**Augmented Macrophages Pro-Inflammatory Response and Apoptosis by Therapeutic Insulin Doses used in Insulin Pump Therapy: An In Vitro Cell-Material Interaction Model**

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1. INTRODUCTION

Continuous subcutaneous insulin infusion (CSII), or insulin pump therapy, is a pivotal method of managing Type 1 diabetes, that relies upon insulin infusion sets (IIS) to provide reliable delivery and absorption of insulin at infusion sites [1]. However, IIS face challenges marked by short wear time and high failure rates [2]. Emerging evidence idicates that the local inflammatory response to the IIS cannula may play a role in both the limited wear time and unreliable insulin absorption [3-5]. However, a comprehensive understanding of the underlying mechanisms drive infusion site inflammation remains elusive.

To address this gap, we studied the effect of insulin analogue Humulin-N over a range of therapeutic concentrations predicted to be present within the local infusion site on macrophage activation using an established protein adsorption model that mimic macrophage activation at a biomaterial implant surface. The primary objective was to determine the effect of therapeutic local Humulin-N concentrations on macrophage activation, including nuclear factor-κB (NF-κB) and activating protein 1 (AP-1) activity, ROS accumulation, as well as assessments of cell density, viability, and apoptosis. The findings aim to improve our understanding of the limitation of CSII therapy and potentially inform strategies for enhancing the reliability of IIS in managing Type 1 diabetes.

1. METHODS

3T3 fibroblast lysate were pre-adsorbed on tissue culture polystyrene (TCPS) surfaces to model the damage-associated molecular patterns (DAMPs) released from tissue injury and activates macrophages via Toll-like receptor 2 [6]. A relevant insulin concentration range (0.1-10 U/ml) was chosen based on the simulation of the local steady-state insulin concentration profile at the infusion site using the COMSOL Multiphysics software (Stockholm, Sweden). Mouse Raw-Blue reporter macrophages were seeded on TCPS surfaces pre-conditioned with either lysate or FBS with or without Humulin-N (Eli Lilly), and incubated for up to 24 hours. NF-$κ$B/AP-1-dependent secreted embryonic alkaline phosphate (SEAP) activity using the Quanti-Blue assay (InvivoGen) and normalized to cell density via QuantiFlour dsDNA assay (Promega). Intracellular ROS was measured with ROS-ID Total Detection Kit (Enzo Life Science) and normalized to adherent cell density. Real-time apoptosis assay was conducted using the IncuCyte® Zoom imaging system (Essen BioScience) and Caspase-3/7 green dye to stain apoptotic cells. Statistical analysis employed a two-way ANOVA with Dunnett’s post hoc (n= 6 - 9) in GraphPad Prism.

1. RESULTS AND DISCUSSION

High insulin concentration (0.5-10 U/ml) enhanced the NF-kB/AP-1 activity of macrophages on lysate-adsorbed surfaces (Figure 1A). Significant differences in the intracellular ROS accumulation were observed at 18h with 5 U/ml insulin compared to no insulin, and at 24h with 1 U/ml insulin, compared to macrophages cultured on lysate adsorbed surfaces without insulin (p < 0.05). Conversely, insulin had no significant impact on NF-$κ$B/AP-1 nor intracellular ROS accumulation in the absence of the inflammatory stimulus. Notably, high concentrations (e.g., 5 U/ml) of Humulin-N decreased macrophage densities over 24 hours (Figure 1B) in both the lysate and control conditions. Significant caspase-3/7 activity in Humulin-N treated macrophage cultured suggest the high 5 U/ml Humulin induced apoptosis, independent of an inflammatory stimulus.



*Figure 1. The effect of high insulin concentrations on the (A) NF-κB/AP-1-dependent SEAP activity per cell and (B) cell density at 24 hours. Mean ± SD (n = 9). \* p < 0.05 compared to control of each group, # p < 0.05 for lysate control compared to media control.*

In summary, the study demonstrates that insulin concentration ($<$ 10 U/ml) had no significant effect on macrophage pro-inflammatory response, as evidenced by NF-$κ$B/AP-1 dependent macrophage pro-inflammatory activity. However, high insulin concentration ($\geq $ 0.5 U/ml) enhanced the activity of pro-inflammatory NF-kB/AP-1 factors transcription and showed significant increased intracellular ROS accumulation when combined with an inflammatory stimulus. Additionally, elevated macrophage apoptosis was observed in both groups, with and without lysate stimulation, following treatment with 5 U/ml insulin. This study contributes to the emerging evidence that infused insulin at therapeutic doses contributes to an enhanced local tissue inflammatory response to IIS, consequently limiting their lifespan in practical usage.

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