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Molecular Characterization of Gut Microbiota in Obese and Lean Hypertensive Patients

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Abstract

Hypertension (HTN) and obesity are the major risk factors for cardiovascular diseases, and the gut microbiota has emerged to play a role in the development of many of the diseases including hypertension. In this study we hypothesized that obesity in hypertension could be linked to a specific gut microbiota. Our aim was to quantify and evaluate the differences in the composition of gut microbiota between obese and lean hypertensive patients and compared them with healthy controls. Fecal samples from 30 obese and 30 lean hypertensive patients and 30 samples from healthy individuals were collected. The samples were analyzed by PCR-Denaturing Gradient Gel Electrophoresis (DGGE) using primers, specifically targeting the V3 region of the bacterial 16s ribosomal RNA gene. Absolute quantification of the bacterial species such as *Prevotella spp*., *Bacteroides spp*., *Clostridium spp*. and *Escherichia coli* was performed by quantitative real time PCR (qPCR). DGGE results revealed that the intra-group similarity was significantly different in the obese hypertensive patients than that of the lean and control groups, and there was a significant decrease of *Bacteroides spp*. in the obese patients. While an elevated level of *Clostridium spp*. was observed in the both hypertensive groups as calculated by qPCR. Collectively, these findings support our hypothesis that obesity in hypertension is associated with compositional changes within gut microbiota and strategies could be developed to manage the gut dysbiosis in obesity linked hypertension and other related diseases.

Keywords: Obesity; Hypertension; Gut Microbiota; DGGE; Real Time qPCR

Introduction

According to a WHO report published in 2012, the total count of overweight adults in 2008 aged 20 years or above surpassed 1.4 billion, and among them more than 200 million males and about 300 million females were obese. Among the children, more than 40 million aged 5 years or below were overweight up to the year 2011[1]. Over the past 30 years, obese population has increased to double or even triple in many countries. A prevalence study conducted in United States from 1999-2004 reported that in 2004 two-third of the adult population was overweight and almost half were obese [2]. In China there has also been observed a rapid increase in obesity during the recent years. It has been reported that from 1992 to 2002, there was a rise from 14.6% to 21.8% in the prevalence of overweight or obese population, which will become more alarming in the coming years [3].

American Medical Association (AMA) has recognized obesity as a disease which is characterized by combining the phenotypes of many diseases including hypertension, hyperlipidemia, a fatty liver, insulin resistance and the accumulation of excess intra-abdominal adipose tissue. In addition, there may be a low-grade systemic and chronic inflammation associated with many other chronic diseases [4]. It has been reported that the overweight or obese adults are at more risk of getting many chronic diseases including mainly the heart problems, hypertension, osteoarthritis, diabetes and certain types of cancers like breast and colon etc. [5]. The children with obesity are more susceptible to hypertension, bone fractures, have breathing problems and psychological stress [1], and are predisposed to obesity and other cardiovascular diseases in the adulthood [6,7].

Globally, at least 2.8 million people die every year due to obesity and it is the fifth major cause of death in the world [1]. It also causes a heavy burden on economy. It is estimated that total costs in providing the health care facilities to obese or overweight patients will double every decade and by 2030 may reach up to 860.7–956.9 billion US dollars in the United States, accounting for 16–18% of the total health care costs in US [8]. This extraordinary burden has become a major concern to public health dealers and the steady increase of CVD common risk factors like obesity, type 2 diabetes and the metabolic syndrome, have become the primary public health concerns during the last decade [9,10].

Gut microbiota has been proposed as an environmental factor responsible for obesity and the altered energy metabolism. Gut microbiota is known to affect the host metabolism by altered lipid metabolism, increased energy extraction, and modulation of the immune system. Physical presence of bacteria and the bacterial metabolites are blamed for these effects [11,12]. Gut is exposed to external environment by receiving food from outside on daily basis and hence can potentially influence the risk factors of cardiovascular diseases (CVD), and it is of no surprise that a lot of studies in recent years have focused to investigate the involvement of Gut microbiota in cardio metabolic problems and have proposed the microbiota

manipulations as a therapeutic tactic to manage CVD and other metabolic diseases [13]. In this study we have characterized the gut microbiota composition in lean and obese hypertensive human patients and compared them with the healthy controls and identified the major fluctuating bacterial populations between the study groups.

Materials and Methods

Study participants and design

60 fecal samples were collected from the overweight / obese (OW/OB, BMI>25) and lean (LN, BMI<25) hypertensive patients (30 OW/OB, 18 male and 12 female; 30 LN, 13 male and 17 female) from the Xi'an, Shaanxi Province, China. All of the patients were between 40 and 70 years old and have Grade 2 or Grade 3 hypertension. Patients were excluded if they had any other acute or chronic inflammatory diseases or infectious diseases at the time of sample collection. All of the patients have received no antibiotic treatment, probiotics and prebiotics, within 30 days prior to sampling. 30 healthy volunteers (16 males and 14 females, aged 40-70 years) were enrolled for this study as a control group. All of the volunteers were in good health with no history of chronic metabolic diseases. Fecal samples were collected from all of the individuals in sterile cups and were stored immediately at -80°C until the DNA extraction was carried out.

Ethical statement

The study was performed under the guidelines of the World Medical Association and Declaration of Helsinki. The sampling and experimental processes were performed with the approval of the Ethical Committee of the Xi'an Jiaotong University, School of Medical Sciences; Reference No: 2018-525. The informed written consent was obtained from all of the participants including obese and lean hypertensive patients as well as healthy volunteers.

Anthropometric measurements

Body weight and height were measured according to the standardized procedures [14]. Blood pressure and Lipid profile analysis data of each patient was collected from the cardiology dept. of the 1st affiliated hospital of Xi'an Jiaotong University, China. The characteristics of the study participants are summarized in Table 1.

	Obese patients (n=30)	Lean patients (n=30)	Control group (n=30)	P (OB-CG)	P (LN-CG)
Male/Female	18/12	13/17	16/14	–	–
Age (yrs)	59.22 ± 12.28	63.26 ± 9.26	60.3 ± 9.88	0.40	0.37
BMI, kg m2	30.3 ± 5.43	21.8 ± 2.31	22.01 ± 3.31	0.002	0.23
SBP, mmHg*	175.45 ± 16.63	160 ± 9.23	118.13 ± 6	<0.0001	<0.0001
DBP, mmHg*	108.84 ± 11.2	100 ± 10.1	77.19 ± 7.36	<0.0001	<0.0001
FBG, mmol/L	4.78 ± 0.81	4.21 ± 0.96	4.5 ± 0.67	0.04	0.10
TC, mmol/L	4.31 ± 1.15	3.76 ± 0.83	3.76 ± 0.83	0.56	0.98
TG, mmol/L	1.70 ± 0.96	1.40 ± 0.49	1.31 ± 0.49	0.008	0.01
HDL, mmol/L	1.02 ± 0.24	1.07 ± 0.28	1.09 ± 0.28	0.21	0.42
LDL, mmol/L	2.54 ± 1.00	2.10 ± 0.65	1.87 ± 0.53	0.04	0.07

Table 1: Characteristics of study participants; The data for age, Weight, SBP, DBP, FGB, TG, TC, HDL and LDL were presented as mean ± SD. P-values for age, weight, SBP, DBP, FGB, HDL, LDL, TG, and TC were calculated using Unpaired t-test, (P <0.05). SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, total triglyceride; and TC, total cholesterol.*Grade 2: current blood pressure 160/~0 ~ 179/109; Grade 3: current blood pressure ≥180/110

DNA Extraction

For the bacterial DNA extraction, the Qiagen QIAamp MiniStool Kit (QIAGEN, Hilden, Germany) was used, according to the manufacturer's instructions. The concentration of extracted DNA was estimated by absorbance at 260 nm (A260), and the purity was determined by calculating the A260 to A280 ratio with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Analysis of gut Microbiota by PCR-DGGE

Total fecal bacterial DNA was used as a template for PCR-DGGE analysis and universal primers for V3 region of 16S rRNA gene were used for amplification of the bacterial DNA of the study samples by applying touchdown PCR in an automated thermo cycler (ABI2720, USA). Each 50 µl PCR reaction mixture contained 1 µl of each primer as shown in Table 2, 1 µl of deoxynucleotide triphosphate (dNTPs) mix (10 mM), 5 µl of MgCl2 (25 mM), 5 µl of 10x buffer, 0.4 µl of Taq DNA polymerase (TaKaRa, Japan), and 2 µl total fecal DNA. The PCR thermal profile conditions include; initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min. The annealing temperature was decreased by 1°C every second cycle until a touchdown 55°C reached, at which 10 additional cycles were carried out, followed by final extension at 72°C for 10 min [15]. Post-PCR gel electrophoresis was performed to confirm the amplicons. Negative controls without a template DNA were included in each reaction.

After PCR, 15µl of PCR products were mixed with 6µl of loading dye prior to loading. DGGE was performed using the DCodeTM Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA)

on 16 cm × 10 cm × 1 mm gels. The sequence-specific separation of the amplicons was obtained with 10% (w/v) polyacrylamide (acrylamide-bis, 37.5:1) gels in 1 × Tris-acetate EDTA (TAE) buffer, containing 65–35% linear denaturant gradient. The 100% denaturing solution contained 7.0 M Urea and 40% (vol/vol) deionized formamide. Electrophoresis was performed at a constant voltage of 90 V at 60°C for 13 h. The gels were stained with 5 µg/ml ethidium bromide solution for 30 min, washed by deionized water and then viewed under BIO-RAD Gel Doc 2000. Comparison of DGGE profiles in different gels was performed using a standard reference DNA Marker (DL2000). No band was detected in the negative controls. The bacterial diversity of the study groups was evaluated by the number of bands and the bands intensities of DGGE profiles using Quantity One software (Bio-Rad, USA). The Shannon–Weaver index (H′) was used to determine the diversity of taxa present in fecal microbiota of the obese, lean hypertensive patients and healthy control group. The similarity score and cluster analysis of DGGE profiles were performed using the UPGMA method based on the Dice similarity coefficient (band-based). Nonparametric statistical analysis was performed using IBM SPSS Statistics (v20) software. Results are expressed as mean values and standard deviations.

Groups	Target Gene	Primer	Sequence(5'-3')	Product Size(bp)	Ref.
PCR-DGGE	Bacteria 16s rRNA gene V3 region	341 Fa	CCTACGGGAGGCAGCAG	193	[15]
		534 R	ATT ACCGCGGCTGCTGG		
Real-time PCR	Bacteroides	Bac F	AAGGGAGCGTAGATGGATGTTTA	193	[27]
		Bac R	CGAGCCTCAATGTCAGTTGC		
	Prevotella	Pre F	ACAGTAAACGATGGATGCC	513	[28]
		Pre R	GGTCGGGTTGCAGACC		
	Clostridium	Clos F	CGGTACCTGACTAAGAAGC	429	[29]
		Clos R	AGTTTGATTCTTGCGAACG		
	Escherichia coli	E.coli F	CATTGACGTTACCGCAGAAGAAGC	190	[29]
		E.coli R	CTCTACGAGACTCAAGCTTGC		

Table 2: Primers used in this study; a A 5′ GC-clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) was added for DGGE analysis.

Sequencing of selected bands from DGGE gels

The dominant and common bands were cut from the gel with a sterile scalpel, washed with RNAse Free water, re-suspended in 20 µl of the RNAse Free water and stored at 4°C overnight for the diffusion of DNA. PCR was performed again with the diffused DNA using V3 region primers 341F/534R as previously used for DGGE analysis but this time without GC clamp (initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min.). Re-amplified PCR products were sequenced by Shanghai Sangon Biological Engineering Technology Service Co. Ltd. (Shanghai, China). Sequences were analyzed with Chromas v2.23 (Technelysium, Tewantin, Australia) and their similarity was confirmed by using BLAST and Seqmatch soft wares.

Quantitative PCR analysis

To evaluate the accurate copy number of *Bacteroides spp*, *Prevotella spp*, *Clostridium spp*, and *Escherichia coli* in the tested samples, real-time quantitative PCR was performed with QuantiFast SYBR Green PCR kit (Qiagen) using the StepOne v2.3 real-time PCR detection system (ABI, USA). Each reaction mixture (20 µl) composed of 10 µl of 2 x SYBR Green PCR mix (Takara Japan), 0.8 µl of each specific primer (Table 2), 2 µl of sample DNA and 6.4 µl of sterile deionized water. The copy numbers within each sample were counted by comparing with a standard curve derived from a serially diluted plasmid DNA ranging from 10^2 to 10^8 copies/g. Negative controls was run along with each analysis. The resultant bacterial population was expressed as Log10 bacterial replica counts in 1g of fecal mass. Triplicate samples were used in each experiment and the mean value was reported. Unpaired t-test was used to calculate the significance among the three groups using IBM SPSS (v20).

Results

Biochemical measurements

The biochemical variables of the study groups are shown in Table 1. Other than the systolic blood pressure (SBP) and diastolic blood pressure (DBP) which were obviously higher in the both (obese and lean) patient groups, BMI was significantly higher in the obese hypertensive patients. The lipid profile data of the three groups was different i.e. low density lipoprotein (LDL) and fasting blood glucose (FBG) levels were significantly increased in the obese hypertensive patients as calculated by the Unpaired t-test. On the other hand, an elevated level of triglycerides (TG) was observed in both hypertensive groups as compared to the control group.

DGGE Analysis

Denaturing gradient gel electrophoresis analysis was performed with the PCR products obtained after amplification with universal primers targeting the V3 region of the 16S rRNA gene from the study

groups as shown in Figure 1. Each lane of the PCR-DGGE gel represented one sample which was selected from the study groups at random. The band number of DGGE profiles, as well as Shannon diversity index (H′), was calculated to estimate the diversity of gut microbiota in the three groups as shown in Table 3. The similarity level of DGGE band patterns was measured using the Dice similarity coefficient and UPGMA dendrogram as shown in Figure 2. Intra-group similarity was significantly different in the obese hypertensive patients than that of the lean and control group. When all the samples were compared, the inter-group similarity index was less than the intra-group similarity index of the study groups.

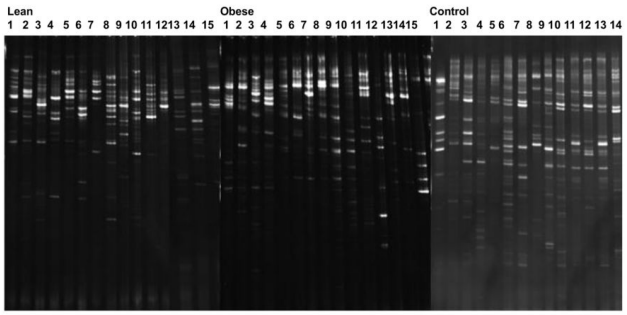

Figure 1: DGGE profiles of the three groups obtained by using the universal primer of V3 region of 16S rRNA gene.

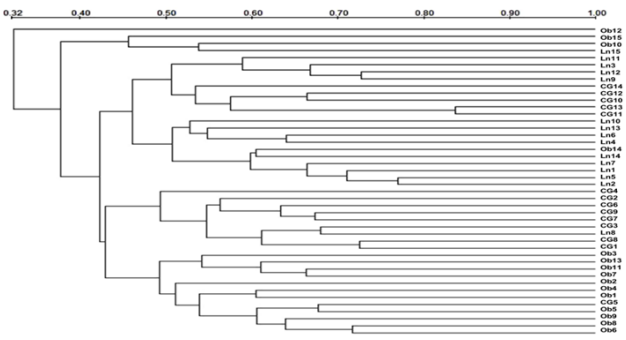

Figure 2: Clustering of DGGE profiles obtained with universal primer (V3) of the three groups using Dice coefficient and UPGMA.

Group	Microbiota diversity		Microbiota similarity	
	DGGE bands (mean ± SD)	Shannon index (mean H'/H' max ± SD)	Intra-group	Inter-group
Obese	16.5± 4.74	2.71± 0.26	33.14 ± 10.2	32.71±10.68
Lean	15.8±4.39	2.62± 0.28	38.76±11.2	
Healthy	20.9± 4.13	2.95±0.22	36.65± 10.5	
P value OB-CG	0.66	0.308	<0.0001	/
P value LN-CG	0.85	0.312	0.06	

Table 3: Microbiota diversity and similarity of hypertensive patients compared with normal controls; Values are presented as means ± SD. Data were analyzed using the unpaired t-test. Values are significantly different, with P <0.05.[a]Number of DGGE bands produced by each sample analyzed. [b]Shannon Diversity Index as calculated using the relative intensities of all DGGE bands in each sample and expressed as a ratio of H′ to H′max, where H′max is the maximum value of the Shannon index for a given sample. [c]Dice similarity coefficients comparing DGGE band profiles within individuals of the same group. [d]Dice similarity coefficients comparing DGGE band profiles between members of the three groups.

Sequence analysis of the selected DGGE bands

Dominant bands from different positions in DGGE profiles were examined by sequencing. The sequences were analyzed through BLAST. The sequencing results showed that the Bacteroidetes and Firmicutes were the dominant bacterial phyla in the study groups as shown in Table 4.

Bacterial Genus (Bands sequencing)	Obese Group (n=32)	Lean Group (n=26)	Control Group (n=35)
Phylum Bacteroidetes			
Genus Prevotella	12(37.5%)	6(23.07%)	5(14.28%)

Genus Barniesiella	0	2(7.69%)	0
Genus Alistipes	4(12.5%)	2(7.69%)	1(2.85%)
Bacteroides vulgatus	2(6.25%)	1(3.8%)	5(14.28%)
Genus Bacteroides	2(6.25%)	8(30.76%)	11(31.42%)
Phylum Firmicutes			
Genus Clostridium	4(12.5%)	3(11.5%)	2(5.7%)
Genus Faecalibacterium	0	1(3.8%)	4(11.4%)
Genus Pseodobutyrivibria	1(3.1%)	0	2(5.7%)
Genus Megasphaera	3(9.37%)	1(3.8%)	1(2.85%)
Phylum Proteobacteria			
Escherichia coli	3(9.37%)	1(3.8%)	2(5.7%)
Genus Klebsiella	1(3.1%)	0	2(5.7%)
Phylum Actinobacteria			
Genus Propionibacterium	0	1(3.8%)	0

Table 4: Sequences of PCR amplicons derived from DGGE gels and identities based on the BLAST database.

Real-time PCR analysis

Real-time PCR analysis was performed to observe the gut microbial quantitative alterations for the *Bacteroides spp.*, *Prevotella spp.*, *Clostridium spp.*, and *Escherichia coli* species in fecal samples of obese / lean hypertensive groups and healthy group. Six serial dilutions of the standard plasmids were used simultaneously for each of the detections. The results showed a significant decrease of *Bacteroides spp.* in OBOW patients as compared to LN patients ($P<0.05$) as shown in Table 5. The *Prevotella spp.* was increased in the obese group though the difference was not significant. No other significant difference was observed among the patient groups. *Clostridium spp.* was significantly higher in the both patient groups (obese and lean respectively) as compared to the control group. *E.coli* was also increased in the hypertensive groups but not significantly different from the control group. The triplicate samples were used to calculate the mean value in each experiment.

Bacteria	Obese Group	Lean Group	Control	P value Ob-CG	Ln-CG
Bacteroides	5.67 ± 1.83	7.26 ± 0.90	6.96 ± 1.06	0.04	0.89
Prevotella	5.70 ± 1.92	5.02 ± 2.15	5.08 ± 1.58	0.35	0.93
Clostridium	6.79 ± 0.79	6.46 ± 0.59	5.59 ± 0.38	0.02	0.04
Escherichia coli	5.45 ± 0.97	5.32 ± 1.27	4.91 ± 1.01	0.78	0.60

Table 5: Quantitative analysis of bacterial groups in fecal samples by real-time PCR (qPCR); Data were reported as the average estimate of Logarithms of fecal PCR target genetic amplicon copy numbers present in 1 g of feces. Results which are significantly different (Unpaired t-test), with $P<0.05$

Discussion

In this study, we have observed if there was any difference in the gut microbial composition of obese and lean hypertensive patients compared to the healthy individuals (controls). After the PCR-DGGE analysis of V3 region of bacterial 16S rRNA gene with imaging and sequencing, and the real-time PCR quantitation followed by statistical analysis was performed. DGGE analysis data revealed that the fecal samples were occupied by different bacterial communities. The total bacterial count as observed through DGGE did not show any link with obesity or the healthy status which means the obesity or the healthy status may be associated with change in the gut microbiota composition instead of a single microbial population responsible for the increased or decreased diversity. In other words we can say that in our study groups no significant alteration was observed in the gut bacterial diversity. It should be noted that using the PCR based approach; only dominant bacterial populations are detected. So in simple words, it is not the measure of total diversity, instead a relative diversity comparison between the samples. We observed that DGGE profiles similarity within-groups was significantly higher than that in-between groups. These results showed that the composition of gut

microbiota among the hypertensive individuals was similar and so was in the healthy group. However the dissimilarity could be markedly observed form the DGGE profiling data between the study groups suggesting that obesity may alter specific subpopulations of the gut microbial community. Sequence analysis of all the dominant bands obtained by DGGE analysis made it possible to link certain bacterial species with obese or lean individuals, and investigate further. Although this association also depends on the accuracy of the sequence obtained from the technique and validation of community analysis. So, in order to verify the reliability and accuracy of the technique, the bands were excised at the same migration positions and sequence analysis was carried out to confirm the same identification.

The microbial diversity exhibits complex DGGE patterns, the bands appearing in close proximity. Microbes with relatively high concentrations (~10^8 CFU/g stool) are only detectable on the gel [16]. As we know that DGGE is not a truly quantitative technique, so we also used real time quantitative PCR to evaluate the mircobiota diversity. Real Time PCR results showed a significant decrease of *Bacteroides spp*. in the obese hypertensive group. Dysbiosis is defined as the imbalance in microbial community associated with certain type of pathology. Firstly it was demonstrated in mice that altered gut microbiota was associated with obesity. Ley et al. examined 5088 bacterial 16S rRNA gene sequences from fat ob/ob, lean ob/+ and wild-type mice, given the same polysaccharide-rich diet [11]. They observed a 50 % reduction in the abundance of Bacteroidetes in the obese mice, and a proportional increase of Firmicutes [11]. *Bacteroides* are thought to be beneficial bacteria and sustain a complex relationship with the host in the gut where they act as commensals [17]. These bacteria are known to cause the fermentation of a wide range of sugar derivatives from plant materials (which are common in the human colon and are potentially toxic) making them beneficial for humans. *Bacteroides* along with other intestinal bacteria cause carbohydrates fermentation and produce volatile fatty acids which are then reabsorbed through the large intestine and consumed by the host as an energy source and provides a considerable proportion to meet the host's daily energy requirement [18].

A number of reports published in prominent journals during the recent years have mentioned the respective proportions of the two main phyla, the Bacteroidetes and the Firmicutes, which are linked to obesity, both in humans and mice [11,12,19,20]. And they consider carbohydrate metabolism as the major factor of getting obesity. They report that microbiota of obese individuals are more significantly enriched with phylum Firmicutes and less with Bacteroidetes. And they assume that this bacterial proportion in the obese individuals may extract energy more efficiently from a given diet than the microbiota of the lean individuals, who have opposite proportion [12]. Together, the results obtained from the studies conducted on humans and mice suggest that obesity modifies the gut microbial proportionate and shifts it towards a raised Firmicutes/Bacteroidetes ratio. Thus they propose a strategy to cure obesity by manipulating the gut microbiota towards a lower Firmicutes/Bacteroidetes ratio. However, if several reports confirm the increased Firmicutes/Bacteroidetes ratio in obese individuals [21-23], there are other studies that report no differences in the abundance of Firmicutes and Bacteroidetes in obese and lean subjects [24-26]. In our study we also do not find any significant difference in the *Clostridium* spp. (which belongs to the phylum Firmicutes) between obese and lean patients. On the other hand, we have observed a significant increase in the *Clostridium* spp. levels in both of the hypertensive groups (obese and lean) as compared to the control group. Therefore, this changed (F/B ratio) in the gut microbiota composition may not be associated with obesity instead it may potentially be related to hypertension.

Keeping in mind the certain environmental and physiological factors which possibly can influence the gut microbial composition such as diet, age, gender and genetic background, we tried our best to minimize the effect of such variables by selecting the obese and lean hypertensive and healthy group individuals of the same age group, gender, sharing almost the similar dietary conditions and living in the same environment. However, diet could not be strictly restricted to the individuals of the both groups, although it's the major risk factor associated with obesity and probably the primary cause of metabolic disorders including obesity, and intestinal microbiota is the part of this process.

Conclusion

We have observed a higher inter-group variability in the gut microbiota composition of hypertensive (obese and lean) groups compared with healthy (control) group. Real-time PCR analysis has shown significant fluctuations in the copy number of *Bacteroides spp*. and *Clostridium spp*. within the study groups. *Bacteroides spp*. is significantly decreased in obese hypertensive group but remain unchanged in lean hypertensive group compared to the control group. While an overall abundance of *Clostridium spp*. is seen in hypertensive (obese and lean) groups as compared to the control group. We propose the next generation sequencing techniques to elucidate the real impact of gut microbiota association with hypertension and obesity.

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Compliance with Ethical Standards

This study as approved by Biomedical Ethics Committee of Xi'an Jiao Tong University, Reference No: 2018-525.

Conflict of interests

All authors declare no conflict of interest.

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