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148

Metformin Alleviates Doxorubicin-induced Liver Injury in Rats via Reducing Oxidative Stress, inflammation and Excessive Cell Death

Metformin, Sıçanlarda Oksidatif Stresi, Enflamasyonu ve Aşırı Hücre Ölümünü Azaltarak Doksorubisin Kaynaklı Karaciğer Hasarını Azaltır

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Abstract

Objective: The liver is one of the most important internal organs in the human body and has high regenerative properties. It performs the functions of protein synthesis, intake, storage, and distribution of nutrients and vitamins from the blood. In this study, we investigated the curative effect of metformin (Met) on doxorubicin (DOX)-induced liver damage.

Materials and Methods: A total of 32 Wistar-albino rats were divided into four groups: control, Met, DOX, and DOX + Met groups. The DOX and DOX + Met groups received four doses of DOX. Met was gavaged daily for 15 days in the DOX + Met and Met groups. Structural liver injury was evaluated with hematoxylin-eosin, picro-sirius, TUNEL, and nuclear factor kB (NF-kB) antibody staining. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total antioxidative status (TAS), total oxidative status (TOS), and hydroxyproline levels were measured as biochemical parameters.

Results: The DOX group was found to have a significant structural liver injury characterized by hyperchromatic nuclei in hepatocytes, widespread sinusoidal dilatations, and granular and vacuolar degeneration. Increased NF-kB staining and the apoptotic index were also detected in the DOX group. Biochemical tests revealed an increase in ALT, AST, and TOS levels and a decrease in TAS levels in the DOX group. Met administration provided a significant improvement in the structural changes caused by DOX. In addition, the DOX + Met group had lower NF-kB staining, apoptotic index, ALT, and TOS levels and a higher TAS level compared to the DOX group.

Conclusion: Our findings indicate that Met alleviates DOX-induced structural liver injury by reducing oxidative stress, inflammation, and excessive cell death.

Keywords: Doxorubicin, metformin, liver injury, apoptosis, oxidative stress

Öz

Amaç: Karaciğer, yenilenme özelliği yüksek olan insan vücudundaki en önemli iç organlardan biridir. Protein sentezi, besin maddelerinin ve vitaminlerin kandan alınması, depolanması ve dağıtılması işlevlerini yerine getirmektedir. Bu araştırmada, doksorubisin (DOX) ile oluşturulan karaciğer hasarı üzerindeki metforminin (Met) iyileştirici etkisinin araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Toplam 32 Wistar-albino sıçan dört gruba ayrıldı: kontrol, Met, DOX ve DOX + Met grupları. DOX ve DOX + Met gruplarına dört doz DOX verildi. DOX + Met ve Met gruplarında 15 gün süreyle günde Met verildi. Yapısal karaciğer hasarı, hematoksilen-eozin, pikro-sirius, TUNEL ve nükleer faktör kB (NF-kB) antikor boyaması ile değerlendirildi. Biyokimyasal değerlendirmeler için alanin aminotransferaz (ALT), aspartat aminotransferaz (AST), total antioksidan durum (TAS), total oksidatif durum (TOS) ve hidroksiprolin düzeyleri ölçüldü.

Bulgular: DOX grubunda, hepatositlerde hiperkromatik çekirdekler, yaygın sinüzoidal dilatasyonlar, granüler ve vakuoler dejenerasyon ile karakterize önemli yapısal karaciğer hasarı olduğu bulundu. DOX grubunda artmış NF-kB boyama ve apoptotik indeks de tespit edildi. Biyokimyasal testler DOX grubunda ALT, AST ve TOS düzeylerinde artış ve TAS düzeyinde azalma gösterdi. Met uygulaması,

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DOX'un neden olduğu yapısal değişikliklerde belirgin bir iyileşme sağladığı gözlemlenmiştir. Ayrıca DOX + Met grubunda DOX grubuna göre NF-kB boyama, apoptotik indeks, ALT ve TOS düzeyleri daha düşük ve TAS düzeyi daha yüksekti.

Sonuç: Bulgularımız, Met'in oksidatif stresi, enflamasyonu ve aşırı hücre ölümünü azaltarak DOX kaynaklı yapısal karaciğer hasarının etkisini hafiflettiğini göstermektedir.

Anahtar Kelimeler: Doksorubisin, metformin, karaciğer hasarı, apoptoz, oksidatif stres

Introduction

Doxorubicin, a broad-spectrum anticancer agent, has a limited clinical usage due to its cardiotoxic and hepatotoxic effects (1). Doxorubicin toxicity is largely caused by an overabundance of reactive oxygen species (ROS) or a reduction in antioxidant defenses, resulting in an imbalance in oxidative status. Increased ROS induces excessive calcium retention in the mitochondria, increasing mitochondrial permeability and resulting in mitochondrial enlargement and adenosine triphosphate depletion, finally leading to cell death (2).

Numerous drugs with antioxidant and anti-inflammatory effects have been utilized to treat doxorubicin-induced liver damage. Metformin is a biguanide derived from a perennial plant (Galega officinalis) extensively used to treat type 2 diabetes (3). In addition to its effects on glucose metabolism, it has anti-oxidative, anti-inflammatory, and anti-apoptotic effects. Thus, metformin inhibits lipid peroxidation and has protective effects against toxic agents in several tissues (4).

In the current study, we aimed to investigate the possible protective effects of metformin on doxorubicin-induced liver damage in a rat model.

Materials and Methods

Animals

Thirty-two male Wistar albino rats, 8-week-old, weighting between 250-300g, were obtained from a private company. All animals were housed in transparent cages with a relative humidity of 40-60% and an ideal temperature (22 °C) during the study period. They were exposed to 12 hours of light/darkness. Throughout the study, rats were fed with a regular rat diet, and clean tap water ad libitum. The study protocol was approved by the Aydın Adnan Menderes University Animal Experiments Local Ethics Committee (Ref. No: 64583101/2018/112, date: 23.10.2018).

Study Protocol

The study was planned on 4 groups: control, metformin (Met), doxorubicin (DOX) and DOX + Met (DOX + Met). For the group, Met (200 mg/kg) was given every day for 15 days by gavage. The study protocol for each group and timeline was given in Table 1.

The rats were sacrificed with exsanguination under general anesthesia [xylazine (5 mg/kg) and ketamine (100 mg/kg)] at the end of the study protocol. Rat liver tissues were preserved in 10% neutral formalin and blood samples were collected into biochemistry tubes for further analyses.

Routine Histopathological Evaluation

Routine tissue follow-up procedure was performed after the fixation material was removed. Liver tissues were embedded in paraffin blocks and sections with 5 µm thickness were prepared with a microtome. Hematoxylin-eosin, Masson's trichrome, and picro-sirius staining were performed for routine histopathological evaluation.

Light microscopic assessment of liver sections was performed with an optical microscope (Olympus BX50, Tokyo, Japan). Structural liver injury was graded with hepatic injury score calculated via evaluation of 4 different areas selected from the liver tissues of four groups by two experienced histologists (5).

Light microscopic observations were converted into quantitative data using the hepatic damage score created by Jacevic et al. (6).

Immunohistochemical Staining

Immunohistochemical staining was performed on 5 µm thick sections mounted on polylysine coated slides. Immunohistochemical staining with nuclear factor kB (NF-kB) primary antibody (Santa Cruz Biotechnology sc-8008, Texas, USA) was performed to reveal inflammatory activity in the liver specimens. Sections were then visualized with chromogen 3,3'-diaminobenzidine solution and Mayer's hematoxylin was used for nuclear background staining.

TUNEL Staining

Extent of cell death in the liver specimens were determined using TUNEL method. Sections were stained according to the kit protocol (Millipore, 2470976, USA). TUNEL positive cells were counted at 400x magnification. Apoptotic index scoring was used to analyze the data. Hundred cells in five distinct locations were counted, and proportion of apoptotic cells were identified to calculate apoptotic index (7).

Biochemical Analyses

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were defined as biochemical markers of liver injury. Measurements were performed using commercially available kits in the biochemistry laboratory.

As a marker of collagen turnover and fibrotic activity, hydroxyproline levels were studied in hydrolyzed liver tissue samples using a colorimetric assay (Elabscience, E-BC-K061, Wuhan, China).

Assessment of Oxidative Status

In order to assess oxidative status in liver specimens, total oxidative status (TOS) and total antioxidative status (TAS) were measured. TOS and TAS in liver tissues were measured using commercial kits (Rel Assay Diagnostics).

Statistical Analysis

Normally distributed parameters were presented as mean ± standard deviation, the remaining parameters were given as median (minimum-maximum). One-way ANOVA and Kruskal-Wallis H analysis were used to compare the normally distributed and abnormally distributed variables, respectively. The SPSS 21 program (SPSS Inc., Chicago, Illinois, USA) was used in the application of this protocol. A p value <0.05 was set statistically significance level.

Results

Body and Liver Weights

Initial body weights of the studied rats were similar between the four groups (p=0.981). On the other hand, there was a statistically significant difference regarding final body weights (p<0.001). DOX group had lower final body weight compared to control and Met groups. DOX + Met group had lower body weight than the control group (Table 2). Liver weights were also found to be different between four groups (p=0.013). DOX group had lower liver weight compared to control and Met groups (Table 2).

Histopathological Evaluation of Structural Liver Injury

Light microscopy revealed typical histological findings in the control and Met groups (Figure 1a, b). On the other hand, evaluation of liver specimens from DOX group demonstrated significant structural damage characterized by mononuclear cell infiltrations, hyperchromatic nuclei in hepatocytes, sinusoidal dilatations and granular and

Table 1. Detailed timeline of the study protocol

Days	Control group (IP + G)	DOX (IP)	DOX (IP) + Met (G)	Met (G)
1	Dw 2 mL/kg (IP) + Dw 1 mL (G)	5 mg/kg dox (IP)	5 mg/kg dox (IP) + 200 mg/kg Met (G)	200 mg/kg (G)
2	Dw 1 mL (G)		200 mg/kg Met (G)	200 mg/kg (G)
3	Dw 1 mL (G)		200 mg/kg Met (G)	200 mg/kg (G)
4	Dw 1 mL (G)		200 mg/kg Met (G)	200 mg/kg (G)
5	Dw 2 mL/kg (IP) + Dw 1 mL (G)	5 mg/kg dox (IP)	5 mg/kg dox (IP) + 200 mg/kg Met (G)	200 mg/kg (G)
6	Dw 1 mL (G)		200 mg/kg Met (G)	200 mg/kg (G)
7	Dw 1 mL (G)		200 mg/kg Met (G)	200 mg/kg (G)
8	Dw 1 mL (G)		200 mg/kg Met (G)	200 mg/kg (G)
9	Dw 2 mL/kg (IP) + Dw 1 mL (G)	5 mg/kg dox (IP)	5 mg/kg dox (IP) + 200 mg/kg Met (G)	200 mg/kg (G)
10	Dw 1 mL (G)		200 mg/kg Met (G)	200 mg/kg (G)
11	Dw 1 mL (G)		200 mg/kg Met (G)	200 mg/kg (G)
12	Dw 1 mL (G)		200 mg/kg Met (G)	200 mg/kg (G)
13	Dw 2 mL/kg (IP) + Dw 1 mL (G)	5 mg/kg dox (IP)	5 mg/kg dox (IP) + 200 mg/kg Met(G)	200 mg/kg (G)
15	Dw 1 mL (G)		200 mg/kg Met (G)	200 mg/kg (G)
16	Sacrification	Sacrification	Sacrification	Sacrification

DOX: Doxorubicin, Met: Metformin, Dw: Distilled water, G: Gavage, IP: Intraperitoneally

vacuolar degeneration (Figure 1c). Met administration significantly attenuated structural changes induced by DOX (Figure 1d). Hepatic injury score was significantly higher in the DOX group compared to control and Met groups. Met administration significantly reduced hepatic injury score in DOX treated animals.

Assessment of Inflammation and Apoptosis

Immunohistochemical staining for NF-kB was used to assess inflammatory activity in liver specimens. In the DOX group, there was intense NF-kB positivity around the central vein, compared to control and Met groups. In the DOX + Met group, a significant decrease in NF-kB staining intensity was observed compared to DOX group (Figure 2).

Apoptotic index was significantly higher in the DOX group compared to control and Met groups. Met administration significantly reduced apoptotic index in the DOX administered animals (Figure 3, Table 2).

Biochemical Evaluation of Liver Injury

Serum ALT values were substantially higher in the DOX group compared to control and Met groups. Although not statistically significant, ALT values were slightly lower in the DOX + Met group than those in the DOX group. The DOX group had considerably higher AST values than control, Met, and DOX + Met groups. When DOX + Met group was compared to the control group, no significant difference was detected regarding AST values (Table 2).

Assessment of Oxidative Status

TOS was significantly higher in the DOX group compared to control, Met and DOX + Met groups. There were no significant difference between control, Met and DOX + Met groups regarding TOS. On the other hand, TAS was significantly lower in the DOX group compared to control, Met and DOX + Met groups. There were no significant difference between control, Met and DOX + Met groups regarding TAS (Table 2).

Assessment of Liver Fibrosis

In the Picro-sirius-stained sections, small fibrotic areas around the central vein were observed in the DOX group (Figure 1g). There was no statistically significant difference between the groups regarding hydroxyproline levels, although the DOX group values were tended to be higher (Table 2).

Discussion

In the present study, we investigated the possible effects of oral Met administration on DOX induced liver injury for the first time in the literature. Our findings revealed that Met alleviates structural and biochemical markers of liver damage in a rat model of DOX-induced hepatotoxicity. We also demonstrated that these beneficial effects were possibly related to a reduction in oxidative stress, inflammatory activity and excessive cell death in the liver tissue.

Table 2. Body and liver weight of rats. Hepatic Injury Score and Apoptosis Index of liver specimens. Serum ALT, AST levels and liver tissue oxidative stress and fibrosis markers

	Control group	Met	DOX	DOX + Met	p-value
Body and liver weight of rats					
Initial weight (g)	295.75±13.13	296.50±11.53	295.38±12.85	297.63±8.72	0.981
Final weight (g)	364.88±33.65	350.88±36.37	270.63±31.38[a,b]	308.13±36.54[a]	<0.001*
Liver Weight (g)	12.64±1.23	12.63±1.20	9.09±2.63[a,b]	11.07±2.64	0.013*
Hepatic Injury Score and Apoptosis Index of liver specimens					
Hepatic Injury Score	0 (0-1)	1 (0-1)	7 (4-8)[a,b,d]	3 (2-4)[a,b,c]	<0.001*
Apoptosis Index	2.6 (1.8-3.2)	4.6 (4.2-8.0)[a]	40.2 (37.8-47.6)[a,b,d]	14.8 (13.4-18.2)[a,b,c]	<0.001*
Serum ALT, AST levels					
ALT	44.00±16.88	47.14±8.21	63.75±16.07[a,b]	52.57±6.99	0.033*
AST	110.00±17.46	119.14±17.69	195.62±49.23[a,b,d]	117.57±25.61[c]	<0.001*
Liver tissue oxidative stress and fibrosis markers					
TOS	1.11±0.45	0.66±0.52	1.76±0.25[a,b,d]	1.17±0.33[c]	<0.001*
TAS	2.05±1.00	1.94±0.17	1.45±0.41[a,b,d]	1.95±0.17[c]	<0.001*
Hydroxyproline	0.94±0.40	0.92±0.32	1.06±0.42	0.86±0.17	0.747

[a]p<0.05 vs control, [b]p<0.05 vs Met, [c]p<0.05 vs DOX, [d]p<0.05 vs DOX + Met, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, TAS: Total antioxidative status, TOS: Total oxidative status, Met: Metformin

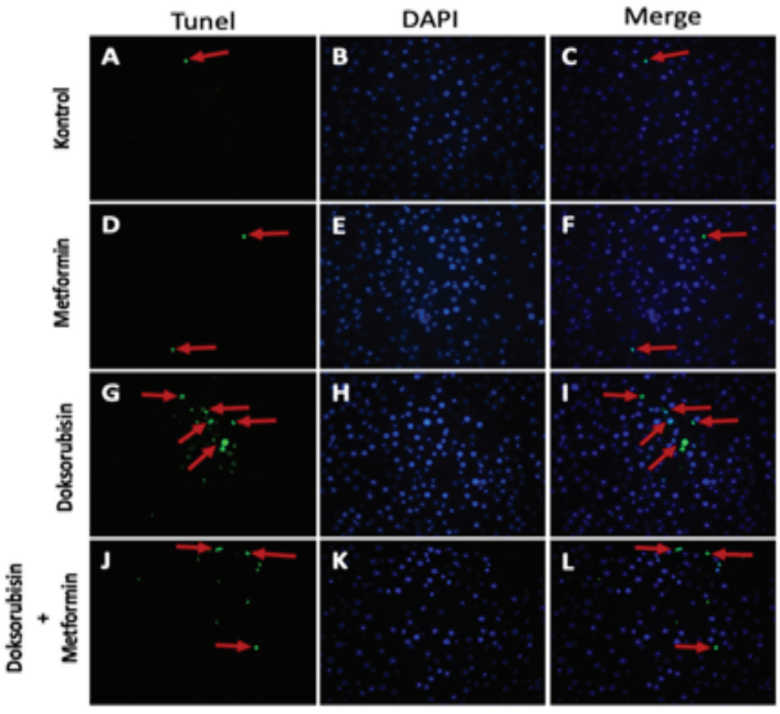

Figure 1. Liver sections stained with hematoxylin-eosin. Control group (a, x200), Met group (b, x200), DOX group (c, x200) and DOX + Met group (d, x200). Liver sections stained with Picrosirius red. Control group (e, x200), Met group (f, x200), DOX group (g, x200) and DOX + Met group (h, x200)

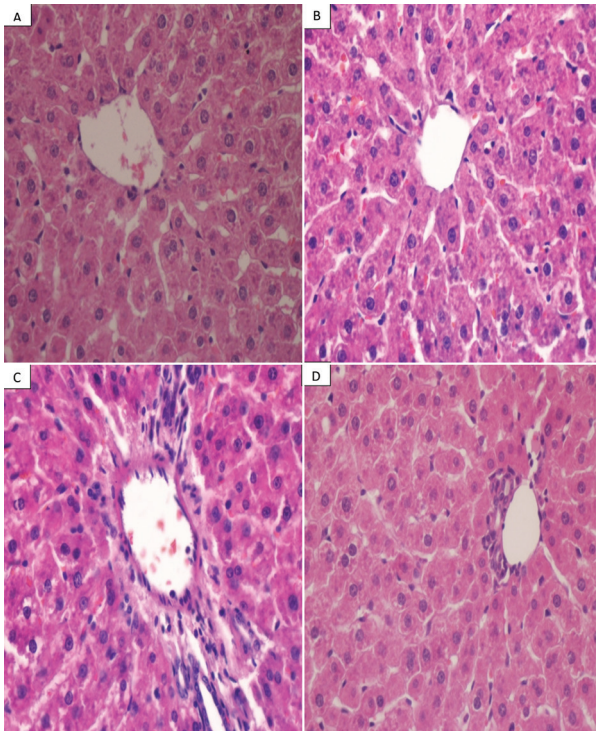

Figure 2. Liver sections immunostained for NF-κB. Control and Met groups demonstrate light staining for NF-κB (a and b, x200). DOX group demonstrates intense staining for NF-κB (c, x200). DOX + Met group demonstrates less intense staining for NF-κB compared to the DOX group (d, x200)

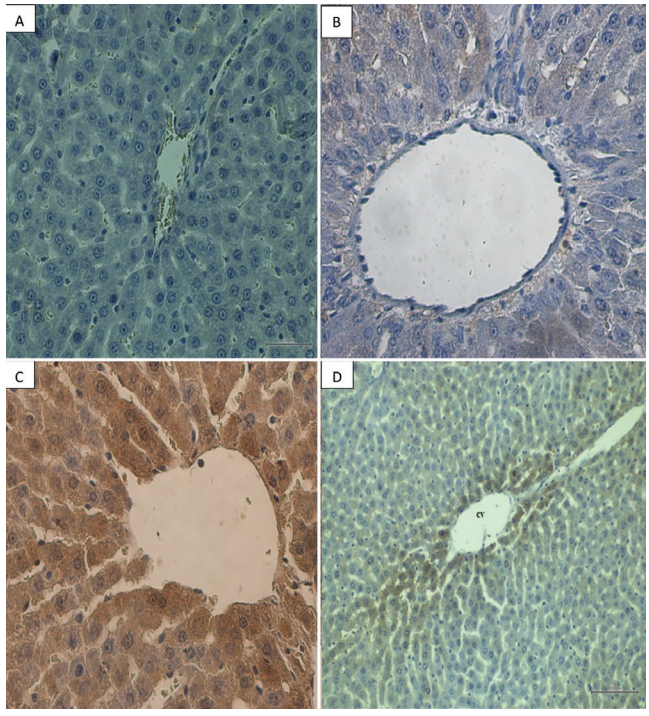

Figure 3. Fluorescent microscopic images of liver sections stained with TUNEL assay to detect cell death

Histopathological evaluation of liver tissues revealed significant structural changes in the DOX group characterized by mononuclear cell infiltrations, hyperchromatic nuclei, sinusoidal dilatations, granular and vacuolar degeneration in hepatocytes. Similar structural alterations were also observed in sections stained with Masson's trichrome. Many previous studies also reported similar structural changes in the histopathological assessment of liver tissues following DOX administration (8-10).

We observed a decrease in sinusoidal dilatations, mononuclear cell infiltrations, and a substantial decrease in the number of pyknotic cells in liver tissue samples taken from DOX + Met group rats compared to the DOX group alone. Numerous researchers have reported similar beneficial effects with Met administration in different hepatotoxicity models (11-13). Met exerts these effects by activating AMP-activated protein kinase in the injured cells, suppressing "cyclin d1" expression, halting mitotic cell division in the G1 phase, and therefore halting the increase in the number of damaged cells (14).

In the DOX group, we observed NF-kB positivity in and around the central vein reflecting an increase in the inflammatory activity. Met administration reduced NF-kB expression in the DOX treated animals. In line with these findings, Rizk et al. (15) reported that 200 mg/kg Met substantially lowered NF-kB, DOX-2, and Caspase 3 expression in rats treated with methotrexate for seven days. Nguyen et al. (16) also demonstrated that Met inhibited ROS generation generated by lithocholic acid (LCA) in HCT116 CRC cells and thereby inhibited NF-kB activity, abolishing LCA-mediated IL-8 overexpression.

A number of *in vivo* and *in vitro* studies have demonstrated that DOX promotes cell death in various tissues by increasing oxidative stress (17-19). We detected a significant increase in TOS and apoptotic index in the liver tissue following DOX administration. Met significantly reduced TOS and apoptotic index in DOX treated animals. Similar to our results, Li et al. (20) reported a decrease in the number of apoptotic cells after Met administration in a cisplatin-induced kidney injury model.

Significant increase in serum AST and ALT levels were detected following DOX administration. Met treatment led to a significant decrease in AST but not in ALT levels. In a similar study, Rizk et al. (15) used Met in a methotrexate-induced liver injury model. When they compared AST and ALT levels before and after treatment, they found that Met -treated rats had substantially lower AST and ALT values (15). Tripathi et al. (21) likewise observed a significant decrease in AST and ALT levels in the Met treatment group in their study. Discrepancy in our findings regarding ALT level may be possibly due to the small number of animals in our groups or due to diffuse toxic effects of DOX in different tissues.

Conclusion

To conclude, our findings have revealed that Met administration attenuates DOX induced structural liver injury via reducing oxidative stress, inflammation and excessive cell death. These data suggest that Met may be useful for prevention of DOX induced hepatotoxicity. Exact mechanisms underlying these beneficial effects of Met on DOX induced liver injury and its potential for clinical application need to be elucidated in further studies.

Ethics

Ethics Committee Approval: The study protocol was approved by the Aydın Adnan Menderes University Animal Experiments Local Ethics Committee (Ref. No: 64583101/2018/112, date: 23.10.2018).

Informed Consent: Informed consent is not required.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: C.G., A.G., E.B., F.B., K.M.G., Concept: C.G., A.G., K.M.G., Design: C.G., A.G., K.M.G., Data Collection or Processing: C.G., A.G., E.B., K.M.G., Analysis or Interpretation: C.G., A.G., F.B., K.M.G., Literature Search: C.G., A.G., K.M.G., Writing: C.G., A.G., K.M.G.

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