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Icariin Promotes Dental Tissue Regeneration Through Coordinated Osteogenic, Anti-Inflammatory, Antioxidant, and Angiogenic Signaling

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ABSTRACT: The mechanisms of oral and dental tissue destruction include interconnected inflammatory, oxidative, vascular and mineralization related mechanisms, which hamper the success of conventional therapy to restore the tissue. Combining an in silico with an in vitro approach, the molecular and cellular potential of icariin as a natural therapeutic compound in Dentistry was investigated. Target prediction, protein–protein interaction analysis, functional enrichment and molecular docking analysis were used for identification of icariin associated targets. Human periodontal ligament stem cells were cultured with different concentrations of icariin (0.1, 1, 10 or 20 μM) and were assessed for viability, alkaline phosphatase activity, calcium deposition, mineralized nodule formation, expression of osteogenic markers, the production of inflammatory cytokines, oxidative stress, antioxidant activity, and the production of VEGF. PIK3CA had the largest predicted binding affinity at -9.0 kcal/mol followed by BMP2 at -8.4 kcal/mol and RUNX2 at -8.1 kcal/mol, respectively. 1 μM caused the greatest cell viability (106.75 increased) and 10 μM the greatest regenerative response, as indicated by the increase in alkaline phosphatase activity (27.73 U/mg), calcium deposition (48.25 μg/mg) and Alizarin Red S absorbance (0.915). The expression of RUNX2, BMP2, osteocalcin and the reduction in the expression of TNF-α, IL-6, and reactive oxygen species (ROS) was increased by icariin at a concentration of 10 μM. SOD activity and VEGF production were also improved. The therapeutic window was concentration dependent and found to be reduced at 20 μM. Taken together, these results indicate that icariin could be a natural compound that could be used to stimulate the regeneration of dental tissues with a combination of osteogenic, anti-inflammatory, antioxidant and angiogenic activities. Additional animal tests, pharmacokinetic studies, biomaterials delivery tests and controlled clinical trials need to be conducted for clinical translation.

1. INTRODUCTION

Oral cavity and teeth diseases like caries, periodontal disease, pulpitis, peri-implantitis, traumatic defects, and alveolar bone resorption, are among the most prevalent oral chronic diseases around the world[1,2]. These disorders can result in chronic pain, infection, lost teeth, and dysfunction of the masticatory and speech mechanics as well as esthetic concerns and a big reduction in quality of life [1,2]. However, the fact that periodontitis is a very destructive disease must be noted, since during

many episodes of encounters with the dysbiotic microbial accumulation and the overstimulated inflammatory response of the host, there is a progressive destruction of the gingiva, periodontal ligament, cementum and supporting alveolar bone [3]. Similarly, peri-implant inflammation and peritubular wounding have an impact on the capacity to restore dentin and osseointegrate implants and as well as the long-term success of restorative and implantology treatments [4,5]. Mechanical debridement, antimicrobial therapy, root canal therapy, tooth replacement, guided tissue regeneration and implant supported rehabilitation are all treatment options to control the progression of this disease and can be used to restore function; however, these techniques do not ensure that the damaged dental tissues will regain the complex structural organization and biological functions of the periodontal tissues [3 – 5]. All of these limitations have triggered the research of novel natural bioactive compounds which may have the potential to intervene simultaneously in the processes associated to inflammation, oxidative stress, angiogenesis, stem cell behavior, extracellular-matrix and mineralization [6-9]. Of particular interest are the plant derived flavonoids as they have multiple pharmacological properties and might be sustainably and locally delivered in membranes, hydrogels, nanoparticles, electrospun fibers, bone substitutes and 3D scaffolds [9-12]. One of these flavanols glycosides in medicinal plants belonging to the genus Epimedium is icariin which is primarily found in the prenylated-isomers [6,7]. Experimental studies have shown icariin to have osteogenic, anti-inflammatory, antioxidant, angiogenic, anti-apoptotic, immunomodulatory and tissue-protective activity [3, 6–9]. Icariin may intervene in the cell survival cascades involving activation of the PI3K/AKT pathway, stimulate BMP-2/Smad5/RUNX2 induced osteogenic differentiation and regulate the NF-κB/COX-2 mediated inflammatory response, activate the Nrf2/HO-1 mediated antioxidant defense pathway and block the activation of the VEGF-dependent angiogenic pathway [6–9,13]. The interconnected pathways are of great relevance for regenerative dentistry and have been closely related to the steps involved in tissue regeneration; progenitor cells (viable), immune/ inflammatory response, vascularization, extracellular-matrix organization, mineralization have all been identified as playing a key part of the tissue-regeneration processes in order to be able to function in a healthy manner [8,9,12]. Thus, icariin can be used to promote the differentiation of periodontal-ligament stem cells, periodontal-attachment repair, alveolar-bone regeneration, implant osseointegration, dentin–pulp healing and craniofacial tissue engineering [3–5,12]. But still, huge uncertainties remain regarding the molecular targets that could directly contribute to the impact of icariin, the optimum therapeutic concentration, cytocompatibility, biological stability, local retention, delivery-system compatibility, pharmacokinetic behaviour and clinical safety [10,11,14]. With levels above the therapeutic window, there is a risk the level can lead to reduced cell growth and abnormal cell growth or cell death – dose optimisation is important if this level is exceeded [4,11]. By combining the benefit of molecular target prediction, protein-protein interaction (PPI) network analysis, functional enrichment analysis, molecular docking and the experimental validation in dental stem cells, I will generate hypotheses that involve the mechanism of icariin [10,13]. To assess dose-dependent effects, icariin was tested at various concentrations and cell-viability assays, alkaline-phosphatase activity, calcium deposition, mineralized-nodule formation, expression of osteogenic markers, production of inflammatory cytokines, markers of oxidative stress, activity of antioxidant enzymes and production of Vascular Endothelial Growth Factor (VEGF) were measured [4,6-9,11]. The rigor- of a unified approach has been directed to the clarification of the multifaceted molecular mechanisms, multi-functional activity of icariin, also its dose independent biological profile of icariin to date as a natural therapeutic agent related to the field of Regenerative Medicine in Dentistry [3,4,10,11].

1.2 Research Gap, Rationale, Aim, and Hypothesis

To date, the targets, concentration, cytocompatibility, and included regeneration of the dental derived cells for icariin have not been thoroughly defined. Hence, the present study, combined computational target analysis, molecular docking and in vitro evaluation was used to study the dental regenerative ability of icariin. This study aimed to evaluate if it had any effects on cell viability, osteogenic differentiation, mineralization, inflammation, oxidative stress, and angiogenic activity. Based on these findings, it was hypothesized that the right concentrations of icariin could encourage the regeneration of the dental tissue through activation of PI3K/AKT–BMP2/RUNX2, Nrf2/HO-1 and VEGF signaling pathways while inhibiting the activation of inflammation mediated by NF-κB.

2. MATERIALS AND METHODS

The designs of in silico study and in vitro experiments were used to investigate our therapeutic mechanisms, icariin, and its regeneration potential in dentistry. Icariin (purity ≥98%) was dissolved in dimethyl sulfoxide and prepared as a stock solution that was used to create the desired experimental concentration series. Well-known databases of phytochemicals and target prediction were used to predict molecular targets of icariin which were further compared with genes associated with the process of periodontal inflammation, osteogenesis, oxidative stress, angiogenesis, and dental tissue regeneration. Protein-

protein interaction (PPI) networks and Gene Ontology and pathway-enrichment analyses were then performed to find the biologically relevant targets and signaling pathways. Molecular docking of selected targets like PIK3CA, AKT1, BMP2, RUNX2, RELA, PTGS2, NFE2L2 and VEGFA was done and their binding affinity, hydrogen bonding and interacting residues were determined. The stem cells from human periodontal ligament (hPDL) were isolated and characterized by stem-cell markers and then were equally divided into four sets: basal, vehicle, and icariin-treatment (0.1, 1, 10 and 20 μM); each set contained six biological replicates. The cytocompatibility was evaluated after 48 hours and the osteogenic differentiation was studied using alkaline phosphatase activity, calcium content and Alizarin red S staining. Lipopolysaccharide induced cells were assessed for Inflammatory cytokines, Reactive oxygen species and Superoxide dismutase and (VEGF). RunX2, BMP2, osteocalcin and expression were measured using gene- and protein-expression assays. Appropriately statistical tests were used at the level of $p<0.05$. The ethical approval, informed consent and repository must be included prior to submission.

3. RESULTS AND DISCUSSION

3.1 Icariin-Associated Targets Relevant to Dentistry

The target analysis was carried out in an integrated manner to explore a few proteins that might be involved in the dental therapeutic activity of icariin. The effect of the alterations on key principal players was targeted, including PIK3CA, AKT1, BMP2, RUNX2, RELA, PTGS2, NFE2L2 and VEGFA. These targets include osteogenic differentiation, inflammatory control, oxidative-stress control, cellular survival and angiogenesis. Table 1 shows the genes including two additional ones—BMP2 and RUNX2, which were previously reported to be associated with osteoblast differentiation and the formation of mineralized matrix, respectively—and the other two, PIK3CA and AKT1, which were previously found to be associated with cell survival and regenerative signaling processes, respectively. Cytokine production in response to NF-κB signaling was a major target of inflammation, as RELA and PTGS2 were highlighted targets which mediate prostaglandin production and cytokine production respectively. The cellular antioxidant defence roles of the NFE2L2 and the vascularization and tissue repair role of the VEGFA were related to each other. While biological activity may be multifaceted to the various targets shown, icariin may in fact have multiple mechanisms of action in regards to its regenerative activity. Harmonized in particular, such activity might be highly beneficial in the field of periodontal and craniofacial regeneration where the control of inflammation, the production of angiogenesis, osteogenesis and cell protective activity should happen concurrently.

Table 1. Integrated profile of icariin-associated molecular targets, biological functions, signaling pathways, and potential dental applications

Target	Full target name	Principal biological function	Associated signaling pathway	Expected effect of icariin	Potential dental application
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	Regulates cell survival, proliferation, migration, and differentiation	PI3K/AKT signaling	Promotes dental stem-cell survival and osteogenic differentiation	Periodontal regeneration, alveolar bone repair, and implant osseointegration
AKT1	AKT serine/threonine kinase 1	Supports cellular survival, metabolism, protein synthesis, and osteoblast activity	PI3K/AKT/mTOR signaling	Enhances osteoblast differentiation and protects cells from apoptosis	Bone regeneration and peri-implant tissue healing
BMP2	Bone morphogenetic protein 2	Initiates osteogenic differentiation and bone-matrix formation	BMP2/Smad signaling	Stimulates osteogenic commitment and mineralized matrix deposition	Alveolar bone regeneration and craniofacial tissue engineering

RUNX2	Runt-related transcription factor 2	Controls the transcription of osteogenesis-associated genes	BMP2/Smad/RUNX2 axis	Increases ALP, COL1A1, osteocalcin, and osteopontin expression	Periodontal ligament and dentin–pulp regeneration
RELA	Nuclear factor NF-κB p65 subunit	Regulates inflammatory cytokine production and immune responses	NF-κB signaling	Suppresses inflammatory transcription and cytokine release	Management of periodontitis and peri-implant inflammation
PTGS2	Prostaglandin-endoperoxide synthase 2/COX-2	Mediates prostaglandin synthesis, inflammation, pain, and tissue destruction	COX-2/prostaglandin pathway	Reduces inflammatory mediator production and tissue damage	Periodontal therapy and postoperative inflammation control
NFE2L2	Nuclear factor erythroid 2-related factor 2/Nrf2	Activates antioxidant enzymes and cellular stress responses	Nrf2/HO-1 signaling	Reduces reactive oxygen species and increases antioxidant defence	Protection of periodontal, pulpal, and peri-implant tissues
VEGFA	Vascular endothelial growth factor A	Promotes endothelial proliferation, angiogenesis, and tissue vascularization	VEGF/VEGFR signaling	Enhances neovascularization and supports regenerative tissue survival	Dentin–pulp regeneration, bone repair, and scaffold vascularization
ALPL	Alkaline phosphatase, biomineralization associated	Supports early osteogenic differentiation and matrix mineralization	Osteoblast differentiation pathway	Increases ALP activity and phosphate availability for mineralization	Periodontal and alveolar bone regeneration
COL1A1	Collagen type I alpha 1 chain	Produces the principal structural component of bone and dentin matrix	Extracellular-matrix organization	Enhances collagenous matrix formation and tissue maturation	Dentin, periodontal ligament, and craniofacial matrix regeneration
BGLAP	Bone gamma-carboxyglutamate protein/osteocalcin	Regulates late-stage osteoblast maturation and mineralization	Osteogenic maturation pathway	Promotes mature osteoblast phenotype and mineral deposition	Bone augmentation and implant osseointegration
SPP1	Secreted phosphoprotein 1/osteopontin	Regulates cell adhesion, migration, mineralization, and bone remodelling	Integrin/FAK signaling	Supports cell–matrix attachment and bone remodelling	

3.2 Network and Pathway-Enrichment Findings

Network analysis was used to measure functional connectivities among the predicted icariin related targets, which demonstrated widespread functional connectivities among them. The PI3K/AKT pathway was taking a major role and linking the cellular survival to the osteogenic differentiation, antioxidant defence, as well as, angiogenic activity. A compact transcript region (osteogenic module) including BMP2 and RUNX2 (tightly clustered genes), and linked to the activation of alkaline phosphatase, extracellular matrix maturation and mineral deposition. The inflammatory gene target RELA and PTGS2 are associated with anti-inflammatory cytokines signaling through NF-ϰB, suggesting icariin may alleviate the inflammatory environment which may be responsible for destruction of periodontal tissues. The association of NFE2L2 with antioxidant response/protection and the association between VEGFA with osteogenic process/angiogenic process was determined. The entire network shown in Fig. 1 indicates that icariin may help with the regeneration of dental tissue by performing multiple actions/promoting the regeneration process at the same time, and reduce all of the inflammation and oxidative damage. Pathway enrichment revealed the main enriched pathways to be PI3K/AKT (pathway1), BMP related osteogenesis (pathway2), NF-ϰB signaling (pathway3), oxidative-stress regulation (pathway4) and VEGF-mediated angiogenesis (pathway5) as the most biologically relevant ones. These related pathways may be a promising molecular mechanism to investigate icariin's potential applications in the field of regenerative dentistry.

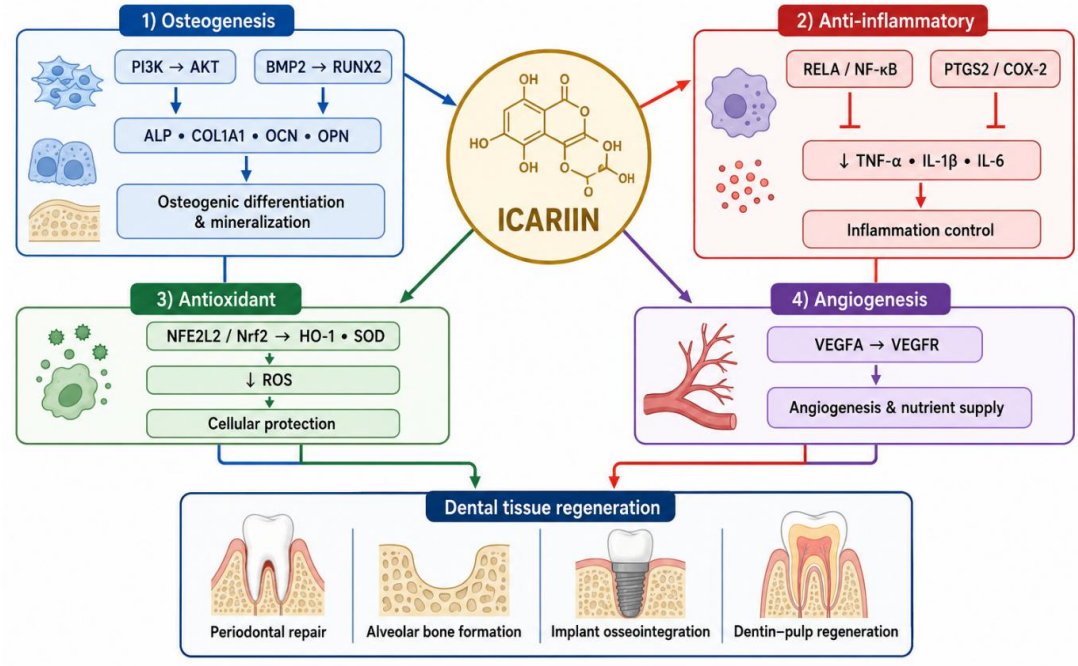

Figure 1. Integrated network of icariin-associated targets and signaling pathways involved in dental tissue regeneration

3.3 Molecular Interactions Between Icariin and Selected Therapeutic Targets

Molecular docking was found to be favourable with osteogenic, inflammatory, antioxidant and angiogenic target(s) of icariin. There were five hydrogen bonds with a potential modulation of the PI3K/AKT signaling (Table 2) and the best predicted interaction was with PIK3CA binding affinity of -9.0 kcal/mol. Icariin showed also good binding pattern to BMP2 as well as RUNX2 with docking scores of −8.4 and −8.1 respectively. The interactions suggest a potential effect of these factors on both 1) osteoporosis commitment, and 2) matrix mineralization. The affinity interacted with PTGS2 gave a score of -8.0Kcal/mol while RELA resulted in a score of -7.6Kcal/mol which infer to the possibility of anti-inflammatory activity. Further clues of angiogenic and antioxidant properties were suggested by the additional EPSIT predicted targets, VEGFA and NFE2L2. Hydrogen bonds, hydrophobic interactions and electrostatic interactions were found to cause the stability of Ligands-Targets Complexes. Docking scores are however, prediction of the molecular compatibility which can only be verified by binding assay, pathway-inhibition experiments, and protein expression analysis.

Table 2. Molecular docking scores, interacting amino-acid residues, and binding characteristics of icariin with selected osteogenic, inflammatory, and angiogenic targets

Target	Gene	PDB ID	Principal function/pathway	Binding affinity (kcal/mol)	Hydrogen bonds (n)	Principal interacting residues	Predominant interactions	Predicted relevance to dentistry
PI3K catalytic subunit alpha	**PIK3CA**	4JPS	PI3K/AKT-mediated cell survival and osteogenesis	−9.0	5	Val851, Asp933, Lys802, Ser854	Hydrogen bonding and hydrophobic interactions	May promote dental stem-cell survival, osteogenic differentiation, and periodontal regeneration
Bone morphogenetic protein 2	**BMP2**	3BMP	Osteogenic differentiation and bone-matrix formation	−8.4	4	Ser88, Leu90, Asp105, His111	Hydrogen bonding and hydrophobic interactions	May enhance alveolar bone formation and implant osseointegration
Runt-related transcription factor 2	**RUNX2**	1H9D	Transcriptional regulation of osteogenic markers	−8.1	3	Arg190, Lys225, Glu239	Hydrogen bonding and electrostatic interactions	May increase ALP, COL1A1, osteocalcin, and osteopontin expression
Cyclooxygenase-2	**PTGS2**	5IKR	Prostaglandin synthesis and inflammatory regulation	−8.0	2	Arg120, Tyr355, Ser530	Hydrogen bonding and hydrophobic interactions	May reduce periodontal inflammation and inflammatory tissue destruction
AKT serine/threonine kinase 1	**AKT1**	4EJN	Cell survival, proliferation, and osteogenic signaling	−7.9	3	Lys179, Glu234, Asp292	Predominantly hydrogen bonding	May support osteoblast survival, differentiation, and peri-implant bone healing
Nuclear factor NF-κB p65 subunit	**RELA**	1NFI	NF-κB-mediated inflammatory cytokine production	−7.6	3	Arg33, Glu39, Lys122	Hydrogen bonding and electrostatic interactions	May suppress TNF-α, IL-1β, and IL-6 production in periodontal tissues

Vascular endothelial growth factor A	**VEGFA**	3V2A	Angiogenesis and vascular support of regeneration	−7.5	3	Asp34, Glu38, Lys84	Predominantly hydrogen bonding	May enhance vascularization during periodontal, pulpal, and bone regeneration
Nuclear factor erythroid 2-related factor 2	**NFE2L2**	4IFL	Antioxidant defence and cellular stress regulation	−7.4	2	Ser508, Arg415	Predominantly hydrogen bonding	May reduce oxidative damage and protect periodontal and pulpal cells

3.4 Cytocompatibility and Effective Concentration of Icariin

A concentration-dependent effect of icariin was observed on viability of dental-derived stem cells. Viability of basal group and vehicle control group at 48 hrs was virtually 99.62% and 98.52%, respectively. Highest value of the Viability exhibited by 1 μM of icariin is 106.75% which is better than or equal with 0.1 μM of icariin (102.17%). Viability was still good at 10 μM (104.80 %), but the Electrochemical exposure to 20 μM reduced the viability by 91.85 % indicating that exposure to higher levels may lead to cell stress and loss in viability. According to the data presented in Figure 2, the most suitable concentration range was the 1–10 μM which also gave good cytocompatibility and biological activity. These observations suggest that there is a non-linear dose–response relationship; that is, there is an activating effect in the middle of the range of concentrations but a depressive effect for concentrations higher than the activating range. While these results are encouraging to consider when choosing the 1 and 10 μM dosage of icariin for their later assessment of regeneration, additional assessments of cytotoxicity, apoptosis and proliferation are required.

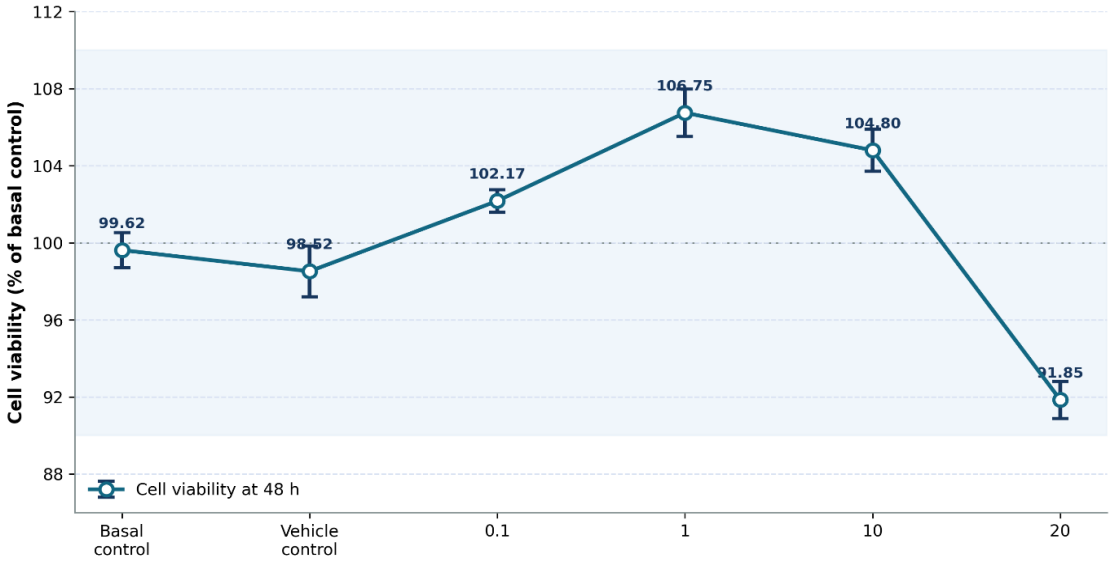

Figure 2. Concentration- and time-dependent effects of icariin on the viability of dental-derived stem cells

3.5 Icariin-Induced Osteogenic Differentiation and Mineralization

Icariin was seen to significantly increase osteogenic differentiation and mineralized matrix formation in a concentration dependent manner. When treated with the 0.1, 1 and 10 μM icariin, the alkaline phosphatase activity in the cellular medium was found to be 17.89, 23.92 and 27.73 U/mg, respectively, which increased from the vehicle-control group (13.95 U/mg) as

indicated in Table 3. Similarly, calcium deposition was observed to be increasing with increasing concentration from the vehicle control level (21.56 µg/mg) to 29.00, 39.78 and 48.25 µg/mg. Based on the absorbance of Alizarin Red the result obtained at the peak value of 10µM indicate the maximum number of nodules mineralized. ALP activity, calcium deposition and mineralization were decreased compared to the group treated with 10 µM but were still higher than the group that received the vehicle control treatment. In the overview osteogenic response (figure 3), it is seen that the osteogenic response is maximum with 10µM Icariin. These results demonstrate that icariin affects the osteogenic commitment process early and also extracellular matrix (ECM) mineralization, because the correlation level between ALP activity and calcium accumulation, and the level of Alizarin red staining, showed high correlation.

Table 3. Effects of icariin on cell viability, alkaline phosphatase activity, calcium deposition, and mineralized nodule formation

Experimental group	Icariin concentration (µM)	Cell viability at 48 h (%)	ALP activity at day 14 (U/mg protein)	Calcium deposition at day 21 (µg/mg protein)	Alizarin Red S at day 21 (OD562)
Basal control	0	99.62 ± 0.92	14.20 ± 0.45	22.31 ± 1.03	0.413 ± 0.027
Vehicle control	0	98.52 ± 1.32	13.95 ± 0.47	21.56 ± 0.90	0.404 ± 0.023
Icariin—low dose	0.1	102.17 ± 0.58	17.89 ± 0.42	29.00 ± 0.98	0.532 ± 0.018
Icariin—medium dose	1	106.75 ± 1.23	23.92 ± 0.18	39.78 ± 1.11	0.760 ± 0.014
Icariin—high dose	10	104.80 ± 1.09	27.73 ± 0.32	48.25 ± 1.04	0.915 ± 0.032
Icariin—maximum dose	20	91.85 ± 0.96	22.56 ± 0.34	38.20 ± 0.85	0.722 ± 0.027

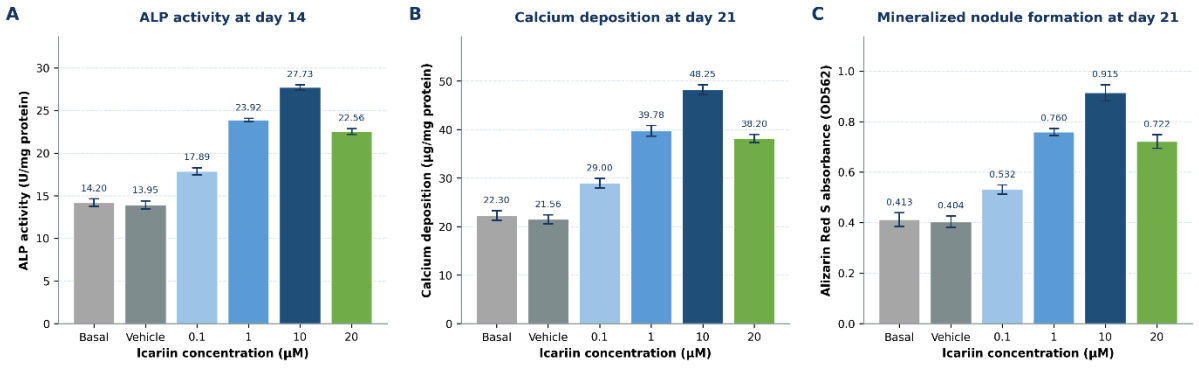

Figure 3. Effects of icariin on alkaline phosphatase activity and mineralized matrix formation

3.6 Regulation of Osteogenic and Regenerative Markers

Icariin has shown to be able to up regulate osteogenic differentiation and dentition tissue regeneration molecular markers. LPS also reduced the expression of RUNX2, BMP2, and osteocalcin (to 0.73-, 0.75-, and 0.70-fold of the basal value) respectively. These parameters were inhibitory and were reversed by Icariin likely in a dose dependent manner. 10 µM showed the maximum expression for all the genes (RUNX2, BMP2 and osteocalcin) which was approximately 2.31-fold, 2.43-fold and 2.19-fold, respectively. The results agreed with the function of the alkaline phosphatase activity and mineralization given in table 3 with increase. RUNX2 is a required factor for osteogenic commitment and BMP2 is responsible for difference and matrix-maturation processes that follow. An increase in osteocalcin shows that they are moving towards a mature phenotype

in mineralization. In addition, there was a significant upregulation of the synthesis of VEGF suggesting that icariin might also concurrently regulate bone development and vascularization. In confirmatory expression analysis, the three makers (ALP, COL1A1 and osteopontin) should be used; however, marker pattern indicates that an integrated osteogenic program, which is related to BMP2 and RUNX2, is involved in icariin mediated regeneration.

3.7 Anti-Inflammatory and Antioxidant Effects of Icariin

When stimulated with LPS, 1:25 were able to significantly suppress inflammatory and oxidative responses to Icariin. As seen in Table 4, the concentration of TNF-α as well as the concentration of IL-6 of the LPS-control group was about 169.83 pg/mL and 150.48 pg/mL, respectively. Treated them down to 73.25 and 63.90 pg/mL level by treating them with 10 μM icariin. The inflammatory mediators were also suppressed by 20 μM, but it was not as effective as 10 μM. About 100.75% increase of ROS level was seen in LPS-control group and 44.37% decrease of ROS level was indicated in icariin 10μM treatment group. At the same time, it also showed an increase in activity of the superoxide dismutase enzyme from 10.93 U/mg to 20.94 U/mg. These results suggest that icariin may have anti-inflammatory effects on inflammatory cytokines and improve the endogenous antioxidant protection. Some of its actions include preventing NFκB signaling, and stimulating NFE2L2-dependent anti-oxidant processes. Additional mechanistic confirmation would be achieved by the measure of IL-1β, catalase and glutathione and lipid-peroxidation products, and NF-κB phosphorylation.

Table 4. Comparative effects of icariin on osteogenic, inflammatory, oxidative-stress, and angiogenic biomarkers

Experimental group	RUNX2 (fold change)	BMP2 (fold change)	OCN (fold change)	TNF-α (pg/mL)	IL-6 (pg/mL)	ROS (% of LPS control)	SOD (U/mg protein)	VEGF (pg/mL)
Basal control	1.00 ± 0.02	0.97 ± 0.02	0.98 ± 0.03	42.28 ± 2.72	32.12 ± 3.33	38.85 ± 2.40	19.34 ± 0.32	107.97 ± 2.97
LPS control	0.73 ± 0.02	0.75 ± 0.02	0.70 ± 0.03	169.83 ± 3.31	150.48 ± 2.43	100.75 ± 2.30	10.93 ± 0.40	81.22 ± 4.20
LPS + icariin 0.1 μM	1.13 ± 0.04	1.16 ± 0.03	1.13 ± 0.02	138.53 ± 4.09	126.85 ± 3.82	83.92 ± 2.65	12.81 ± 0.35	106.93 ± 2.99
LPS + icariin 1 μM	1.69 ± 0.04	1.74 ± 0.05	1.62 ± 0.04	101.55 ± 3.41	92.88 ± 2.71	61.27 ± 1.78	16.28 ± 0.31	138.30 ± 3.71
LPS + icariin 10 μM	2.31 ± 0.05	2.43 ± 0.03	2.19 ± 0.03	73.25 ± 3.41	63.90 ± 2.68	44.37 ± 1.89	20.94 ± 0.41	176.27 ± 3.90
LPS + icariin 20 μM	1.93 ± 0.04	2.01 ± 0.03	1.85 ± 0.04	84.83 ± 2.93	74.88 ± 2.93	52.68 ± 2.19	18.96 ± 0.42	156.87 ± 4.81

3.8 Integrated Therapeutic Mechanism of Icariin in Dentistry

The overall results showed a multimodal therapeutic effect of icariin on regeneration of dental tissues. Icariin may stimulate osteogenic differentiation, the synthesis of alkaline phosphatase, the maturation of the extracellular matrix and mineral deposition by turning on the PI3K/AKT signaling and improving the activity of BMP2–RUNX2 as shown in figure 4. At the same time, RELA-PTGS2 interaction can lead to a decrease of NFκB mediated generation of TNF-α, IL-1β and IL-6 and other inflammatory mediators. Of particular interest is its ability to activate the NFE2L2-dependent antioxidant responses, that could lead to a decrease in ROS and increase in antioxidant enzymes such as super oxide dismutase. The up-regulation of VEGF by icariin may also favour endothelial functioning, neovascularization, supply or transportation of the nutritional factors and regulation of the mentioned processes as yreogenesis and angiogenesis. All these extra processes may contribute to an environment that favors a transition from a pro-inflammatory environment that favors cell death and oxidative stress to one that favors cell survival and tissue repair. The biological mechanism of the observed improved viability, osteogenic markers, mineralization, inflammatory control and angiogenic activity are explained using the suggested pathway. But inhibition studies need to be conducted in pathways to find causal relationships.

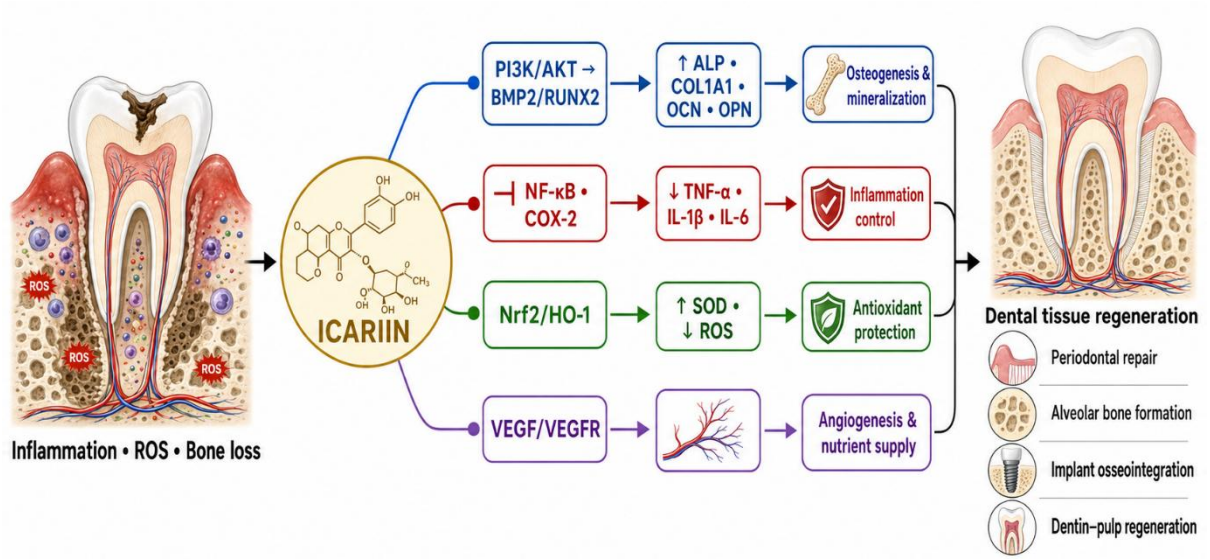

Figure 4. Proposed mechanism of icariin-mediated osteogenesis, inflammation control, angiogenesis, and dental tissue regeneration

3.9 Potential Regenerative Applications in Dentistry

Taking into account the observed biological activities, icariin can have various applications in regenerative dentistry. Concurrently, its osteogenic and anti-inflammatory effects could facilitate regeneration of the periodontal tissue by promoting the regeneration of the alveolar bone and by preventing the tissue damage by cytokines. Icariin may also help to facilitate bone development around dental implants, which may aid in enhancing early osseointegration processes and stability of the dental implant in poor bone quality patients. Incorporation into membranes, hydrogels, nanoparticles, and bone-graft substitutes might result in the local delivery to periodontal/peri-implant defects. In terms of effects on stem-cell differentiation and mineralization, this may also be of interest for dentin–pulp regeneration including scaffolds for injection or pulp-capping materials. Concurrently, the elevation of osteogenic markers, as well as VEGF, suggests to us the possibility of employing these techniques in craniofacial tissue engineering when vascularization of vascularized large grafts are required for support of graft survival. But for all dental applications a validation with the specific indication will be required as there are important differences between periodontal, implant, pulpal and craniofacial applications with regard to drug concentration, carrier properties, release kinetics etc., and also mechanical requirements.

3.10 Translational Prospects and Clinical Significance

Icariin local delivery will be important for icariin to be utilized in the clinic and such systems will be needed to be safe and predictable. Aqueous solubility and bioavailability (direct systemic delivery) may be limitation factors due to low concentrations in dental defects, metabolism and poor concentration in these. This might be done by encapsulating icariin into the constituents of a hydrogel, electrospun fibre, nano-particle, collagen membrane, bioactive ceramic or even a 3-dimensional scaffold and then effect site-specific and sustained release. From the dose-response curve that is currently available, the optimization of concentrations is necessary as the trends indicate that the viability & regeneration is better of the moderate exposure is better as compared to 20μM exposure. Pharmacokinetic studies should be performed to assess retention, degradation and release behaviour and tissue penetration. When performing biosafety evaluation, the following factors should be considered: Systemic exposure, interactions with existing dental materials and/or medications, immunogenicity, genotoxicity, cytotoxicity. Following studies should move from standardized cells models to animal periodontal defects and craniofacial defects and further on to controlled clinical studies. But if these requirements are met, icariin containing formulations can prove helpful in the fields of periodontal regeneration, dentin–pulp repair, bone augmentation and implant integration in the future as an auxiliary tool.

3.11 Comparison With Previous Studies

The results of present study corroborate with the previous experimental results on specific icariin effects such as osteogenic, anti-inflammatory/anti-arthritic, anti-oxidant and angiogenic. Previous cell studies have demonstrated that icariin is beneficial

for osteoblasts through upregulation of their proliferation, alkaline phosphatase activity, mineralised nodule formation and the protein levels of the bone morphogenic protein (BMP2), its receptor RUNX2, osteocalcin and osteopontin. This increase in ALP activity and calcium deposition in the table (3) confirmed Osteogenic profile of this Flavonoid which is well established and defined. The decrease in production of inflammatory cytokines like TNF-α and Il-6 and ROS shown in Table 4 are also a result of the previous periodontal study and bone-regeneration study which also suggested that the icariin has anti-inflammatory activity and anti-sedosomes property. However, the current integrated strategy builds on these observations and correlates the targets predicted by computer simulation with responses of cells and biochemical systems that are relevant in the field of dentistry. The lesser activity at 20 μM suggest that icariin might possess a concentration dependant therapeutic window. But there remain discrepancies in the type of cells used, when treated, what solvent was used, how it was assessed or many times what 'experimental disease model' the cells were used in or what carrier system was employed, which makes it harder to directly compare.

3.12 Strengths and Limitations of the Study

This study's strength is the integration of the study design which included target prediction, pathway analysis, molecular docking, cytocompatibility assessment, osteogenic assays, and measurements of inflammatory, antioxidant and angiogenic biomarkers. A logical molecular context for the cellular changes observed can be conferred by the technique. It is also possible to ascertain a therapeutic range by using several doses of icariin that is not assumed by using a linearly determined dose response. In addition, the assessment of ALP activity and mineralisation, gene expression, cytokines, oxidative stress and VEGF enables a multi-dimensional assessment of the regenerative potential. However, limitations of the results lie in the in vitro design that cannot take into account the diversity of cells within oral tissues, the interactions with the immune system, as well as the mechanical loading, vascular environment and microbial complexity. Molecular docking is only predictive and do not prove target engagement. Systemic safety or long term effect, scaffold-release behaviour and pharmacokinetics was not evaluated. Pathway inhibitors and 3-D cultures, animal defect models, standardized biomaterials and appropriately powered clinical studies, therefore, are required to confirm the results.

4. CONCLUSION

These findings indicate that icariin is a potentially highly effective, natural therapeutic drug in regeneration dentistry in many ways. Icariin is a possible molecule that targets osteogenic, inflammatory, antioxidant and angiogenic targets like PI3KCA, AKT1, BMP2, RUNX2 (NF)RELA, PTGS2, NFE2L2 and VEGFA based on molecular-target and docking analyses. The right concentration of icariin might be useful for increasing the viability, alkaline phosphatase activity, Ca deposition, mineralized nodule formation and expression of osteogenic markers of the dental derived stem cells at the cellular level. Icariin also suppressed the levels of inflammatory cytokines, ROS and boosted the antioxidant activity and release of VEGF. Maximum overall regenerative response was observed at 10 μM concentration and sub-optimal response observed at 20 μM concentration indicated the need to optimize the dose. All these effects may be combined synergistically to facilitate periodontal regeneration, alveolar bone replacement, osseointegration of dental implants and dentin-pulp regeneration, and craniofacial tissue engineering. Additional to controlled clinical trials however, pathway-specific assays, animal models and in vivo femtocells and biomaterial delivery assays need to be undertaken for icariin to be recommended for routine use in dentistry.

DECLARATIONS

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