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Anti inflammatory Effect of Natural Honey on Bovine Thrombin-induced Oxidative Burst in Phagocytes

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Thrombin, hyperglycemia and reactive oxygen species (ROS) have been discovered to play a pivotal role in the pathogenesis of cardiovascular disease (CVD). The aim of the study was to evaluate the direct effect of bovine thrombin (BTh) on ROS production by human neutrophils and rodent macrophages and to investigate the effect of honey on BTh-induced ROS production from phagocytes.

Professional phagocytes, i.e. neutrophils and macrophages, were stimulated by BTh and ROS production was measured in luminol/lucigenin enhanced chemiluminescence (CL) assays. In another experiment the effects of honey treatment on BTh-induced ROS production by phagocytes was tested using a CL assay.

The results indicate that BTh directly activates phagocytes. A significant generation of ROS was noted with the luminol/lucigenin enhanced chemiluminescence (CL) system. Honey treatment of phagocytes activated by bovine thrombin showed effective suppression of oxidative respiratory burst monitored by the CL assay.

In conclusion, it can be assumed that this direct action of BTh on phagocytes causing ROS production might exaggerate the inflammatory response at the site of atheromatous plaques. The suppressive activity of honey towards thrombin-induced ROS production by phagocytes could be beneficial in the interruption of the pathological progress of CVD and may play a cardioprotective role. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: natural honey; thrombin; professional phagocytes; reactive oxygen species; cardiovascular diseases.

INTRODUCTION

Cardiovascular disease (CVD) remains a leading cause of death worldwide. Traditional cardiovascular risk factors include diabetes, hypertension, dyslipidemia, smoking etc. (Wang 2008). Thrombin, hyperglycemia and reactive oxygen species (ROS) have been discovered to play a pivotal role in the pathogenesis of CVD. The development of the atherosclerotic lesion, which is the primary lesion of CVD, depends on the presence of CVD risk factors. These risk factors initially damage the vascular endothelial cell lining, which initiates a cascade of events and as part of this chain of events, thrombin activates endothelial and smooth muscle cells which causes ROS production. The ROS augment cell signaling as well as causes oxidation of low density lipoprotein (LDL) thus providing a nidus for the development of atherosclerotic plaque (Cathcart, 2004). In addition, thrombin has been reported to have interactions with professional phagocytes, i.e. neutrophils and macrophages, for example thrombin induces chemotaxis, adherence and recruitment of professional phagocytes. Indirect stimulation of neutrophils with platelets and thrombin has been reported to generate ROS by neutrophils (Gorlach, 2004, 2005; Herkert *et al.*, 2004; Thannickal and Fanburg, 2000; Strukova, 2001; Zimmerman *et al.*, 1985; Gando *et al.*, 2002; Cunningham *et al.*, 1999; Bizios *et al.*, 1986; Cohen *et al.*, 1991; Kaur *et al.*, 2001; Lippuner *et al.*, 2007; Szaba and Smiley, 2002; Chan *et al.*, 1988; Losche and Temmler, 2001).

A large body of evidence exists on dietary benefits on cardiovascular health. Postprandial hyperglycemia is reported to increase oxidative stress, which, in turn, has negative consequences on blood pressure, blood clot formation, endothelial function, heart failure (Kalergis *et al.*, 2005; Giordano, 2005). A low glycemic index diet and reducing daily energy intake by 15–40% showed an improvement in glucose tolerance, insulin action, reduced blood pressure, decreased heart rate, reduced oxidative damage to lipids, protein and DNA (Minamiyama *et al.*, 2007; Nakano *et al.*, 2001; Weiss *et al.*, 2006; Park *et al.*, 2005; Lin *et al.*, 2002; Zhu *et al.*, 1999; Qin *et al.*, 2006; Wiggins *et al.*, 2005; Sears and Bell, 2004; Ceriello *et al.*, 1998, 1999; Rao and Agarwal, 1999). Natural honey which is a solution of simple sugars and bioactive natural products is reported to have antioxidant, ROS scavenging and quenching actions and has been classified as a low GI food thus producing an improved glycemic response in non-diabetic as well as in the diabetic population (Al-Mamary *et al.*, 2002; Gheldof *et al.*, 2002; Henriques *et al.*, 2006; Schramm *et al.*, 2003; Tonks *et al.*, 2003; Samanta *et al.*, 1985; Chepulis and Starkey, 2008; Al-Waili, 2004; Shambaugh *et al.*, 1990; Agrawal *et al.*, 2007; Chepulis, 2007).

This study was therefore conducted to determine the direct effect of bovine thrombin on ROS production

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in professional phagocytes, i.e. human neutrophils and rodent macrophages, during the process of phagocytosis recorded by the luminol/lucigenin enhanced chemiluminescence assay and the effect of natural honey treatment was examined in bovine thrombin-induced production of reactive oxygen species from phagocytes.

MATERIAL AND METHODS

In this study, six different types of commercially available natural honey samples were used, obtained from local supermarkets (Karachi, Pakistan). These samples included Clover (America), Capilano (Australia), Langnease (Germany), Al-Shafa, Swat and Sidder (Pakistan) honeys. The floral sources of all these honeys were not mentioned. Luminol, Hanks balanced salts solution (HBSS), lucigenin and bovine thrombin were obtained from Research Organics, Cleveland; Gibco, UK; Sigma, USA; Human Diagonista Gmbh, Germany, respectively. Lymphocyte separation medium (LSM), phorbol myristate acetate (PMA) and zymosan-A were obtained from MP Biomedicals, Inc., Germany. The luminometer R.S. was procured from Labsystem, Finland. NMRI mice (20–30 g) of either sex, were bred in the animal house of the International Center for Chemical and Biological Sciences (University of Karachi).

The luminol and lucigenin were prepared to give 1 mM and 5.0 mM, respectively, as working concentrations. Bovine thrombin was prepared according to the manufacturer's instructions and was used in different concentrations. The opsonization of zymosan particles was carried out as described earlier (Cohen and Gray, 1984). PMA (1 mg) was dissolved in DMSO and diluted to give 3.2×10^{-9} M as a working concentration. Throughout the studies, every time, fresh human blood (10 mL) was obtained from healthy human volunteers who were not on any regular medications and had not consumed any medicines in the past 2 weeks. The blood was drawn by clean vein puncture into heparin containing tubes and was used immediately. Peritoneal macrophages were isolated 3 days after injecting fetal bovine serum into a mouse peritoneal cavity, as reported earlier (Yeskaliyeva *et al.*, 2006). Phagocytes were separated by the ficoll gradient centrifugation method (Ferrante and Thong, 1980). The phagocytosis kinetic studies were monitored using a luminometer in a 50 min repeated scan mode. Peak and total integral chemiluminescence readings were expressed as the relative light unit (RLU) (Helfand *et al.*, 1982; Haklar *et al.*, 2001).

Effect of bovine thrombin on professional phagocytes. These studies were performed using human neutrophils and rodent peritoneal macrophages. Essentially these phagocytes were stimulated by various concentrations of bovine thrombin ranging from 0.5 to 0.0002 units/mL. ROS production was measured in luminol and lucigenin enhanced chemiluminescence assays, in 96-well flat-bottom plates (Helfand *et al.*, 1982; Haklar *et al.*, 2001). Measurements were taken at zero time incubation and after 20 min incubation. Cells with luminol or lucigenin in the absence of thrombin were taken as a positive control. To validate every experiment with luminol and lucigenin, serum opsonized zymosan and PMA respectively, were used as standard activators.

Effect of natural honey on bovine thrombin-induced phagocyte oxidative burst. A luminol or lucigenin enhanced chemiluminescence assay was performed by using neutrophils and macrophages separately. Briefly, different types of honey sample were placed, 25 μL, in each well. Later either 25 μL neutrophils (3×10^6/mL) or macrophages (4×10^6/mL) was added to each well. This mixture was incubated for 30 min at 37 °C temperature. After incubation, 25 μL thrombin was added to each well. Final concentrations of thrombin with neutrophils and macrophages for the luminol enhanced chemiluminescence (LmECL) assay were 0.03 and 0.12 units/mL, respectively. Whereas in the lucigenin enhanced chemiluminescence (LuECL) assay with neutrophils and macrophages, thrombin at 0.015 and 0.25 units/mL was used. Cells without activator (no thrombin) were taken as a positive control.

Statistics. All data are presented as mean ± standard deviation of the mean. Where needed, statistical analysis of results were carried out using Student's *t*-test. Significance was attributed to probability values of $p \leq 0.05$. The IC_{50} was calculated using an Excel based program.

RESULTS

Effect of bovine thrombin on ROS production

In the first part of the study, the effect of bovine thrombin action on phagocytes was examined. Upon stimulation with thrombin at different concentrations, both neutrophils and macrophages showed an increase in ROS production compared with the control (cells without thrombin). Table 1 shows the results of significant ($p \leq 0.05$) thrombin concentrations in LmECL and LuECL, at zero time incubation and after 20 min incubation.

Effect of honey on thrombin-induced neutrophil oxidative burst

In the study, with luminol, thrombin at a concentration 0.03 units/mL was used as a cell activator. A strong dose-dependent suppressive activity was observed with all six honey samples (Figs 1, 2). A complete (100%) suppression of ROS production (results not shown) was noted at honey concentrations more than 1 mg/mL. Subsequently the doses of honey were decreased to 0.8–0.03 mg/mL. IC_{50} values were found to be 0.1–0.03 mg/mL. In the lucigenin enhanced study thrombin at 0.015 units/mL was used. The use of honey ≥15 mg/mL showed complete (100%) suppression in ROS production (results not shown). Subsequently the doses of honey samples were decreased to 15–0.4 mg/mL. IC_{50} values were found to be 1.8–3.5 mg/mL.

Effect of honey on thrombin-induced macrophage oxidative burst

In the luminol enhanced study, thrombin at 0.12 units/mL concentration was used as the cell activator. A strong dose-dependent suppressive activity was observed with all six types of honey samples (Figs 3, 4).

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Table 1. Effect of bovine thrombin concentrations (2.5×10^{-2}–2×10^{-5} units/mL) on oxidative burst and ROS production

CLp	Time	Cells	2.5×10^{-2}	1.2×10^{-2}	6×10^{-3}	3×10^{-3}	1.5×10^{-4}	7×10^{-4}	3.9×10^{-5}	1.9×10^{-5}	9×10^{-6}	4×10^{-5}	2×10^{-5}
Lm	0	PMN	7144 ± 823.1[a]	12897 ± 636.4	23201.5 ± 5161.2	19301 ± 1970	21019 ± 1833[a]	21160 ± 3838	24017 ± 2052[a]	19773 ± 3887	19678 ± 2840	14936 ± 769.3	12979.5 ± 416.4
	20		20178 ± 3046.2	26262 ± 7164.4	25705 ± 2435.3[a]	26633 ± 2968.4[a]	26831.5 ± 1997.6[a]	22514.5 ± 7580.9	22186.5 ± 2692	18479 ± 105.7	14681.5 ± 2437.4	16182 ± 1698.5	17279.5 ± 1884.4
Lu	0	PMN	319 ± 49.5	407.5 ± 0.7	406 ± 59.4	401.5 ± 2.1	457 ± 117.4	471 ± 8.5	445.5 ± 26.2[a]	426 ± 12.7	318 ± 14.1	231.5 ± 21.9	305.5 ± 17.7
	20		735.5 ± 24.7[a]	546 ± 15.6[a]	357 ± 1.4	331 ± 17	412 ± 19.8	454 ± 26.9	433 ± 12.7	330 ± 58	333.5 ± 36.1	333.5 ± 55.9	301.5 ± 2.1
Lm	0	MQ	2905.5 ± 140.7[a]	3087 ± 35.4[a]	2110.5 ± 190.2[a]	2056.5 ± 82.7[a]	2136.5 ± 16.3[b]	2065.5 ± 40.3[b]	1591 ± 193.7[a]	2329.5 ± 38.9[b]	2255.5 ± 20.5[b]	1836.5 ± 99.7[a]	1229.5 ± 19.1[a]
	20		1070 ± 65.1[a]	1520 ± 124.5[b]	1207.5 ± 62.9[a]	1065.5 ± 128	613 ± 2.8[a]	425.5 ± 95.5	420 ± 29.7	328 ± 22.6[a]	385.5 ± 27.6[a]	461 ± 66.5	615 ± 24
Lu	0	MQ	2269.5 ± 228.4[a]	2114.5 ± 68.6[a]	1694.5 ± 111[a]	980 ± 12.7[a]	892.5 ± 47.4[a]	1198 ± 36.8[a]	908.5 ± 65.8[a]	1180 ± 46.7[a]	1134 ± 65.1[a]	1007 ± 32.5[a]	870.5 ± 81.3[a]
	20		643.5 ± 14.8	1082 ± 9.9[a]	1293.5 ± 70[a]	928.5 ± 62.9[a]	918.5 ± 40.3	840 ± 46.7	531.5 ± 26.2	237 ± 18.4	279 ± 35.4	394.5 ± 30.4[b]	239 ± 50.9[a]

This table represents the effect of various concentrations of bovine thrombin on professional phagocytes after zero min and 20 min incubation. Student's *t*-test was used to calculate significance ([a] $p \leq 0.05$, [b] $p \leq 0.005$). Bovine thrombin concentrations in luminol/lucigenin enhanced chemiluminescence system. Each reading represents mean and standard deviation (SD ±) of three observations of relative light units [RLU]. CLp, chemiluminescence probe; Lm, luminol; Lu, lucigenin; PMN, neutrophils; MQ, macrophages.

A complete suppression (100%) in ROS production (results not shown) was noted at honey concentrations of more than 1 mg/mL. IC_{50} values were found to be 0.1–0.03 mg/mL. In the lucigenin enhanced study, thrombin at a 0.25 units/mL concentration was used. Honey at ≥15 mg/mL showed complete suppression (100%) in ROS production (results not shown). IC_{50} values were found to be 2–0.4 mg/mL.

DISCUSSION

In the pathogenesis of cardiovascular diseases (CVD), the development of atherosclerotic plaque is the fundamental step (Toschi *et al.*, 1997). CVD risk factors, diabetes, hypertension, dyslipidemia, smoking etc. (Wang 2008) initiate vascular endothelial cell damage, this begins a complex interplay between cells of inflammation, coagulation factors, lipids etc. As a part of this process, at the site of endothelial cell damage, accumulation of professional phagocytes, ROS production and thrombin activation takes place. Thus investigating the *direct* effect of thrombin (Tracy, 2003; Strukova, 2001) on professional phagocytes and recording the release of ROS by chemiluminescence would be of great value in further understanding the molecular changes that take place during the pathogenesis of atherosclerotic plaque formation (Fig. 5). In this study, the effect of bovine thrombin compatible with the human body (Lundblad *et al.*, 2004) was examined on the phagocyte respiratory oxidative burst response which showed that bovine thrombin directly stimulates professional phagocytes (i.e. human neutrophils and rodent macrophages) and causes a significant release of ROS (Table 1). The results indicate that the effect of bovine thrombin was more pronounced on macrophages, signifying that probably macrophages were more responsive to bovine thrombin action. In addition, increased ROS production was recorded in studies at zero time incubation compared with studies after 20 min incubation which might be because of the short half-life of thrombin (Bungay *et al.*, 2003). It was noted that both luminol as well as Lucigenin based CL assays recorded the ROS production, implying that upon activation with bovine thrombin, both macrophages and neutrophils showed MPO-dependent, as well as MPO independent systems become activated and cause a wider range of ROS release. Phagocyte activation commences a chain reaction which gives rise to the production of reactive oxygen species (ROS) due to a sequence of events collectively known as the oxidative burst (Halliwell, 2006). The generation of highly reactive superoxide anion ($O_2^{\bullet -}$) by the NADPH-oxidase complex of phagocytes is the primary event of the oxidative burst. Dismutation of $O_2^{\bullet -}$, either spontaneously or catalysed by superoxide dismutase, results in the formation hydrogen peroxide (H_2O_2), which acts as a substrate for the myeloperoxidase system (MPO) and this H_2O_2 converts to hypochlorous acid (HOCl) (Arnhold *et al.*, 2004). Emission of photons which takes place during ROS production can be measured easily as chemiluminescence (CL). This CL effect can be amplified by the use of chemiluminescent probes, i.e. luminol/lucigenin (Allen, 1986; Faulkner and Fridovich, 1993). Lucigenin detects superoxide radical ($O_2^{\bullet -}$) which is the initial event

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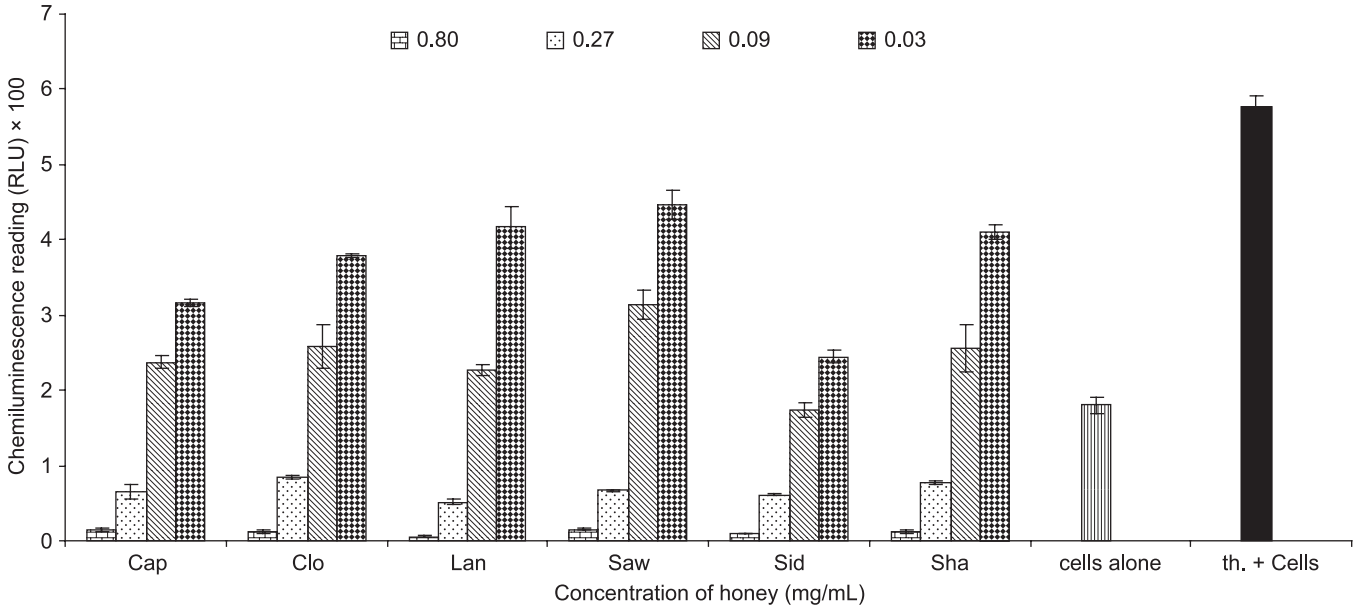

Figure 1. Effect of honey on human neutrophils in thrombin (0.03 units/mL) induced luminol enhanced chemiluminescence assay. Cells alone (no activator) and cells with thrombin (no honey) were used as controls. IC_{50} was calculated compared with control (cells with thrombin). Each plot and error bar represents reading ± SD of three repeats. Honey samples studied were cap, Capilano; clo, Clover; lan, Langnease; swa, swat; sha, Al-Shafa; sid, Sidder.

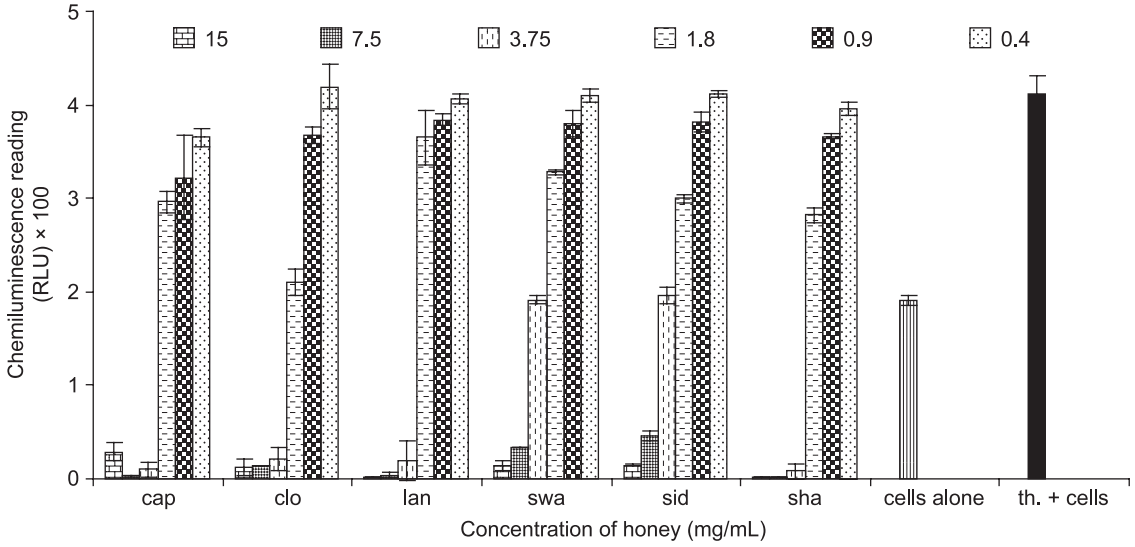

Figure 2. Effect of honey on human neutrophils in thrombin (0.015 units/mL) induced Lucigenin enhanced chemiluminescence assay. Cells alone (no activator) and cells with thrombin (no honey) were used as controls. IC_{50} was calculated compared with control (cells with thrombin). Each plot and error bar represents reading ± SD of three repeats. Honey samples studied were cap, Capilano; clo, Clover; lan, Langnease; swa, swat; sha, Al-Shafa; sid, Sidder.

in the formation of ROS by an MPO-independent mechanism. On the other hand, luminol is specific for the detection of hypochlorous acid, hydroxyl free radical etc., which are primarily produced at the later phase of oxidative burst by phagocytic myeloperoxidase (MPO) (Edwards, 1987; Faulkner and Fridovich, 1993; Gerber *et al.*, 1996; McNally and Bell, 1996; Dahlgren *et al.*, 1985; Costa *et al.*, 2006). Here it can be assumed that ROS generated after the interaction of thrombin with professional phagocytes might take part in the process of cell signaling as well as LDL oxidation at the site of vascular endothelial cell damage and could participate in the progress in the atheromatous plaque.

Treatment of professional phagocytes (activated by bovine thrombin) showed that all honey samples of different origin showed effective suppression of the bovine thrombin-induced oxidative respiratory burst of phagocytes monitored by the luminol/lucigenin-enhanced CL system. Further it was noted that lower concentrations of honey could effectively suppress the ROS production with IC_{50} values in the range 0.03–3.5 mg/dL. The results of this present study support the previous data which reported that honey caused scavenging and quenching of ROS (Al-Mamary *et al.*, 2002; Gheldof *et al.*, 2002; Henriques *et al.*, 2006). Human consumption of honey has also been reported to increase plasma antioxidant levels (Schramm *et al.*, 2003). Moreover, honey has been shown to decrease the zymosan-stimulated human monocyte cell line-based ROS production (Tonks *et al.*, 2003). The discovered

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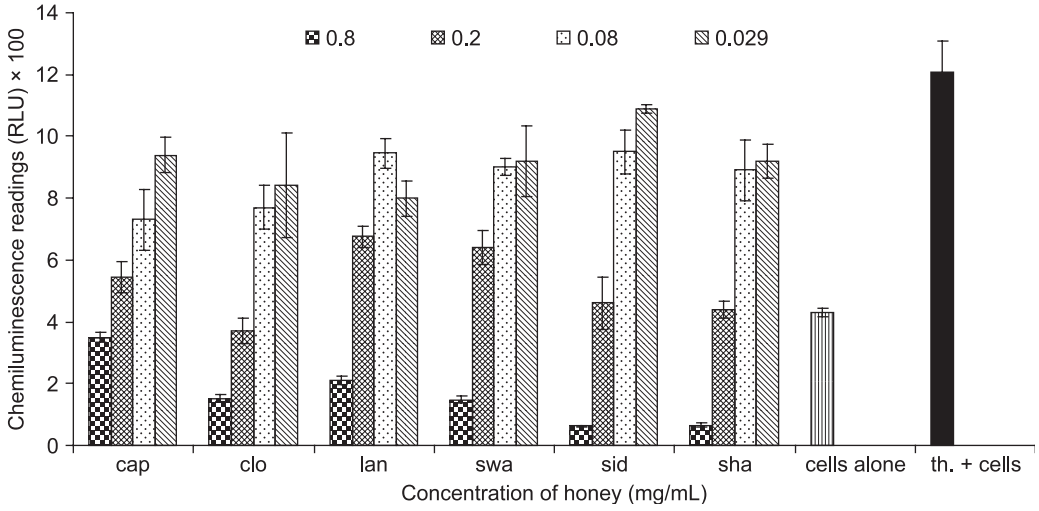

Figure 3. Effect of honey on rodent macrophages in thrombin (0.12 units/mL) induced luminol enhanced chemiluminescence assay. Cells alone (no activator) and cells with thrombin (no honey) were used as controls. IC_{50} was calculated compared with control (cells with thrombin). Each plot and error bar represents reading ± SD of three repeats. Honey samples studied were cap, Capilano; clo, Clover; lan, Langnease; swa, swat; sha, Al-Shafa; sid, Sidder.

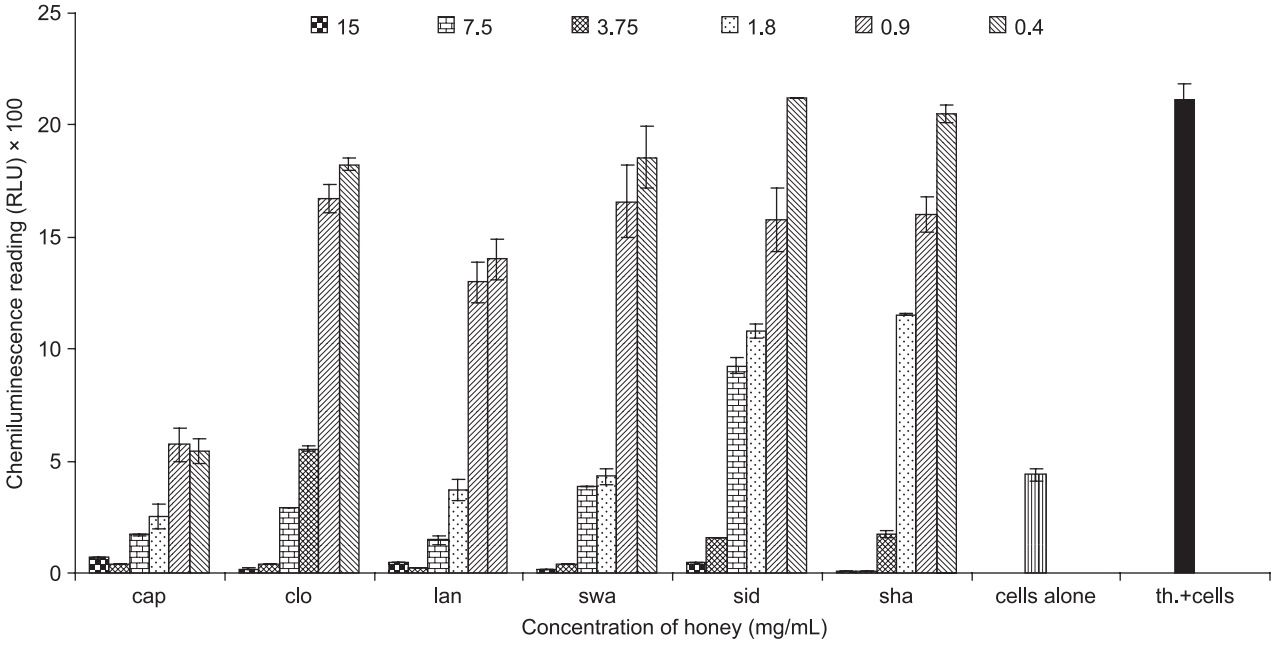

Figure 4. Effect of honey on rodent macrophages in thrombin (0.25 units/mL) induced lucigenin enhanced chemiluminescence assay. Cells alone (no activator) and cells with thrombin (no honey) were used as controls. IC_{50} was calculated compared with control (cells with thrombin). Each plot and error bar represents reading ± SD of three repeats. Honey samples studied were cap, Capilano; clo, Clover; lan, Langnease; swa, swat; sha, Al-Shafa; sid, Sidder.

honey natural products are oligosaccharides, flavonoids, isoflavones, glycosides, phenolics, peptides/proteins, waxes, pollen grains etc. (Merken and Beecher, 2000; Martos *et al.*, 2000a,b; Jimenez *et al.*, 2000; Scarselli *et al.*, 2005; Chow, 2002; Wang and Gibson, 1993; Sanz *et al.*, 2004; Postmes *et al.*, 1995) and they exhibit a wide range of biological effects, including antibacterial, antiinflammatory and antithrombotic action. It can be postulated that the presence of bioactive natural products might play a role in the suppression of either the direct action of bovine thrombin on phagocytes or the battery of enzymes involved in the MPO-dependent and MPO-independent systems. This repressive effect of honey noted by the luminol and lucigenin CL assay highlights that honey suppressed all initial, intermediate as well as late reactive oxygen species. Here it is worth mentioning that honey might affect ROS-induced cell signaling and LDL oxidation. Honey can inhibit LDL oxidation directly as well as indirectly through the suppression of ROS (Hegazi and El-Hady, 2007; Honey–Health and Therapeutic Qualities). It is also noteworthy that honey has a low GI value (Samanta *et al.*, 1985) and antithrombotic properties (Gheldof *et al.*, 2002; Henriques *et al.*, 2006) which could additionally contribute in slowing down the progress of CVD.

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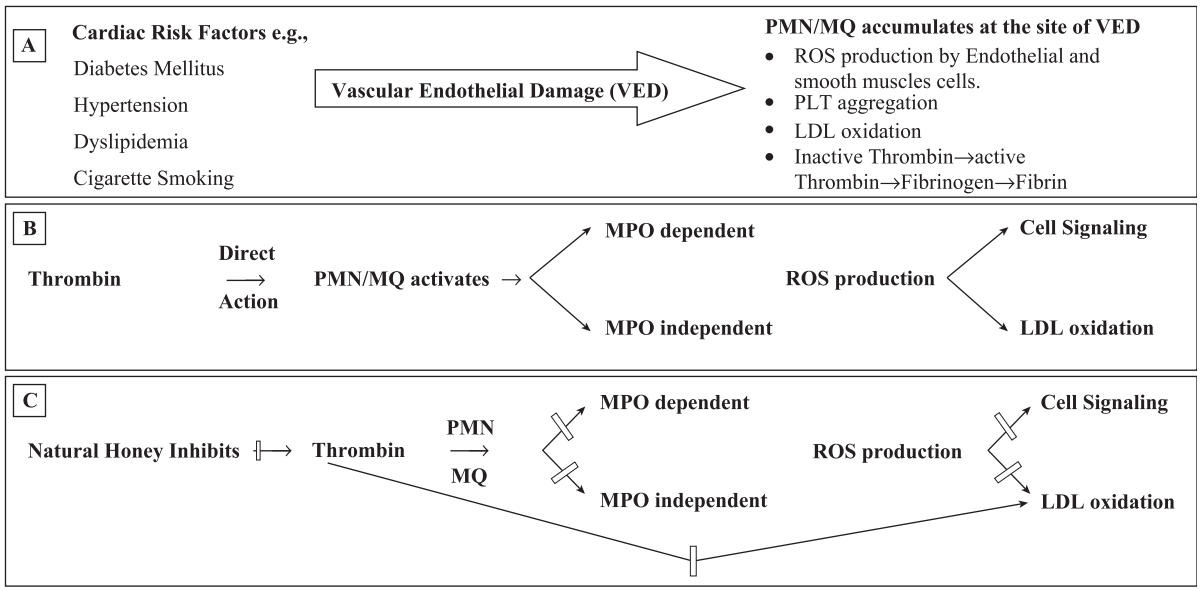

Figure 5. Schematic representation of the complex interplay between cardiac risk factors, vascular endothelium, phagocytes, thrombin, ROS and honey. (**A**) Presence of cardiac risk factors causes vascular endothelial cell lining damage which initiates cascade of events. As a part of these events professional phagocytes accumulate, platelets aggregate and ROS production, LDL oxidation, formation of active thrombin etc. takes place at the site of vascular damage. (**B**) Active thrombin acts on phagocytes and MPO-dependent and –independent production of ROS takes place. The ROS generated by phagocytes participate in cell signaling and LDL oxidation, which ultimately augments the process of atherosclerotic plaque formation. (**C**) Natural honey suppresses thrombin-induced phagocytes, (MPO dependent and independent) production of ROS. This effect of honey could interrupt the progress of the atherosclerotic plaque formation by affecting either thrombin action directly or the subsequent formation of ROS under the influence of thrombin, which in turn induces cell signaling and LDL oxidation. Honey can inhibit LDL oxidation directly as well as indirectly through the suppression of ROS. PMN, polymorph neutrophils; MQ, macrophages; ROS, reactive oxygen species; PLT, platelets; LDL, low density lipoprotein; MPO, myeloperoxidase.

CONCLUSIONS

In this study it was noted that different concentrations of thrombin in the range 2.5×10^{-2}–2×10^{-5} units/mL activated professional phagocytes and increased the production of reactive oxygen species. Macrophages were found to be more responsive compared with neutrophils and probably due to the short half-life of thrombin, more ROS production was recorded at zero time incubation. Based on the present data, it can be hypothesized that (1) upon activation with bovine thrombin, phagocytes produce ROS and this ROS might take part in the exaggeration of the inflammatory response at the site of atheromatous plaques. (2) Natural honey suppressed the bovine thrombin-induced phagocytic oxidative burst. Reactivity of honey towards the phagocytes leading to the suppression of ROS could be extremely beneficial in the interruption of the pathological progress of cardiovascular disease and play a cardioprotective role particularly related to the production of ROS-induced LDL oxidation and cell signaling.

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