Mechanism of nonshivering thermogenesis activation by pepper and cinnamon extracts

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Abstract

A previous study from our laboratory established the thermogenic potential of pepper and cinnamon extracts. In this study we tried to further understand the mechanism by which the two spices were exhibiting their thermogenic potential by studying the expression level of specific genes, (UCP1, PRDM16, PGC1α and CPTC1) in specific organs. We also measured the levels of norepinephrine (NOR), free fatty acids (FFA), thyroid hormone levels in circulation and lastly measured the shivering intensity in skeletal muscles of cold exposed rats. We observed that treatment with the spices increased the expression levels of studied genes in specific organs. The up-regulated genes were indicative of increased BAT content and activity, browning of WAT and an overall increase in metabolism.

We also noted an increased NOR, FFA and thyroid hormone levels in circulation. Shivering intensity in muscles of cinnamon treated animals decreased more in comparison to pepper. Based on our results, we concluded that the two spices increase the overall heat generation in cold exposed animals by increasing BAT activity, BAT content, causing browning of WAT and increasing oxidative metabolism. Further understanding of this orchestered response might throw new light into the existing knowledge of regulation of the thermogenic process.

Keywords: Thermogenesis, pepper, cinnamon, brown adipose tissue, white adipose tissue, thyroid hormones.

Introduction

A previous report from our laboratory established that two spice extracts namely, pepper and cinnamon are effective in preventing a fall in core body temperature during cold exposure. In this study, we further evaluate and understand the mechanism by which the spices are aiding in increasing the physiological heat generation leading to an effective reduction in fall in core body temperature. As indicated in literature, factors beyond the sympathetic tone are now identified that directly or indirectly affect BAT activity[24]. Enhanced physiological heat production is an orchestered response involving a cross talk between many organs viz. muscle[6], liver[26], heart[5] etc. In light of these facts, the current study aims at understanding the effect of pepper and cinnamon on organs beyond BAT viz. muscle, WAT, liver and the thyroid system.

Material and Methods

Preparation of extracts: Lyophilized extracts of the two spices, pepper and cinnamon were prepared. Briefly, pepper and cinnamon were purchased from the local market, shade dried and ground to a fine powder using a lab mill grinder. 100 g of the powder was suspended in 500 ml of 70% aqueous ethanol and shaken for 24 hours using a rotary shaker at room temperature. The solution was then filtered and the extraction process was repeated for 3-4 times. The filtrate was concentrated under vacuum using a flash evaporator. The concentrate was then lyophilized and stored in an air tight container at -20˚C until further use. Epigallocatechin gallate (EGCG) rich green tea extract was procured from Indo Vedic Nutrients Pvt. Ltd. and used as positive control as its thermogenic properties are well reported.

Experimental design: Animal handling and experimentation were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). 48 animals were divided into two major groups, 24 animals exposed to room temperature (RT) and remaining 24 animals exposed to cold temperature (CT). Each major group was further divided into 4 sub groups (n=6 animals) control (receiving water), green tea, pepper and cinnamon respectively. The extracts were administered orally at a dosage of 250 mg/kg for a period of 7 days.

Measurement of core body temperature: Prior to beginning the treatment, all the CT group animals were subjected to a cold challenge, where they were exposed to 4˚C for 140-180 minutes. Changes in core body temperature (CBT) were recorded continuously using rectal probes inserted approximately 1.5 cm into the rectum and held in place using an adhesive tape. The rectal probe was connected to a T-type thermister pod and an eight channel power lab data acquisition system (AD Instruments, Australia) for continuous recording of temperature. To hold the rectal probes in place and to avoid erroneous measurement, the rats were placed in a restrainer.

The restrainer was placed in the cage 3-4 days before commencing the experiment with both ends open allowing free in and outward movement for the animal. This facilitated familiarization of the animals with the restrainer and caused minimal stress during experimentation. The size

of the restrainer used was such that it prevented the animal from turning around but allowed forward and backward movement. After 7 days of treatment, the animals were again exposed to 4˚C and the changes in CBT were recorded. Difference in the fall in CBT before and after treatment when exposed to the same period of cold, was used as an index of cold endurance.

Estimation of norepinephrine (NOR): NOR in plasma was quantified using High Performance Liquid Chromatography with an electrochemical detector[1]. Plasma was purified with aluminum oxide. The levels of NOR were expressed as ng/dL of plasma.

Estimation of free fatty acids (FFA): Free fatty acids (FFA) in serum were estimated as described by Falholt et al[9].

Relative quantification of target gene expression by western blot analysis: Tissue homogenates were prepared in lysis buffer containing 10 mM HEPES 42 mM KCl, 50 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 5 mM DTT, 2 mM PMSF, 1X protease inhibitor cocktail. The homogenate was then centrifuged at 10,000 *g* for 30 min and supernatant was used for further analysis. Protein concentration was estimated by the method of Lowry et al[13] using bovine serum albumin as the standard. Known amount of each sample and pre-stained marker was mixed with equal volume of 2X sample buffer, boiled for 5 min and cooled prior to loading.

Tissue homogenates containing 60 mg of proteins were separated on SDS-PAGE and transferred onto a nitrocellulose membrane using an electro blotting apparatus (Bio Rad systems, UK). After transfer, the membranes were probed with primary antibodies against UCP1, PRDM16, PGC1α, CPTC1, Cytochrome C2 and GAPDH (Santa Cruz Biotechnology, CA, USA) at 1: 1000 dilution and incubated at room temperature for 3 h. The membranes were then washed 7 times in PBST at 5 minutes interval for a period of 35 minutes followed by incubation for 80 minutes in horse radish peroxidase conjugated to respective secondary antibody used at 1: 10,000 dilution. The membranes were washed again and developed in a Syngene G-box apparatus using ECL reagent. Western blot band intensity was measured using NIH image J software.

Quantification of target gene expression by quantitative RT-PCR analysis

RNA isolation: RNA was isolated using trizol method. With a tissue homogenizer, 100 mg of tissue was homogenized with 1ml of trizol on ice and incubated at room temperature for 5-10 minutes. The homogenate was then centrifuged at 12,000 g for 10 minutes at 4ºC. The resulting pellet was discarded whereas the supernatant containing RNA was used for further separation.

To the supernatant, 0.2ml chloroform was added and shaken vigorously for 15-30 minutes and incubated at room temperature for 15-30 minutes. This mixture was centrifuged at 12,000 g for 15-20 minutes at 4ºC.The supernatant contains RNA which is separated and transferred to a clean tube. 0.5ml of isopropyl alcohol was added to the supernatant and incubated at room temperature for 10 minutes and centrifuged at 12,000 g for 10 minutes. The pellet was collected and washed with 75% ethanol 2-3 times by centrifugation at 12,000 g for 5 minutes. The pellet was then dried and resuspended in water and stored at -80ºC until further use. Purity and concentration of RNA were determined using nano drop and measuring the absorbance at 260/280 nm.

Conversion of RNA to cDNA: RNA was converted to cDNA using the high capacity RNA to cDNA conversion kit provided by Thermo Fisher Scientific. Briefly, a master mix was prepared based on the number of reactions to be performed. Each reaction mix contained 10µl of buffer, 1µl of enzyme and 2µl of RNA sample. The volume of the mixture was made up to 20µl using nuclease free water. This mixture was gently mixed and placed on ice till the reaction was performed. The condition used for performing reverse transcription was 37ºC for 60 minutes, 95ºC for 5 minutes and 4ºC until the sample was removed from the cycler. The cDNA was stored at -20ºC until further use.

RT-PCR analysis: Abundance of cDNA of the targeted gene was examined by RT PCR analysis. The PCR reaction mix contains 2 ng of total RNA converted c-DNA, 2X Eva green ready mix (containing dNTP`s, Taq-Polymerase, 10 µl) and 10 pmol forward and reverse primers. The final volume of PCR reaction mixture was made up to 20 µl with PCR grade water. The cycling programs were as follows: thermal profile consisted of 10 min of activation at 50°C and 5 min of polymerase activation at 95°C followed by 45 cycles of PCR at 95°C for 10s, 60°C for 30 s. Melt curve analysis was performed at 65°C to 95°C at an increment of 0.5°C for 10 s to verify the authenticity of the amplified product by its specific melting temperature (Tm) with the melting curve analysis software of the Bio- Rad CFX-96. The threshold cycle (Ct) of gene of interest and housekeeping gene and the difference between their Ct values (ΔCt) were determined. Relative and normalized gene expression was calculated as per Bio-Rad software.

Measurement of shivering intensity by electromyography (EMG): Intramuscular EMG of skeletal muscle was measured using the eight channel power lab data acquisition system (AD Instruments, Australia). The fine needle electrodes were inserted into the two leg skeletal muscle. The animal was anesthetized during the whole procedure to prevent any movement and erroneous recordings. The EMG was recorded continuously for a period of 140 minutes. The initial EMG when exposed to cold temperature was recorded and EMG after treatment with green tea/spices for a period of seven days was recorded as well. The shivering intensity and time of onset of shivering were noted before and after treatment with both the spices and green tea.

Estimation of thyroid hormone status: Thyroid hormones were estimated by enzyme linked immune sorbent assay Elisa using the commercially available kit from Weldon biotech India Pvt. Ltd.

Statistical analysis: The data are expressed as mean ± standard deviation (SD). Green tea, pepper and cinnamon treated groups were compared with control group. Within each group, differences, if any, before and after treatment were also compared. In addition, where applicable, respective room and cold temperature groups were compared. Data were analyzed by One-way ANOVA followed by Tukey's Post hoc test using Graphpad prism 5 for Windows software. Differences at $p < 0.05$ were considered significant.

Results

Effect of cold exposure on core body temperature before and after spice treatment: Consistent with our previous findings, treatment with pepper and cinnamon reduced fall in CBT. The results are summarized in figure 1.

Effect of cold exposure and spice treatment on plasma NOR and serum FFA levels: Cold exposure alone increased NOR and FFA levels as indicated by the difference in RT and CT control groups. Cold exposure and treatment with both the spices significantly increased NOR and FFA levels in comparison to RT and CT control groups. Results are summarized in table 1.

Effect of cold exposure and spice treatment on expression of various organs and genes: The effects of treatment of green tea, pepper and cinnamon on BAT, WAT, muscle and liver were studied. In BAT, effects on levels of UCP1, PRDM16, PGC1α, CPTC1, Cytochrome C2 were studied. In WAT, effects on levels of UCP1, PRDM16, CPT1C, Cytochrome C2 were studied. In muscle, effects on levels of CPT1C, Cytochrome C2 were studied. In liver, effect on the expression level of FGF21 was studied. UCP1 is the uncoupling protein found in the inner mitochondrial membrane that is a hall mark of nonshivering thermogenesis in BAT[12]. PRDM16 is a master regulator of BAT development[25]. PGC1α is a master regulator of mitochondrial biogenesis, induces BAT features in WAT and it is a cold inducible protein governing adaptive thermogenesis[10]. CPT1C increases mitochondrial activity and fatty acid oxidation[23]. Cytochrome C2 plays a key role in oxidative phosphorylation[14,23].

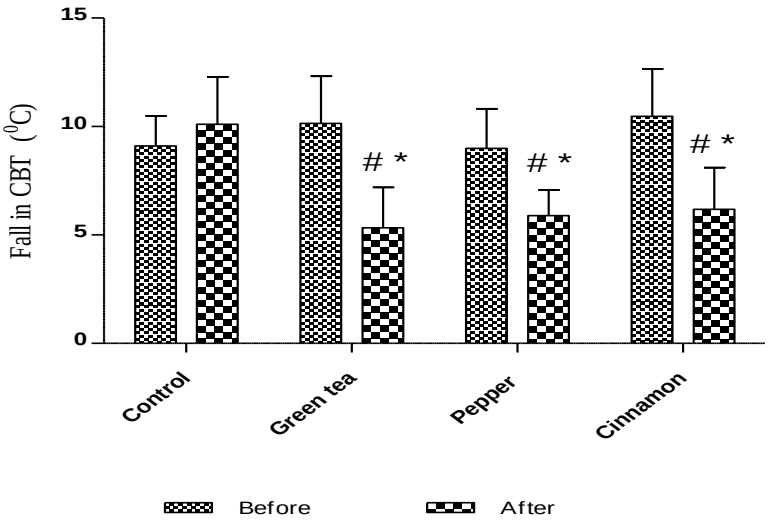


Fig. 1: Effect of cold exposure on core body temperature before and after spice treatment.
Results are expressed as mean ± SD (n=6). * indicates significantly different from control group, *p<0.05*.
 # indicates significantly different before and after treatment, *p< 0.05*

Table 1
Changes in plasma NOR and serum FFA levels after treatment with spices.
Results are expressed as mean ± SD (n=6). * indicates significantly different from room temperature control, p<0.05; # indicates significantly different from cold control, *p<0.05*.

Groups	Plasma Norepinephrine (ng/dL)	Serum Free fatty acids (µM/dL)
Room temperature Control	30.16 ± 2.79	250.14 ± 10.9
Cold temperature control	41.46 ± 1.83*	300.89 ± 14.7*
Green Tea	64.32 ± 3.19*#	546.19 ± 13.8*#
Pepper	67.14 ± 2.33*#	502.76 ± 16.6*#
Cinnamon	69.18 ± 1.84*#	515.34 ± 11.1*#

Effect of pepper and cinnamon on expression of specific genes in BAT, WAT, muscle and liver

Brown adipose tissue: Both green tea and pepper had a profound positive effect on BAT. All five genes studies were up regulated following treatment with both green tea and pepper. Cinnamon up regulated UCP1, PGC1 α levels considerably. However, the up regulation of PRDM16, CPT1C and cytochrome C2 was not as high as pepper and green tea. It significantly increased in comparison to the control group though.

White adipose tissue: Green tea and pepper upregulated the expression of all 4 genes studied. Green tea caused an increased expression when compared to pepper. Cinnamon upregulated the expression of UCP1 and CPT1C only.

Muscle: Green tea, pepper and cinnamon upregulated the expression of both the genes studied. The increased expression caused by treatment with pepper was the highest. However, treatment with all three extracts was significantly greater than the control group.

Liver: Green tea upregulated the expression of FGF21 in liver. Pepper also upregulated the expression of FGF21 but the degree of expression was lower than green tea. Cinnamon did not have a significant effect on FGF21 levels in liver.

Effect of cold exposure and spice treatment on levels of mRNA levels of genes: The abundance of mRNA of the genes studied in the four organs followed a similar pattern as the western blot results. Fold increase in expression of each gene in each organ when exposed to RT or CT, is summarized in figure 2.

Effect of cold exposure on EMG after green tea/spice treatment: Time of onset of shivering and amplitude of shivering (mV) was used to represent EMG. EMG was measured for animals in all four groups before treatment. However, since the onset of shivering for all groups was found to be within the initial 5 minutes of cold exposure and consistent henceforth, the results are not discussed. After treatment for seven days, the time of onset of shivering was significantly reduced in comparison to control group. In green tea treated group, time of onset of shivering was approximately 39 minutes, where as in pepper and cinnamon it was approximately 12 and 22 minutes respectively. The total duration of cold exposure was 120 minutes.

Effect of cold exposure and spice treatment on thyroid hormone status: Cold exposure itself increased T3 and T4 hormones in circulation when compared to room temperature control. Cold exposed animals treatment with green tea and both the spices increased thyroid hormone levels in comparison to control groups. Results are summarized in table 2.

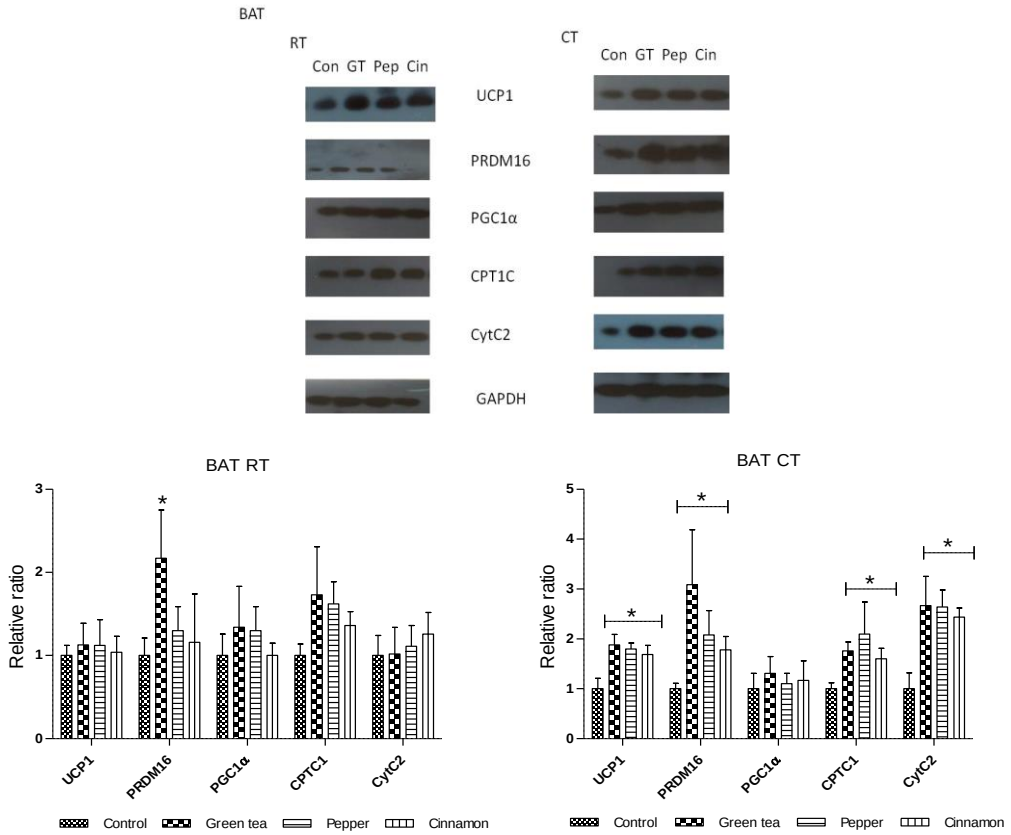


Fig. 2: Effect of cold exposure, green tea and spice treatment on expression levels of genes in BAT. * indicates significantly different from control group. *p<0.05*.

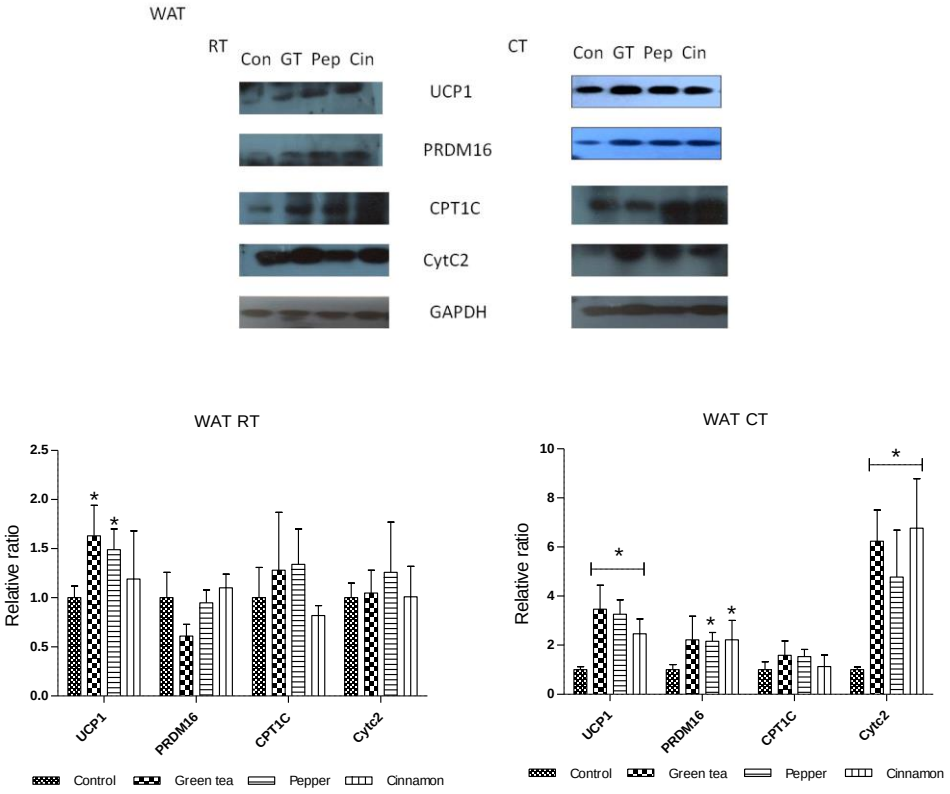


Fig. 3: Effect of cold exposure, green tea and spice treatment on expression levels of genes in WAT. * indicates significantly different from control group. *p<0.05*.

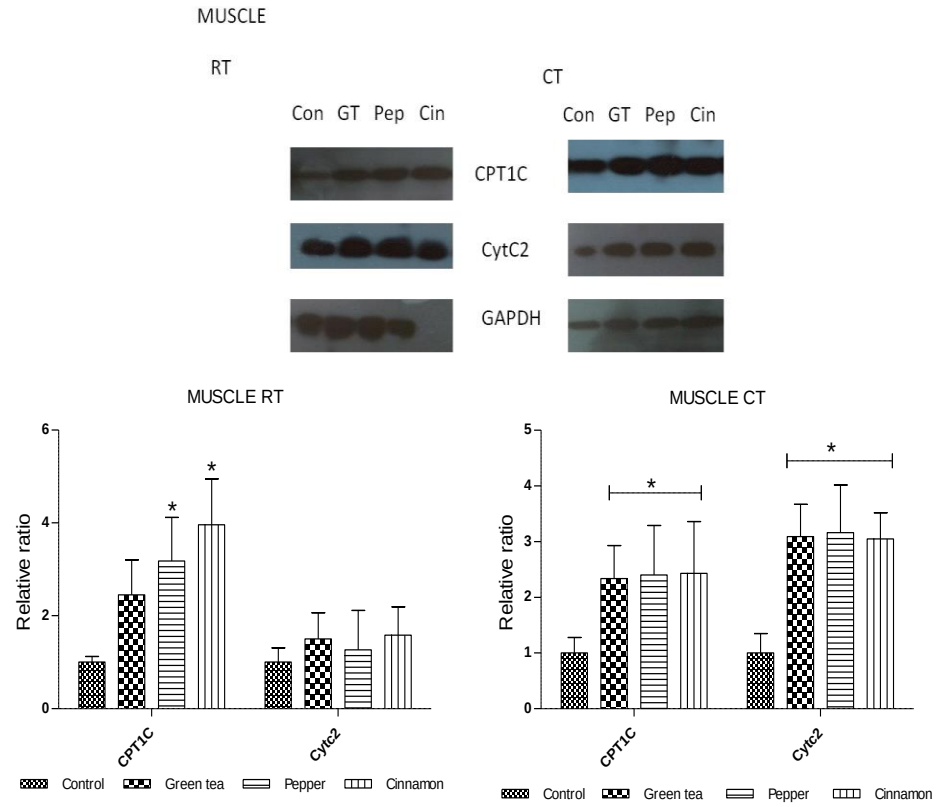


Fig. 4: Effect of cold exposure, green tea and spice treatment on expression levels of genes in muscle. * indicates significantly different from control group. *p<0.05*.

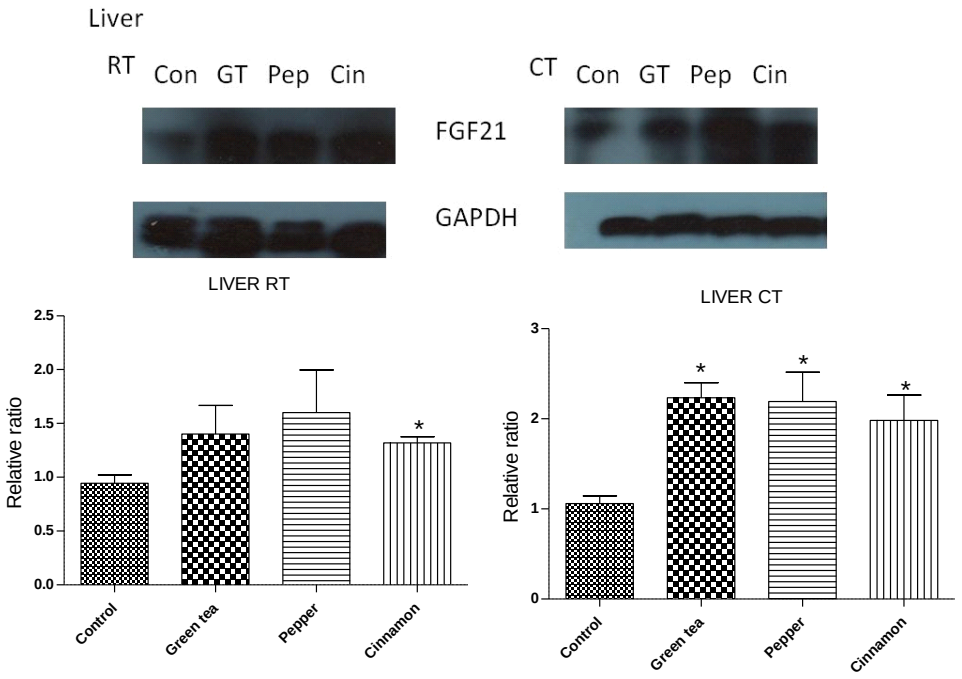


Fig. 5: Effect of cold exposure, green tea and spice treatment on expression levels of genes in liver. * indicates significantly different from control group. *p<0.05*.

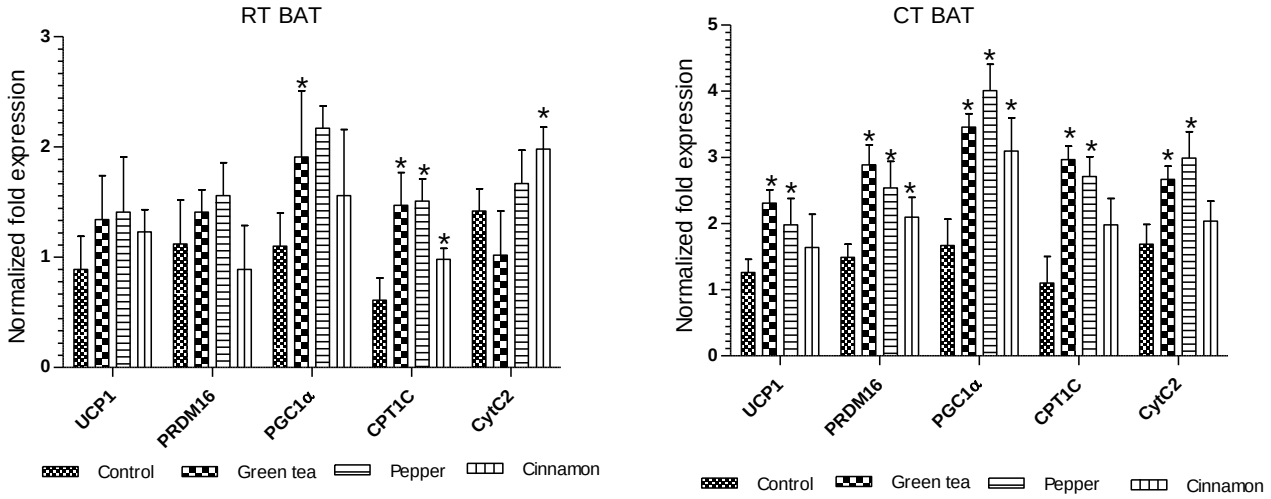


Fig. 6: Effect of cold exposure, green tea and spice treatment on mRNA levels of genes in BAT. * indicates significantly different from control group. *p<0.05*.

Table 2
Effect of cold exposure and spice treatment on thyroid hormone status. * indicates significantly different from control group. # indicates significantly different from room temperature control. *p<0.05*.

Groups	T3 (nmol/L)	T4 (nmol/L)	TSH (ng/ml)
Room temperature control	0.72 ± 0.22	30.8 ± 4.1	3.43 ± 0.97
Cold temperature control	1.47 ± 0.29$^{\#}$	36.1 ± 6.2	2.10 ± 0.70
Green tea	2.19 ± 0.30$^{*\#}$	44.2 ± 4.7$^{\#}$	1.43 ± 0.47
Pepper	1.94 ± 0.19$^{*\#}$	42.7 ± 2.8$^{\#}$	1.26 ± 0.68
Cinnamon	2.01 ± 0.27$^{\#}$	45.8 ± 7.4$^{\#}$	1.22 ± 0.75

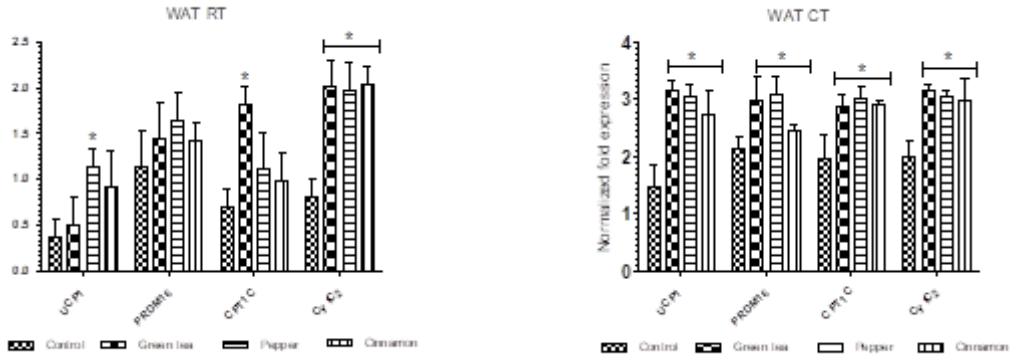


Fig. 7: Effect of cold exposure, green tea and spice treatment on mRNA levels of genes in WAT. * indicates significantly different from control group. *p<0.05*.

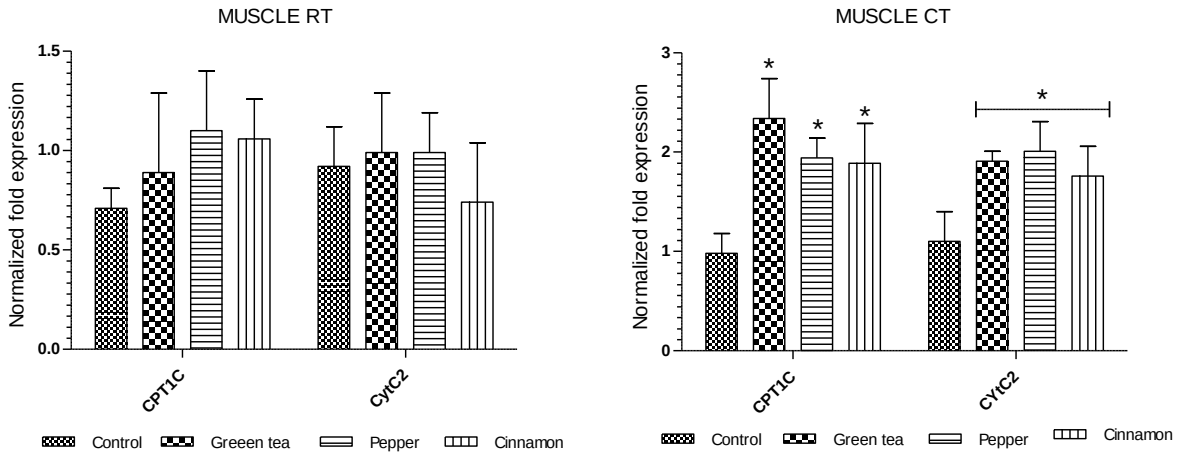


Fig. 8: Effect of cold exposure, green tea and spice treatment on mRNA levels of genes in muscle. * indicates significantly different from control group. *p<0.05*.

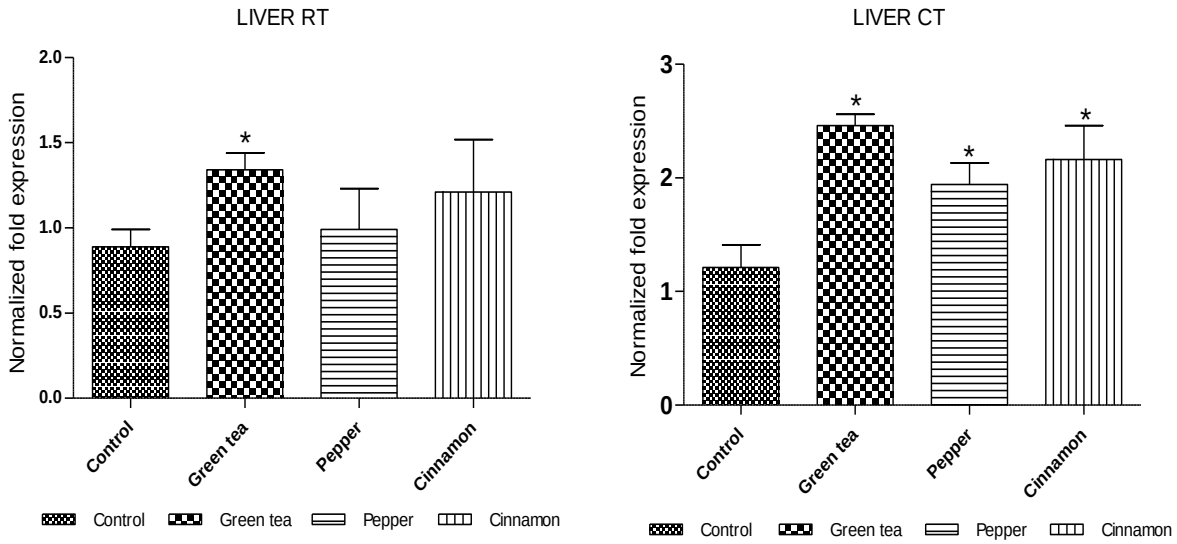
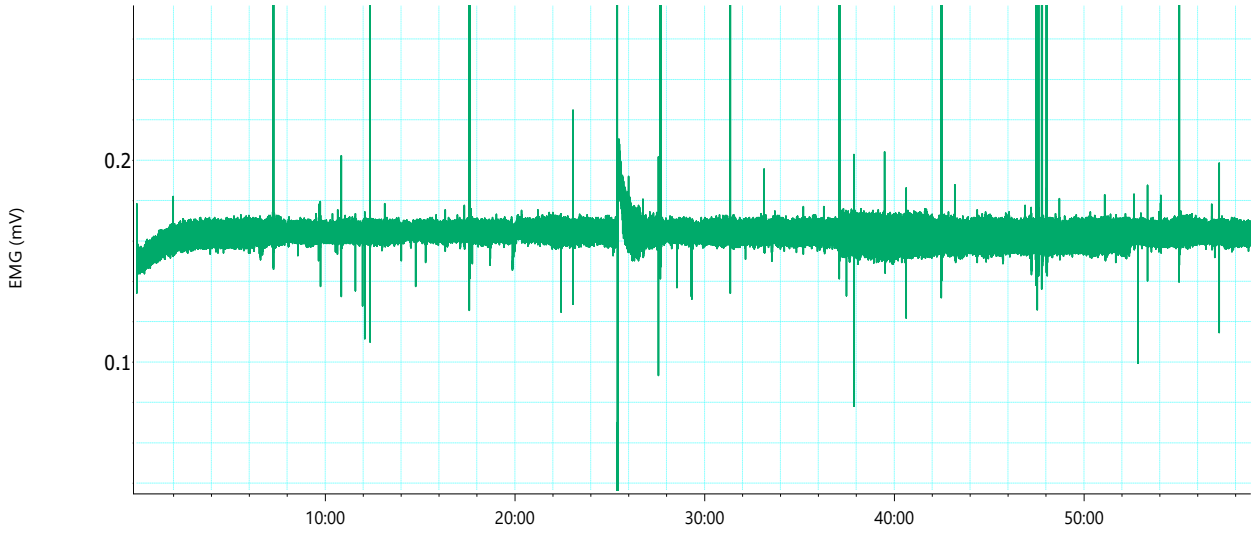
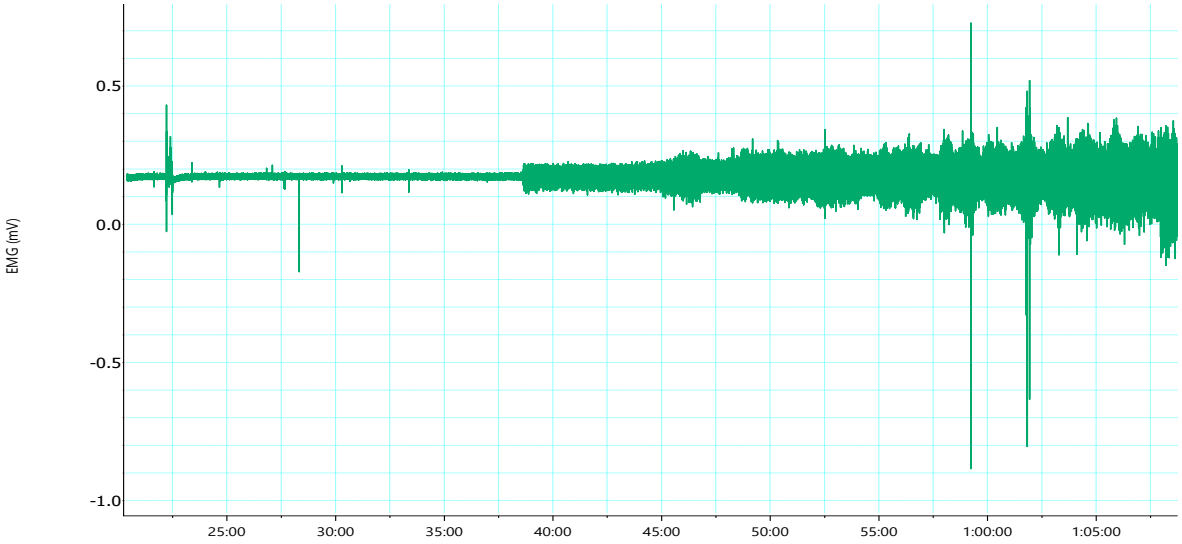


Fig. 9: Effect of cold exposure, green tea and spice treatment on mRNA levels of genes in liver. * indicates significantly different from control group. *p<0.05*.

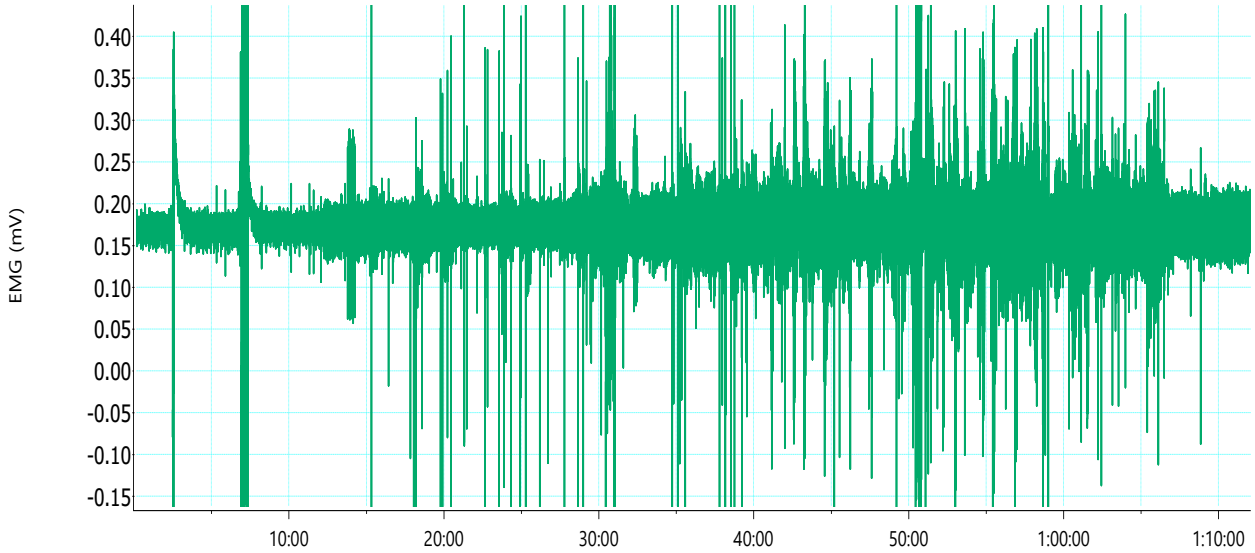
Control



Green tea



Pepper



Cinnamon

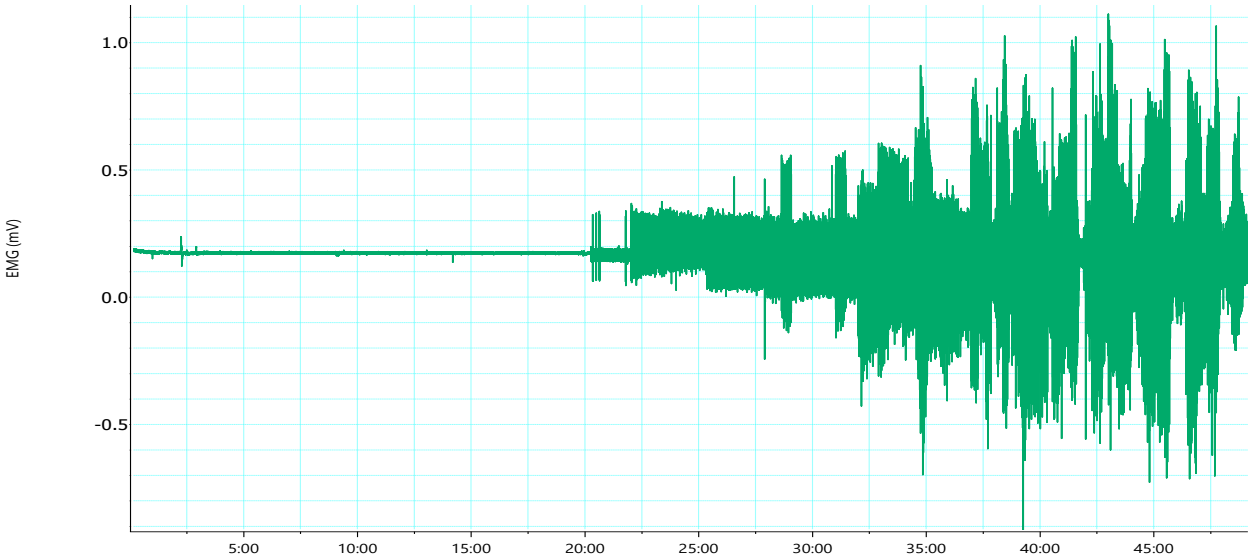


Fig. 10: Effect of cold exposure on EMG following green tea and spice treatment

Discussion

Both pepper and cinnamon extracts mediate their thermogenic action mainly by the sympathetic nervous system through norepinephrine. In the current study, we tried to understand the effect of the two spices on expression levels of specific genes in four organs namely BAT, WAT, muscle and liver. We also studied the effect of the spices on circulating levels of thyroid hormones, NOR and FFA. Although BAT is the primary site of non shivering thermogenesis, research today has enabled us to identify organs beyond BAT that can aid in physiological heat production[24]. Ability of various compounds to cause browning of WAT[2,4,11] leads us to question if the two spices also had any effect on WAT, muscle and liver. As green tea extract is the positive control for this study and its mode of action of thermogenesis has been reported by many researchers, it is not discussed in detail here.

We observed that green tea and pepper followed a similar pattern of action whereas cinnamon affected the studied organs differently. Distinctive RT and CT groups were used for both the spices. This was so as to learn the effect of spices alone on the studied genes as well as to understand the effect of cold alone on the expression level of the studied genes. It is interesting to note that in the RT group of animals, in comparison to the control group, treated (green tea/both spices) animals exhibited an increased expression of genes in the studied organs. This pattern of upregulation was observed in all four organs for most of the genes studied.

However, unlike indicated in literature[15], the increased expression was not significant in most cases. This implies that treatment with green tea and both the spices does have an effect on non shivering thermogeneis under room temperature conditions. But the degree of up regulation achieved was way less in comparison to their respective cold treated groups. It is also noteworthy that cold exposure alone increased the expression of the studied genes to a degree greater than the treated RT groups. This is suggestive that although spices and green tea are more effective in potentiating an already activated thermogenic cascade, cold temperature is the best inducer of non shivering thermogenesis in the body leading to an enhanced heat generation[8]. Treatment with pepper and cinnamon upregulated the expression of all five genes studied in BAT viz. UCP1, PRDM16, PGC1α, CPTC1 and Cytc2.

Up regulation of PRDM16 implies an increased BAT differentiation and quantity which is a pre-requisite for improving the cold endurance. UCP1 is the main protein responsible for uncoupling oxidative phosphorylation from ATP synthesis leading to generation of heat. Upregulation of PGC1α resulted in increased mitochondrial content, activity and promoted pathways involved in adaptive thermogenesis. Enhanced expression of CPT1C and cytc2 led to an increased β-oxidation and over all metabolic rates in BAT, increasing physiological heat production. Thus upregulation of the above mentioned genes results in an increased BAT content, activity and metabolic rate. This enables BAT to act as a heat sink providing physiological heat to rest of the body. Although both pepper and cinnamon affected BAT, it is important to note that the upregulation of genes resulting from pepper was to a higher degree than cinnamon.

The ability of spices to cause browning of WAT under room temperature conditions has been reported by many groups of researchers[17]. Consistent with this, we also noted that treatment with pepper and cinnamon in the room temperature animals caused browning of WAT, uncoupling of oxidative phosphorylation and increased overall metabolic activity of the organ. However, the BAT like activity was more pronounced in CT treated group than the RT groups. This again is assertive of the fact that although green tea and both the spices possess thermogenic potential and can promote heat generating pathways, cold temperature is the best inducer of thermogenic pathways. Treatment with

spices following cold exposure can effectively potentiate an already activated thermogenic pathway.

A careful observation of the pattern of genes upregulated in the four organs studied indicates that pepper majorly acts via the BAT and WAT organs whereas cinnamon majorly affects BAT and muscle. This is a very interesting point as it indicates that although the net result of a reduction in fall in CBT was similar in both the spices, their mode of action is varied at least to a certain degree. These results again assert and support the findings of involvement of organs other than BAT in non shivering thermogenesis[24].

Interaction of the sympathetic nervous system with thyroid hormones is essential for the complete activation of thermogenesis in BAT[19,20].Thyroid hormone is essential for sustaining the NOR mediated cascade in BAT and other organs[7,16,22]. It also interacts synergistically with NOR regulating the expression of many genes[3].involved in non shivering thermogenesis specially UCP1[21].Upon perceiving the cold temperature, the SNS responds by releasing NOR which then might have acted on thyroid gland resulting in an increased thyroid hormone status. The hyper thyroid status increases the basal metabolic rate in the body contributing to physiological heat production. The NOR released by SNS also acts on other organs viz. BAT, WAT and muscle. In BAT, this initiates the cAMP pathway increasing the FFA levels by the hormone sensitive lipase. Activation of WAT by NOR might have initiated the browning process. In muscle and liver also, it appears that NOR is the main initiator of the changes observed following cold exposure.

Shivering intensity as measured by EMG greatly decreased upon treatment with both the spices. As indicated previously, treatment with cinnamon affected muscles more than pepper. Consistently, we observed that in cinnamon treated group the onset of shivering was at a later time than pepper. Also the shivering intensity was less when compared to the pepper group. We speculate that this could be due to the increased β oxidation and overall metabolic rate as indicated by an upregulation of CPT1C and Ctyc2 genes. The increased metabolic rate might lead to physiological heat production of such magnitude that the requirement from shivering to maintain CBT is effectively shared and hence the need for shivering is decreased. This is only a hypothesis and needs further validation.

Conclusion

In conclusion, results from our study indicate that pepper and cinnamon affect organs beyond BAT namely WAT, muscle, liver and thyroid organ following a cold challenge. Treatment with pepper majorly affected BAT and WAT whereas cinnamon majorly affected BAT and muscle. Although treatment with both the spices upregulated the expression of the genes studied in RT group of animals, the upregulation was not significant in comparison to their respective control groups. Cold exposed and treated animals showed an increased expression level of the genes studied in the four organs in comparison to their respective cold controls. Increased upregulation of CPT1C and cytc2 in muscle following treatment with cinnamon when compared to pepper, might be the basis for the delayed onset of shivering observed in cinnamon treated cold exposed animals.

The results from this study clearly point towards the involvement of organs beyond BAT in responding to a cold challenge. Our results confirm that undoubtedly cold exposure remains the best activator for upregulation of genes involved in the thermogenic process, be it BAT or other organs. Our studies have shown that in addition to cold, certain thermogenic compounds can accelerate this response tremendously. Further understanding of this orchestered response might throw new light into the existing knowledge of regulation of the thermogenic process of the human body

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