

**EFFECT OF FUNGAL CULTURE BROTHS ON K⁺ - AND FIELD
STIMULATION-RESPONSES OF *Bufo tibiamicus* STOMACH
AND HEART MUSCLES. 1- IN NORMAL AMPHIBIAN SALINE.**

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EFFECT OF FUNGAL CULTURE BROTHS ON K^+ - AND FIELD STIMULATION-RESPONSES OF *Bufo tibiamicus* STOMACH AND HEART MUSCLES. 1- IN NORMAL AMPHIBIAN SALINE.

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ABSTRACT: The present investigations were conducted to study the effect of fungal culture broths of both *Aspergillus flavus* (*A. flavus*) and *Aspergillus niger* (*A. niger*) on K^+ -contractures and field stimulation (FS) responses of the toad (*Bufo tibiamicus*) stomach and heart muscles. Addition of *A. flavus* culture broth enhanced normal activities of toad stomach muscles, while it inhibited natural activities of the heart muscles. This broth slightly inhibited K^+ -contractures of the stomach muscle affecting the tonic response, but was without significant effect on heart muscles K^+ -contractures and FS-responses. On the other hand, *A. niger* culture broth inhibited normal activities and severely inhibited K^+ -contractures and both phasic and tonic responses of toad stomach muscles, while it enhanced natural activities of the heart muscles, but was without significant effect on heart muscles K^+ -contractures and FS-responses. In field stimulation, the heart muscles responded to K^+ -additons. This could be due to nerve ending stimulation which may activate cell membrane and ion-channels located there. In general, both fungal culture broths exerted toxic effect on toad stomach and heart muscles. *A. flavus* culture broth was more effective on the heart as an inhibitory agent, whereas it has an excitatory action on the stomach muscle. Whereas, *A. niger* culture broth has an inhibitory effect on the stomach muscle and an excitatory effect on the heart muscle. The two different actions of both fungal culture broths on both muscle preparations may indicate that they may affect different ion-channels and / or different receptors due to their differences in muscular and innervation systems.

INTRODUCTION

The widespread occurrence of fungi in our environment and their toxic products have stimulated many investigators to study their toxicity. Many species of *Aspergillus* and *Penicillium* spoil food stuffs especially when they are stored in hot and moist conditions (Sargent, et al, 1961; Nesbitt, et al, 1962; Hesseltine, et al, 1966; Gupta, 1981).

In the course of screening several strains of *A. flavus* isolated from contaminated corn and other food stuffs such as: oat, millet and rice or potatoes, a peculiar toxin syndrome was seen in mice after oral administration of the crude fungus extract (Wilson, 1971). Mice-trembling was caused by substances extracted from the mycelial mat and sclerotia of *A. flavus* but not from spores or from the culture broth (Betina, 1984).

Ikegwonu (1983) reported that aflatoxin-B₁ produced by *A. flavus*, resulted in neurotoxicity in rats. *Citreoviridin* is produced by *Penicillium citreoviridea*, *P. citrinum* as well as *A. terreus*. Moreover, *citreoviridin* is neurotoxin to several animals causing the same symptoms as in acute cardiac beriberi (Engel & Teuber, 1980).

Most of the studies on the amphibian muscles were concentrated on skeletal muscles (Lorkovic, 1967; Stuesse, et al, 1974; Frank, 1979) and cardiac muscles (Tjioe & Bianchi, 1969; Hussein, et al, 1992). However, little work was carried on the smooth muscles (Kitazawa, et al, 1986).

Most of the work done on *A. flavus* was in the field of *Aflatoxin production*. However, to the best of our knowledge no previous work was obliged on the tremogenic effect exerted by *A. niger* extracts. The aim of the present investigation is to study the toxic effect of the culture broths of both *A. flavus* and *A. niger* on the toad (*B. tibiamicus*) stomach and heart muscles K⁺ - and FS-responses.

MATERIALS AND METHODS

Experimental animals: Sexually mature male and female toads *Bufo tibiamicus* collected from Makkah area, Saudi Arabia, were used throughout this study. They

were transported to the laboratory and kept in large glass aquaria with small amount of tap water (50ml) which were changed twice daily. Each toad was kept separately in 500ml glass jar containing 50ml of normal amphibian saline which has the following compositions (g/l) : NaCl (20.16), KCl (0.555), Na₂HPO₄ (0.915), Na₂H₂PO₄ (0.398) and CaCl₂ (0.6615) adjusted to pH 7.1 using NaOH at room temperature (~27°C).

The methods to record tentions, normal activities, K⁺-responses and field stimulation (FS) were those adopted by Oldfield and Huddart (1982), Mutwally and Jamel Al-Layl (1992 & 1993) with little modifications to suite the present study. All the drugs and chemicals used in this study were obtained from Sigma Chemical Co., and were added to the organ baths from standard laboratory concentrates and freshly made up in distilled water just before use.

Muscle preparations: A toad was stunned and bled, and then the whole stomach and heart were removed and immediately immersed in normal amphibian saline. Each specimen was ligated and suspended vertically in an aerated organ bath (25ml) whose contents could be rapidly changed. To record the isometric contractions, both tissues were connected to an isotonic transducer whose output was fed into a Washington Instruments MD 400/2 ink-writing oscillograph via an FC 137 coupler, and these were adjusted to put a slight passive tension on the preparations.

For stomach muscle, the pharyngeal region was connected to the transducer while the proventricular ligature was vertically attached to the glass hook in an organ bath (25ml). Whereas, for heart muscle, the auricular region was ligated to the transducer and the ventricular region was connected vertically to the glass hook. All experiments were run at room temperature (~27°C).

The preparations were then allowed to equilibrate for 30-60 minutes in the organ bath, while normal saline was changed every 10-20 minutes. Saline could be removed from the organ bath by vacuum and replaced by gravity feed in less than 5 seconds. The contents of the organ bath were constantly aerated. All preparations were left in normal amphibian saline (1.5mM / 0.6615g/l) (control) for 30-60 minutes to develop spontaneous activity (Sp.Act) naturally before introducing the test solutions.

There was a 10 minutes washout interval, with normal saline between each experiment.

The test fungi: The test strains of *Aspergillus flavus* and *Aspergillus niger* were isolated from imported Pakistani and Indian rice samples respectively. The isolation medium was potato dextrose agar (Difco). It has the following composition (g/l): Potato infusion from 200g, dextrose 20, and agar 15, with a final pH 5.1. The purified strains were kept on slants of the same medium. Both *A. flavus* and *A. niger* were identified according to Domsch, et al (1980).

Both *A. flavus* and *A. niger* were grown on Czapek's Dox broth medium which had the following composition (g/l): sucrose (30), NaNO_3 (3), K_2HPO_4 (1), MgSO_4 (0.5), KCl (0.4) and FeSO_4 (0.01) with pH 7.3. The test fungi were incubated at 25°C for 10 days; then only the filtrate of culture broths were used.

To study the effect of Czapek's broth medium (G.M.) before and after fungal growth on normal activities of toad stomach and heart muscles, they were separately added to the organ bath 3-5 minutes on both tissues before K^+ -contractures and FS-responses were initiated. There was a 10 minutes washing interval between each response. Dose-response curves for K^+ -responses and FS-responses were determined by a series of separated drug trails and not by serial addition. Data presented in this study show the mean, the standard error and number of replicates.

RESULTS

Figure 1 showed the effect of series of separate K^+ -additions on the stomach muscle of control toads at ascending concentration (5-200mM), the K^+ -contractures were dose-dependent. Data presented in this study showed the mean, the standard error and number of replicates of K^+ -additions (Figure 2).

After one day of animal collections, addition of growth medium (G.M.) (control) did not affect spontaneous activities (Sp.Act) of toad stomach muscles, but they were increased slightly with the addition of *A. flavus* (0.5-1ml) culture broth.

On the contrary, these normal responses were decreased with the addition of the same amount of *A. niger* culture broth. The effect of both fungal culture broths on Sp.Act of toad stomach muscles were also dose-dependent. After washing with normal saline the muscles resumed their normal activities (Figure 3).

After 3 days of animal collections, toad stomach muscles became quicent. Additions of 0.5-1ml of G.M. to the preparation slightly induced Sp.Act. Moreover, the addition of 0.5ml *A. flavus* culture broth induced small contracture, but at 1ml it inhibited these responses. However, additions of 0.5-1ml of *A. niger* culture broth induced muscle contractures. After washing with normal saline the muscle also induced contracture (Figure 4). The G.M. did not affect 40mMK⁺-responses, whereas, addition of *A. flavus* culture broth inhibited K⁺-responses slightly and slowed tonic phase responses. Moreover, *A. niger* culture broth induced small contraction and severely inhibited K⁺-responses (Figure 5). Data presented in this study showed the mean, the standard error and number of replicates of K⁺-additions (Figure 6). On the other hand, addition of 40mMK⁺ on this quicent stomach muscle caused very small contracture, which was abolished with the addition of 1ml of G.M. and both fungal cultur broths (Figure 7). In addition, FS-responses of stomach muscles were slightly decreased with 20mMK⁺. However, addition of 1ml of G.M. and both fungal culture broths did not affect FS-responses nor K⁺-contractures (Figure 8).

The toad heart muscle produced natural activites which were inhibited with K⁺-additions up to 40mM, while 80mMK⁺-induced small contracture. After this treatment, the muscle did not retrun to normal even after several washing with normal saline (Figure 9).

The heart muscle responses were slowed slightly by the additions of 0.5-1ml of G.M. On the other hand, *A. flavus* culture broth inhibited heart muscle responses, while *A. niger* culture broth increased heart responses and caused disturbance to their sequences. Moreover, Figure 10 showed also that the addition of 1ml of *A. niger* culture broth induced small contracture and caused more acceleration. Figure 11

showed the effect of 20mMK⁺ on heart muscle responses in the presence of the G.M. and 0.5-1ml of the fungal culture broth. Moreover, additions of both G.M. and 20mMK⁺ did not affect heart responses, while additions of *A. flavus* culture broth slowed heart responses. However, pretreatment with *A. niger* culture broth, 20mMK⁺ dropped base line tension and decreased heart responses. After washout with normal saline the heart muscles resumed their normal activities.

In field stimulation (FS), K⁺-additions (5-200mM) induced heart muscle contractions, while normal heart activities were inhibited (Figure 12). Data presented in this study showed the mean, the standard error and number of replicates of K⁺ additions (Figure 13). However, additions of G.M. increased FS-responses of heart muscle, but additions of *A. flavus* and *A. niger* culture broths and 20mMK⁺ were without any effect (Figure 14).

DISCUSSION

It is a common observation that most smooth muscles develop in response to high K⁺-saline, a contracture exhibiting obvious phasic and tonic components. The development of tension in mammalian smooth muscle reflects an increase in the free ionized Ca²⁺-concentration of the sarcoplasm (Langton & Huddart, 1987). The strong dependence of visceral muscles on [Ca²⁺]_o for the maintenance of activity has led to the view that influx of Ca²⁺ to the cells is an essential requirement for the excitation-contraction (EC) coupling mechanisms (Cheng, 1976; Huddart, et al, 1984). The present study showed that the stomach muscles of the toad (*B. tibiamacus*) were responded to K⁺-additions and the responses were dose-dependent. However, above 80mMK⁺, the relationship between excitation-contraction was uncoupled.

The low K⁺-induced twitch activity is initiated by Ca²⁺-media influx by a population of fast readily-inactivating Ca²⁺-channels. Langton and Huddart (1987) reported that as [K⁺] is elevated twitch is initiated and with incremental addition there

is an increase in amplitude and frequency of the twitches. At higher concentration twitches activity is inhibited and tension falls to a stable quiescent tonic phase. Similar results were seen in rat vas deferens by Huddart, et al (1984). In rat ileal smooth muscle, Syson and Huddart (1976) showed that low K^+ -concentration initiated a burst of Ca^{2+} -dependent spike firing, while higher K^+ -levels suppressed the spike as the membrane significantly depolarized. Huddart, et al (1984) reported that with progressive depolarization that fast-channel population becomes voltage inactivated and the slow voltage-dependent Ca^{2+} -channel population opens, this being the population responsible for sustained tonic tension.

Hurwitz, et al (1980) and Hurwitz (1986) reported that many smooth muscles, can be stimulated to contract by exposing them to a membrane-depolarizing high K^+ -bathing medium, which activated at least two different types of voltage-dependent (or K^+ -activated) Ca^{2+} -channels in the smooth muscle cell. Ratz and Flaim (1982) concluded that Ca^{2+} -channels in a high K^+ -medium may play an important role in smooth muscle cells that normally contract in response to graded depolarizations of the plasma membrane. Batra (1976) reported that the final outcome in the contractile activity of the muscle will be governed by the level of the free Ca^{2+} in the myoplasm.

Huddart and Butler (1986) and Mutwally (1990) reported that FS-technique provide another Ca^{2+} -dependency of both rat urinary bladder and locust gut divisions respectively. Natural activities, K^+ -contractures and FS-responses of rat smooth muscle and locust visceral muscles are dependent upon $[Ca^{2+}]_o$ (Huddart & Butler, 1986; Mutwally & Jamel Al-Layl, 1992 & 1993). The results of this present study also goes in harmony with previous studies and confirmed above conclusions.

The action potential of the frog heart was reported to be affected by alterations of external K^+ (Pappano & Rembish, 1970). At low concentration, the normal heart activities in this study were inhibited, while 80mMK⁺-induced large contraction. It was reported that the total Ca^{2+} -content of heart muscle is close to that found in the plasma (Sands & Winegrad, 1970). It is this Ca^{2+} were ionized and free in the

sarcoplasm due to K^+ -depolarization and field stimulation, the contractile apparatus would be fully activated (Chapman, 1979). There is evidence of two mechanisms which operate to maintain a low $[Ca^{2+}]_i$; an active Ca^{2+} -pump and an ionic exchange system in the cardiac system which are: (a) The membrane Ca^{2+} -pump, and (b) The Na^+ - Ca^{2+} exchange (Chapman, 1973 & 1979).

When moulds such as *Aspergillus* are grown on relatively rich media in laboratory culture it is frequently observed that they produced a range of metabolites, some of which are retained in the mycelium and fruiting structures, but the majority of which are secreted into the media. *A. niger* was found to produce malformin- A_1 , which have been shown to be toxic to mammals (Anderegg, et al, 1976; Moss, 1977). They reported that the isolation and characterisation of malformin from *A. niger* with a significantly higher malforminic toxicity (i.p. LD_{50} 0.9mg / Kg b.w. newborn rats and 0.87mg / Kg b.w. in the case of 28 days).

Betina (1984) reported that mice-trembling was caused by substances extracted from the mycelial mat and sclerotia of *A. flavus* but not from spores or from the culture broth. The present study also showed that both preparations contracted naturally in normal saline, but the addition of the G.M. slightly slowed the rhythm of these normal activities. This action may be due to differences in ionic composition between the G.M. and the normal amphibian saline.

The addition of *A. flavus* culture broth on stomach muscle accelerated the Sp.Act and inhibited heart muscle responses. On the contrary, *A. niger* culture broth inhibited Sp.Act and accelerate the heart beats. These results indicated that both tissues may depend on $[Ca^{2+}]_o$ and may also suggest that the G.M. may slow ions-exchange through cell membrane, while both fungal cultural broths may affect cell membrane and other cellular loci. Moreover, it could be speculated that *A. flavus* culture broth may contain one or more factors (may be aflatoxin- B_1) (Lancaster, et al, 1961), which may activate intracellular Ca^{2+} -stores of the toad stomach muscle, whereas, it may inactivate these intracellular Ca^{2+} -stores of the toad heart muscle. However, *A. niger* culture broth may contain one or more factors (may be malformin-

A₁) (Anderegg, et al, 1976), which may affect Ca²⁺-channels, cellular membrane, cellular loci, and / or other ion-channels.

The initiation of contraction occurs when Ca²⁺ is released in the vicinity of the myofilaments from some elements of the sarcoplasmic reticulum (SR) and relaxation follows depletion of myofibrillar Ca²⁺ and its reaccumulation in the SR (Weber, et al, 1963). The electrical stimulation and excitation-contraction coupling in the frog ventricle is modified by the adrenergic and cholinergic nerve fibers (Tjioe & Bianchi, 1969). In field stimulation, the heart muscles responded to K⁺-additoin this could be due to nerve ending stimulation which may activate cell membrane and ion-channels located their. These results goes in harmony with the conclusions of Tjioe & Bianchi (1969) and Batra (1976).

In general, the cultural broths of both fungi have toxic effect on toad stomach and heart muscles. *A. flavus* culture broth was more effective on the heart as an inhibitory agent, whereas it has an excitatory action on the stomach muscle. On the other hand, *A. niger* culture broth inhibited stomach muscle responses but it increased heart muscle responses. These two different actions of both fungal culture broths on both muscle preparations may indicate that they may affect different ion-channels and / or different receptors which may confirm previous studies of young (1933) and Tjioe & Bianchi (1969). In the following paper, the authors will study the effect of G.M. and both fungal culture broths on the same animal preparations in Ca²⁺-free and in different solutions.

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FIGURES LEAGENDS

Figure 1 (a-j): Show normal spontaneous activity (Sp.Act) (a, control) of toad stomach muscle, the effect of 5-200mMK⁺ (1-points). Washout (W) with normal amphibian saline (2-points). Upper calibration apply to traces from (a-c) and lower calibration apply to traces from (d-j). Time scales apply to all traces.

Figure 3: Additions of growth medium (G.M.) and both fungal culture broths of *A. flavus* (A.F.) and *A. niger* (A.N.) (0.5-1ml) (1-point) on toad stomach Sp.Act after one day of animal collection. The inhibitory effect was stronger with A.N. culture broth. After washout (W) (2-points) with normal saline the stomach muscles resumed their normal activities. Calibration and time scales apply to all traces.

Figure 4: After 3 days of animal collection, addition of G.M. (1 ml) induced Sp.Act, it also shows that treatment with *A. flavus* (A.N.) and *A. niger* (A.N.) culture broths induced muscle contraction. After washing (W) with normal saline the muscle returned its normal activity. Calibration and time scale apply to all traces.

Figure 5: The effect of G.M., A.F. and A.N. (1ml) culture broths additions on 40mMK⁺-contracture of toad stomach muscles. The responses were strongly inhibited with A.N. culture broth. Calibration and time scales apply to all traces.

Figure 7: The effect of 5 minutes pretreatment of G.M. and both fungal culture broths on 40mMK⁺-responses of toad stomach muscles after 3 days of animal collections. Calibration and time scales apply to all traces.

Figure 8: The effect of 10 minutes of G.M. and both fungal culture broths on 20mMK⁺-responses of toad stomach muscles in FS-responses. Calibration and time scales apply to all traces.

Figure 9: The effect of ascending K⁺-concentrations (5-80mM) on toad heart muscle responses. Calibration and time scales apply to all traces.

Figure 10: The effect of G.M. and both fungal culture broths (0.5-1ml) on toad heart muscle normal activities. Calibration and time scales apply to all traces.

Figure 11: The effect of 5 minutes pretreatment of G.M. and both fungal culture broths (0.5-1ml) on 20mMK^+ -responses of toad heart responses. Calibration and time scales apply to all traces.

Figure 12: The effect of elevated K^+ -concentrations (10-200mM) on FS-responses of toad heart muscle. Calibration and time scales apply to all traces.

Figure 14: The effect of 10 minutes pretreatment with G.M. and both fungal culture broths (1ml) on 20mMK^+ -responses of FS-responses of toad heart muscles. Calibration and time scales apply to all traces.

أثر الوسط الغذائي الفطري على الانقباضات المستحثة بالبوتاسيوم والمجال الكهربائي لعضلات المعدة والقلب للضفدعة المصرية بوفو تيبيامكس في المحلول الفسيولوجي للبرمانيات الطبيعي.

حامد محمد عبدالقارء متولي و محمد ابراهيم محمود

قسم الاحياء كلية العلوم التطبيقية-جامعة أم القرى-ص ب ٣٧١١-مكة-المملكة العربية السعودية

الملخص:

هذا البحث أهتم بدراسة أثر الوسط الغذائي الفطري على الانقباضات المستحثة بالبوتاسيوم والمجال الكهربائي لعضلات المعدة والقلب للضفدعة المصرية بوفو تيبيامكس في المحلول الفسيولوجي للبرمانيات الطبيعي. إضافة الوسط الغذائي الذي نمي عليه فطرة/سيرجس فلافس زاد من تقلصات الانقباضات الطبيعية لمعدة الضفدعة في حين أنها قد ثبطت انقباضات القلب الطبيعية، كذلك ثبط نفس الوسط انقباضات المعدة المستحثة بالبوتاسيوم خصوصا على فترة مابعد النشاط (تونيك)، ولكنها كانت غير مؤثرة على انقباضات عضلات القلب المستحثة بالبوتاسيوم وعديمة الأثر على كلا العضلتين في وسط المجال الكهربائي. من جهة أخرى، فإن الوسط الغذائي الذي نمي عليه فطرة/سيرجس نايجر قد ثبط الانقباضات الطبيعية وكذلك انقباضات المعدة المستحثة بالبوتاسيوم بشدة خصوصا على فترتي النشاط (فيسك) ومابعد النشاط (تونيك)، ولكنها نشطت انقباضات القلب الطبيعية إلا أنها لم تؤثر على انقباضات القلب المستحثة بالبوتاسيوم ولا على العضلتين في وسط المجال الكهربائي. في هذا البحث وجد أن عضلات القلب لم تتأثر بأضافات البوتاسيوم إلا في حالة استخدام المجال الكهربائي. يحتمل أن يكون ذلك بسبب تنبيه التيارات الكهربائية للنهايات العصبية المنتشرة هناك والتي بدورها قد تنشط غشاء الخلية والقنوات الأيونية المتواجدة هناك. أجمالا، يحتمل أن كلا وسطي النمو للفطرتين قد أحدثا أثرا سميا على انقباضات عضلات المعدة والقلب للضفدعة المصرية بوفو تيبيامكس. لكن كان أثرهما متضادان على انقباضات كلا العضلتين، يحتمل أن يكون سبب ذلك لتأثيرهما على قنوات أيونية مختلفة أو على نهايات المستقبلات العصبية المنتشرة على كلا العضلتين والتي يكون سببها هو الاختلافات الجوهرية في التركيب العضلي والتوزيع العصبي على كلا العضلتين.