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ORIGINAL ARTICLE

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Immune profile of hyaluronic acid hydrogel polyethylene glycol crosslinked: An in vitro evaluation in human polymorphonuclear leukocytes

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Abstract

Neauvia hydrogel (N-Gel) is a hyaluronic acid-based dermal filler, cross-linked with polyethylene glycol. This filler contains sodium hyaluronate at different concentrations, poly(ethylene glycol) diglycidyl ether cross-linked, glycine, and L-prolyne. Assessing any effects of N-Gel on immunity and inflammation is of crucial importance. The aim of the study was to characterize the ability of N-Gel to modulate human polymorphonuclear leukocyte (PMN) functions, including migration, oxidative metabolism, and production of proinflammatory mediators. N-Gel was tested on isolated human PMN. Spontaneous and N-formylmethionyl-leucyl-phenylalanine (fMLP)-stimulated migration were examined using the Boyden Chamber technique, whereas the oxidative metabolism was assessed through spectrofluorometric measurement of reactive oxygen species (ROS) production under resting conditions and after stimulation with fMLP. Tumor necrosis factor (TNF)-α and interleukin (IL)-8 mRNA levels were measured by real-time PCR after stimulation with fMLP or *Escherichia coli* lipopolysaccharide. This study showed that N-Gel reduced fMLP-induced migration and ROS production without affecting these functions in resting cells. In addition, incubation of PMN with N-Gel effectively reduced both TNF-α and IL-8 mRNA levels. N-Gel modulates critical functions of human PMN such as migration and oxidative metabolism, indicating its potential as an anti-inflammatory agent.

KEYWORDS

cell migration, interleukin-8, Neauvia hydrogel, polymorphonuclear leukocytes, reactive oxygen species, tumor necrosis factor-α

1 | INTRODUCTION

Glycosaminoglycans of the extracellular matrix can link chemokines and cytokines thus modulating the activation and migration of immune and inflammatory cells.[1,2] Glycosaminoglycans-based engineered materials could therefore provide major therapeutic benefit mitigating chronic inflammation, such as in wounds and lesions,[3] due to their potential to modulate immune and inflammatory processes. The tested Neauvia hydrogel contains sodium hyaluronate at a concentration of 26 mg/mL, poly(ethylene glycol) diglycidyl ether cross-linked, glycine, and L-prolyne. N-Gel is used as a dermal filler for the correction of congenital or acquired soft tissue deficits. Rheology, chemical property, biocompatibility, and bio-integration of N-Gel were previously assessed[4-10]; however, so far its effect on immunity and inflammation has never been examined.

Polymorphonuclear leukocytes (PMNs) are crucial players in the immunoinflammatory response. PMNs are the first cells involved in tissue defense against invading microorganisms[11] being able to detect

and respond to bacterial by-products as well as to endogenous cytokines and chemokines.[12] PMNs migrate to inflamed tissues, where they produce and release proinflammatory and antimicrobial mediators, as well as oxidative metabolites such as reactive oxygen species (ROS). While resolution of inflammation leads to tissue repair, chronicization of the process may result in tissue damage.[13] Testing the effects of chemical compounds on PMN function therefore provides useful information to estimate the potential impact of these compounds on the local and systemic inflammatory responses involved in tissue homeostasis and in many immunoinflammatory diseases.[14,15]

The aim of the present study was to characterize the ability of N-Gel to modulate human PMN functions, including migration, oxidative metabolism as well as the expression of tumor necrosis factor (TNF)-α and interleukin (IL)-8.

2 | METHODS

2.1 | Product and preparation

N-Gel is composed of HA that is polyethylene glycol diglycidyl ether (PEGDE) cross-linked to obtain a 3D hydrogel matrix.[5-7,9] N-Gel can be considered completely biocompatible and degradable hydrogel. The chemical properties and techniques used to obtain N-Gel are reported in the literature.[4,7,8,10,16]

2.2 | Hydration process

N-Gel was fully hydrated by incubation with Roswell Park Memorial Institute medium (RPMI). Briefly, 0.2 mL of gel were hydrated with 4.8 mL of RPMI at 37°C for 30 minutes, followed by centrifugation at 1400*g* for 10 minutes; finally, the excess supernatant was removed and gel was used for subsequent dilutions. To test the direct effects of N-Gel on cell functions, we used the filler at the concentrations of 0.5 mg/mL and 1.0 mg/mL, respectively.

2.3 | Dilution protocol

The concentration of hyaluronic acid in N-Gel is about 26 mg/mL. To obtain a N-Gel concentration of 0.5 mg/mL, we placed 25 mg of gel directly into a sterile tube (50 mL) added with 50 mL of sterile RPMI, while to obtain a N-Gel concentration of 1 mg/mL, we placed 50 mg of gel directly into a sterile tube (50 mL) added with 50 mL of sterile RPMI. The tubes were then placed on the magnetic agitator for 15 minutes. The final product was stored at 4°C. Before the use, gel was warmed at 37°C.

2.4 | PMN isolation and culture

PMNs were isolated standard density-gradient centrifugation from buffy coats from the local blood bank (Ospedale di Circolo, Fondazione Macchi, Varese, Italy) as previously described.[17] Briefly, blood was allowed to sediment on dextran at 37°C and after 45 minutes incubation, supernatant was recovered and PMN were isolated by Ficoll-Paque Plus (GE Healthcare Uppsala, Sweden) density-gradient centrifugation.

The cell suspension was deprived from erythrocytes by hypotonic lysis (10 minutes incubation in distilled water with added [g/L]: 8.25 NH_4Cl, 1.00 $KHCO_3$, 0.04 EDTA). Cells were then washed three times in 0.15 M NaCl. The cells were subsequently used for the functional studies only in the case that purity and viability were always ≥95% and no platelets or erythrocytes could be detected by either light microscopic examination or flow cytometric analysis (morphological parameters, side-scatter [SSC] and forward-scatter [FSC]).

Finally, the isolated PMN were cultured in RPMI 1640 medium (Euroclone S.p.A., Milan, Italy) at 37°C under resting or activated conditions with N-formylmethionyl-leucyl-phenylalanine (fMLP) 0.1 μM or lipopolysaccharide (LPS) 1 μg/mL (Sigma, Milan, Italy), as appropriate and, as below described, for different times according to the specific experimental procedures.

2.5 | Cytotoxicity assay

N-Gel cytotoxicity was assessed on PMN by means of the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] reduction method.[15] Briefly, freshly isolated PMNs were resuspended (1 × 106 cells/mL) in RPMI 1640 medium added with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were then seeded in duplicate in a 96-well round bottom plate and cultured for 24 hours alone or in the presence of N-Gel (37°C in 5% CO2 atmosphere). The absorbance was measured using a microplate spectrophotometer with a 570 nm test wavelength and a 690 nm reference wavelength and finally the results were expressed as mean OD value of duplicates.

2.6 | Cell migration

PMN migration was measured according to the Boyden chamber assay as previously described.[17] PMNs were resuspended at 1×10^6/mL in RPMI medium with 0.5% BSA or in N-Gel diluted with medium as before described. The chemotactic peptide fMLP (0.1 μM) was used to activate PMN in both conditions and was placed in the bottom compartment of the chamber; finally, a 3-mm-pore cellulose nitrate filter (Millipore Corporation, Bedford, Massachusetts) was placed between the two compartments. After an incubation period of 90 minutes at 37°C, the filter was recovered, dehydrated, fixed, and finally stained with hematoxylin/eosin. PMN migration (in the different conditions used) was measured and quantified microscopically measuring the distance (measured in μm) from the surface of the filter to the leading front of cells and finally expressed as distance of migration (measured in μm).

2.7 | ROS generation

The redox sensitive dye C-DCDHF-DA (Molecular Probes, Eugene, Oregon) was used to measure the intracellular ROS levels according to our previous study.[17] To this end, 1×10^6 cells/mL were resuspended in HBSS/HEPES medium or in HBSS/HEPES plus N-Gel (0.5 mg/mL) medium and incubated for 1 hour with 2 μmol/L C-DCFH-DA at 37°C in the dark. Cells were then washed twice with HBSS/HEPES, centrifuged (400 *g*, 20°C, 5 minutes) and fluorescence was detected by means of a spectrofluorometer (Perkin-Elmer LS-50B, Perkin-Elmer Instruments, Bridgeport, Connecticut; [excitation wavelength: 488 nm, fluorescence emission: 525 nm]). ROS levels were assayed under resting conditions and after activation with fMLP (0.1 μM).

ROS generation was monitored up to 30 minutes or 60 minutes and finally expressed as difference (Δ) between resting values measured at 60 seconds and levels measured after 30 (or 60) min monitoring or as area under the curve (AUC) of the whole intervals of measurement (after the first resting 60 seconds, the 29 [or 59] min analysis).

2.8 | Cell culture for TNF alpha and IL-8 measurement

TNF alpha and IL-8 were measured in cultured PMN at the concentration of 10×10^6 cells/mL in RPMI 1640 alone or in the presence of N-Gel (0.5 mg/mL) alone (resting) or together with fMLP (0.1 μM) or LPS (1 μg/mL) at 37°C for 1 hour. Thereafter, cells were centrifuged at 1400 *g*, 5 minutes, 20°C, and pellets and supernatants were collected and stored at −80°C until time for analysis.

2.9 | RNA isolation and real-time polymerase Chain Reaction for TNF alpha and IL-8 mRNA expression

Total mRNA was extracted from 1×10^6 cells by PerfectPure RNA Cell Kit (5 Prime GmbH, Hamburg, Germany); the amount and the quality of RNA extracted were estimated by spectrophotometry (260/280 nm ratio). Finally, the RNA was reverse transcribed using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions and Real-time PCR was performed (StepOne System; Applied Biosystems) using assay-on-demand kits. Gene expression level was represented as Δ Ct = [Ct (sample) - Ct (housekeeping gene)] and the relative expression was determined by normalization to 18S cDNA (analyzed by StepOne software 2.2.2—Applied Biosystems).

2.10 | Statistical analysis

Data are presented as means ± SD of the mean, with n indicating the number of observations. Statistical significance of the differences was assessed by Student's *t* test for paired or unpaired data as appropriate and *P* was set at <.05. Calculations were performed using a commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California; www.graphpad.com).

3 | RESULTS

3.1 | Cytotoxicity

Treatment of human PMN for 3 hours with N-Gel 0.5 or 1.0 mg/mL did not result in any significant effect on PMN viability. In comparison to PMN in culture medium alone, cell viability with N-Gel 0.5 or 1.0 mg/mL was, respectively, 100.6 ± 5.8% and 104.8 ± 5.9% in resting PMN and 112.6 ± 13.5% and 107.8 ± 5.9% in fMLP-activated cells (n = 4, *P* always >.05 vs respective control).

3.2 | Cell migration

Spontaneous migration was 13.6 ± 4.9 (n = 6) in resting PMN and 28.6 ± 12.6 in the presence of fMLP (n = 6, *P* = .006 vs resting). Coincubation with N-Gel did not affect resting cell migration,

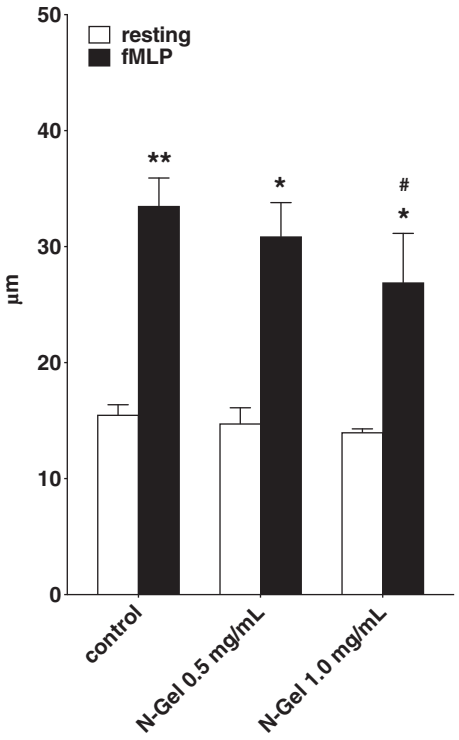

FIGURE 1 Effect of Neauvia gel on human neutrophil functions. Human neutrophil migration cultured alone (control) or in the presence in the cell medium of two different concentrations (0.5 and 1.0 mg/mL) of Neauvia Intense in resting conditions (empty columns) and after stimulation with fMLP (0.1 μM, filled columns)

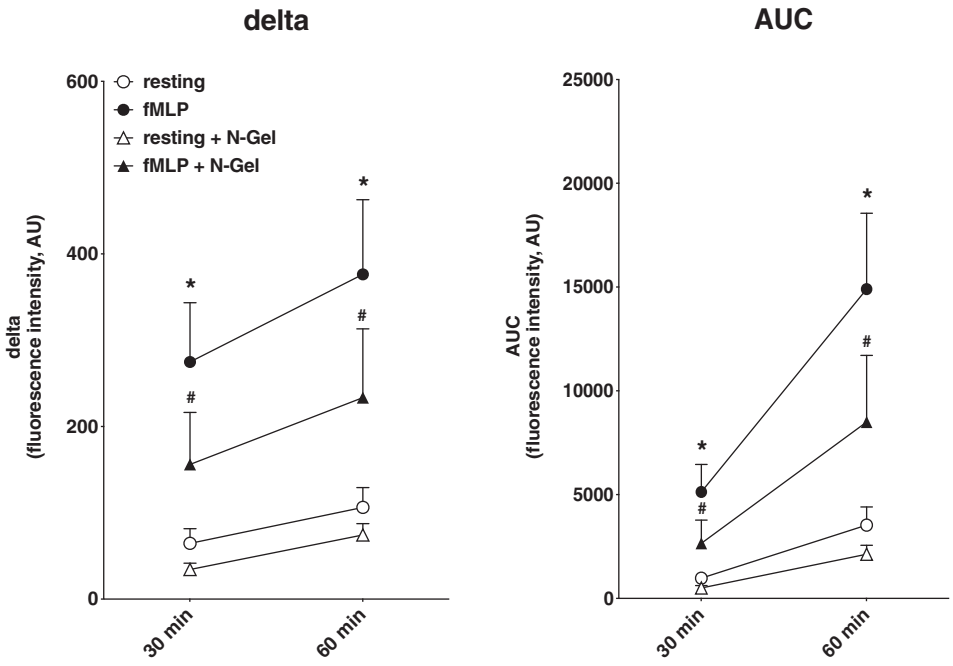

FIGURE 2 Effect of Neauvia gel on human neutrophil oxidative metabolism. ROS generation measured as delta (left panel) or AUC (right panel) in human neutrophil cultured for 30 and 60 minutes alone (circles) or in the presence of Neauvia Intense 0.5 mg/mL (triangles) under resting (empty symbols) or after stimulation with fMLP (0.1 μM, filled symbols). AUC, area under the curve; fMLP, N-formylmethionyl-leucyl-phenylalanine; ROS, reactive oxygen species

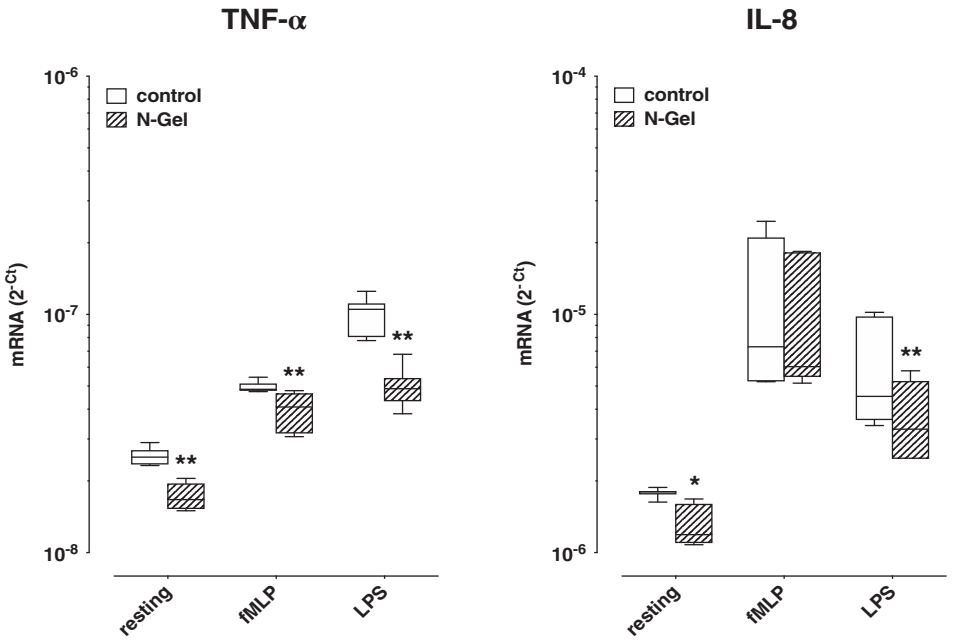

FIGURE 3 Effect of Neauvia gel on human neutrophil cytokine/chemokine mRNA. mRNA levels for TNF-α (left panel) and IL-8 (right panel) measured in human neutrophil cultured alone in RPMI medium (empty symbols) or in medium in the presence of Neauvia Intense 0.5 mg/mL (filled symbols) under resting conditions or after stimulation with fMLP (0.1 μM) and LPS (1 μg/mL). fMLP, N-formylmethionyl-leucyl-phenylalanine; IL-8, interleukin-8; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α

however at 1.0 mg/mL, it significantly reduced fMLP-induced migration by 23% (n = 6, *P* = .01 vs fMLP alone) (Figure 1).

3.3 | ROS generation

ROS levels in resting PMN was 64.6 ± 34.3 (delta 30 minutes), 106.2 ± 46.1 (delta 60 minutes), and 973.4 ± 535.0 (AUC 30 minutes; n = 4), 3535.0 ± 1740.0 (AUC 60 minutes; n = 4) and both parameters were increased in the presence of fMLP by 423-527% after 30 minutes and by 354-421% after 60 minutes (Figure 2). Coincubation with N-Gel 0.5 mg/mL did not affect resting ROS levels; however, it effectively reduced fMLP-induced ROS generation at both 30 minutes and 60 minutes (Figure 2).

3.4 | TNF-α and IL-8 mRNA levels

Both TNF-α and IL-8 mRNA were detected in resting PMN, and their levels increased after cell stimulation with either fMLP or LPS (Figure 3). N-Gel 0.5 mg/mL significantly reduced both resting as well as LPS-induced TNF-α and IL-8 mRNA expression, while it affected only fMLP-induced TNF-α mRNA but not IL-8 mRNA (Figure 3).

4 | DISCUSSION

The present results show that in human, PMN N-Gel reduces stimulated migration, resting and stimulated ROS production, as well as resting and stimulated levels of TNF-α and IL-8 mRNA, suggesting as a whole not only that N-Gel does not carry significant risks of pro inflammatory effects on PMN, but also that it can effectively modulate PMN functions resulting in anti-inflammatory activities, which may contribute to the beneficial effects of N-Gel in its different medical and cosmetic applications.

N-Gel is an original proprietary product composed of HA and PEGDE, with peculiar chemical properties.[4] HA is a ubiquitous glycosaminoglycan widely distributed in human tissues, and its ability to modulate PMN function has been the subject of several studies. HA has been reported to effectively inhibit PMN migration and chemotaxis[18]; however, stimulation of adherence, migration, chemotaxis, and phagocytosis has been also reported.[19] In particular, the stimulatory effect of HA on PMN may occur in the presence of fibronectin.[20] The effects of HA on PMN oxidative metabolism are on the contrary apparently much more reproducible throughout the studies,[14,21,22] an effect that may include also direct scavenging activity and has been suggested as relevant also for cell protection against oxidative stress.[14] Nevertheless, it should also be considered that only recently it has been pointed out that different glycosaminoglycans may exert distinct effects on PMN, possibly also resulting in pro inflammatory effects including increased ROS production, phagocytosis and IL-8 expression.[13,15] Moreover, HA as a dermal filler has received relative attention only recently and mainly in histopathologic studies, which however did not assess its effects on cell function.[23] Even less is known about the possible effects of PEG on PMN function, although circumstantial evidence suggests potential inhibitory effects on cell motility,[11,12] while promoting the release of the microbicidal peptides α-defensins.[24]

Available evidence clearly suggests that the effects of glycosaminoglycans-based hydrogels like N-Gel on immune and inflammatory cells cannot be predicted based just on their chemical composition, as minor differences in the chemical structure and proportions of the components may eventually result in different and sometimes even opposite effects. On the other side, although HA-based hydrogels have been generally considered safe and well-tolerated, recent evidence increasingly points to emerging safety issues related to their immune effects, including delayed hypersensitivity as well as granulomatous reactions.[25,26] Assessment of the immune effects of HA-based hydrogel should be therefore carefully considered as part of the general safety evaluation of these products.

Our results concerning the ability of N-Gel to modulate human PMN functions, including migration, oxidative metabolism, and TNF alpha and IL-8 expression, suggests that N-Gel carries a very low risk of immune-mediated adverse effects, particularly granulomatous reaction and associated cellulitic processes. In dermal cosmetics, as well as in aesthetic medicine, the use of products not only devoid of proinflammatory effects but also able to modulate any local inflammatory process, represents a significant opportunity, thus strengthening previous evidence and supporting N-Gel as new frontier in dermal cosmetic.[5,6,8-10]

5 | CONCLUSIONS

Exposure of human PMN to N-Gel results in reduced migration and oxidative metabolism, as well as in reduced mRNA levels of key pro-inflammatory mediators like TNF-α and IL-8. While a whole characterization of the immune effects of N-Gel still requires a wide array of studies, for example, on monocytes and T lymphocytes, nonetheless results in PMN indicate that N-Gel can efficiently modulate some critical immune and inflammatory mechanisms.

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