Carvacrol inhibits osteoclast differentiation induced by RANKL in RAW264.7 cells

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**Article History:**

Received on: 09 Oct 2020
Revised on: 26 Nov 2020
Accepted on: 20 Dec 2020

**Keywords:**

RAW264.7 cells, RANKL, Osteoclast, TRAP, Cathepsin K

**Abstract**

RAW264.7, a murine macrophage cell line, an osteoclast model used for the differentiation of Osteoclast by inducing RANKL. Carvacrol, a monoterpenoid phenol that possess various medicinal property, was used for the treatment of osteoclast cells in this study. We investigate the effect of carvacrol in osteoclast cells induced by RANKL in RAW264.7 cells. RAW264.7 cells, cultured along with 40ng/ml of RANKL, on day 5, the cells were treated with TRAP staining to assess the formation of osteoclast cells, the presence of multinucleated TRAP positive cells were visualised using an inverted light microscope. The osteoclast cells were treated with varying concentrations of carvacrol (0-200 μM) respectively for 48 hours. By MTT assay, it was found that there was no cytotoxic effect induced by carvacrol in RAW264.7 cells, whereas in RANKL induced osteoclast cells, there was a significant change in a dose dependent manner for 48 hours. By western blot and agarose gel electrophoresis, the levels of osteoclastogenic marker genes such as TRAP and cathepsin k were assessed and the developed osteoclast cells were treated with 150 μM of carvacrol and found a significant change on treatment with carvacrol when compared with control. The present study reveals the anti-osteoclastogenic effect of carvacrol on osteoclast cells induced by RANKL in RAW264.7 cells.

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ISSN: 0975-7538
DOI: [https://doi.org/10.26452/ijrps.v11iSPL4.4371](https://doi.org/10.26452/ijrps.v11iSPL4.4371)

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**INTRODUCTION**

In a healthy individual, bone remodelling is coupled with the resorption and formation of bone via Osteoclast and osteoblast (Raggatt and Partridge, 2010). Pathologies related to bone such as osteoporosis, rheumatoid arthritis, periodontitis are linked with increased osteoclast formation and activity (Blair and Athanasou, 2004). Osteoclast is the cell with multinuclei from hematopoietic progenitor of macrophage lineage involved in the resorption of bone (Charles and Aliprantis, 2014). The resorption by Osteoclast takes place by creating a microenvironment rich in minerals and organics, which dissolve the bone and degrade it (Bar-Shavit, 2007). A sealing zone is formed by the osteoclast cells, which tightly seal the bone matrix by encircling the ruffled border and thereby increase the bone resorption (Bar-Shavit, 2007). RANKL, a receptor activator of the necrosis factor kappa B ligand, binds to the receptor RANK, an important step involved in the osteoclastogenesis in which the mononuclear osteoclast precursor cells are fused to form mature multinucleated osteoclasts (Nakashima and Takayanagi, 2011). Multinucleated mature osteoclast cells secrete an enzyme known as TRAP, tar-
trate resistant acid phosphatase, which is used as a marker to detect the presence of the mature Osteoclast, which has the capacity to breakdown the collagen and undergo resorption of the bone matrix (Halleen, 2003). Activation of RANK-RANKL leads to activation of various signalling cascades such as NF-κB, MAPK, ERK. Activation of these pathways leads to the promotion of various transcriptional factors such as NFATc1, Cathepsin K (CTSK), tartrate resistant acid phosphatase (TRAP), matrix metalloproteinase-9 (MMP-9) (Kasonga et al., 2015; Kim et al., 2008). Cathepsin K and MMP-9 are the lysosomal enzymes which degrade the organic matrix (Henriksen et al., 2011; Väänänen and Laitala-Leinonen, 2008; Takayanagi, 2007).

Numerous studies proved that RAW264.7 cells could stimulate multinucleated cells with characteristics required for osteoclast differentiation (Xu et al., 2012). RAW264.7, a murine macrophage an osteoclast precursor cell line which expresses the receptor c-FMS, which stimulate M-CSF and undergo differentiation and develops multinucleated osteoclast bone resorbing cells when prompt with RANKL (Collin-Osdy and Osdoby, 2012). As RANKL, a primary signal required for the osteoclast differentiation, which triggers the downstream signalling molecules required for the formation of Osteoclast. Targeting the RANKL signalling pathway may be an effective approach to inhibit bone resorption and osteoclastogenesis. This study aims to evaluate the effect of carvacrol, a natural monoterpenoid with various medicinal properties that can inhibit the RANKL induced osteoclast differentiation in RAW264.7 cells.

MATERIALS AND METHODS

Cell lines and reagents
RAW264.7 cells were purchased from NCCS, Pune. RANKL, Cathepsin k, Trap antibody and Carvacrol were purchased from Sigma Aldrich.

Osteoclast differentiation
RAW264.7 cells were cultured in 24-well dishes at a density of 1.3 × 10^5 cells per well and allowed to adhere overnight. DMEM was then replaced, and the cells were treated with 40 ng/mL of RANKL. On Day 5, cultures were stained to detect TRAP expression using an acid phosphatase kit and the total number of TRAP-positive multinucleated osteoclasts (3 nuclei).

MTT assay
After the formation of osteoclast cells, the cells were treated with varying concentrations of carvacrol ranging from 0-150 μM respectively for 48 hours. About 20μl of MTT solution was added and kept at incubation for 3 hours at room temperature. 150 μl of DMSO per well was used to dissolve the formazan and the absorbance was measured at 490nm using a microplate reader (Bio-Rad, USA).

TRAP staining
Osteoclast cells were washed using PBS and using 37% formaldehyde the cells were fixed for 30 sec, then cells were incubated for 10 minutes with Triton X-100 (0.1%). Cells were then washed and incubated at room temperature in the dark for 1 hour with the solution containing the mixture of sodium nitrite, naphthol AS-BI phosphoric acid, acetate, fast garnet GBC and tartrate leukocyte acid phosphatase Assay kit by following the manufacturer’s instruction. Using a hematoxylin solution, the counterstaining was carried out for 2 min. After washing the cells gently, the TRAP positive multinucleated cells were counted using an inverted phase contrast microscope.

Western blot
Using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), about 30μg of proteins were separated and then transferred to (NC) nitrocellulose membrane using a semi dry transferring unit. By using TBS, the membrane was blocked at room temperature for 1 hour with 5% BSA. Then the membrane was washed thrice with TBST. After washing, the membrane was incubated with primary antibody (TRAP and Cathepsin- K) dilution is 1:500 and secondary antibody dilution 1:5000 with BSA overnight at 4°C. Membrane after incubation was washed thrice with TBST and incubated with secondary antibodies conjugated Horseradish- Peroxidase at room temperature for 1 hour. Finally, the band chemiluminescence intensity was observed using the ECL kit solution from Sigma Aldrich and exposed to chemidoc XRS System (Bio-Rad).

RNA Extraction and synthesis of cDNA
Osteoclast cells after treatment with carvacrol, total RNA was isolated using TRizol reagent and the RNA was quantified using Bio Photometer. About 1 μg of RNA was used for cDNA synthesis and primers are tabulated in Table 1. By using Iscript cDNA synthesis Kit from Bio-Rad, PCR polymerase chain reaction amplifications were carried out 30 cycles for Cathepsin K (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min), 40 cycles for TRAP (95°C for 60 s, 55°C for 30 s, and 60°C for 60 s), 40 cycles for GAPDH (94°C for 60 s, 60°C for 60 s, and 72°C for the 60s). After amplification, the PCR product was then electrophoresed by using agarose gel of 2% and
visualised in XRS plus gel doc (Bio-Rad, USA). Using image lab software (Bio-Rad, USA), the densitometric analysis was carried out.

RESULTS

Effect of Carvacrol on RAW264.7 cells and in Osteoclast cells

Raw264.7 cells and RANKL induced osteoclast cells were treated with different concentrations of carvacrol 0.25, 50, 75, 100, 125, 150, 200 \( \mu \)M respectively for 48 hours. We found that osteoclast cells developed from RANKL decreased significantly on treatment with carvacrol in a dose dependent manner, whereas no significant changes were observed in RAW 264.7 cells alone. Carvacrol shows a significant difference in dose dependent manner, as shown in Figure 1.

Effect of Carvacrol in RANKL induced Osteoclast

An important stimulator of Osteoclast is the RANKL, the cytokine. RAW264.7 cells on treatment with 40 ng/ml of RANKL stimulate osteoclast cells on day 5. After the formation of osteoclast cells, the cells were treated with various concentration of carvacrol such as 75, 100, 125 and 150 \( \mu \)M respectively for 48 hours and Trap staining assay was carried out and found that the multinucleated osteoclast cells were reduced with respect to the increasing concentration of carvacrol as shown in Figure 2.

Effect of Carvacrol on TRAP and Cathepsin K

We further analysed the effect of carvacrol on specific markers related to Osteoclast using mRNA and protein. Osteoclastogenic marker genes include cathepsin K and TRAP. Cathepsin K, a cysteine protease, plays an important role in bone resorption related to Osteoclast on treatment with carvacrol, the level of cathepsin K was found to be reduced both in mRNA and protein level when compared with the RANKL treated group. TRAP, an osteoclast marker during bone resorption, is found to be elevated and found abundantly in bone resorption, whereas in carvacrol treated groups, it was found to be decreased, as shown in Figure 3. Carvacrol at 150 \( \mu \)M downregulated the expression of both Trap and cathepsin K.

DISCUSSION

In an individual, the remodelling of bone takes place throughout their life (Rucci, 2008). During pathological conditions, resorption of bone will be more than bone formation, which leads to disruption in the activity and formation of Osteoclast (Feng and McDonald, 2011). Osteoclast formation is regulated by the cytokine RANKL (Wada et al., 2006). In a disease condition like rheumatoid arthritis, osteolysis induced by cancer and osteoporosis in which the signalling pathway involved in the regulation of bone was disturbed (Helfrich, 2003). And increase the risk of fractures which alter the formation of Osteoclast (Redlich and Smolen, 2012).

By targeting the formation of Osteoclast by a natural compound that exhibits anti-inflammatory activity can inhibit osteoclastogenesis. In the present study, a murine RAW 264.7 macrophage cell line was used as an osteoclast model. RAW264.7 cell lines were cultured in the presence of RANKL and differentiated the same into Osteoclast and were used for our further studies, which is well supported by the previous findings (Xu et al., 2012).

Carvacrol found to exert an antioxidant, anti-inflammatory and anti-osteoclastogenic property due to its vast medicinal values the efficacy of carvacrol was assessed on Osteoclast and found that carvacrol shows no morphological changes in RAW264.7 cells when compared with RANKL treated group, which was evaluated by cytotoxic assay. TRAP is an enzyme that is expressed highly in Osteoclast (Ljusberg et al., 2005). Increasing concentration of carvacrol reduced the multinucleated TRAP cells when compared with control which was evaluated by TRAP staining assay. TRAP staining assay assessed at varying concentrations of carvacrol and at higher concentration, it was more efficient when compared with low dosage. To further assess the effect of carvacrol, the level of TRAP enzymes was evaluated in both mRNA and protein level and found that increased concentration of carvacrol reduced the expression of TRAP. Cathepsin K, a collagenase that degrades the collagen I in the bone matrix and it was found that processes the proteolytic and trafficking of intracellular enzymes TRAP (Ishikawa et al., 2001). To check the previous findings of Ljusberg J et al., we assessed the expression of cathepsin K both in mRNA and protein level and found that carvacrol at increased concentration reduced the activity of cathepsin K. Our preliminary findings showed that carvacrol exerts an anti-osteoclastogenic property as it reduces the levels of TRAP positive multinucleated cells as well as TRAP and Cathepsin K expression in both mRNA and protein levels. This was a preliminary study conducted. Furthermore, experiments will be carried out to assess the exact molecular mechanism involved in osteoclastogenesis and the efficacy of carvacrol in Osteoclast.

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Table 1: List of Primers

| Target   | Forward Primers          | Reverse Primers          | Size (bp) |
|----------|--------------------------|--------------------------|-----------|
| Cathepsin-K | CCGCAGTAATGACACCCTTT   | AAGGCATTGGTCATGTAGCC   | 258       |
| TRAP     | CCAATGCCAAAGAGATCGCC    | TCTGTGCAGAGCTGGCCAAG    | 216       |
| Gapdh    | TCAAGCTCATTTTCTGGTAT    | GTGAGGGTCTCTCTCTTCCT    | 141       |

Figure 1: Represents the cytotoxic effect of carvacrol in osteoclast cells induced by RANKL in RAW264.7 cells by MTT assay.

Figure 2: RAW264.7 cells were differentiated along with 40 ng/ml of RANKL and carvacrol for 5 days and multinucleated osteoclast cells were visualised using an inverted microscope.

Figure 3: Cathepsin-K and TRAP with significant *p < 0.05; #p < 0.01, $p < 0.001. The values were normalised with internal control GAPDH (mean ± SEM) of three independent experiments.
CONCLUSIONS

In this study, we conclude that carvacrol exhibits an anti-osteoclastogenic property and inhibits the RANKL induced osteoclastogenesis and it possesses an inhibitory effect on the formation of Osteoclast. Further studies based on molecular level should be carried out both in vivo and in vitro model.

ACKNOWLEDGEMENT

I would like to thank our director Dr P. Rasappan for providing us with the opportunity to carry out the research work in his esteemed institution.

Conflicts of Interest

The authors declare that they have no conflict of interest for this study.

Funding Support

The authors declare that they have no funding support for this study.

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