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BRADYKININ FORMING CAPACITY OF OVERSULFATED CHONDROITIN SULFATE CONTAMINATED HEPARIN: *IN VITRO* STUDIES.

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ABSTRACT

Oversulfated chondroitin sulfate (OSCS) contaminated heparin has been associated with

severe anaphylactoid reaction (AR), mainly in dialysed patients. Although attributed to

bradykinin (BK) released during contact system activation by OSCS, no definitive

evidence exists until now for a BK release during incubation of contaminated heparin

with human plasma. In this study, we investigated the kinin forming capacity of OSCS

and OSCS contaminated heparin when incubated *in vitro* with a pool of human plasma.

At 100µg/mL, OSCS liberates BK in a profile similar but not identical to dextran sulfate,

a well known activator of the plasma contact system. The results have highlighted that

the quantity of BK accumulated during contact system activation depends not only on the

concentration of OSCS but also on the plasma dilution and the presence of an angiotensin

converting enzyme inhibitor. We demonstrate a highly significant correlation between the

concentration of OSCS present in the contaminated heparin and BK released

concentration. In conclusion, for the first time, we show that OSCS contaminated heparin

incubated with human plasma has the capacity to liberate BK at a concentration that

could explain the role of this inflammatory peptide in the pathophysiology of AR

associated with OSCS contaminated heparin.

Disclaimer: The findings and conclusions in this article have not been formally

disseminated by the Food and Drug Administration and should not be construed to

represent any Agency determination or policy.

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1. Introduction

Anaphylactic-like or anaphylactoid reactions (AR) have been associated with the

injection of heparin manufactured in China, late in 2007 and in 2008 [1, 2]. Hundreds of

AR were observed mainly in the United States and in Germany, and 159 deaths were

reported to the Food and Drug Administration (FDA) [3]. These AR, observed mainly in

hemodialysis patients at the beginning of the dialysis session, were characterized by

hypotension, tachycardia, angioedema, urticaria and nausea. Similar symptoms have been

reported in patients dialyzed with a negatively charged membrane (made primarily of

polyacrylonitrile) and simultaneously treated with an angiotensin converting enzyme

inhibitor (ACEi) [4, 5].

Physicochemical investigation of the different batches of heparin associated with these

AR revealed that these heparin solutions were contaminated with oversulfated

chondroitin sulfate (OSCS) [6], a synthetic product obtained by oversulfatation of the

natural chondroitin sulfate A (CSA) [7]. Synthetic OSCS and the OSCS present in

contaminated batches of heparin obtained from the marketplace by the FDA were

investigated for their capacity to activate the plasma contact system which can lead to the

release of bradykinin (BK), a vasoactive peptide potentially responsible for the symptoms

associated with these AR. These authors measured a serine protease activity using a

synthetic substrate to assay the contact system activation [8]. As noted by Ramaciotti *et*

al., however, no definitive evidence has existed for BK release during human plasma

contact with OSCS and/or OSCS contaminated heparin [9]. In this work we demonstrate

the BK release by OSCS in the presence of heparin.

The contact system of plasma consists in 3 proenzymes: Hageman factor (factor XII,

FXII), factor XI (FXI) of the coagulation and prekallikrein (pKK). PKK and FXI

circulate in plasma complexed with a cofactor, high-molecular-weight kininogen (HMW-kininogen). The contact of plasma with a negative surface like dextran sulfate (DxS) [10, 11], glass beads [12], or polyacrylonitrile membrane [13, 14] leads to the binding of FXII and HMW-kininogen. This binding of FXII is responsible for its auto activation. Activated FXII (FXIIa) in turn activates FXI and transforms pKK into an active serine protease kallikrein (KK). Both FXIIa and KK are responsible for the amidolytic activity reported earlier by Kishimoto *et al.* [8]. KK hydrolyses HMW-kininogen and releases BK. BK is a powerful vasodilator and proinflammatory nonapeptide which exerts its pharmacological activities by stimulation of B2 receptors. Importantly, BK has a very short half life due to the presence of peptidases in the circulation. In human plasma, BK is primarily metabolised by angiotensin converting enzyme (ACE) and secondarily by aminopeptidase P (APP) or carboxypeptidase N (CPN) [12].

The objective of our study was to measure the kinetics of BK release and to characterize BK immunoreactivity in human plasma incubated with OSCS. As a control we compared the values observed for OSCS with DxS, a well known contact system activator [10, 11]. As the AR associated with OSCS contaminated heparin occurred mainly in the first minutes of dialysis session [1], we also studied the effect of the plasma dilution on the amount of BK released in the presence of an ACE inhibitor, a cardiovascular drug commonly used in dialysed patients. Finally, we quantify the BK forming capacity of different batches of OSCS contaminated heparin responsible for AR and we compare this effect with reference heparin spiked or not with increasing concentrations of synthetic OSCS.

2. Material and methods

The experimental protocol was approved by the Ethics Committee of the Université de Montréal

2.1. Reagents

OSCS was synthesized by the US Food and Drug Administration/Division of Pharmaceutical Analysis (FDA/DPA) laboratory in St-Louis [7]. Different batches of OSCS contaminated heparin were collected from the marketplace by FDA consumer safety officers and sent to the FDA/DPA in St-Louis for analysis. OSCS levels were measured using a strong-anion-exchange-HPLC method [15, 16]. DxS, chondroitin sulfate A (CSA) and chondroitin sulfate B (CSB, aka dermatan sulfate or DS) and vehicle (phosphate buffered saline; PBS) were from Sigma Aldrich (Oakville, ON, Canada). The ACEi, enalaprilat, was obtained from Merck Frost Canada (Kirkland, QC, Canada). Reagents of analytical grade were purchased from Fisher Scientific (St-Laurent, QC, Canada) unless specified otherwise.

2.2. Plasma collection

The different activation processes were assayed on a pool of plasma samples (n=5) obtained from healthy volunteers. Eighteen millilitres of total venous blood were collected using a polypropylene catheter from the forearm of each donor into a polypropylene tube containing 2 mL of 0.1 M sodium citrate pH 7.4. After centrifugation (22 °C, 15 min, 2500 g) the plasmas were decanted, pooled and stored at -80 °C until *in vitro* experiments. On the day of experiment, the pool of plasmas was thawed and vortexed carefully. This protocol has been shown to avoid *in vitro* contact system activation [12].

2.3. Preparation of stock solutions of the different activators of the contact system

A stock solution of the different activators to be tested (DxS, OSCS, CSA, CSB, non contaminated and OSCS contaminated heparin) was prepared in standardized conditions: Solutions of 10 mg/mL in PBS buffer in polypropylene tubes were made. These stock solutions were stored at -80 °C until use. At the time of use, these different solutions were thawed in the same conditions: 60 min at room temperature. Then, after vortexing 30 sec, they were diluted to the tested concentrations.

2.4. Incubations of plasma

The different incubation processes were made at 37 °C in polypropylene tubes and used 1 volume of activator solution for 9 volumes of plasma pre-incubated for 20 min with an ACEi, enalaprilat at a final concentration of 520 nM [12].

2.4.1. Comparative effect of DxS and OSCS on BK release during plasma activation

900 µL of pre-incubated plasma was mixed with 100 µL of OSCS or DxS stock solution to obtain a final concentration of 1, 10, 100 or 1000 µg/mL and incubated with agitation at 37 °C. At different intervals (0, 5, 10, 20, 40, 60 and 120 min), 50 µL of the incubation mixture was added into 2 mL of ice-cold 80% ethanol. Samples were incubated for 1 hr on ice before centrifugation for 15 min at 3000 g and 4 °C. Supernatants were collected and evaporated to dryness over-night in a Speed Vac Plus (Savant, Farmingdale, NY, USA). A similar incubation procedure was used to test the kinin forming capacity of CSA or B and heparin at a final concentration of 100µg/mL. This time course of incubation and samples processing were used for the different incubations described here after.

In each case, 9 volumes of plasma added with 1 volume of PBS buffer, preincubated with

enalaprilat and incubated in polypropylene tube under the same conditions were used as a

control.

2.4.2. Effect of plasma dilution and ACE inhibition on the profile of BK released

during contact system activation by OSCS.

Successive dilutions of plasma (90,80,60,40 and 20% with Tris 50mM/NaCl 150 mM pH

7.4), pre-incubated with enalaprilat as described above were mixed with OSCS or DxS to

obtain a final concentration of 100 µg/mL. To define the effect of ACE inhibition, 900

µL of diluted plasma was pre-incubated or not with the ACEi and these samples were

incubated with OSCS at the 100 µg/mL final concentration.

2.4.3. Kinin forming capacity of non contaminated heparin and of the different

batches of OSCS contaminated heparin

 900 µL of diluted plasma pre-incubated with enalaprilat were incubated with the

different batches of heparin summarized in Table 1. Among the different batches of

active pharmaceutical ingredients (API) obtained from the marketplace, heparin API

samples containing 24.3, 16.7 and 3.1 weight percent OSCS had been used to formulate

drug products which have been associated with severe AR. Here, the different samples

were tested at a total 410 µg/mL concentration of heparin in the incubation medium. The

410 µg/mL concentration of heparin corresponds to 100 µg/mL of OSCS for the most

(24.3%) contaminated sample (sample #10).

2.5. Quantification of immunoreactive BK

Residues of evaporated ethanolic extracts were re-suspended in 50 mM Tris/HCl buffer,

pH 7.4, containing 100 mM NaCl and 0.05% Tween-20. After re-suspension, BK was

quantified by a specific competitive enzyme immunoassay, as described previously [17, 18]. This method has been validated and its analytical performances reported [19].

2.6. Physicochemical and pharmacological characterization of immunoreactive BK

Residues of the evaporated ethanolic extracts containing immunoreactive BK following diluted plasma activation by DxS or OSCS (100 µg/mL), contaminated heparin (sample #10, 100 µg/mL of contaminant) or without any reagent in presence of enalaprilat were used for the physicochemical and the pharmacological characterization. This physicochemical characterization was obtained by HPLC separation combined with competitive enzyme immunoassay as described in Moreau et al. [20]. Retention time of each immunoreactive fraction was compared to that of standard peptides.

The BK-like pharmacologic activity of immunoreactive BK was measured using an ERK1/2 MAP kinase phosphorylation assay in HEK 293 cells stably expressing the rabbit B_2 receptor conjugated to green fluorescent protein (B_2R-GFP) [21]. This assay is responsive to nanomolar BK concentration and, as a B_2 receptor mediated response, should not respond to any of BK fragments which exhibit very low affinity at this receptor. Briefly, HEK 293 cells stably expressing B_2R-GFP grown in $25 cm^2$ flasks were treated for 10 min with the dried extracts of the treated plasmas, diluted in sterile saline, combined or not with the B_2 receptor antagonist LF16-0687 (gift from Laboratoires Fournier, Daix, France[22]), in the regular, serum-containing culture medium. Total cell lysates were recovered and analyzed after SDS/PAGE (9% gel, general methods as in [23]) and protein transfer using anti-phospho-ERK1/2 and -total ERK 1/2 (respectively, monoclonals and polyclonals, Cell Signaling Technology, 1:1000 for each) that were

used in conjunction with appropriate horseradish peroxydase-conjugated secondary

antibodies.

2.8. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The quantity of BK

released in different experimental conditions was used to calculate the area under curve

(AUC) of the kinetic profile. The AUC for BK was calculated by summing the area of the

different quadrilaterals obtained by joining kinin concentrations measured at consecutive

times calculated by GraphPad Prism 4.0 software. Two sample t tests were used to

compare the AUC and the peak concentration of BK released. A $p \le 0.01$ was considered

significant for the different comparisons [24].

3. Results

3.1. Comparative BK forming capacity of OSCS and DxS in human plasma.

Figure 1A shows the kinetic profiles of BK released in the pool of plasma when

incubated with DxS or OSCS at a 100 µg/mL final concentration. The peak concentration

of BK released during the activation of plasma by OSCS (115 ± 28 pmol/mL) is

significantly lower than this measured with DxS (377 ± 42 pmol/mL). Moreover, this

peak is observed later (19.2 ± 0.6 min) than that of DxS (7.2 ± 0.4 min). The area under

the curve (AUC) which represent the total BK released during the 120 min incubation

process with OSCS at concentrations ranging from 1 to 1000 µg/mL are illustrated in

figure 1B along with these measured for DxS. These total BK released showed that

incubations with DxS concentrations of 1 or 10 µg/mL leads to a BK accumulation in the

nmol range (1.86 ± 0.02 nmol and 1.58 ± 0.03 nmol). These BK accumulations reach

rapidly a plateau with the highest DxS concentrations in plasma (11.38 ± 0.72 nmol and 10.76 ± 0.15 nmol for 100 µg/mL and 1000 µg/mL DxS respectively). In contrast, the AUC of BK released in plasma incubated with 1, 10, 100 and 1000 µg/mL of OSCS demonstrated a dose-dependent profile (respectively 1.31 ± 0.02, 2.84 ± 0.02, 5.31 ± 0.49 and 9.15 ± 0.09 nmol of BK). A concentration of both activators as low as 1 µg/mL leads to the release of a quantity of BK (1.86 ± 0.02 and 1.31 ± 0.02 nmol) significantly higher than this measured in the plasma control incubated in the same conditions (0.38 ± 0.02 nmol of BK).

With the same incubation conditions, the quantity of BK measured in presence of 100 µg/mL of CSA, CSB and heparin (respectively 0.57 ± 0.01, 0.63 ± 0.01 nmol and 2.38 ± 0.06 nmol of BK) was significantly lower than that observed for OSCS at 100 µg/mL (5.31 ± 0.49 nmol) although significantly higher than that measured in absence of any activator (0.38 ± 0.02 nmol) (Figure 2B). However, for CSA, CSB and heparin, no peak concentrations in the kinetic profiles were observed, in contrast to the OSCS activation.

3.2. Effect of plasma dilution on the BK forming capacity of OSCS

The quantity of BK released during the incubation of plasma pool with OSCS (from 5.31 ± 0.49 to 31.88 ± 0.50 nmol) or DxS (from 11.38 ± 0.72 to 40.4 ± 1.14 nmol) increases progressively when plasma is successively diluted from 90 to 20% (Figure 3). Because dilutions in the range of 40 to 60% mimic the dilution of plasma in the dialyser at the connection during a hemodialysis session we chose a 50% dilution of plasma for subsequent experiments.

3.3. Effect of the ACE inhibition on the BK forming capacity of OSCS

With a 50% dilution of plasma, the presence of an ACEi significantly modifies the

metabolic profile of BK released when plasma is incubated with 100 µg of OSCS (Figure

4). Effectively, the second part of these BK kinetic profiles demonstrated that ACEi

slowed BK degradation and concomitantly leads to BK accumulation over a longer

period of time. Due to the inhibition of the main pathway of BK degradation, the AUC in

presence of enalaprilat (12.26 ± 0.21 nmol) was significantly higher than in absence of

this inhibitor (3.93 ± 0.06 nmol).

3.4. Kinin forming capacity of non-contaminated heparin and of the different

batches of OSCS contaminated- or synthetic OSCS spiked-heparin

As illustrated in Figure 5, incubation of 50% diluted plasma in presence of an ACEi and

of 410 µg of heparin containing 100 µg of OSCS leads to a kinetic profile of BK which is

totally different from these obtained in presence of the same concentration of

uncontaminated heparin and for the control plasma. The AUC observed in presence of

heparin containing 24.3% of OSCS (7.4 ± 0.15 nmol) is more than 2.5 times higher than

this measured for heparin alone (2.9 ± 0.33 nmol).

Moreover, no peak concentration of BK could be observed in the kinetic profile of

plasma incubated with non-contaminated heparin, contrarily to plasma incubated with

contaminated heparin. When the BK concentration reached a peak value (92.0 ± 3.7

pmol/mL) in plasma incubated with OSCS contaminated heparin, the maximum

concentration of BK observed in presence of non-contaminated heparin was more than

3.5 times lower (25.4 ± 2.5 pmol/mL).

As shown in figure 6, a correlation could be calculated between the weight % OSCS in

heparin the maximum concentration ($y = 2.04x + 38.10$; $R^2 = 0.93$) or the total quantity (y

$= 0.150x + 3.535; R^2 = 0.87$) of BK released in plasma during the incubation process.

Moreover, batches of OSCS contaminated heparin which were associated with cases of

AR have all led to a significantly higher release of BK in plasma.

3.5. Physicochemical and pharmacologic characterization of immunoreactive BK

released during plasma contact system activation.

After HPLC chromatography of ethanolic extracts of plasma incubated with DxS, OSCS

or contaminated heparin, immunoreactivity could be detected only at the retention time

(24.1 min) which corresponds to the native BK.

HEK 293 cells that stably expressed a recombinant BK receptor (B_2R-GFP) were

exposed to human plasmas (pre-reacted with OSCS, contaminated heparin or DxS)

extracted with ethanol and the ERK1/2 MAP kinases phosphorylation probed via specific

antibodies (Figure 7A). A similar ethanol extraction from control incubated human

plasma did not activate ERK1/2 phosphorylation. Extracts from contaminated heparin- or

OSCS-treated plasmas lost effectiveness for p-ERK1/2 upon dilution of the extracts

(Figure 7B). A calibration with authentic BK estimated the kinin content of the 1:45

diluted plasmas extracts around 10 nM of BK, congruent with the concentration

determined with the BK immunoassay run in the same samples. The pharmacological

activity of treated plasmas was essentially lost when cells were pre-treated with the BK

B_2 receptor antagonist LF16-0687 (Figure 7C).

4. Discussion

In this work, we provide evidence for the first time that OSCS has the capacity to liberate

BK when incubated with human plasma. We show that the quantity of BK released in

presence of OSCS depends not only on ACE inhibition but also on plasma dilution.

Moreover, experiments quantifying plasma activation by different batches of OSCS

contaminated heparin and heparin spiked with different OSCS quantities demonstrated a

correlation between the OSCS contaminant level and the quantity of BK released.

Our results complete a former report which presented an indirect evidence for the

participation of BK in the pathogenesis of these acute and potentially lethal AR

associated with different batches of heparin contaminated with OSCS. *Kishimoto et al.*

[8] measured an increase in amidolytic activity reflecting the enzyme activity of KK and

to a lesser extent FXIIa, during contact system activation when either OSCS or different

batches of contaminated heparin were incubated *in vitro* with human plasma. Increased

enzyme activity doesn't indicate that BK is present in plasma during the incubation

processes, however. In fact, the plasma concentration of BK potentially responsible for

these AR depends not only on BK release but also on the metabolism of the peptide in the

circulation which is decreased in presence of an ACEi [12].

In preliminary assays, we have carefully defined the experimental conditions that yield

reproducible results with the different activators. Because these activators are of

biological origin, heterogeneity can be present and affect the precision and accuracy of

the assays.

In a first set of experiments, we have compared the kinin forming capacity of OSCS with

that of DxS. DxS is a well known activator of the plasma contact system and is currently

used in coagulation tests at a µg/mL concentration [25, 26]. Earlier, we also used DxS at

a similar concentration to measure the amidolytic activity of kallikrein during the

activation of plasma [27, 28].

These incubations were performed in the presence of an ACEi for 3 reasons. First, ACEi blocks the main degrading pathway of BK in plasma leading to a higher accumulation of the B2 agonist during the kinetic contact system activation. Second, as the symptoms of AR associated with OSCS contaminated heparin mimic AR reported in ACEi-treated patients who were dialysed with a negatively charged membrane [2], we proposed that the presence of ACEi could play a role in the pathophysiology of OSCS associated AR because of the similarity in the negative charge density of OSCS and negatively charged dialysis membranes [4, 5]. Finally, Blossom *et al.* [29] have reported that 25 % of dialysed patients who presented with an AR associated with OSCS contaminated heparin were treated with an ACEi.

In support of the hypothesis that both kinds of AR share a similar mechanism, we show a correlation between OSCS concentration and the concentration of BK released in the presence of an ACEi. In addition, the quantity of BK released in the presence of OSCS (final concentration: 100 μg/mL) was lower than that measured in presence of DxS, but was higher than that measured in presence of CSA or CSB and heparin at the same concentration. For concentrations of OSCS as low as 1 μg/mL accumulation of BK in the nmol range could still be measured. These concentrations are higher than these measured in experimental rat models of inflammation triggered by contact system activation [17, 30].

In our studies, the time of BK peak concentration occurs later for OSCS than for DxS. This could suggest the activation of fibrinolysis, another important kinin forming system [31]. Although *Carranza et al.* [32, 33] have recently reported that OSCS enhances activation of plasminogen by tissue plasminogen activator (tPA), we could not show that

OSCS *per se* triggers the kinin forming capacity of plasminogen, contrarily to rtPA (data not shown). These differences could be due to the structural differences between DxS and OSCS. Dextran sulfate is primarily sulfated α-1,6 linked glucose while OSCS is primarily a glucuronic acid 1,3 linked to N-acetylglucosamine disaccharide unit with the disaccharide units 1,4 linked to form the polymer. In addition, the DxS used in this study was 17% by weight sulphur while OSCS was 14% sulphur. Finally the DxS used here has a larger average molecular weight of >500 kDa while OSCS is approximately 18 kDa. As the AR associated with OSCS contaminated heparin occurred in the first minutes of the dialysis session [1, 2], we have tested whether plasma dilution affected the BK quantity detected during the activation process. We observed a progressive increase in BK concentrations when the plasma was diluted from 90 to 20%. Two main causes could explain this accumulation. First, there could be an imbalance between the level of proteases (FXIIa and KK) and the anti-proteases responsible for the inhibition of their serine protease activity (mainly C1 inhibitor) [34]. Second, there could be a progressive decrease in the two pathways responsible for the metabolism of BK in presence of an ACEi: APP and CPN [12].

For testing the different batches of OSCS contaminated or spiked heparin, we chose a plasma dilution of 50% compatible to the dilution of blood in the dialyser at the time of patient connection. Under these conditions, we observed a significant correlation between the concentration of OSCS contaminant in heparin or the added synthetic OSCS, the peak concentration and the total quantity of BK released during the activation of the contact system. Moreover, we found the same correlation for both types of adulterated heparin samples suggesting the ingredient identified in contaminated heparin releases BK in

human plasma in a similar manner to synthetic fully oversulfated CSA (4 sulfates/disaccharide unit).

The physicochemical and pharmacological characterisations of immunoreactive BK also support the participation of BK in the pathophysiology of OSCS associated AR. Previous investigators reported that contaminated heparin induces a transient hypotensive reaction when injected in a small number of pigs [8]. Here we measured BK by immunodetection following HPLC separation, confirming that the immunoreactivity measured during the activation of the contact system corresponds to the native B2 receptor agonist. More importantly, we established the specificity of the OSCS mediated pharmacological effect by demonstrating that the effect was suppressed with a B2 receptor of kinin antagonist, B2 receptor antagonism also prevented the AR associated with polyacrylonitrile membrane [14].

The final question we have to answer is: "Are our results relevant to the pathophysiology of OSCS contaminated heparin adverse events?" The total quantity of pharmacologically active BK released by incubation of plasma with the different batches of OSCS contaminated heparin is significantly higher than non contaminated heparin for a plasma concentration of heparin compatible with that obtained *in vivo* with a bolus injection [35]. These values are the total amount measured by the AUC and correlate with the peak concentrations observed with the different incubation conditions and correspond to the hydrolysis of an equal nmol number of HMW-kininogen during plasma contact system activation. Such quantities of hydrolysed HMW-kininogen (or BK released) are in the same range as those measured in patients treated with an ACEi and presenting an AR while dialysed with a negatively charged membrane [36]. Similarly, *in vivo*, we have

reported that DxS intravenously injected in rabbit treated with an ACEi leads to an important hypotensive reaction corresponding specifically to the hydrolysis of HMW-kininogen and to BK release, as shown by the suppression of the hypotensive reaction by injection of a B2 receptor antagonist [37].

5. Conclusions

In conclusion, the globalization of the heparin supply chain and the OSCS contamination of heparin in 2007-2008 highlight the need for careful screening of heparin for biomaterials that could lead to adverse reactions in patients. In this work, for the first time, we show that OSCS, a synthetic contaminant added to heparin, has the capacity to liberate BK during *in vitro* activation of the contact system at a concentration that is compatible with its participation in the pathophysiology of the severe and potentially lethal AR associated with contaminated heparin. In addition, the experimental approach developed in this paper could be useful in the future to explore any bradykinin-link adverse reactions associated with biomaterials added to heparin for economic gain. Finally, this method could also be used to test for and prevent any unforeseen side effects that could be associated with the bio-engineered heparin now in development [38]

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3

Table I: OSCS concentration in the different samples of heparin.

Sample #	OSCS concentration (w/w)
Heparin spiked with OSCS	
1	0
2	9.7
3	4.7
4	1.1
Heparin contaminated with OSCS	
5	1.3
6*	3.1
7	5.9
8	10
9*	16.7
10*	24.3

* OSCS contaminated heparin causing AR

Captions

LEGENDS TO FIGURES

Figure 1: **Panel A-** Mean kinetic profiles (n=6) of BK obtained when plasma (90%) was incubated with 100 μg/mL DxS (●), 100 μg/mL OSCS (○) and PBS (□) in the presence of an ACEi. **Panel B** shows representative AUCs (n=3) corresponding to kinetic profiles of the control plasma, DxS (■) and OSCS (□) at 1, 10, 100 and 1000 μg/mL. Error bars correspond to SEM; if values are small, bars are missing. *p<0.0001 compared to control plasma.

Figure 2: Panel **A-** Mean kinetic profiles (n=3) of BK measured when plasma was activated with 100 μg/mL OSCS (■), 100 μg/mL CSA (□), 100 μg/mL CSB (○) or 100 μg/mL of non-contaminated heparin (●) in the presence of an ACEi. **Panel B** represents AUCs (n=3) corresponding to the kinetic profiles of panel A. Error bars correspond to SEM; if values are small, bars are missing. *p<0.002 compared to control plasma, ∞p<0.0001 compared to OSCS.

Figure 3: Representative AUCs (n=3) corresponding to total BK released during 120 minutes when 100 μg/mL of DxS (■) or OSCS (□) activates different dilutions of plasma (90,80,60,40 and 20%) in presence of an ACEi. Error bars correspond to SEM.

Figure 4: Mean kinetic profile (n=3) of BK obtained when diluted plasma (50%) was activated by 100 μg/mL OSCS in the absence (●) or in presence (○) of an ACEi. The upper right panel illustrates AUCs corresponding to both kinetic profiles. Error bars correspond to SEM. *p<0.0001.

Figure 5: Mean kinetic profile (n=6) of BK obtained when diluted plasma was incubated with PBS (□), 410 μg/mL of non-contaminated (●) or 100 μg OSCS contaminated (○) heparin in presence of an ACEi. The lower left and right panels represent respectively the AUCs and the maximum concentration corresponding to both kinetic profiles. Error bars correspond to SEM. *p<0.0001.

Figure 6: Plots that demonstrate the effect of heparin contamination (percentage of OSCS) on the peak concentration (A) and total (B) BK released during 120 min incubation of diluted plasma (50%) with 410 μg/mL of non-contaminated heparin spiked with OSCS(■) contaminated heparin(●) or contaminated heparin causing AR (○) with an ACEi (n=6). Error bars correspond to SEM; if values are small, bars are missing. *p<0.01 compared to non contaminated heparin.

Figure 7: Signaling responses induced by human plasma extracts in lysates of HEK 293 cells stably expressing B$_2$R-GFP. **A**. Immunoblots for phospho-ERK1/2 and total ERK1/2 in response to diluted reconstituted plasma extracts (1:45) previously incubated with OSCS, contaminated heparin (CHep), DxS or with no reagent (plasma) corresponding respectively to a separately measured and calculated immunoreactive BK concentration of 5.6, 4.8, 18.1 and 0.0 pmol/mL for the 1:45 dilution of the reconstituted extract. Cells were stimulated for 10 minutes before lysis. Controls (Ctrl) are extracts of unsimulated Hek 293 cells. **B.** Effect of authentic BK or of various dilutions of extracts from contaminated heparin- or OSCS-treated plasmas (10 min stimulations). **C.** Antagonist effect of LF 16-0687 (1 μM; applied 10 min before extracts) on the activation of ERK1/2 induced by human plasma extracts.

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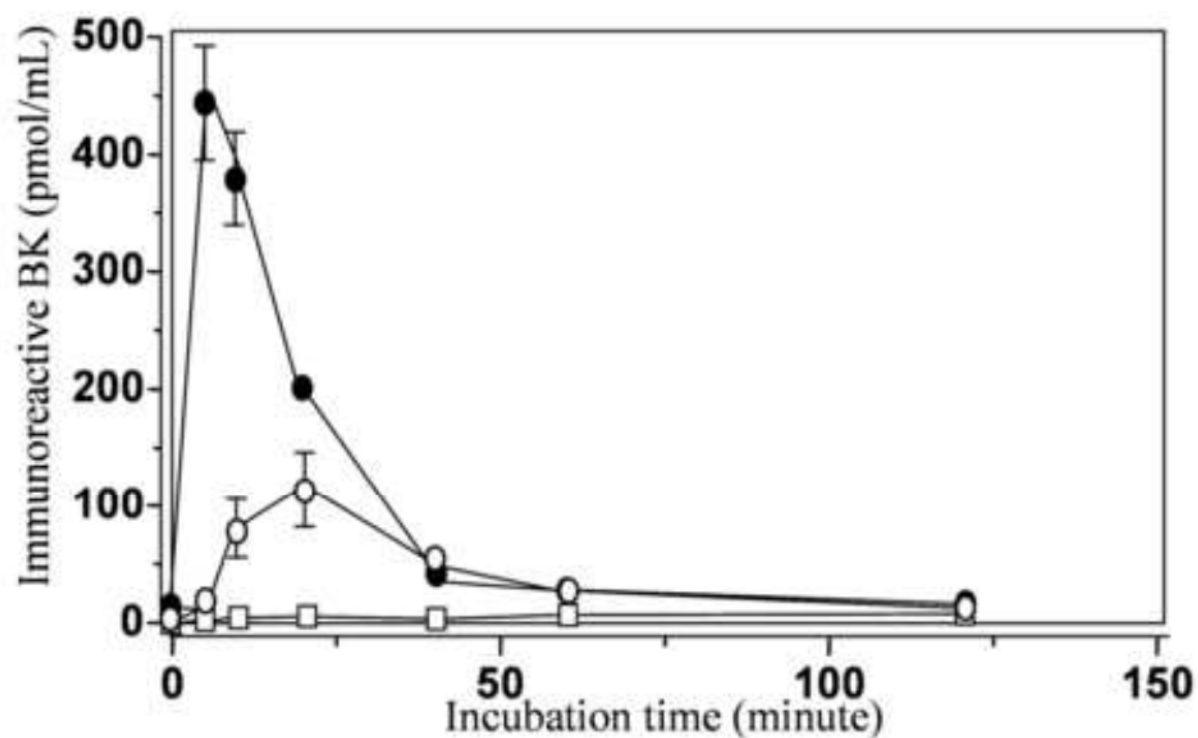
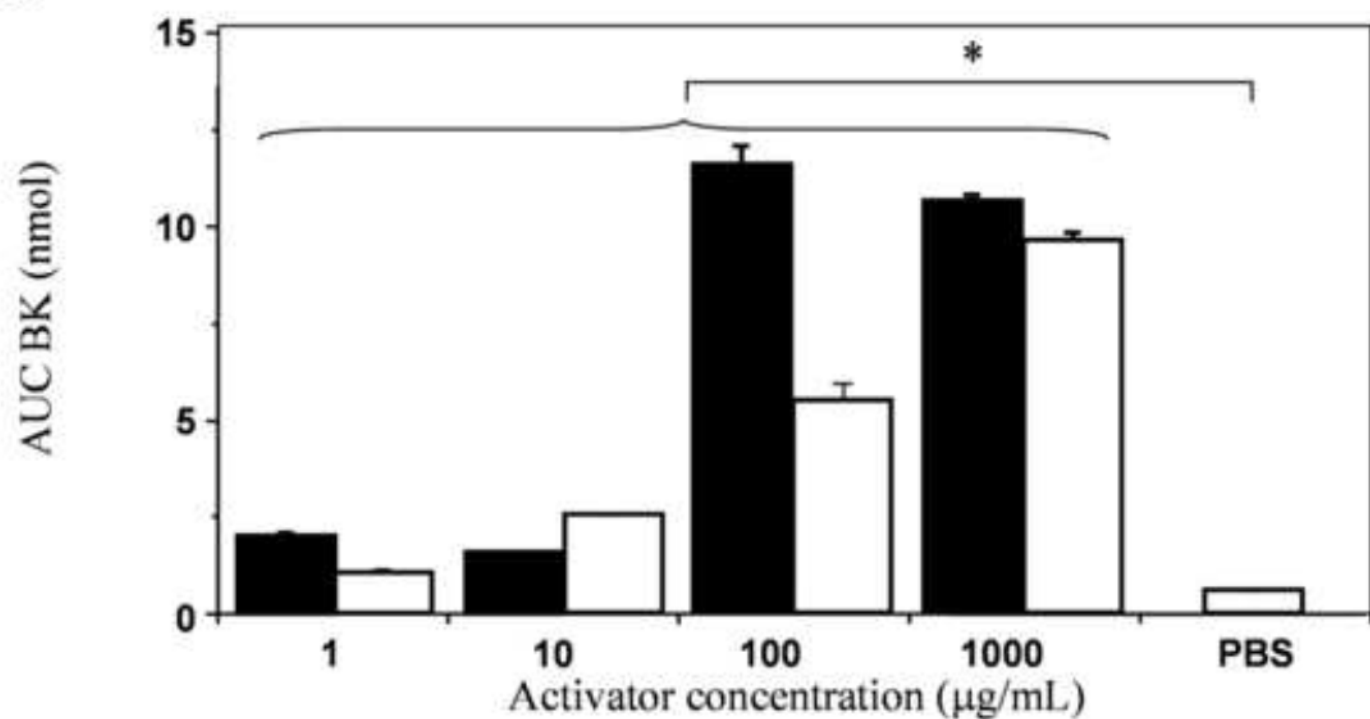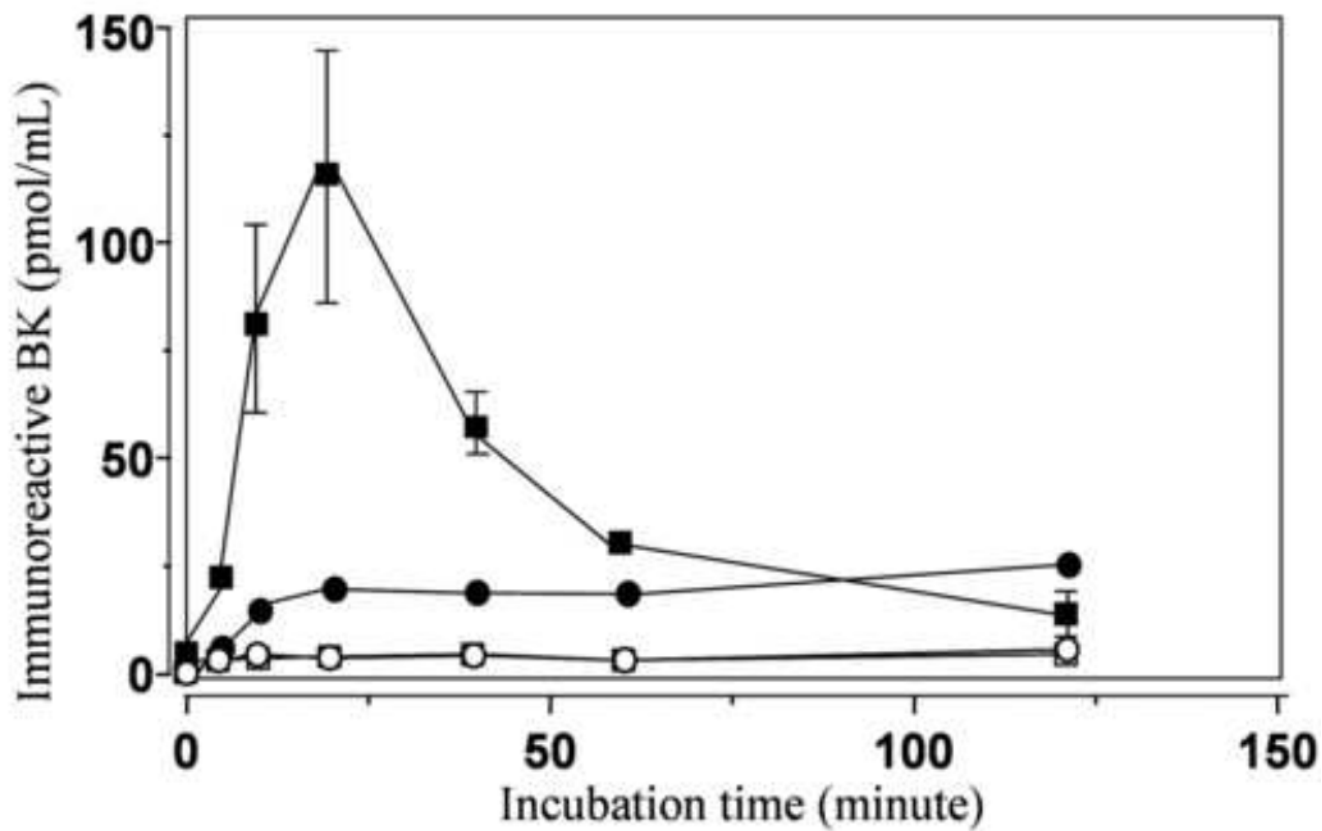**Figure 2**
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A

B

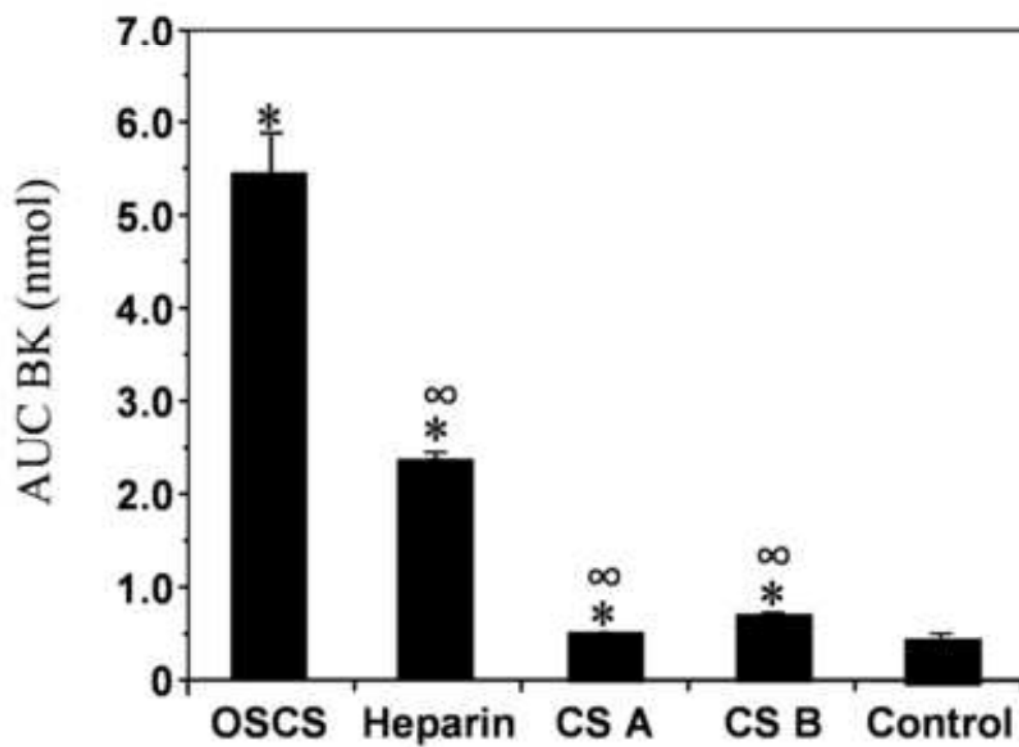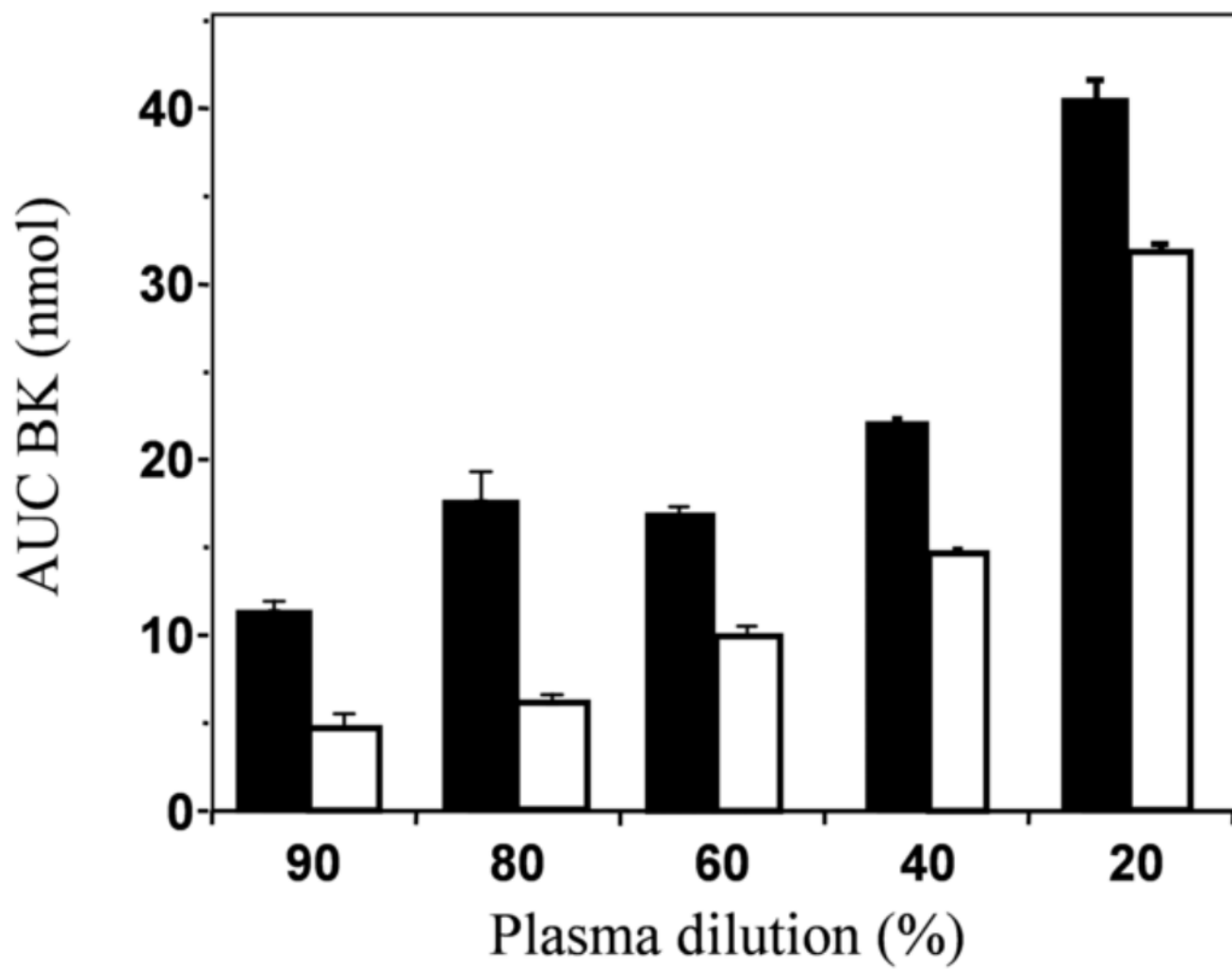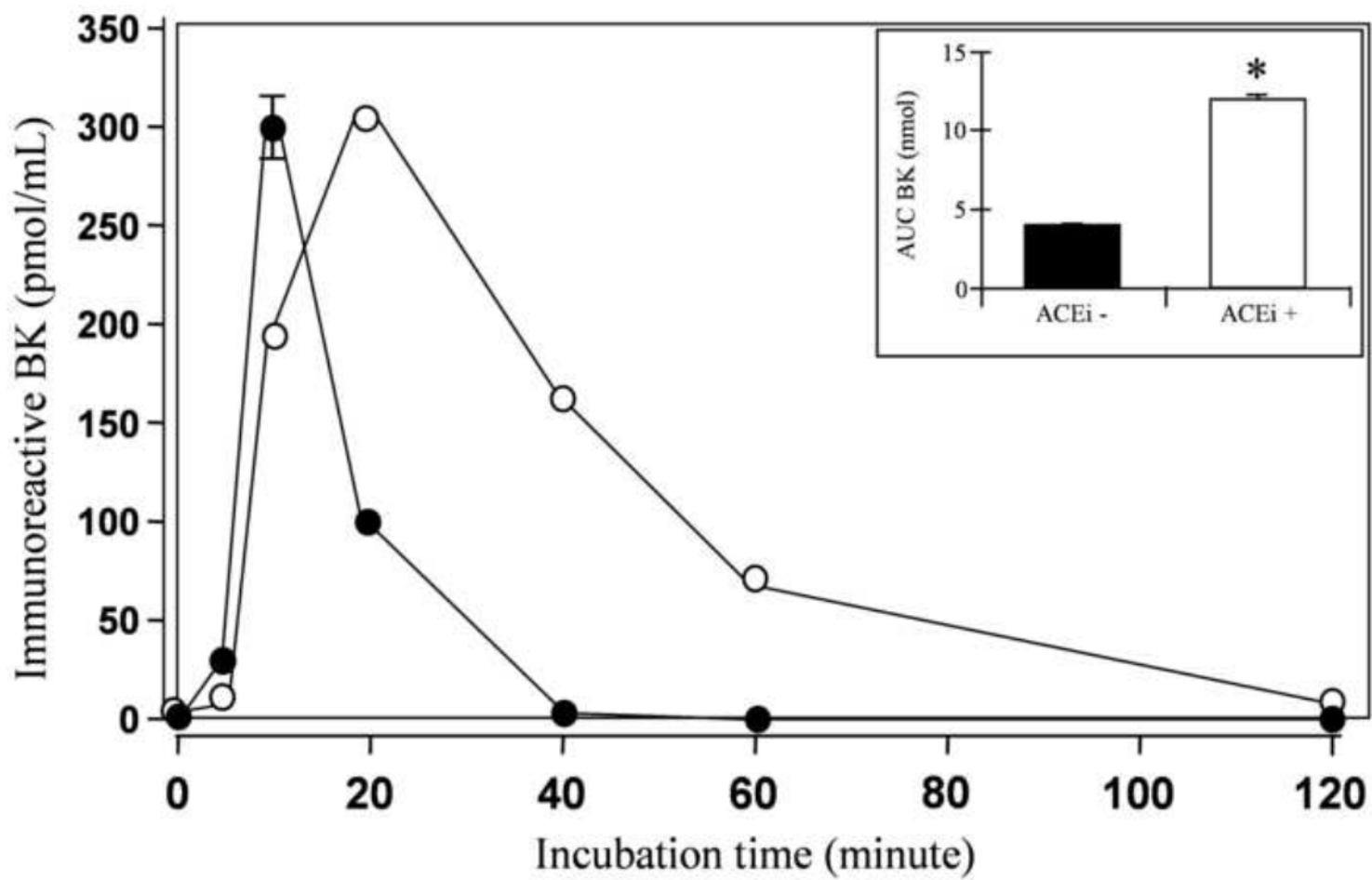**Figure 4**
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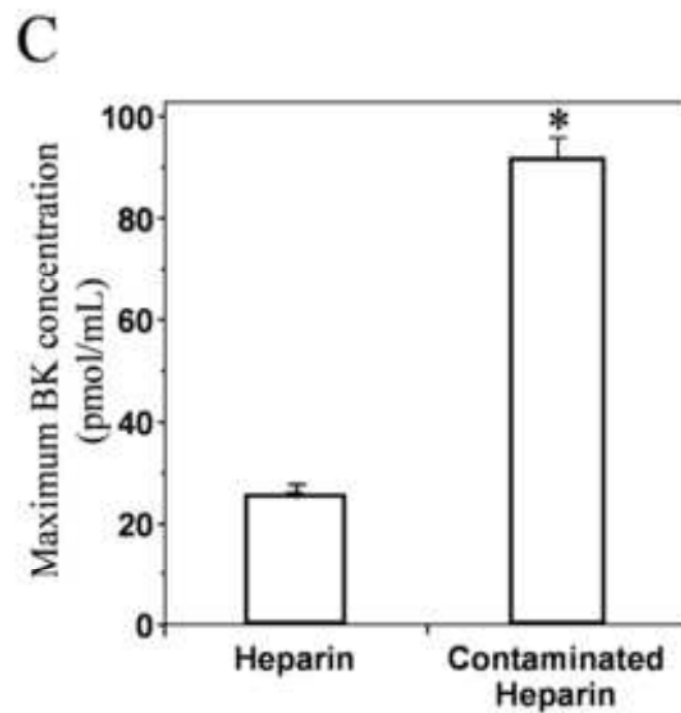
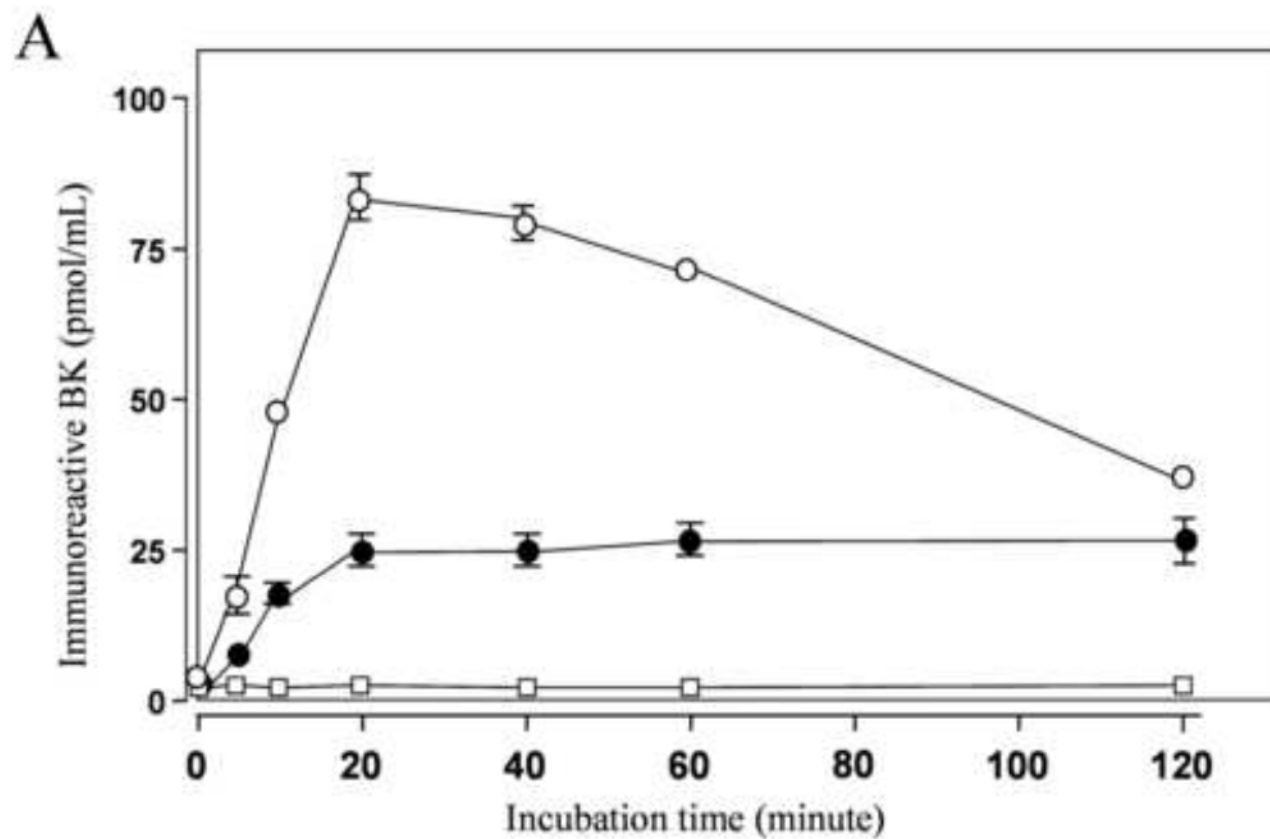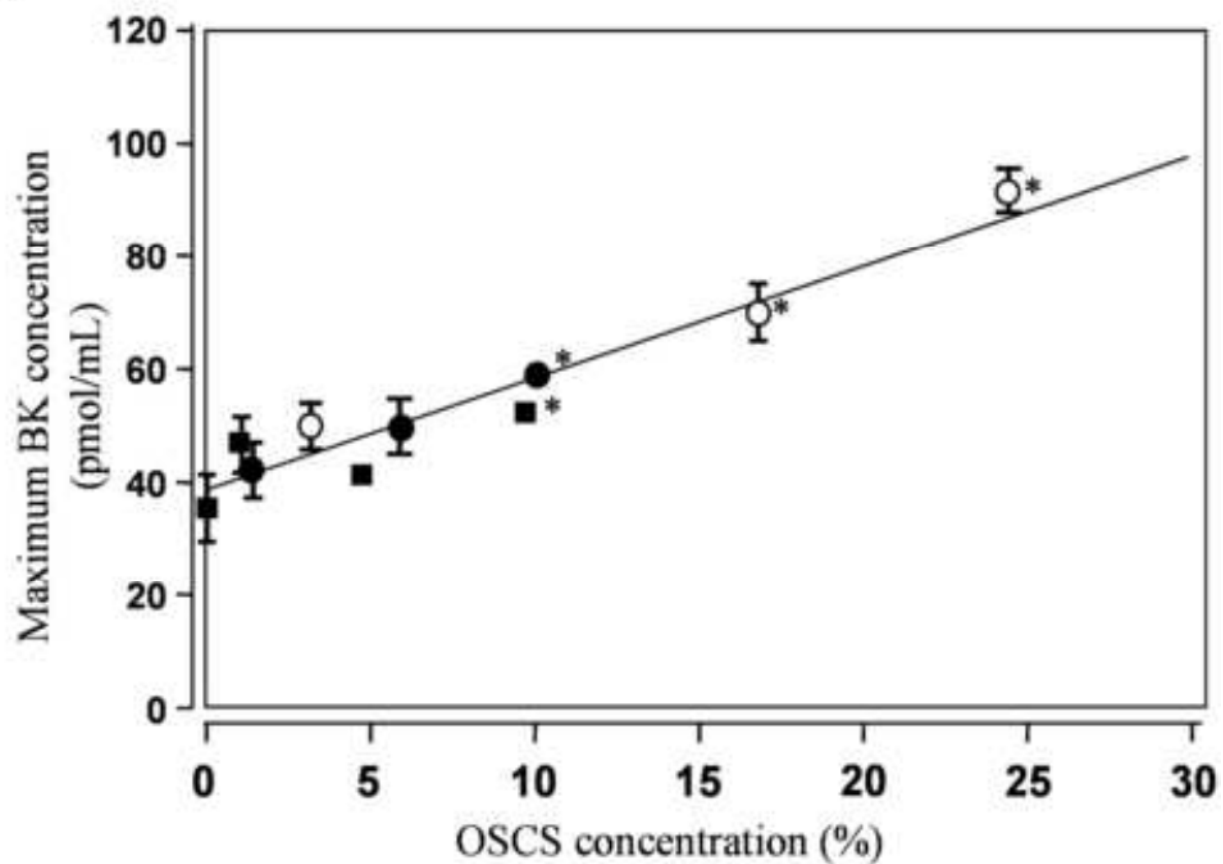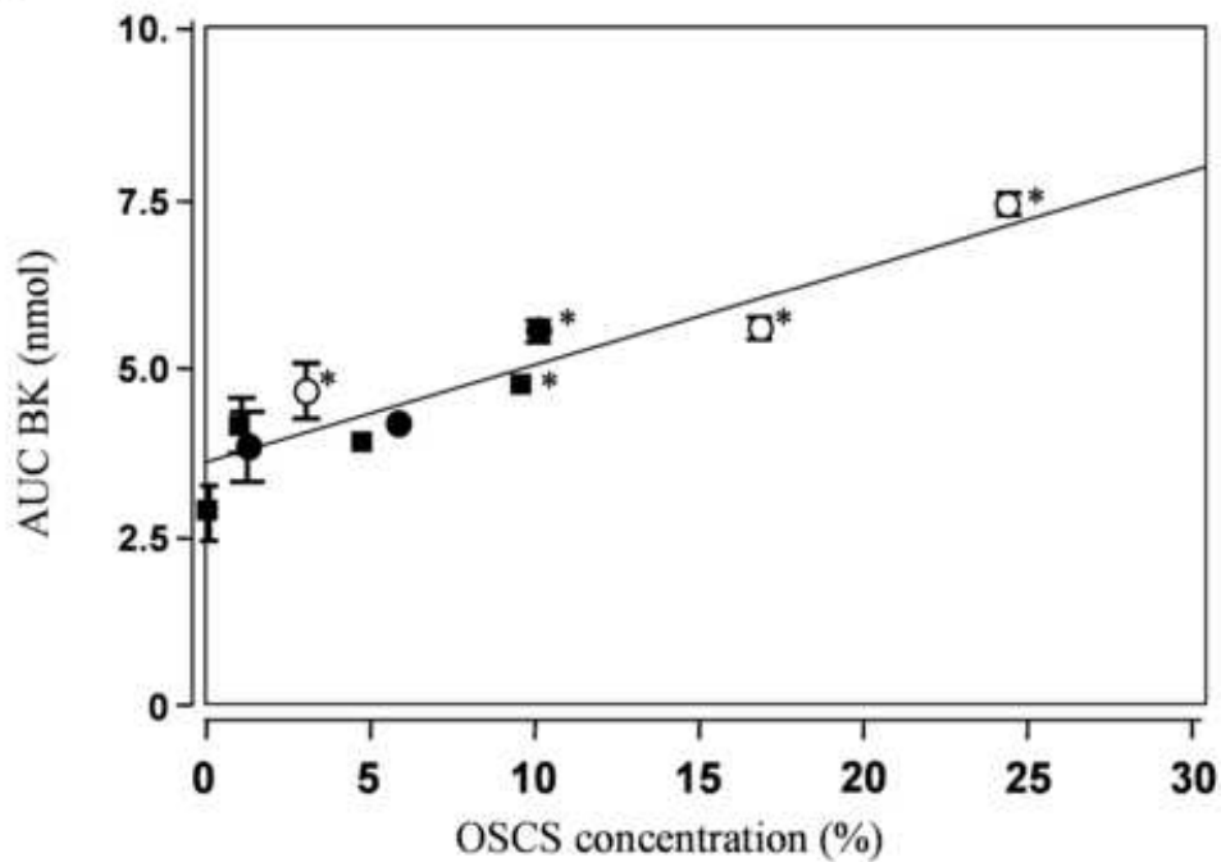**Figure 5**
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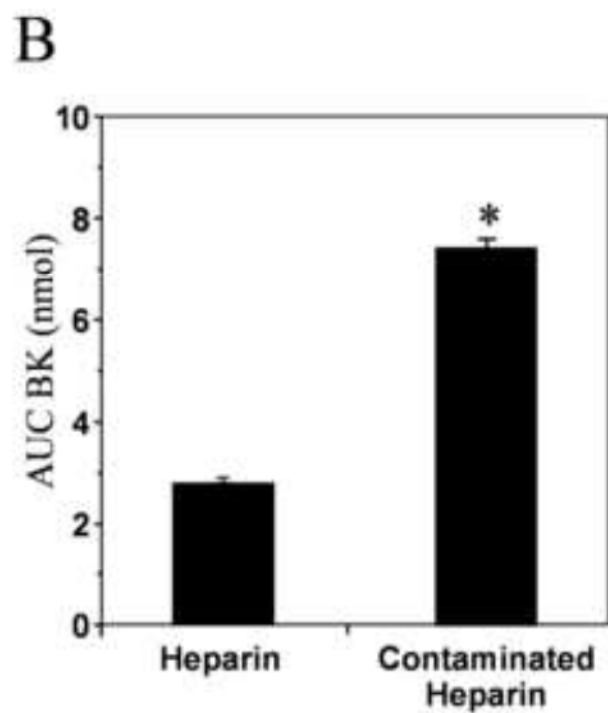
Figure 6
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