Federazione Erboristi Italiani

AUDIZIONE PRESSO LA14ª COMMISSIONE

POLITICHE DELL'UNIONE EUROPEA

SENATO DELLA REPUBBLICA

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REGOLAMENTO (UE) 2021/468 DELLA COMMISSIONE EUROPEA DEL 18 MARZO 2021 CHE MODIFICA L'ALLEGATO III DEL REGOLAMENTO (CE) N.1925/2006 DEL PARLAMENTO EUROPEO E DEL CONSIGLIO PER LE SPECIE BOTANICHE CONTENENTI DERIVATI DELL'IDROSSIANTRACENE

Dott. Angelo Di Muzio

Presidente F.E.I.

Federazione Erboristi Italiani

Confcommercio Imprese per l'Italia

CONFALI ALIMENTARE, INSIEME

Federsalute CONFCOMMERCIO IMPRESE PER L'ITALIA

CONFCOMMERCIO IMPRESE PER L'ITALIA PROFESSIONI

Federazione Erboristi Italiani

Audizione della Federazione Erboristi Italiani sugli effetti dell'entrata in vigore del Regolamento (UE) 2021/468 della Commissione europea del 18 marzo 2021 che modifica l'allegato III del regolamento (CE) n.1925/2006 del Parlamento europeo e del Consiglio per quanto riguarda le specie botaniche contenenti derivati dell'idrossiantracene.

Preg.mo Presidente Stefàno,

Onorevoli Senatori,

con la presente nota il sottoscritto Dott. Angelo Di Muzio, Presidente Nazionale della Federazione Erboristi Italiani e del Settore Produzione Feder Botanicals Italia aderente a Confcommercio Imprese per l'Italia, intende mettere al corrente il Presidente Sen. Dario Stefàno e gli Onorevoli Senatori della 14ª Commissione Permanente Politiche dell'Unione Europea delle forti criticità, riguardanti la categoria rappresentata, emerse a seguito dell'approvazione e dell'entrata in vigore del citato Regolamento (UE) 2021/468. Intendiamo infatti evidenziare le carenze di applicazione e le gravi conseguenze derivanti, riportando le evidenze scientifiche che non supportano le decisioni relative di messa al bando di numerosi prodotti naturali.

A tale scopo è necessario fare riferimento all'Opinione Scientifica EFSA, su richiesta della Commissione europea, del 22 novembre 2017 e pubblicata sull'EFSA Journal il 23 gennaio 2018, che in sintesi evidenzia che:

"Alcune sostanze appartenenti a un gruppo di ingredienti vegetali noti come derivati dell'idrossiantracene possono danneggiare il DNA ed eventualmente causare il cancro al colon retto", questo è quanto afferma l'EFSA a seguito della valutazione della sicurezza quando queste sostanze vengono aggiunte agli alimenti.

Ma di quali sostanze si sta parlando?

Si tratta di derivati comunemente presenti in molti estratti vegetali di ampio e lungo impiego come aloe, rabarbaro, senna, frangula e cascara. Rimedi notissimi e di largo consumo, citati già nell'antichità e nella nostra tradizione erboristica, studiati a livello botanico farmaceutico e fitoterapico e ancora oggi largamente impiegati sotto forma di tisane, estratti in forma di capsule, compresse e utilizzati da un gran numero di persone per regolarizzare la funzionalità intestinale.

Alcuni di questi estratti sono presenti anche sotto forma di farmaci OTC di libera vendita per i loro effetti lassativi e anche in questa forma si tratta di rimedi ampiamente presenti sul mercato, la cui sicurezza è, quindi, confermata da anni di impiego. Non dimentichiamo infine il loro uso soprattutto nel caso di aloe e rabarbaro come aromi e piante da estrazione per amari e digestivi di largo consumo.

Per quale motivo quindi si è arrivati al loro divieto?

Il divieto si basa su un possibile problema di cancerogenicità e genotossicità. In sintesi l'EFSA nella sua Scientific Opinion conclude il suo rapporto ipotizzando che potrebbe esserci un reale rischio per le droghe contenenti derivati idrossiantracenici contenuti nelle precedenti droghe vegetali e considerati responsabili degli effetti tossici. Si riporta tuttavia che permane l'incertezza scientifica di tali effetti che pertanto andrebbero verificati. Infatti, la stessa EFSA non è stata in grado di indicare la dose minima di impiego, ovvero una dose sicura per la salute riguardo i derivati idrossiantracenici. L'assenza di questa indicazione è contraria a qualsiasi approccio farmacologico basato sul rapporto dose/attività. Tutto questo perché la valutazione del rischio effettuata dall'EFSA si basa su studi pubblicati che riguardano l'impiego di sostanze isolate e non all'impiego delle piante come tali. È questo stesso approccio che sta mettendo in discussione anche l'impiego di altre piante officinali come il basilico o il finocchio, e quindi la tisana al finocchio utilizzata anche nei bambini neonati per ridurre le coliche gassose, oppure il vino da diluire per ridurre il danno alcolico e tutto questo basandosi sul fatto che contengono sostanze potenzialmente tossiche o cancerogene ma se assunte isolatamente come tali e non così come invece realmente ingerite nelle preparazioni erboristiche o sotto forma di integratori alimentari.

A livello europeo con il regolamento "Novel food - Regolamento (UE) 2015/2283) - è stato chiarito e condiviso che una sostanza isolata e altamente purificata da un estratto vegetale è da ritenersi "novel food" in quanto il suo profilo biochimico può sensibilmente cambiare. Nonostante ciò si continua a valutare la sicurezza secondo schemi di farmacologia non adatti ad una valutazione obiettiva di composti molto complessi come appunto i botanicals. Non c'è quindi da stupirci se con tale approccio i risultati siano contradditori e le conclusioni assolutamente opinabili.

A seguito della Scientific Opinion Efsa e tralasciando ulteriori passaggi, che ci hanno visti

protagonisti nel presentare ampia documentazione scientifica, la Commissione ha emanato il Regolamento (UE) 2021/468 senza tuttavia prendere in considerazione nessuna argomentazione prodotta che sulla base di evidenze scientifiche metteva in discussione il parere scientifico EFSA.

È inoltre necessario ricordare che almeno un italiano su tre utilizza regolarmente integratori alimentari a base naturale e che l'Italia è leader mondiale nel settore erboristico con migliaia di aziende di produzione e trasformazione e attività commerciali di vendita al dettaglio.

La situazione che si è venuta a creare è piuttosto complessa ed allarmante e alla richiesta da parte delle nostre Autorità di spostare le varie preparazioni di Aloe dalla parte A (divieto) alla parte C (periodo di monitoraggio di 4 anni) dell'Allegato III del Regolamento (CE) n.1925/2006, l'Autorità europea ha opposto un fermo diniego non includendo neanche eventuali soglie di utilizzo, metodiche analitiche validate e, soprattutto, negando qualunque periodo di smaltimento dei prodotti che pertanto ha determinato con l'entrata in vigore del regolamento, l'8 aprile scorso, l'immediata eliminazione dal mercato di tutti i prodotti contenenti aloe creando danni economici a molte aziende che da tanti anni vendono e hanno venduto preparati a base di questa pianta. In altre parole, dopo migliaia di anni di utilizzo e innumerevoli persone, nei secoli come tutt'oggi, che hanno trovato giovamento da questa pianta medicinale, improvvisamente si scopre una enorme pericolosità tossica, che ne determina la necessità di una immediata scomparsa, sebbene stranamente solo dal mercato erboristico.

Infatti quali prodotti sono stati eliminati?

Tutti quelli contenenti aloe o come aroma, o come farmaco o come integratori alimentari. La Commissione europea ha ritenuto che l'unico impiego dei derivati idrossiantracenici che è da ritenersi pericoloso per la salute pubblica sia solo quello sotto forma di integratori alimentari e pertanto l'uso degli stessi come aroma nei liquori e tanto meno come farmaco è da ritenersi sicuro. Come è possibile tutto questo se una sostanza è ritenuta genotossica o cancerogena perché è tale solo se presente in una tipologia di prodotto e non in un'altra anche se le quantità e le concentrazioni sono le stesse o anche superiori?

Quali sono quindi le contraddizioni del regolamento?

Nella parte A delle sostanze vietate sono state inserite oltre l'aloe anche le sostanze:

“Aloe-emodina e tutti i preparati in cui questa sostanza è presente”, “Emodina e tutti i preparati in cui questa sostanza è presente”, e nel decidere questo non si è tenuto in debito conto che l’aloe-emodina e l’emodina sono principi attivi, come confermato dall’EFSA stessa, presenti comunemente in molte diverse altre specie vegetali, utilizzate normalmente a livello alimentare, quali piselli, fagioli, lattuga, cicoria, cavolo, ecc.. Tale decisione quindi dovrebbe comportare l’automatica esclusione dall’utilizzo alimentare di comuni alimenti oltre delle piante officinali oggetto di valutazione.

E’ da notare inoltre che non esistono metodiche analitiche ufficiali per la determinazione di tali sostanze e soprattutto che nessuna metodica analitica sarebbe in grado di discriminare l’origine delle sostanza riscontrate. In pratica è impossibile capire a livello analitico se aloe-emodina e emodina derivino da una pianta presente nella parte A o C dell’Allegato o da qualsiasi altra matrice non indicata nel regolamento stesso. Questo attualmente e alla luce di quanto riportato nel regolamento in esame rappresenta un problema irrisolvibile.

Senza metodiche analitiche ufficiali per ogni singola matrice si incorre in gravi errori di valutazione rendendo il regolamento inapplicabile e aprendo la strada ad un sicuro e costoso contenzioso, rendendo oltremodo complessa ed insicura anche l’attività di controllo.

Con questo regolamento non solo si vanno a penalizzare piante officinali ed integratori alimentari favorenti in modo fisiologico il transito intestinale ma, potenzialmente, tutta una larga serie di prodotti alimentari, dalle marmellate, ai the istantanei a gran parte del settore delle erbe infusionali, bevande varie, ecc. in cui le sostanze incriminate potrebbero essere naturalmente contenute.

Il regolamento, in aggiunta, sembrerebbe escludere le cosiddette sostanze aromatizzanti (es. per la produzione liquoristica) perché disciplinate da altro regolamento, ma sappiamo tutti che l’aloe e il rabarbaro sono per eccellenza le sostanze amare più impiegate nell’industria liquoristica e pertanto la loro presenza, pur sapendo che contengono sostanze potenzialmente cancerogene e genotossiche oltre a generare un’incomprensibile disparità di trattamento, porrebbe le basi per numerose controversie applicative e legali. E’ evidente che se una sostanza è tossica, la sua pericolosità permane indipendentemente dal tipo di prodotto che la contiene, per cui la conseguenza logica è che tutti i prodotti interessati siano in futuro banditi, a meno che non si voglia operare in modo discrezionale, a questo punto non basandosi su evidenze scientifiche, ma su altri piani, ad esempio per puri interessi commerciali di parte.

Ne consegue che è necessario valutare la ricaduta economica che l'applicazione di tale regolamento avrebbe con ripercussioni negative rilevanti su tutta la filiera agroalimentare ed erboristica, dalla coltivazione, raccolta, trasformazione e commercializzazione di importanti specie vegetali come ad esempio l'aloe investendo non solo le aziende italiane, ma producendo ingenti danni anche al mercato extra europeo in particolare per quei Paesi la cui economia è in larga parte basata sulla coltivazione di aloe, senna, frangula, ed altre specie botaniche contenenti derivati idrossiantracenici. L'attuale nuova regolamentazione non tiene in alcun conto il rilevante impatto economico che verrebbe a prodursi.

Nella documentazione tecnico-scientifica che allegheremo a questa nota sintetica andremo ad evidenziare la presenza di numerosi studi clinici a supporto della sicurezza dell'aloe e delle piante coinvolte dal Regolamento 2021/468 dalla cui analisi evidenziamo una mancanza di correlazione tra l'uso di derivati idrossiantracenici e insorgenza di neoplasie, in particolare colon-rettali.

Le dosi normalmente utilizzate per stimolare il transito intestinale, pari a 20 – 30 mg di derivati idrossiantracenici/die non hanno provocato trasformazioni tumorali anche con l'abuso nell'uomo, inoltre, sono di gran lunga inferiori a quelle che non hanno comunque provocato alcun effetto indesiderato negli studi su animali.

A titolo esemplificativo vorrei però qui citare i risultati di uno studio clinico sperimentale *in vivo* prodotto dalla collaborazione tra i Dipartimenti di farmacologia e tossicologia delle Università di Napoli, Salerno e Genova e l'Istituto di anatomo-patologia dell'Università di Genova, in cui si investiga sulla presunta induzione di tumore a livello del colon retto mettendo a confronto l'azione di una pianta contenente derivati dell'idrossiantracene come la cascara e il farmaco bisacodile attualmente in commercio. Lo studio conclude che mentre il bisacodile al dosaggio di 43 mg/kg (43 ng/g) è in grado di indurre tumori al colon, la cascara somministrata a dosaggi di 140 e 420 mg/kg non induce sviluppo di tumori al colon. In particolare i risultati del presente studio indicano un possibile effetto promotore del bisacodile sulla carcinogenesi del colon di ratto (specialmente a dosi più elevate) e assenza di qualsiasi attività promotrice o di avvio di cancerogenesi di una dose ad azione lassativa e diarroica di cascara. Un ulteriore studio della durata di due anni eseguito presso il Dipartimento di Farmacologia dell'Università di Napoli diretto dal Prof. Francesco Capasso, uno dei massimi esperti internazionali a livello di piante contenenti idrossiantraceni, ha messo in evidenza che la somministrazione per due anni di senna, alle dosi di 25, 100 e 300 mg / kg / die nei ratti di ambo i sessi, non ha indotto statisticamente significative differenze a livello di valutazioni istologiche di alcuni tessuti e

nel rapporto di mortalità rispetto al gruppo di controllo. In particolare lo studio in oggetto ha confermato quanto riportato in un precedente studio del 1993 che non esiste alcuna correlazione tra la somministrazione a lungo termine della senna con lesioni neoplastiche intestinali.

Un approccio idoneo alla risoluzione della problematica da parte della Commissione europea, vista la permanenza sul mercato di sostanze aromatizzanti, liquori e medicinali contenenti le piante oggetto del regolamento 2021/468 sarebbe stato quello di individuare un preciso valore soglia rispetto al contenuto di emodina ed aloe-emodina tale da assicurare l'azione fisiologica prevista e delle precise regole di etichettatura degli integratori alimentari favorenti il transito intestinale corredate da avvertenze specifiche e modalità di assunzione eventualmente non superiore a un determinato numero di giorni. In mancanza di tale approccio ragionevole e sicuro la rimozione dal mercato degli integratori alimentari contenenti le specie antrachinoniche sarebbe immediatamente sostituita da medicinali contenenti le medesime piante oltre a dare luogo ad un pericoloso "fai da te" come peraltro già in atto e ad una proliferazione incontrollata di prodotti non regolamentati, ma contenenti le specie botaniche oggetto del regolamento, in vendita su siti internet che si avvantaggerebbero immediatamente della mancata reperibilità di prodotti sicuri e da sempre utilizzati. Ad ogni buon conto giova ricordare che i medicinali contenenti le specie botaniche citate sono largamente venduti in farmacia come medicinali da banco, senza prescrizione medica e liberamente acquistabili, il che non risolverebbe affatto la problematica emersa con l'entrata in vigore del regolamento (UE) 2021/468 **se lo scopo era quello di eliminare dal mercato sostanze cancerogene capaci di generare cancro al colon**.

Ciò che colpisce negativamente chi come noi svolge la professione di erborista e lavora quotidianamente con le piante medicinali e con i loro derivati raccogliendo un'eredità millenaria da ogni parte del mondo è che la Commissione europea possa prendere decisioni così restrittive pur in assenza di evidenze scientifiche omogenee e soltanto per una categoria di prodotti quali gli integratori alimentari.

La/le contraddizioni del regolamento mi auguro possano ora essere chiare.

Ribadisco se davvero ci fosse un pericolo di cancerogenicità/genotossicità riguardante gli idrossiantraceni presenti in piante e alimenti, non quindi isolati, quale ratio ci sarebbe nel proibirli in una sola tipologia di prodotti e lasciarli in libera circolazione in tutti gli altri quali alimenti di largo consumo tipo lattuga e piselli ed altri ortaggi, nei liquori ed amari di

amplissima diffusione, confetture, medicinali vegetali, medicinali vegetali tradizionali, medicinali?

Come è possibile che nella protezione della salute la Commissione proceda a compartimenti stagni?

Questa insolubile contraddizione apre al dubbio che in casi come questo non la salute del cittadino europeo sia stata messa al primo posto, ma che la scelta proibizionistica sia solo il frutto di una contrapposizione commerciale in atto tra i paesi del nord Europa e quelli del sud, tra industria del farmaco e dell'integratore alimentare, quindi tra lobby di interessi industriali. Gli integratori alimentari infatti sono una tipologia merceologica che si è sviluppata soprattutto nei paesi mediterranei ed in particolare in Italia dove vi è una grande tradizione d'uso di piante officinali e dei loro derivati grazie anche alla presenza diffusa in tutto il territorio nazionale di erboristerie con al loro interno erboristi professionisti, di numerose aziende grandi, medie, piccole e micro, che producono integratori alimentari vegetali o preparazioni erboristiche alimentari a base proprio di aloe e delle altre piante prese in considerazione dal regolamento.

Il settore agricolo italiano consta di numerosissime piccole e medie aziende, che grazie al favorevole clima mediterraneo hanno avviato coltivazioni di varie specie di aloe investendo in esse risorse economiche ed impegno lavorativo.

Tutto il settore qui rappresentato sarebbe il primo a non utilizzare piante e derivati dannosi per la salute, qualora emergessero dati scientifici certi e inequivocabili. Abbiamo una professione millenaria alle spalle che ha nella pianta medicinale il proprio centro ed è proprio per la serietà e la preparazione tradizionale e scientifica con cui continuiamo a praticare il nostro lavoro che la nostra professione continua ad avere un senso ed un'utilità sociale anche in società complesse come quella attuale.

Tuttavia il metodo usato dalla Commissione europea sui "botanicals", come dimostra il caso Aloe, non è accettabile in quanto basato su evidenze scientifiche parziali e non tiene conto alcuno della millenaria sperimentazione d'uso negli esseri umani, che finora non ha registrato o documentato alcun singolo effetto, quali quelli alla base della decisione della Commissione europea.

Non solo questo metodo di procedere danneggia a livello economico e professionale ingiustamente chi opera con le piante in scienza e coscienza, ma anche i cittadini europei

che si vedono privati del loro diritto a poter continuare ad usare piante e derivati che vantano una "sperimentazione" d'uso sul campo a livello mondiale.

Dopo l'Aloe incombe la scure europea su preparazioni a base di rabarbaro, ovvero *Rheum palmatum* L., *Rheum officinale* Baillon e i loro ibridi contenenti derivati dell'idrossiantracene, ed ancora su quelle note come senna, a base di foglie o frutti di *Cassia senna* L. ed altre specie cogeneri, ed ancora su quelle note come frangola, a base di corteccia di *Rhamnus frangula* L. o *Rhamnus purshiana* D.C., tutte quante da bandire dal consueto notissimo impiego come blandi regolatori del transito intestinale, in quanto contenenti aloina e aloe emodina, metaboliti secondari caratteristici del loro fitocomplesso che tuttavia non è equivalente alle sostanze pure in esso contenute e prese come base per il divieto indifferenziato dell'utilizzo di queste usatissime specie botaniche. Queste preparazioni sono state poste sotto monitoraggio per quattro anni alla fine dei quali la Commissione europea prenderà una decisione in merito. Non vorremmo che si ripetesse il copione dell'aloe, cioè che non venissero tenuti in alcun conto studi che ne dimostrano la mancanza di rischio per la salute, oltre che il consolidato e sicuro impiego. Affinché questo non avvenga occorre rivendicare con forza che tutti gli studi siano presi in considerazione da parte dell'EFSA e dalla Commissione europea e che si tenga sempre presente la differenza esistente tra il principio attivo isolato ed il fitocomplesso di una pianta che non sono assolutamente interscambiabili, ma indicano una fondamentale differenza sotto tutti i profili, sia quelli di efficacia che quelli tossicologici.

La Federazione ritiene il regolamento approvato sia completamente inapplicabile in quanto i provvedimenti contenuti si manifestano contraddittori, incongruenti e difficilmente applicabili sia dal punto di vista eminentemente tecnico che giuridico, tali da generare una ricaduta assolutamente negativa su tutto il comparto erboristico/alimentare.

Entro il termine di 18 mesi dall'entrata in vigore del Regolamento 2021/468 dovremo presentare alla Commissione europea la documentazione scientifica che attesti la sicurezza d'uso delle piante messe in sorveglianza, ma che comunque esclude l'aloe e pertanto ci stiamo attrezzando nel produrre studi a livello sperimentale e una documentazione bibliografica assolutamente completa ed aggiornata contrariamente a

quella dell'EFSA, ma sarebbe importante anche un'azione più forte da parte del Governo italiano al fine di richiedere l'annullamento del regolamento stesso così come per iniziativa di aziende private si sta organizzando un ricorso presso la Corte di Giustizia europea.

Alla luce di quanto sinteticamente esposto e con l'aggiunta della documentazione scientifica allegata, la Federazione Erboristi Italiani e il Settore produzione Feder Botanicals Italia – Confcommercio Imprese per l'Italia auspicano una particolare attenzione della 14ª Commissione atta a stimolare un deciso intervento del Governo italiano a supporto delle migliaia di imprese rappresentate al fine di ottenere il ritiro del regolamento (UE) 2021/468 attraverso l'affiancamento del Governo italiano nel ricorso presso la Corte Europea di Giustizia promosso da alcune importanti aziende del settore per abuso del principio di proporzionalità ed incoerenza nella stesura della norma che ingiustamente, indebitamente e impropriamente punisce solo il settore degli integratori alimentari e delle piante officinali mettendo seriamente a rischio la stessa sopravvivenza di gran parte delle aziende del settore, dalla coltivazione, alla trasformazione alla vendita all'ingrosso e al dettaglio, che hanno già subito consistenti ripercussioni economiche nel ritirare dal mercato i prodotti a base di aloe ed in particolar modo in un periodo assai complesso e delicato come quello generato dall'attuale emergenza sanitaria.

La Federazione Erboristi Italiani – Confcommercio Imprese per l'Italia rimane a completa disposizione per qualsiasi chiarimento in merito alle posizioni espresse, con spirito costruttivo e collaborativo.

Si ringrazia il Presidente Sen. Stefàno e la Commissione per l'attenzione.

All. 01 Relazione tecnica FEI
02 Tabella effetti antitumorali
Alcuni studi pubblicati

Federazione Erboristi Italiani - FEI
00153 Roma - Piazza G.G. Belli, 2
tel. 065866345 – 305 - fax 065812750
feiconfcommercio@gmail.com
fei@confcommercio.it -
www.feierboristi.org
Membro EHTPA
European Herbal & Traditional Medicine Practiotioners Association
Membro SIF – Società Italiana di Fitochimica

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Alla 14ª Commissione
Politiche dell'Unione Europea

Audizione della Federazione Erboristi Italiani – Confcommercio Imprese per l'Italia.

Relazione tecnico - scientifica

Regolamento (UE) 2021/468 – Specie botaniche contenenti derivati dell'idrossiantracene.

Osservazioni della Federazione Erboristi Italiani - F.E.I. – Confcommercio Imprese per l'Italia per mantenere la commercializzazione di alimenti e integratori alimentari a base di derivati idrossiantraceni largamente impiegati in Italia ed Europa.

L'uso di prodotti per favorire il transito intestinale è un'abitudine piuttosto diffusa, soprattutto tra la popolazione anziana. Questo impiego ha radici molto antiche nella Materia Medica, e non si registrano nella documentazione antica effetti negativi, mentre ne viene lodato l'impiego.
Il 20% delle persone di età superiore ai 60-65 anni assume almeno una volta alla settimana lassativi, che nel 70% dei casi sono costituiti da piante tal quali in polvere o taglio granetta, o loro preparati, contenenti derivati idrossiantracenici. La loro assunzione è consigliabile non solo in caso di stipsi, ma anche per ottenere una evacuazione con feci molli in individui con emorroidi, ragadi anali, che hanno subito interventi all'addome, o ipertesi e cardiopatici.
Metabolismo e farmacocinetica di questo tipo di lassativi sono stati studiati in vivo e sono riportati in letteratura.

FARMACOCINETICA
I derivati idrossiantracenici nei vegetali sono presenti per la maggior parte come glicosidi, solo in minima parte come agliconi.
I glicosidi antrachinonici sono i principali responsabili dell'attività favorente il transito intestinale, somministrati per via orale non vengono degradati nello stomaco (pH 1-3) e non sono neanche assorbiti dal primo tratto dell'intestino, tuttavia , una vota raggiunto il colon i glicosidi si comportano come pro farmaci, liberando gli agliconi (aloe-emodina, reina, emodina, crisofanolo) ad azione lassativa. Tali sostanze , soprattutto reina ed aloe-emodina, agiscono sinergicamente sia sulla motilità che sulla secrezione/assorbimento intestinale di liquidi.

METABOLISMO
E' stato dimostrato che gli agliconi allo stato libero eventualmente assorbiti sono coniugati, nell'epitelio intestinale e nel fegato, nei corrispondenti glucuronidi e solfati e sono escreti attraverso bile e urine (1).

MECCANISMO D'AZIONE

I derivati dell'idrossiantracene in forma glicosidica rimangono invece intatti fino a quando non sono idrolizzati dalla flora batterica, (che è capace di rompere il legame C-.glicosidico della barbaloina , nel caso dell'Aloe, come dimostrato da Che et al. (1991) a livello dell'intestino crasso, nei rispettivi agliconi, che agiscono localmente, svolgendo la desiderata attività lassativa, attraverso lo stimolo della sintesi o del rilascio di sostanze endogene, quali prostaglandine, serotonina e ossido di azoto, attive sulla muscolatura liscia e sulla secrezione intraluminale.

Il mancato o ridotto assorbimento dei derivati idrossiantracenici li rende sicuri se usati a dosaggio consigliato di 20-30 mg e utilizzati 1-2 volte a settimana.

La Commissione E del Ministero della Salute Tedesco raccomanda preparazioni equivalenti ad una dose giornaliera di 20-30 mg di derivati idrossiantracenici in un'unica somministrazione.

SICUREZZA DEI DERIVATI IDROSSIANTRACENICI

Per quanto riguarda l'estratto di aloe è noto che contiene anche aloctine, sostanze che migliorano l'attività mitogena nei linfociti umani, attivano il complemento e si comportano da immunomodulatori. L'aloctina si è mostrata capace di ridurre il fibrosarcoma indotto nei topi da metilcolanturano: questa risposta è stata attribuita all'effetto immunomodulante dell'aloctina A e solo in parte al suo effetto citotossico. Anche gli antrachinoni possiedono attività antiflogistiche, immunomodulanti e antitumorali. E' stato tra l'altro osservato che l'aloe-emodina è attiva contro la leucemia P-388 nel topo e inibisce selettivamente la crescita di un tipo di cellula tumorale umana in colture tissutali e in modelli animali. L'aloe-emoidina inibisce, inoltre la proliferazione di cellule tumorali MCC (*Merkel cell carcinoma)* (meno attiva è l'emodina), più attiva è l'aloina) e induce apoptosi. Alcuni studi sono stati effettuati per determinare se i derivati idrossiantracenici possono essere usati come adiuvanti per incrementare gli effetti degli agenti antitumorali. Gli studi sono stati condotti su una linea cellulare MCC. Gli agenti antitumorali utilizzati (cisplatino, doxorubicina, 5-fluorouracile) hanno inibito la proliferazione delle cellule in esame. Tra i derivati antracenici utilizzati, l'aloe-emodina, ma non l'emodina e l'aloina, ha potenziato gli effetti degli antitumorali di sintesi, soprattutto quando questi venivano utilizzati a basse concentrazioni (Fenig et al. 2004).

I risultati di questi studi mostrano che l'attività antitumorale dell'aloe non dipende soltanto da un suo effetto immunomodulante, bensì anche da una diretta inibizione della proliferazione delle cellule tumorali da parte dei derivati antracenici. Effetti antitumorali sono anche esibiti dal dietilesilftalato (DEHP) un altro componente dell'aloe che ha manifestato un potente effetto antileucemico *in vitro* su cellule umane e un effetto antimutageno sulla salmonella. La contemporanea presenza di tutte queste sostanze, espressione del fitocomplesso, può essere sufficiente a spiegare l'effetto antitumorale dell'estratto di aloe, che è risultato attivo anche contro la leucopenia causata da esposizione al cobalto 60, il sarcoma 180 e l'ascite di Ebelich. Altri studi hanno poi mostrato un effetto inibente dell'estratto di aloe sulle lesioni epatocellulari preneoplastiche indotte nei ratti e una regressione del tumore da parte dell'aloe succo.

I risultati ottenuti su modelli sperimentali o su cellule *in vitro* hanno incoraggiato l'impiego dell'aloe nel trattamento del cancro. La preparazione utilizzata prevede che la foglia dell'aloe provenga da una pianta di almeno 5 anni e che sia utilizzata allo stato fresco. La foglia viene omogeneizzata con miele ed alcol (Tomasin et al. 2011). La presenza del miele migliora la palatabilità della preparazione, ma sinergizza anche l'azione antitumorale dell'aloe grazie a un suo componente, il CAPE (*caffeic acid phenetyl ester*), che è risultato essere un potente chemopreventivo, utile nelle patologie con una forte componente infiammatoria, come per esempio alcuni tumori. Secondo Tomasin et al. (2011) l'aloe riduce la massa tumorale. Anche in studi sperimentali è stata documentata un'attività antimetastatica dell'aloe nei roditori. E' noto che i tumori promuovono l'aggregazione piastrinica stimolando la produzione di trombossano e/o inibendo la produzione di prostaciclina. L'aloe gel inibisce le metastasi inibendo la produzione di trombossani, e questo potrebbe essere uno dei meccanismi della sua attività antimetastatica. L'aloe gel e gli altri prodotti naturali che inibiscono la produzione o la degradazione di chinine possono infine ostacolare l'angiogenesi indotta dalle chinine. L'aloe gel e le glicoproteine isolate da *A. arborescens* e *A. saponaria* degradano la bradichinina *in vitro* e inibiscono la formazione di istamina, sempre *in vitro.* L'aloe gel si è

dimostrato attivo come antiangiogenico in un modello sperimentale nel topo (*pouch* sinoviale). (Capasso R. Laudato M. Grandolini G. Capasso F. , 2013).

Ipotesi su possibili effetti di mutagenicità e cancerogenicità conseguente all'uso di lassativi antrachinonici sono nate a seguito dell'osservazione della *melanosis coli,* una pigmentazione bruna della mucosa del colon e del retto, conseguente ad abuso di lassativi antrachinonici.
E' comunque ormai accertato che si tratta di un fenomeno innocuo e reversibile, non correlato alla comparsa di tumore colon-rettale (2) (4).
Il fenomeno si produce dopo 9-12 mesi di utilizzo e non in tutti gli individui (8) (10), e scompare, previa interruzione del trattamento senza che ci sia evidenza di fenomeni di carcinogenesi o genotossicità (9).
Prove fatte con Cascara (*Rhamnus purshiana* D.C.) hanno mostrato una reversibilità della pigmentazione del colon in 5-15 mesi nella maggior parte dei pazienti senza che sia stato rilevato un legame con mutagenicità o cancerogenicità (8).

In uno studio su 2000 pazienti con carcinoma colon-rettale, le coloscopie non hanno evidenziato alcuna associazione a presenza di *melanosis coli* o uso di lassativi (11) (12).

Uno studio prospettico di coorte sul rischio dell'uso di lassativi antrachinonici di sviluppare tumori colon-rettali, indica una correlazione statisticamente non significativa: una *melanosis coli* macroscopica o di grado elevato non rappresenta un fattore di rischio per lo sviluppo di adenomi o carcinomi (13).

A tal proposito è di interesse anche uno studio clinico di D. Loew et al. (3) che conclude: "Da nuove prove in vivo non si deduce alcun rischio evidente di genotossicità e cancerogenesi a carico di Senna (*Senna alexandrina* Mill.) frutti e dei relativi derivati idrossiantracenici.
In 4 studi clinici si è potuta escludere una relazione tra assunzione di lassativi antrachinonici e carcinoma rettale". Alle stesse conclusioni, gli stessi autori, erano giunti in uno studio precedente del 1994.

Considerando che i dati tossicologici sui derivati idrossiantracenici sono stati ottenuti prevalentemente con prove di mutagenesi in vitro e di cancerogenesi nell'animale (5), va tenuto presente che i risultati di prove di mutagenesi in vitro raramente sono predittivi degli stessi effetti sull'uomo.
A questo proposito si esprime Loew, farmacologo clinico dell'Università di Francoforte: "In diverse pubblicazioni Ames e Gold hanno sottolineato che la sola genotossicità non basta per affermare l'esistenza di un rischio di carcinogenesi." Una sostanza cancerogena è sempre mutagena, una sostanza mutagena non sempre è cancerogena.
Addirittura, in alcuni test in vitro, qualche derivato idrossiantracenico è risultato mutageno (6), mentre un estratto standardizzato di Senna (*Senna alexandrina* Mill.) ha inibito invece l'azione mutagena dell'aflatossina B1 (7).
Il volume "Farmaci genotossici" edito dall'Università La Sapienza di Roma nel 1990 elenca centinaia di prodotti normalmente usati in terapia.
Test a dosi elevate nell'animale hanno mostrato che le cinetiche degli effetti cancerogeni non sono lineari, ma aumentano spesso con la dose in maniera esponenziale.

Non è stata osservata una induzione di tumori colon-rettali in topi a cui era stata somministrata quotidianamente una dieta contenente lo 0,03% di sennosidi per 20 settimane; non sono stati evidenziate significative modifiche di valori di elettroliti nel siero o di parametri correlati a epatotossicità e nefrotossicità (14).

Non è stata osservata induzione di foci di cripta aberranti in ratti cui erano stati somministrati 30 o 60 mg/kg di un estratto di follicoli di Senna per due anni.
La somministrazione di estratto di Senna follicoli inibisce lo sviluppo di tumori in animali trattati con un agente promotore tumorale (15).

Un incremento nella comparsa di tumori chimicamente indotti è stata osservata in ratti cui era somministrata una dose giornaliera di 100 mg/kg di estratto di follicoli di Senna (una dose che induce diarrea cronica) per 13 - 28 settimane. Non sono state invece evidenziate foci di cripta aberranti o induzione di tumore in ratti cui erano somministrati giornalmente 10 mg/kg di estratto di follicoli di Senna, una dose che provoca effetto lassativo (16).

La somministrazione giornaliera di 5-15 o 25 mg/kg di un estratto purificato di Senna con l'acqua da bere per due anni non ha incrementato l'incidenza di tumori del tratto gastrointestinale, del rene o delle ghiandole surrenali (17).

In uno studio durato due anni, ratti F/344N maschi e femmine hanno ricevuto per via orale 280, 830, o 2500 ppm di emodina, corrispondenti ad una dose media giornaliera di emodina pari a 110, 320 o 1000 mg /kg di peso corporeo nei ratti maschi e 120, 370 o 1100 mg/kg di peso corporeo nelle femmine. Non sono stati riscontrati effetti cancerogeni dell'emodina nei ratti maschi. Un aumento marginale dell'incidenza di carcinoma della ghiandola di Zymbal si è osservato nei ratti femmina trattati col dosaggio elevato, ma è stato valutato come opinabile (19).

In un ulteriore studio condotto per due anni sul topo B6C3F1, i maschi sono stati esposti a 160, 312 o 625 ppm di emodina (corrispondenti ad una dose giornaliera media di 15, 35 o 70 mg/kg di peso corporeo) e le femmine a 312, 625 o 1250 ppm di emodina (pari a 30, 60 o 120 mg/kg di peso corporeo, al giorno). Non si sono osservati effetti cancerogeni sul topo femmina. Una bassa incidenza di neoplasie dei tubuli renali nei maschi esposti non è stata considerata rilevante (19).

In anni recenti anche l'aloe come del resto la senna (Borrelli et al. 2005), è stato oggetto di studi per verificare la sua potenziale carcinogenicità. Uno studio condotto *in vivo* su ratti ha mostrato che l'aloe somministrata quotidianamente per 13 settimane non induceva lo sviluppo di lesioni preneoplastiche o neoplastiche, o foci di cripte aberranti (ACF, *aberrant crypt foci*); e quindi non promuoveva alcuna attività tumorigena nel colon del roditore. In aggiunta, quando l'aloe veniva somministrata a ratti trattati con un agente carcinogeno, l'azossimetano, non incrementava il numero di ACF e quello dei tumori indotti dall'agente carcinogeno (Capasso, 2012).
Così pure è stato osservato, che contrariamente a quanto supposto, l'aloina somministrata ai topi (aggiunta al mangime) per venti settimane non produce tumori e non incrementa l'incidenza dei tumori al colon-retto. E' stato tra l'altro osservato che il LOAEL (*lower observed adverse effect level*) per l'aloina è di 11.8 g/kg (Zhou et al. , 2003). Diversi studi condotti *in vitro* e su animali hanno evidenziato che anche l'aloe-emodina, piuttosto che indurre, inibisce la crescita dei tumori. Questi studi sono in accordo con precedenti studi condotti su altre droghe antrachinoniche.

Tossicità acuta: La DL_{50} per la somministrazione orale di sennosidi è pari a 5 g/kg in topi e ratti, la cui morte viene attribuita a perdita di acqua e elettroliti dopo diarrea severa (18).

Tossicità a breve termine: Non sono stati osservati effetti indesiderati in ratti cui erano state somministrate oralmente dosi di sennosidi pari a 5, 10, o 20 mg/kg per 4 settimane. Nel gruppo trattato con 20 mg/kg è stato evidenziato un effetto lassativo di basso livello insieme ad un aumento del peso renale (18).

Non sono stati osservati effetti indesiderati in cani cui era stata somministrata quotidianamente una dose di 500 mg/kg di sennosidi per 4 settimane (18).

Per concludere si può affermare che i dati riportati evidenziano un'assenza di effetti indesiderati e, in particolare, una mancanza di correlazione tra l'uso di derivati idrossiantracenici e insorgenza di neoplasie, in particolare colon-rettali.

Le dosi normalmente utilizzate come lassativo, pari a 20-30 mg di derivati idrossiantracenici/die non hanno provocato trasformazioni tumorali anche con l'abuso nell'uomo, inoltre, sono di gran lunga inferiori a quelle che non hanno comunque provocato alcun effetto indesiderato negli studi su animali.
Va poi evidenziato che se una sostanza è considerata pericolosa per la salute, come si ipotizza per i derivati idrossiantracenici, essa dovrebbe essere vietata all'uso in generale e non solo in una categoria di prodotti, come alimenti e integratori, come anni fa fu fatto per il Kawa kawa (*Piper methysticum* Forster).

L'opinion EFSA presenta delle evidente criticità in quanto le sue conclusioni si basano di fatto su due lavori pubblicati in letteratura effettuati 1 vitro su un estratto di aloe (Guo 2014) che mostra un effetto mutageno e uno in vivo dell'aloemeodina realizzato su topo con somministrazioni pari a 2000 mg/ kg mai impiegate in pratica dal momento che i dosaggi utilizzati non superano i 30-50 mg giornalieri.
Gli effetti poi riscontrati in vitro su sostanze isolate come aloe-emodina, emodina e diantrone vanno prese in considerazione ma non possono da sole rappresentare un rischio all'impiego della droga in toto senza un' evidenza clinica. Inoltre esistono tanti lavori pubblicati in letteratura che dicono esattamente l'incontrario.

Di seguito si riporta in forma di tabella gli effetti antitumorali riscontrati per i derivati idrossiantracenici (tabella I).

Pertanto la valutazione del rischio va effettuata tenendo conto di tutti i dati a disposizione e dal momento che gli estratti di piante lassative presentano un pool di costituenti similari l'efficacia e sicurezza sono paragonabili e non riteniamo corretto mettere inserire una droga come aloe ed alcuni costituenti, fra l'altro ubiquitari, nella parte A dell'allegato III del regolamento 1925/2006 e inserire le altre droghe nella parte C. Ci sembra inoltre importante sottolineare che i dosaggi impiegati sono ben lontani da quelli saggiati in vitro e, spesso, anche a tali dosaggi non sempre si riscontrano evidenze di tossicità/cancerogenicità.

Piante contenenti derivati idrossiantracenici presenti in commercio e usate per favorire il transito intestinale:

Senna foglie e follicoli (*Senna alexandrina* Mill.)
Cascara corteccia (*Frangula purshiana* (DC) J.G. Cooper)
Frangola corteccia (*Frangula alnus* Mill.)
Rabarbaro rizoma (*Rheum officinale* baill.)
Aloe succo essiccato (*Aloe ferox* Mill.) *usato sempre in combinazione con altre piante contenenti derivati idrossiantracenici.*

Riguardo allo specifico impiego come botanicals e altri integratori alimentari possiamo mettere in evidenza i seguenti punti di riferimento:

a) il raw material, ovvero la droga vegetale, è la stessa impiegata in tutti i settori, dall'integratore alimentare fino al prodotto fitoterapico presente nelle farmacopee;
b) i principi attivi presenti nelle droghe antrachinoniche sono tutti dello stesso tipo e presenti in combinazione, con eventuale prevalenza dell'uno o dell'altro;
c) nelle farmacopee ed altri riferimenti normativi nazionali si stabilisce un limite relativo alla concentrazione dei principi antrachinonici, riferiti ad una sostanza marker;
d) i principi su cui si fonda la farmacologia e la tossicologia prescrivono la definizione di una soglia di concentrazione/attività, per distinguere l'effetto curativo da quello tossico;
e) Non ci sono motivi per considerare e trattare a livello normativo sostanze dello stesso tipo in modo differente. Tale orientamento sarebbe in evidente contrasto sia da un punto di vista scientifico che legale.

In particolare non si capisce perché nell'analisi del rischio di un botanical si tenga conto solo degli studi effettuati sulla singola sostanza senza valutare l'estratto in toto così come realmente viene tradizionalmente impiegato.

A livello europeo con il regolamento "Novel food" - Regolamento (UE) 2015/2283) - è stato chiarito e condiviso che una sostanza isolata e altamente purificata da un estratto vegetale è da ritenersi "novel food" in quanto il suo profilo biochimico può sensibilmente cambiare. Nonostante ciò si continua a valutare la sicurezza secondo schemi di farmacologia obsoleti non adatti ad una valutazione obiettiva di composti molti complessi come appunto i botanicals. Non c'è quindi da stupirci se con tale approccio i risultati siano contradditori e le conclusioni assolutamente opinabili.

Il Presidente

Dott. Angelo Di Muzio

Allegato Tabella - Effetti antitumorali idrossiantraceni

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Federazione Erboristi Italiani - FEI
00153 Roma - Piazza G.G. Belli, 2
tel. 065866345 – 305 - fax 065812750
feiconfcommercio@gmail.com
fei@confcommercio.it -
www.feierboristi.org
Membro EHTPA
European Herbal & Traditional Medicine Practiotioners Association
Membro SIF – Società Italiana di Fitochimica

Attività antitumorale in Vitro di Emodina, Aloe emodina e Reina.

ANTRACHINONI	LINEE CELLULARI	TRATTAMENTO	OUTCOMES	RIFERIMENTO
Emodina	Cellule A549 adenocarcinoma polmonare umano	10-50 µM	Generazione di ROS, inattivazione ERK e Akt, riduzione Bcl-2,apoptosi	Su YT, Chang HL, Shyue SK, Hsu SL. Emodin induces apoptosis in human lung adenocarcinoma cells through a reactive oxygen species-dependent mitochondrial signaling pathway. Biochem Pharmacol 2005;70:229 – 241.
Emodina	Cellule HL-60 leucemia mieloide umana	10-80 µM	Riduzione di Mcl-1,apoptosi	Chen YC, Shen SC, Lee WR, Hsu FL, Lin HY, Ko CH, Tseng SW. Emodin induces apoptosis in human promyeloleukemic HL-60 cells accompanied by activation of caspase 3 cascade but independent of reactive oxygen species production. Biochem Pharmacol 2002;64:1713.
Emodina	Celulle umane HeLa carcinoma cervicale	0,5-10 µM	Generazione di ROS, inattivazione NfKβ e AP-1,	Yi J, Yang J, He R, Gao F, Sang H, Tang X, Ye RD. Emodin enhances arsenic trioxide-induced apoptosis via generation of reactive oxygen species and inhibition of survival signaling. Cancer Res 2004;64: 108.
Aloe emodina	Cellule umane HepG2 e Hep3B epato-carcinoma	37-74 µM	Attivazione p53 e p21, aumento densita recettoriale FAS, aumento espressione BAX, induzione apoptosi	KuoPL,LinTC,LinCC.Theantiproliferativeactivityofaloe-emodinisthroughp53-dependentandp21- dependent apoptotic pathway in human hepatoma cell lines. Life Sci 2002;71:1879–1892.
Aloe emodina	Cellule gliali trasformate U-373MG e SVG	40 µM	Ritardo inizio e terminazione fase S (replicativa),riduzione attività PKC, induzione apoptotica	Acevedo-Duncan M, Russell C, Patel S, Patel R. Aloe-emodin modulates PKC isozymes, inhibits proliferation, and induces apoptosis in U-373MG glioma cells. Int Immunopharmacol 2004;4:1775–1784.
Reina	Cellule epatoma umano Hep-G2	0-400 µM	Aumento p53,aumento legame CD95, apoptosi	Kuo PL, Hsu YL, Ng LT, Lin CC. Rhein inhibits the growth and induces the apoptosis of Hep G2 cells. Planta Med 2004;70:12–16.
Reina	Cellule leucemia mieloide umana HL-60	0-100 µM	Perdita del potenziale di membra mitocondriale,apoptosi	Lin S, Fujii M, Hou DX. Rhein induces apoptosis in HL-60 cells via reactive oxygen species-independent mitochondrial death pathway. Arch Biochem Biophys 2003;418:99–107

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Effect of bisacodyl and cascara on growth of aberrant crypt foci and malignant tumors in the rat colon

F. Borrelli[a], E. Mereto[b], F. Capasso[a,*], P. Orsi[b], D. Sini[b], A.A. Izzo[a], B. Massa[c], M. Boggio[c], N. Mascolo[d]

[a]*Department of Experimental Pharmacology, University of Naples "Federico II", Naples, Italy*
[b]*Department of Internal Medicine, Pharmacology and Toxicology Unit, University of Genoa, Genoa, Italy*
[c]*Institute of Anatomo-Phatology, University of Genoa, Genoa, Italy*
[d]*Department of Pharmaceutical Sciences, University of Salerno, Salerno, Italy*

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Abstract

Laxatives abuse has been associated with an increased risk for colon cancer. However, little is known about laxatives long-term carcinogenic potential in experimental studies. The present study was designed to investigate the effects of bisacodyl (4.3 and 43 mg/kg) and cascara (140 and 420 mg/kg) on azoxymethane (AOM)-induced aberrant crypt foci (ACF) and tumors. Animals, divided in 10 groups were treated with AOM and laxatives (alone or in combination) for 13 weeks. At the end of treatment animals were killed and the colon removed and analysed for the determination of ACF and tumors. Bisacodyl (4.3 and 43 mg/kg), given alone, did not induce the development of colonic ACF and tumors. Bisacodyl (4.3 mg/kg) coupled with AOM increased the number of crypt per focus, but not the number of tumors. Bisacodyl (43 mg/kg) significantly increased the number of crypt per focus and tumors. Cascara (140 and 420 mg/kg) did not induce the development of colonic ACF and tumors and did not modify the number of AOM-induced ACF and tumors. The results of the present study indicate a possible promoting effect of bisacodyl on rat colon carcinogenesis (especially at higher doses) and absence of any promoting or initiating activity of a laxative and diarrhoeal dose of cascara.

Keywords: Laxative; Cascara; Bisacodyl; ACF; Tumors; Intestine

Introduction

Chronic constipation is a common symptom of many patients, especially elderly people. The initial therapeutic approach is to add fiber and bulk-forming agents to the diet to insure

* Corresponding author. Department of Experimental Pharmacology, via D. Montesano 49, 80131 Naples, Italy. Tel.: +39-81-678415; fax: +39-81-678403.
E-mail address: franborr@unina.it (F. Capasso)

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adequate hydration [1]. However, these measures are often inadequate, and additional medications are added such as anthraquinone drugs (senna, cascara, aloe etc.) and diphenolic derivatives (phenolphthalein and bisacodyl). These laxatives increase intestinal motility and secretion through multiple mechanisms, including stimulation of prostaglandins, serotonin, platelet activating factor and nitric oxide synthesis [2–5]. Anthraquinone laxatives are viewed as harmless drugs by western society and are freely available for self-medication. If taken on an occasional basis they produce abdominal complaints (meteorism, flatulence, cramping abdominal pain). However, ease of access to laxatives renders them liable to abuse, and chronic administration may produce severe complications like dehydration, electrolyte depletion and melanosis coli.

In recent years, the risk of colon cancer (the second most common cancer in the western countries) has been found to be related to constipation and to use of anthraquinone laxatives [6–8], but epidemiological studies are in disagreement. Jacobs and White [9], have examined the associations of colon cancer with constipation and the use of commercial laxatives in a case-control study among men and women each 30–62 years. When constipation and commercial laxative use were adjusted for each other, the association with commercial laxative use disappeared, whereas the association with constipation remained strong. These results suggest that frequent constipation may be an important risk factor for colon cancer among middle-aged adults. Other retrospective data related to laxative use did not show any significant increase of colon cancer incidence [10–12], but in these studies differences among several classes of laxatives were not taken into account. In a prospective study, Siegers demonstrated a positive correlation between pseudomelanosis coli (a marker of chronic abuse of laxatives anthranoids) and colon carcinoma [13], but this correlation failed in an other retrospective study [14]. Several data have outlined the possible genotoxic and tumorigenic activity of anthranoids (the most used laxatives) [15–17], but some authors analysing the current literature suggest a sufficient margin of safety for these laxatives [18, 19].

In the light of these findings, we were interested to verify the possible role of these drugs in colon carcinogenesis, in an experimental animal model. In previous studies we analysed the carcinogenic effect of some anthranoids on the rat colon [20–21]. The aim of this study was to verify the ability of bisacodyl and cascara to induce the development of Aberrant Crypt Foci (ACF) in the rat colon, which are generally considered as putative preneoplastic lesions [22], and of tumors.

Materials and methods

Chemicals

Azoxymethane (AOM) and bisacodyl were purchased from Sigma (Milan, Italy); cascara was obtained from Indena (Milan, Italy). AOM was dissolved in saline, cascara in water and bisacodyl was suspended in 1% arabic-gum.

Animals

Experiments were performed on male Wistar rats (Harlan-Nossan, Correzzana, Italy) weighing 120 ± 10 g at the start of the study. They were housed in an air-conditioned room

(23±2°C; 50±10% relative humidity) with a 12 hr light-dark cycle, and had free access to tap water and rat chow (Mucedola, Milan, Italy).

Treatments

Animals were randomly divided into ten groups as follows: group 1 was treated with AOM (30 mg/kg); groups 2 and 4 were given 4.3 and 43 mg/kg/day of bisacodyl; groups 3 and 5 received AOM + bisacodyl 4.3 and 43 mg/kg/day; groups 6 and 8 received 140 and 420 mg/kg/day of cascara; groups 7 and 9 received AOM + cascara 140 and 420 mg/kg/day; group 10 served as the untreated control and was given the arabic-gum solution (1%) used as vehicle for bisacodyl. Two doses of bisacodyl (4.3 and 43 mg/kg/day) and cascara (140 and 420 mg/kg/day) were selected; the low doses, which induces a laxative effect (increase in faecal and water content) and the high doses which produces a diarrhoeal response (presence of characteristic unformed water faeces) as experienced by a single treatment.

AOM (30 mg/kg in total, ip) used as initiating agent to evaluate whether laxatives act as tumor promoters, was administered intraperitoneally on day 1 (15 mg/kg) and 5 of treatment (15 mg/kg). The two test laxatives were given six times a week, for 13 successive weeks, by intragastric gavage in the morning. All animals were killed by asphyxiation with CO_2 13 weeks after AOM initiation. This time is associated with the higher incidence of ACF [22].

ACF technique

For ACF determination, the colons were rapidly removed after sacrifice, washed with saline, opened longitudinally, laid flat on a polystyrene board, and fixed with 10% buffered formaldehyde solution before being stained with 0.2% methylene blue in saline. They were examined using a light microscope at 40× magnification. The criteria used to identify the aberrant crypts were previously described [23]; briefly, in comparison to normal crypts, they have greater size, larger and often elongated openings, thicker lining of epithelial cells, compression of adjacent crypts, and are more darkly stained with methylene blue. The detection and quantitation of ACF were performed on the distal 9 cm of the colon, starting from the rectal end. According to the number of constituent crypt, ACF were divided into two groups: small ACF containing 1–3 crypts per focus, and large ACF containing 4 or more crypts per focus. To determine crypt multiplicity, the number of aberrant crypts in each focus was recorded.

Tumor evaluation

For tumor evaluation, the colons of all rats were discoloured with 70% ethanol and embedded in paraffin; serial sections 3μm thick were obtained and stained with hematoxylin-eosin. Either benign tumoral lesions (adenomas) or malignant ones (adenocarcinomas) were scored, and for adenomas the degree of dysplasia was recorded (as of low and high grade) [9].

Statistics

Statistical analysis was performed by using ANOVA followed by the Dunnett's test. The number of rats with tumors was analyzed by Fisher exact test.

Results

ACF and tumors

The number and type of ACF and tumors observed after 13 weeks of treatment with the two laxatives in either the presence or absence of the AOM initiating treatment are shown in Tables 1 and 2, respectively.

Table 1 shows that AOM given alone (group 1) induced the expected appearance of ACF. In contrast bisacodyl 4.3 mg/kg and cascara 140 and 420 mg/kg, when given without the AOM-initiating treatment (groups 2, 6 and 8), were clearly unable to induce ACF. One animal treated with 43 mg/kg of bisacodyl alone (group 4) displayed two ACF. Bisacodyl (group 3 and 5) at both doses used (4.3 and 43 mg/kg) coupled with the initiating treatment with AOM, did not modify significantly the appearance of ACF but increased the number of crypts/focus. Cascara plus AOM (group 7 and 9) did not cause either a significant increase of ACF or an increase of crypt/focus (Table 1).

Table 2 shows that AOM given alone (group 1) induced the expected appearance of tumors. When bisacodyl and cascara, at both doses used, were given without the AOM initiating treatment (groups 2, 4, 6 and 8) any evidence of tumors development was absent. When the treatment with bisacody 4.3 mg/kg and cascara 140 and 420 mg/kg was coupled with the initiating treatment with AOM (groups 3, 7 and 9) any significant increase of tumors was absent. On the contrary, AOM + bisacodyl at 43 mg/kg (group 5) caused a significant increase of the number of tumors approximately 10-fold higher than in rats given AOM alone (group 1).

Discussion

In recent years the use of laxatives has been associated with the development of intestinal cancer. However, conflicting reports have been published in the literature in this respect. This may be explained by the fact that laxatives (i) are a heterogeneous family of compounds, (ii)

Table 1
Induction of aberrant crypt foci (ACF) in rats exposed for 13 weeks to bisacodyl and cascara in presence and absence of azoxymethane (AOM) initiating exposure

Group	Treatment conditions and dose	N of rats	ACF/rat[#]		Crypt/focus (N)[#]
			Total N	N with ≥ 4 crypts	
1	AOM 15 mg/kg, twice	20	135.2 ± 49.04	41.8 ± 26.5	3.0 ± 0.8
2	Bisacodyl 4.3 mg/kg	10	0	0	0
3	AOM+bisacodyl 4.3 mg/kg	10	100 ± 46.3	41.2 ± 21.5	4.37 ± 0.63*
4	Bisacodyl 43 mg/kg	8	0.25 ± 0.71	0.12 ± 0.35	0.50 ± 1.4
5	AOM+bisacodyl 43 mg/kg	9	105.1 ± 34.8	62.0 ± 23.3	4.44 ± 0.93*
6	Cascara 140 mg/kg	10	0	0	0
7	AOM+cascara 140 mg/kg	10	151.6 ± 44.9	39.3 ± 27.3	2.95 ± 0.91
8	Cascara 420 mg/kg/day	10	0	0	0
9	AOM+cascara 420 mg/kg	10	139.1 ± 44.1	43.3 ± 27.5	3.94 ± 0.90
10	Arabic gum	10	0	0	0

[#] Means ± SD.

* $p<0.05$ vs group 1 (ANOVA followed by Dunnett's test).

Table 2
Number and aspect of tumors induced in colonic mucosa of rats by 13 weeks of treatment with bisacodyl and cascara in presence and absence of azoxymethane (AOM) initiating exposure

Group	Treatment conditions and dose	N of rats	Rats with tumors (%)	Tumors/rat (N)	Type of tumors	
					Adenomas[#]	Adeno-carcinomas
1	AOM 15 mg/kg, twice	20	20	0.25 ± 0.55	2(↑)1(↓)	2
2	Bisacodyl 4.3 mg/kg	10	0	0	0	0
3	AOM+Bisacodyl 4.3 mg/kg	10	30	0.50 ± 0.85	2(↑)	3
4	Bisacodyl 43 mg/kg	8	0	0	0	0
5	AOM+Bisacodyl 43 mg/kg	9	77.7	2.33 ± 1.87*	0	21
6	Cascara 140 mg/kg	10	0	0	0	0
7	AOM+Cascara 140 mg/kg	10	30	0.40 ± 0.70	2(↑)	2
8	Cascara 420 mg/kg	10	0	0	0	0
9	AOM+Cascara 420 mg/kg	10	30	0.50 ± 0.97	3(↑)	2
10	Arabic gum	10	0	0	0	0

[#] All adenomas were tubulo-villous. (↓) low, (↑) high grade of dysplasia.
* $p<0.05$ vs group 1 (ANOVA followed by Dunnett's test).

involve different mechanisms of action and (iii) are given for a long period of time at different doses. We have recently shown that an oral dose of senna that caused a mild laxative effect in rats does not influence the carcinogenesis of rat colon [21]. In order to further clarify the possible role of laxatives in colon carcinogenesis we have examined the ability of cascara and bisacodyl to induce both ACF and tumors in the rat colon mucosa. In our experiments, the low doses of cascara (140 mg/kg) and bisacodyl (4.3 mg/kg) induced a weak laxative effect and the higher doses (cascara 420 mg/kg; bisacodyl 43 mg/kg) induced diarrhoea during the whole treatment.

The results obtained demonstrate that a laxative dose of bisacodyl (4.3 mg/kg) coupled with AOM increases the number of crypt/focus, but not tumors. This effect is due to a reduction (not significant, from 135.2 ± 49.04 to 100 ± 46.3) of total number of ACF/rat rather than an increase of the number of foci with less than 4 crypt. In order to further clarify this aspect, we have performed a second series of experiment, by treating rats with a higher dose of bisacodyl (43 mg/kg, able to induce a diarrhoeal response). This dosage coupled with AOM treatment caused a significant increase of ACF with four or more crypts and tumors, showing a promoting effect on colon carcinogenesis. There are weak and conflicting data in the literature about the possible genotoxic and/or promoting effect of bisacodyl. In an *in vivo* study bisacodyl did not influence the Labelling Index [24] in the rat colon and should not be regarded as tumor-promoting substances, but another similar study put in evidence a significative cell proliferation in the entire intestinal epithelium [25]. Recently Schorkhuber [26] observed in primary cultures of colon adenoma cells a strong increase of DNA synthesis. Phenolphthalein, another synthetic biphenolic laxative, was recently found to be a carcinogen in animal model [27]. Besides, in spite of human data suggested a laxative-colon cancer association, Longnecker et al. [28] reported that use of phenolphthalein-containing laxatives does not increase risk of adenomatous colorectal polyps in the man.

The present results also shown that cascara (140 and 420 mg/kg), given alone for 13 weeks, did not induce the development of preneoplastic or neoplastic lesions; therefore, this laxative, at both doses used, did not display any tumor initiating activity in the rat colon. In addition, when cascara was administered coupled with AOM, it did not produce any significant increase of the parameters considered. Therefore, it is possible to exclude a promoting effect on colon carcinogenesis.

In our previous study [20] we found a weak but significant increase of number crypt/focus in the same experimental model and we supposed a possible promoting effect of cascara on colon carcinogenesis. This discrepancy between the negative findings of this study and our previous results could be due to the different initiating treatment (dimethilhydrazine instead of AQM), to the different modality of administration of drug (diet instead of intragastric) and to the different rat strain (Sprague Dawley instead of Wistar). Any way crysophanol, a metabolite of cascara, does not display genotoxic activity in the Reuber hepatoma H4-II-E and in Balb/3T3 cells [29], and other data suggest that it exerts an antimutagenic activity in Salmonella typhimurium [30].

Recent studies have also shown that anthraquinone compounds (e.g. emodin) block selectively the retardation of oncogene-modulated signal transduction through the inhibition of protein kinases [31].

However it is worth noting that some data are in favour of a potential genotoxic activity. Chrysophanol was weakly genotoxic in mouse lymphoma cells [32]. Obviously, care should be taken to extrapolate the findings of *in vitro* studies to the human situation.

Conclusion

In conclusion our results outline the clear-cut possible promoting effect of bisacodyl on colon rat carcinogenesis especially at higher (diarrhogenic) dose and absence of any promoting or initiating activity of a laxative and diarrhogenic dose of cascara.

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Anti-proliferative effect of rhein, an anthraquinone isolated from *Cassia* species, on Caco-2 human adenocarcinoma cells

Gabriella Aviello [a], Ian Rowland [b], Christopher I. Gill [c], Angela Maria Acquaviva [d], Francesco Capasso [a], Mark McCann [c], Raffaele Capasso [a], Angelo A. Izzo [a], Francesca Borrelli [a, *]

[a] Department of Experimental Pharmacology, University of Naples Federico II, Naples, Italy
[b] Department of Food Biosciences, University of Reading, Reading, UK
[c] Northern Ireland Centre for Food and Health (NICHE), Biomedical Sciences, University of Ulster, Coleraine, Northern Ireland
[d] Department of Biology and Cellular and Molecular Pathology 'L. Califano', University of Naples Federico II, Naples, Italy

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Abstract

In recent years, the use of anthraquinone laxatives, in particular senna, has been associated with damage to the intestinal epithelial layer and an increased risk of developing colorectal cancer. In this study, we evaluated the cytotoxicity of rhein, the active metabolite of senna, on human colon adenocarcinoma cells (Caco-2) and its effect on cell proliferation. Cytotoxicity studies were performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red (NR) and trans-epithelial electrical resistance (TEER) assays whereas ^{3}H-thymidine incorporation and Western blot analysis were used to evaluate the effect of rhein on cell proliferation. Moreover, for genoprotection studies Comet assay and oxidative biomarkers measurement (malondialdehyde and reactive oxygen species) were used. Rhein (0.1–10 μg/ml) had no significant cytotoxic effect on proliferating and differentiated Caco-2 cells. Rhein (0.1 and 1 μg/ml) significantly reduced cell proliferation as well as mitogen-activated protein (MAP) kinase activation; by contrast, at high concentration (10 μg/ml) rhein significantly increased cell proliferation and extracellular-signal-related kinase (ERK) phosphorylation. Moreover, rhein (0.1–10 μg/ml): (*i*) did not adversely affect the integrity of tight junctions and hence epithelial barrier function; (*ii*) did not induce DNA damage, rather it was able to reduce H_2O_2-induced DNA damage and (*iii*) significantly inhibited the increase in malondialdehyde and reactive oxygen species (ROS) levels induced by H_2O_2/Fe^{2+}. Rhein was devoid of cytotoxic and genotoxic effects in colon adenocarcinoma cells. Moreover, at concentrations present in the colon after a human therapeutic dosage of senna, rhein inhibited cell proliferation *via* a mechanism that seems to involve directly the MAP kinase pathway. Finally, rhein prevents the DNA damage probably *via* an anti-oxidant mechanism.

Keywords: rhein • human colon adenocarcinoma cells • mitogen-activated protein kinase • genoprotection • antioxidant

Introduction

Constipation, a complaint conceptually regarded as disordered movement of stool through the large intestine, afflicts many people in the Western countries [1, 2]. The first approach for the treatment of constipation consists in lifestyle changes including increased fibre (about 30 g/day), and water (about 2 l/day) intake and physical exercise. If these measures are ineffective, laxative therapy has to be considered. Among laxatives, anthranoids are the oldest used drugs in clinical practice and as self-medication [3]. Senna is the most known anthranoid laxative; it is obtained from the dried leaves and pods of *Cassia acutifolia* Delile and *Cassia angustifolia* Vahl (*Caesalpinaceae* family). Senna contains sennosides that are not absorbed in the small intestinal tract; in the colon, sennosides are metabolized by the bacterial β-glucosidase and reductase to the pharmacologically active compound, rhein anthrone [4, 5]. Rhein anthrone is poorly absorbed and produces a laxative effect throughout two independent mechanisms: (*i*) changing of colonic motility leading to an accelerated large intestinal transit and (*ii*) alterations in colonic adsorption and secretion of water and electrolytes resulting in fluid accumulation [6].

In the last years, there has been the concern that therapeutic or chronic use of anthranoid laxatives can cause structural damage

*Correspondence to: Francesca BORRELLI,
Department of Experimental Pharmacology,
University of Naples Federico II, Via D. Montesano 49,
80131 Naples, Italy.
Tel.: +39-081678665
Fax: +39-081678403
E-mail: franborr@unina.it

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to colonic tissue and induce colonic neuropathy and colorectal cancer. However, human and animal data in this area are inconsistent. Three clinical studies have evaluated the potential side effects of long-term use of anthraquinone laxatives on colonic nerve tissue [7–9]. One study found a direct association between stimulant laxative use and anatomic changes in the colon [7] while two clinical trials did not support the hypothesis that anthraquinone containing laxatives are able to provoke relevant degenerative changes in the colonic nerve tissue [8, 9]. van Gorkom and colleagues reported three clinical studies showing increased cell proliferation in colonic tissue of patients treated with a single high dose of sennosides [10–12]. Most epidemiological studies failed to find any association between anthranoid laxative use and colorectal cancer [13–17], although a prospective study showed an increased cell proliferation (interpreted as higher risk for developing colorectal cancer) in patients using anthranoid laxatives [18]. Finally, two single case reports identified an increased cell proliferation in patients using anthranoid laxatives [19, 20]. Similar conflicting data have been reported on preclinical studies. As a consequence of these data in 1995, the German Federal Institute for Drugs and Medical Devices revised the product information of all medicinal products containing anthranoids and in 1998 the Food and Drug Administration (FDA) classified the stimulant laxatives (including senna) from category I (generally recognized as safe and effective) to category III (further testing is required). In 2002, the FDA re-classified senna, but not aloe and cascara, to category I. Although there is no conclusive clinical evidence that senna is dangerous, there are still doubts and unresolved questions on the safety of this drug and its components. On this basis, physicians often underuse anthraquinones laxatives and are more willing to use far more expensive drugs that have a much shorter track record concerning potential long-term consequences to the constipated patient.

Therefore, the aim of this study was to investigate the potential damaging effects of the active anthranoid metabolite of senna, rhein, on a human colon adenocarcinoma cell line, Caco-2.

The cytotoxicity of rhein on proliferating and differentiating cells has been evaluated as well as its effects on cell proliferation, genotoxicity and epithelial integrity, all of which are thought to be associated with colorectal carcinogenesis.

Materials and methods

Chemicals

The 4,5-Dihydroxyanthraquinone-2-carboxylic acid (rhein), deoxycolic acid (DCA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1,1,3,3-tetramethoxypropane (malonaldehyde bis dimethyl acetal], standard MDA) were purchased from Sigma Aldrich S.r.l. (Milan, Italy). Monoclonal primary antibodies for phosphorylated extracellular-signal-related kinase $ERK_{1/2}$ ($pERK_{1/2}$) and ERK_2 were obtained from Santa Cruz Biotechnology Inc. (CA, USA) while peroxidase-conjugated (HRP) anti-mouse IgG antibody was obtained from Amersham Biosciences Inc. (GE Healthcare, Milan, Italy). All reagents for Western blot analysis and cell culture were obtained from Sigma Aldrich S.r.l. (Milan, Italy), Bio-Rad Laboratories (Segrate, Italy) and Microglass Heim S.r.l. (Naples, Italy). Rhein was dissolved in dimethylsulphoxide (DMSO) to obtain a final concentration of 0.1% (v/v) in the culture medium. The concentration range for rhein (0.1–10 μg/ml) used in the study was selected on the basis that a human therapeutic dosage (20–40 mg of dianthrones derivatives) or an overdosage (80–100 mg) of senna generate rhein concentrations of approximately 0.1–1 μg/ml (0.35–3.5 μM) or 10 μg/ml (35 μM), respectively [3].

Cell culture

Caco-2, A-431 cells (American Type Culture Collection, LGC Promochen, Italy) and human fibroblasts were cultured in 75 cm^2 polystyrene flasks (Falcon, Microglass Heim, Naples, Italy) as monolayers in modified Eagle medium (MEM) containing 10% foetal bovine serum (FBS), 100 Units/ml penicillin, 100 μg/ml streptomycin and 1% non-essential amino acids. Cells were cultured at 37°C in a humidified 5% CO_2 and 95% filtered air and the culture medium was replaced every 2 days. After washing in phosphate buffered saline (PBS), cells were trypsinized with 0.25% trypsin-EDTA at 37°C for 5 min., centrifuged at 1000 × *g* for 3 min. and then re-suspended in the appropriate medium. Cell viability was determined by trypan blue staining.

Cytotoxicity assay

MTT assay

The effect of rhein on cell survival was measured using the CellTiter 96® proliferation assay (MTT assay) (Promega, Madison, WI, USA) [21]. Caco-2 cells (passage between 26 and 29) were seeded in 96-wells plates at a concentration of 3×10^4 cells/well. After 48 hrs of culturing, the medium was removed and the cells were treated with rhein (0.1–10 μg/ml) at 37°C for 24 hrs. Following treatment, cells were washed and fresh medium was replaced. After 48 hrs of culturing at 37°C, 15 μl MTT dye solution were added to each well for 4 hrs. Finally, 100 μl of solubilization/stop solution were added to dissolve the purple crystals; absorbency of formazan was measured at a wavelength (λ) of 570 nm using a multiwell reader (Rainbow, SLT, Austria). Treatments were compared with a positive control, deoxycolic acid (250 μM). Experiments were repeated independently three times. The results are expressed as percentage of cell viability.

Neutral red (NR) assay

The NR assay system, one of the most used and sensitive cytotoxicity test, is a mean of measuring living cells *via* the uptake of the vital dye neutral red. Viable cells will take up the dye by active transport and incorporate the dye into lysosomes, whereas non-viable cells will not take up the dye. An increase or decrease in the number of cells or their physiological state results in a concomitant change in the amount of dye incorporated by the cells in the culture [22]. Caco-2 cells (passage between 26 and 29) were seeded in 96-wells plates at a concentration of 1×10^4 cells/well. After 48 hrs of culturing, the medium was removed and the cells were treated with rhein (0.1–10 μg/ml) at 37°C for 24 hrs. Following treatment, cells were washed and 200 μl NR dye solution (50 μg/ml in DMEM) were added to each well for 3 hrs at 37°C. After washing in PBS, 100 μl of 1% acetic acid were added and the absorbency was measured at a wavelength (λ) of 532 nm using a multiwell reader (Perkin-Elmer Instruments Waltham, MA, USA). Treatments were compared with a positive control, deoxycolic acid (250 μM). The results are expressed as percentage of cell viability.

Trans-epithelial electrical resistance (TEER) assay

TEER was monitored as an indication of tight junction formation and epithelial monolayer integrity as previously described [23]. Falcon®-Transwell inserts (0.4 μm pore size; BD Bioscience, Buccinasco, Italy) were coated with 0.01% type I rat-tail collagen (Sigma) and left to dry overnight under ultraviolet (UV) lights in 6-well plates. Caco-2 cells (passage between 57 and 63) were seeded into these inserts in 2.5 ml aliquots at a concentration of 2.5×10^5 cells/ml. Culture medium (1.5 ml) was added to the basolateral compartment of each well. The cells were grown on these inserts and the medium was changed every 2 days. After 7 days of culturing TEER, readings (expressed as Ωcm^2) were taken using an EVOM epithelial voltohmmeter with chopstick electrodes (World Precision Instruments Inc., Stevenage, UK). Readings were taken every 24 hrs until they stabilized (at days 14–16). At this point the cells, maintained at 37°C in a humidified 5% CO_2 and 95% filtered air, were deemed fully differentiated. The culture medium was replaced every other day for 21 days. A final TEER reading was taken immediately before adding rhein (0.1–10 μg/ml) and after 24 and 48 hrs. Treatments were compared with a positive control, deoxycholic acid (250 μM). The results are expressed as percentage change in TEER compared with the TEER value at the start of the experiment (time 0). Three independent experiments were conducted.

Paracellular permeability assay

Caco-2 monolayers in 6-well plates were incubated with rhein (0.1–10 μg/ml) in presence of 10 μM fluorescein (Sigma). Samples (200 μl) were removed from the apical compartment at the beginning of the experiment (time 0) and from the basolateral compartment after 24 and 48 hrs. The samples fluorescein content was measured using a microplate reader (Tecan SPECTRA Rainbow, PAA Ltd, UK) (488 nm excitation and 510 nm emission). The paracellular permeability represents the amount of fluorescein in the basolateral compartment expressed as percentage of the amount added to the apical compartment at the start of the experiment, to give a measure of the relative amount of fluorescein travelling through the Caco-2 monolayer tight junctions (percentage of monolayer permeability). Three independent experiments were conducted.

Proliferation assay

^{3}H-Thymidine incorporation

Caco-2 cells (passage between 28 and 34) were seeded in 24-well plates at a density of 1.0×10^4 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. After 48 hrs of incubation, cells were treated with rhein (0.1–10 μg/ml) in the presence of ^{3}H-thymidine (1 μCi/well) for 24 hrs. Medium was removed by aspiration, and after washing in PBS, cells were scraped in 1 M NaOH (100 μl/well) and collected on glass fibre filter mats using an LKB (Bromma, Sweden) automatic cell harvester prior to liquid scintillation counting. The effect of rhein on cell proliferation was expressed as count per minute/mg of protein (CPM/mg protein) of incorporating ^{3}H-Thymidine cells. The treatments were carried out in triplicate and three independent experiments were performed.

Preparation of cytosolic fractions and Western blot analysis

Caco-2 cytosolic extracts were prepared as previously described [24]. Briefly, after rhein (0.1–10 μg/ml) incubation for 24 hrs, the medium was removed and cells were washed with ice-cold PBS. The cells were collected by scraping for 10 min. at 4°C with lysis buffer (50 mM Tris-HCl pH = 7.4, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1% NP-40, 1mM PMSF, 1 mM Na_3VO_4 containing complete protease inhibitor cocktail [Roche Diagnostics, Mannheim, Germany]). After centrifugation at 16,200 *g* for 15 min. at 4°C, the supernatants were collected and protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, Milan, Italy). For Western blot analysis, lysate aliquots containing 70 μg of proteins were separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Protran®, Protran Nitrocellulose Transfer Membrane Schleicher & Schuell Bioscience, Dassel, Germany) using a Bio-Rad Transblot (350 mA, 3 hrs). Proteins were visualized on the filters by reversible staining with Ponceau-S solution (Sigma) and de-stained in PBS containing 0.1% Tween 20. Membranes were blocked at 4°C in milk buffer (5% non-fat dry milk in PBS/Tween 0.1%) and then incubated overnight at 4°C with mouse monoclonal antibodies for $pERK_{1/2}$ and ERK_2 (Santa Cruz, DBA S.r.l, Italy). The mouse monoclonal anti-$pERK_{1/2}$ and anti-ERK_2 were used at 1:1000 dilution in milk buffer (5% non-fat dry milk in PBS/Tween 0.1%). Subsequently, the membranes were incubated for 1 hr at room temperature with 1:2000-dilution of antimouse IgG-HRP-conjugated secondary antibody (Amersham Biosciences, UK). After washing in PBS/Tween 0.1%, the membranes were analysed by enhanced chemiluminescence's (ECL; Amersham Biosciences). The optical density of the bands on autoradiographic films was determined by an image analysis system (GS 700 Imaging Densitometer, Bio-Rad) equipped with a software Molecular Analyst (IBM, Milan, Italy). The effect of rhein on the mitogen-activated protein (MAP) kinase activation was expressed as ratio of densitometric analysis of $pERK_{1/2}$/total ERK bands.

Genotoxicity assay

DNA damage was examined by single-cell gel electrophoresis (Comet assay) [25]. Caco-2 cells (passage between 33 and 37) were seeded in 25 cm^2 polystyrene flasks (Falcon) and grown to ~70% confluence. Then, cells were incubated with rhein (0.1–10 μg/ml) for 24 hrs and subsequently they were trypsinized to obtain a suspension of 1.5×10^5 cells/ml. Aliquots of cell suspension were incubated with and without H_2O_2 challenge (75 μM for 5 min. on ice) and then centrifuged at $1000 \times g$ for 5 min. at 4°C. The supernatants were discarded and the pellets were mixed with 85 μl of 0.85% low melting point agarose in PBS. Cells were added to previously prepared gels of 1% normal melting point agarose in PBS. The gels on frosted slides were suspended in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris and 1% Triton X-100, pH 10) at 4°C for 1 hr, and then electrophoresed in buffer (300 mM NaOH, 1 mM Na_2EDTA, pH>12) at 26 V, 300 mA for 20 min. After electrophoresis, gels were immersed in 0.4 M Tris–HCl, pH 7.5 (3×5 min. washes) to neutralize the alkaline pH, and stained with 20 μl of ethidium bromide (2 μg/ml) before scoring. Images were analysed using a fluorescence microscope (Nikon, Florence, Italy) interfaced with a computer. DNA damage (percentage of tail DNA) was quantified using Komet 5.0 image analysis software (Kinetic Imaging, Liverpool, UK). Positive (H_2O_2; 75 μmol/L) and negative (PBS) controls were included for all experiments. The mean percentage of tail DNA was calculated from 100 cells per gel (each sample in triplicate) and the mean of each independent experiment ($n = 3$) was used in the statistical analysis.

Antioxidant assay

TBARs assay

Lipid peroxidation products from Caco-2 cells were measured by the thiobarbituric acid colorimetric assay [26]. The reaction of lipid peroxides

(formed after the oxidative stress) and the thiobarbituric acid (TBA) leads to the formation of malondialdehyde that can be measured by colorimetric detection of the chromogen (pink colour complex). Briefly, Caco-2 cells were seeded in 6-well plates at a density of 3.0×10^6 in DMEM supplemented with 10% FBS. After treatment with rhein (0.1–10 μg/ml) for 24 hrs, cells were washed with PBS and incubated with the Fenton's reagent (Fe^{2+}/H_2O_2 1 mM) for 3 hrs at 37 °C. After incubation, cells were washed and scraped in PBS. Cells were lysed by six cycles of freezing and thawing in PBS and then centrifuged at 12,600 × *g* for 10 min. at 4°C. To 150 μl of cellular lysate were added 300 μl of 10% (w/v) trichloroacetic acid (TCA) and, after centrifugation at 16,200 × *g* for 10 min., 0.67% (w/v) thiobarbituric acid was added and the mixture was heated at 80°C for 30 min. After cooling, malondialdehyde (MDA) production was recorded (A_{490nm}) in a Beckman DU-62 spectrophotometer (Beckman, Milan, Italy) and the results are presented as μmol of MDA/mg of cell proteins determined by the Bio-Rad protein assay. A standard curve of MDA was used to quantify the MDA levels formed during the experiments.

Intracellular reactive oxygen species (ROS) measurement

The generation of intracellular ROS was estimated using the fluorescence probe 2',7'-dichlorfluorescein-diacetate (H_2DCF-DA) [27]. The H_2DCF-DA is able to diffuse passively into cells, where the acetate is cleaved by intracellular esterases to the non-fluorescent H_2DCF that thereby traps it within the cell. In the presence of intracellular ROS, H_2DCF is rapidly oxidized to the highly fluorescent 2′,7′- dichlorofluorescein (DCF). The DCF fluorescence intensity is paralleled to the amount of ROS formed intracellularly. For the experiments, cells were plated in 96 multi-well black plates (Corning, USA) at the density of 1×10^4 cells per well and led to the differentiation. Confluent Caco-2 cell monolayers were incubated for 24 hrs at 37°C with rhein (0.1–10 μg/ml). After washing in PBS, cells were incubated for 30 min. with 200 μl of 100 μM DCFH-DA in Hank's balanced salt solution (HBSS) containing 1% FBS. Finally, cells were rinsed and incubated with the Fenton reagent (H_2O_2/Fe^{2+} 2 mM) for 3 hrs at 37°C. The DCF fluorescence intensity was detected using a fluorescent microplate reader (Perkin-Elmer Instruments) at the excitation wavelength (λ) of 485 nm and the emission wavelength (λ) of 538 nm. The ROS levels were expressed as fluorescence intensity (picogreen).

Statistical analysis

Results are expressed as mean ± S.E.M of *n* experiments. The analyses of results were carried out using GraphPad Prism Software (GraphPad Software, Inc. San Diego, CA, USA). Comparisons between two sets of data were made by Student's t-test for paired data. When multiple comparisons against a single control were made, one-way ANOVA was used, followed by Turkey-Kramer multiple comparisons test. A *P*-value less than 0.05 was considered significant. For Comet assay the differences between means were evaluated by ANOVA, followed by *post hoc LSD*.

Results

MTT and NR assay

In the MTT assay, rhein in the concentration range of 0.1–10 μg/ml had no significant cytotoxic effect on Caco-2 cells after 24 hrs exposure (control: 100 ± 0.0; rhein 0.1 μg/ml: 94.69 ± 3.45; rhein 1 μg/ml: 95.47 ± 2.49; rhein 10 μg/ml: 93.70 ± 0.42). Deoxycholic acid (DCA, 250 μM), a secondary bile acid used as positive control, significantly ($P < 0.001$) reduced the cell viability in proliferating Caco-2 cells (control: 100 ± 0.0 and DCA 250 μM: 69.04 ± 2.21). These data have been confirmed by the NR assay (control: 100 ± 0.0; rhein 0.1 μg/ml: 102.5 ± 2.02; rhein 1 μg/ml: 93.26 ± 7.14; rhein 10 μg/ml: 91.21 ± 5.07; DCA 250 μM: 50.5 ± 0.32).

TEER and fluorescein assay

Figures 1 and 2 show the effect of rhein on Caco-2 epithelial monolayer integrity. Differentiated Caco-2 cells exhibit different phenotypes characteristic of villus tip cells or crypt base cells and they are a well-known experimental model to examine the maintenance of junctional integrity according to a procedure previously described [28]. All tested rhein concentrations (0.1–10 μg/ml) had no significant adverse effects on the epithelial monolayer, as indicated by TEER value measurement after 24 and 48 hrs of rhein exposure (Fig. 1A). As expected, the incubation of differentiated Caco-2 cells with DCA (250 μM), resulted in a marked decrease in TEER value (~30%; $P < 0.001$), reflecting disruption of enterocytes tight junctions (Fig. 1). The absence of detrimental effects of rhein on tight junction integrity as assessed by TEER, was confirmed by the fluorescein flow assay (Fig. 1B) where no increase in fluorescein flow from the apical to the basolateral compartment of the Caco-2 monolayer occurred.

^{3}H-Thymidine incorporation

Thymidine is a precursor of DNA and it is incorporated into new DNA in proliferating cells. For this reason, the ^{3}H-Thymidine incorporation into DNA during 24 hrs has been measured as an indicator of cell proliferation, and thereby DNA synthesis. Rhein, at the concentrations of 0.1 and 1 μg/ml, significantly ($P < 0.001$) reduced the ^{3}H-Thymidine incorporation in proliferating Caco-2 cells (Fig. 2). By contrast, rhein at the higher concentration (10 μg/ml) significantly ($P < 0.001$) increased Caco-2 cells proliferation (Fig. 2). Moreover, rhein, at all concentrations used (0.1–10 μg/ml) did not modify the ^{3}H-Thymidine incorporation in proliferating A-431 cells (control: 548.5 ± 60.48 CPM/mg; rhein 0.1 μg/ml: 541.3 ± 56.26 CPM/mg; rhein 1 μg/ml: 612.7 ± 56.63 CPM/mg; rhein 10 μg/ml: 596.2 ± 76.49 CPM/mg) and human fibroblasts (control: 684.0 ± 10.0 CPM/mg; rhein 0.1 μg/ml: 585.5 ± 22.50 CPM/mg; rhein 1 μg/ml: 644.5 ± 29.50 CPM/mg; rhein 10 μg/ml: 670.0 ± 12.0 CPM/mg).

Western blot analysis

The possible molecular mechanism of rhein on cell proliferation was investigated by studying its effect on the MAP kinase (MAPK)

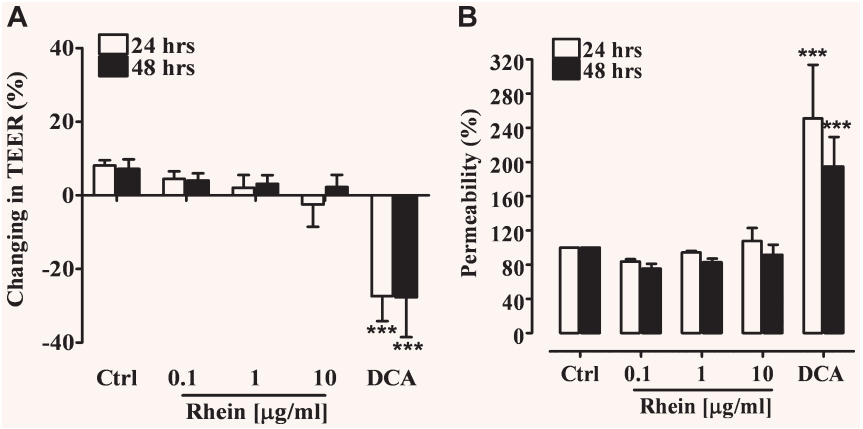

Fig. 1 Trans-epithelial electrical resistance (TEER) of polarized Caco-2 monolayer exposed to rhein (0.1–10 μg/ml) and deoxycholic acid (DCA, 250 μM) for 24 and 48 hrs (**A**). Effect of rhein (0.1–10 μg/ml) and deoxycholic acid (DCA, 250 μM) on the colonic monolayer permeability after 24 and 48 hrs exposure (**B**). *** $P <$ 0.001 *versus* control (Ctrl), $n = 3$, mean ± S.E.M.

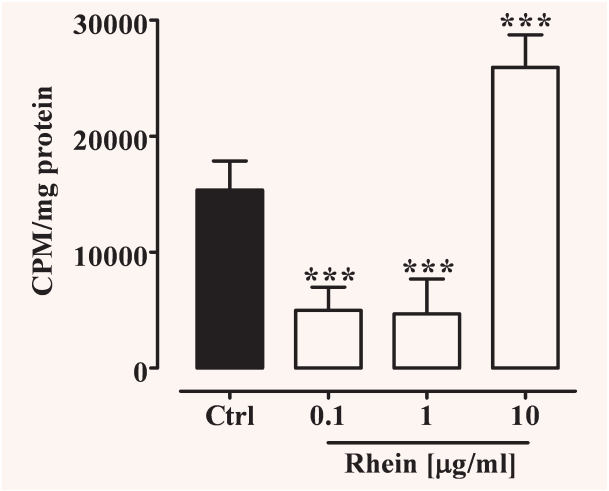

Fig. 2 Effect of rhein (0.1–10 μg/ml) on Caco-2 cell proliferation after 24 hrs of incubation. *** $P < 0.001$ *versus* control (Ctrl), $n = 3$, mean ± S.E.M.

signalling pathways. The MAPK pathway involves two closely related kinases, known as ERK 1 (ERK_1, p44) and 2 (ERK_2, p42) that come from dimerization of total cytosolic ERK. The modulation in ERK_1 and ERK_2 phosphorylation after 24 hrs rhein exposure was consistent with the observed effect of rhein on cell proliferation. At concentrations of 0.1 and 1 μg/ml, a significant ($P <$ 0.001) reduction in the expression of phosphorylated ERK_1 ($pERK_1$) and ERK_2 ($pERK_2$) was observed (Fig. 3). By contrast, rhein at the higher concentration (10 μg/ml) significantly ($P <$ 0.001) increased $pERK_1$ and $pERK_2$ expression, resulting in MAP kinase activation (Fig. 3).

Genotoxicity assay

The comet assay is a sensitive and valid method to evaluate the potential genotoxic effect of a substance. Rhein (0.1–10 μg/ml) alone did not significantly affect DNA damage after 24 hrs exposure suggesting the absence of a genotoxic effect. Exposure of Caco-2 cells to hydrogen peroxide (75 μM) produced a significant ($P < 0.001$) increase in the percentage of DNA in the comet tail, indicating an increase in single-strand breaks (Fig. 4). Pre-treatment Caco-2 cells with rhein for 24 hrs reduced, in a concentration-dependent manner, the H_2O_2-induced DNA damage. The decrease was significant ($P < 0.05$) at a rhein concentration of 10 μg/ml (Fig. 4).

MDA and intracellular ROS measurement

Fenton's reagent, hydrogen peroxide (1 mM) in the presence of iron (II) ions (1 mM), induced an oxidative stress in Caco-2 cells after 3 hrs exposure, resulting in malondialdehyde production ($P < 0.01$) (Fig. 5). Pre-incubation of Caco-2 cells for 24 hrs with high concentration of rhein (10 μg/ml) significantly ($P < 0.001$) reduced the increase of H_2O_2/Fe^{2+}-induced malondialdehyde cytosolic levels (Fig. 5). Rhein at the concentrations of 0.1 and 1 μg/ml was inactive.

The exposure of the Caco-2 cells to H_2O_2/Fe^{2+} (2 mM) produced a significant ($P < 0.0001$) increase in ROS formation (Fig. 6). A pre-treatment for 24 hrs with rhein (10 μg/ml) reduced significantly ($P < 0.05$) the ROS formation as measured by the inhibition of DCF fluorescence intensity (Fig. 6). Consistent with the results obtained in the MDA assay, rhein at the concentrations of 0.1 and 1 μg/ml was not active (Fig. 6).

Discussion

Gastrointestinal tract mucosa shows a rapid cell turnover rate determined by cell proliferation and cell loss (apoptosis) processes [29]. The integrity of the epithelial cell layer is maintained by intercellular junctional complexes composed of tight

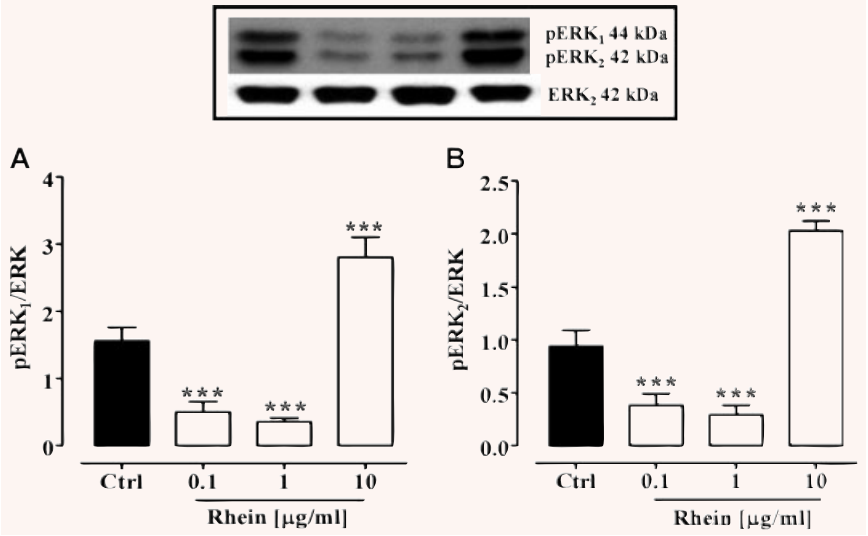

Fig. 3 pERK$_1$ (**A**) and pERK$_2$ (**B**) expression (MAP-kinase activation) in Caco-2 cells after 24 hrs of rhein (0.1–10 μg/ml) incubation. Insert: representative Western blot analysis. *** $P < 0.001$ *versus* control (Ctrl), $n = 3$, mean ± S.E.M.

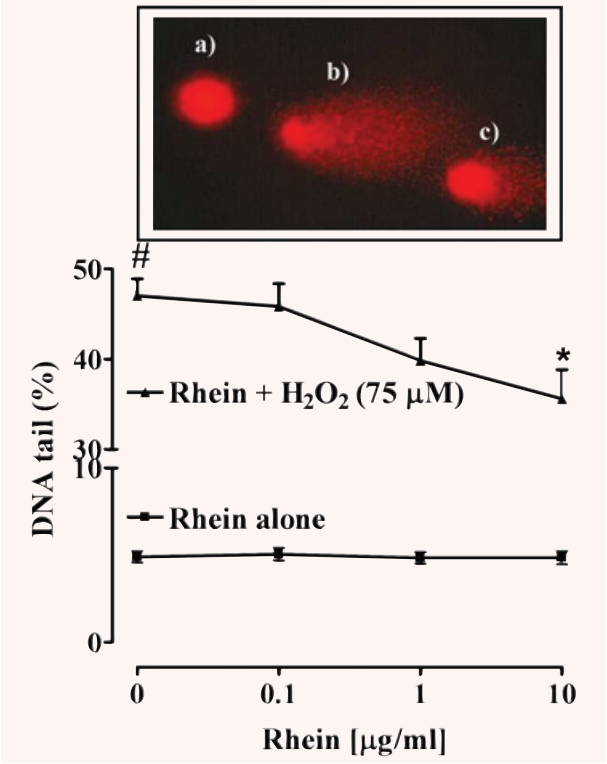

Fig. 4 Effect of rhein (0.1–10 μg/ml) on Caco-2 cells exposed (▲) or not (■) to 75 μM hydrogen peroxide (H_2O_2). Insert: representative comet images of a control cell (**a**), a severally damaged cell (**b**) and a partially genoprotected cell (**c**). # $P < 0.001$ *versus* untreated cells; * $P < 0.05$ *versus* H_2O_2 alone, $n = 4$, mean ± S.E.M.

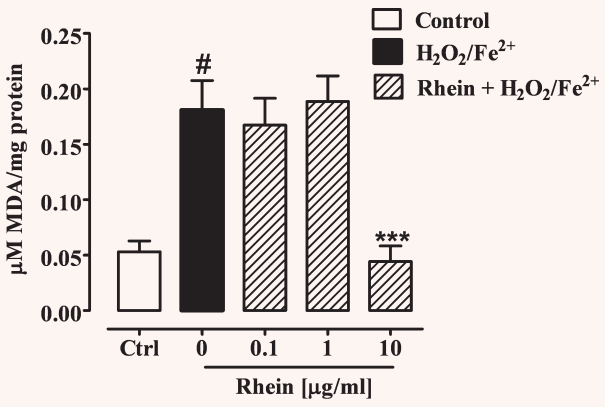

Fig. 5 Effect of rhein (0.1–10 μg/ml) on Fenton's reagent (H_2O_2/Fe^{2+} 1 mM)-induced malondialdehyde (MDA) production after 24 hrs exposure in 7 days differentiated Caco-2 cells. # $P < 0.01$ *versus* control (Ctrl) and *** $P < 0.001$ *versus* H_2O_2/Fe^{2+} alone, $n = 3$, mean ± S.E.M.

junctions, adherent junctions and desmosomes. Tight junctions are essential structures for the physiological functions of epithelial and endothelial cells, and have been suggested to have both barrier and defence functions, including the regulation of paracellular pathways. Changes in epithelial cell turnover and alterations of the epithelial monolayer permeability can occur during many gastro-intestinal diseases such as colon cancer. In fact, colon cancer cells exhibit both abnormal cell turnover and impaired tight junction morphology, leading to increase of paracellular permeability [30]. Furthermore, in the latter phases of carcinogenesis, the disruption of cell-cell junctions with concomitant changes in the junctional proteins expression represents a hallmark of cancer cell invasion and metastasis [31].

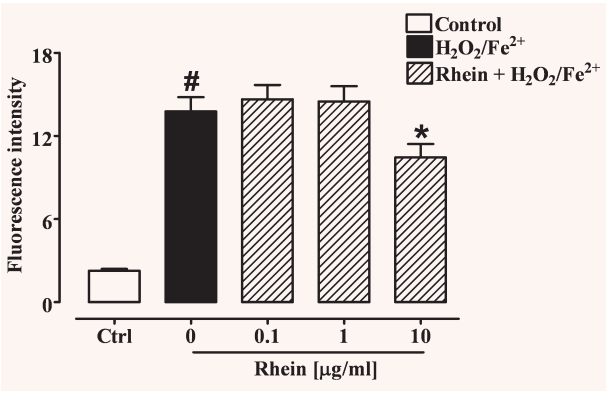

Fig. 6 Effect of rhein (0.1–10 μg/ml) on Fenton's reagent (H_2O_2/Fe^{2+} 2 mM)-induced reactive species reagents production after 24 hrs exposure in differentiated Caco-2 cells. $^{\#}$ $P < 0.001$ *versus* control (Ctrl) and * $P < 0.05$ *versus* H_2O_2/Fe^{2+} alone, $n = 3$, mean ± S.E.M.

In the last decade, the use of anthraquinone laxatives, in particular the use of senna, has been associated with a damage of the intestinal epithelial layer and an increased risk to develop colorectal cancer. In this paper, we have demonstrated that rhein, the active metabolite of senna, does not trigger cytotoxic or genotoxic effects in a human colon adenocarcinoma cell line; moreover, rhein shows modulatory effects on cellular proliferation depending on the concentration used and possesses anti-genotoxic activity.

Caco-2 cells, which undergo differentiation in culture, are widely used as a model for colorectal cancer; particularly to assess effects on epithelial function [32, 33], genotoxicity [34, 35] and cell proliferation [36].

Although there are conflicting data, it has been suggested that the mechanism of the laxative effect of anthranoids is strictly correlated to cell damage. Anthranoids laxatives have been proposed to increase: (*i*) the number of macrophages in the connective tissue of the colonic mucosa, (*ii*) the intensity of lysosomal activity and (*iii*) the number of lysosomes in macrophages, Schwann cells and neurones of the sub-mucosal plexus of the colonic mucosa [37, 38]. In addition, recently, it has been reported that rhein in Caco-2 cells produces changes of membrane fluidity either *via* a reduction of adenosine-5'-triphosphate (ATP) production or *via* an intercalation into cell membranes [39]. Using the MTT and NR assays, we demonstrated that rhein did not affect the vitality of proliferating Caco-2 cells; moreover, TEER and the fluorescein flow assays showed that rhein did not alter the integrity of membrane barrier function, thus excluding cell damage in the mechanism of rhein laxative effect. Our results are in disagreement with some previous studies; here, we have used rhein concentrations (0.35 and 3.5 μM) corresponding to concentrations reached in the colonic lumen after therapeutic doses of anthranoid laxatives. Previous studies have reported a cytotoxicity effect of rhein on intestinal mucosal cells using very high concentrations (>50 μM) [11, 40].

It is well known that colorectal cancer is promoted by an uncontrolled proliferation of mucosa cells in the terminal portion of intestine. Conflicting results have been reported on the effect of rhein on cell proliferation. Two pre-clinical *in vivo* studies reported no effect of rhein on intestinal cell proliferation [41, 42] whereas other authors showed a stimulatory effect [40], and an inhibitory effect [43]. In this paper, for the first time we report a modulatory action of rhein on cell proliferation, depending on the concentration used. In fact, our experiments showed that rhein, at the concentrations of 0.1 and 1 μg/ml (rhein concentrations present in the colon after a human therapeutic dosage of senna) reduced the cell proliferation of human colon adenocarcinoma cells; whereas, rhein at the high concentration (10 μg/ml, concentration corresponding to rhein present in the colon after a dosage of senna ten-fold higher then therapeutic dosage) produced a significant increase of the human colon adenocarcinoma cell proliferation. Interestingly, long-term *in vivo* studies have shown that senna, used at laxative dosage, reduced the appearance of preneoplastic lesions and tumours, whereas a diarrhogenic dose increases the appearance of tumours induced by a carcinogenic agent [44, 45]. Whether or not the effect of rhein is time dependent has been not determined in this study. Also, it would be interesting to investigate in future studies if the effect of rhein involves c-Jun N-terminal kinase (JNK) or related pathways.

Using the normal human fibroblast cells and an epidermoid carcinoma cell line from human skin (A-431), we have demonstrated that the effect of rhein on cell proliferation is tumour and colon specific, respectively.

One of the most important pathway in cell proliferation is represented by the MAP kinases. MAPKs encompass a group of enzymes recruited in response to cellular stress, including heat and osmotic stress, cytokines and UV irradiation [46, 47]. The accepted role of MAPKs is to produce cellular response to those stresses by integrating multiple stimuli and activating transcription factors. In most cell lines, MAPKs have been implicated in regulating mitogenic responses and in the synthesis of stress response proteins [42]. The three major MAPK families have been recognized as p38 MAPK, ERK_1 and ERK_2 and stress-activated protein kinase (SAPK/JNK). Although the molecular mechanisms inducing MAPKs pathways activation are not totally clear, it is well known that $ERK_{1,2}$ phosphorylated proteins stimulate the cell growth and inhibit the apoptotic processes [48, 49]. In this study, we observed that rhein at concentrations of 0.1 and 1 μg/ml reduced ERK_1 and ERK_2 phosphorylated proteins expression. By contrast, rhein at the concentration of 10 μg/ml determined an increase of ERK_1 and ERK_2 activation. These findings suggest that rhein modulates cell proliferation *via* an involvement of the MAPK pathway either inhibiting or stimulating the phosphorylation of ERK proteins. Our results are consistent with a recent study showing that rhein modulates cell proliferation of articular chondrocytes by acting on ERK and JNK-AP-1-dependent pathways [50]. Interestingly, other compounds isolated from wild plants, such as *Saxifraga stolonifera* and rhubarb have been reported to inhibit the proliferation of cancer cells by apoptosis induction or ERK phosphorylation inhibition [51, 52].

Studies in human beings and animals implicate oxidative DNA damage as an important factor in mutagenis and carcinogenesis

[53–55]. In our experiments, rhein did not induce DNA damage in Caco-2 cells suggesting that it is not a genotoxic agent. In fact, rhein exerted a protective effect on hydrogen peroxide-induced DNA damage in human colon carcinoma cells at the concentration of 10 μg/ml. This is consistent with a previous report showing a rhein-mediated inhibition of DNA damage induced by Trp-P-1 in human lymphocytes [56].

In order to investigate the mechanism responsible for the genoprotective effect of rhein on H_2O_2-induced DNA damage, we investigated MDA and ROS cytosolic levels in Caco-2 cells. In accordance with other papers, exposure of Caco-2 cells to hydrogen peroxide and ferrous sulphate (Fe^{2+}) resulted in oxidative damage, assessed as increased cytosolic levels of MDA and ROS [57, 58]. Pre-treatment with rhein significantly reduced the Fenton reaction-induced MDA and ROS formation, suggesting a potential antioxidant mechanism in the anti-genotoxic effects of rhein.

In conclusion, this paper provides *in vitro* evidence that rhein, the active metabolite of senna, does not possess cancer-inducing potential at concentrations corresponding to human therapeutic doses of senna. At such concentrations, rhein was not cytotoxic in both proliferating and differentiated cells, did not disrupt tight junctions and in fact inhibited colon adenocarcinoma cell proliferation. Such results indicate a lack of tumour-promoting activity. The molecular mechanism of the rhein anti-proliferative effect seems to involve directly the MAP kinase pathway. Furthermore, genotoxicity studies have shown that rhein did not induce DNA damage; by contrast, it was able to protect the DNA against the genotoxic action of hydrogen peroxide, probably because of an anti-oxidant action.

Funding

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Competing interest

None.

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LETTER TO THE EDITOR

Senna: a laxative devoid of carcinogenic effects

F. Borrelli · G. Aviello · R. Capasso · F. Capasso

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In the last years, the use of anthranoid laxatives has been associated with the development of intestinal cancer. Recently, Mitchell et al. (2006) have reported results from a 2 years study on senna (Tinnevelly senna fruit) tolerability and safety. Senna, administered for 2 years at doses of 25, 100 and 300 mg/kg/day in male and female rats, did not induce statistically significant differences on several outcome measures (or assessments) (e.g., body weight, hematology analyses, histology evaluation of some tissues and mortality ratio), compared to control group. The only differences observed in the highest dose of senna (dose producing a laxative effect) were an increase of water consumption, changes in electrolytes in serum and urine and increase of tubular basophilia and epithelial hypertrophy in the kidneys; these effects, which are reversible, are typical physiological events produced by laxatives. Very interesting, in this paper, is the absence of both alterations in the colonic nervous plexus and increase of cell proliferation in the large intestine. This study confirms pre-existing results on the potential cancerogenicity of senna (Lyden-Sokolowski et al. 1993). In particular, Lyden-Sokolowski et al. (1993) have shown that there is no relationship between long-term administration of senna and intestinal neoplastic lesions.

Two recent studies, not mentioned by Mitchell et al., report the same conclusions (absence of an initiating effect on colorectal carcinogenesis) and highlight the absence of a promoting effect on colorectal carcinogenesis (Mascolo et al. 1999; Borrelli et al. 2005). These papers have shown that senna, administered at laxative doses, is devoid of any carcinogenic potential, when it was given to rats over a 2-year period. Indeed, senna is not able to induce the development of aberrant crypt foci (preneoplastic lesions) and tumors in healthy rats. In contrast, the development of aberrant crypt foci and tumors in rats treated with an initiating tumor agent, azoxymethane, was significantly reduced by the laxative dose of senna. These results seem to suggest that senna possesses a potential anti-tumoral activity on rat colon carcinogenesis instead of a carcinogenic effect.

In conclusion, there is no evidence to support the hypothesis that senna influences the carcinogenesis of colon when used at therapeutic dosages; in contrast, an anticancer action could exist. Further studies are necessary to evaluate the potential anti-tumoral activity of senna.

F. Borrelli (✉) · G. Aviello · R. Capasso · F. Capasso
Department of Experimental Pharmacology,
University of Naples Federico II, Via D. Montesano 49,
80131 Naples, Italy
e-mail: franborr@unina.it

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Does Senna Extract Promote Growth of Aberrant Crypt Foci and Malignant Tumors in Rat Colon?

NICOLA MASCOLO, EUGENIO MERETO, FRANCESCA BORRELLI, PATRIZIA ORSI, DANIELA SINI, ANGELO A. IZZO, BARBARA MASSA, MAURIZIO BOGGIO, and FRANCESCO CAPASSO

Current evidence suggests that aberrant crypt foci (ACF) can be used to evaluate agents for their potential colon carcinogenic activity. The aim of the present study was to determine whether senna pod extract (SE) itself induces ACF and tumors in the rat colon or increases the development of ACF and tumors induced by azoxymethane (AOM). A daily administration of SE 10 mg/kg by mouth for 13–28 weeks produced a weak laxative effect but did not itself cause the appearance of ACF or tumors. The numbers of ACF and tumors induced by AOM were, however, increased by a dose of SE (100 mg/kg) able to induce chronic diarrhea over three months. These results suggest that SE does not cause the appearance of ACF or tumors in the rat colon nor does it have a promoting effect when given to rats at a dose that produces laxation (10 mg/kg), whereas a diarrhogenic dose (100 mg/kg) increases the appearance of tumors induced by AOM.

KEY WORDS: anthraquinones; aberrant crypt foci; tumors; intestine; laxative; senna.

In the modern society use and abuse of laxatives have been rapidly increasing in last decades. This is due to the increasing age of the population (laxative use being higher among elderly), to particular sociocultural habits (sedentary life-style, low-fiber diet, psychological need of daily evacuation) and to the easy availability of such drugs that do not need any medical prescription (1). Among the various laxatives, anthraquinones stand out as being effective laxatives (2). As a matter of fact they decrease fluid and electrolyte absorption and increase secretion in the small intestine and especially in the colon, an effect due in part to nitric oxide generation (3, 4). They also alter motility of the large intestine, and this action contributes to their laxative effect (2). During the late 1970s some anthraquinone derivatives (1-hydroxy- and 1,8-dihydroxyanthraquinones) were reported to be mutagenic in bacteria (5), the supposed mechanism of action being a DNA intercalation similar to that of acridine mutagens (6). Since nonelectrophilic intercalating agents tend to be less effective genotoxins toward mammalian DNA than to prokaryotic DNA (7), the anthraquinones were not considered dangerous for human health. Later on, many authors pointed out a genotoxic effect on mammalian cells (8) and a carcinogenic effect after long-term administration in rats (9, 10). A higher risk of colonic cancer in humans linked to prolonged use of such substances had also been reported (11, 12). In addition to a possible direct genotoxic effect, anthraquinones were

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From the Department of Pharmaceutical Sciences, University of Salerno, Salerno; Department of Internal Medicine. Pharmacology and Toxicology Unit, University of Genoa, Genoa; Department of Experimental Pharmacology, University of Naples "Federico II", Naples; and Institute of Anatomo-Pathology, University of Genoa, Genoa, Italy.

Address for reprint requests: Professor Francesco Capasso, Department of Experimental Pharmacology, via D. Montesano 49, 80131 Naples, Italy.

reported to act as cancer-promoting agents (13), by inducing an increase in the proliferative activity of the colonic epithelium (14–16).

This work was undertaken to examine senna, the anthraquinone drug most frequently employed as a laxative, for its ability to induce both aberrant crypt foci (ACF), which are regarded as putative preneoplastic lesions in colon carcinogenesis (17–20), and tumors in the rat colon mucosa.

MATERIALS AND METHODS

Chemicals and Animals. Azoxymethane (AOM) was purchased from Sigma (Milan, Italy), senna pod extract (from *Cassia angustifolia* containing about 50% of sennoside B; SE) was obtained from Indena (Milan, Italy). AOM was dissolved in saline; SE was dissolved in water.

Experiments were performed on male Wistar rats (Harlan-Nossan, Correzzana, Italy) weighing 120 ± 10 g at the start of the study. They were housed in an air-conditioned room (23 ± 2°C, 50 ± 10% relative humidity) with a 12-hr light–dark cycle, and had free access to tap water and rat chow (Mucedola, Milan, Italy). The animals were randomly divided into nine groups as follows: group 1 served as the untreated control; groups 2 and 7 were treated with AOM (15 mg/kg); groups 3 and 4 were given 10 and 100 mg/kg of SE for 13 weeks; groups 5 and 6 received AOM + SE 10 and 100 mg/kg; and groups 8 and 9 received SE 10 mg/kg and AOM + SE 10 mg/kg, respectively, for 28 weeks. The SE doses were selected to induce laxation (10 mg/kg) or diarrhea (100 mg/kg) as experienced by a single treatment. The body weight of rats was controlled weekly and the laxative effect was measured on the first day of treatment and after 13 and 28 weeks of treatment. For collection of feces, animals were kept individually in cages with a wire mesh floor through which feces fall onto blotting paper. After treatment, feces were collected every 2 hr for 10 hr. The amount of normal and soft feces was counted by visual observation for each period and wet and dry weight determined. Dry weight was evaluated after drying at 60°C until constant weight.

Treatments, ACF Technique, and Tumor Evaluation. AOM, used as initiating agent to evaluate SE acting as tumor promoter, was administered intraperitoneally on day 1 and 5 of treatment. SE was given six times a week by intragastric gavage in the morning. All animals of groups 2–6 were killed by cervical dislocation under ether anesthesia 13 weeks after AOM initiation. This time is associated with the higher incidence of ACF (20). Rats of groups 7, 8, and 9 were killed at week 28 after the beginning of treatment in order to verify the number of ACF that had developed into tumors.

For ACF determination, the colons were rapidly removed after death, washed with saline, opened longitudinally, laid flat on a polystyrene board, and fixed with 10% buffered formaldehyde solution before staining with 0.2% methylene blue in saline. They were examined using a light microscope at 40× magnification. The criteria used to identify the aberrant crypts were as previously described (21); briefly, in comparison to normal crypts, they have greater size, larger and often elongated openings, thicker lining of epithelial cells, compression of adjacent crypts, and are more darkly stained with methylene blue. The detection and quantitation of ACF were performed on the distal 9 cm of the colon, starting from the rectal end. According to the number of constituent crypts, ACF were divided into two groups: small ACF containing 1–3 crypts per focus, and large ACF containing 4 or more crypts per focus. To determine crypt multiplicity, the number of aberrant crypts in each focus was recorded.

For tumor evaluation, the colons of all rats were discolored with 70% ethanol and embedded in paraffin; serial sections 3 μm thick were obtained and stained with hematoxylin–eosin.

Benign tumoral lesions (adenomas) and malignant ones (adenocarcinomas) were scored. Adenomas were classified in tubular, tubulovillous, and villous tumors, and the degree of dysplasia was recorded (identified as of low and high grade).

Since ACF and tumors were detected only in AOM-treated rats, statistical analysis was performed only among groups 2, 5, and 6 (13 weeks treatment) and groups 7, 8, and 9 (28 weeks treatment).

Statistical analysis was carried out by using ANOVA followed by the Dunnett's test. The number of rats with tumors was analyzed by Fisher exact test.

RESULTS

Although no evident early signs of toxicity were observed, one rat died during the first week of treatment in each of groups 2, 7, and 8. Weight gain in rats given AOM was 244 ± 20 g and 265 ± 23 g after 13 and 28 weeks, respectively, and did not significantly differ from the control groups (242 ± 19 g and 260 ± 22 g). Within the 13–28 weeks of experiments, weight gain in low-dose SE-treated rats was 217 ± 15 g and 256 ± 20 g (groups 3 and 8), whereas in AOM + SE-treated rats it was 229 ± 16 g and 257 ± 20 g (groups 5 and 9). In the high-dosage group (group 4), weight gain was only 169 ± 11 g, which is a significant reduction (30%; $P < 0.05$) similar to that of the group 6 (158 ± 10 g) (35%, $P < 0.05$).

The mean water content of feces excreted during the first day of treatment and after 13–28 weeks was about 50% in the control group during the whole experimental period. Water content in feces of rats treated with a low dose of SE was between 54 (last treatment) and 60% (first treatment) during the 28 weeks. The high dose of SE increased fecal water content significantly up to 71% ($P < 0.05$) at the first treatment and 65% at the last treatment. There were no significant differences between rats given AOM + SE and rats given SE alone throughout the experimental period.

Table 1. Induction of Aberrant Crypt Foci (ACF) in Rats Exposed for 13 and 28 Weeks to Senna Pod Extract (SE) in Presence and Absence of Azoxymethane (AOM) Initiating Exposure

Group	Treatment conditions and dose (length of treatment)	Effective N of rats	ACF/rat*		Crypt/focus (N)*
			Total N	N with ≥ 4 crypts	
1	Vehicle control (28 weeks)	10	0	0	0
2	AOM 15 mg/kg × 2 (13 weeks)	19	131.1 ± 56.1	29.3 ± 17.4	2.5 ± 0.3
3	SE 10 mg/kg/day (13 weeks)	10	0	0	0
4	SE 100 mg/kg/day (13 weeks)	10	0	0	0
5	AOM 15 mg/kg × 2 + SE 10 mg/kg/day (13 weeks)	10	103 ± 55.7	24.3 ± 14.1	2.5 ± 0.2
6	AOM 15 mg/kg × 2 + SE 100 mg/kg/day (13 weeks)	10	97.5 ± 19	43.8 ± 11.7a	4.2 ± 0.8b
7	AOM 15 mg/kg × 2 (28 weeks)	9	126.4 ± 46.2	30.7 ± 14.8	2.5 ± 0.6
8	SE 10 mg/kg/day (28 weeks)	9	0	0	0
9	AOM 15 mg/kg × 2 + SE 10 mg/kg/day (28 weeks)	10	157.9 ± 71.9	41.5 ± 20.7	2.8 ± 0.5

* Means ± SD.
† a, $P < 0.05$; b, $P < 0.01$ vs group 2 (ANOVA followed by Dunnett's test).

The number and type of ACF and tumors observed after 13 and 28 weeks of treatment with SE in either the presence or absence of the AOM initiating treatment are shown in Tables 1 and 2, respectively. In comparison to untreated animals, AOM given alone induced, after 13 and 28 weeks (groups 2 and 7), the expected appearance of ACF and tumors. In contrast SE, when given without the AOM-initiating treatment (groups 3 and 8), was clearly unable to induce ACF and tumor growth after both 13 and 28 weeks of treatment, thus demonstrating that it is devoid of tumor-initiating activity.

When the treatment with SE was coupled with the initiating treatment with AOM, its effect was modified. However, in rats killed after 13 weeks, any enhancing effect was absent at low SE dose (10 mg/kg; group 5), whereas both the number of large ACF and of crypts per focus were increased to a significant extent in rats given 100 mg/kg of SE (group 6), and in the same rats also the percentage of rats with tumors, the total number of tumors, the number of tumors per rat, and the frequency of adenocarcinomas were markedly higher than in rats given AOM alone. In rats killed after 28 weeks, evidence of a promoting effect of the SE 10 mg/kg was borderline; as a matter of fact the increase in the percentage of large ACF and the number of crypts per focus did not reach the level of statistical significance and the number and type of tumors were similar to those observed in rats given AOM alone.

Table 2. Number and Aspect of Tumors Induced in Colonic Mucosa of Rats by 13 and 28 Weeks of Treatment with Senna Pod Extract (SE) in Presence and Absence of Azoxymethane (AOM) Initiating Exposure

Group	Treatment conditions and dose (length of treatment)	Effective N of rats	Rats with tumors (%)	Tumors/rat (N)	Type of tumors	
					Adenomas*	Adenocarcinomas
1	Vehicle control (28 weeks)	10	0	0	0	0
2	AOM 15 mg/kg × 2 (13 weeks)	19	10.5	0.11 ± 0.3	1 (↑)	1
3	SE 10 mg/kg/day (13 weeks)	10	0	0	0	0
4	SE 100 mg/kg/day (13 weeks)	10	0	0	0	0
5	AOM 15 mg/kg × 2+ SE 10 mg/kg/day (13 weeks)	10	10.0	0.10 ± 0.3	1 (↓)	0
6	AOM 15 mg/kg × 2+ SE 100 mg/kg/day (13 weeks)	10	90.0†	3.20 ± 2.4‡	10 (↑) 3 (↓)	19‡
7	AOM 15 mg/kg × 2 (28 weeks)	9	55.5	1.44 ± 1.6	1 (↑)	12
8	SE 10 mg/kg/day (28 weeks)	9	0	0	0	0
9	AOM 15 mg/kg × 2+ SE 10 mg/kg/day (28 weeks)	10	80.0	1.50 ± 1.5	0	15

* All adenomas were tubulo-villous. (↓) Low, (↑) high grade of dysplasia.
† $P < 0.001$ vs group 2 (Fisher exact test).
‡ $P < 0.01$ vs group 2 (ANOVA followed by Dunnett's test).

DISCUSSION

In our experiments, the low dose of SE (10 mg/kg) induced a weak laxative effect and the higher dose (100 mg/kg) clear diarrhea during the whole treatment. Leng-Peschlow et al (22) have shown that the laxative effect induced in rats by a low dose of pure sennosides (10 mg/kg) was very weak and not significantly different from controls after two to six months of treatment. The discrepancy between these and our results may be a consequence of different senna preparations used. It is well known that senna pod extract has a more powerful laxative action than the pure sennosides (23–25). Another possible factor is the young age of rats used in our experiments. However, only rats with chronic diarrhea present a lower body weight gain (30–35%) compared to control rats.

In a previous study we have demonstrated a probable promoting effect of senna on rat colon carcinogenesis when administered in the diet at high doses (26). However, the doses used and modality of administration do not reflect the employment of senna in experimental and clinical studies.

In order to clarify the possible role of senna in colon carcinogenesis, an experimental protocol capable of demonstrating both initiating and promoting activity was selected. In fact, the occurrence of colon ACF or tumors in rats treated with SE in the absence of AOM would have indicated an initiating activity, while an increase of ACF and tumors in animals treated with SE + AOM would have indicated a promoting activity. The results suggest that SE does not act as initiating agent of colon carcinogenesis after 13 weeks of treatment. The lack of initiating activity is also evident at both dose levels. Our data also show that SE did not act as promoting agent of colon carcinogenesis at a dose of 10 mg/kg given for 13 weeks. A promoting effect seems to occur at the highest dose (100 mg/kg) tested, presumably due to prolonged distress at the level of the colonic mucosa as a consequence of diarrhea. The great increase of large ACF and tumors induced by 13 weeks of treatment with 100 mg/kg SE + AOM might be due, as suggested by Ames and Gold (27) and Cohen and Ellwin (28), to the induction by this extremely high dose of a nonspecific cell proliferation. However, in our study (28 weeks) the low dose of SE (10 mg/kg), which is able to induce laxation, still did not act as both initiating and promoting agent.

In recent years Siegers et al (12) have studied the relationship between colorectal cancer and abuse of anthranoid-containing laxatives, because certain anthranoid laxatives show genotoxic potential in mammalian and bacterial test systems (5, 29, 30) and because danthrone and 1-hydroxyanthraquinone , two anthranoid derivatives, have carcinogenic activity in animals (9, 14). In light of this study, a long-term treatment of anthraquinone laxatives has been associated with an increased risk for colon cancer. However, the clinical relevance of this epidemiological study is still not clear because it does not consider the possibility that chronic constipation *per se* might increase the risk for colorectal cancer (31). On the other hand Jacobs and White (32) have recently demonstrated that frequent constipation may be an important risk factor for colon cancer among middle-aged adults. Different studies have shown that an ethanol extract of Senokot tablets inhibits the mutagenicity of aflatoxin B_1 and other toxic substances, suggesting that an antimutagenic principle(s) is (are) present in the complex plant material (33). More recently Brusick and Mengs (7) have also submitted senna products to a large number of genetic tests; their study does not support concern that senna poses a genotoxic risk to human when used periodically at therapeutic doses.

In conclusion the present study provides no evidence to support the hypothesis that SE influences the carcinogenesis of rat colon when used at dose that produces laxation. In contrast, a SE treatment at diarrhogenic doses increases the appearance of tumors induced by AOM.

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SHORT COMMUNICATION

Senna and the formation of aberrant crypt foci and tumors in rats treated with azoxymethane

F. Borrelli, R. Capasso, G. Aviello, G. Di Carlo, A.A. Izzo, N. Mascolo, F. Capasso*

Department of Experimental Pharmacology, University of Naples Federico II, Naples, Via D. Montesano 80131 Naples, Italy

Abstract

Chronic use of anthraquinone laxatives has been blamed for the induction of habituation and the development of colonic cancer, but there are no definitive studies which have demonstrated this. To evaluate the carcinogenic potential of anthraquinones, the effect of long-term senna pod extract (SE) treatment on either healthy rats or rats treated with an initiating tumor agent (azoxymethane – AOM) has been studied. SE (30 and 60 mg/kg), administered for 110 weeks, did not induce the development of aberrant crypt foci (ACF) and tumors in healthy rats. The development of ACF and tumors in rats treated with AOM were significantly reduced by SE (30 and 60 mg/kg). These results suggest that a chronic SE use does not predispose to colon cancer. By contrast, SE might exert an anti-tumoral activity on rat colon carcinogenesis.

Keywords: Anthraquinones; Laxative; Senna; Tumors; Aberrant crypt foci

Introduction

Anthranoids are a group of naturally occurring laxatives, which are commonly used in clinical practice and as self-medication for chronic constipation (Thorpe, 2001; Gattuso and Kamm, 1994). These laxatives increase intestinal motility and secretion (Capasso and Gaginella, 1997), inducing laxation or a water diarrhoea. The short-term use of these laxatives is generally safe; they occasionally produce flatulence, meteorism and bowel cramps. However, ease of access to laxatives renders them liable to abuse, and chronic administration can lead to complications like dehydration, hypokalaemia and pseudomelanosis coli (Capasso and Gaginella, 1997; Xing and Soffer, 2001).

In recent years, the risk of colon cancer has been found to be related to constipation and to use of anthraquinone laxatives, but experimental and clinical studies are in disagreement (Brusick and Mengs, 1997). In previous studies we have shown that senna, the anthraquinone drug most frequently employed as a laxative, and cascara, do not influence the carcinogenesis of rat colon when given for a period of 13–28 weeks (Mascolo et al., 1999; Borrelli et al., 2001).

The aim of the present work was to study the carcinogenic potential of senna pod extract (SE), administered orally to rats for a period of 110 weeks.

Materials and methods

Chemicals

Azoxymethane (AOM) was purchased from Sigma (Milan, Italy); SE (from *Cassia angustifolia* containing about 50% of sennosides B) was obtained from Indena

*Corresponding author. Tel.: +39 81 678415; fax: +39 81 678403.
E-mail address: fcapasso@unina.it (F. Capasso).

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(Milan, Italy). AOM and SE were dissolved in saline and water, respectively.

Animals

Experiments were performed on male Wistar rats (Harlan-Nossan, Correzzana, Italy) weighing 120 ± 10 g at the start of the study. They were housed in an air-conditioned room (23 ± 2 °C; 50 ± 10% relative humidity) with a 12 h light dark cycle, and had free access to tap water and rat chow (Mucedola, Milan, Italy).

Treatments

A total of 120 animals were randomly divided into six groups as follows: group 1 was treated with AOM (15 mg/kg); groups 2 and 3 were given 30 and 60 mg/kg/day of SE; groups 4 and 5 received AOM + SE 30 and 60 mg/kg/day; group 6 served as the untreated control and was given water used as vehicle for SE. Two doses of SE (30 and 60 mg/kg/day) were selected; a low dose, which induces a laxative effect (increase in fecal and water content) and a high dose which produces a more pronunciated laxative response (presence of characteristic unformed water feces) as experienced by a single treatment. The body weight of rats was controlled weekly and the laxative effect was measured on the first day of treatment and after 55 and 110 weeks of treatment. For collection of feces, animals were kept individually in cages with a wire mesh floor through which feces fall into blotting paper. The feces were collected after 10 h of treatment on the first day and after 55 and 110 weeks. The amount of normal and soft feces was counted by visual observation and wet and dry weight determined. Dry weight was evaluated after drying at 60 °C until weight constant weight.

AOM (15 mg/kg in total, ip) used as initiating agent to evaluate whether laxative act as tumor promoters, was administered intraperitoneally on day 1 (7.5 mg/kg) and 5 of treatment (7.5 mg/kg). Senna was given six times a week, for 110 successive weeks, by intragastric gavage in the morning. All animals were killed by asphyxiation with CO_2 110 weeks after AOM initiation.

ACF technique and tumor evaluation

For ACF determination, the colons of 10 animals randomly selected from groups 1 to 6 were rapidly removed after sacrifice, washed with saline, opened longitudinally, laid flat on a polystyrene board, and fixed with 10% buffered formaldehyde solution before being stained with 0.2% methylene blue in saline. They were examined using a light microscope at 40 × magnification. The criteria used to identify the aberrant crypts were previously described (McLellan et al., 1991); briefly, in comparison to normal crypts, they have greater size, larger and often elongated openings, thicker lining of epithelial cells, compression of adjacent crypts, and are more darkly stained with methylene blue. The detection and quantitation of ACF were performed on the distal 9 cm of the colon, starting from the rectal end. According to the number of constituent crypt, ACF were divided into two groups: small ACF containing 1–3 crypts per focus, and large ACF containing 4 or more crypts per focus. To determine crypt multiplicity, the number of aberrant crypts in each focus was recorded.

For tumor evaluation, the stained colons were discolored with 70% ethanol and embedded in paraffin; serial sections 3 μm thick were obtained and stained with hematoxylin–eosin. Either benign tumoral lesions (adenomas) or malignant ones (adenocarcinomas) were scored, and for adenomas the degree of dysplasia was recorded (as of low and high grade) (Jacobs and White, 1998).

Statistics

Statistical analysis was performed by using ANOVA followed by Dunnett's test. The number of rats with tumors was analyzed by Fisher exact test.

Results

Body weight and laxative effect (water content in feces)

Weight gain in rats given AOM was 555 ± 25 and 725 ± 24 g after 55 and 110 weeks, respectively, and did not significantly differ from the control groups (560 ± 21 and 743 ± 38 g). After 55 and 110 weeks of experiments weight gain in low-dose SE-treated rats was 530 ± 23 and 685 ± 21 g, respectively (group 2) whereas in AOM + SE-treated rats was 540 ± 16 and 690 ± 32 g (group 4). In the high dosage group weight gain was only 500 ± 14 g and 635 ± 20 (group 3) and 495 ± 16 g and 630 ± 17 (group 5).

The mean water content in feces excreted during the first day of treatment and after 55–110 weeks was about 50% in the control and AOM-treated group. The low and high dose of SE increased fecal water content significantly up to 65% and 68% during the whole experimental period (Fig. 1). There were no significant differences between rats given AOM + SE and rats given SE alone throughout the experimental period.

ACF and tumors

The number and type of ACF and tumors observed after 110 weeks of treatment with the two doses of

laxative in either the presence or absence of the AOM initiating treatment are shown in Tables 1 and 2, respectively.

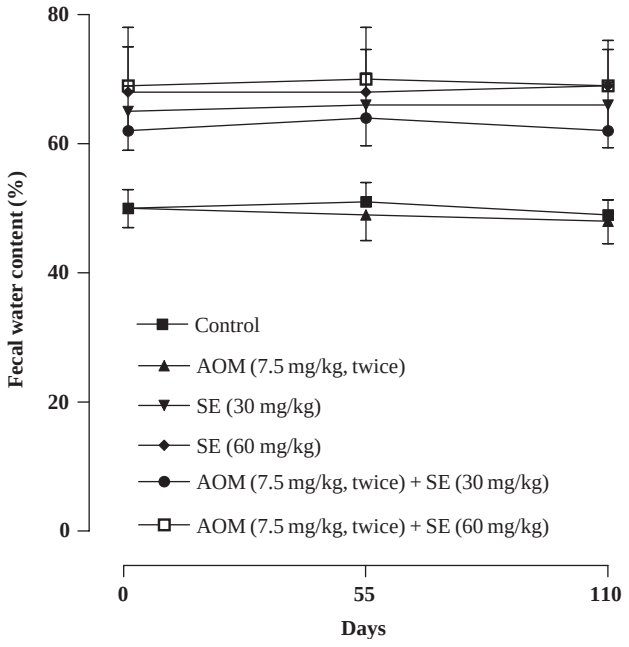

Fig. 1. Effect of AOM, senna pod extract (SE) and azoxymethane (AOM) + SE on percentage of fecal water content.

Table 1 shows that AOM given alone (group 1) induced the expected appearance of ACF. In contrast SE 30 and 60 mg/kg, when given without the AOM-initiating treatment (groups 2 and 3), were clearly unable to induce ACF. SE (group 4 and 5) at both doses used (30 and 60 mg/kg) coupled with the initiating treatment with AOM, reduced significantly the appearance of ACF. Vehicle alone did not show any effect (Table 1).

Table 2 shows that AOM given alone (group 1) induced the expected appearance of tumors. When SE, at both doses used, was given without the AOM initiating treatment (groups 2 and 3) any evidence of tumors development was absent. When the treatment with SE 30 and 60 mg/kg was coupled with the initiating treatment with AOM (groups 4 and 5) a significant reduction of the number of tumors was observed (Table 2).

Discussion

A long-term therapeutic use of laxatives is usually thought to result in habituation, i.e. the reduction or even disappearance of laxative response. However, this assumption, has not been properly demonstrated in patients (Leng–Peschlow, 1992). Clinical studies do not show a loss of effect to laxatives (Muller Lissner, 1993)

Table 1. Induction of aberrant crypt foci (ACF) in rats exposed for 110 weeks to senna pod extract (SE) in presence and absence of azoxymethane (AOM) initiating exposure

Group	Treatment conditions and dose	*N* of rats	ACF/rat[a]		Crypt/focus (*N*)[a]
			Total *N*	*N* with ⩾4 crypts	
1	AOM 7.5 mg/kg, twice	10	90.3 ± 12.6	49.1 ± 6.39	4.92 ± 0.45
2	SE 30 mg/kg	10	0	0	0
3	SE 60 mg/kg	10	0	0	0
4	AOM + SE 30 mg/kg	10	25.0 ± 4.22**	18.7 ± 3.88**	5.93 ± 0.61
5	AOM + SE 60 mg/kg	10	34.2 ± 7.04**	22.1 ± 5.96**	6.29 ± 0.71
6	Vehicle alone	10	0	0	0

[a]Means ± SE; $^{**}p < 0.01$ vs. group 1 (ANOVA followed by Dunnett's test).

Table 2. Number and aspect of tumors induced in colonic mucosa of rats by 110 weeks of treatment with senna (30 and 60 mg/kg) in presence and absence of azoxymethane (AOM) initiating exposure

Group	Treatment conditions and dose	*N* of rats	Rats with tumors (%)	Tumors/rat (*N*)[a]	Type of tumors	
					Adenomas[b]	Adenocarcinomas
1	AOM 7.5 mg/kg, twice	10	80	2.20 ± 0.29	8(↑)6(↓)	8
2	SE 30 mg/kg	10	0	0	0	0
3	SE 60 mg/kg	10	0	0	0	0
4	AOM + SE 30 mg/kg	10	50	1.20 ± 0.18**	4(↑)5(↓)	3
5	AOM + SE 60 mg/kg	10	50	1.30 ± 0.21*	5(↑)5(↓)	3
6	Vehicle alone	10	0	0	0	0

[a]Means ± SE.
[b]All adenomas were tubulo-villous. (↓) low, (↑) high grade of dysplasia; $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. group 1 (ANOVA followed by Dunnett's test).

and studies carried out in rats also suggest that long-term sennoside treatment in diarrhogenic doses does not induce habituation in the sense of a reduced laxative effect and does not lead to secondary hyperaldosteronism (Leng-Peschlow et al., 1993). Our present results indicate that both doses of senna used (30 and 60 mg/kg) induced a laxative effect during the whole treatment. Moreover, the low dose produced a reduction in body weight gain during the second years of about 7% while the high dose was associated with a reduction in body weight gain during the first and second year; The reduction was over 10%: 10.7% and 14.5% for the first and second year of treatment with senna 60 mg/kg, respectively (similar reductions in body weight have been observed in the rat treated with AOM plus senna). This could suggest that the high dose used was close to the maximally tolerated dose. Nevertheless, the dose levels used in our experiments are approximately 20–40 times the highest recommended clinical dose (Schiller, 2001) suggesting a sufficiently high margin of safety.

In the last years the use of anthranoid laxatives (e.g. aloe, senna, rhubarb and cascara) has been associated with the development of intestinal cancer. However, experimental and clinical studies are contradictory in this regard. *In vitro* studies have reported a mutagenic activity from rhein, aloe-emodin, crude senna leaves and senna in bacterial strains (Brown and Brown, 1976; Brown, 1980; Morimoto et al., 1982; Westendorf et al., 1990; Sandnes et al., 1992), whereas sennosides A and B failed to induce mutation in strains (Mengs, 1988). In addition, in mammalian cell test systems, neither rhein nor sennosides A or B induced mutations (Westendorf et al., 1990; Mengs, 1988). Among anthranoids laxatives, only 1-hydroxyanthraquinone and danthrone have shown carcinogenic activity in animal studies (Mori et al., 1985, 1992). Laxative doses of senna extract did not show any carcinogenic effect when it was administered to animals either for 3–6 months or over a 2 years period (Siegers et al., 1993a; Lyden-Sokolowski et al., 1993; Mascolo et al., 1999); The carcinogenic activity of senna was only seen after 3–6 months exposure to very high dosages (Mascolo et al., 1999). The lack of a tumorigenic potential from laxative doses of senna is also supported by the present study that showed no evidence tumorigenicity in the colon of rats treated with senna for a period of 110 weeks. Few clinical studies have evaluated the possible carcinogenic effects of anthranoid laxatives (Gardiner et al., 1982; Nakamura et al., 1984; Kune et al., 1988; Nascimbeni et al., 2002). It is widely known that a long-term use of anthranoid-containing laxatives is the cause of melanosis coli, a brownish pigmentation of the colon caused by the accumulation of dark brown pigment in macrophages of the lamina propria (Capasso and Gaginella, 1997). In addition, it has been also demonstrated that after discontinuation of anthranoids intake the pigmentation disappears apparently without noxious effects, including carcinogenicity and genotoxicity. Siegers et al. (1993b) assessed a carcinogenic risk from anthranoids laxatives in man considering the fact that they found a high incidence of melanosis coli in patients with colorectal carcinomas. However there are not clear evidences of a relationship between melanosis coli and colon cancer whereas there are a strong associations between aberrant crypt foci and colon cancer in humans.

The constipation itself has been demonstrated as a risk factor for colorectal cancer. Recent studies report a positive association between constipation and increased risk for colon cancer (Roberts et al., 2003). By contrast, no association between laxative use and increased risk for colon cancer was found (Jacobs and White, 1998; Roberts et al., 2003). However, in these studies no distinction was made between anthranoids and other laxatives.

Our study also shows that senna suppressed the occurrence of colonic ACF and adenocarcinomas induced by AOM, a carcinogen agent. In conclusion, the results of our study suggest that senna (i) is devoid of any carcinogenic potential, when it was given to rats over a 2-year period, and (ii) may act as an anti-tumoral agent against colon carcinogenesis. Further studies are now in progress to clarify the precise mechanism of such effects.

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Commentary: Risk assessment of anthranoid laxative abuse during chronic constipation

The major anthranoid-containing herbal drugs that are used as stimulant laxatives are senna, aloe, cascara, frangula and rhubarb. Anthranoid laxatives may play a role in the induction of colorectal cancer in humans, for a review see TIPS (Vol. 13), 229–231, 1992. This risk is particularly important in view of the wide abuse of laxatives self-administered for the control of chronic constipation. Case reports and clinical epidemiological studies have considered the cancer risk assessment in patients abusing anthranoid laxatives over a long period. The results of a recent prospective clinical study in 1095 patients undergoing coloscopic control are based on the coincidence of Pseudomelanosis coli (PMC; a brown/black pigmentation of the colonic mucosa) as a reliable marker of chronic anthranoid laxatives abuse and pathological diagnoses. In patients without pathological alterations a PMC incidence of 6.9% was detected. In patients with colorectal adenomas this was enhanced to 9.8% ($p = 0.068$) and in those with colorectal carcinomas to 18.6% ($p = 0.0008$); from these data a relative risk of 3.04 (1.18–4.90, 95% confidence interval) for colorectal cancer in patients with anthranoid laxatives abuse can be calculated (GUT 34, 1099–1101, 1993). Risk assessment for anthranoid laxatives is based on accumulated evidence for a genotoxic potential of some aglycosidic anthranoids and on *in vivo* carcinogenicity studies in rodents. Chronic constipation per se does, in combination with dietary factors like low fibre and high fat intake as confounding factors increase the risk for colorectal cancer in humans. Nevertheless, experimental data alone suggest a carcinogenic risk from anthranoid laxatives in humans. On the other hand, chronic constipation itself may be a risk factor for intestinal carcinogenesis.

Siegers, C.-P.
Department of Experimental and
Clinical Pharmacology and Toxicology,
UK S-H, Campus Lübeck, Germany
E-mail address: siegers@medinf.mu-luebeck.de