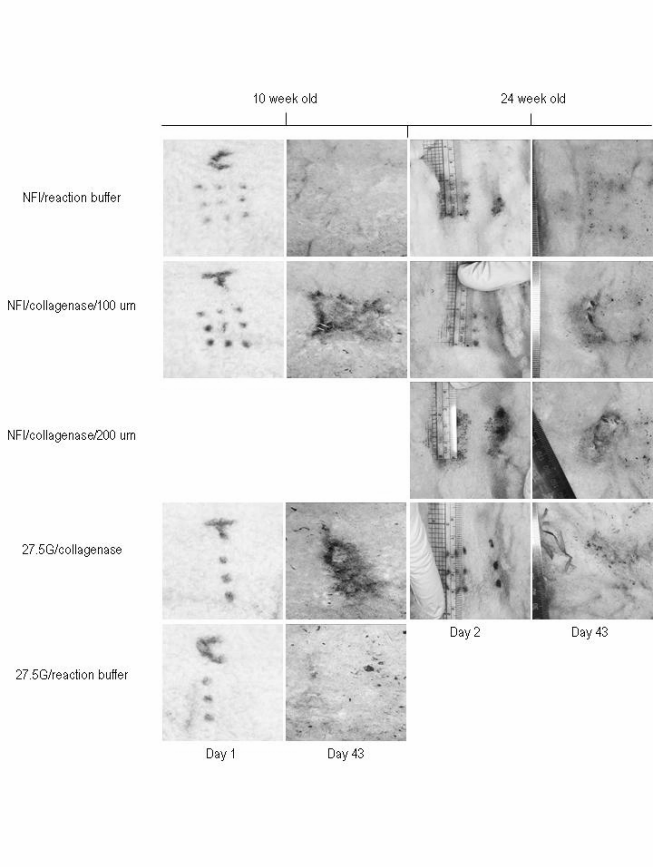


Fig. 3. Photographs of physical changes observed in the wether's skin as a result of injecting a blend of bacterial collagenases or 1x RB using the NFI or a conventional syringe with a 27.5 gauge needle.

*B2. Biochemical evidence for drug activity post-injection*

Biopsies were assayed for enzyme activity using both the EnzCheck assay and zymography (Fig. 4). The activity detected using the fluorescent assay was clearly influenced by the choice of substrate (Fig. 4a). Marginal to no activity, relative to the activity detected in the absence of enzyme given a specific substrate, was observed in crude extracts from biopsied tissue that had been injected with 1x RB (negative controls). Similarly, biopsies from sites injected with collagenase using the 27.5 gauge needle showed marginal to no activity when collagen type IV or type I were used as substrate. However, activity was observed when gelatin was used as the substrate.

Crude extracts from biopsied tissue injected with collagenase using the NFI showed marginal activity when collagen type IV was used as substrate, moderate activity with collagen type I, and considerable activity with gelatin. In fact, the drug activity detected in NFI injected tissue extracts was almost 10 x greater than that observed in tissue injected using the 27.5 gauge needle.

As noted earlier, the activity observed in post-NFI injected tissue extracts, when gelatin was used as a substrate was less than that observed in pre-NFI injected sample and most probably reflects loss due to extraction as discussed.

The gelatin zymogram confirmed the differential level of

enzyme activity observed in the fluorescence based assay (Fig. 4b). It also suggested the presence of endogenous enzyme activity in all tissue extracted samples. That this activity is more enhanced in the crude extracts from biopsied skin injected with collagenase suggests that injection of the collagenase may be causing an increase in putative endogenous MMP activity. This increased activity may be related to the wound response. That the base activity is not due to the properties of the injectate or the delivery mode is suggested by the fact that it is present in all tissue derived samples regardless of the injectate or mode of delivery and absent in non tissue derived samples (C7926 or *Clostridium histolyticum* lanes). These latter samples contain bacterial collagenases only. Antibodies may prove useful in differentiating these MMPs from the injected collagenases.

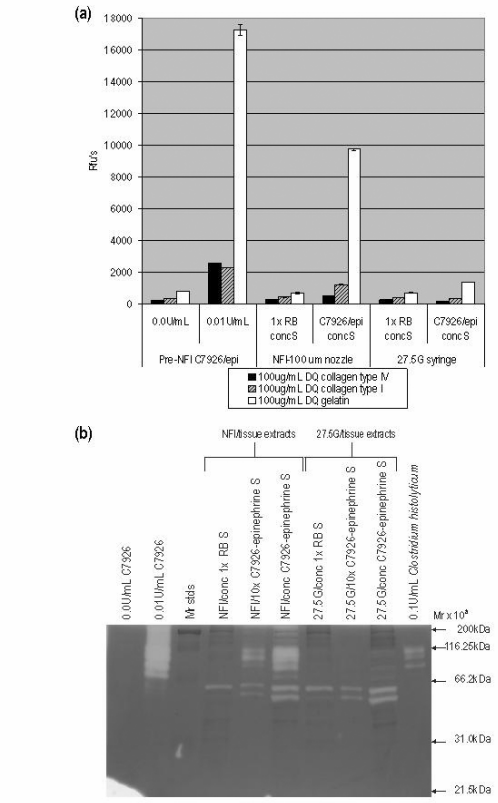


Fig. 4. Bacterial collagenase (C7926) activity in protein extracts from biopsies obtained from the mid-side of a wether 7 weeks post injection. (a) EnzCheck assay using collagen type IV-, collagen type I-, or gelatin-fluorescein conjugate as substrate. (b) Gelatin zymogram showing enzyme activity in each of the extracts in (a).

Comparable results, albeit not as pronounced, were observed in the second trial (data not shown).

*B3. Histological evidence for drug activity*

Clear differences were observed in the trichrome stained sections from the negative controls and the positive control and NFI injected tissue (Fig. 5). Biopsies from the collagenase injected skin sites showed marked thickening of the follicular bulbs and disruption of the hair follicles, scabbing (consistent with the physical observations), expansion of disorganized collagen in the deep dermis, and reactive proliferation of collagen.

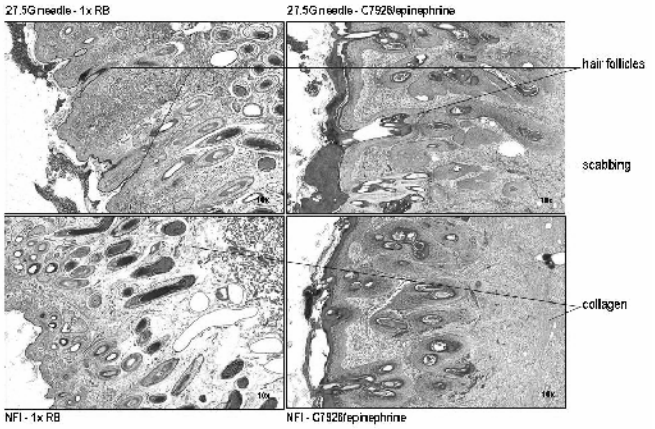


Fig. 5. Photomicrographs of 5 μm lamb skin paraffin sections stained with Masson's trichrome. Panels on the left represent biopsies from sites on the skin injected with 1x RB (control) while panels on the right represent biopsies from skin sites injected with collagenase.

While sections from sites injected with collagenase using a 27.5 gauge needle exhibited greater disorganization than did corresponding sections injected using the NFI, it is important to note that these sections represent only a single biopsy. In addition, the more pronounced scabbing in the positive control sample may reflect a subtle difference in skin morphology between the rib and the area posterior to the rib (consistent with the histological differences observed in the negative controls even though the overall cellular morphology was similar), some loss of collagenase activity when it penetrated the tissue, or differential turnover of the drug given the delivery mode/location. If location/morphology affects absorption, it is possible that the more pronounced scabbing and lower activity in the assay but increased disorganization in the histological section associated with the grid/biopsy injected using a conventional syringe may reflect the phase of wound healing [17][18].

## IV. CONCLUSION

This report demonstrates use of a novel Lorentz-force actuated NFI to deliver active collagenase to the skin. More specifically, we have shown that drug delivered through a nozzle orifice of 100 or 200 μm retains activity comparable to that observed with a conventional syringe. Approximately 86% of drug activity is retained regardless of the delivery mode when compared to pre-ejected drug. While it is more difficult to determine the change in activity when assaying tissue extracts from skin injected with collagenase because of losses due to the extraction procedure, we can conclude that the level of activity retained is as high, if not better, than that observed using a conventional syringe.

Our pharmacodynamic studies indicate that the drug delivered using the NFI is active post injection. Necrosis and hair loss are observed at sites injected with collagenase using either the NFI or a conventional syringe. Bacterial collagenases, as defined by relative molecular mass, are only observed in extracts from biopsies where tissue was injected with the collagenase blend. The proposed induction of endogenous collagenase in response to wounding needs to be further characterized. However the changes in tissue morphology in sections from collagenase injected tissue are characteristic of the wound response [26][27]. Moreover, these changes which occur in both tissue injected using the NFI or a conventional syringe are comparable.

## ACKNOWLEDGMENT

N.C. Hogan would like to thank both the Comparative Pathology Laboratory and members of the Division of Comparative Medicine Animal Resource Program at MIT for their guidance and training.

## REFERENCES

[1] Bogner, R.H., and M.F. Wilkosz, "Transdermal Drug Delivery – PART 2; Upcoming Developments," U.S Pharmacist 28:05, 2003. Available: http://www.uspharmacist.com
[2] Wilkosz, M.F., and R.H. Bogner, "Transdermal Drug Delivery – PART 1: CURRENT STATUS," U.S. Pharmacist: A Jobson Publication 28:04.,2003. Availabe: http://www.uspharmacist.com
[3] www.cdc.gov/niosh/2000-108.html#5
[4] Corgentech Inc. Available: http://www1.corgentech.com
[5] Bioject Needle-free Injection Systems. Available: http://www.bioject.com
[6] Antares Pharma, Inc. Available: http://www.antarespharma.com
[7] Medical House Products Ltd. Available: http://www.insulinjet.com
[8] Pradesh, A., "Needle-free Insulin device developed," The Hindu, Jan 03, 2005. Available: http://www.shreya.co.in
[9] Romanowski P., "Needle-free injection system," How Products Are Made: Volume 6, Needle-free Injection System forum. Available: www.makehow.com
[10] Chen, D., and L.G. Payne, "Targeting epidermal Langerhans cells by epidermal powder immunization," Cell Res 12: 97-104, 2002.
[11] Chiron Corp 2006. Available: http://www.chiron.com
[12] Osorio, J.E., C.L.Zuleger, M.Burger, Q. Chu, L.G. Payne, and D. Chen, "Immune responses to hepatitis B surface antgen following epidermal powder immunization, "Immunology and Cell Biology 81: 52-58, 2003.
[13] Sinha, V.R., A. Trehan, and P. Tiwari, "Needle free injection technology," Technology Trendz, June 02, 2005. Available: http://www.expresspharmapulse.com/20050602/technologytrendz01.shtml
[14] Baichwal, A., and D.A. Neville, "Adding value to product's life-cycle management: Product enhancement through drug delivery systems," Drug Delivery Technology 1: 1-3, 2001.
[15] Harrison, R.G., "Needle-free drug delivery technology," Drug Delivery & Formulation: Innovations in Pharmaceutical Technology.
[16] Hitchens, K., "New technology is spawning novel ways to deliver drugs, from orals and IVs to patches and implants," Drug Topics 2003.
[17] Sadowski, P.L, D.M. DeBoer, C.L. Berman, P.L. Lesch, Jr., and M.L. Holland, "Needle assisted jet injector," US Patent No. 6746429, 2004.
[18] Gross, M., and R. Jaenicke, "Proteins under pressure: The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes," Eur. J. Biochem. 221: 617-630, 1994.
[19] Benedek, K., E. Walker, L.A. Doshier, and R. Stout, "Studies on the use of needle-free injection device on proteins," J Chromatogr A., 1079: 397-407. 2005.
[20] Hemond, B.D, D.M. Wendell, C. Hogan, A.J. Taberner, I.W. Hunter, "A Lorentz-Force Actuated Autoloading Needle-free Injector," Proceedings of the 28th Annual International Conference of the IEEE EMBS, 2006.
[21] Wendell, D.M., "The Effect of Jet Parameters on Jet Injection," Proceedings of the 28th Annual International Conference of the IEEE EMBS, 2006.
[22] Ahtikoski, A.M., E-M. Riso, S.O.A. Koskinen, J. Risteli, T.E.S.Takala, "Regulation of type IV collagen gene expression and degradation is fast and slow muscles during dexamethasone treatment and exercise," Pflugers Arch. – Eur. J. Physiol. 448: 123-130, 2004.
[23] Vellard, M., "The enzyme as drug: application of enzymes as pharmaceuticals," Curr. Opin. Biotech. 14: 444-450, 2003.
[24] Ozcan, C., O. Ergun, A. Celik, N. Corduk, G. Ozok, "Enaymatic debridement of burn wound with collagenase in children with partial-thickness burns," Burns 28: 791-794, 2002.
[25] Falanga, V., "Wound bed preparation and the role of enzymes: A case for multiple action of therapeutic agents," Wounds 14: 47-57, 2002. Available: http://www.medscape.com/viewarticle/430899
[26] Keast, D., and H. Orsted, "The basic principles of wound healing." Available: http://www.pilonidal.org/pdfs/Principles-of -Wound-Healing.pdf
[27] Cockbill, S., "Wounds: The healing process," Hospital Pharmacist 9: 255-260, 2002. Available: http://www.pjonline.com/pdf/hp/200210/hp_200210_wounds_healing.pdf