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Leptin-induced Osteogenic Differentiation and Heterotopic Ossification of Achilles Tendon in TDSCsis Inhibited by Rapamycin, an Inhibitor of mTORC1 Signaling Pathway

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KEYWORDS Heterotopic Ossification. Leptin. Osteogenic Differentiation. Rapamycin. Tendon Stem Cells

ABSTRACT Present investigation estimated that osteogenic separation and Achilles tendon solidification (HO) stifled byrapa mycin may be initiated by leptin evoked tendon stem cells (TDSCs). To demonstrate this, rodent TDSCs was separated and refined for osteogenesisand articulation of alkaline phosphatase (ALP), Runt related interpretation factor 2 (Runx2), osteogenic related translation factor (OSX) and Osteocalcin (OCN) was identified by qRT-PCR after TDSCs L4 treatment with leptin. Articulations of Runx2, OSX, leptin and PS6, the downstream markers of mTORCl were distinguished by Western Blot and immunohisto chemical method. Expression of osteogenic factors ALP, OSX, Runx2, OCN mRNA, Runx2, OSX, and mTORCl signaling pathways of PS6K1 and PS6 expanded altogether with the expansion of leptin focus in vitro, and PS6K1, PS6 protein, protein, and rapamycin were essentially higher in the leptin group. Rapamycin, an inhibitor of mTORC1 signaling pathway, can viably repress osteogenic separation and heterotopic solidification of TDSCs in Achilles tendon instigated by leptin.

INTRODUCTION

Tendon stem cells (Tendon-derived stem cells, TDSCs) are principle undifferentiated cells, have the ability of multilineage separation, for example, osteoblast, chondroblast, lipoblast, tendon cells (Zhang et al. 2015; Guo et al. 2015). The changed neighborhood condition got from harmed Achilles tendon can cause osteogenic separation of stem cells in the Achilles tendon tissue, bringing about the development of Heterotopic solidification (HO). Leptin is a hormone-like protein emitted by fat cells and assumes a significant job in bone metabolism and bone rebuilding, neuroendocrine, insusceptible guideline, and wound fix (Xu et al. 2018; Zhao et al. 2016). TDSCs and mesenchymal stem cells (MSCs) have similar biological components rightly working for tissue recovery and fixation of small-scale injuries of tendons. Earlier research have also shown that TDSCs osteogenic differentiation plays a fundamental role in HO tendon. Leptin can promote osteogenic separation of osteoblasts, and its behavior is very small in normal tendon tissue, but it is considered to be particularly upwardly controlled in tendon HO tissue (Gao et al. 2015). Leptin effectively work on the osteogenic separation of TDSCs and the development of HO in tendons, just as downstream intracellular molecular mechanism,

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has not been effectively understood (Zhang et al. 2016).

It has been accounted for that leptin could actuate themTORC1 pathway in numerous other cell types. The mammalian rapamycin target protein complex 1 (mTORC1) signaling pathway is a key pathway controlling the sign inside and outside the cell. The hindrance of the mTORC1 pathway can obstruct the transduction of different development factor unusual signs. The rapamycin is a particular inhibitor of mTORCl signaling pathway. Various literary works revealed that restraint of mTORCl signaling pathway can stifle osteogenic separation of multiple cells (Zhang et al. 2016). A past study showed that mTOR complex 1 (mTORC1) plays a fundamental role in post-natal bone alignment and bone repair (Long and Chen 2014). Its initiation stimulates the recruitment of mesenchymal stem cells to differentiate into osteoblasts (Xian et al 2012; and the inhibition of mTORC1 will reduce the formation of osteoblasts (Singha et al. 2008; Isomoto et al, 2007). Since HO implies cartilage in the separation of cells and osteoblasts (Ranganathan et al. 2015), the mTORC1 pathway may provide the necessary signal prompt for HO. Therefore, it stays hazy whether leptin can stifle leptin-incited osteogenic separation of TDSCs and ectopic calcification of Achilles tendon by hindering mTORCl signaling pathway. Present investigation estimate that leptin assumes job in osteogenic separation of TDSCs and ectopic hardening of Achilles tendon, and study the impact of mTORC1 signaling pathway inhibitor rapamycin on osteogenic separation of TDSCs actuated by leptin and ectopic solidification.

MATERIAL AND METHODS

Animal Materials and Grouping

Seventy six sound male Sprague-Dawley rats, matured 6 years with a solitary weight of 241-265 g, bought from Beijing WeitongLihua Experimental Animal Technology Co., Ltd. [permit number: SCXK 2007-0001 (Beijing)] were chosen. Rats were pre-hospitalized in animal research center individual ventilated confines, 6 for each cage, and all rats were taken care of with the equivalent sterile food without limiting drinking water. All rats were fasted for 12 h before medical procedure. Rat TDSCs were extricated for osteogenic enlistment culture, and 76 rats were haphazardly partitioned into typical group, HO+RA group, HO group, HO+LEP+RA group and HO+LEP group utilizing irregular table strategy. There was no critical distinction in all information between these groups (P>0.05). This investigation was accounted for to the Animal Protection Association and the Medical Ethics Committee for endorsement.

Experimental Drugs and Reagents

Rat recombinant leptin (Shanghai Soobao Biotechnology Co., Ltd.), PBS cradle (Beijing Lanbosite Biotechnology Co., Ltd.), rapamycin (Tianjin Xinmei Biotechnology Co., Ltd.), type I collagenase (Shanghai Limin Industrial Co., Ltd.), TRIzol reagent (Xiamen Research Biotechnology Co., Ltd.), invert interpretation unit (Beijing SuoLaibao Technology Co., Ltd.), â-mercaptoethanol (Wuhan RongenWeiye Chemical Co., Ltd.), xylene (Shandong Maojun Chemical Technology Co., Ltd.); OCN counter acting agent (Shanghai Jianglai Biotechnology Co., Ltd.) citrate support (Beijing Huakesheng Fine Chemical Products Trading Co., Ltd.), EnVisionTM (Nanjing Jingda Biotechnology Co., Ltd.), formalin arrangement (Hunan Kang Pharmaceutical Co., Ltd.), hematoxylin (Shanghai Shifeng Biotechnology Co., Ltd.), ALP Primer (Shanghai Yingjun Biotechnology Co., Ltd.), OCN Primer (Shanghai Quanyang Biotechnology Co., Ltd.), Runx2 immune response (Shanghai Anzhen Bio-Limited Company), OCN Primer (Shanghai Quanyang Biotechnology Co., Ltd.)OSX neutralizer (Shanghai Anzhen Biological Co., Ltd.),

Experimental Model Establishment

Normal Group: Basic skin cut at the two sides of the Achilles tendon was performed, trailed by stitching; multi week after medical procedure, 0.1 ml typical saline was infused around the Achilles tendon once every week; and 1 mg/kg saline was intraperitoneally infused daily.

HO Group: Bilateral Achilles tendon was cut away in midcourse, multi week after medical procedure, 0.1 ml typical saline was infused around the Achilles ligament once per week; and

1 mg/kg saline was intraperitoneally infused, daily.

HO+RA Group: Bilateral Achilles tendon was cut away in midcourse, multi week after medical procedure, 0.1 ml typical saline was infused around the Achilles tendon once per week; and 1 mg/kg rapamycin was intraperitoneally infused, daily.

HO+LEP Group: Bilateral Achilles tendon was cut off in midcourse, multi week after medical procedure, 0.1 ml recombinant leptin protein was infused around the Achilles tendon once every week; and 1 mg/kg saline was intraperitoneally infused, daily.

HO+LEP+RA Group: Bilateral Achilles tendon was cut off in midcourse, one week after medical procedure, 0.1 ml recombinant leptin protein was infused around the Achilles tendon once every week; and 1 mg/kg rapamycin was intraperitoneally infused, daily.

qRT-PCR

After treatment of TDSCs with various concentrations of leptin (Sigma-Aldrich,Beijing, China) (1ng/m1, 10 ng/m1, 100 ng/m1) for 14 days, qRT-PCR was utilized to distinguish the declaration of osteoblasts ALP, OSX, Runx2 and OCN mRNA in TDSCs. The overall RNA of rat P2 age TDSCs was extricated and solidified in - 80 °C temperature. The strategy was performed by the directions in the opposite interpretation unit. The reverse transcription items were identified by qRT-PCR for the statement of TDSCs osteogenic factor ALP, OSX, Runx2, OCN mRNA.

Western Blot

Western Blot was applied to identify TDSCs osteogenesis factor Runx2, OSX protein articulation and articulation of the downstream factors PS6K1, PS6 protein in the mTOIC1 signaling pathway. After treatment of TDSCs with 100 ng/ml leptin + 10nM rapamycin and 100 ng/ml leptin for fourteen days, Western Blot was utilized to recognize the statement of PS6 and PS6K1 proteins in the downstream of mTOIC1 signaling pathway and the declaration of osteoblasts OSX and Runx2 in TDSCs. All cells were cleaned with PBS buffer, and cell lysate was included for 15 min. At that point it was accelerated, and the supernatant was gotten, trailed by electrophoresis, transfer memberane, and block. The primary antibody (Abcam, USA) was added, shaken at 4° C overnight, an secondary antibody was included, and shaken at 25° C for 1 h. Subsequent to washing the membrane, introduction and improvement were done, and the dim estimation of each strip was broke down by a Quantity-one gel imaging analysis (BioRad, China) framework.

Immunohisto Chemical Method

The outflow of leptin in the HO group and the normal group was recognized by immunohistochemistry. The statement of OSX and Runx2 in HO group, RA+HO group, HO+LEP+RA group and HO+LEP group and the outflow of mTORCl downstream marker PS6 were recognized by immunohistochemistry. The Achilles tendon tissue was fixed with formalin solution, and afterward got dried out, installed in paraffin, cut into 4 ìm sequential areas, trailed by dewax, hydration, and endogenous hydrogen peroxide catalyst disposal, heat repair, PBS flushing, serum blocking. At that point it was incubated with primary antibody (Abcam, china) overnight at 4° C, flushed with PBS, and a universal secondary antibody (Abcam, China) was included at 25° C for 30 minutes, at that point washed with PBS, trailed by coloring, counterstaining, dehydration, stain-clearing, mounting. The outflow of leptin, OSX, Runx2, PS6 was checked.

Statistical Processing

Every single factual investigation were performed utilizing Statistical Package SPSS 22.0. Estimation information were communicated as the m± SD (Mean ± standard deviation), examination between two samples was estimated by Student's t-test. Examination between groups was performed utilizing ANOVA (one way analysis of variance) or Mann-Whitney U test. $P< 0.05$ was considered measurably huge.

RESULTS

Leptin (LEP) Promoted the Expression of Osteogenesis Factors ALP, OSX, Runx2, mRNA, OCN in TDSCs

The aftereffects of qRT-PCR indicated that the declaration of osteogenic factors ALP, OSX, Runx2 and OCN mRNA expanded fundamentally with the expansion of leptin concentration (P<0.05) (Table 1).The articulation was expanded gardually with the increase concentration of leptin showing that the osteogenic fators articulation were dose dependent. Western Blot results indicated osteogenic factors OSX and Runx2 expanded essentially with the expansion of leptin focus, and the thing that matters was factually significant (P<0.05) (Fig. 1).Simialr patterns were seen for leptin on the statement of osteoblasts OSX and Runx2 in TDSCs, as the leptin fixation was expanded the articulation were additionally upregulated (Table 2).

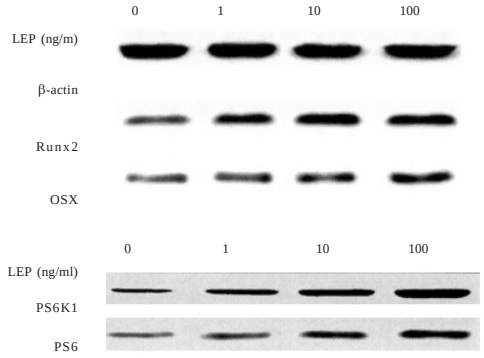

Fig. 1. Leptin endorses oosteogenesis protein expression in TDSCs; leptin effects on mTORCl signaling pathway during TDSCs osteogenic differentiation of TDSCs

Table 2: Effects of various concentrations of leptin on osteoblasts Runx2 and OSX expression in TDSCs

Group	*Runx2*	*OSX*
Control group	1.04± 0.03	1.03± 0.03
1ng/m1leptin group	1.66± 1.21*	1.56± 0.10*
10ng/m1 leptin group	2.33± 0.15*#	1.99± 0.14*#
100ng/m1 leptin group	3.02± 0.19*# and	2.89± 0.14*# and

Note: Statistically compared with control (*P<0.05); with 1 ng/m1leptin group (#P<0.05); with 10 ng/m1leptin group (*#P<0.05)

Leptin Activated mTORCI Signaling Pathway in the Course of Osteogenesis Differentiation of TDSCs

Various groupings of leptin was minded the statement of downstream factors PS6 and PS6K1 of mTOIC1 signaling pathway. The declaration of these components were excessively expanded with leptin treatment in portion subordinate manner (Table 3). Western Blot results demonstrated that the outflow of PS6 and PS6K1 protein expanded altogether with the expansion of leptin concentration, and the thing that matters was factually critical (P<0.05) (Fig. 1).

Table 3: Effect of various concentrations of leptin on downstream factors PS6K1 and PS6 expression on mTOIC1 signaling pathway

Group	*PS6K1*	*PS6*
Control group	1.06± 0.09	1.07± 0.10
1ng/m1leptin group	1.96± 0.19*	1.88± 0.20*
10ng/m1 leptin group	2.89± 0.12*#	2.53± 0.18*#
100ng/m1 leptin group	4.26± 0.15*#and	3.67± 0.15*# and

Note: Statistically compared with control (*P<0.05); with 1 ng/m1leptin group (#P<0.05); with 10 ng/m1leptin group (*#P<0.05)

Table 1: Effects of various concentrations of leptin on ALP, Runx2, OSX and OCN mRNA expression

Group	*ALP*	*Runx2*	*OSX*	*OCN*
Control group	1.03±0.02	1.41±0.02	1.04±0.02	1.02±0.02
1ng/m1leptin group	1.35±0.09*	1.95±0.17*	1.80±0.19*	1.38±0.09*
10ng/m1 leptin group	1.98±0.16*#	2.49±0.12*#	2.28±0.22*#	1.89±0.17*#
100ng/m1 leptin group	2.91±0.17*# and	3.54±0.19*# and	3.15±0.20*# and	2.62±0.21*# and

Note: Statistically compared with control (*P<0.05); with 1 ng/m1leptin group (#P<0.05); with 10 ng/m1leptin group (*#P<0.05)

Rapamycin (RA) Inhibited mTORCI Signaling Pathway in the Course of Osteogenic Differentiation of TDSCs

Articulation of downstream factors PS6 and PS6K1 was expanded in portion subordinate way as it was indicated in previous results, yet strikingly when it was treated with rapamycin and leptin the articulation was restrained (Table 4).This outcome was bolstered by western Blot results which demonstrated outflow of PS6 and PS6K1 protein in leptin group was fundamentally higher than that in leptin + rapamycin group and control group, and the thing that matters was factually noteworthy (P<0.05) (Fig. 2).

Table 4: Effect of rapamycin on mTORCl signaling pathway during osteogenic differentiation of TDSCs

Group	*PS6K1*	*PS6*
Control group	1.06±0.04	1.01±0.02
100ng/m1leptin	4.26±0.17*	3.66±0.12*
100ng/m1 leptin + 10nM rapamycin	0.16±0.04*#	0.09±0.01*#

Note: Statistically compared with control group (*P<0.05); with 100 ng/m1leptin group (#P<0.05)

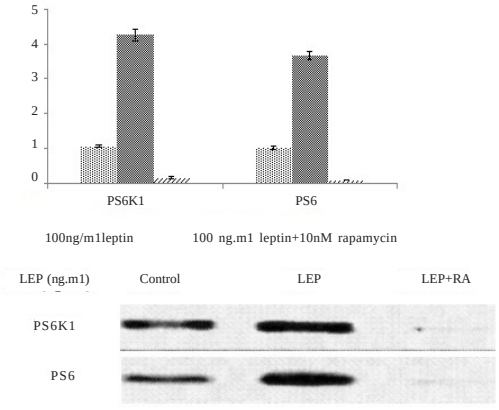

Fig. 2. mTORCl signaling pathway during morphogenesis of TDSCs by rapamycin

Rapamycin Inhibited the Expression of Osteogenic Factors in TDSCs

Articulation was osteogenic factors was downegualted in presence of rapamycin which modulate the impact of leptinon these components (Table 5). Western Blot results demonstrated that the outflow of OSX and Runx2 protein in the leptin group was fundamentally higher than that in the leptin + rapamycin group and control group, and the thing that matters was measurably huge (P<0.05) (Fig. 3).

Table 5: Effect of rapamycin on the expression of osteoblasts Runx2 and OSX in TDSCs

Group	*Runx2*	*OSX*
Control group	1.09±0.03	1.03±0.02
100ng/m1leptin	3.02±0.09*	2.91±0.12*
100ng/m1 leptin + 10nM rapamycin	1.51±0.12*#	1.54±0.09*#

Note: Statistically compared with control group (*P<0.05); with 100 ng/m1leptin group (*#P<0.05)

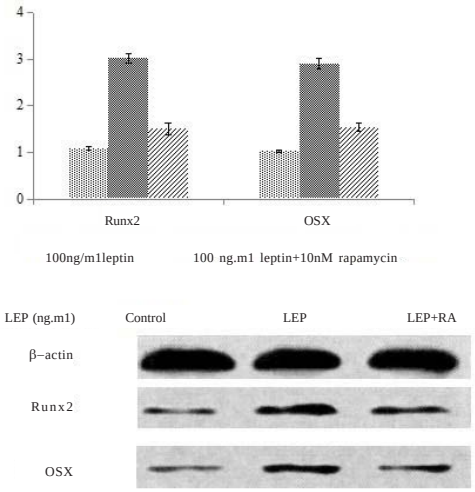

Fig. 3. Effect of rapamycin on TDSCs osteogenic differentiation

Immunohistochemical Staining-Expression of Leptin in Normal Group and HO Groups and Its Effect on Expression of Osteogenic Factors and Ectopic Ossification of Achilles Tendon

Immunohistochemistry demonstrated that the declaration of leptin was altogether lower in the normal group than HO group (P<0.05) (Fig. 4). The consequences of immunohistochemistry demonstrated OSX and Runx2 in HO+RA group was altogether lower than HO gathering (P<0.05). Runx2 and OSX in HO group was essentially low-

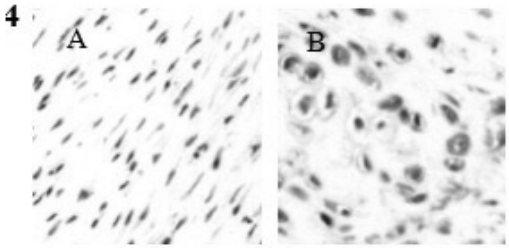

Fig. 4. Expression of leptin in normal and HO groups-the expression of leptin in the normal group; B. the expression of leptin in the HO group

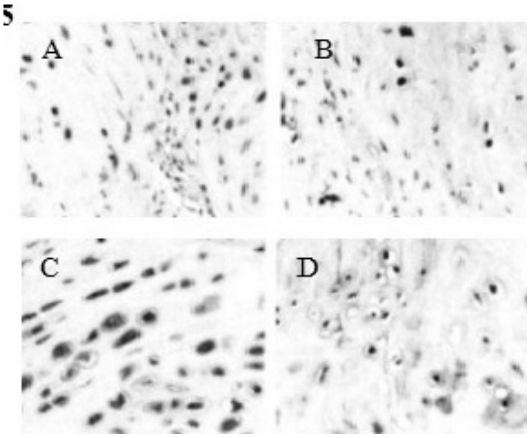

Fig. 5. Effect of leptin and rapamycin on the expression of heterogenous ossified Runx2 in Achilles tendon.
A. the expression of Runx2 in HO group;
B. the expression of Runx2 in HO+RA group;
C. the expression of Runx2 in HO+LEP group;
D. the expression of Runx2 in HO+LEP+RA group

er than HO+LEP group and HO+LEP+RA group (P<0.05). There was no critical contrast in the outflow of Runx2 and OSX between HO+LEP+RA group and HO group (P>0.05) (Figs. 5 and 6).

Effects of Leptin on the Heterotopic Ossified mTORCI Signaling Pathway in Achilles Tendon

The consequences of immunohistochemistry demonstrated that the declaration of PS6 in HO group was essentially higher than HO+RA group (P<0.05). The outflow of PS6 in HO group was altogether lower than HO+LEP group (P<0.05). The statement of PS6 in HO+LEP+RA group was significantly lower than HO+LEP group (P<0.05) (Fig. 7).

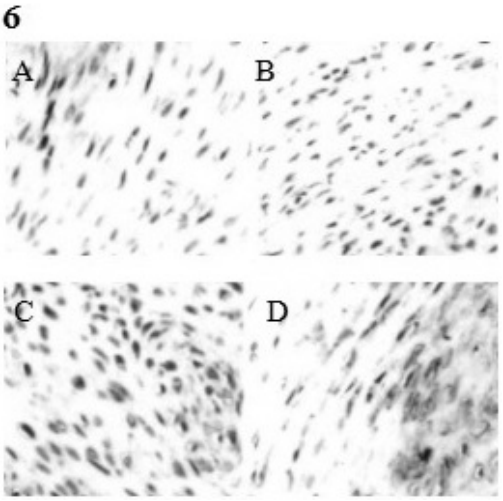

Fig. 6. Effect of leptin and rapamycin on the expression of ectopic ossified OSX in
A. Achilles tendon- the expression of OSX in the HO group;
B. the expression of OSX in the HO+RA group;
C. the expression of OSX in the HO+LEP group;
D. the expression of OSX in the HO+LEP+RA group

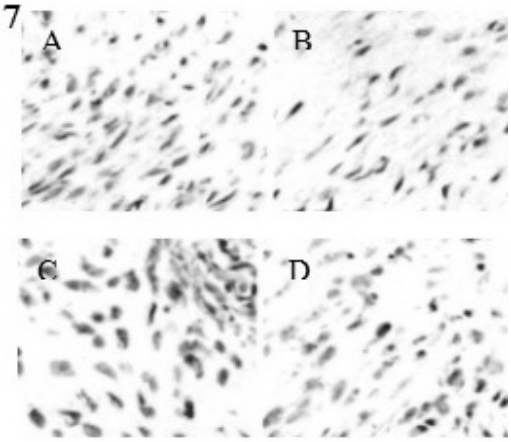

Fig. 7. Effect of leptin and rapamycin on the expression of PS6 downstream marker of ectopic ossified mTORCl in Achilles tendon-
A. the expression of PS6 in the HO group;
B. the expression of PS6 in the HO+RA group;
C. the expression of PS6 in the HO+LEP group;
D. the expression in the HO+LEP+RA group

DISCUSSION

Tendon is a string like or membranous thick connective tissue made out of fiber collagen bundles that associate muscles and bones, and its footing causes muscle contraction to drive body movement (Spang et al. 2016). They can separate into tendon cells and change into nontend cells, for example, fat cells, chondrocytes and bone cells under specific conditions, and can likewise take an interest in the repair process of tendon injury (Stolk et al. 2017; Liu et al. 2018; Li et al. 2015a). Information indicated that the likelihood of heterotopic hardening in patients with Achilles tendon injury is 14 to 62 percent (Li et al. 2015a; Li et al. 2015b). This examination planned to investigate the job and instrument of leptin in TDSCs osteogenic separation and the development of ectopic hardening of Achilles tendon, which is of clinical importance for the avoidance and treatment of heterotopic ossification. It is accounted that leptin can actuate mTORCl flagging pathway in an assortment of cells, and initiation of mTORCl signaling pathway can advance osteogenic separation of mesenchymal stem cells (Jiang et al. 2017; Li et al. 2016). Hence, leptin is related with heterotopic ossification and it is a significant factor in heterotopic ossification. Blood leptin levels are constantly raised at various level in obesity patients (Rostami et al. 2017). Low degrees of leptin lead to an assortment of cardiac dysfunction, for example, calcium guideline issue, diminished myocardial contractility, and prolonged relaxation time in mice cardiomyocytes (Sullivan et al. 2018). This investigation uncovered that leptin articulation levels in patients with heterotopic ossification are fundamentally higher than that in normal Achilles tendon tissue.

mTOR assumes a centralized role in prompting and actuating different interlinked pathways. In this way, it goes about as a signaling center and manages certain physiological highlights of the cell (Ganesan et al. 2019). mTOR is a mammalian objective of rapamycin, which exists as synergist subunits of mTORC1 and mTORC2. The mTORCl signaling pathway is engaged with the guideline of bone metabolism and bone formation, and assumes a vital job in cell quality interpretation, ribosome biosynthesis, protein interpretation commencement and apoptosis (Chen and Long 2014). Present examination found that with the expansion of leptin fixation, osteogenic factors ALP, Runx2, OCN mRNA, OSX and Runx2, OSX protein and mTORCl signaling pathway downstream factors PS6K1, PS6 protein articulation expanded essentially, recommending that leptin can adequately actuate the mTORCl signaling pathway over the span of osteogenesis separation of TDSCs. The expression of PS6, PS6K1 protein, OSX and Runx2 protein in the leptin group was altogether higher than control group and the leptin + rapamycin gathering, proposing that rapamycin can fundamentally repress leptin-prompted osteogenic separation of TDSCs in the wake of blocking mTORCl signaling pathway.

In-vivo explores demonstrated that the declaration of leptin in the HO group was altogether higher than that in the normal group, recommending that leptin is profoundly communicated during the ectopic solidification of Achilles tendon. The after effects of immunohistochemistry indicated that the statement of OSX and Runx2 in HO group was altogether higher than HO+RA group. The declaration of Runx2 and OSX in HO group and HO+LEP+RA group was essentially lower than HO+LEP group. There was no critical distinction in the outflow of OSX and Runx2 between HO group and HO+LEP group. It is recommended that leptin can advance the declaration of Runx2 and OSX during the heterotopic ossification of Achilles tendon, and rapamycin can advance the outflow of OSX and Runx2 through repressing leptin (Jiang et al. 2018).The consequences of immunohistochemistry uncovered that the outflow of PS6 in HO group was essentially higher than that in HO+RA group. The statement of PS6 in HO group was altogether lower than that in HO+LEP group. The outflow of PS6 in HO+LEP group was essentially higher than that in HO+LEP+RA group. Leptinis firmly identified with heterotopic ossification, engaged with regulating body feeding, neuroendocrine, angiogenesis, energy metabolism and different activities. Jiang et al. (2018) found that serum leptin is profoundly communicated in patients with heterotopic ossification. After the arrangement of heterotopic ossification when medicate treatment fails, medical procedure is required, however the repeat rate and expenses is high (Ganesan et al. 2019).

CONCLUSION

The mTORC1 signaling pathway inhibitor rapamycin can adequately restrain the osteo-

genic separation of leptin-actuated TDSCs and the development of ectopic calcification of Achilles tendon. This recommend leptin can actuate the mTORCI signaling pathway throughout heterotopic ossification of Achilles tendon, and the utilization of rapamycin to hinder the mTORCl signaling pathway can successfully restrain PS6 articulation.

RECOMMENDATIONS

Leptin may advance differentiation of TDSC osteogenic separation and arrangement of heterotopic bone *in-vitro* and *In-vivo* model by means of mTORC1 signaling, which gives another possible therapeutic target for HO anticipation.

ABBRREVIATIONS

Tendon derived stem cells (TDSCs)
Heterotopic ossification (HO)
Mesenchymal stem cells (MSCs)
Mammalian rapamycin target protein complex 1 (mTORC1)
Alkaline phosphatase (ALP)
Runt related transcription factor 2 (Runx2)
Osteogenic related transcription factor (OSX)
Osteocalcin (OCN)

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