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Original Article

Rhamnetin decreases the expression of HMG-CoA reductase gene and increases LDL receptor in HepG2 cells

[Ramnetina disminuye la expresión del gen de la HMG-CoA reductasa y aumenta los receptores de LDL en las células HepG2]

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Abstract

Context: Rhamnetin is a naturally occurring methylated derivative of quercetin. This flavonoid is abundant in *Syzygium aromaticum*, *Coriandrum sativum Prunus cerasus,* and *Rhamnus* spp.

Aims: To evaluate the effects of rhamnetin on HMG-CoA reductase and low-density lipoprotein receptor (LDLR) gene and protein expressions in the HepG2 hepatoma cell line.

Methods: The expression of HMG-CoA reductase and LDLR genes and proteins were studied in HepG2 liver cancer cell line by PCR, Western blot, and indirect ELISA, as well as their antioxidant activity.

Results: Rhamnetin was non-toxic up to 200 µM on HepG2 at 24, 48, and 72 h. Rhamnetin (25 µM) upregulated LDLR gene expression by 1.66 folds compared to 3.12 folds exerted by the well-known hypocholesterolemic drug simvastatin. Rhamnetin (100 µM) increased the expression of LDLR protein at the cell membrane, while the other concentrations produced no significant change from the control (vehicle-treated). In HepG2 cell lysate, LDLR was increased by 50 µM of rhamnetin. Also, rhamnetin increased SOD activity significantly by 100.98, 86.28, and 100.98% by the concentrations 25, 50, and 100 µM, respectively. Using the same concentrations, rhamnetin reduced H_2O_2 levels by 50, 67, and 76.34%, respectively.

Conclusions: This study demonstrated for the first time that rhamnetin reduced HMG-CoA reductase gene expression and increased LDLR in HepG2 cells.

Keywords: HepG2; hydroxymethylglutaryl CoA reductase; LDL; rhamnetin; receptors.

Resumen

Contexto: La ramnetina es un derivado metilado natural de la quercetina. Este flavonoide abunda en las especies *Syzygium aromaticum*, *Coriandrum sativum*, *Prunus cerasus* y *Rhamnus* spp.

Objetivos: Evaluar los efectos de la ramnetina en las expresiones génicas y proteicas de la HMG-CoA reductasa y el receptor de la lipoproteína de baja densidad (LDLR) en la línea celular de hepatoma HepG2.

Métodos: Se estudió la expresión de los genes y proteínas de la HMG-CoA reductasa y del LDLR en la línea celular de hepatoma HepG2 mediante PCR, Western blot y ELISA indirecto, así como su actividad antioxidante.

Resultados: La ramnetina fue no tóxica hasta 200 µM en HepG2 a las 24, 48 y 72 h. La ramnetina (25 µM) aumentó la expresión del gen LDLR en 1,66 veces en comparación con 3,12 veces ejercida por el conocido fármaco hipocolesterolemiante simvastatina. La ramnetina (100 µM) aumentó la expresión de la proteína LDLR en la membrana celular, mientras que las demás concentraciones no produjeron cambios significativos con respecto al control (tratado con vehículo). En el lisado de células HepG2, el LDLR aumentó con 50 µM de ramnetina. Asimismo, la ramnetina aumentó significativamente la actividad de la SOD en 100,98; 86,28 y 100,98% mediante las concentraciones de 25, 50 y 100 µM, respectivamente. Utilizando las mismas concentraciones, la ramnetina redujo los niveles de H_2O_2 en 50, 67 y 76,34%, respectivamente.

Conclusiones: Este estudio demostró por primera vez que la ramnetina redujo la expresión del gen de la HMG-CoA reductasa y aumentó el LDLR en células HepG2.

Palabras Clave: HepG2; hidroximetilglutaril CoA reductasa; LDL; ramnetina; receptores.

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INTRODUCTION

Atherosclerosis is one of the main causes of morbidity and mortality worldwide (Khan et al., 2020). Elevated blood low-density lipoprotein (LDL) levels increase monocyte adhesion onto arterial endothelial cells, and enhance the entry of LDL into the intima, thereby accelerating atherosclerosis. LDL-laden foam cells accumulate in the wall of blood vessels, promote inflammation and cause more endothelial injury leading to advanced lesions or thrombus (Hansson, 2001). Therefore, increased plasma LDL cholesterol is considered a risk factor for atherosclerosis (Nimkuntod and Tongdee, 2015). To control blood LDL cholesterol levels, statins are used to inhibit HMG-CoA reductase, a key regulatory enzyme in the cholesterol biosynthetic pathway. Since most of plasma LDL cholesterol is removed by the liver through LDL receptor (LDLR)-mediated uptake (Brown and Goldstein, 1986), statins lead to upregulation of the LDLR gene and, therefore, increase blood LDL cholesterol removal (Nawrocki et al., 1995).

Despite the approved advantages of LDL-cholesterol lowering drugs, many patients do not reach the recommended LDL level as defined in guidelines (Kotseva et al., 2008). Recent studies indicated that many coronary patients with dyslipidemia are still inadequately treated (Kotseva et al., 2008). The use of lipid-lowering drugs, particularly statins, over the past decade has significantly increased to 79.8% (Reiner et al., 2013). Unfortunately, statins have many adverse effects, usually dose-related, including elevated liver enzymes and muscle pain (Grundy, 2005). Furthermore, some patients are statin intolerant or can not tolerate higher statin doses. In hyperlipidemia that does not respond adequately to a single statin therapy, combinations of other drugs are usually employed to lower plasma LDL cholesterol (Reiner, 2010). Therefore, the search for new hypocholesterolemic drugs continues.

Rhamnetin is a naturally occurring methylated derivative of quercetin. This flavonoid is abundant in *Syzygium aromaticum* (Vosgen and Herrmann, 1980), *Coriandrum sativum* (Chaudhry and Tariq, 2006) *Prunus cerasus* (Szabo et al., 2004) and *Rhamnus* spp. (Cuoco et al., 2014). Rhamnetin showed antioxidant (Jiang et al., 2008) and anti-inflammatory properties (Jnawali et al., 2014). Furthermore, it exhibited multiple cardiovascular protective effects, such as preventing myocardial ischemia, angina, and arrhythmia (Lee et al., 2015; Mattarei et al., 2010). In an *in vivo* study, rhamnetin lowered serum cholesterol of rats fed a cholesterol-enriched diet (Igarashi and Ohmuma, 1995). However, its mechanism of action was not investigated. This work was designed to study the effect of rhamnetin on HMG-CoA reductase and LDLR gene and protein expression in HepG2 hepatoma cell line. Furthermore, its antioxidant activity in HepG2 was investigated.

MATERIAL AND METHODS

Drugs

Rhamnetin was purchased from Sigm-Aldrich (USA). A stock solution of rhamnetin was prepared by dissolving in 10% DMSO and was diluted with media to obtain the required concentrations.

Cells and culture media

Human HepG2 hepatocarcinoma cells were grown as monolayer cultures in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% fetal bovine serum, penicillin, streptomycin (100 U/mL), in a humidified 5% CO_2 atmosphere at 37°C.

Cytotoxicity assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Promega, USA) was performed according to the manufacturer directions as described earlier (Abbas et al., 2020). Percentage cell viability was calculated using the formula [1].

$$\text{Cell viability (\%)} = \frac{(OD_{Sample} - OD_{Blank})}{(OD_{Vehicle} - OD_{Blank})} \times 100 \quad [1]$$

Where: OD is the optical density.

RNA extraction and quantitative real-time polymerase chain reaction (PCR)

The HepG2 cell line (10^6 cells) was cultured in a DMEM medium. The next day, the culture medium was removed and replaced with a fresh one. Rhamentin (25, 50, and 100 µM) or simvastatin (100 µM) were then added so that the final concentration of DMSO in culture media was less than 0.1%.

Analysis of gene expression was performed after 24 h of rhamnetin or simvastatin treatment. Cells were washed with PBS and then trypsinized. Total RNA was extracted using a Direct-zol RNA Miniprep Kit (Zymo, USA Cat. # R2050) according to the manufacturer's instructions. About 1µg total cell RNA, extracted from the vehicle, simvastatin, or rhamnetin-treated cells, was used for cDNA synthesis. Reverse transcription was carried out according to the manufacturer's instructions using a Takara kit (Cat. # RR036A). The β-actin gene was utilized as an internal control. Real-time PCR was carried out using GoTaq®

1-Step RT-qPCR (Promega, Cat. # A6020) and iCycler Thermal Cycler System apparatus (Thermo Fisher) using the following parameters: 90°C for 10 s, followed by 60°C for 30 s, and 72°C for 30 s for 40 cycles. A further melting curve step at 55-95°C was performed. Primers were purchased from Alpha DNA (Canada). Sequences of sense and antisense primers for HMG-CoA reductase, LDLR, and β-actin genes are shown in Table 1. All expression data were normalized by dividing the target amount by the amount of β-actin used as an internal control for each sample.

Table 1. Sequences of primers.

Gene	Primer
HMG-CoA reductase	(R) AGCCATAAGAGGAAGGCACCG
	(F) TCGGCCTTTCACTCTCGATG
LDL receptor	(R) TCATCCATCGCTCCAGGTA
	(F) CTTACGGGCCCTGGACAAAT
β-Actin	(R)TGAAGGTAGTTTCGTGGATGC
	(F) CGGGACCTGACTGACTACC

Protein extraction

HepG2 cells (10^6) were cultured in a 10 mL culture medium in a 25 cm flask for 24 h. Then, cells were subjected to different treatments of rhamnetin (25, 50, and 100 µM) in triplicates and incubated for 24 h. The cells were washed twice with PBS and incubated with lysis buffer (iNtRON biotechnology Cat. # 17081) for 20 min at 4°C and centrifuged at 15,000 rpm for 5 min. Protein concentration was measured using Pierce BCA Protein Assay Kit (ThermoScientific, USA). The supernatant was stored at -20°C until analysis for SOD, catalase activity, Western blot, and ELISA.

Western blot

After 24 h of treatment with different concentrations of rhamnetin, simvastatin, or vehicle, HepG2 cells were harvested. A total of 30 µg of each protein sample was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (Thermofisher, USA). The membranes were blocked overnight at room temperature with a 5% bovine serum albumin (BSA) (Biotechne/UK) in Tris-buffered saline (Chemcruz, USA). Then, the membranes were incubated for 1 h at room temperature with 1:000 rabbit monoclonal antibodies specific for HMG-CoA reductase (Abcam, USA). The blots were washed 3 times for 10 min each with Tris-buffered saline with Tween-20. The membranes were then incubated for 1 h with anti-rabbit secondary antibody conjugated with horseradish peroxidase (Bio-techne, Minneapolis, USA). Protein bands were detected using 3,3',5,5'-tetramethylbenzidine substrate (Sigma, USA).

Indirect enzyme-linked immunosorbent assay (ELISA) for the detection of LDLR

HepG2 cells were cultured with vehicle, simvastatin (100 µM), or different rhamnetin concentrations (25, 50, 100 µM). ELISA plate was coated with 100 µL/well of cell lysate (10 µg/mL protein extract) in triplicates overnight at 4°C. At the end of the incubation period, the plate was blocked with 200 µL of 5% BSA (Bio-techne/UK) in PBS for 2 h at room temperature. After washing, anti-LDLR primary antibody (Bio-techne/UK) (1:1000) dilution was added at room temperature for 1 h. At the end of the incubation period, three washes with 0.05% PBS-Tween were performed. One hundred microliters of 1:1000 HRP-conjugated goat anti-mouse immunoglobulin G (Promega, USA) were added and incubated at room temperature for 1 h. At the end of the incubation period, three washes with 0.05% PBS-Tween were done. Then, one hundred microliters of TMB substrate solution (Biotech, USA) were added to each well and incubated for 15 min at room temperature before adding the stopping solution. The plate was finally read with an ELISA plate reader (Biotech, USA) at 450 nm.

Cellular ELISA was also performed using 1×10^4 cells monolayer cultured for 24 h in a 96-well plate and treated with vehicle, simvastatin, or different concentrations (25, 50, 100µM) of rhamnetin in triplicates. Then anti-LDLR primary antibody (Biotechne/UK) (1:1000) dilution was added at room temperature for 1 h to bind with the target receptor on HepG2 cells. At the end of the incubation period, three washes with 0.05% PBS-Tween were performed. The cells were then fixed with 4% paraformaldehyde and blocked with 200 µL of 5% BSA. 100 µL of 1:1000 HRP-conjugated goat anti-mouse immunoglobulin G (Promega, USA) was added and incubated at room temperature for 1 h. Then, 100 µL of TMB substrate solution was added to each well and incubated for 15 min at room temperature before the stop solution was added. Finally, the plate was read with an ELISA plate reader (Biotech, USA) at 450 nm.

Superoxide dismutase (SOD) activity assay

SOD activity was measured using the Abcam superoxide dismutase assay kit (Abcam, US, Cat. # ab65354). The test was performed according to the manufacturer's directions. Percentage change in SOD activity was calculated using the formula [2].

$$\text{SOD activity (\%)} = \frac{(SOD_{Sample} - SOD_{Control})}{SOD_{control}} \times 100 \quad [2]$$

Catalase activity assay

The catalase assay test (Abcam Cat. # ab83464, US) was performed according to the manufacturer's directions.

Statistical analysis

GraphPad Prism version 8 was used in all tests to perform a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. P≤0.05 was regarded as significant.

RESULTS

Cell cytotoxicity assay

In HepG2 cells, rhamnetin was non-toxic up to 200 µM after 24, 48 and 72 h (Fig. 1).

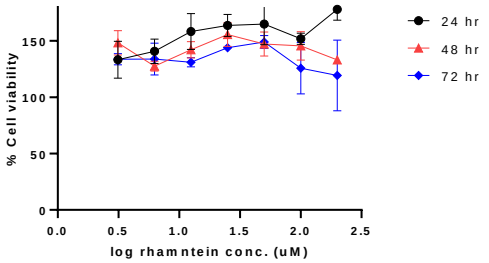

Figure 1. Cell cytotoxicity after treatment with rhamnetin at 24, 48, and 72 h.

HMG-CoA reductase gene and protein expression

Rhamnetin 25, 50, 100 µM, and simvastatin decreased HMG-CoA reductase gene expression significantly by 42.0, 88.4, and 80.4%, respectively (Fig. 2A). As shown in Fig. 3, rhamnetin lowered HMG-CoA reductase protein in HepG2 cells compared to the control.

LDLR gene and protein expression

Rhamnetin (25 µM) upregulated LDLR gene expression by 1.66 folds compared to 3.12 folds exerted by the well-known hypocholesterolemic drug simvastatin (Fig. 2B). Results of indirect ELISA suggested that LDLR expression in tissue homogenate increased by 2.1 folds by rhamnetin (100 µM) compared with the untreated control HepG2 cells. At the same time, no significant difference was found in simvastatin-treated cells (Fig. 4A). Similarly, LDLR expression at the cell surface showed a significant increase by rhamnetin treatment (50 µM) by 3.8 folds compared to 2.5 fold increase by simvastatin. The other concentrations of rhamnetin produced no significant change from the control (Fig. 4B).

Antioxidant activity

Rhamnetin increased SOD activity significantly by 100.98, 86.28, and 100.98 % for the concentrations of 25, 50, and 100 µM, respectively (Fig. 5). Also, rhamnetin reduced H_2O_2 levels by 50, 67, and 76.34% using the same rhamnetin concentrations (Fig. 5B).

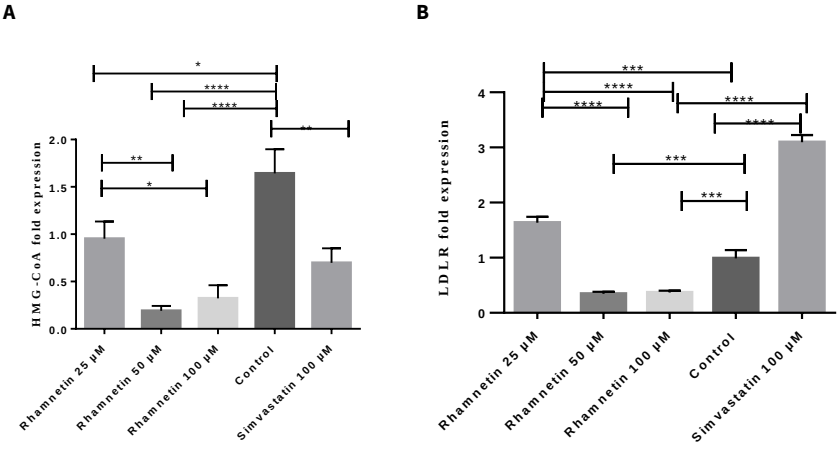

Figure 2: Gene expression of HMG-CoA reductase enzyme **(A)** and LDLR **(B)** in the vehicle or rhamnetin-treated HepG2 cells.

*P≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

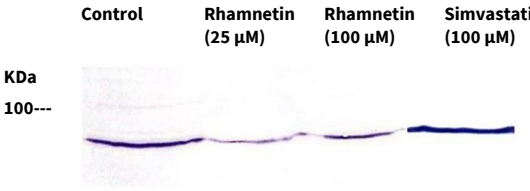

Figure 3. Immunoblot of HMG-CoA reductase enzyme in vehicle-treated, simvastatin, and rhamnetin-treated HepG2 cells.

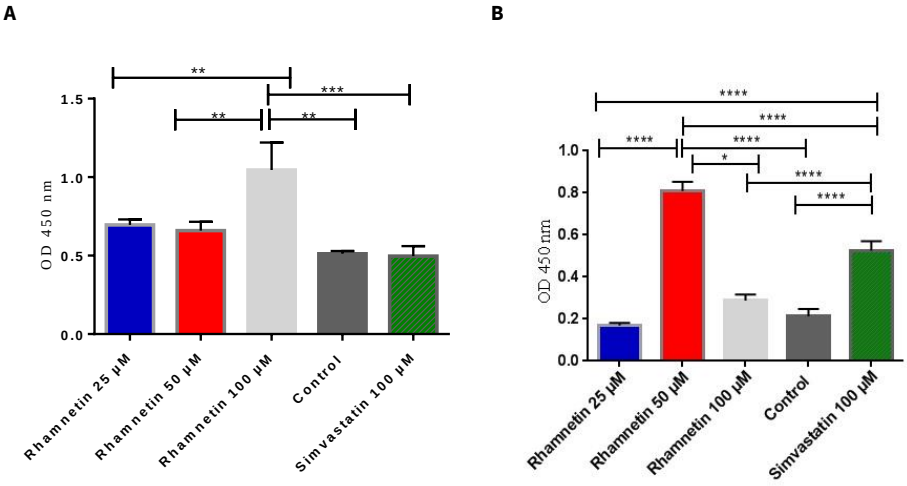

Figure 4. Detection of LDL receptor by cell homogenate ELISA **(A)** or cellular ELISA **(B)** after treatment with vehicle or rhamnetin.

*P≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

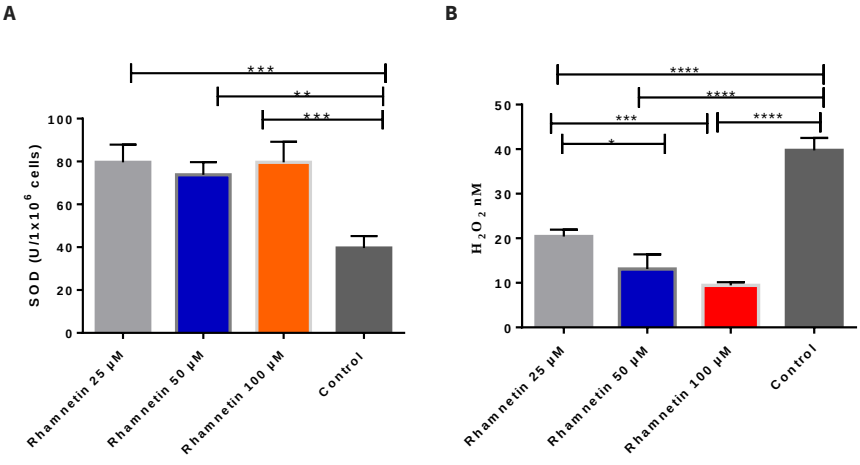

Figure 5. (A) SOD activity after rhamnetin treatment. **(B)** H_2O_2 reduction after rhamnetin treatment, which reflects catalase activity.

*P≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

DISCUSSION

Cell cytotoxicity assay

It has been suggested that the presence of a methyl group at the A ring in the rhamnetin structure is responsible for the absence of cytotoxicity (Novo Belchor et al., 2017). In this study, rhamnetin was non-toxic up to 200 µM on the HepG2 liver cancer cell line. This may indicate that rhamnetin is relatively safe.

HMG-CoA reductase gene and protein expression

HMG-CoA reductase - the time-limiting enzyme in cholesterol biosynthesis - is the main contributor to

cholesterol biosynthesis and regulation (Goldstein and Brown, 1990). Currently, HMG-CoA reductase inhibitors, such as statins, are commonly used to lower blood cholesterol levels (Reiner, 2010). Oral administration of rhamnetin extracted from *Rhamnus alaternus* leaf decreased the level of cholesterol in hyperlipidemic rats by 60% (Tacherfiout et al., 2018). In our study, HMG-CoA reductase gene and protein expression were significantly lower in HepG2 cells treated with rhamnetin compared with the untreated HepG2 cell line. It is well established that many flavonoids lower HMG-CoA reductase expression (Mahdavi et al., 2020) and activity (Lee et al., 2021). To our best knowledge, this is the first report that rhamnetin can decrease the expression of HMG-CoA reductase.

LDLR gene and protein expression

LDL cholesterol clearance from cells to the liver is regarded as crucial to avoid the formation of atherosclerotic lesions. In fact, about 75% of the circulating cholesterol is removed by LDLR endocytic circulation (Yang et al., 2020). It is well established that statins – the most commonly used anti-hypercholesterolemic drugs available in the markets – upregulate LDLR (Vogel, 2012). The present study showed that rhamnetin (25 µM) upregulated LDLR gene expression by 1.66 folds compared to 3.12 folds exerted by the well-known hypocholesterolemic drug simvastatin, which agrees with the findings of previous studies (Morikawa et al., 2000). Up to our best knowledge, this is the first report that rhamnetin was able to increase the expression of LDLR.

In an *in vivo* study, orally administered rhamnetin possessed a hypocholesterolemic effect in rats (Igarashi and Ohmuma, 1995). Therefore, testing the effect of rhamnetin on LDLR gene expression in vivo is needed to clarify the mechanism by which rhamnetin lowers blood cholesterol levels.

In the present study, rhamnetin (50 µM) increased membranous LDLR by 3.8 folds, while simvastatin (100 µM) increased membranous LDLR by 2.5 folds. This agrees with previous studies that reported simvastatin upregulates LDLR at the protein level (Mayne et al., 2008).On the other hand, a 100 µM dose of rhamnetin increased the total LDLR in cell lysate. This may indicate that, in addition to the upregulation of the LDLR gene by rhamnetin, a possible effect on LDLR recycling and degradation may exist.

Antioxidant activity

In the present work, rhamnetin had higher SOD and catalase enzyme activities than the vehicle-treated control. Similar results were obtained by Park et al. (2014) using the H9c2 cell line in which rhamnetin reduced ROS production and enhanced catalase and SOD expression.

CONCLUSION

Rhamnetin lowered the expression of HMG-CoA reductase and increased LDLR gene expression. Furthermore, it exerted antioxidant effects on HepG2 cells by increasing the activity of SOD and catalase. Future preclinical and clinical studies are needed to thoroughly investigate this flavonoid's effect on cholesterol and lipid metabolism and to study their toxicity *in vivo*.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Al-Yousef RR	Abbas MM	Obeidat R	Abbas MA
Concepts or ideas	x	x		x
Design		x		x
Definition of intellectual content		x		x
Literature search	x			x
Experimental studies	x		x	
Data acquisition	x		x	
Data analysis		x		
Statistical analysis		x		x
Manuscript preparation	x			x
Manuscript editing		x		x
Manuscript review	x	x	x	x

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