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Effects of Taurine and *Ginkgo biloba* Extract on Platelet Aggregation

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Abstract

Effects of the leaf extract *Ginkgo biloba*, ginkgolides A and B, and the amino acid taurine (2-aminoethane sulfonic acid) on platelet aggregation was studied. All potential anitcoagulants were incubated in day one platelet-rich plasma and subjected to various agonist-induced clotting and tests and other measures of platelet viability. *G. biloba* extract and its respective ginkgolides had no effect on platelet aggregation in response to ADP and thrombin, while taurine exhibited prolongation of thrombin time (TT) and reduced thrombin-induced aggregation by 10%. Taurine prolonged time of initial clot formation on throm- bolestographic tests, but overall clot viability remained unaffected. These data suggest that *G. biloba* and its active ginkgolides do not inhibit platelet aggregation induced by ADP and thrombin, while taurine mildly inhibits thrombin-induced platelet aggregation. These findings correlate with taurine's osmoregulatory and cryoprotective properties and indicate a role as a hemostasis stabilizer rather than an anticoagulant. Possible mechanism of taurine action is suggested.

Abbreviations: EGb 761, *Ginkgo biloba* extract; GA, ginkgolide A; GB, ginkgolide B; PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; ESC, extent of shape change; HSR, hypotonic shock response; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

Introduction

Leaves from the plant *Ginkgo biloba* have been an integral part of Chinese herbal medicine for over 5,000 years, but it is only fairly recently that Western medicine has acknowledged it's physiological effects and pharmacological implications. Similarly, taurine, an amino acid ubiquitous throughout the animal kingdom, has an ancient evolutionary history, but only in recent decades has it stimulated interest in the biomedical community. Many biological effects of *G. biloba* have been reported in current literature including cerebrovascular and memory enhancement,[1, 2] antioxidant action,[2, 3] neurotransmitter stimulation,[2, 3] and inhibition of platelet aggregation.1-5 Taurine exerts a similarly copious influence on biological phenomena with effects including bile conjugation, [6-8] osmoregulation, [6, 8-9] membrane stabilization, [6,9] cellular calcium modulation, [6, 10] antioxidation, [6,7] and inhibition of platelet aggregation.[6, 10-13] *G. biloba* and taurine's influence on platelet aggregation is the exclusive focus of this study.

The number of studies on *G. biloba* is rapidly increasing, due in no small part to the American public's increasing fascination with complimentary and alternative medicine and accordingly, the traditional healer's sensitivity to the needs of his or her patients. Due to its classification as a supplement, *G. biloba* extract (EGb 761), the commercially available form of the herb, is not regulated by the FDA, and because it can be purchased over-the counter, adverse side effects and/or drug-interactions are usually not considered before taking the supplement. Similarly, physicians may not realize their patients are using herbal extracts until a potentially dangerous situation presents itself, such as in situations of perioperative care.[3]

EGb 761 is purified and concentrated 1:50 (extract: leaves) and consists of 24%

flavonoid glycosides compounds, 2.4%
bilobalide, and 2, 0.8, and 1% ginkgolides
A, B, and C, respectively (Fig. 1). The
extract is processed as a powder and
manufactured in pill-form by various
pharmaceutical companies.

Figure 1. Chemical structures of *Ginkgo biloba*
extract (EGb 761) constituents
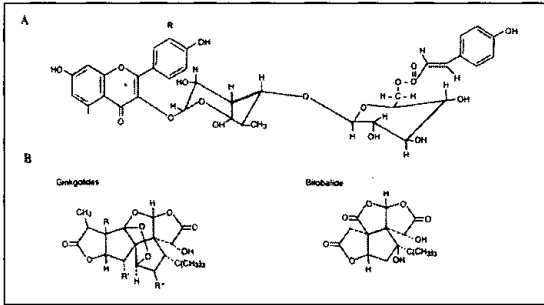
A.   Glucorhamnoside ester of
     kaempferol (R=H) and quercetin
     (R=OH): Flavenoid fraction
B.   Ginkglodides and bilobalide:
     Terpene lactone fraction
     Ginkgolide A: R=OH, R =H, R =H
     Ginkgolide B: R=OH, R'=OH,
     R''=H
     Ginkgolide C R=OH,   R'=OH,
     R''=OH

Taurine (Fig. 2) has been drawing the
attention of researchers since it was first
isolated from the bile of the ox that carries
its name (*Bos taurus*). A quick glance at its
phylogenic distribution and physiological
influence reveals why. Taurine is present in
surprisingly high concentrations in algae
and in the majority of the animal kingdom,
including arthropods. Except for trace
amounts, taurine is not found in the
bacterial and plant kingdoms, mostly due to
the fact that such organisms possess a cell
wall and are less likely in need of the
osmoregulatory protection taurine provides.

Figure 2. Chemical structure of taurine
(2-aminoethane sulfonic acid)
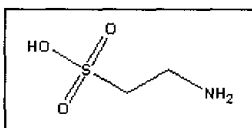
Taurine is not utilized in protein synthesis
but rather is found as a free ?-amino acid in
plasma and in tissues, with highest
concentrations found in the excitable
tissues of the heart, brain, and retina.[6, 8]
It is also found in increasingly high
concentrations in platelets. Taurine is
extremely important in mammalian
development, and dietary deficiencies have
been linked to pathological conditions such
as cardiomyopathy, retinal degeneration,
growth retardation, hypercholesterolemia,
hepatic disorders, and cystic fibrosis.[14]
Taurine's developmental importance is
reinforced by the fact that since 1984 the
U.S. Department of Agriculture has
sponsored addition of taurine to human
infant formulas.[15] Virtually all infant
formula manufacturers worldwide now
add taurine to their products.

Another interesting aspect of taurine's
role in physiological phenomena is its little
explored or understood role in hibernation
and freeze tolerance. Hatchlings of the
painted turtle *Chrysemys picta marginata*
are the highest vertebrate life known to
tolerate the natural freezing of extracellular
body fluids during winter hibernation.
When exposed to sub-optimal temperatures,
it has been shown that the total amino acid
pool increases 2.25-fold in the liver,
muscle, and blood, with taurine accounting
for 52% of this increase.[16] Elevated levels
of GABA and taurine have been detected
during hibernation-induced hypothermia in
hedgehogs,[17] and it has been shown that in
cold acclimation of garter snakes
(*Thamnophis* sp.), plasma levels of all
amino acids except taurine significantly
decrease, while taurine itself increases in
concentration.[6] Such research has provided
an impetus for studying taurine's role in
cryopreservation, and it has recently been
shown that taurine pretreatment and its
continued presence during islet
cryopreservation impoves post-thawing
viable recovery of islets.[18]

Because of the significant increase in
plasma taurine levels exhibited by several
species during hibernation, a period during

which the cardiovascular system becomes sluggish, resulting in decreased blood flow and increased risk of intravascular thrombus formation, taurine's role as a potential anticoagulant or hemostatic stabilizer is probable. It has already been shown that during hibernation in the frog *Ratna tigrina* clotting tests indicate prolongation of several hemostatic factors including whole blood clotting time, plasma recalcification time, cephalin time, and prothrombin time.[19]

While differing markedly in chemical structure and mechanism of action, EGb 761 and taurine both effect similar biological processes, notably platelet aggregation. The mechanism of EGb 761's inhibitory action in the aggregation response has been linked to the inhibition of Platelet Activation Factor. It has also been linked to inhibition of aggregation induced by oxidative stress, with ginkgolides A and B of the terpene fraction exerting the greatest inhibitory effect.[1-3] Taurine's inhibition of platelet aggregation is associated with its effects on intercellular calcium ion translocation, but a universally agreed upon mechanism has yet to be proposed.

While several studies have addressed *G. biloba* and taurine's inhibitory effects on platelet aggregation *in vitro*, a direct comparison of both compounds and their effects on platelet aggregation and other factors of hemostasis remains to be studied. EGb 761, ginkgolides A and B, and taurine were examined in relation to their effects on aggregation of platelet rich plasma through agonist-induced lumi-aggregation and thrombelastography methods with the goal of contributing to this understanding.

Materials and Methods

Materials

G. biloba leaf extract (standardized to 24% flavonoid glycosides, 2.4% bilobalide, and 2, 0.8, and 1% ginkgolides A, B, and C, respectively) was purchased in powdered pill form from Rite-Aid pharmacy

(Washington, D.C.). Purified ginkolides A and B, taurine, ADP, thrombin, and all other reagents were purchased from Sigma Chemicals (St. Louis, MO).

Platelet supply and cell adjustment

Donor plasma units were obtained from Walter Reed Army Medical Center under a protocol approved by the Institutional Review Board. Plasma (101 mL containing approximately 17 mL ACD-A anticoagulant) was frozen at −70°C. At the time of experiments plasma was rapidly thawed at 37°C and processed for STA Compact coagulation assay. For all other assays, unfrozen, unthawed, day one (post-donation) Platelet Rich Plasma (PRP) was used. Units were kept under standard blood banking conditions (22°C with rotation) until testing. PRP cell count was performed on an automated cell counter (Cell-Dyne 1700, Abbott Diagnostics Division, Mountain View, CA) and later adjusted to 300×10^3 cells/uL with HEPES-buffered platelet-poor plasma (PPP). PPP was prepared by centrifugation of PRP at 3000 x g for 20 minutes at 22°C. HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1mM $MgCl_2$, 5.6 mM glucose, and 3.8 mM HEPES, pH7.4) was added to PPP for a final concentration of .0152 HEPES (%vol.)

Stock Solution Preparation

Powdered *G. biloba* extract was partially dissolved in 1x Phosphate-Buffered Saline and filtered through a 20 mosmol porosity hypodermic syringe filter to a final concentration of 1000 mg/mL. Ginkgolides A and B were partially dissolved in 1x PBS and placed in an ultrasound water bath for complete dissolution to a final concentration of 1000 mg/mL. Taurine was dissolved in 1x PBS and adjusted to a concentration of 250 mM.

Solution Incubation and sample preparation

PRP was incubated 10:1 with EGb 761 and ginkgolides (final concentration 100 mg/mL), and 10:1 and 50:1 with taurine (25 mM, 5 mM final concentrations,

respectively) at 37°C for 1 hour. Cell count of incubated PRP was then adjusted to 300 x 10^3 cells/ul with HEPES-buffered PPP. Based on initial PRP cell count, EGb 761, ginkgolides A and B, and taurine stock solutions were added to HEPES-buffered PPP before dilution in order to retain their respective concentrations in solution.

Clotting Time

Platelet samples were stimulated with various agonist-induced clotting tests and other measures of platelet viability. *In vitro* testing of coagulation was measured using the SPA Compact electromechanical clot detection system (Chronolog, Havertown, PA). All assays were carried out according to standard procedures developed by American Bioproducts. The following measures of the extrinsic clotting cascade were measured: Prothrombin Time (PT)— a mixture of thromboplastin with calcium is added to citrated plasma, Activated Partial Thromboplastin Time—involves the recalcification time of plasma in the presence of a standardized amount of cephalin and activator silica, and Thrombin Time (TT)—in the presence of 0.5 u/ml thrombin, plasma will consistently clot in a finite time unless there is insufficient or abnormal fibrin formation. Taurine was prepared in 5 and 25 mM concentrations in plasma.

Extent of Platelet Shape Change induced by ADP and Hypotonic Shock Response

Extent of ADP-induced platelet Shape Change (ESC) and osmotic Hypotonic Shock Response (HSR) was measured as the change in optical density of the reaction mixture using the SPA-2000 instrument (Chronolog, Havertown, PA). The sample contained EDTA (2 mM final concentration) to prevent aggregation. ADP (0.02 mM final concentration) induces

platelet shape change from discoid to spherical shape with pseudepodial projections, causing an increase in optical density. The difference is computed automatically and given as a percentage. Duplicate measurements were compiled from five donors.

Platelet aggregation by thrombin

1.0 u/mL thrombin-induced aggregation was performed and monitored with a luminescent aggregometer (Chronolog, Havertown, PA), also utilizing change in optical density as an indication of platelet response. Aggregation results were measured as a percentage of maximum light transmittance as determined by PPP. Using AGGRP/LINK software, the slope (S) and amplitude (A) of the generated curves were computed. Duplicate measurements were compiled from five donors.

Thrombelastography

To gain a better understanding of the additive's effects on hemostasis as a whole, thrombelastograpy (TEG), which measures the kinetic changes associated with clotting and clot retraction, was performed on the TEG Coagulation Analyzer 5000 (Haemoscope, Skokie, IL). A normal thrombelastographic tracing with associated values is represented in Fig. 3. Disposable cups and pins were used in accordance with manufacturer guidelines. Coagulation was induced by thrombin (0.6 U/mL final concentration) and $CaCl_2$ (6.25 mM final concentration) While thrombelstagraphic tracings evaluate many aspects of platelet response, Maximum Amplitude (MA) and R time were focused on in this experiment. Duplicate runs were compiled from five donors.
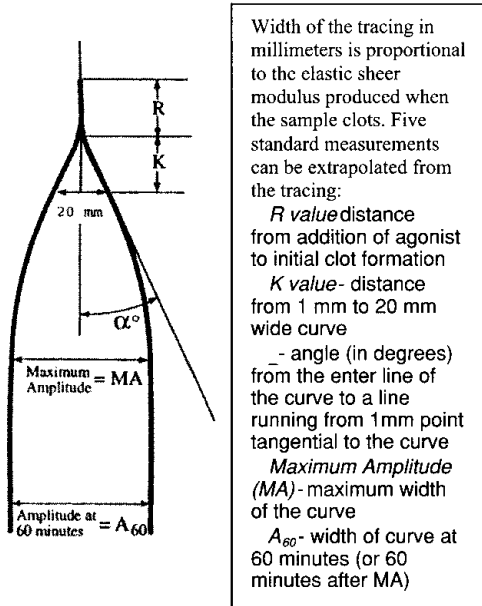
Figure 3. Diagrammatic thrombelastographic
tracing[20]

Width of the tracing in millimeters is proportional to the elastic sheer modulus produced when the sample clots. Five standard measurements can be extrapolated from the tracing:
R value distance from addition of agonist to initial clot formation
K value - distance from 1 mm to 20 mm wide curve
_ - angle (in degrees) from the enter line of the curve to a line running from 1mm point tangential to the curve
Maximum Amplitude (MA) - maximum width of the curve
A_{60} - width of curve at 60 minutes (or 60 minutes after MA)

Stastical Analysis

Mean values ±SE are shown throughout this report. Statistical significance of differences between means was calculated using a paired t test analysis (MINITAB software, Minitab Inc., State College, PA). P values are expressed at the 95% confidence level (P>0.05)

Results

Effect of EGb 761 and Ginkgolides A and B

EGb 761 and ginkgolides A and B exhibited no significant effect on ADP-induced shape change, and little to no effect on hypotonic shock response of platelet samples (Fig. 4). Similarly, EGb 761 and its respective ginkgolides had no observable effect on lumi-aggregation (Fig. 5). Ginkgolide B, the most potent inhibitor of aggregation response based on current literature, is shown as indicative of this null response. No significant effect was observed on thrombelastography and plasma clotting times.

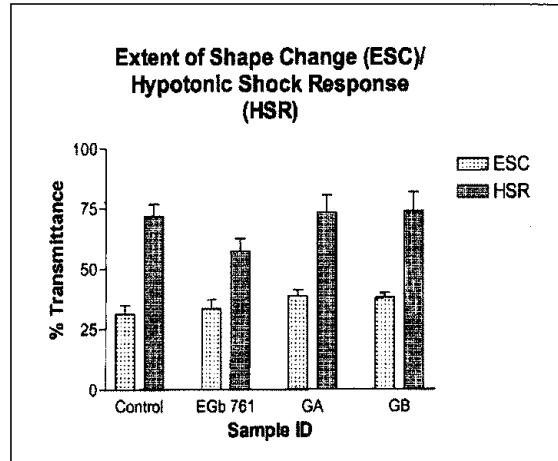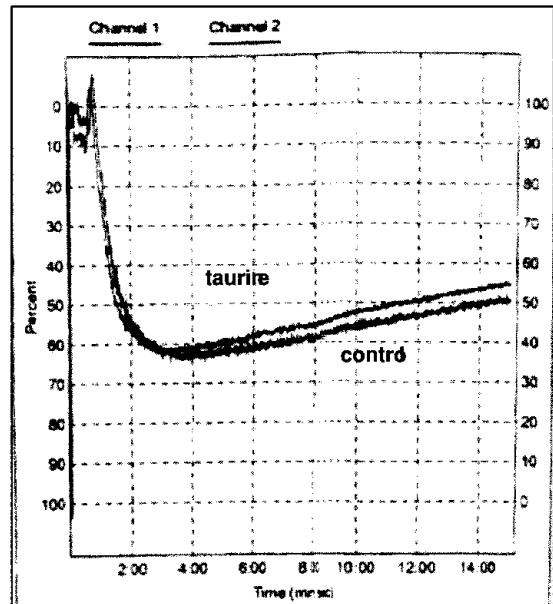Figure 4. Effects of *Ginkgo biloba* and ginkgolides A and B on ADP-induced Extent of Shape Change and Hypotonic Shock

Figure 5. Effect of ginkolide B on thrombin-induced lumi-aggregation.

Taurine

Taurine also had no significant effect on ADP-induced shape change (Fig. 6). ESC for control platelets was 30.02% ± 6.81, while ESC for platelets incubated with 5 and 25 mM taurine was 28.85% ± 4.64 and 33.57 ± 4.9, respectively. Both values were not statically different from the control.

Figure 6. Effect of taurine on ADP-induced Extent of Shape Change.

B.
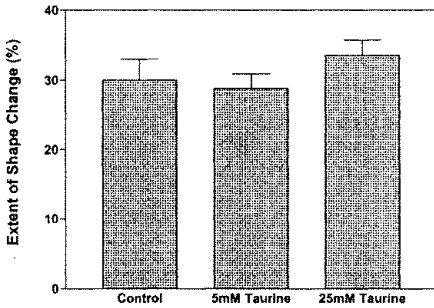
Taurine exhibited significant inhibition of platelet aggregation in lumi-aggregation (Fig. 7). Aggregation of control platelets is characterized by A = 94% and S = 119. 25 mM taurine displayed notable decrease in amplitude, with A = 81% and S = 114. 5 mM taurine displayed a similar 10% reduction in aggregation response, indicating a saturating effect and a lack of dose-dependence inhibition in concentrations greater than 5 mM.

Figure 7. Effects of taurine on thrombin-induced lumi-aggregation: A. Effect of taurine concentration on inhibition response. B. Representative 25 mM taurine tracing exhibiting aggregation inhibition.
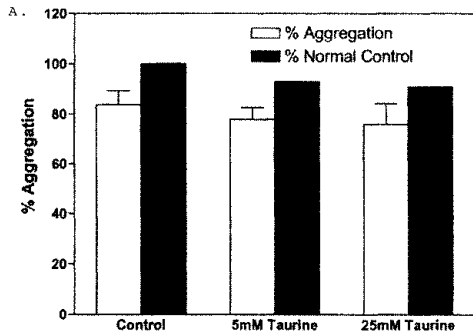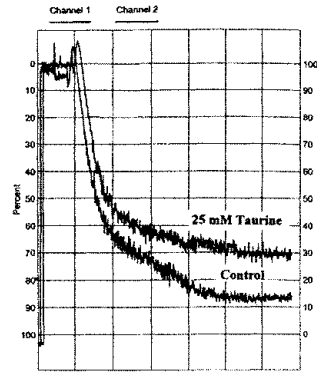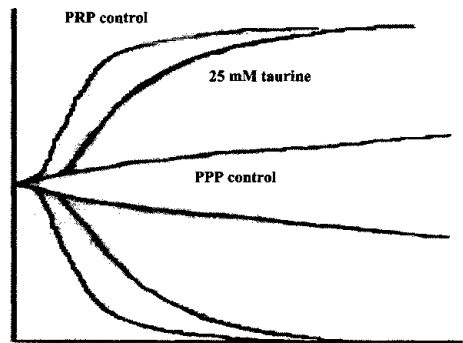

While taurine produced an increase in time of initial clot formation (R), overall clot viability (MA) was unaffected. Control MA = 73.95 ± 2.30 mm, while 25 mM taurine produced a stastically insignificant decrease with an MA = 72.60 ± 2.60 mm. This effect is observable in figure 8.

Figure 8. Thrombelastographic effects of 25 mM taurine on thrombin-induced aggregation.

Results of plasma clotting tests are presented in figure 9. A significant increase of TT was observed at 25 mM concentration, prolonging TT by 9%. While this observation is stastically significant, (P<0.05), it is not clinically significant, as the data obtained still falls in the normal TT range. PT and APTT remained unaffected by taurine.

Figure 9. Effect of taurine on plasma clotting time.
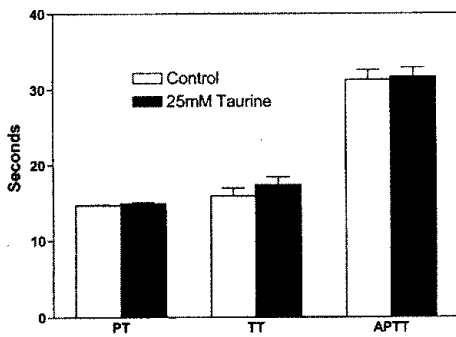
Discussion

Although several studies have demonstrated inhibition of platelet aggregation by *G. biloba* and taurine, none have used thrombelastography as a comparative hemostatic measurement. The results obtained support those present in the literature, as EGb 761 and ginkgolides A and B had no effect on ADP or thrombin-induced aggregation. These findings support the specificity of *G. biloba's* anticoagulant action, which purportedly acts by inhibiting Platelet Activating Factor and coagulation induced by oxidative-stress. The slight decrease in HSR produced by EGb 761 is more an indication of faulty reagent preparation than osmoregulatory action. As the only readily obtainable form of EGb 761 was the pill form, cellulose and other pharmaceutical additives prevented dissolution in PBS, and filtration was necessary to prevent hyperosmotic conditions that might have effected aggregation studies. The filtration procedure was obviously not successful, and future studies would warrant obtaining EGb 761 in hydrophilic liquid form to prevent such sources of error. Ginkgolides A and B, which did dissolve in PBS after ultrasound treatment, did not show any significant change in osmoregulatory response.

While Taurine also exhibited a null effect on ADP-induced shape change (Fig.6), it did inhibit thrombin-induced lumi-aggregation by 10% (Fig. 7).

Increasing concentration from 5 to 25 mM did not contribute to further augmentation of the inhibitory effect. It has been assumed that taurine's inhibitory action on platelet aggregation can be attributed to its regulation of intracellular Ca^{2+} movement. It has been postulated that platelet shape change is mediated by both calcium dependent and independent pathways.[21] When agonist-induced cytosolic Ca^{2+} increases were prevented by a cytosolic Ca^{2+} chelator, platelets still underwent shape change. Thus it is possible that in the presence of taurine ESC remains unchanged as calcium independent pathways compensate for its inhibitory action on intracellular translocation.

Thrombelastographic analysis revealed a significant decrease in R value distance, which is associated with initial clot formation, while MA value remained unaffected. This indicates an initial inhibition of platelet response being eventually counteracted by other plasma coagulation elements. MA represents the maximum clot elastic shear modulus of the developed clot, and is an indication of the interaction between platelets and fibrin strands. Initial clot formation was mildly inhibited while overall clot viability remained unaffected. As MA represents an integral function of several hemostatic elements, the 10% reduction in thrombin-induced platelet aggregation observed in lumi-aggregation tests is not enough to significantly impact the overall strength of the clot.

Taurine was found to have a marginal effect on TT, while leaving PT and APTT unaffected (Figure 9). The minor effect on TT is not clinically significant, as TT remained within the normal range. Due to the lack of a significant impact on the clotting tests taurine cannot be considered an anticoagulant. However, due to its statistically significant effect on TT it may be pictured as a TT regulator during conditions of hypothermia when its concentration in plasma profoundly increases. Interestingly, Ahmad *et al.* report

prolongation of several clotting tests but no change in TT during hibernation in frogs, indicating that there is no significant decrease in the plasma fibrinogen level.[19] Boral et al. even reported an increased plasma fibrinogen level of blood in the hibernating toad.[21]

It can be inferred from these results and the results obtained that taurine regulates TT by modifying the sensitivity of the Ca^{2+} binding site of fibrinogen to Ca^{2+}. Ca^{2+} ions are known to accelerate polymerization of fibrin monomers, and it has been recently shown that the function of the polymerization site depends on the structural integrity of its nearby Ca^{2+} site.[23] It is suggested that taurine acts by modifying the sensitivity of the Ca^{2+} binding site of fibrinogen to Ca^{2+}, thus indirectly impairing polymerization. The result is a slight prolongation of TT that may be of a special importance under hypothermic conditions or conditions of elevated fibrinogen levels.

It is not clear yet what mechanisms are at work in hibernating animals that are able to sustain sluggish blood flow without risk of microvascular blockade, however this research supports taurine as a probable hemostatic stabilizer rather than an anticoagulant. Increased levels of taurine in plasma during hibernation can be attributed to an increased requirement for hemostasis regulation and prevention of hyper-cogulative states by modifying the Ca^{2+} binding site of fibrinogen and thus regulating polymerization during hyperthermic conditions.

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