Inhibition of Nf-κb prevents trauma-induced heterotopic ossification in rat model

Jinyong Ju[1*],Du Yu[2*],Feng Xue[2],Yong Zhao[2],Weizhe Shi[2],Mingmang Pan[2],Guo Tang[2] and Haijun Xiao[2]

1.Department of Orthopaedics,Fenxian District Central Hospital Graduate Training Base, Jinzhou Medical University,Shanghai,China

2.Department of Orthopaedics,Fenxian District Central Hospital ,Shanghai,,China

*These authors contributed equally to this work.

Corresponding author:

Haijun Xiao

Department of Orthopaedics,Fenxian District Central Hospital ,Shanghai,,China

Tel: +86-13564695890

Fax: +86-021-64085875

E-mail: xiaohaijun89@126.com

Abstract

Background. To find a better prophylactic regimen, the pathogenesis of acquired heterotopic ossification (AHO) must be more understood. To date, AHO formation is largely thought to be related to inflammation, which is activated by trauma, resulting in AHO by up-regulation of pro-osteogenic genes.

Methods. Brain-traumatic/burn/tenotomy model is firstly used in experiment. At first ,44 rats were randomly divided into two groups: E group and C group.Two rats in every group were euthanized during second,third,fourth,sixth,eighth,tenth weeks for collecting tendon. The remaining rats survived until tenth week for X-Ray radiation examination to confirm the size of AHO.Then,124 rats were randomly divided into four group: P group, L group, M group, H group. The three rats of every group were euthanized during every week of the first seven weeks for collecting tendon to detect P65 protein.The remaining rats survived until tenth week for X-Ray examination to confirm the size of AHO.

Results. The success rate of Brain-traumatic/Burn/Tenotomy model is 100%. Difference of P65 expression in E group and in C group are statistically significant,and that in E group is higher.Pharmacologic inhibition of Nf-κb signaling pathway limits AHO formation, and that The bone formation content of M group is decreased.

Conclusion. Brain-traumatic/Burn/Tenotomy model is highly reliable.Results indicate that the Nf-κb /p65 signaling response occurs in the forming process of AHO. PDTC limits formation of AHO. The most effective concentration is 6mg/ml for local injection.

Subject Orthopaedic

Key word Nf-κb/P65,inflammation,heterotopic ossification ,Pharmacologic inhibition ,PDTC

Introduction

Heterotopic ossification (HO) ,the ectopic formation of bone in soft tissues, is a musculoskeletal disorder. Acquired heterotopic ossification (AHO) offten occurs following traumatic injury, burn injury, spinal cord injury ,traumatic brain injury and orthopaedic surgery,(Kraft, 2015; Amar et al., 2015) which is commonly observed around hip, knee, shoulder and elbow joints,(Griffin et al., 2013)leading to pain ,reduction in range of motion .To prevent AHO, Indomethacin, celecoxib and radiation are generally accepted by patients as prophylactic treatment in spite of many side effects.(Baird & Kang, 2009)Hewever, surgical resection is the only choice once it come into being,even thought, a high recurrence rate is observed.

To find a better prophylactic regimen, the pathogenesis of AHO is not completely understood. To date, AHO formation is largely thought to be related to inflammation. The inflammation is activated by traumatic, which results in AHO by up-regulation of pro-osteogenic genes.(Kan & Kessler, 2014; Davis et al., 2013; Korboi N. Evans, 2012) The predominant cytokine of the inflammation is the lagand of receptor activator of nuclear factor κb(Nf-κb). The inflammatory reponse induces a cascade of Nf-κb , which not only promotes angiogenesis and osteogenic differentiation, but also induce osteoprogenitor cells to release bone morphogenetic proteins (BMPs).(Mountziaris & Mikos, 2008) Forsberg etal has demonstrated that IL-3, IL-12p70 and IL-13 were associated with AHO,(Forsberg et al., 2014)They also activated Nf-κb . More than that, it has been demonstrated that hypoxia inducible factor-1α(HIF-1α), a key mediator of cellular a adaptation to hypoxia , plays a pivotal role in AHO.(Agarwal et al., 2016) TAK1-IKK axis is the major mechanism of

underlying the activation of the Nf-κb pathway following hypoxia stimultion.(D Ignazio & Rocha, 2016) So, Nf-κb plays an important role in AHO formation. It is a very important to detect the effect of Nf-κb in AHO.

Many rodent modes exist in studying AHO. In according to clinical high risk factors,we create a Brain-traumatic/burn/tenotomy model, which undergos Achilles' tendon transection with concomitant partial-thickness dorsal burn injury and moderate traumatic brain injury.(Peterson et al., 2014; Dizdar et al., 2013)In this experiment,we aim to research if Nf-κb is an important signaling pathway of AHO formation.

Materials and Methods

Reagents and antibodies

Anti-Nf-κb P65 antibody was purchased from Abcam（ab16502,Shanghai,China).Anti-Gapdh rabbit polyclonal antibody was purchased from Sangon Biotech(D110016,Shanghai, China).HRP-conjugated Goat Anti-Rabbit IgG was purchased from Sangon Biotech(D10058, Shanghai,China）Ammonium pyrrolidinedithiocarbamate(PDTC) was purchased from Abcam (ab141406,Shanghai,China).Trizol reagent, SYBR Green PCR Master Mix and RT Kit were purchased from Invitrogen.

Animal experiment

Male Sprague-Dawley rats(4 weeek,200±5g), purchased from Shanghai SLAC Laboratory Animal Co.,Ltd(Animal Quality Certificate:2007000562918), were kept in the Laboratory Animal Center, East China Normal University(Animal Experment License:SYXK2010-0094), Shanghai.All rats were kept in the cage in an SPF-grade Lab.

The whole operation was performed under general anesthesia. The rats were anaesthetized using Isoflurane(Model：R510-22,RWD,Shanghai), followed by skin preparation for surgery and routine disinfection using ethanol on the parietal lobe of the rat, covered with drapes.The scalp was cut open with 1cm incision.Then a bone window was polished by dental drill with a diameter of 5 mm on the parietal lobe of the rat for the dura intact. A 25-g weight on the free fall instrument fell from 30 cm height and hit bone window of the rat resulting in mild brain injury. Incision was smeared with amoxicillin (5 mg) before it was closed by 4-0 silk suture. Then,the skin of the rat legs were prepared for surgery and disinfected with ethanol .The rats underwent bilateral midpoint Achilles tenotomy though a posterior approach. Incision was routinely closed with a 4-0 silk suture after it was smeared with amoxicillin (5 mg).At last,the rat received a 30％ of back surface-area partial-thickness dorsal burn injury after the skin was prepared.Because burn patients are a critical patient population at risk for trauna-induced HO development.(Downey et al., 2015; Peterson et al., 2014)(Figure 1).

The experiments were divided into two parts. In the first part, 44 rats were randomly divided into two Groups: experiment group (E group) and Control group (C group).The rats of C group were operated for exposing achilles' tendon though a same approach without tenotomy.Then the
incision was routinely closed with 4-0 silk suture.Two rats in E group and C group were euthanized during second,third,fourth,sixth,eighth,tenth weeks for collecting tendon. The remaining rats survived until tenth week for X-Ray radiation examination to confirm the size of AHO.

In the second part, 124 rats were randomly divided into four group: positive control group (P group), low dosage group(L group), mediate dosage group(M group) and high dosage

group(H group). All rats were operated for AHO model.The PDTC was dissolved in normal saline(NS) for three concentration: low dosage(2mg/ml), mediate dosage(6mg/ml) and high dosage(10mg/ml). P group ,L group, M group and H group was respectively administered normal saline, low dosage, mediate dosage and high dosage of PDTC at tendon transection site via local injection for 0.1 ml. They were administered every day for a total of two weeks. Three rats in each were euthanized during every week of the first seven weeks for collecting tendon to detecting P65 protein.The remaining rats survived until tenth week for X-Ray radiation examination to confirm the size of AHO.

Figure 1 Trauma-induced model of HO in which mice receive a 30% of back surface-area partial-thickness dorsal burn injury with bilateral midpoint Achilles tenotomy and mild brain injury, resulting in HO formation in the soft tissue of transection site.

X-Ray radiation examination

The remaining rats anesthetized with isoflurane were imaged at tenth week of postoperation using the cabinet X-Ray system(MX-20,America) with the following setting:32-KV X-Ray beam ,current of 250 μA and an scans time of 6 seconds.The image of AHO is analyzed with Quantity One software.

Western blot analysis

The tendon was collected at the indicated time. Every tissue specimen was harvested 2.0mg, which was lysised for 30 minutes in RIPA lysis buffer supplemented with protease inhibitors after being grinded in glass blender on ice. Protein concentration was measured by BCA protein assay kit(Beyotime,Shanghai).Then it was run on SDS-PAGE gels and electrotranferred to nitrocellulosemembrane at 4 ℃ for 60 minutes. The blots were probed with anti-P65 at 1:2000 and GAPDH at 1:5000 dilation Overnight at 4℃.The proteins were detected by electro-chemi-luminescence . GAPDH was used as an internal control.The result was analyzed with Quantity One software.

Quantitative real-time RT-PCR analysis

Total RNA of the specimens was extracted with Trizol reagent(Sangon Biotech,Shanghai) and quantified by spectrophotometer(NANODROP 2000C, Thermo). cDNA synthesis was performed using RNA(1μg) as a template by AB Applied Biosystems(Veriti Thermal Cycler).Primers specificfor murine P65 and GAPDH were used.For quantitative real-time PCR, P65 was amplified using 5’-CACTGTCACCTGGAAGCAGA-3’ and

5’-GACCTGGAGCAAGCCATTAG-3’. GAPDHwas amplified using 5’-AGGTCGGTGTGAACGGATTTG-3’ and 5’-TGTAGACCATGTAGTTGAGGTCA-3’. SYBR green was used for detection of the product using the SYBR Green PCR Master Mix assay(Applied Biosystems). The standard curve used series of duplicate dilutions of plasmid for P65 gene and GAPDH cDNA.The amplification reaction was performed for 40 cycles with denaturation at 95℃ for 10 minutes,followed by annealing at 95℃ for 15 seconds and extension and detection at 60℃ for 1 minutes.The relative RNA abundance of P65 Gene transcript was normalized against endogenous gene control.

Histological and immunohistochemical staining

Histologic evaluation was performed at certain time point .The tendon tissues were fixed in 10％ neutral formaldehyde solution for one day and subsequently decalcified in 15％ EDTA solution for 2-3 weeks at 4℃. After that, the tissues were dehydrated using ethanol and embedded in paraffin to cut 5μm thick section.The section was mounted on the slide.HE staining was performed on some sections for light microscopy. However, immunostaining was performed with the goat anti-rat anti-P65. We achieve the final image at appropriate dilutions(1:1000).

Statistical analysis

Western blot parameter analysis and statistical analysis were performed with Quantity One 4.62(Bio-Rad Inc. America) and SPSS 17.0(SPSS Inc.America). Difference between the two groups or four groups were analyzed via independent-samples t Test and analysis of variance. Data was Express as mean ± standard deviation (SD). $p<0.05$ was considered statistically significant.

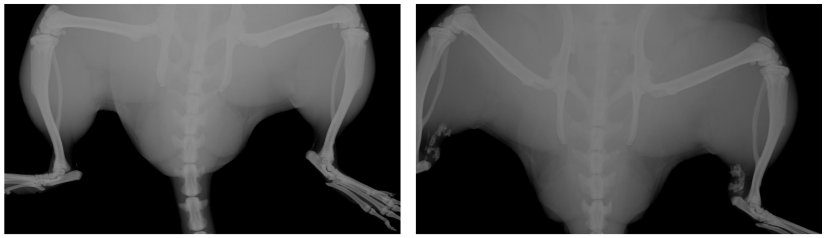
Ethics statement

The animal experiment protocol was approved by the Bioethics Committee of East China Normal University (Animal Experiment License:SYXK 2010-0094)

The result of first part

The success rate of Brain-traumatic/Burn/Tenotomy model is 100%.

In the first part, 22 rats were operated for AHO model in E group, two of whom were dead after the operation. 22 rats operated in C group survived. After ten weeks, the survival rats is 8 in E group and it is 10 in C group.Then the remaining 18 rats receive X-Ray radiation examination .The 8 rats all develop heterotopic bone in E group, and none of 10 rats develop that at tendon transection site in C group (Figure 2).There is a deal of high density heterotopic bone at tendon transection site in E group, but there is nothing at the same site in C group. So the success rate of Brain-traumatic/Burn/Tenotomy model is 100％.



C group *E group*

Figure 2.At the tenth week, the result of X-Ray radiation examination in E group and C group.There is a deal of high density heterotopic bone at tendon transection site in E group, but there is nothing at the same site in C group.

Difference of P65 expression in E group and in C group are statistically significant.

To verify activation and dynamic variation pathway in E group and C group, we examined the Expression of the Nf-κb subunit p65 at second,third,fourth,sixth,eighth,tenth week by western-blotting technique in E group and C group .(Figure 3,4) We also analysis the results of western blot parameter by software.(Table 1) Obviously, the expression of Nf-κb/p65 in E group is higher than that in C group during the ten weeks.It reached a peak at sixth week in E group,then it began to slide.But it always kept a low level in C group.It was the same level in two groups at tenth week. At second,third,fourth,sixth,eighth week,the difference of p65 is significant in E group and C group . To further verify the results, we also examined it by immunohistochemical staining.（Figure 5) Immunohistochemical analysis is consistent with Western- blot result.These results indicate the an Nf-κb /p65 signaling response occurs in the forming process of AHO.

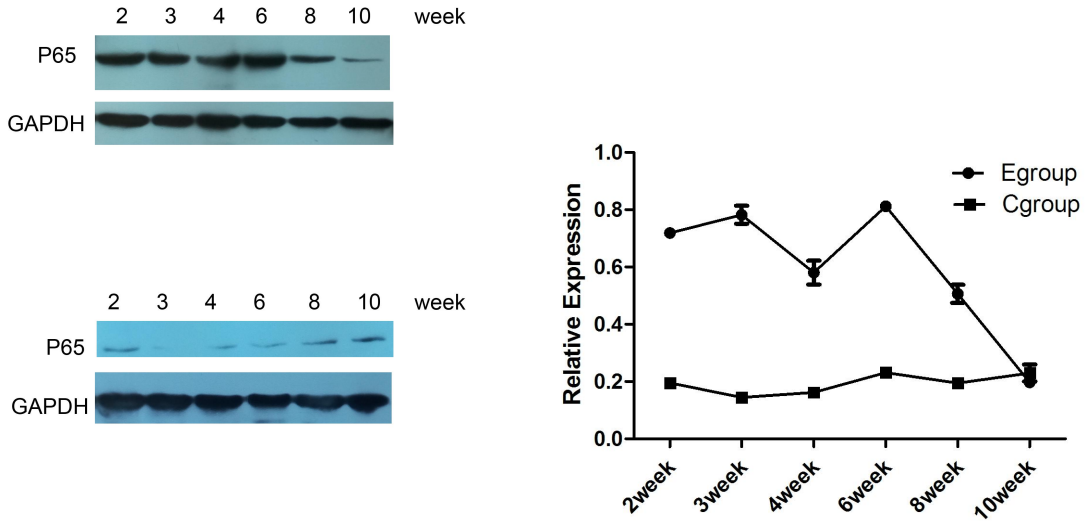


Figure 3,4 The expression of Nf-κb /p65 in E group and C group (Total extract).The expression of Nf-κb /p65 in E group reached a peak at sixth week,then it began to slide.However,it is always low and stable in C group .At tenth week ,it was the same level in two groups.

Table 1

week	2th	3th	4th	6th	8th	10th
E Group	0.72±0.03	0.78±0.05	0.58±0.07	0.81±0.01	0.51±0.05	0.20±0.01
C Group	0.02±0.03	0.14±0.02	0.16±0.02	0.23±0.02	0.19±0.01	0.23±0.05
t	20.17	19.60	9.6	44	9.6	-1.09
p	0.000	0.000	0.001	0.000	0.001	0.381

Table 1 Data are based on analysis of three replicates on four different samples(mean±SD).At second,third,fourth,sixth,eighthweek,Difference of P65 expression in E group and in C group

are statistically significant.

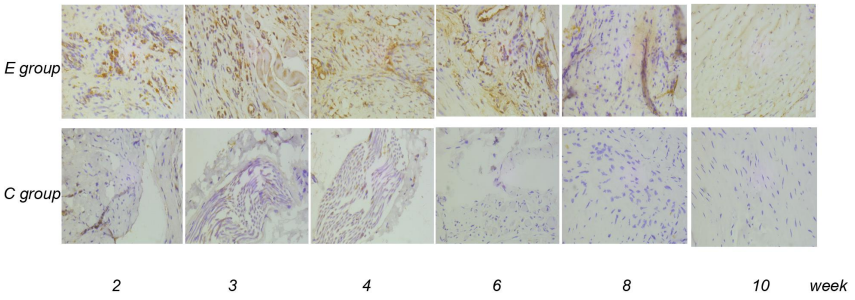


Figure 5 The result of immunohistochemical staining of Nf-κb /p65 in E group and C group.(10×4)It is consistent with Western- blot result.Difference of the expression in two groups is significant.

The result of second part

Pharmacologic inhibition of Nf-κb signaling pathway limits AHO formation after Brain-traumatic/Burn/Tenotomy

To verify whether Nf-κb inhibition can prevent AHO , we used the drug PDTC, which is proved to be a high efficient inhibitor. Then we tested whether treatment of PDTC decrease Nf-κb/p65 expression and cartilage formation.We have four group(P,L,M,H group) for exploring the effect of different concentrations on Nf-κb Signaling pathway and AHO formation. We detected the dynamic variation of Nf-κb/p65 continuously in the first seven weeks. As a result, we found that PDTC diminished Nf-κb/p65 expression in the first seven week in L,M and H group, especially in M group (Figure 6,Table 2) Immunohis-tochemical staining is coincident with western blot.(Figure 7) Further more, In agreement with Nf-κb/p65 protein decrease, Nf-κb/p65 mRNA expression is inhibited in four groups in the first seven week ,especially M group. (Figure 8) So, Nf-κb/p65 signing pathway is inhibited by PDTC and the effect of M group(6 mg/ml) is best.

The X-Ray radiation Image of different groups is shown that the bone formation content of M group is least in four groups.(Figure 9,Table 3) Histologic evaluation after ten weeks confirmed a substantial decrease in the cartilage and bone anlagen in four groups.(Figure 10)

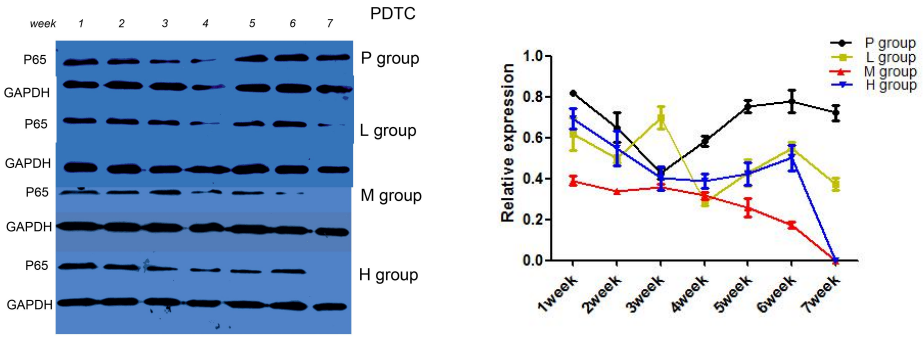


Figure 6A and 6B Total protein was extracted from tendon tissue in four groups and

western-blot analysis for Nf-κb/p65 and Gapdh was performed.Obviously, the expression of Nf-κb/p65 were inhibited in three groups,and it is the most significant in M group.

Table 2

week	1	2	3	4	5	6	7
P group	0.82 ± 0.02	0.65 ± 0.13	0.43 ± 0.05	0.58 ± 0.04	0.75 ± 0.05	0.78 ± 0.10	0.72 ± 0.06
L group	0.62 ± 0.14	0.50 ± 0.03	0.70 ± 0.09	0.28 ± 0.03	0.43 ± 0.11	0.55 ± 0.05	0.37 ± 0.05
M group	0.34 ± 0.05	0.34 ± 0.02	0.44 ± 0.11	0.31 ± 0.31	0.26 ± 0.08	0.17 ± 0.03	0.00 ± 0.00
H group	0.62 ± 0.20	0.54 ± 0.14	0.41 ± 0.09	0.39 ± 0.06	0.43 ± 0.11	0.50 ± 0.11	0.00 ± 0.00
F	16.409	5.308	7.86	31.657	17.641	31.789	230.759
p	0.001	0.026	0.009	0.000	0.001	0.000	0.000

Table 2 Evaluation of Nf-κb/p65 expression in four groups in first seven week.(mean±SD). Four groups are statistically significant in every week..

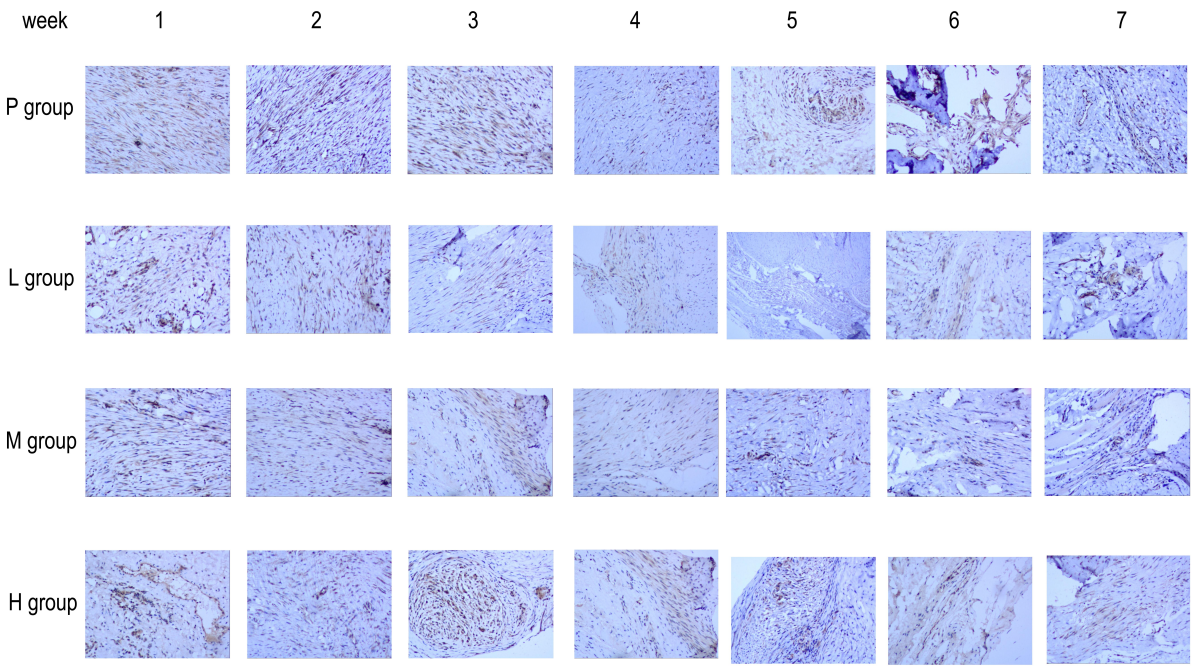


Figure 7 Immunohis-tochemical staining result in four groups in first seven week.Obviously L,M,H group are decreased than P group, especially M group.

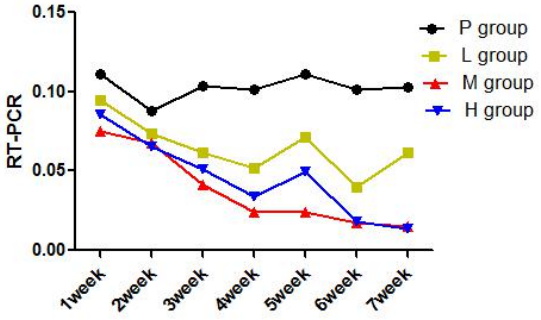


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Figure 8 In agreement with Nf-κb/p65 protein decrease, Nf-κb/p65 mRNA expression is inhibited in four groups in the first seven week ,especially M group

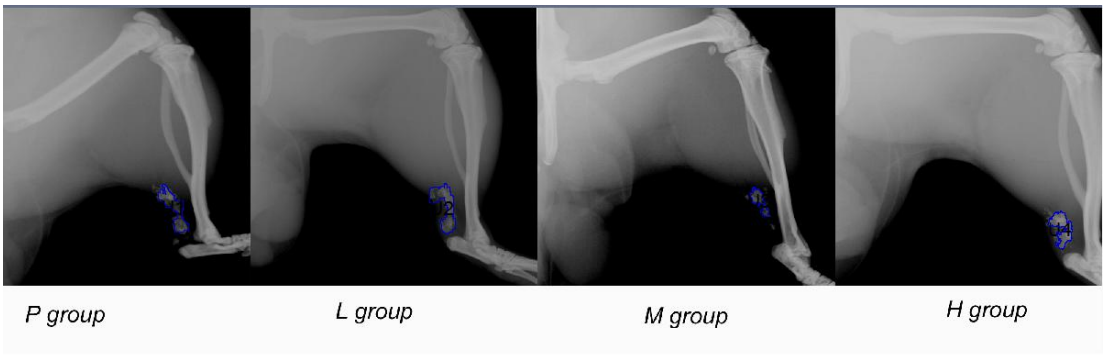

Figure 9 The X-Ray radiation Image of four groups at tenth week.

	Bone mass	Bone mass	Bone mass
P group	4408.25±676,74	4408.25±676,74	4408.25±676,74
L group	4055.00±535.36		
M group		2206.25±175.11	
H group			3045.25±121.64
t	0.819	6.300	3.965
p	0.444	0.001	0.007

Table 3 Ectopic bone mass in four group at tenth week.Difference of bone mass is statistically significant between P group and M group and between P group and H group. Difference is not statistically significant between P group and L group.

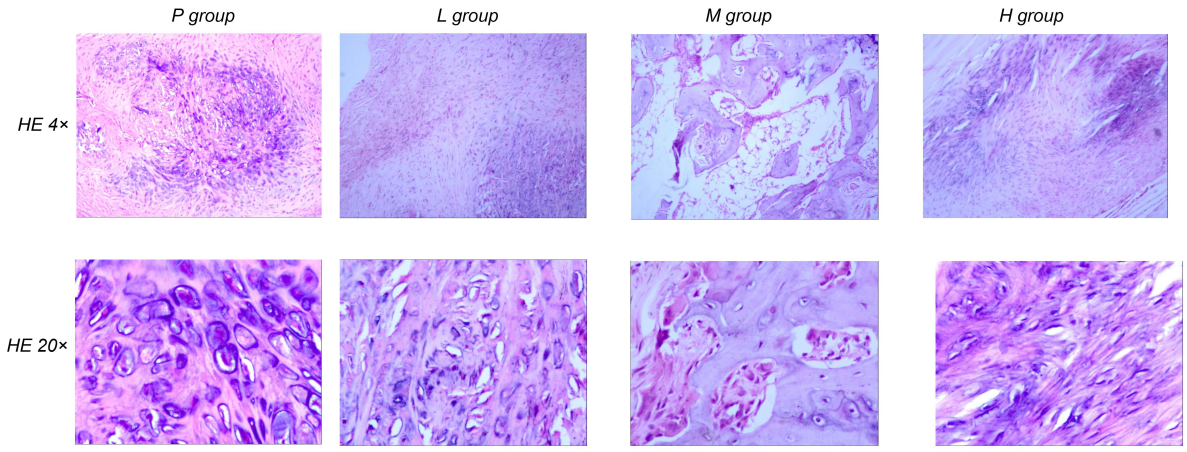

Figure 10 Histologic evaluation in four group at tenth week

Discussion

AHO is a pathologic process of bone formation in the patient population with severe burns and central nervous system injury and musculoskeletal trauma.To date,Celecoxib is used to prevent the formation of AHO, and it has been proved to be effective . So the inflammation stimulation play a significant role in the process. Now, we leverage our knowledge to demonstrate that Nf-κb/p65 represents the pivotal target for ectopic bone formation. Pharmacologic inhibition is effective to reduce AHO. These results suggest that targeting the phase of inflammation about Nf-κb/p65 is a effective solution to reduce AHO.

The Brain-traumatic/Burn/Tenotomy model is a kind of reliable AHO model. These are a lot of kinds of models of AHO that have been reported.(Zotz et al., 2012; Kan & Kessler, 2011) Achilles tenotomy model is widely used. McClure applied the model to mice and found that AHO developed by 10th weeks in 1983.(MCCLURE, 1983) In according to clinical high risk factors and to ensure the success rate, we designed the Brain-traumatic/Burn/tenotomy model, which is a comprehensive model. X-Ray examination in 10th week proved the reliability of the model. The success rate of Brain-traumatic/Burn/Tenotomy model is 100%.

In the first part,AHO model exhibits up-regulation in expression of Nf-κb/p65 protein.Protein analysis shows significant overexpression of Nf-κb/p65 in tendon tissue isolated from E group in the formation process of AHO at different time point. Immunohistochemical analysis is consistent with Western- blot result. These initial finding prompted our interest in evaluating Nf-κb/p65 as it is highly expressed in response to trauma in tissues of AHO, and it is critical for AHO development. AHO formation is largely thought to be related to the inflammatory response to trauma, which in turn cause ectopic bone formation through the up-regulation of pro-osteogenic gene and activation of osteopotent progenitor cell.(Winkler et al., 2015; Pavey et al., 2015) Nf-κb signal path is important in inflammatory response,which is activated by many cytokines, such as TNF-α,IL-1. Guo and Caron have proved that the activation of Nf-κb promote the expression of Sox9, which induce endochondral ossification.(Marjolein M. J. Caron, 2012; Guo et al., 2015)Shailesh Agarwal demonstrated that the process of AHO occurs through endochondral Ossification.[10] The result is consistent with our histologic evaluation result.These findings suggest that Nf-κb signal path play the crucial role in the process of AHO formation.

Our results showed that pharmacologic inhibition of Nf-κb/p65 (PDTC,Ammonium pyrrolidinedithiocarbamate) significantly reduced AHO formation. To evaluate the effect of Nf-κb/p65 in AHO formation, we used the drug, PDTC, to inhibit Nf-κb/p65.(Zhao et al., 2014; Ding et al., 2015; Wei et al., 2014)PDTC has been shown to decrease Nf-κb/p65 by decreasingNf-κb/p65 mRNA levels and blocking translation. PDTC is a kind of Nf-κb/p65-activator-inhibitor,which is across cellular membranes,and it is also antioxidant.We designed four drug concentrations to observe the effect.When we tested the Nf-κb/p65 in the tendon ,we observed a significant decrease. The X-Ray radiation Images of different groups show that the ectopic bone formation content significantly became reduced,especially M group. The results indicate that PDTC decreased the formation of AHO through inhibiting Nf-κb/p65 expression and pharmacologic inhibition of Nf-κb/p65 is a viable therapeutic strategy to prevent AHO. We suppose that PDTC inhibited Nf-κb/p65 signal path ,then the expression of Sox9 is inhibited. which results in inhibiting endochondral ossification. In according to the dynamic change of Nf-κb/p65 protein for first 7 weeks, we found that M group is greatly inhibited. So 6mg/ml is the best medical concentration.This is the first demonstration that the target of Nf-κb/p65 plays a developmental role in endochondral ossification of AHO. These results also suggest that pharmacologic inhibitor of Nf-κb/p65,PDTC,may serve as a therapeutic option for AHO . In our study, we chose local injection,but it is impossible to determine the accurate site of AHO formation. It is a difficult problem for accurate local treatment,therefore needs further study.

Conclusions

Our study suggests that a new target for treatment of AHO. We found that pharmacologic inhibitor of Nf-κb/p65, using PDTC, can significantly reduce ectopic bone formation through decreasing Nf-κb/p65 expression in Brain-traumatic/Burn/Tenotomy model. 6mg/ml is the appropriate medical concentration for local injection.

Additional information and declarations

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