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The Effect of Dark Chocolate on C-Reactive Protein Level: A Systematic Review and Meta-Analysis of Randomized Controlled Clinical Trials

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SYSTEMATIC REVIEW and META-ANALYSIS

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ABSTRACT

Background: This systematic review and meta-analysis of randomized controlled trials (RCTs) was conducted to determine the effect of dark chocolate on C-Reactive Protein (CRP) levels as one of the inflammatory factors. **Methods**: A literature search was conducted in PubMed, ISI Web of Science, Scopus, and Google Scholar up to April of 2020. The registration number of study in PROSPERO was CRD4202020072, which was conducted over 5 eligible RCTs containing a total of 330 participants. The weighted mean difference (WMD) with 95% confidence intervals was calculated for the pool effect size of CRP. The heterogeneity of studies was examined by Cochran's Q test and I-squared (I^2) statistic. **Results**: Effect sizes from 330 participants based on random effect model showed no effect between consumption of dark chocolate on CRP levels compared to the control group (WMD: 0.01 mg/dl; 95% CI: −0.19, 0.22 mg/dl; P = 0.89; Cochran's Q test, Q statistic = 21.97; P < 0.001; I^2 = 81.80%). The results of subgroup analysis based on intervention duration and dosage showed no significant effect on CRP levels (WMD = 0.05 mg/dl, 95% CI: −0.30, 0.42 mg/dl; P = 0.76). Meta-regression for the intervention duration (slope: −0.0033, 95% CI: −0.0089, 0.0022; P = 0.24) and dosage (slope: 0.00006, 95% CI: −0.0036, 0.0037; P = 0.97) indicated no significant relationship with the mean difference of CRP. **Conclusion**: The results of the present meta-analysis showed that consuming dark chocolate had no significant effect on the CRP level. More clinical trials are required with higher quality and bigger sample sizes to verify the positive impact of dark chocolate on the reduction CRP level.

Keywords: *Dark chocolate; CRP; Inflammation*

Introduction

Inflammation increased the risk of cardiovascular diseases, certain cancer, metabolic diseases, such as obesity, metabolic syndrome, and type 2 diabetes (Hotamisligil *et al.*, 1993, Mizuno *et al.*,

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2011, Nahrendorf and Swirski, 2015, Wellen and Hotamisligil, 2005). Previous studies have shown that inflammation can develop atherosclerosis, insulin resistance, endoplasmic reticular stress, and alternative gut microbiota. It also plays a role in the pathology of diseases, such as diabetes, cardiovascular disease, and obesity (Fernández-Real and Ricart, 2003, Hotamisligil, 2010, Tlaskalová-Hogenová *et al.*, 2011). Oxidative stress is associated with inflammation in the body, leading to activation of nuclear factor-kB (NF-kB) and increasing transcription genes related to inflammatory cytokines production (Javadi *et al.*, 2017). C-reactive protein (CRP) is a recognized marker of systemic inflammation that decreases the production of nitric oxide by endothelial cells and also affects the renin-angiotensin system. Reports have shown that CRP could affect progressive endothelial inflammation and atherosclerosis (Cottone *et al.*, 2006).

Dark chocolate (DC) is one of the cocoa products, including cocoa butter and bean solids (up to 80% of the total weight) (Montagna *et al.*, 2019). Results of meta-analysis have shown that consuming chocolate or cocoa improved endothelial function and reduced insulin resistance and blood pressure in adults (Hooper *et al.*, 2012). Cocoa and dark chocolate contain flavonoids that have antioxidant and anti-inflammatory properties (Magrone *et al.*, 2017). Several in vitro studies have reported that cocoa polyphenols could reduce the transcription and secretion of pro-inflammatory cytokines in human peripheral blood mononuclear cells (PBMCs), macrophages, and lymphoid cell lines (Mao *et al.*, 2003, Ramiro *et al.*, 2005). Results of some studies have shown that consumption of DC improves oxidative stress marker and has the potential to reduce oxidative stress (Malhi and Loomba, 2016, Nanetti *et al.*, 2012).

Several clinical trials have reported the protective effects of DC on blood pressure (Desch *et al.*, 2010, Haber and Gallus, 2012, Sudarma *et al.*, 2011), glycemic factors (Grassi *et al.*, 2008), and lipid profiles (Nanetti *et al.*, 2008, Rezazadeh *et al.*, 2015), while there are some controversial findings regarding the effect of DC on CRP. A study assessed the effect of DC on CRP level in 28 healthy volunteers. Results of this study showed that DC reduced high sensitivity-CRP levels (Hamed *et al.*, 2008). However, some studies have represented no significant changes in CRP level after consuming DC (Di Renzo *et al.*, 2013, Rezazadeh *et al.*, 2015). To the best of the authors' knowledge, no systematic review and meta-analysis of randomized controlled trials (RCTs) have examined the effect of DC supplementation on CRP. Therefore, this meta-analysis was conducted to summarize the up-to-date current evidence of RCTs, estimating the effect of DC supplementation on CRP as a biomarker of inflammation.

Materials and Methods

Search strategy: The present systematic review and meta-analysis was accomplished according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Guidelines (PRISMA) (Moher *et al.*, 2015). The protocol of this study was registered in PROSPERO, an International Prospective Register of Systematic Reviews (http://www.crd.york.ac.uk/ PROSPERO) with the registration number of CRD42020200722. PubMed, ISI Web of Science, Scopus, and Google Scholar were searched up to 4 February 2022 without any restrictions to find eligible studies. Medical Subject Heading (MeSH) terms and non-MeSH terms were applied to investigate the effect of DC supplementation on CRP. The following keywords were used to search: ((chocolate[mesh] OR chocolate[tiab] OR "dark chocolate"[tiab]) AND ("c-reactive protein"[mesh] OR "c-reactive protein"[tiab] OR CRP[tiab] OR "hs-CRP"[tiab] OR inflammation[mesh] OR inflammation[tiab] OR "inflammatory markers"[tiab] OR "inflammatory biomarkers"[tiab] OR "inflammatory factors"[tiab] OR "inflammatory mediators"[tiab] OR "inflammatory indices"[tiab]))

To widen the search scope, the trial registries of the Iranian Registry of Clinical Trials and ClinicalTrials.gov were checked to identify unpublished trials in this context. In addition, the references list of the included trials was checked for further possible sources to ensure the

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comprehensiveness of searches.

Selection criteria: The selected studies had the following criteria: (1) had RCT design, (2) evaluated the impact of DC supplementation on CRP versus placebo/control, (3) presented the prescribed dosage, (4) included participants aged ≥18 years, and (5) provided sufficient information on CRP levels (serum or plasma) at the beginning and end of study (or its changes).

Study selection: The initial screening was carried out via two independent researchers (Yaghoubi F and Darabi Z), who studied the articles titles and abstracts. Then, the full texts of all related articles were evaluated via reviewers to select the trials about the effect of supplementation on CRP levels. Eventually, any possible disagreement was solved by consulting with the other researchers (Ahmadi Vasmehjani A and Sangsefidi ZS) (**Figure 1**).

*Data extraction***:** Data extraction was conducted from the selected studies based on the following items: authors' family names; publication year; sample size; loss to follow-up; a dose of intervention; study duration; crossover or parallel study design; participants' gender, age, and health status; mean and standard deviation (SD) of CRP level at the beginning and at the end of the study, as well as the mean changes and SD of CRP level.

Risk of bias assessment: Evaluating the risk of bias of the included trials was conducted according to the Cochrane criteria (Higgins *et al.*, 2019). The following items were applied for assessing the risk of bias of each research: (1) random sequence generation; (2) allocation concealment; (3) blinding of participants and personnel; (4) blinding of outcome assessment; (5) incomplete outcome data; (6) selective outcome reporting; and (7) other potential sources of bias. Based on the Cochrane Handbook recommendations, studies were rated on each item as "yes" demonstrating the low risk of bias, "no" indicating a high risk of bias, or "unclear" when the risk of bias was unclear or unknown. Additionally, by identifying the six domains as the most important ones ('key domains'), each trial was specified an overall risk of bias in terms of low risk (low for all key domains), high risk (high for one or more key domains.), and unclear risk (unclear for one or more key domains).

Ethics approval and consent to participate: This is a systematic review and meta-analysis which was conducted based on prior studies, so ethical approval is not mentioned. The protocol of this study was registered in PROSPERO, an International Prospective Register of Systematic Reviews (http://www.crd.york.ac.uk/PROSPERO) with the registration number of CRD42020200722.

Data synthesis and analysis: The difference in means was determined as effect sizes. The weighted mean differences (WMDs) were computed as follows: mean divided by the standard deviation of a difference between two random values each from one of two groups (Higgins, 2011). In studies that the standard error (SE) value had been presented, SD was obtained by the following formula: $SD = SE \times \sqrt{n}$ (n=number of participants in each group). The random-effects model was used to calculate the WMDs with 95% confidence intervals (CIs) for conducting the meta-analysis, which took the between-study heterogeneity into account (Borenstein *et al.*, 2011). Moreover, heterogeneity of studies was examined by Cochran's Q test and I-squared (I^2) statistic. Heterogeneity was specified as follow: Q statistic P-value of < 0.1; weak heterogeneity: I^2 = 25-50, relatively high heterogeneity: I^2 = 50-75, high heterogeneity: I^2 = 75-100 (Higgins and Thompson, 2002). To identify the possible sources of heterogeneity in the included studies, Subgroup analysis was performed. Given that the administered intervention dosage and duration trial might affect the findings regarding the effect of DC, sub-group analysis was accomplished based on these variables. Publication bias was also examined by assessing the funnel plot and asymmetry tests, including Begg's rank correlation test and Egger's regression test (using P-value of < 0.05) (Duval and Tweedie, 2000). Furthermore, a sensitivity analysis was performed to determine the effect of a specific study or a particular group of studies via individual removal of each study or a specific group and recalculation of the pooled

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estimates. Meta-regression was also performed to evaluate the association of the estimated effect size with dark chocolate dosage and study duration. Statistical analyses were accomplished by CMA software, version 11.2 (STATA Corp.). Statistically significant levels were specified as $P < 0.05$.

Results

Study selection: In the initial search, according to the search designs from electronic medical databases, 647 articles were found (**Figure 1**). The search strategy is shown in the supplementary material. After removing duplicates, 421 studies remained. From this number, 416 articles were excluded because they were not clinical trials (n = 376) or did not meet the inclusion criteria (n = 37) and or not found in medical databases (n = 3). Finally, 5 eligible studies included in this meta-analysis.

Characteristics of the studies: The details of the 5 included studies are presented in **Table 1**. Studies were listed based on the following information: country, age (y), gender, sample size (intervention), RCT design, intervention name, the dosage of DC, name control, duration, outcome, the health status of participants. Studies were published from 2005 to 2018 and the sample size ranged from 19 to 110 participants. A cross-over design was in three studies (da Costa Teixeira *et al.*, 2017, Grassi *et al.*, 2008, Grassi *et al.*, 2005), one study had parallel -4 armed design (Kurlandsky and Stote, 2006) and so one was a randomized clinical trial (Jafarirad *et al.*, 2018). The intervention duration in the trials was 15-56 days. The dose of DC in the intervention groups was from 30 to 100 g/day. All studies except one trial reported mean ± SD for CRP levels at the start and end of the trial in two groups of intervention and placebo. Two studies were conducted in Italy (Grassi *et al.*, 2008, Grassi *et al.*, 2005) and one in every one of the following countries, Brazile (da Costa Teixeira *et al.*, 2017), USA (Kurlandsky and Stote, 2006), Iran (Jafarirad *et al.*, 2018).

Quality assessment of the studies: The quality assessment of the included articles is summarized in **Table 2**. Briefly, all studies were categorized as low risk of bias for incomplete outcome data and selective reporting. Random sequence generation was described only in one study (Jafarirad *et al.*, 2018), but a lack of information was observed in other studies. Three studies were considered low risk of bias for allocation concealment (Grassi *et al.*, 2008, Jafarirad *et al.*, 2018, Kurlandsky and Stote, 2006) and the remaining two studies did not express any method for allocation concealment; therefore, they were regarded as unclear risk of bias (da Costa Teixeira *et al.*, 2017, Grassi *et al.*, 2005). Two studies did not report the use of any method for blinding of participants and personnel and selective reporting (Grassi *et al.*, 2005, Kurlandsky and Stote, 2006). The blinding of outcome assessment was unclear in all studies. Three included studies were at least 1 of the 6 key domains of unclear risk of bias, thus overall quality was unclear (da Costa Teixeira *et al.*, 2017, Grassi *et al.*, 2008, Jafarirad *et al.*, 2018). Two studies had at least 1 of the 6 key domains of high risk of bias; therefore, their overall quality became the high risk of bias (Grassi *et al.*, 2005, Kurlandsky and Stote, 2006).

Finding from meta-analysis: Combining 5 effect sizes from 5 studies (n = 376) based on random effect model showed no effect of consuming DC on CRP levels compared to the control group WMD: 0.014 mg/dl; 95% CI: −0.19, 0.22 mg/dl; $P = 0.89$; Cochran's Q test, Q statistic = 21.97; $P < 0.001$; $I^2 = 81.80\%$] (**Figure 2**). Sensitivity analysis was accomplished to evaluate the effect of each study individually on the overall findings; each trial was removed but did not change meta-analysis results. The findings of subgroup analysis based on intervention duration and dosage disclosed no significant effect on CRP levels (WMD = 0.05 mg/dl, 95% CI: −0.30, 0.42 mg/dl; $P = 0.76$) and (WMD = −0.03 mg/dl, 95% CI: −0.27, 0.20 mg/dl; $P = 0.76$), respectively (**Figures 3 and 4**).

Meta-regression analysis based on all studies was implemented to investigate the linear association between DC duration and dosage with the mean difference of CRP levels. Meta-regression for the intervention duration (slope: −0.0033, 95% CI: −0.0089, 0.0022; $P = 0.24$) and dosage (slope:

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0.00006, 95% CI: −0.0036, 0.0037; *P* = 0.97) did not indicate any significant relevance with the mean difference of CRP (**Figures 5 and 6**).

Although the asymmetric statistical tests did not show the existence of a publishing bias for the meta-analysis of CRP (Begg's test, *P* = 1; Egger's regression test, *P* = 0.51), the funnel plot indicated a visual publication bias (**Figure7**). After adjusting the effect size for publication bias by the" trim and fill" correction, no potentially missing surveys were needed in the funnel plot (WMD= 0.01 mg/dl, 95%CI= -0.19-0.22 mg/dl).

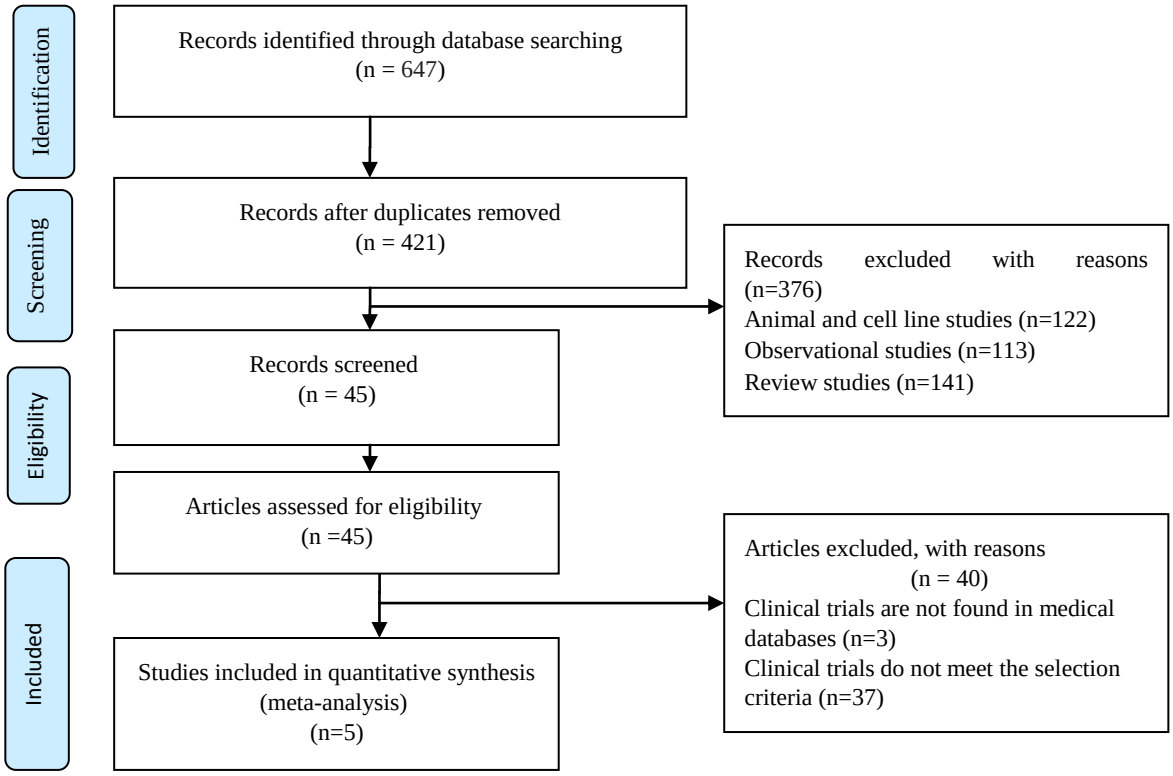


Figure 1. Flowchart of selection process of the studies.

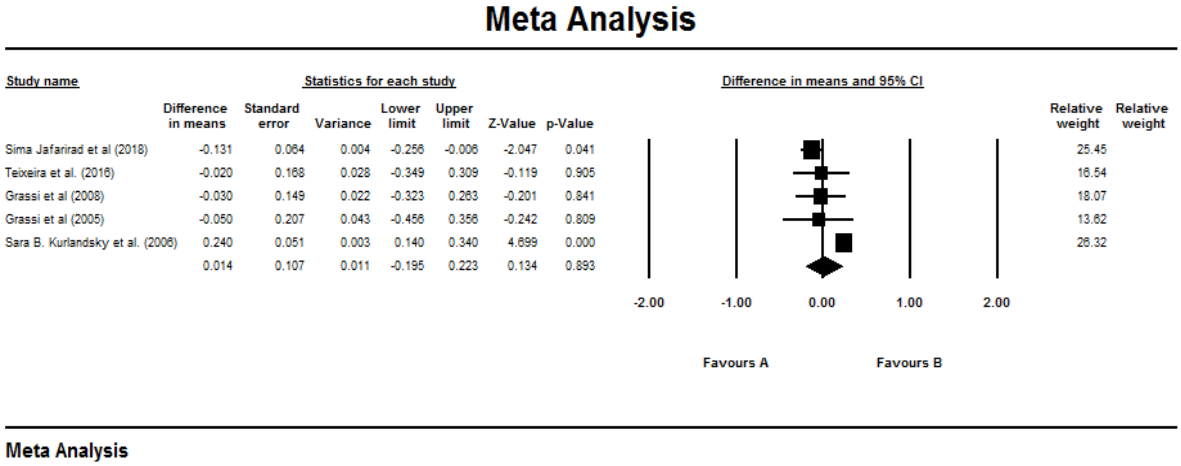


Figure 2. Forest plot illustrates weighted mean difference (represented by the black square) and 95 % CI (represented by horizontal line) for concentration of CRP and dark chocolate. Weights are from random effects analysis.

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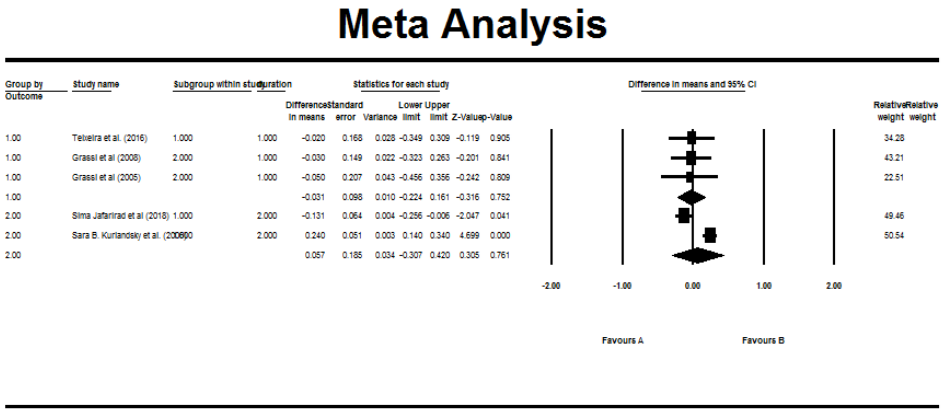


Figure 3. Forest plot illustrates weighted mean difference (represented by the black square) and 95 % CI (represented by horizontal line) for CRP levels and duration of trail. Weights are from random effects analysis.

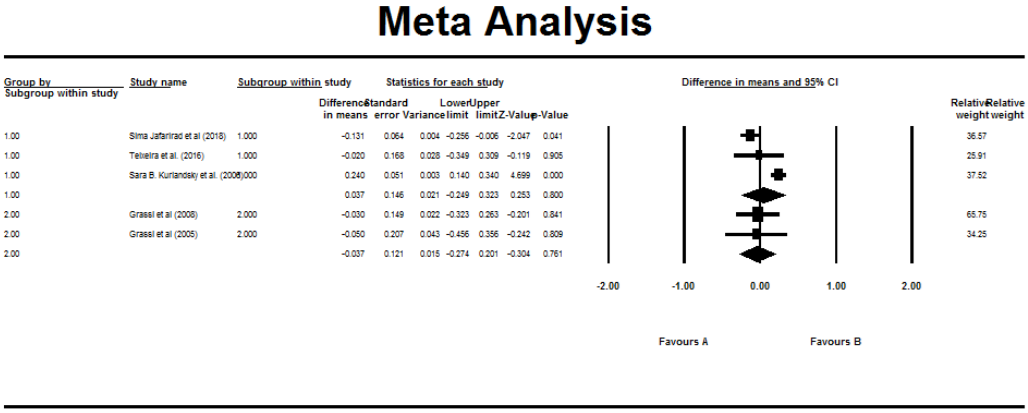


Figure 4. Forest plot illustrates weighted mean difference (represented by the black square) and 95 % CI (represented by horizontal line) for concentration of CRP and dose of dark chocolate. Weights are from random effects analysis.

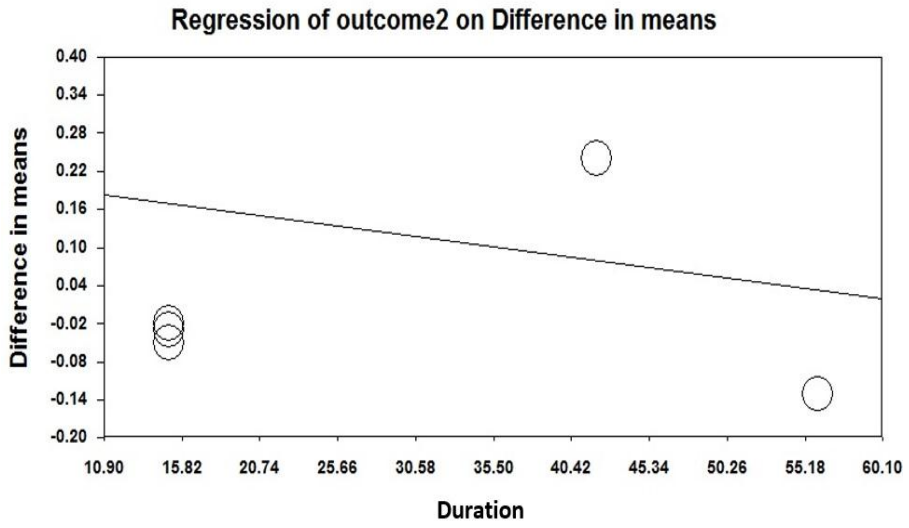


Figure 5. Meta-regression plot of the association between differences in means of CRP levels with duration of trail. The size of circle is inversely proportional to the variance of change.

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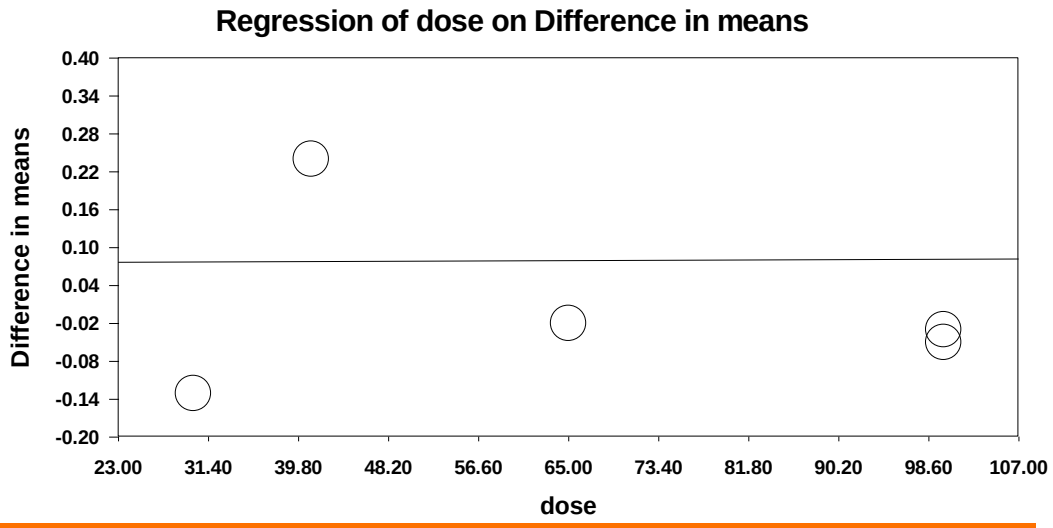


Figure 6. Meta-regression plot of the association between differences in means of CRP levels with dose (gram) of dark chocolate. The size of circle is inversely proportional to the variance of change.

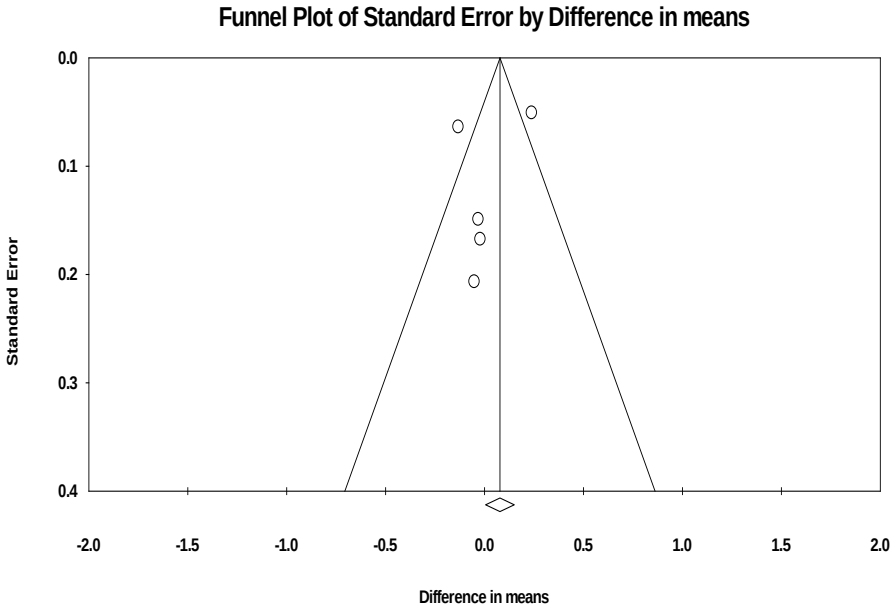


Figure 7. Funnel plot for publication bias.

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Table 1. General characteristics of the included studies.

First Author (year)	Country	Age (y)	Gender	Sample size(int)	RCT Design	Int name and dose	Dosage of dark chocolate (g/day)	Con	Outcome		Duration (day)	Status of health
									Int	Con		
Grassi et al. (2005)	Italy	25-60	F/M	35(20)	Crossover	Flavanol-rich dark chocolate	100	Flavanol-free white chocolate	CRP before: 0.37 ± 0.39[a] mg/dl; after: 0.33 ± 0.39 mg/dl	CRP before: 0.35 ± 0.31 mg/dl; after: 0.36 ± 0.31 mg/dl	15	Never treated essential hypertensive
Sara B. Kurlandsky et al. (2006)	USA	22-65	F	24(12)	Parallel 4-armed	Dark chocolate daily with a self-selected diet	41	Self-selected diet avoiding nuts and chocolate	1.1±1.3 [b]	[b] -1.3±1.2	42	Healthy
Grassi et al. (2008)	Italy	mean: 44.8	F/M	38(19)	Crossover	Flavanol-rich dark chocolate	100	Flavanol-free white chocolate	CRP before: 0.38 ± 0.26 mg/dl; after: 0.34 ± 0.33 mg/dl	CRP before: 0.37 ± 0.23 mg/dl; after: 0.36 ± 0.14 mg/dl	15	Hypertensive with IGT
Teixeira et al. (2016)	Brazil	Range: 19-59	F/M	110(53)	Crossover double-blind	Chocolate bar containing 36 g cocoa (2864 mg polyphenols	65	White chocolate	CRP before: 0.33 ± 0.81 mg/dl; after: 0.28 ± 0.34 mg/dl	CRP before: 0.33 ± 0.81 mg/dl; after: 0.30 ± .57 mg/dl	15	HIV/AIDS undergoing ART
Sima Jafarirad et al. (2018)	Iran	30-60	F/M	44(21)	RCT Parallel	Dark chocolate	30	Only TLC guidelines	CRP before: 0.42 ± 0.12 mg/dl; after: 0.39 ± 0.21 mg/dl	CRP before: 0.32 ± 0.13 mg/dl; after: 0.42± 0.16 mg/dl	56	Diabetes

F: female, M: male; RCT: randomized controlled trial; int: intervention; con: control; CRP: C-reactive protein; IGT: ımpaired glucose tolerance; ART: Antiviral therapy; TLC: Therapeutic lifestyle change; [a]: mean ±SD; [b]: Changes in circulating levels of CRP

Table 2. Risk of bias assessment according to the Cochrane collaboration tool.

First author (year)	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Summary of overall assessment
Grassi et al. (2005)	U	U	H	U	L	L	H
Sara B.Kurlandsky et al. (2006)	U	L	H	U	L	L	H
Grassi et al. (2008)	U	L	L	U	L	L	U
Teixeira et al. (2016)	U	U	L	U	L	L	U
Sima Jafarirad et al. (2018)	L	L	L	U	L	L	U

H: high risk; L: low risk; U: unclear

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Discussion

The study findings indicate that DC supplementation has any significant effects on the level of CRP. This result was independent of doses and duration of chocolate intake. Altogether among 5 surveys, the results of 4 studies (da Costa Teixeira *et al.*, 2017, Grassi *et al.*, 2008, Grassi *et al.*, 2005, Jafarirad *et al.*, 2018) showed a negative relationship between DC and the level of CRP. In contrast, the study by Sara B. Kurlandsky et al. is inconsistent with the total result of the present study (Kurlandsky and Stote, 2006). However, sensitivity analysis did not represent any sensitivity to these studies.

To the best of the authors' knowledge, the present systematic review and meta-analysis is the first study on the effects of dark chocolate on CRP. Inconsistent with the present study results, a recent meta-analysis assessed the effect of cocoa/dark chocolate supplementation on body weight, body mass index, and waist circumference, which reported no significant effect (Kord-Varkaneh *et al.*, 2019). Another meta-analysis examined the effect of cocoa products as a source of DC on serum lipids. Intervention with cocoa products significantly reduced serum low-density lipoprotein (LDL) and total cholesterol (TC) levels, while it did not have any significant effects on high-density lipoprotein (HDL) similar to the current study, although no significant change was observed in LDL cholesterol in high-quality studies (3 studies) (Jia *et al.*, 2010). Mehrabani et al. conducted a meta-analysis on the impact of cocoa consumption on markers of oxidative stress. They reported that malondialdehyde and 8-iso-prostaglandin F2α were significantly reduced, but not in other markers of oxidative stress (Mehrabani *et al.*, 2020).

Consequently, several factors can lead to these controversial results, including different dosages and duration of DC, sample sizes of studies, the diverse status of subjects' health, and initial levels of CRP (Haghighat *et al.*, 2013, Sangsefidi *et al.*, 2020). Also depending on the concentration of dark chocolates' polyphenols and free radical sources, the role of polyphenols could differ between antioxidants and pro-oxidants and subsequently act as either anti-inflammatory or pro-inflammatory compounds (Cao *et al.*, 1997, Di Giuseppe *et al.*, 2008).

The role of inflammation in the pathogenesis of many chronic diseases, such as different cancers diabetes and cardiovascular disease is undeniable (Libby, 2007). On the other hand, many studies represent the protective role of flavonols on the inflammatory pathway (Kuebler *et al.*, 2016, Selmi *et al.*, 2008). The underlying mechanisms by which DC can attenuate inflammatory responses include inhibition of cyclooxygenase-2, prevention of the activation of T-cell and B-cells, reducing the expression and secretion of Interleukin 2, and reducing the amount and properties of inflammatory cytokines (IL5, TNF-α, TGF-β) (Haghighat *et al.*, 2013, Mao *et al.*, 2000, Sanbongi *et al.*, 1997). Possibly DC can mediate these events via transcription factors, such as nuclear transcription factor-kB (NF-kB), affecting the transcription and secretion of many cytokines (Mao *et al.*, 2000).

The advantages of the present study include using advanced search strategies, lack of linguistic restrictions in the search process, quality assessment based on Cochrane criteria, and different subgroup analyses according to duration and dosage of supplementation. However, there are some limitations, including the low quality of the related clinical trials and the significant heterogeneity, resulting from different doses of DC (22-100 g/day), supplemental period (15-56 days), different study size (20-158), and diverse characteristics of participants, such as health status, age, and gender. On the other hand, owing to the high publication bias, caution needs to be considered about the result of this study.

The current study results reveal that the treatment with DC had no significant effect on the CRP level. Due to the controversial finding of the conducted clinical trials, more studies with more quality and bigger sample sizes are required to verify the positive impact of DC in reducing CRP level.

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Conflict of interest

The authors declare no conflict of interest.

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There was no role of the funding in the preparation of this study.

Authors' contributions

Yaghoubi F and Darabi Z prepared the proposal, obtained ethical approvals, applied for funding and Yaghoubi F, Ahmadi A, Darabi Z and Sangsefidi ZS wrote the manuscript and performed data analysis. Hosseinzadeh M conceived the idea and supervised the study. All authors read and approved the final manuscript.

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