RESEARCH ARTICLE

Impact of Metformin on Pro-inflammatory Cytokines and Histological Evaluation of the Liver Induced Diabetes on Albino Male Rats

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ABSTRACT

This study aimed to assess metformin effect with different concentration and duration on pro-inflammatory factors serum and tissue of liver and its impact upon the liver of diabetic albino rats. Metformin is administered in multi doses at various times to diabetic rats. Alloxan injected to induced diabetes in the rats, and then metformin was administered by gavage; then blood and tissue collected to analyze the biochemical parameters (a pro-inflammatory cytokine), serum blood glucose, body weight blood, and tissue were collected. Liver tissues were collected for histologic analysis at the beginning of experiments day 0, days 7, days 21, and days 35 (end of experiments) and sacrificed three animals for each period. The findings metformin-treated diabetic rat showed significant changes observed in terms of body weight and food intake behavior. Metformin treatment dropped serum blood glucose levels in diabetic rats. Following metformin treatment, an ELISA assay indicated dropped levels of pro-inflammatory markers in the liver tissues and serum. Further, metformin treatment reduced the intensity of liver inflammation measured by pathologists blindly.

Conclusion: The results of reducing pro-inflammatory cytokines in the liver tissues and serum recommend the anti-inflammatory effects of metformin to improve inflammation.

Keywords: Diabetes mellitus, Liver inflammation, Metformin, Pro-inflammatory cytokines.

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INTRODUCTION

Diabetes mellitus (DM) is a variety of physiological disorders celebrated by hyperglycemia produced by excessive glucagon secretion, inadequate insulin secretion or increased insulin resistance four types as type 1 diabetes (T1D), type 2 diabetes (T2D), diabetes in pregnancy, and other types.[1] In developing countries 85 to 95% of all diabetes is of type (2).[2] The importance of lifestyle measures in diabetes therapy did not forget the value pharmacotherapy to control blood glucose levels. Different oral hypoglycemia has been in use to aid in controlling blood glucose level.[3] There are many classes of hypoglycemia agents, including sulfonylureas, alpha glycosidase inhibitor, thiazolidinedione, metformin and synthetic insulin injection, also therapy against type 1 DM.[4] Metformin lowered blood glucose level by downgrade glucose output from liver and by elevating glucose uptake in peripheral tissue. These effects are mediated by the action of liver kinase B1(LKB-1), an upstream kinase that adjusts the downstream kinase adenosine monophosphate and protein kinase (AMPK), AMPK phosphorylates a transcriptional co-activator (TORC2), rendering it inactive and resulting in cytoplasmic down-regulation. Transcriptional processes aid gluconeogenic enzyme production.[5] Obesity is a key risk factor for type (2) diabetes mellitus (T2DM), a crucial health problem worldwide. T2DM is associated to low-grade chronic inflammation, which is exacerbated by innate immune system activation. This activation in obesity results in the production of pro-inflammatory cytokines, including tumor necrosis factor-1, interleukin-1, and interleukin-6, which inhibit key anabolic pathways downstream of insulin signalling, disrupting insulin homeostasis and function. Acute-phase reactants such as C-reactive protein, plasminogen activator inhibitor-1, serum amyloid-A, and haptoglobin are also produced in response to cytokines. Pro-inflammatory cytokines and acute-phase proteins are raised in synthesis. (Inflammatory network) describes the early (or pre-clinical) phases of T2DM and shows a progressive rise as the illness progresses. Metformin's multifunctional profiles, such as anti-cancer, cardiovascular defense, and anti-inflammatory actions, have been indicated in recent research.[6] One of the many actions researched, metformin's anti-inflammatory properties have received much attention, and its clinical implications are showing promise.[7]

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Metformin has been shown to activate the AMPK/PI K/Act pathway resulting in anti-inflammatory effects and decreased pro-inflammatory cytokine expression.[8] Furthermore, Metformin therapy appears to reduce the production of various pro-inflammatory cytokines by inflammatory cells in individuals with impaired glucose tolerance, according to medical studies.[9] Inflammation is becoming more well recognized as a major contributor to DM Because of its anti-inflammatory properties.

The aim of this study we focused at the drug's anti-inflammatory properties and how they contribute to its anti-hyperglycemic properties. The aim of this study was whether metformin could reduce inflammation in diabetic rat's and metformin's impact upon on liver in diabetic rats.

MATERIALS AND METHODS

Subject

The Council of the College of Medicine, Mosul University established the research project approval committee. The scientific committee of the Department of Pharmacology at the College of Medicine approved the research project and approved it by Medical Research Ethics Committee (Ref.no: UOM/COM/MREC/20-21,34).

Animal Experiments

This study was held at an animal's house in the College of Veterinary Medicine, University of Mosul. In this study using Male albino rats, weighing (175–200 gm), were obtained from Laboratory Animal at 2 to 3 months of age and housed at 23 ± 2°C, relative humidity (55%) with a 12 hours cycle of light/dark.[10] Water and food were given ad libitum. After a one-week acclimatization period, a veterinarian performed examinations to confirm its safety, health and free from diseases.

Experimental Designs Induction of Diabetes Mellitus

Diabetic rats induced by alloxan were used. The rats were split into four diabetes groups: one control group and four diabetic groups. The animals were randomly assigned to five group's (n = 15).Diabetes was induced by a single dose of alloxan, intra-peritoneal injection of alloxan (AVONCHEM. CAS NO.50-71-5, made in UK)at the dose of (200 mg/kg) of the body weight overnight fasted rats. Alloxan was dissolved in 1 mL of distilled water The mode of administration, species of animals, and nutritional condition have all been addressed when calculating the optimum dosage of alloxan for diabetes induction.[11] To avoid drug-induced hypoglycemia, rats were taken a (5%) glucose solution to consume overnight.[12] The animals were kept in the blood until diabetes for a week, and during this time, fluctuations in blood glucose levels were noticed until blood glucose levels were stable Diabetic rats are characterized as animals having a random blood glucose level of more than 200 mg/dL. An alloxan-induced diabetes by a process that involves the destruction of pancreatic islet yet reduces insulin released by beta cells.[13]

Rats were randomly divided into five groups via random glucose and body weight. All animals were who were routine feed and Tap water for all group.[14]

The rat was then divided into five groups:

- Control Group (C): It is the healthy control group, which is composed of fifteen (15) healthy rats who had never been injected with alloxan. The healthy control group, which were routine feed, were left all alone in cages until the end of the experiment, and clear water was provided to them.
- Diabetic Group (D): It is the diabetic control group which includes (15) rats injecting alloxan for 30 days in cages
- Metformin Group (M100): A group of diabetic rats that were dosed with oral metformin at a concentration of 100 mg/kg
- Metformin Group (M200): A group of diabetic rats that were dosed with oral metformin at a concentration of 200 mg/kg
- Metformin Group (M300): A group of diabetic rats that were dosed with oral metformin at a concentration of 300 mg/kg

Blood and tissue collected were analyzed for the biochemical parameters at the beginning of experiments day 0, Days 7, Days 21, and Days 35 (end of experiments) and sacrificed three animals for each of this period

Metformin dose administered to rat in this study based on calculating based on the surface area of the body. Drug metformin (Samara company) dissolved in D.W was administered by gavage at 8:30 to 9:00 p.m. every day sacrificed three animals for each period.

Observation of the Animals

The Rats were frequently checked to provide with a suitable living environment was done on a regular schedule though had sufficient feed and water, regular cleaning and removal of feces together along with feed in their cages ensured good hygiene and monitoring the weight of each group.[15]

Biochemical Assessment

Samples Collection

- *Blood Samples:* Blood samples were collected in clean dry centrifuge tubes from the orbital plexus of vein allowed clotting; After 15 minutes of centrifugation at 1500 rpm, serum was separated.
- *Tissue Collection:* Rat was died by cervical decapitation at each period. The livers were then removed, isolated from the fat immediately, and carefully washed then separated into two sections and fixed in a 10% buffer. These implanted samples were sliced into thin slices (5 m) and stained with hematoxylin and eosin (H&E) for analysis. Histological evaluations kept the tissue by buffer formalin to evaluate by pathologists the assent consist of descriptive histology via to inflammation score and weight one gram per tissue as per kit protocol, clean tissues completely in PBS (pH 7.4) to eliminate excess blood, and weigh before homogenization rat tissues and homogenize them in Phosphate-buffered saline (PBS (pH7.4)), a type of saline that has been buffered with phosphate,commonly used in biological research. On the ice, with a glass homogenizer Freeze at -20°C or thaw at 2–8°C. For around 20 minutes, centrifuge at 2000–3000 RPM. The serum samples and tissues were kept at a temperature of -80°C.[16]

Blood Glucose and Body Weight Determinations

The serum obtained from blood samples was used to estimate blood glucose concentration using a blood glucose monitoring system (TOYCOO, Hamburg, Germany). The body weight of each group was determined using special balance 0 at day 0, day 7, day 21, day 35).[17] Blood and tissue were analyzed as mentioned in sample collection for pro-inflammatory cytokines (TNF-α and IL-1β) using ELISA kits TNF-α (Cat no. 0722 Ra, assay range 15 mg/L–300 mg/L, sensitivity 2.8 mg/L, made in china) following the manufacturer's instructions. This sandwich kit is for quantitative detection of rat Tumor Necrosis Factor Alpha (also known as TNF α) in serum and tissue estimation of IL-1β ELISA kits (Cat NoSL0402Ra, assay range 1 pg/L–80 pg/L, sensitivity 0.1 pg, Made in China). This sandwich kit is for the accurate quantitative detection of Rat Interleukin 1 Beta (also known as IL-1B) in serum, tissue. These calculations are best done with computer-based curve-fitting software, and regression analysis can be used to identify the optimal fit line. An ELISA plate reader was used to take all of the readings (BioTek 50TS microplate Washer). The results of all samples, standards and blanks were examined.

RESULTS

Monitoring of the Animals

The rats in the control group had good observable behaviours. Hair color and luster were fine. The rats in the DM group had a flabby spirit and lags in response and actions, and their hair was color-disordered and lackluster. However, in rats treated with metformin,there are improvements in behavior, response, movement, and hair.

Table 1: Effect of metformin on fluid and food intake

Groups	*Food intake*	*Fluid intake*
Control group (C)	18.55 ± 0.37a	21.80± 0.41a
Diabetic group (D)	32.90 ± 0.47c	115.60 ± 3.13c
Metformin group (M_{100})	22.80 ± 0.44b	36.10 ± 1.51b
Metformin group (M_{200})	22.40 ± 0.30b	32.70 ± 0.67b
Metformin group (M_{300})	22.10 ± 0.27b	32.40 ± 0.16b
p-value	0.000*	0.000*

*The mean with S.E. of the mean values is usednon-identical superscripts small letter with different superscript (a,d) are significantly at ($p \leq 0.05$)

Effect of Metformin on Fluid and Food Intake

The rats in the control group had excellent general characteristics. During the experiment, there was no noticeable difference in water or food consumption, food intake and body weight also increased steadily and consistently. For rats in DM group, flagging spirit and lags in response and action were present, with the typical symptom of polyphagia(increase food intake significantly than control group), polyuria, loose stools and loss of body weight the hair was color-disordered and lackluster polydipsia (increase water intake significantly than control group). In contrast, improvements in spirit, response, action, food intake, water intake, and hair in rats treated with metformin decrease significantly food intake and water intake compared to DM, as Table 1.

Table 2 shows a decrease in weight of diabetic rat rather than control healthy group and metformin treatment 100, 200, 300 mg/kg increase in weight of rats comparison between weight at day 0 and 35 days showed significant difference ($p = 0.003$).

Influence of Metformin on Blood Glucose Level

The effect of metformin on blood glucose levels with dosages of metformin is seen in Table 3. The control group has normal blood glucose levels, whereas the diabetic group has high blood glucose levels Statistical comparison between control group and metformin group before induction of diabetes showed the non-significant difference. After induction of diabetic before administration of metformin insignificant differences were shown between control group and other groups ($p=0.000$) after addition of metformin, a significant difference was found with rat of diabetic group day 21 and day 35 ($p =0.000$).

Effect Metformin on Serum TNF-α Level

Table 4 shows effect of metformin drug different Dose (100, 200, 300 mg/Kg/Day) on Serum TNF-α Level in Diabetic Rats A substantial increase of the TNF-α level In diabetic rats, it was discovered. Compared to the control group (Table 4). Metformin treatment reduced the generation of inflammatory cytokines in diabetic rats. Serum levels of TNF-α with various tiny letters change considerably between groups in the same period, indicating

Table 2: Effect metformin drug different dose onbody weight

Duration / *Group*	*Before experimentation starts (Day 0)*	*End of experimentation (Day 35)*	*Difference*
Control group (C)	216.80 ± 0.37 (A)	268.66 ± 2.43 (A)	52
Diabetic group (D)	229.75 ± 21.98 (A)	187.75 ± 8.17 (B)	42
Metformin group (M_{100})	198.75 ± 12.12 (A)	206.50 ± 0.940 (B)	7.75
Metformin group (M_{200})	179.75 ± 6.86 (A)	191.62 ± 10.27 (B)	11.87
Metformin group (M_{300})	199.0 ± 16.57 (A)	219.50 ± 16.98 (B)	20.5
p-value	0.293	0.003 *	

*Various superscripts capital letters with different superscript A,B are significantly at ($p \leq 0.05$) at period begging and end of study values are expressed as mean S.E.M.

Table 3: Effect metformin drug different dose (100, 200, 300 mg/kg/day) on blood glucose levels in diabetic rats caused by alloxan

Duration / Group	*Before experimentation starts(Day 0)*	*After induced diabetic(Day 7)*	*Treatment with Metformin (Day 21)*	*Treatment with Metformin(Day 35)*	*p-value*
Control group (C)	98 ± 2.48 (A,a)	102.8 ± 2.35 (A,a)	98.30 ± 1.83 (A,a)	101.0 ± 2.00 (A,a)	0.375
Diabetic group (D)	97.60 ± 2.65 (A,a)	344.90 ± 24.74 (B,c)	340.20 ± 33.48 (B,c)	335.10 ± 29.20 (B,c)	0.000*
Metformin group (M_{100})	98.90 ± 2.33 (A,a)	301.90 ± 15.60 (D,b)	229.60 ± 8.28 (C,b)	179.00 ± 16.11 (B,b)	0.000*
Metformin group (M_{200})	99.30 ± 2.04 (A,a)	333.10 ± 23.47 (C, b)	227.60 ± 22.83(B, b)	173.00 ± 20.87 (B,b)	0.000*
Metformin group (M_{300})	98.50 ± 2.23 (A, a)	302.90 ± 26.54 (C,b)	213.80 ± 26.63 (B,b)	157.20 ± 26.82 (AB,b)	0.000*
p-value	0.995	0.000*	0.000*	0.000*	

*Serum glucose levels are reported as mean S.E.M. values with distinct superscripts (capital letters with various superscript AD in the horizontally same row different period different group) are considerably different at ($p \leq 0.05$). Means with various superscript small letter (ae) are difference between group in the same period (Dunken test, $p < 0.05$)

Table 4: Effect metformin drug different dose (100 mg/kg/day, 200 mg/kg/day, 300 mg/kg/day) on serum level in tnf-α diabetic rats

Duration / Group	*Before experimentation starts (Day 0)*	*After induced diabetic (Day 7)*	*Treatment with metformin (Day 21)*	*Treatment with metformin (Day 35)*	*p-value*
Control group (C)	82.21 ± 0.27 (A,a)	81.70 ± 0.290 (A,a)	82.11 ± 0.29 (A,a)	82.01± 0.39 (A,a)	0.697
Diabetic group (D)	81.81 ± 0.34 (A,a)	197.75 ± 3.46 (B,b)	196.65 ± 3.566(B,e)	191.85 ± 2.86 (B,d)	0.000*
Metformin group (M_{100})	81.40 ± 0.268 (A,a)	199.15 ± 4.61 (D,b)	142.31 ± 3.70 (C,d)	129.50 ± 2.19 (B, c)	0.000*
Metformin group (M_{200})	82.01 ± 0.33 (A,a)	191.85 ± 2.86 (D,b)	129.49 ± 3.80 (C,c)	108.63 ± 3.62 (B,b)	0.000*
Metformin group (M_{300})	82.31 ± 0.28 (A,a)	194.55 ± 2.601 (D,b)	109.99 ± 2.58 (C,b)	101.93 ± 1.650 (B,b)	0.000*
p-value	0.250	0.000*	0.000*	0.000*	

*Distinct superscripts (capital letters with different superscript A,D in the horizontally same row different time different group) are substantially different at ($p < 0.05$). Serum Level in TNF-Values are expressed as mean S.E.M. Means with a different superscript small letter (ae) are significantly different between different groups in the same period (Dunken test, $p < 0.05$).

that metformin lowered inflammatory cytokine production in diabetic rats.

Metformin's Impact on IL-1 β Levels in Diabetic Rats' Serum

Table 5 illustrates the impact of metformin on IL-1 β levels in diabetic rats' serum. At day 0, no difference between the groups was obtained ($p = 0.974$). At day 7, after induction of diabetes before administering significant metformin difference was found between all groups and control group ($p = 0.000$) comparison between group of treatment with metformin at day 21 and day 35 and diabetic group showed a significant difference ($p = 0.000$). This indication the treatment with metformin lead to decrease the IL-1β level.

Effect of Metformin on Pro-inflammatory Cytokines (TNF-α and IL-1β) Determination on the Liver Tissue

Figure 1 (A) demonstrates the effects of different doses of metformin on reduced pro-inflammatory cytokines (TNF-α.) on the liver tissue in diabetic rats and with increased time (decrease pro-inflammatory cytokines TNF-α with increased dose and time).

Figure 1 (B) Shows metformin's impact on tissue IL-1β values that significantly reduced treatment of metformin between groups, while within one group reduced pro-inflammatory cytokines with increased time

Metformin's Impact on the Histopathology of Induced Diabetic Rats' Liver

Effect on the Liver's Histopathology The hepatic tissue of non-diabetic people who have not been treated control group (C) rats exhibited radially distributed hepatocytes around the central vein, among other histological findings. Acidophilic cytoplasm and rounded central vesicular achromatic nuclei with well-defined nucleoli characterize the cells. Blood sinusoids with thin walls and flat endothelial cells separate the hepatocyte plates. Von Kupffer cells with prominent nuclei were occasionally seen. Untreated diabetic control rats

Table 5: Effect metformin drug different dose (100 mg/kg/day, 200 mg/kg/day, 300 mg/kg/day) on serum level in IL-1β diabetic rats

Duration / *Group*	*Before experimentation starts (Day 0)*	*After induced diabetic (Day 7)*	*Treatment with metformin (Day 21)*	*Treatment with metformin (Day 35)*	*p-value*
Control group (C)	14.35 ± 0.32 (A,a)	13.85 ± 0.25 (A,a)	14.53 ± 0.28 (A,a)	14.13 ± 0.27 (A,a)	0.379
Diabetic group (D)	14.13 ± 0.31 (A,a)	33.99 ± 0.62 (B,b)	33.89 ± 0.64 (B,d)	32.89 ± 0.66 (B,e)	0.000*
Metformin group (M_{100})	14.15 ± 0.29 (A,a)	34.25 ± 0.48 (D,b)	29.15 ± 0.36 (C,c)	24.11 ± 0.70 (B,d)	0.000*
Metformin group (M_{200})	14.03 ± 0.47 (A,a)	33.69 ± 0.54 (D,b)	27.43 ± 0.51 (C,b)	22.25 ± 0.62 (B,c)	0.000*
Metformin group (M_{300})	14.15 ± 0.20 (A,a)	33.29 ± 0.58 (D,b)	26.24 ± 0.520 (C,b)	18.94± 0.33 (B,b)	0.000*
p-value	0.974	0.000*	0.000*	0.000*	

Different superscripts (capital letters with different superscript AD in the horizontally same row different time different group) are substantially different at ($p<0.05$). Serum Level in IL-1βin Values are presented as mean S.E.M. Means with different superscript tiny letters (ae) differ substantially between groups in the same period (Dunken Test, $p<0.05$).

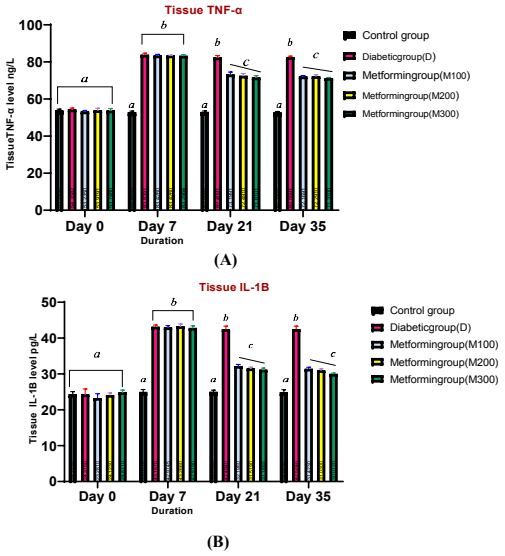

Figure 1 A and B: (A) Effects of different doses of metformin on reducedpro-inflammatory cytokines (TNF-α.) on the liver tissue; (B) Effect of metformin on tissue IL-1β levelTNF

increased apoptotic hepatocytes in a portion of their hepatic tissue (shrunken and dark-stained cells with small degenerated nuclei).

Histopathology Analysis

Inflammation Findings (score): Histological slides were prepared from each specimen under a light microscope for inflammation scored separately by three histopathologists. The mean was calculated and settled as a final score for statical analysis as Figures 2 and 3.

On Friedman Test For Inflammation, highest mean rank recorded diabetic group the least mean rank for control group For 7th day of experiments (before treatment), 21st, 35th Days of experiments and least mean rank of treatment than diabetic it appears to reduce significantly of inflammation.

In Kruskal-Wallis Test and p-value for inflammation between days 7th, 21st, 35th different duration of metformin significantly difference reduce than diabetic group.

DISCUSSION

The general characteristics induced diabetes by alloxan in rats. An elevation of serum glucose in alloxan caused diabetic over 200 mg/dL defined as diabetic rat. Alloxanes cause the production of oxidizing agents that break down beta cells in the pancreatic gland. We used rat albino as model. Since various species and background strains have different susceptibilities to diabetes, and treatments, Male rat were involved because many knock-outs and transgenic models of diabetes show a gender bias, strain, and species differences. It should be carefully examined when selecting a model because the permanent damage of pancreatic cells in alloxan-induced diabetic rats, increases blood glucose levels and leads to a fall in serum insulin levels.[18] It has been suggested that this is due to the effects of sex hormones in some cases.[19] In alloxan-induced diabetic rats, the blood glucose levels raised because of permanent destruction of pancreatic β-cell resulting in reduced serum insulin level.[20] The present study showed that metformin reduced the pro-inflammatory mediator of IL-1β and TNF-α in diabetic rats, indicating metformin's anti-inflammatory characteristics were proven to be effective regardless of whether or not the individual developed diabetes.[21] Anti-inflammatory properties should be regarded a potentially major element of metformin's pharmacology. According to evidence from cells, animal models, patient records, and randomized clinical trials, metformin is used as the first-line agent for treating T2DM. Metformin's pleiotropic effects have been thoroughly established, in addition to its well-known role in

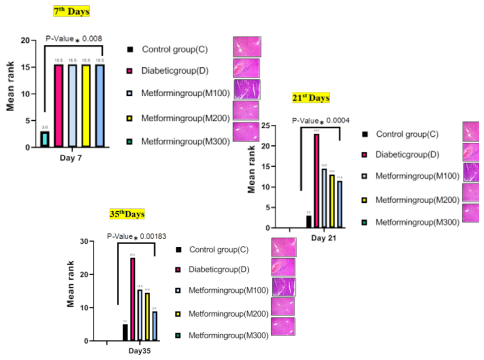

Figure 2: Mean rank table and p-value of Friedman test for inflammation for 7th, 21st, 35th days of treatment

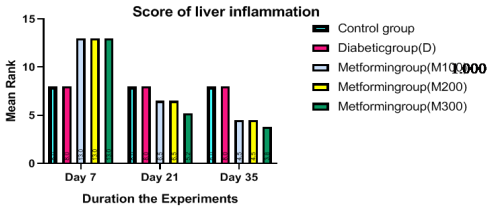

Figure 3: Kruskal-Wallis Test and p-value for inflammation between days

DM management. One of the most noteworthy findings from in vitro and in vivo research is that metformin, regardless of its ability to manage glucose, can produce potent inflammation-inhibitory effects. Metformin has been shown in numerous pre-clinical and clinical trials to alleviate chronic inflammation by improving metabolic parameters such as hyperglycemia, insulin resistance, and atherogenic dyslipidemia. However, nuclear factor kB has a direct anti-inflammatory response (NF-kB) Through independent and dependent AMP-activated protein kinase (AMPK) pathways

CONCLUSION

Lowering pro-inflammatory cytokines, including IL-1 and TNF- in liver tissues and serum implies that metformin's anti-inflammatory effects may aid inflammation relief. This study suggests that metformin could be used to reduce inflammation in people with diabetes, implying a clinical benefit of metformin during anti-diabetic therapy.
More research was done to figure out how metformin works as an anti-inflammatory agent.

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