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Original article

Neurokinin-1-tachykinin receptor agonist promotes diabetic fracture healing in rats with type 1 diabetes via modulation of Wnt/β-catenin signalling axis

Xiaohui Wang [a], Ning Su [b,*]

[a] Department of Endocrinology, Gansu Provincial of Traditional Chinese Medicine, Lanzhou, Gansu 73000, China
[b] Department of Geriatrics, Hengshui People's Hospital, Hengshui, Hubei 053000, China

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ABSTRACT

Diabetes mellitus is an ill-famed metabolic disorder with varied repercussions including delayed fracture healing. Wnt/β-catenin axis is known to play a tight pivotal role in the bone healing process. Substance P (SubP) is a neuropeptide with established positive modulatory functions in fracture healing and associated neuronal milieu. In this study, we performed local delivery of recombinant adenovirus of Dickkopf-1 (DKK1) into the fracture site to understand the antagonizing the role of DKK1 against substance P. Rats were segregated into 4 groups: (i) Fractured non-diabetic rats; (ii) Fractured T1D rats; T1D was provoked by using STZ 50 mg/kg for 5 consecutive days; (iii) Fractured T1D + SubP (50 mg/ml/Kg; i.p.; 30 min prior to fracture procedure); (iv) Fractured T1D + SubP + Ad-DKK1. Bone radiographs were taken using a Faxitron X-ray machine and the residual gap size was measured using an electric caliper. Western blotting was also performed to determine the protein expression levels of osteogenic markers (RUNX2, OSTX and OSTC) bone resorption markers (OPG, RANKL and RANK) and also Wnt-signalling markers (β-catenin, LRP5 and GSK-3β). We observed that SubP promoted osteogenesis (as indicated by RUNX2, OSTX and OSTC upregulation) and mitigated the bone resorption (as indicated by optimized OPG/RANKL/RANK axis) via activated Wnt signalling (manifested by upmodulated β-catenin and LRP5, with downmodulated GSK-3β levels. Activation of endogenous SubP or administration of exogenous mimics might counterprotect the fractured bone against the deforming effects of T1D.

1. Introduction

Diabetes mellitus is an ill-famed metabolic disorder orchestrated by lifestyle modifications of population across the global arena. Estimates reflect that about 450 million diabetics in 2017 and this figure is projected hike up to around 700 million diabetics in 2045 (Cho et al., 2018). Type 1 diabetes mellitus (T1D) is an autoimmune form of diabetes with higher risk profile than T2D. Bone fracture-and-delayed fracture healing is one of the crucial, yet underrated complications of T1D. T1D patients depicted increased/3-6-fold greater risk of bone fracture, in contrast to the T2D/non-diabetic population (Shah and Snell-Bergeon, 2019). Shockingly, there is no robust evidence to exemplify the notion that supplemental calcium and vitamin D coupled with/without aerobic exercise regimen might alleviate skeletal fragility in T1D (Gil-Díaz et al., 2019). This clearly emphasizes that further exploratory drive with respect to the pathoamechanisms and the therapeutic discovery is obligatory in the T1D-fracture continuum.

Fracture occurs due to bone turnover deficit along with deprived bone mineral density (BMD) and flawed bone microarchitecture. Kalaitzoglou et al. (2016), proposed that T1D hampers the bone formation process through negative regulation of osteoblasts and osteocytes, along with hyperactivated osteoclast functions, culminating in bone resorption and delayed fracture healing. The fracture healing occurs in two stages (i) anabolic stage: fractured bone healing and repair of the tissue in skeletal vicinity result in stem cell-based osteovascular regeneration, tissue volume accrual and subsequent callus formation; (ii) catabolic stage: resorption of bony callus tissue results in the metamorphosis of callus into cor-

* Corresponding author at: Department of Geriatrics, Hengshui people's hospital, No. 2, Renmin Road, Hengshui, Hubei 053000, China.
E-mail address: xfjjya@163.com (N. Su).
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tical bone through "coupled remodelling", a process manifested by coupled cycles of osteoblast and osteoclast activities (Einhorn and Gerstenfeld, 2015; Guru et al., 2020).

There exists a robust positive relationship between bone remodelling and callus innervation in complete fracture healing. Based on the neurotransmitter phenotype, an array of nerve fibres emerging from sensory, autonomic, and opioid nervous systems, innervate the bone tissue. In this line, Madsen and his colleagues underscored that integral sensory innervation is obligatory for mechanically robust bony callus formation and that pertinent neuronal injury culminates in malformed callus (Madsen et al., 1998). The precise interplay between fracture healing and nerve damage, albeit remains elusive, is known to be mediated through nerve-derived efferent molecular signals termed as "neuropeptides". Many neuropeptides including substance P (SubP), vasoactive intestinal peptide, and tyrosine hydroxylase, neuropeptide Y and calcitonin gene-related peptide (CGRP) are reported to have a regulatory role in the bone homeostasis (Ma et al., 2015; Venkatadri et al., 2020).

Among the neuropeptides, substance P has attracted our attention due to its documented pancreatic β-cell protective and bone remodelling regulatory functions in T1D/T2D and fracture healing respectively (Issac et al., 2020; Um et al., 2018; Hofman et al., 2019; Sannasimuthu et al., 2020). In fact, an interesting study by Kunt et al., (2000) revealed that SubP is diminished in the serum of T1D patients with associated neuronal dysfunction. Another study illustrated that SubP ameliorates wound healing by modulating the inflammatory mechanisms in neuroischemic diabetic models (Leal et al., 2015; Mani et al., 2020). A gamut of research studies underlined that activation of canonical Wnt/β-catenin signalling axis has positive implications in the fracture healing process, while perturbed signalling leads to delayed/distorted callus formation in T1D/non-diabetic models (Jin et al., 2015; Arasu et al., 2019; Chen et al., 2007; Cai et al., 2018; Valsalam et al., 2019). Dickkopf-1 (DKK1), a soluble glycoprotein, is a potent Wnt antagonist known to negatively regulate the fracture healing process by hampering the osteoblastogenesis in T1D (Hie et al., 2011; Tsentidis et al., 2017). Further, an interesting piece of evidence, based on a cell-model, indicated that DKK1 impedes the role of SubP in Wnt/β-catenin-mediated osteogenesis (Mei et al., 2014; Kumaresan et al., 2018). These evidences tempted us to explore the missing link between SubP and its plausible fracture healing effect in T1D mouse model.

2. Materials and methods

2.1. Animals and treatment

This research study was performed in harmony with the guidelines for animal handling and care in the laboratory prescribed by the institutional ethical committee and also, by the National Institutes of Health. Male Wistar rats were kept in a temperature and humidity monitored milieu. All the rats offered with rodent chow and tap water *ad libitum*. Rats were segregated into 4 groups with 10 animals in each group: (i) Fractured non-diabetic rats; (ii) Fractured T1D rats; T1D was provoked by using STZ 50 mg/kg for 5 consecutive days; (iii) Fractured T1D + SubP (50 mg/ml/Kg; i.p.; 30 min prior to fracture procedure); (iv) Fractured T1D + SubP + Ad-DKK1 (200 μL Ad-Dkk1 (10^9 particles) was locally injected into the fracture site, 30 min prior to fracture procedure). After study, all the animals were humanely sacrificed to collect the bone tissue for molecular studies.

2.2. Femoral segmental bone defect model

To study the fracture healing, we replicated the femoral segmental bone defect model as reported earlier. Succinctly, the rats were subjected to anesthesia, and the patella-lateral femoral joint was exposed through 1-cm incision. Then, by using surgical scissors, transverse osteotomy was performed through the middle femoral shaft. Then to fix the fractures, a metallic clip was placed in an anterio-posterior manner around the fracture and the wound was sutured.

2.3. X-ray photography and histology

Radiographs were taken using a Faxitron X-ray machine (Wheeling, IL) at 5.0 kV by an exposure of up to 6 s. The residual gap size between the fractured sections was gauged by using an electric caliper. The histological analysis was done using Masson-Goldner staining.

2.4. Western blot analysis

The bone tissue sections around the fracture site were cut and homogenized in ice-cold RIPA buffer using an electric homogenizer.

2.5. Statistical analysis

Data quantification was performed using SPSS software (V13.0; SPSS, Inc., USA) and the statistical evaluation was done using one-way analysis of variance (ANOVA) by applying Tukey's post-hoc test for comparisons among different animal groups. Significant level was kept at P value less than 0.05.

3. Results

3.1. SubP improved the fracture healing in T1D rats

Residual gap size of the fractured bone area was measured on the X-ray photograph using an electric caliper. We found that the residual gap size was increased in the T1D rats indicating delayed fracture healing in T1D rats, while SubP treated T1D rats displayed significantly ($P < 0.05$) reduced residual gap size. However, ad-DKK1 cotreatment with SubP inhibited its healing effects in T1D rats, as indicated by the increased residual gap size when compared to the SubP-alone treated T1D rats (Fig. 1A-B). Histological analysis showed more mineralized bone components (indicated by green) in the control and also the SubP-treated groups, while T1D rats and rats with ad-DKK1 and SubP cotreatment indicated red/orange unmineralized organic portions (osteoids) (Fig. 1C).

3.2. SubP ameliorated the fracture healing via Wnt/β-catenin signalling in T1D rats

To ascertain whether Wnt/β-catenin has a robust role in the fracture healing process, we blunted the pathway using adenoviral DKK1 overexpression. SubP has a beneficial role in the fracture healing. In this view, we observed that SubP treated T1D rats displayed significantly ($P < 0.05$) upmodulated levels of β-catenin and LRP5, with downmodulated GSK-3β expressions. However, ad-DKK1 cotreatment with SubP inhibited its healing effects in T1D rats (Fig. 2).

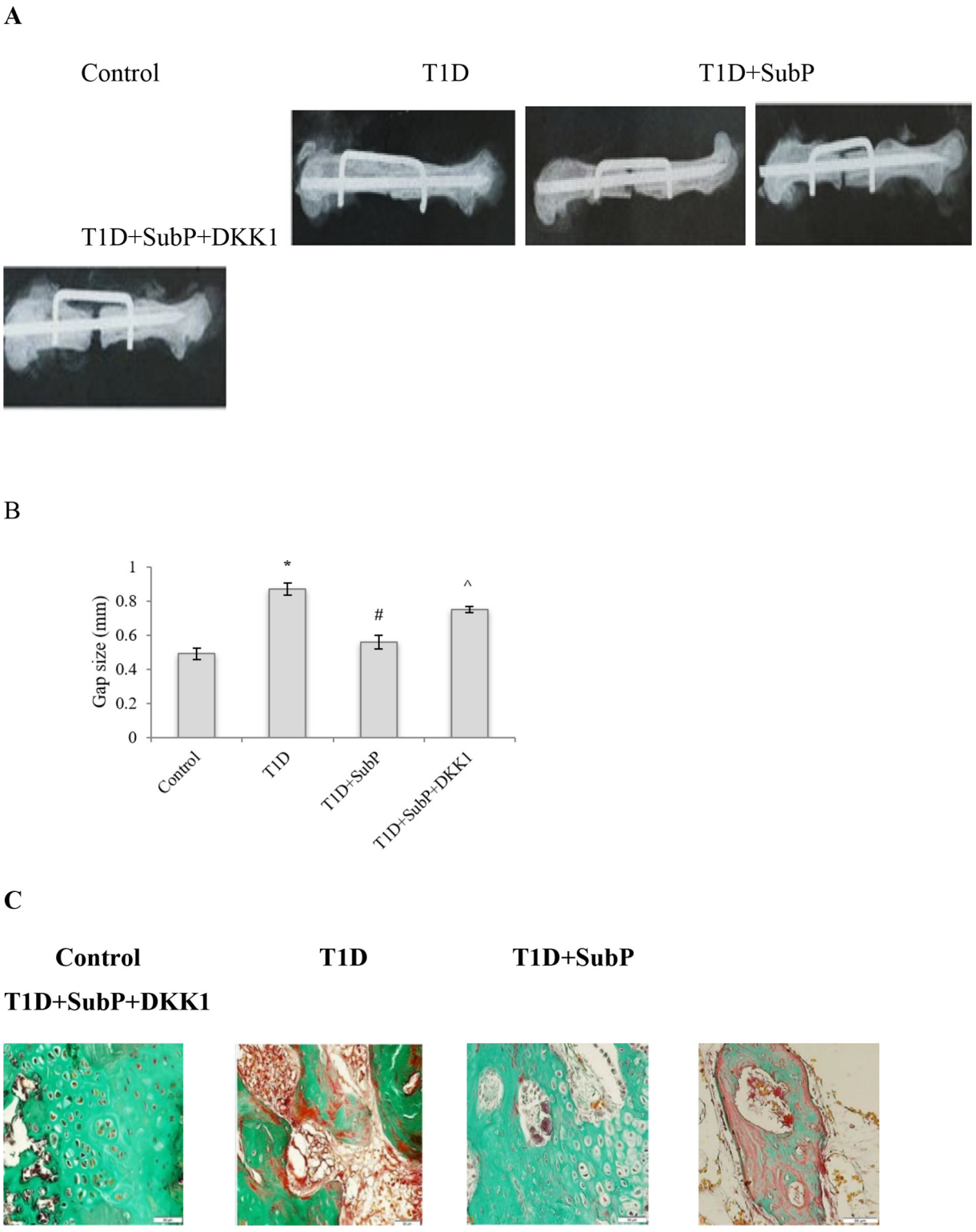

Fig. 1. SubP improved the fracture healing in T1D rats. A) Representative X-ray photographs of the fractured area; B) Residual gap size measured on the X-ray photograph using an electric caliper. C) Histological sections showing Masson–Goldner trichrome staining. Mineralized bone components were indicated by green, osteoids (unmineralized organic portions) were indicated by red/orange. *P < 0.05 (Control vs T1D); #P < 0.05 (T1D + SubP vs T1D); ^P < 0.05 (T1D + SubP + DKK1 vs T1D + SubP).

3.3. SubP positively modulated the OPG/RANKL/RANK pathway in fractured T1D rats

The ratio of OPG/RANKL is a decisive factor in the bone resorption activity by osteoclasts. We observed that OPG was significantly (P < 0.05) upregulated in the SubP treatment group, while RANKL/RANK were significantly (P < 0.05) downregulated against T1D in fractured rats. However, ad-DKK1 thwarted these positive effects of SubP, indicating that Wnt signaling is involved in the anti-resorption effects of SubP (Fig. 3).

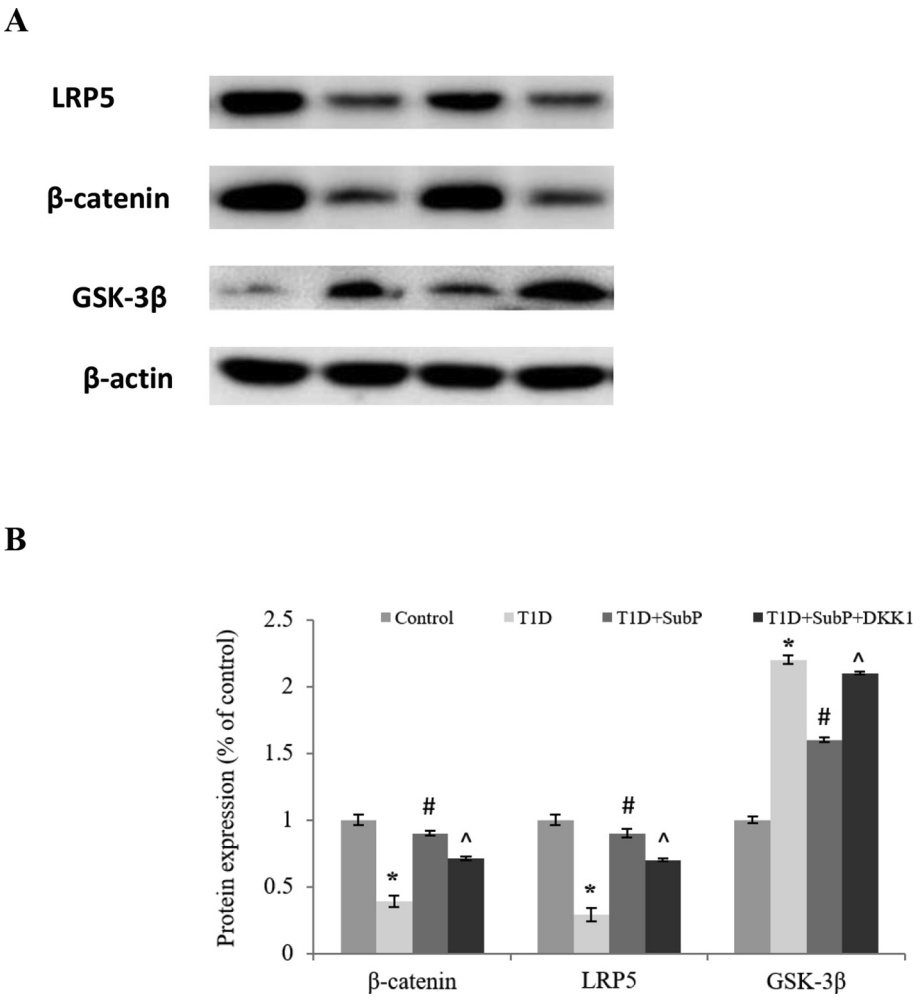

Fig. 2. SubP ameliorated the fracture healing Wnt/β-catenin signalling in T1D rats. A) Indicative western blot images of β-catenin, LRP5 and GSK-3β expressions in each group using β-actin control. C) Relative β-catenin, LRP5 and GSK-3β protein expression levels in each group. *P < 0.05 (Control vs T1D); #P < 0.05 (T1D + SubP vs T1D); ^P < 0.05 (T1D + SubP + DKK1 vs T1D + SubP).

3.4. SubP enhanced the osteogenic markers in fractured T1D rats

The protein expression of osteogenic markers including RUNX2, OSTX and OSTC were significantly (P < 0.05) improvised by the exogenous administration of SubP against T1D-provoked delayed healing of fractured bones. On the other side, ad-DKK1 blocked the osteogenic activities of SubP in the fractured T1D rats (Fig. 4).

4. Discussion

Delayed healing, malunion or non-union of fractured bone, an under-appreciated repercussion of diabetes, is associated with physical, psychological and socioeconomic commotion leading to worsened quality of life. Especially, patients with diabetic neuropathy experience severe fracture healing repercussions (Shibuya et al., 2013). In this backdrop, this study was designed in an endeavour to disentangle the signalling knots involved in the T1D-fracture healing continuum in a neurological perspective. We utilized the femoral fracture rat model with streptozotocin (STZ)-provoked T1D to analyse the mechanistic effects of exogenous SubP administration on diabetic fracture healing in the context of DKK1-mediated Wnt/β-catenin signalling.

Various Wnt signalling component molecules have been illustrated to play a tight interactive role in the fracture healing process. Administration of recombinant murine DKK1 protein dramatically downmodulated the expression patterns of Wnt signalling cascade including Wnt3A, along with Wnt10B (Lu et al., 2016). SubP is a positive modulator of Wnt signalling and hence, we observed Wnt pathway proteins were upmodulated in the SubP-treated T1D fracture bearing rats. In fact, expression of Wnt pathway coordinators including β-catenin and LRP5 were downsized in the DKK1 fractured group, while GSK-3β expression was up surged in that group. Nevertheless, SubP heralded the activation of Wnt signalling and shaped up the Wnt signalling pathway and allowed the upregulation of β-catenin and LRP5 along with repression of GSK-3β expression to enable proper fracture healing (Wang et al., 2018).

DKK-1 repression was known to enhance the expression of osteoprotegerin (OPG), a molecule which signals to tune down the bone resorption mechanism mediated by receptor activator of NF-kB ligand (RANKL) (Diarra et al., 2007). Accretion of bone is blocked by the overexpression of DKK1, while conversely, blunting of DKK1 activity by gene ablation, antibody of DKK1 treatment reciprocates the bone resorption or antagonizes the slowdown of bone accrual. Boyce and Xing demonstrated that binding of OPG to RANKL, thwarts the binding of the latter to the RANK (Boyce and Xing, 2008). Delos and his colleagues illustrated that reported albeit the deficient bone remodeling was observed in the mice, RANKL repression did not alter the mechanical strength of the fractured bone (Delos et al., 2008). In the light of these evidences, we

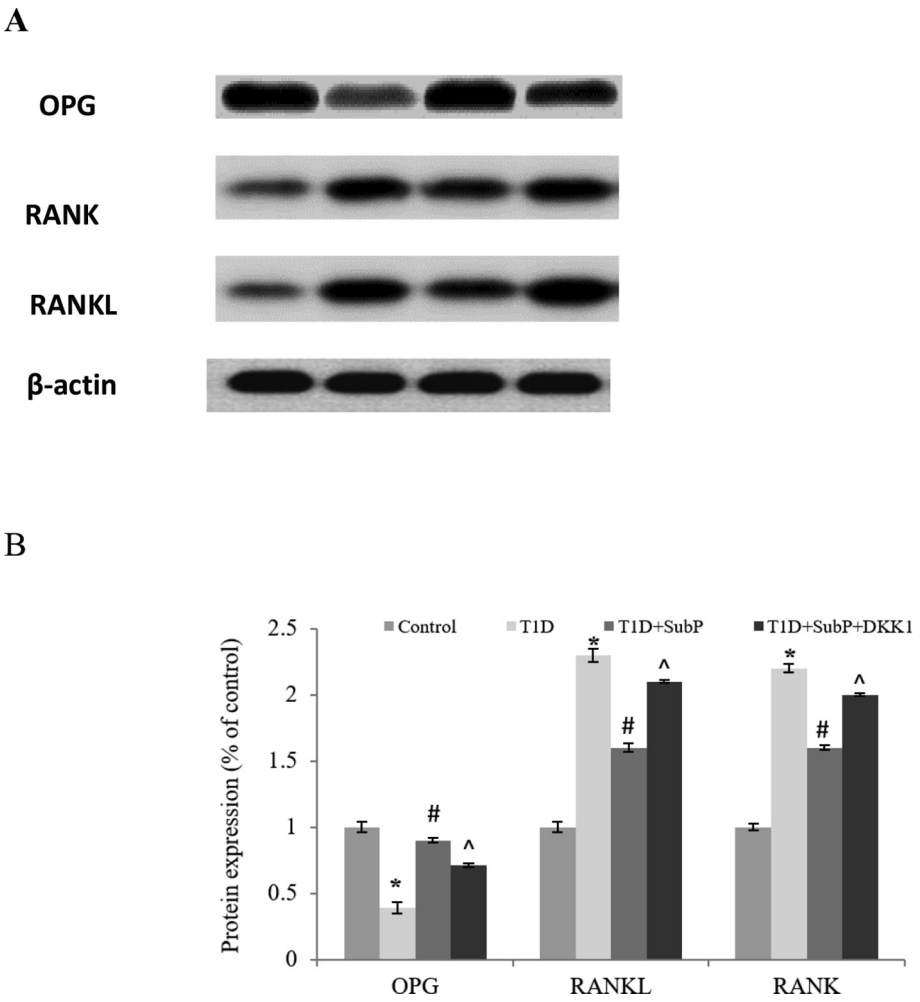

Fig. 3. SubP positively modulated the OPG/RANKL/RANK pathway in fractured T1D rats. A) Indicative western blot images of OPG, RANKL and RANK expressions in each group using β-actin control. C) Relative OPG, RANKL and RANK protein expression levels in each group. *P < 0.05 (Control vs T1D); #P < 0.05 (T1D + SubP vs T1D); ^P < 0.05 (T1D + SubP + DKK1 vs T1D + SubP).

have also observed similar results that downmodulation of OPG expression was effectively thwarted by SubP, while it hindered the RANKL/RANK protein expressions implicating that osteoclast functions were decelerated. However, DKK1 group reflected reversal effects that underscored that anti-resorptive potential of SubP was mediated through suppression of DKK1 function in the fracture healing phase.

With these evidences proffering a winning hope for the utilization of SubP as an osteogenic candidate, we further analysed the expression of osteogenic markers runt-related transcription factor 2 (RUNX2), osterix (OSTX) and osteocalcin (OSTC). Very candidly, we observed that DKK1 treatment stalled the upregulation of these osteogenic markers, while SubP depicted an effective increase in the expression of these markers. These observations were supported by various reports: Fujita et al. (2004) illustrated that Runx2 promotes osteoblast differentiation and triggers their migration in the fracture site. In various fractures, even clinically, it has been reported that OSTC levels were amplified around the fracture site, indicating that it is an essential marker for bone healing post-fracture period (Akesson et al., 1995). Notably, the maturity of pre-osteoblasts into osteocytes and developed osteoblasts is fuelled by the transcriptional activation of the precursor bone cells by OSTX (Sinha and Zhou, 2013). Interestingly, Sun et al. (2010) illustrated that SubP triggers the osteoblast differentiation in a murine model through upmodulation of OSTX expression.

5. Conclusions

These evidences accentuate that SubP might be a turbine drive to pamper the accelerated, yet normal healing of the fractured bone. Of note, SubP directs the mitigation of osteoclastic, bolstering of the osteoblastic functions, mediated through the elicitation of Wnt/β-catenin in the T1D fracture model. Hence, it could an interesting finding that activation of endogenous SubP or administration of exogenous mimics might counter-protect the fractured bone against the deforming effects of T1D.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

None.

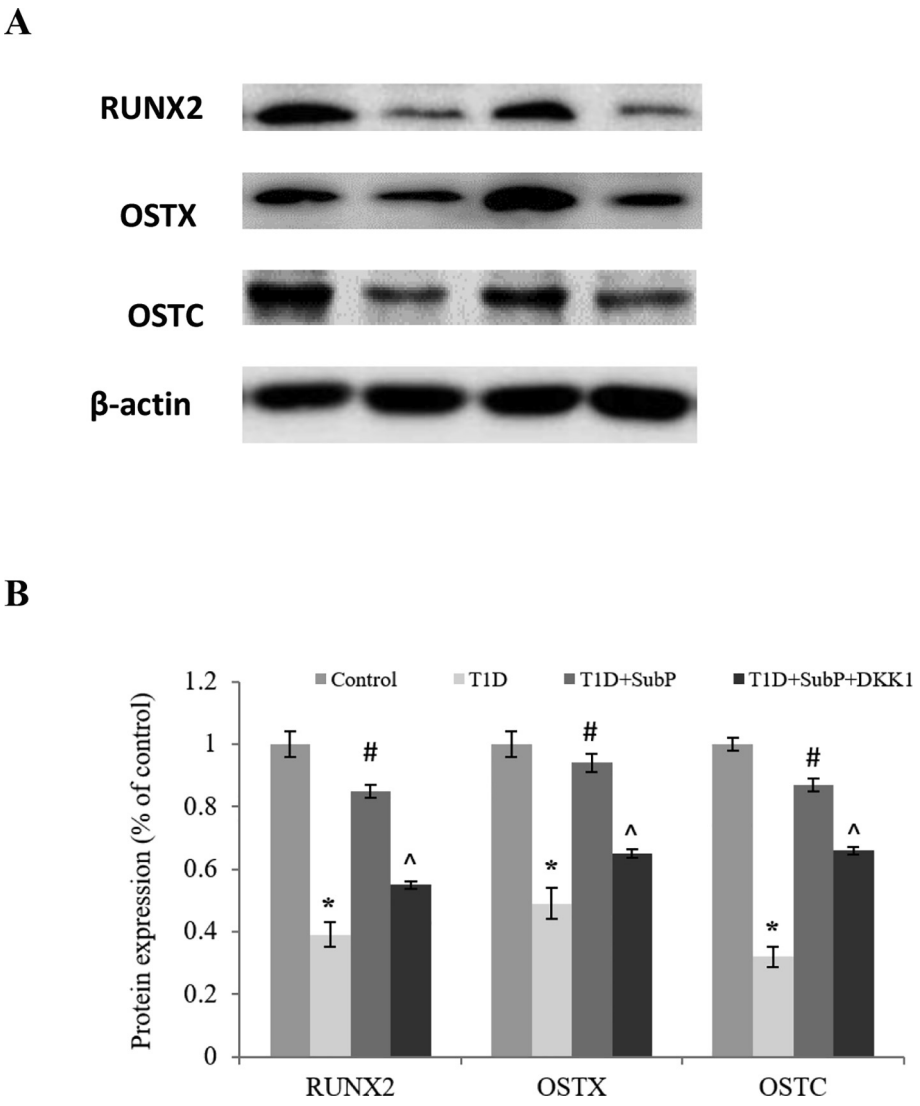

Fig. 4. SubP enhanced the osteogenic markers in fractured T1D rats. A) Indicative western blot images of RUNX2, OSTX and OSTC expressions in each group using β-actin control. C) Relative RUNX2, OSTX and OSTC protein expression levels in each group. *P < 0.05 (Control vs T1D); #P < 0.05 (T1D + SubP vs T1D); ^P < 0.05 (T1D + SubP + DKK1 vs T1D + SubP).

Statement of Ethics

No human subjects were involved in this research. Animal studies were conducted as per the international NIH guidelines for animal care and handling.

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References

Akesson, K., Vergnaud, P., Delmas, P.D., Obrant, K.J., 1995. Serum osteocalcin increases during fracture healing in elderly women with hip fracture. Bone 16, 427–430. https://doi.org/10.1016/8756-3282(95)90187-6.

Arasu, M.V., Madankumar, A., Theerthagiri, J., Salla, S., Prabu, S., Kim, H.-S., Al-Dhabi, N.A., Arokiyaraj, S., Duraipandiyan, V., 2019. Synthesis and characterization of ZnO nanoflakes anchored carbon nanoplates for antioxidant and anticancer activity in MCF7 cell lines. Mater. Sci. Eng.: C 102, 536–540.

Boyce, B.F., Xing, L., 2008. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch. Biochem. Biophys. 473 (2), 139–146. https://doi.org/10.1016/j.abb.2008.03.018.

Cai, J., Li, W., Sun, T., Li, X., Luo, E., Jing, D., 2018. Pulsed electromagnetic fields preserve bone architecture and mechanical properties and stimulate porous implant osseointegration by promoting bone anabolism in type 1 diabetic rabbits. Osteoporos Int. 29 (5), 1177–1191. https://doi.org/10.1007/s00198-018-4392-1.

Chen, Y., Whetstone, H.C., Lin, A.C., Nadesan, P., Wei, Q., Poon, R., Alman, B.A., Horton, W., 2007. Beta-catenin signaling plays a disparate role in different phases of fracture repair: implications for therapy to improve bone healing. PLoS Med. 4 (7), e249. https://doi.org/10.1371/journal.pmed.0040249.

Cho, N.H., Shaw, J.E., Karuranga, S., Huang, Y., da Rocha Fernandes, J.D., Ohlrogge, A. W., Malanda, B., 2018. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. Diabetes Res. Clin. Pract. 138, 271–281. https://doi.org/10.1016/j.diabres.2018.02.023.

Delos, D., Yang, X., Ricciardi, B.F., Myers, E.R., Bostrom, M.P.G., Camacho, N.P., 2008. The effects of RANKL inhibition on fracture healing and bone strength in a mouse model of osteogenesis imperfecta. J. Orthop. Res. 26 (2), 153–164. https://doi.org/10.1002/jor.v26:210.1002/jor.20469.

Diarra, D., Stolina, M., Polzer, K., Zwerina, J., Ominsky, M.S., Dwyer, D., Korb, A., Smolen, J., Hoffmann, M., Scheinecker, C., van der Heide, D., Landewe, R., Lacey, D., Richards, W.G., Schett, G., 2007. Dickkopf-1 is a master regulator of joint remodeling. Nat. Med. 13 (2), 156–163. https://doi.org/10.1038/nm1538.

Einhorn, T.A., Gerstenfeld, L.C., 2015. Fracture healing: mechanisms and interventions. Nat. Rev. Rheumatol. 11 (1), 45–54. https://doi.org/10.1038/nrrheum.2014.164.

Fujita, T., Azuma, Y., Fukuyama, R., Hattori, Y., Yoshida, C., Koida, M., Ogita, K., Komori, T., 2004. Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. J. Cell. Biol. 166 (1), 85–95. https://doi.org/10.1083/jcb.200401138.

Gil-Díaz, M.C., Raynor, J., O'Brien, K.O., Schwartz, G.J., Weber, D.R., 2019. Systematic review: associations of calcium intake, vitamin D intake, and physical activity with skeletal outcomes in people with Type 1 diabetes mellitus. Acta Diabetol. 56 (10), 1091–1102. https://doi.org/10.1007/s00592-019-01334-5.

Guru, A., Lite, C., Freddy, A.J., Issac, P.K., Pasupuleti, M., Saraswathi, N.T., Arasu, M.V., Al-Dhabi, N.A., Arshad, A., Arockiaraj, J., 2020. Intracellular ROS scavenging and antioxidant regulation of WL15 from cysteine and glycine-rich protein 2 demonstrated in zebrafish *in vivo* model. Develop. Immunol. 114, 103863. https://doi.org/10.1016/j.dci.2020.103863.

Hie, M., Iitsuka, N., Otsuka, T., Tsukamoto, I., 2011. Insulin-dependent diabetes mellitus decreases osteoblastogenesis associated with the inhibition of Wnt signaling through increased expression of Sost and Dkk1 and inhibition of Akt activation. Int. J. Mol. Med. 28, 455–462. https://doi.org/10.3892/ijmm.2011.697.

Hofman, M., Rabenschlag, F., Andruszkow, H., Andruszkow, J., Möckel, D., Lammers, T., Kolejewska, A., Kobbe, P., Greven, J., Teuben, M.(J., Poeze, M., Hildebrand, F., 2019. Effect of neurokinin-1-receptor blockage on fracture healing in rats. Sci. Rep. 9 (1). https://doi.org/10.1038/s41598-019-46278-6.

Issac, P.K., Guru, A., Chandrakumar, S.S., Lite, C., Saraswathi, N.T., Arasu, M.V., Al-Dhabi, N.A., Arshad, A., Arockiaraj, J., 2020. Molecular process of glucose uptake and glycogen storage due to hamamelitannin via insulin signalling cascade in glucose metabolism. Mol. Biol. Rep. 47 (9), 6727–6740.

Jin, H., Wang, B., Li, J., Xie, W., Mao, Q., Li, S., Dong, F., Sun, Y., Ke, H.-Z., Babij, P., Tong, P., Chen, D.i., 2015. Anti-DKK1 antibody promotes bone fracture healing through activation of β-catenin signaling. Bone 71, 63–75. https://doi.org/10.1016/j.bone.2014.07.039.

Kalaitzoglou, E., Popescu, I., Bunn, R.C., Fowlkes, J.L., Thrailkill, K.M., 2016. Effects of Type 1 diabetes on osteoblasts, osteocytes, and osteoclasts. Curr. Osteoporos. Rep. 14 (6), 310–319. https://doi.org/10.1007/s11914-016-0329-9.

Kumaresan, V., Pasupuleti, M., Arasu, M.V., Al-Dhabi, N.A., Arshad, A., Amin, S.M.N., Yusoff, F.M., Arockiaraj, J., 2018. A comparative transcriptome approach for identification of molecular changes in *Aphanomyces invadans* infected *Channa striatus*. Mol. Biol. Rep. 45 (6), 2511–2523.

Kunt, T., Forst, T., Schmidt, S., Pfützner, A., Schneider, S., Harzer, O., Löbig, M., Engelbach, M., Goitom, K., Pohlmann, T., Beyer, J., 2000. Serum levels of substance P are decreased in patients with type 1 diabetes. Exp. Clin. Endocrinol. Diabetes. 108 (03), 164–167. https://doi.org/10.1055/s-2000-7738.

Leal, E.C., Carvalho, E., Tellechea, A., Kafanas, A., Tecilazich, F., Kearney, C., Kuchibhotla, S., Auster, M.E., Kokkotou, E., Mooney, D.J., LoGerfo, F.W., Pradhan-Nabzdyk, L., Veves, A., 2015. Substance P promotes wound healing in diabetes by modulating inflammation and macrophage phenotype. Am. J. Pathol. 185 (6), 1638–1648. https://doi.org/10.1016/j.ajpath.2015.02.011.

Lu, H., Li, X., Mu, P., Qian, B., Jiang, W., Zeng, L., 2016. Dickkopf-1 promotes the differentiation and adipocytokines secretion via canonical Wnt signaling pathway in primary cultured human preadipocytes. Obes. Res. Clin. Pract. 10 (4), 454–464. https://doi.org/10.1016/j.orcp.2015.08.016.

Ma, W.H., Liu, Y.J., Wang, W., Zhang, Y.Z., 2015. Neuropeptide Y, substance P, and human bone morphogenetic protein 2 stimulate human osteoblast osteogenic activity by enhancing gap junction intercellular communication. Braz. J. Med. Biol. Res. 48, 299–307. https://doi.org/10.1590/1414-431X20144226.

Madsen, J.E., Hukkanen, M., Aune, A.K., Basran, I., Møller, J.F., Polak, J.M., Nordsletten, L., 1998. Fracture healing and callus innervation after peripheral nerve resection in rats. Clin. Orthop. Relat. Res. 351, 230–240. https://doi.org/10.1097/00003086-199806000-00028.

Mani, S., Balasubramanian, B., Balasubramani, R., Chang, S.W., Ponnusamy, P., Esmail, G.A., Arasu, M.V., Al-Dhabi, N.A., Duraipandiyan, V., 2020. Synthesis and characterization of proanthocyanidin-chitosan nanoparticles: An assessment on human colorectal carcinoma HT-29 cells. J. Photochem. Photobiol. B: Biol. 210, 111966.

Mei, G., Zou, Z., Fu, S.u., Xia, L., Zhou, J., Zhang, Y., Tuo, Y., Wang, Z., Jin, D., 2014. Substance P activates the Wnt signal transduction pathway and enhances the differentiation of mouse preosteoblastic MC3T3-E1 cells. Int. J. Mol. Sci. 15 (4), 6224–6240. https://doi.org/10.3390/ijms15046224.

Sannasimuthu, A., Ramani, M., Pasupuleti, M., Saraswathi, N.T., Arasu, M.V., Al-Dhabi, N.A., Arshad, A., Mala, K., Arockiaraj, J., 2020. Peroxiredoxin of Arthrospira platensis derived short molecule YT12 influences antioxidant and anticancer activity. Cell Biol. Int. 44 (11), 2231–2242.

Shah, V.N., Snell-Bergeon, J.K., 2019. Fracture risk in type 1 diabetes: Think beyond bone mineral density. J. Diabetes Compl. 33 (11), 107411. https://doi.org/10.1016/j.jdiacomp.2019.107411.

Shibuya, N., Humphers, J.M., Fluhman, B.L., Jupiter, D.C., 2013. Factors associated with nonunion, delayed union, and malunion in foot and ankle surgery in diabetic patients. J. Foot Ankle Surg. 52 (2), 207–211. https://doi.org/10.1053/j.jfas.2012.11.012.

Sinha, K.M., Zhou, X., 2013. Genetic and molecular control of osterix in skeletal formation. J. Cell Biochem. 114 (5), 975–984. https://doi.org/10.1002/jcb.v114.510.1002/jcb.24439.

Sun, H.B., Chen, J.C., Liu, Q., Guo, M.F., Zhang, H.P., 2010. Substance P stimulates differentiation of mice osteoblast through up-regulating Osterix expression. Chin. J. Traumatol. 13, 46–50. https://doi.org/10.3760/cma.j.issn.1008-1275.2010.01.009.

Tsentidis, C., Gourgiotis, D., Kossiva, L., Marmarinos, A., Doulgeraki, A., Karavanaki, K., 2017. Increased levels of Dickkopf-1 are indicative of Wnt/β-catenin downregulation and lower osteoblast signaling in children and adolescents with type 1 diabetes mellitus, contributing to lower bone mineral density. Osteoporos Int. 28 (3), 945–953. https://doi.org/10.1007/s00198-016-3802-5.

Um, J., Jung, N., Kim, D., Choi, S., Lee, S.-H., Son, Y., Park, K.-S., 2018. Substance P preserves pancreatic β-cells in type 1 and type 2 diabetic mice. Biochem. Biophys. Res. Commun. 499 (4), 960–966. https://doi.org/10.1016/j.bbrc.2018.04.028.

Valsalam, S., Agastian, P., Arasu, M.V., Al-Dhabi, N.A., Ghilan, A.-K., Kaviyarasu, K., Ravindran, B., Chang, S.W., Arokiyaraj, S., 2019. Rapid biosynthesis and characterization of silver nanoparticles from the leaf extract of *Tropaeolum majus* L. and its enhanced in-vitro antibacterial, antifungal, antioxidant and anticancer properties. J. Photochem. Photobiol. B: Biol. 191, 65–74.

Venkatadri, B., Shanparvish, E., Rameshkumar, M.R., Arasu, M.V., Al-Dhabi, N.A., Ponnusamy, V.K., Agastian, P., 2020. Green synthesis of silver nanoparticles using aqueous rhizome extract of Zingiber officinale and Curcuma longa: In-vitro anti-cancer potential on human colon carcinoma HT-29 cells. Saud. J. Biol. Sci. 27 (11), 2980–2986.

Wang, X., Luo, E.n., Bi, R., Ye, B., Hu, J., Zou, S., 2018. Wnt/β-catenin signaling is required for distraction osteogenesis in rats. Connect Tissue Res. 59 (1), 45–54. https://doi.org/10.1080/03008207.2017.1300154.