E FAECALIS INDUCES INCREASED PROTEIN EXPRESSION OF CASPASE-1 AND NLRP3 INFLAMMASOME – A SYSTEMATIC REVIEW

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Abstract

Introduction: Enterococcus faecalis, a dominant pathogen in pulpal and periapical diseases, induces an inflammatory response leading to increased interleukin 1 beta (IL-1β). Studies have implicated the role of Porphyromonas gingivalis and elevated levels of human Nucleotide Oligomerizing Domain Like Receptor Protein Pyrin 3 (NLRP3) inflammasome in addition to IL-1β. But due to the dominant role of E. faecalis in retreatment endodontic cases, the purpose of this systematic review was to prove that E faecalis is responsible for the activation of NLRP3 inflammasome and caspase-1 leading to increased levels of IL-1β.

Materials and Methods: This review was registered in the Open Science forum under https://doi.org/10.17605/OSF.IO/GC879. PubMed/Medline, Scopus and EBSCO databases were searched. In-vitro studies that analyzed the levels of NLRP3 inflammasome and caspase-1 using the (RT-PCR) and western immunoblotting tests all in one study were included.

Results: After fulfilling the inclusion criteria and careful scrutinization of the cross-references a total of 6 studies were selected. The results clearly indicate that E faecalis induces increased levels of NLRP3 inflammasome and caspase-1 in addition to interleukin 1β. The risk of bias assessment done using the QUIN tool showed a low risk of bias among the studies included.

Conclusion: The paucity of studies in this field clearly points out the limited research done and the ever-growing need to conduct more and more studies related to NLRP3 inflammasome and its association with pulpal and periapical diseases.

Keywords: NLRP3 inflammasome, E faecalis, ELISA test, RT-PCR, Western immunoblotting

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Introduction

The detection of Enterococcus faecalis in the root canals prior to three-dimensional obturation markedly reduces the prognosis of the case. It is the most commonly detected pathogen in failed root canals and research still continues to develop predictable methods for eradicating this pathogen (Gomes et al., 2004; Sundqvist et al., 1998).

Scientific evidence has further proved that it is difficult to eradicate Enterococcus faecalis as it invades dentinal tubules, forms biofilms and is resistant to intracanal medicaments such as calcium hydroxide (Athanassiadis et al., 2010; Evans et al., 2002; van der Waal et al., 2011). The frequent occurrence of apical periodontitis in dental clinics has been pointed out as bacterial invasion (Rechenberg et al., 2016).

The most commonly detected inflammatory cytokine is Interleukin 1 beta (IL-1β) in the periapical granulation tissue (Barkhordar et al., 1992). The formation of IL-1β comprises two events: Expression of pro-1L-1β and Maturation of 1L-1β (Franchi et al., 2009). The expression of pro-1L-1β is dependent on the Toll-like receptor-mediated nuclear factor Kappa B (NF-κB) while the maturation process is mediated by caspase -1 (Mariathasan et al., 2004; Zhang et al., 2015). Procaspase 1 is activated to caspase-1 by the inflammasome which are multiprotein complexes playing vital roles in innate immunity. These inflammasomes contain Pattern Recognition Receptors (PRRs) typically a Nucleotide Oligomerization domain-like receptor (NLR) or Absent in Melanoma-2 (AIM 2) like receptors.

The NLR and AIM-2 are activated by pathogens leading to the activation of caspase-1 (Ozaki et al., 2015). The most extensively studied NLR is the NLRP3 inflammasome and studies have demonstrated the increase in NLRP3 levels during an inflammatory process.

NLRP3 inflammasomes are cytoplasmic multiprotein complexes that play a vital role in innate immunity. E. Faecalis pathogen has been known to induce apoptosis and pyroptosis in human cells via the NLRP3 inflammasome but the pathway is not clear.

Hence this systematic review was carefully designed to include those in vitro studies that researched the impact of E faecalis on human macrophages and evaluated the levels of caspase-1 mRNA levels in addition to NLRP3 and IL-1β. This would establish the pathway of increased expression of IL-1β.

MATERIALS AND METHODS

The systematic review technique was developed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist (Page et al., 2021). The review has been registered at https://doi.org/10.17605/OSF.IO/GC879

Information sources

PubMed/Medline, Scopus and EBSCO databases were searched until January 2023 without the year's restriction and language. The summary of search results in the databases is summarized in **Table 1**. The references of eligible studies were checked for additional studies by D.A. and A.G. Google scholar and an OpenGrey search was performed for the grey literature as well. Wherever needed the authors of eligible papers were contacted for further information related to the study or any other additional details. Any doubts or clarifications were resolved with a senior researcher (A.S.).

Search Strategy

The research question raised for performing the search was: Do cells infected with E.Faecalis show increased levels of NLRP3 inflammasome?

The PICOS for the eligibility criteria was:

(P): Differentiated cell lines

(I): Differentiated cells infected with E. faecalis

(C): Cells without any E faecalis infection

(O): Increase in the levels of NLRP3 inflammasome and caspase1

(S): In-vitro setting

Keywords used for the literature search

A broad-based search was implemented with individual keywords: "E. faecalis", "NLRP3 inflammasome", and "caspase-1".

This was followed by the application of Boolean Tools AND and OR with the above-mentioned keywords.

The search strategy after eliminating various combinations was finalized as (("e faecalis"[All Fields]) AND ("nlrp3 inflammasome"[All Fields])) AND ("caspase1"[All Fields])

Study Selection and eligibility criteria

Inclusion criteria:

a) In Vitro studies or studies having a combination of animal studies and in vitro components in which differentiated cell lines were used.

b) Studies where the differentiated cells were infected with Enterococcus faecalis
c) Studies that used RT-qPCR tests to analyze the levels of either caspase-1, interleukin 1beta or NLRP3,
d) Studies that used western immunoblotting to analyze the protein levels of either caspase-1, interleukin 1beta or NLRP3,

The publications with their titles and abstracts discovered during the electronic and manual searches were reviewed by two investigators (D.A. and A.G.). The articles that did not meet the criteria for inclusion were weeded out. All of the remaining articles were retrieved and thoroughly screened by two of the above-mentioned reviewers to achieve a consensus.

Exclusion criteria:
a) In vivo studies that did not have in-vitro component
b) in vitro studies that did not use RT-PCR, Western Immunoblotting and ELISA test all in one study.
c) Studies that did not analyze NLRP3 inflammasome, caspase-1 and IL-1β.
d) case reports.
e) narrative reviews.

Data collection process
Based on the characteristics of the studies a Microsoft office 365 powered customized excel sheet was developed that was used to scrutinize each and every inclusion criterion so that homogeneity prevailed among the studies. Two reviewers (D.A. and A.G.) collected the data and entered it into the sheet. In case of any disagreement, it was resolved after consultation with a senior researcher (A.S). The references were managed with the EndNote Basic Software (Thomson, Reuters, New York, NY) and duplicates were removed accordingly.

Statistical Analysis
Considering the heterogeneity of the data, it was analyzed for descriptive characteristics without meta-analysis.

Risk of Bias of Individual Studies
The quality of included studies was assessed with a QUIN (Quality Assessment tools for in vitro studies) [13,14]. Studies that did not report 1–3 of the items were classified as low-risk studies, those which did not report 4–6 items were considered as moderate bias studies, and more than 6 of those with non-reported items were considered as having a high risk of bias. (**Table 2**)

Results
The search conducted in 3 databases identified a total of 09 studies. After the elimination of 3 duplicates and studies that did not fulfil the criteria, only 6 were identified with a detailed review of the cross-references as well. The details of the PRISMA chart are displayed in **Figure 1.**

Study characteristics
Table 3 describes the characteristics of the studies included and the results.
Confirmatory tests: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot procedures were carried out to analyze the levels of NLRP3 inflammasome in addition to IL-1β.

Western immunoblotting test
These tests indicated that there is a significant increase in IL-1β, caspase-1 and NLRP3 inflammasome in cells after exposure to E. faecalis

RT-PCR test
The relative expression of NLRP3 inflammasome messenger RNA (mRNA), caspase-1 and IL-1β were significantly higher than the control group thus reiterating the fact that E faecalis activated the NLRP3 inflammasome via the caspase 1 pathway leading to an increase in IL-1β levels.

Discussion
Of the 700-plus species of bacteria that dominate the oral flora, Enterococcus Faecalis has been known to be the most dominant pathogen, especially in post-treatment apical periodontitis refractory periodontitis (AlShwaimi et al., 2016).
The dental pulp is confined within the walls of the dentin-mineralized tissue and is known to comprise fibroblasts, undifferentiated mesenchymal cells, odontoblasts and macrophages.
The macrophages are the dominant cells in the innate immunity of the pulp which is the first line of defense against any pathogenic insult to the pulp (Anand et al., 2011; Hahn et al., 2000). The macrophages are known to initiate the inflammatory process and hence trigger the pathological processes in the pulp. These macrophages are known to exhibit the Pattern Recognition Receptor which scans the extracellular complex for any pathogenic insult. One such category of the PRRs is the NLRs which recognize the Pathogen-associated Molecular proteins (PAMPs) and

Danger-associated molecular proteins (DAMPs) .

Of the various inflammasomes that have been identified in humans, the Nucleotide Oligomerising Domain Like Receptor Protein Pyrin 3(NLRP3) is the most extensively studied inflammasome and is known to be present in all chronic inflammatory conditions . On activation, NLRP3 assembles into multi-protein cytoplasmic complexes, termed inflammasomes, which regulate the secretion and bioactivity of cytokines belonging to the IL-1 family (IL-1β, IL-18).

Pro interleukin 1 beta can be activated by two pathways and one of them is the caspase -1 mediated pathway which is through the NLRP3 inflammasome. The activation of NLRP3/caspase-1 inflammasome in irreversible pulpitis was systematically documented by Jiang W et al (Jiang et al., 2015). They concluded that NLRP3 protein expression was greater in irreversible pulpits than in the normal pulp. Another study by Song et al demonstrated the presence of NLRP3 in human dental pulp cells and tissues. They mentioned that NLRP3 may be easily detected through western blotting rather than immunohistochemistry (Song et al., 2012).

The research is void of the fact that there is no clear-cut understanding of the mechanism of E Faecalis in activating the NLRP3 inflammasome. Hence this systematic review was designed to include those in-vitro studies that used western immunoblotting studies to detect the presence of NLRP3 inflammasome in E faecalis-infected macrophages.

The results of this review clearly indicate that E. Faecalis has induced the maturation of pro-IL-1β was mediated by the NRLP3 inflammasome/caspase-1 pathway.

Ran S et al (Ran et al., 2021)conducted an in vitro study to investigate the pathogenicity of E. Faecalis and the molecular method of IL-1β secretion. They infected the THP-1 macrophages with E faecalis strains and subjected the samples to ELISA, western blotting and qRT-PCR test. Their results led them to conclude that increased levels of IL-1β were present at increased concentrations of E. Faecalis. Further, the increased levels of CASP-1 mRNA with increased levels of E faecalis confirmed that E faecalis induced the increased levels of IL-1β through the caspase-1 mediated NLRP3 inflammasome pathway. They further proved that silencing the NLRP3 inhibited the E.Faecalis induced IL-1β secretion which clearly leads one to infer that NLRP3 inflammasome is critical for IL-1β maturation and cell death.

Yang HH et al (Yang et al., 2014) analyzed the caspase -1 activation by E faecalis in macrophages. The cultured THP-1 macrophages were infected with E. Faecalis and subjected to ELISA, cell death assay, RT-PCR tests and western immunoblotting tests. Their results displayed increased IL-1β counts with the ELISA test while increased pro-IL-1β was revealed in the immunoblot tests. Their cell death assay tests confirmed the fact that E faecalis induced cell death via the caspase-1 mediated pathway. Hence it can be safely concluded here as well that E faecalis did induce the increased secretion of IL-1β via the caspase-1 mediated NLRP3 inflammasome pathway.

Wang L et al (Wang et al., 2016)conducted an animal model and in-vitro study. Since the invitro study characteristics were similar to our inclusion criteria we included this study. They investigated the effect of the molecular mechanism of E faecalis on the expression of NLRP3 inflammasome. The results of their study led them to conclude that the increased expression of caspase-1, NLRP3 and IL-1β with the confirmatory tests confirmed the role of E faecalis in inducing this via the caspase-1 pathway.

Apart from these, there was a study by Ran S (Ran et al., 2019) where odontoblasts were infected. It is noteworthy to mention here that the odontoblasts were infected with E faecalis and subjected to western blotting, and cell death assay tests. Their results revealed marked levels of NLRP3, IL-1β and caspase -1 through the mentioned confirmatory tests. They concluded that E. Faecalis promoted cell death via the caspase-1 pathway mediated by the NLRP3 inflammasome.

A study by Chi D et al (Chi et al., 2021)studied murine macrophages infected with different strains of E. faecalis. However, the results of this study revealed increased levels of caspase-

1, NLRP3 and IL-1β in the cells after inoculation with E. faecalis. It can be safely concluded that E faecalis induced cell death and increased levels of NLRP3 in addition to IL-1β through the caspase-1 pathway.

Chung IC (Chung et al., 2019) used E faecalis as a probiotic to modulate the effect on NLRP3 inflammasome. Their results led them to conclude that attenuated form of E faecalis can reduce the effect of NLRP3 inflammasome. Hence, we could safely conclude that E faecalis directly activates the NLRP3 inflammasome leading to increased levels of IL-1β which sets off the inflammation cascade.

The highly limited literature further reiterates the fact that more such research needs to be carried out to understand the molecular basis of pulpal inflammation. The NLRP3 inflammasome which has been particularly detected in GCF and Saliva has indicated the role of inflammasome as a "Molecular diagnostic marker". Studies have established the levels of NLRP3 inflammasome in Saliva and GCF for periodontitis cases (Isaza-Guzmán et al., 2017; Yang et al., 2014). Hence with a paradigm shift towards molecular diagnostic aids in pulpal and periapical diseases, the detection of NLRP3 inflammasome levels as diagnostic markers may be the future in clinical diagnosis of pulpal and periapical diseases. Previous studies have already established that NLRP3 is responsible for the stimulation of caspase -1 by L. Monocytogens (Ozören et al., 2006) and P. gingivalis (Park et al., 2014)

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Thus, the main objective of this systematic research was to highlight the role of NLRP3 inflammasome in the endodontic infections caused by E faecalis which is the most dominant pathogen in retreatment cases. The role of E faecalis in endodontic infections and the difficulty in eradicating the same is very well evidenced by rich pioneering research studies. Thus, with this systematic review, although the number of included studies is less and low evidence may be cited but the take-home point is very clear as to the involvement of E faecalis in NLRP3 inflammasome activation and how the detection of NLRP3 inflammasome could provide major breakthrough in diagnosis and treatment planning.

Conclusion

The present systematic review has proved that E faecalis is responsible for inducing increased levels of NLRP3 inflammasome and caspase-1 proteins. Further increased levels of Interleukin 1β which is responsible for the inflammation cascade.

We affirm that we have no financial affiliation (e.g., employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past three years.

Declarations

Competing interests: Not applicable

Authors' contributions

DA, AS and AG designed the experiments;
DA performed experiments and collected data;
DA, AG and AS discussed the results and strategy;
AS Supervised, directed and managed the study;
AS , DA and AG Final approved of the version to be published

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Availability of data and materials

Not Applicable

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Legends

Table 1: Search strategy
Table 2: Risk of Bias assessment
Table 3: Characteristics of the study
Figure 1: PRISMA chart

Table 1 : Search Strategy

Data base	Search strategy (2022)	n
PubMed	(("e faecalis"[All Fields]) AND ("nlrp3 inflammasome"[All Fields])) AND ("caspase1"[All Fields])	05
EBSCOhost	(("e faecalis"[All Fields]) AND ("nlrp3 inflammasome"[All Fields])) AND ("caspase1"[All Fields])	03
Scopus	TITEL-ABS-KEY(("e faecalis"[All Fields]) AND ("nlrp3 inflammasome"[All Fields])) AND ("caspase1"[All Fields])	00
Gray literature	(("e faecalis"[All Fields]) AND ("nlrp3 inflammasome"[All Fields])) AND ("caspase1"[All Fields])	0

Table 2: Risk of bias assessment

	Clearly stated aims and objectives	Detailed explanation of sample size calculation	Detailed explanation of sampling technique	Details of comparison group	Detailed explanation of methodology	Operator details	Randomization	Method of measurement of outcome	Outcome assessor details	Blinding	Statistical analysis	Presentation of results	Overall assessment
Wang L et al J Endod. 2016	+	Unclear	Unclear	+	+	Unclear	+	+	+	+	+	+	Low risk of bias
Yang HH et al J Endod. 2014	+	Unclear	Unclear	+	+	Unclear	+	+	+	+	+	+	Low risk of bias
Ran S et al Microb Pathog. 2021	+	Unclear	Unclear	+	+	Unclear	+	+	+	+	+	+	Low risk of bias
Ran S et al Int Endod J. 2019	+	Unclear	Unclear	+	+	Unclear	+	+	+	+	+	+	Low risk of bias
Chi D et al Front Cell Infect Microbiol. 2021	+	Unclear	Unclear	+	+	Unclear	+	+	+	+	+	+	Low risk of bias
Chi D et al J Clin Med. 2022	+	Unclear	Unclear	+	+	Unclear	+	+	+	+	+	+	Low risk of bias

Table 3: Charecteristics of the study

Author, journal and year	Type of study	Cells used	Bacteria used in the test group to infect the cells	Control group	Western blot analysis	RT-qPCR analysis	Conclusion
Wang L et al J Endod. 2016	In vitro cell culture and in vivo	RAW264.7 cells	E faecalis	Yes	Exposure of the cells to 10μg/mL LTA from E. faecalis led to significant increase in levels of NLRP3, caspase-1 and IL-1β	Relative expression of NLRP3, caspase-1 and IL-1β was significantly higher than the negative control group	E faecalis activate the NLRP3 inflammasome leading to increased levels of caspase-1 and IL-1β
Yang HH et al J Endod. 2014	In vitro study	THP-1 cell line	E faecalis	Yes	Exposure of the cells to E faecalis resulted in increased levels of caspase-1 and IL-1β	Relative expression of NLRP3, caspase-1 and IL-1β was significantly higher than the negative control group	E faecalis activate the NLRP3 inflammasome leading to increased levels of caspase-1 and IL-1β
Ran S et al Microb Pathog. 2021	In vitro study	THP-1 cell line	E faecalis	Yes	Exposure of the cells to E faecalis resulted in increased levels of caspase-1 and IL-1β	Relative expression of NLRP3, caspase-1 and IL-1β was significantly higher than the negative control group	E faecalis activate the NLRP3 inflammasome leading to increased levels of caspase-1 and IL-1β
Ran S et al Int Endod J. 2019	In vitro study	Human osteosarcoma MG63cells	E faecalis	Yes	Exposure of the cells to E faecalis resulted in increased levels of caspase-1 and IL-1β and NLRP3 inflammasome protiens		E faecalis activate the NLRP3 inflammasome leading to increased levels of caspase-1 and IL-1β
Chi D et al Front Cell Infect Microbiol. 2021	In vitro study	RAW 264.7 cell line	E faecalis	Yes	Exposure of the cells to E faecalis resulted in increased levels of caspase-1 and IL-1β and NLRP3 inflammasome protiens	Relative expression of NLRP3, caspase-1 and IL-1β was significantly higher than the negative control group	E faecalis activate the NLRP3 inflammasome leading to increased levels of caspase-1 and I L-1β
Chi D et al J Clin Med. 2022	In vitro study	RAW 264.7 cell line	E faecalis	Yes	Exposure of the cells to E faecalis resulted in increased levels of caspase-1 and IL-1β and NLRP3 inflammasome protiens	Relative expression of NLRP3, caspase-1 and IL-1β was significantly higher than the negative control group	E faecalis activate the NLRP3 inflammasome leading to increased levels of caspase-1 and I L-1β

Identification of studies via databases and registers

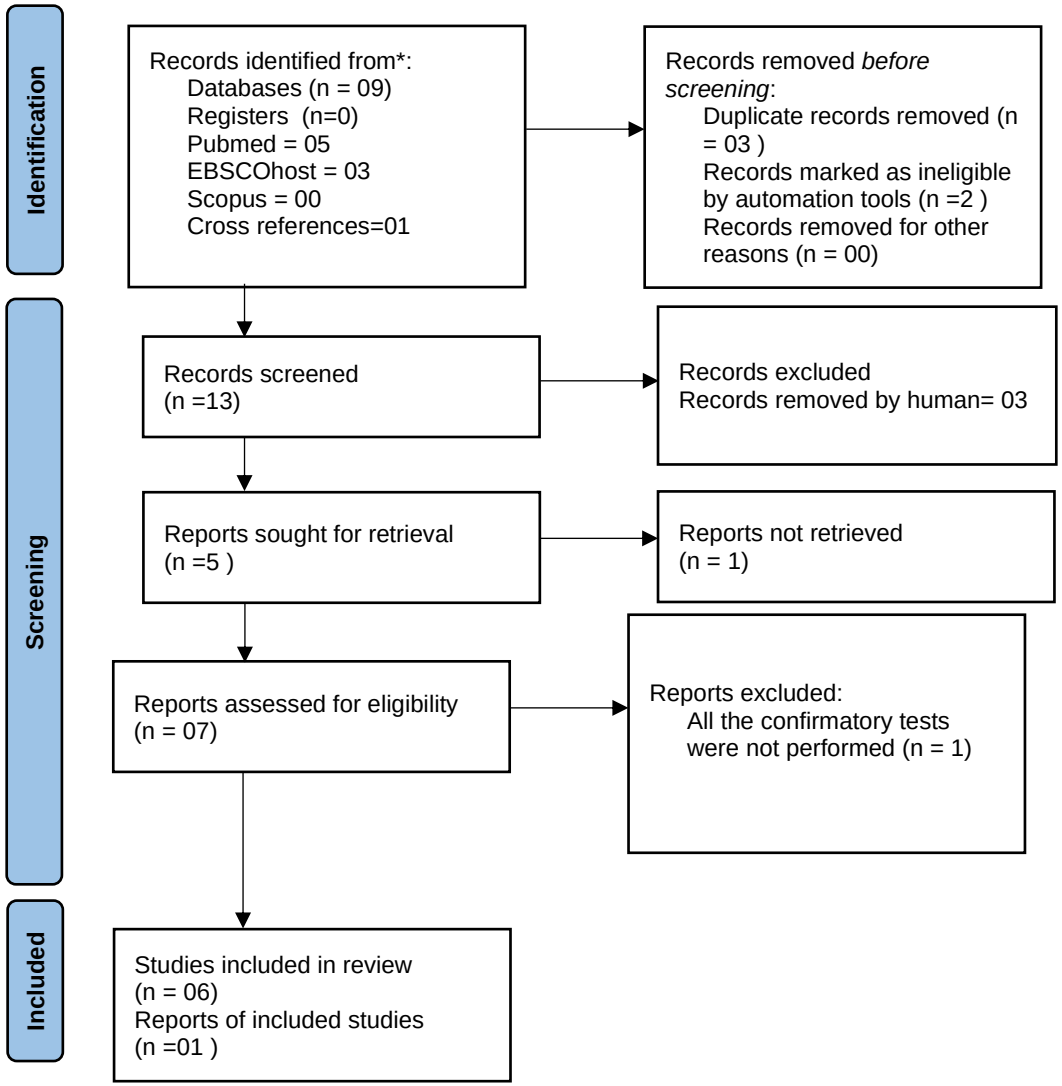

Fig 1: PRISMA FLOW CHART