Effects of APPA on Human Neutrophil Function

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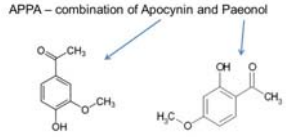
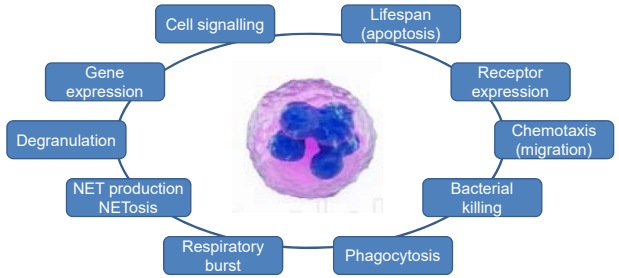
Background, Results

APPA is a synergistic combination of 2 anti-inflammatory molecules (apocyanin, AP and paeonol, PA), which is completely synthetic. APPA has shown efficacy in several animal models of osteoarthritis, decreasing pain and meniscal cartilage damage. Its efficacy is thought to be due to the effects of the constituent components, AP and PA, on various levels of regulation of the transcription factor, NF-κB but as we show here also has effects upon other signalling pathways and neutrophil processes.

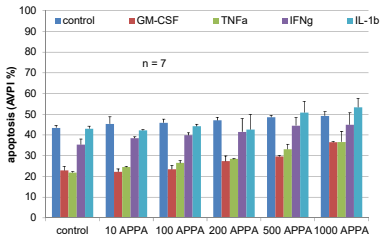


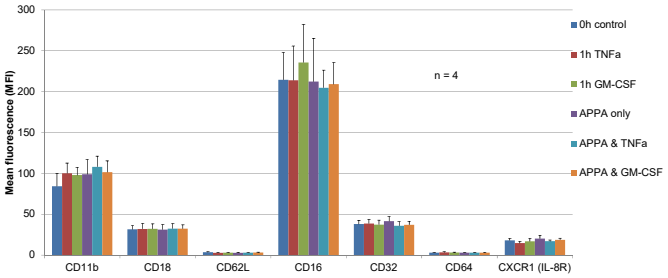
Neutrophils are the first line of defence against invading pathogens and are vital to fight infection. Their function *in vivo* and *in vitro* is regulated by the activities of a number of cytokines. These cells are potent producers of reactive oxidants species (ROS, activators of NF-κB) via an NADPH oxidase and many inflammatory functions are also regulated by NF-κB activation.



Our experimental plan was designed to understand the effects of APPA on human neutrophil functions (shown above) that are activated by individual cytokines or combinations of cytokines that are known to regulate their function *in vivo*.

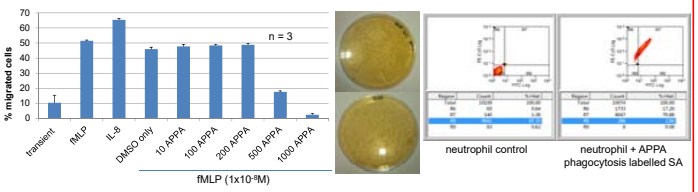
Apoptosis was measured by utilizing Annexin V binding to exposed phosphotidyserine. There was a slight increase in apoptosis in a dose dependent manner but this did not reach significance in control cells or cells treated with cytokines.



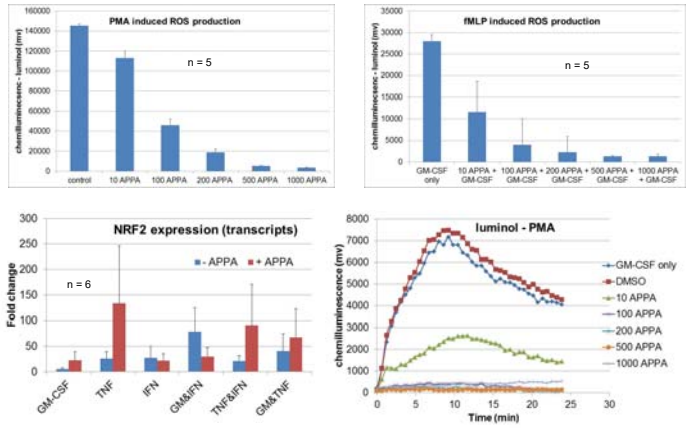


Receptor expression on the surface of neutrophils did not change after pre-incubation with 100 μg/mL APPA. Receptors measured included integrin and chemokine receptors (migration) and Fc receptors (phagocytosis).

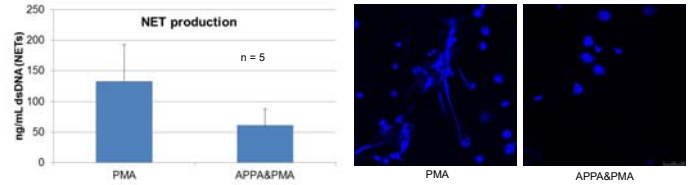
Results



Chemotaxis (migration) was only affected at the highest concentrations of APPA (500 & 1000 μg/mL) and rounding of neutrophils was observed. Both **bacterial killing** of live staphylococcal aureus (SA) and **phagocytosis** of heat-killed, PI labelled, SA were not affected at any concentration used.

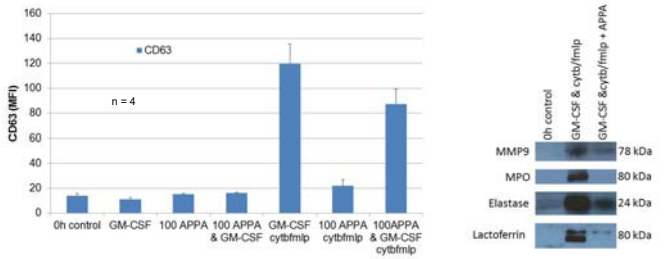


Respiratory burst (ROS production) by neutrophils was measured using luminol-enhanced chemiluminescence. Preincubation of neutrophils with APPA before GM-CSF or TNFα priming appeared to inhibit both receptor dependent (fMLP) and receptor independent (PMA) chemiluminescence in a dose dependent manner. However adding APPA immediately before or during ROS measurements showed that this decrease in respiratory burst is predominantly a scavenging action. APPA did appear to up regulate NRF2, a regulator of cellular resistance to oxidants, at the mRNA level but results were variable and did not reach statistical difference.

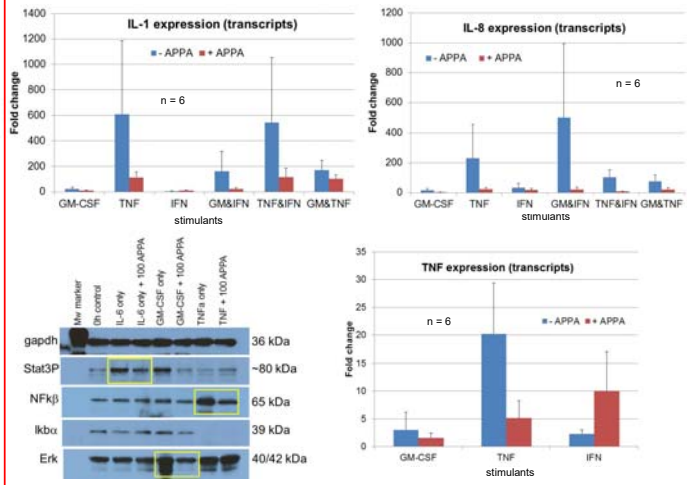


NET production (NETosis) by neutrophils was measured using fluorescent spectroscopy and visualised by microscopy. APPA decreased the level of NETosis induced by both PMA and TNFα. Stimulated neutrophils in the presence or absence of APPA (100 μg/mL) were allowed to NET for a total of 3 h before analysis. Confocal microscopy image shows neutrophil DNA and extracellular (NET) DNA stained by DAPI.

Results, Summary



Degranulation was impaired by APPA. GM-CSF primed neutrophils and degranulation stimulated by fMLP (in the presence of cytochalasin B) was measured using both a marker of degranulation (CD63) and by Western blotting for secreted proteins in the supernatant of stimulated neutrophils.



Gene expression analysis using RT-PCR showed an inhibition of IL-1β and IL-8 stimulated gene expression after pre-incubation with APPA (100 μg/mL). **Cell signalling** was measured utilizing western blotting analysis for active (phosphorylated) forms of signalling proteins. APPA inhibited IL-6-stimulation of STAT3, TNF stimulated NF-κB and GM-CSF-stimulated Erk1/2.

- **APPA** did not interfere with vital neutrophil functions such as bacteria killing and the processes that aid this, such as prolonged lifespan, migration and phagocytosis
- **APPA** is a strong scavenger of reactive oxygen species and also appears to up regulate NRF2, an anti-inflammatory regulator of antioxidant proteins
- **APPA** inhibited the production of neutrophil extracellular traps (NETs)
- **APPA** decreased degranulation of neutrophils
- **APPA** affected several signal transduction pathways both at the gene and protein levels

It is clear that APPA via its two components, acts in an anti-inflammatory manner without affecting host defence mechanisms involved in bacterial killing. This work supports previous work observed here and in other laboratories on cell and animal models.