This Thesis, The effect of acetaminophen on the neutrophil oxidative burst, presented by Fatima Zaman and Submitted to the Faculty of The Harvard Medical School in Partial Fulfillment of the Requirements for the Degree of Master of Medical Sciences in Immunology has been read and approved by:

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The effect of acetaminophen on the neutrophil oxidative burst

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ABSTRACT

The dihydrorhodamine (DHR) assay is used to quantify the neutrophil oxidative burst, which is reduced in primary immunodeficiencies with defective neutrophil function, such as chronic granulomatous disease (CGD) or RAC2 deficiency. Although there are reports of reduced neutrophil oxidative burst after *in vitro* neutrophil exposure to acetaminophen, there are no systematic studies that address whether therapeutic doses of acetaminophen impair the neutrophil oxidative burst. We demonstrate that the neutrophil oxidative index (NOI) is dramatically impaired by acetaminophen in healthy human subjects. This effect which peaked 2 hours after administration dissipated within 24 hours and correlated with inhibition of myeloperoxidase (MPO) activity. Retrospective analysis of 195 hospitalized patients without CGD or RAC2 revealed an abnormal DHR results nearly half of the time (44.8%) when patients had DHR testing within four hours of acetaminophen exposure. No similar effect was seen with ibuprofen, steroids or lidocaine. This data shows that to avoid falsely abnormal testing for CGD, patients should be advised to avoid acetaminophen for at least 24 hours prior to DHR testing.

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CHAPTER 1: BACKGROUND

1.1. INTRODUCTION

Neutrophils are hematopoietic cells and are the most abundant circulating leukocytes in the blood, comprising of 50 to 70% of total circulating leucocytes.[1] They are the first line of host defense against a wide range of pathogens, including bacteria, fungi, and protozoans. Neutrophils have a broad spectrum of defense mechanisms to clear infections, including phagocytosis, release of neutrophil extracellular traps (NETs), production of pro-inflammatory cytokines, production of reactive oxygen species, and activation of the adaptive immune system.[1]

Mechnikov shared a Nobel prize in medicine with Paul Ehrlich in 1908 for describing the process of phagocytosis. This term originated from the Greek word ''phagein'' which means to eat. Phagocytosis holds great importance for host defense against invading microorganisms. It is initiated when neutrophils recognize microbes through opsonic or non-opsonic receptors. Opsonic receptors include C5a and FcγR, and non-opsonic receptors include CD169 and CD33.[2] Receptor engagement initiates a series of signaling cascades that modifies the actin cytoskeleton and lipids within the cell membrane to form pseudopods around the particle. Pseudopods envelop the particle and internalize it, forming a phagosome.[2] Within the phagosome, microorganisms are killed by either oxygen-dependent or independent pathways.[3] Oxygen-dependent bactericidal activity is dependent upon production reactive oxygen species (ROS) through NADPH oxidase.[4]

ROS are reactive molecules of oxygen that include superoxide anion (O2−), hydroxide (HO^-), hydrogen peroxide (H2O2), hypochlorous acid ($HOCL^-$), and ozone (O_3).[4] ROS are produced inside the phagosome through phagocytic NADPH oxidase. Phagocytic NADPH oxidase is a multisubunit complex that has cytosolic and

membrane components.[5] These subunits include $gp91^{phox}$,$p22^{phox}$ $p40^{phox,}$ $p67^{phox}$ and $p47^{phox}$. $gp91^{phox}$ and $p22^{phox}$ are present in the cell membrane or membrane-bound vesicles, and together are known cytochrome b_{558} while $p40^{phox,}$ $p67^{phox}$ and $p47^{phox}$ are present within the cytosol. These subunits assemble to form a complex in response to a wide range of stimuli including bacterial peptide, fMLF, PMA, C5a, opsonized bacteria, opsonized zymosan, and calcium ionophores.[6] All of these agents lead to the assembly of NADPH oxidase by activation of multiple protein kinases, including PKC, PKA, AKT, and MAP kinases (MAPK). In unstimulated neutrophils, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ are present as heterotrimeric complex. $p47^{phox}$ is in an autoinhibitory state, with the two SH3 carboxy-terminal domains occupied by its autoinhibitory region. Conformational changes are induced upon phosphorylation of $p47^{phox}$ that relaxes the protein configuration and makes the SH3 domain available for interaction with $p22^{phox}$. $p67^{phox}$ interacts with GTP bound Rac, and the heterotrimeric cytosolic complex along with Rac translocate to the membrane and associates with cytochrome b_{558}. $gp21^{phox}$ functions as a docking site for the cytosolic complex. $gp91^{phox}$ has a C-terminal NADPH binding domain towards the cytosol and an N-terminus that consists of six alpha-helices localized within the membrane. $gp91^{phox}$ also has a Flavin adenine dinucleotide (FAD) and two hemes which act as an electron carrier. When NADPH binds to the C-terminus of $gp91^{phox}$ it transfers electron through FAD and hemes inside the phagosome. These electrons are taken up by molecular oxygen, which gets converted to superoxide anion.[7]

Azurophilic granules containing myeloperoxidase fuse with phagosomes and release myeloperoxidase (MPO). MPO is heme-peroxidase that catalyzes the conversion of H_2O_2 and Cl^- into $HOCL^-$ within the phagosome.[8] $HOCL^-$ acts as a

bactericidal agent by inhibiting protein synthesis, and causing protein degradation and membrane disruption.[9]

Chronic granulomatous disease (CGD), caused by defects in NADPH oxidase, is a rare disease with an incidence of 1 in 200,000 United States.[10] However, the incidence varies in different ethnicities. X-linked CGD, caused by mutations in $gp91^{phox}$ is common in non-consanguineous populations, whereas autosomal recessive CGD due to mutations in $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, or $p40^{phox,}$ which are encoded by *CYBA*, *NCF1*, *NCF2*, and *NCF4*, respectively, are more common in regions where consanguinity is common. X-linked CGD is typically more severe as there is often complete absence of oxidase activity in these patients, whereas the autosomal recessive patients typically have some residual activity.[11,12]

First described it in 1954 by Janeway and colleagues, patients were found to have high immunoglobulin levels and had a recurrent infection.[13] The disease was initially term "Fatal Granulomatous Disease of Childhood" owing to the high under ten mortality rate[13]. The mortality rate declined with the use of prophylactic antibiotic and antifungal agents[14]. Quie and colleagues discovered that patient-derived neutrophils could phagocytose pathogens but were unable to clear them.[15] Neutrophils from CGD patients fail to produce sufficient neutrophil oxidative burst to kill bacteria, particularly catalase-positive organisms such as *Staphylococcus aureus, Burkholderia cepacian, Serratia marcescens, Nocardia spp., Candida albicans, and Aspergillus spp.* due to the impaired generation of reactive oxygen species (ROS).[10] In contrast, infections with catalase-negative organisms are less common because these organisms are killed within the phagosome by endogenously produced ROS.

Patients with CGD may exhibit a range of symptoms, including severe infections with pyogenic organisms, granulomas, abscesses, and immune dysregulation including inflammatory bowel disease.[16,17]

Clinically CGD can be screened for by measuring the neutrophils' ability to produce ROS upon stimulation with Phorbol 12-myristate 13-acetate (PMA). PMA directly activates protein kinase C (PKC), which phosphorylates the cytosolic subunits of NADPH oxidase and leads to assembly of NADPH oxidase. Baehner and Nathan in 1968 first developed the nitroblue tetrazolium test (NBT) for the detection of CGD.[18] The principle of this test was that neutrophils stimulated with PMA will reduce nitroblue into formazan, which has a purple color that can be detected through a microscope. While cost-effective and a straightforward, this is a qualitative that cannot measure the extent of impairment of ROS production. Thus autosomal recessive CGD in which there is residual NADPH activity can be missed.

In 1990 dihydrorhodamine (DHR) assay, now the gold standard for clinical diagnosis of CGD was first published. PMA-stimulated neutrophils produce ROS, which oxidizes dihydrorhodamine 123 (DHR), to rhodamine 123 which emits green fluorescence. This fluorescence can be measured quantitatively by flow cytometry and expressed as a neutrophil oxidative Index (NOI), the ratio of mean fluorescence activity in stimulated versus unstimulated cells.[19] Measurement of fluorescence produced by this method has been shown to correlate with ROS production capacity, and residual oxidative function to correspond with the survival of patients with CGD.[19]

While DHR testing is the current gold standard for clinical testing of abnormal neutrophil oxidative function, confounding factors may produce artificial deterioration in the respiratory burst. There have also been reports of abnormal neutrophil oxidative index in patients with myeloperoxidase deficiency despite normal superoxide anion

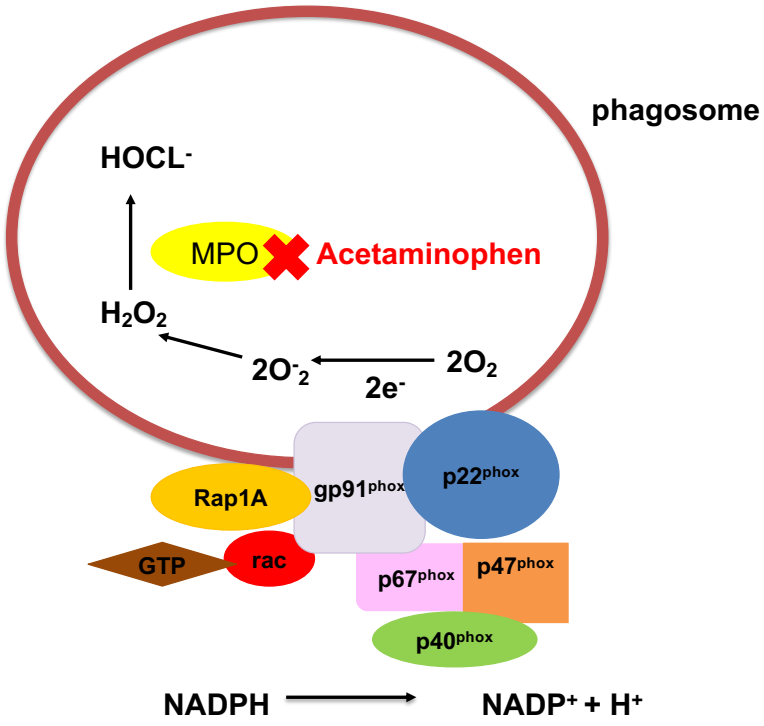
production.[20] Other factors that show abnormal neutrophil oxidative index despite normal neutrophil oxidative burst include delay in processing blood specimens and acute sickness.[21]

Myeloperoxidase (MPO) deficiency is has an autosomal recessive inheritance affecting 1 in 4000 people. These patient have normal ROS production but fail to generate $HOCL^-$. While MPO-deficient PMNs shows impaired bacterial clearance *in vitro*, patients with complete or partial MPO deficiency don't present with CGD-like infections. While the ROS generated are sufficiency to control infection, patients with MPO deficiency have an abnormal DHR as $HOCL^-$ is required for DHR oxidation[22,23]. Of note, MPO-deficient patients have normal nitroblue tetrazolium tests because H_2O_2 alone can reduce nitroblue tetrazolum to formazan.[24]

Acetaminophen (N-acetyl-p-Aminophenol) is the most commonly used over counter analgesic and antipyretic in the children. Acetaminophen has a rapid onset of action of about 3 to11 minutes depending on route of drug delivery[25]. It has a plasma half-life of 1.5-2.5 hours.[26] Although its precise mechanism of action remains elusive, one potential mechanism is through inhibition of the peroxidase activity cyclooxygenase (COX) enzymes.[27] This inhibition reduces prostaglandin production and reduces pain. While there is a extensive literature on acetaminophen-induced oxidative stress after administration of toxic doses due to increased glutathione utilization[27,28], acetaminophen has been shown *in vitro* to scavenge superoxide and impair neutrophil oxidative burst, but not O^{2-} production, suggesting that NADPH oxidase function is preserved[20]. This antioxidant property underlies the ability of acetaminophen to reduce reperfusion injury and ischemia of the heart in guinea pigs by scavenging hydroxyl radicals and peroxynitrite.[29] Acetaminophen has also demonstrated to impair MPO activity and reduces $HOCL^-$.[30]

To our knowledge, there is no documented evidence suggesting that acetaminophen causes false-positive DHR assay. Given that acetaminophen is commonly used for fever or pain in adults and children, we sought to investigate whether the use of acetaminophen is associated with decreased neutrophil oxidative activity by impairing myeloperoxidase, which could lead inaccurate interpretation of the DHR assay in these patients.

1.2. Schematic Figure. Model of acetaminophen impairing HOCL$^-$production.



NADPH oxidase consists membrane-bound heterodimer (gp91^{phox} and p22^{phox}) and heterotrimeric cytosolic subunits (p67^{phox}, p47^{phox} and p40^{phox}). Upon phosphorylation, the cytosolic subunits along with activated rac translocate to phagosome and form NADPH oxidase. Two electrons from NADPH transfers insides the phagosome through gp91^{phox}. These electrons are taken up by O_2 to form O_2^-. O_2^- spontaneously or through superoxide dismutase forms H_2O_2. H_2O_2 is converted to HOCL$^-$ in the presence of MPO. Acetaminophen impairs MPO activity and reduces HOCL$^-$ production.

CHAPTER 2:METHODS AND DATA

2.1. INTRODUCTION

We performed DHR assay immediately prior to, 2, and 24 hours after 1 gram of acetaminophen ingestion in 15 healthy individuals to observe if acetaminophen impairs neutrophil oxidative burst. Ibuprofen just like acetaminophen is also an over the counter analgesic and antipyretic. We performed the DHR assay at similar time points after giving 400 mg of ibuprofen. To understand the mechanism behind acetaminophen, causing false-positive DHR results, we performed an MPO activity assay on the whole blood lysate.

2.2. Materials and methods

Ethical oversight

This study was approved by the Institutional Review Board at Boston Children's Hospital (Protocols P00028149 and P0002975600000).

Study participants for the prospective study

Sixteen adult volunteers with no history of immune deficiency and no history of acetaminophen or ibuprofen use for seven days consumed a single oral dose of either acetaminophen 1 gram (Tylenol, Johnson & Johnson, New Brunswick, NJ) or ibuprofen (Costco Wholesale Company, Seattle, WA). Blood samples for DHR testing were obtained immediately prior to ingestion, then 2, and 24 hours after drug ingestion. The DHR assay was performed immediately after sample collection . Fifteen participants had DHR testing after acetaminophen ingestion. 5 participants had DHR testing after ibuprofen ingestion.

Study participants for retrospective study

To identify patients who had clinical laboratory DHR testing, we queried the electronic medical record using i2b2, an open-source clinical data warehousing, and analytics research platform. We included patients admitted to Boston Children's Hospital who had both: (1) at least one clinical result for dihydrorhodamine testing from 1998-2019, (2) documented medication administration of at least one dose of oral or parenteral acetaminophen, non-steroidal inflammatory drug (NSAID: ibuprofen, naproxen, ketorolac, aspirin), glucocorticoid steroid (prednisone, prednisolone, dexamethasone, methylprednisolone, hydrocortisone), or lidocaine (topical application only) within 72 hours prior to DHR testing. We excluded patients with a history of hematopoietic stem cell transplant or primary immune deficiency affecting neutrophil function (including CGD diagnostic codes ICD9 288.1 or ICD10 D71). For each patient, we recorded age at test, neutrophil oxidative activity on DHR test result(s), and relevant medication administration details (medication, route, dose, time of administration, number of doses). To confirm exclusion criteria, we reviewed clinical documentation for all patients with abnormal DHR obtained at least 8 hours after the last administration of acetaminophen. Patient screening and final cohort were independently reviewed by at least two study team members.

Flow cytometric assessment of neutrophil oxidative activity

Testing was performed as previously described[31]. Briefly, whole blood from healthy volunteers (100 uL) was combined with 3.2 uL of 2 ug/mL dihydrorhodamine 123 (DHR, ThermoFisher Scientific, Waltham, MA. Cat #D632) and (for stimulated samples) 3. 4 uL of 0.4 ug/mL of Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO. Cat# P8139). After 30 minutes incubation at 37ºC, cells were

lysed with 900 uL of BD FACS lysing solution (Beckton-Dickinson, Franklin Lakes, NJ. Cat# 349202) for 15 minutes, then washed and analyzed on a FACSCanto II flow cytometer. Results for granulocytes were analyzed in FloJo software version 10.5.0 (TreeStar). We reported the result as the ratio of mean fluorescence intensity (MFI) of the stimulated sample to the unstimulated sample (neutrophil oxidative index, NOI).

Assessing Myeloperoxidase Activity

Myeloperoxidase activity was measured on whole blood lysate. 2 ml of blood was drawn in a heparinized tube. Blood was lysed with 6 mL of RBC lysing solution for 10 minutes. Lysed blood was centrifuged for 5 minutes at 400 x g, and the supernatant was carefully removed. Leucocyte pellet was washed with 1 mL of PBS and was again centrifuged for 5 min at 400 x g. The pellet was lysed with cell lysing buffer (cat. no. ab105136; Abcam, Cambridge, MA, USA). The cell lysate was centrifuged at 100,000 g for 10 mins to remove the insoluble material. The supernatant was harvested. The MPO activity was assayed using Myeloperoxidase Activity Assay kit (cat. no. ab105136; Abcam, Cambridge, MA, USA) by measuring the absorbance of the sample at 412 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The specific MPO activity in the whole blood cells was measured as units/umol of TNB consumed.

Statistical analysis

Statistical comparisons were performed with Prism 8 (GraphPad, San Diego, CA). For continuous variables where normality could not be assumed, the Wilcoxon signed-rank test (paired tests), Mann-Whitney U test (unpaired tests) and t-test were used.

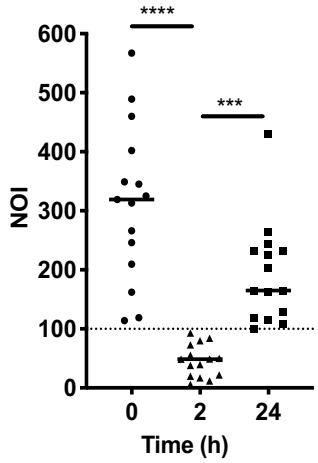
For correlation, we used the Spearman test. All tests were two-tailed. We considered $p<0.05$ significant.

2.3. Results:

Functional assessment of neutrophil oxidative activity is impaired in healthy adults after acetaminophen ingestion.

To assess the effect of medication ingestion on diagnostic testing, we examined neutrophil oxidative activity in 15 healthy adult volunteers who were administered a single oral dose of 1000 mg acetaminophen. None had history of immune deficiency, including chronic granulomatous disease. None consumed acetaminophen or any non-steroidal anti-inflammatory medications (NSAID) for at least seven days prior to testing. All had normal NOI (normal ≥100, median 311) at baseline (Fig. 1). As acetaminophen has an onset of action of 3-11 minutes and half-life of 1.5-2.5 hours[25], we tested neutrophil oxidative function at 2 hours after acetaminophen dose. At two hours after ingestion, NOI was abnormal (<100) in all 15 individuals (median NOI: 48.8). At 24 hours, all individuals had normal NOI (median 192.5).

Figure 1: Neutrophil oxidative burst is impaired in healthy volunteers after acetaminophen ingestion.

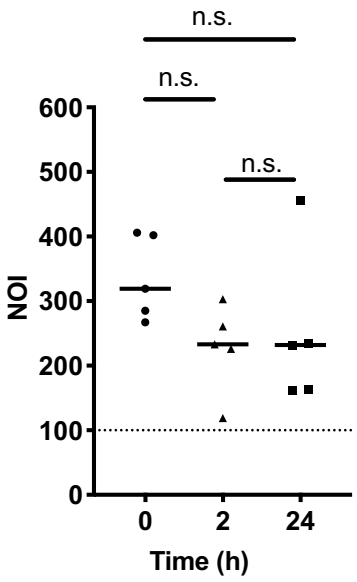


Neutrophil oxidative index (NOI) is shown before, 2 hours after and 24 hours after a single dose of acetaminophen. The dotted line represents an NOI of 100 the cutoff between normal and abnormal DHR test. Bars represent medians. ***p < .001 and ****p <.0001. Wilcoxon signed-rank test, 2 tailed.

Functional assessment of neutrophil oxidative activity is not affected in healthy adults after ibuprofen ingestion.

We tested the effect of a different anti-inflammatory medication, ibuprofen, on neutrophil oxidative activity (Fig. 2). In 5 healthy adults who received a single dose of ibuprofen (400 mg PO, NOI was normal at 2 hours (median 233) and 24 hours (median 232) after receiving ibuprofen. There was no significant difference from NOI at baseline (median 319) at either the 2 hours or 24 hours time point after ibuprofen dosing. Thus, assessment of neutrophil oxidative activity was abnormal in healthy individuals who had received acetaminophen, but not ibuprofen.

Figure 2: Neutrophil oxidative burst is not inhibited in healthy volunteers after ibuprofen ingestion.

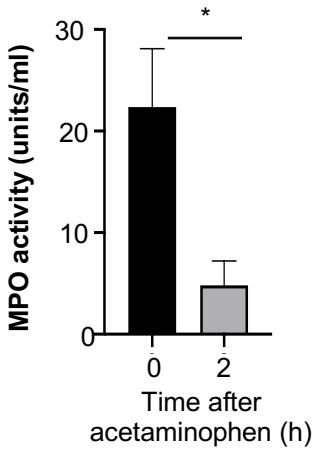


Neutrophil oxidative Index (NOI) is shown before, 2 hours after, and 24 hours after a single dose of ibuprofen. Bars represent medians. ns, Not significant. Wilcoxon signed-rank test, 2 tailed.

Acetaminophen inhibits myeloperoxidase activity in healthy individuals

Acetaminophen and ibuprofen may both exert effects through cyclooxygenase[32,33,34]. As we observed that acetaminophen, but not ibuprofen, dosing resulted in abnormal assessment of neutrophil oxidative activity, this suggested that cyclooxygenase was unlikely to be the mechanism through which acetaminophen affects the DHR. Acetaminophen, but not ibuprofen, has been shown to inhibit myeloperoxidase activity *in vitro*[30]. We thus evaluated myeloperoxidase activity in three healthy individuals who consumed a single dose of acetaminophen (Fig. 3). Two hours after the acetaminophen dose, myeloperoxidase activity, was significantly lower than at baseline ($p= 0.048$).

Figure 3: MPO activity is impaired in healthy individuals after acetaminophen ingestions



Myeloperoxidase (MPO) activity is shown before and 2 hours after hours after a single dose of acetaminophen. Columns and bars represent mean and SEM. *p < .05. Unpaired t test, two tailed.

Acetaminophen use is associated with abnormal neutrophil oxidative functional testing in patients evaluated for CGD.

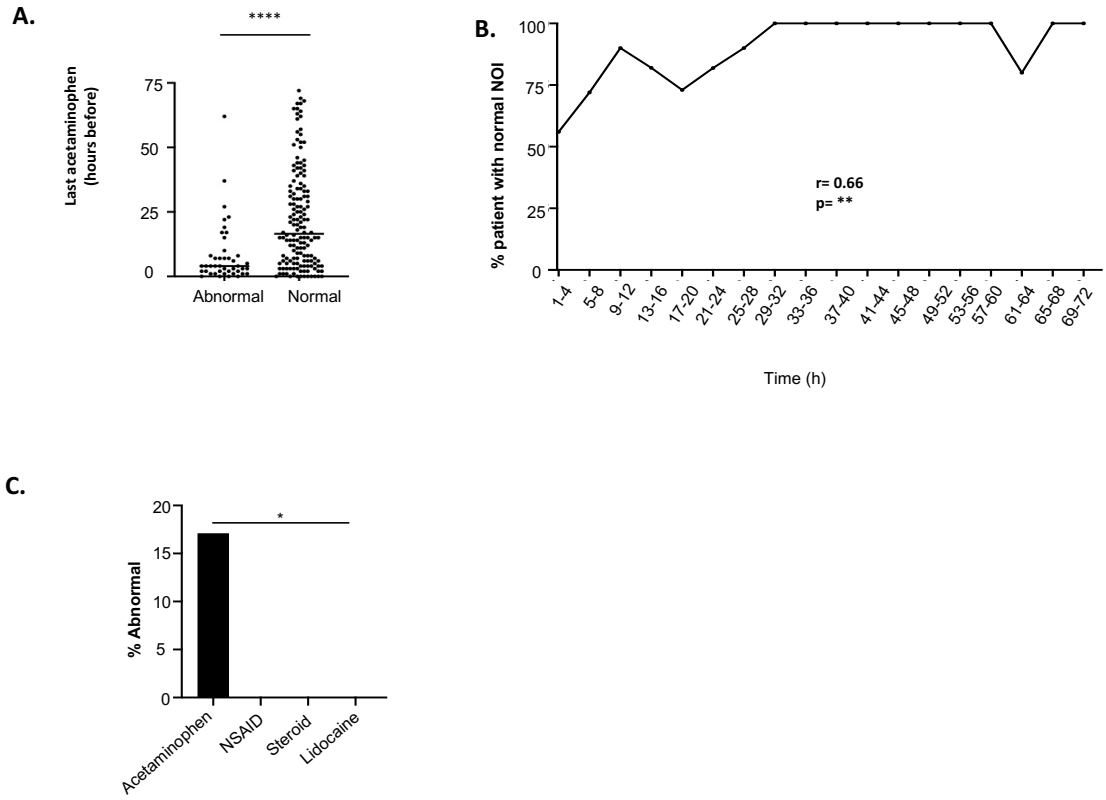
To evaluate the impact of acetaminophen treatment upon clinical diagnostic testing for chronic granulomatous disease and primary neutrophil functional defects, we performed a retrospective chart review of patients who had neutrophil functional testing performed during admission to Boston Children's Hospital (a tertiary care pediatric referral center) from 1996-2019. Our I2B2 database search identified 195 cases of DHR with no history of CGD or Haematopoietic stem cell transplantation (HSCT) and had neutrophil function testing performed within 72 hours after documented administration of at least one dose of acetaminophen. Patient age at testing ranged from less than 1 month to 34.9 years old. 15 patients (7%) received acetaminophen intravenously rather than enterally but did not have significant differences in test results.

Documented acetaminophen administration occurred from immediately before to up to 72 hours prior to sample collection. In patients who had received at least one dose of acetaminophen within 72 hours prior to testing, an abnormal test result (NOI<100) occurred in 43/195 cases (22.1%). We examined the median time from the last dose of acetaminophen prior to testing (Fig. 4A). On aggregate, patients with an abnormal test result had received their last dose of acetaminophen closer to testing (median time 4.00 hours before) than patients with normal test results (16.5 hours), with a median time difference of 12.5 hours. Thus, we examined the effect of acetaminophen dose timing upon neutrophil functional testing (Figure 4B). A strong linear relationship between the increase in the time interval between acetaminophen ingestion and normal DHR assay is observed (Spearman correlation= 0.66, p=0.0025). False-positive testing occurred in nearly half (44.8%) of patients who had

documented acetaminophen administration within 4 hours prior to testing, and in about a third (29.9%) of patients who had received acetaminophen within 24 hours prior to testing. In comparison, testing was abnormal in only 18.4% of cases of testing at 4-24 hours after acetaminophen use. Only 4.9% patient had an abnormal DHR test results 24 hours after acetaminophen use. Thus, testing performed within 24 hours after at least one dose of acetaminophen was significantly more likely to produce an abnormal (false positive) result than testing performed 24 hours or later after acetaminophen.

Acetaminophen is widely used, but many patients undergoing DHR testing may receive other medications for analgesia and antipyresis. To determine if non-steroidal anti-inflammatory medications and corticosteroids might have a similar effect as acetaminophen upon neutrophil oxidative functional testing, we identified cohorts of patients who had received only one type of medication (acetaminophen, NSAID, or corticosteroid, or topical lidocaine as a control), during the 72 hours prior to DHR testing. Due to difficulty identifying a sizeable cohort of patients who had not received any medications prior to testing, we used patients who received topical lidocaine as a control cohort due to the limited systemic absorption of topical lidocaine.[35] No patients who received only NSAID, only corticosteroid, or topical lidocaine had an abnormal DHR result (Fig. 4C). In contrast, patients who received acetaminophen alone had a significantly higher percentage of false-positive testing compared to the control group (lidocaine only).

Figure 4: Acetaminophen use is associated with abnormal neutrophil oxidative functional testing in patients undergoing evaluation for CGD.



Median time from the last dose of acetaminophen prior to testing in patients with normal and abnormal DHR. A. Percentage of patients who had normal NOI after acetaminophen within 72 hours of intake. B. False-positive DHR inpatient who received either acetaminophen or other medications. C. Columns and bars represent medians and SEM. *p < .05, **p < .01,***p < .001, and****p < .0001. ns, Not significant. Mann-Whitney test and Spearman correlation.

2.4.Discussion:

In this study, we demonstrate that the over the counter analgesic and antipyretic acetaminophen, routinely used in inpatient and outpatient care, causes abnormal neutrophil oxidative index by impairing MPO activity. Based on our results, we suggest avoiding acetaminophen 24 hours before DHR assay and being replaced with ibuprofen if required.

Chapter 3: Discussion and Perspectives

3.1. Discussion

The DHR assay is a robust and sensitive screening assay for impaired neutrophil functional defects such as CGD and Rac2 deficiency. However, confounding factors, such as rapid degradation of neutrophil function *ex vivo,*[36] acute illness,[21] or defects in the oxidation of hydrogen peroxide as in myeloperoxidase deficiency,[24,22] have been associated with decreased neutrophil oxidative activity in the DHR assay.

We assessed the effect of systemic administration of acetaminophen on neutrophil oxidative index testing for chronic granulomatous disease using prospective and retrospective approaches. In the prospective part of our study, we found abnormal neutrophil oxidative index is associated single dose of acetaminophen within the first half-life[26] As prostaglandin E1 impairs neutrophil function and decreases ROS production, inhibition of prostaglandin synthesis was considered as a potential mechanism.[37] However we demonstrated that ibuprofen a more potent inhibitor of prostaglandin production failed to significantly alter the NOI, demonstrating that this is not the mechanism.

Acetaminophen-induced inhibition of myeloperoxidase activity was the considered as 1) acetaminophen has been shown to impair myeloperoxidase activity in i*n vitro* studies[30] and as abnormal DHR testing is seen in complete myeloperoxidase deficiency.[38] We demonstrated that myeloperoxidase activity was markedly reduced in test subjects after acetaminophen ingestion. Taken together, these results suggest that acetaminophen causes abnormal DHR testing by inhibiting myeloperoxidase, imitating myeloperoxidase deficiency.

A retrospective review convincingly demonstrated that practical implications of our findings as systemic administration of acetaminophen was frequently associated with an abnormal NOI. In our cohort of almost 200 cases, testing was abnormal nearly one-third (29.9%) of the time when acetaminophen had been administered within 24 hours before testing, and almost half of the time (44.8%) when patients had received acetaminophen within four hours. None of the patients had abnormal testing after 24 hours of acetaminophen. This was not the case with ibuprofen, steroids, or lidocaine, demonstrating that this effect is specific to acetaminophen.

To the best of our knowledge, this is the first *in vivo* study on the effect of acetaminophen on DHR testing. A primary goal of this research was to develop practical recommendations about the timing of DHR testing after acetaminophen, to guide clinicians evaluating patients for suspected CGD. As many patients undergoing screening for CGD are likely to require treatment options for fever or pain before DHR testing, we recommend using ibuprofen or an age-appropriate NSAID rather than acetaminophen. This will increase diagnostic accuracy and reduce the financial burden and patient anxiety associated with repeated DHR testing.

3.2.Limitations

We limited our testing to neutrophil activation with a single stimulus (PMA) as performed by our clinical laboratory throughout the time period of the study. Other reference laboratories may incorporate other stimuli such as fMLP into the assay. We presume that our findings would be relevant to all stimuli used, though we did not formally demonstrate this.

3.3. Future Research

The DHR assay can be transiently abnormal during acute viral illness.[21] One of the possible mechanism behind it could be impaired ROS production. In human population there is increased morbidity and mortality in patients who have bacterial infection superimposed to viral infections.[39,40] It has also been shown in studies done on mice that influenza impairs ROS production by phagocytic NADPH oxidase and decrease bacterial clearance during the recovery stage.[41] Whereas phagocytosis by neutrophils is unaltered.[41] Impaired ROS production could explain the mechanism of abnormal DHR assay during acute viral illnesses in otherwise healthy individuals[42]. To my knowledge, we are not certain which pathogens reduce ROS production and during which phases of its life cycle this effect is seen. Better guidelines for performing DHR assay in relation to acute viral illnesses is needed. Moreover, there is a need to develop techniques for cost-effective genetic testing for screening of CGD to avoid confounding factors, including acute illnesses, drug interactions, and MPO deficiency.

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