表5.3.1 食品添加物の一日摂取量と許容一日摂取量（ＡＤＩ）との比較（平成14・15年度）（文献5より抜粋）

食品添加物名	一日摂取量（mg/人）	許容一日摂取量（ＡＤＩ）（mg/kg/day）	日本人の平均体重（50kg）における一日あたりの許容摂取量（mg/人）	摂取量のＡＤＩに占める割合（%）
食用赤色２号アマランス	0.006	0.5	25	0.02
食用黄色４号タートラジン	0.469	7.5	375	0.13
亜硫酸	0.154	0.7	35	0.44
ソルビン酸	13.56	25	1250	1.08
アスパルテーム	5.853	40	2000	0.29
アセスルファムK	0.736	15	750	0.1
スクラロース	0.31	15	750	0.04

である」ことまたは「遺伝子組換え不分別である」ことを表示することが義務づけられている．

e. 放射線照射食品　食品貯蔵期間延長や殺菌・殺虫の目的のために，X線，ガンマ線や電子線などの放射線を照射することがある．日本ではジャガイモの発芽防止のための放射線照射のみが認められているが，外国では香辛料，食肉，果実等，多くの食品についても認められている．

5.3.2 食品添加物

a. 食品添加物の法規制　食品衛生法において，食品添加物（food additive）は，「この法律で添加物とは，食品の製造の過程においてまたは食品の加工もしくは保存の目的で，食品に添加，混和，浸潤その他の方法によって使用する物をいう」と定義されている．日本の食品添加物は，1995年の食品衛生法の大幅改正以降，法規制上の分類では，下記の4種類に区分される．このうち，2～4が，いわゆる天然添加物に相当する．

1：「指定添加物」ソルビン酸やキシリトールなど393品目（2009年6月現在）．

2：「既存添加物」クチナシ色素，カラメル，ペクチンなど418品目（2009年6月現在）．アカネ色素が2004年に消除された．

3：「天然香料」バニラ香料やカニ香料など約600品目（2009年6月現在）が例示されている．

4：「一般飲食物添加物」正式名：「一般に食品として飲食に供されている物であって添加物として使用されるもの」イチゴジュースや寒天など約100品目（2009年6月現在）が例示されている．

指定添加物は，食品衛生法第10条に基づき，厚生労働大臣が定めたもので，食品衛生法施行規則別表第1に収載されている．原則として，厚生労働大臣が定めたもの以外の製造，輸入，使用，販売等は禁止されており，この指定の対象には，化学的合成品だけでなく天然物も含まれている．ただし例外的に，「天然香料」および「一般飲食物添加物」を，指定制度の対象外としている．また今後新たに使われる食品添加物は，天然，合成の区別なくすべて食品安全委員会による安全性の評価を受け，厚生労働大臣の指定を受けたのち，「指定添加物」となる．既存添加物は，既存添加物名簿に収載されている．1995年に食品衛生法が改正され，指定の範囲が化学的合成品のみから天然物を含むすべての添加物（ただし，天然香料と一般飲食物添加物を除く）に拡大された．既存添加物名簿に新たな品目を追加することは認められず，ヒトの健康を損なうおそれがあると認められるとき，および流通実態がないと認められるときには，既存添加物名簿から消除できる．2004年ヒトの健康を損なうおそれがあるとしてアカネ色素が消除された．天然香料は動植物から得られる天然の物質で食品に香りを付ける目的で使用される添加物，一般飲食物添加物は，一般に飲食に供されているもので添加物として使用されるものと定められている．

食品添加物は必要に応じて食品添加物の品目ごとあるいは対象となる食品ごとに規格（添加物の本質・基原，含量，純度など成分について最低限遵守すべき項目を示した成分規格）や基準（添加物の製造，使用，表示に関する基準）が定められている．個々の食品添加物の成分規格・基準は「食品，添加

物等の現格基準」(厚生省告示，1959年）に収載されている．この内容は，「食品添加物公定書」という名称で冊子体として4～8年ごとに刊行されている．「食品添加物公定書」は1960年にはじめて作成されたが，その後，製造・品質管理技術の進歩および試験法の発達等を受け改訂され，2009年現在，第8版が刊行されている．また，食品に使用した食品添加物は，指定添加物，既存添加物，天然香料，一般飲食物添加物の区別なく，原則としてすべて表示することが義務づけられている．保存料，甘味料等の8用途で使用したものについては，その用途名も併記する必要がある．用途名としては，1）甘味料，2）着色料，3）保存料，4）増粘剤，安定剤，ゲル化剤または糊料，5）酸化防止剤，6）発色剤，7）漂白剤，8）防かび剤または防ばい剤，の8種類が定められている（厚生省生活衛生局長通知「食品衛生法に基づく添加物の表示等について」)．

食品添加物のうち，香料，酸味料，調味料，乳化剤など14種類の目的に使用される食品添加物については，一括名による表示が認められている．米国では，経験や科学的な知見から専門家が判断して一般的な使用法においてリスクがないものとみなされた物質をGRAS（generally recognized as safe）物質として使用できるようにしている[7]．GRAS物質は「一般的に安全と考えられるもの」と訳されている．1958年の食品添加物規制の大幅な改訂の際に設けられた．GRAS物質の届け出には民間の科学的専門家による一定の定められた科学的手順に基づいた適切な評価があればよく，政府（FDA）の判断を除外するわけではないが要求していない点が特徴として挙げられる．米国のFlavor and Extract Manufacturers Association（FEMA）(米国食品香料製造者協会）ではFDAの了解のもとに，食品香料（フレーバー）に関するFEMA GRASと呼ばれる物質リストを作成しており，リスト中の物質は米国で自動的に食品に使用できることとなっている．

b.　食品添加物の安全性評価[5]　食品添加物の安全性評価は，物質の代謝や実験動物を用いた毒性試験結果等の科学的なデータに基づき，食品安全委員会の行う食品健康影響評価（リスク評価）によって審議され，食品添加物ごとに許容一日摂取量（acceptable daily intake：ADI）が設定される．この結果を受けて，薬事・食品衛生審議会において食品添加物としての指定の可否，成分規格，使用基準などにつき審議・決定される．ADIは，ヒトがある物質を毎日一生涯にわたって摂取し続けても，現在の科学的知見からみて，健康への悪影響がないと推定される一日あたりの摂取量であり，毒性試験から求められた無毒性量（NOAEL）を安全係数で除して算出され，通常mg/kg/dayで表される．安全係数は通常，種差と個体差の観点からそれぞれに10倍を見込み，これらを乗じた100が用いられているが，固定されたものでなく，評価に使われたデータが不足している場合や現れた毒性が，神経毒性，発がん性，催奇形性など重篤な場合には追加の係数が加えられることがあり，500など数値は変わりうる．なお，いわゆる健康食品の主成分など，食品自身に由来し薬事法・食品衛生法等の法律で規定されていない化学物質に関しては，通常，安全係数の概念は適用されずADIの算出も行われない．

毒性試験については，標準的実施方法の指示のもと，(1) 28日反復投与毒性試験，(2) 90日反復投与毒性試験，(3) 1年間反復投与毒性試験，(4) 繁殖試験，(5) 催奇形性試験，(6) 発がん性試験，(7) 1年間反復投与毒性/発がん性併合試験，(8) 抗原性試験，(9) 遺伝毒性試験，(10) 一般薬理試験，を検討することが定められている（1996年厚生省通知「食品添加物の指定および使用基準改正に関する指針について」)．こうした毒性試験の整備に伴い，これまでに人体への悪影響を考慮して消除されたものだけで十数種にのぼる．例として，着色料の食用赤色1号（肝障害，肝がん），甘味料のサイクラミン酸ナトリウム（チクロ）(膀胱がん，催奇形性），甘味料のズルチン（肝障害，肝がん），保存料のAF-2（変異原性，染色体異常試験陽性），既存添加物であったアカネ色素（腎がん）がある[6]．

食品添加物の摂取状況については，厚生労働省により，マーケットバスケット方式および食品添加物の生産流通統計量に基づく一日摂取量推定調査が実施されている．マーケットバスケット方式とは，スーパーなどで売られている食品を購入し，そのなかに含まれている食品添加物量を分析して測り，その結果に国民栄養調査に基づく食品の喫食量を乗じて推定摂取量を求める方式をいう．この調査結果の一例をADIとともに表5.3.1に示す[5]．

c.　食品添加物規制の国際標準化[5]　食品添加

表5.3.2 マイコトキシンによる食品汚染[8](一部改変)

マイコトキシンの種類	主な原因カビ	主な汚染食品	毒性様式
	*Aspergillus*属		
アフラトキシン(B_1, B_2, G_1, G_2, M_1, M_2など約20種類の誘導体)	*A. flavus*, *A. parasiticus*	ピーナッツ, トウモロコシ, 麦, 米, 綿実	肝がん, 肝障害
ステリグマトシスチン	*A. versicolor*	穀類	肝がん, 肝硬変, 血管肉腫
オクラトキシン	*A. ochraceus*, *A. carbonarius*	ピーナッツ, トウモロコシ, 麦, コーヒー豆	腎がん, 腎障害
	*Penicillium*属		
ルテオスカイリン	*P. islandicum*	穀類(米)	肝がん, 肝硬変
シトリニン	*P. citrinum*	穀類(米)	腎障害
シトレオビリジン	*P. citreoviridae*	穀類(米)	神経毒性
パツリン	*P. expansum*	麦芽根, 小麦, リンゴ加工品	消化管障害, 腎機能障害
	*Fusarium*属		
ニバレノール	*F. culmorum*	トウモロコシ, 麦, 米	免疫系・造血器・消化管障害
デオキシニバレノール	*F. graminearum*	トウモロコシ, 麦, 米	免疫系・造血器・消化管障害
T-2トキシン	*F. sporotrichioides*	トウモロコシ, 麦, 米	免疫系・造血器・消化管障害
ゼアラレノン	*F. graminearum*, *F. culmorum*	トウモロコシ, 麦	不妊症(エストロゲン様作用)
フモニシン	*F. moniliforme* (*F. verticillioides*)	トウモロコシ, 麦, 大豆, アスパラ	肝がん, 腎障害, ウマの白脳軟化症
	*Claviceps*属		
麦角アルカロイド(エルゴタミンなど)	*C. purpurea*	穀類(麦)	神経毒性, 循環器毒性, 流産

物の規格や基準については, それぞれの国の法律により定められており, 各国間で相違点がある. 他方で, 国際的な貿易が盛んとなり, 食品の輸出や輸入が増大し, 食品の安全性を確保しつつ, 規制を整合化することが, 国際的な課題となっている. 食品添加物については, 国連食糧農業機関 (Food and Agriculture Organization, FAO) / 世界保健機関 (WHO) の合同食品規格委員会 (コーデックス委員会 ; Codex Alimentarius Commission) の食品添加物部会において検討がなされている. また, 食品添加物の安全性について国際的な評価を行う機関としては, 国連食糧農業機関/世界保健機関合同食品添加物専門家会議 (FAO/WHO Joint Expert Committee on Food Additives : JECFA) がある. JECFAは, コーデックス委員会とは独立しているが, コーデックス委員会に対して助言を行っている.

5.3.3 食品汚染物質[2]

有害な化学物質による食品の汚染は, 偶然あるいは過失で混入する場合と, 環境汚染物質として食品に残留する場合が考えられてきたが, 最近ではメラミン添加牛乳事件や事故米の食品への転用で明らかになったように, 意図的な犯罪行為への対応も考慮する必要が出てきた. なお, メラミン混入ペットフードにより2007年に米国を中心に多数の犬や猫が腎不全等で死亡する事件が起きたが, 米国獣医師会は, メラミンとその不純物シアヌル酸との反応から生じた結晶がその原因物質である可能性を報告している.

a. 加工製造中の不純物混入による汚染 この実例として, 粉乳中にヒ素が混入したヒ素ミルク事件 (1955年), 米ぬか油にPCB (ポリ塩化ビフェニル) に混入した事件 (カネミ油症, 1968年) を挙げることができる. ヒ素ミルク事件では, 乳質安定剤として使用された工業用リン酸水素二ナトリウムに含まれていた不純物ヒ素を摂取したために起き, 乳児の死亡を招いた. カネミ油症では, 油を加熱脱臭するために, 熱媒体として使用したPCBが熱交換パイプから漏出し, 油に混入したためとされており, 塩素痤瘡, 色素沈着, 肝障害などを招いた. なお, この油症の原因物質は, PCBに含まれていた

ダイオキシン類の一種であるポリ塩化ジベンゾフラン（polychlorinated dibenzofurabin, PCDF）と考えられている．

その他，食品容器に由来する化学物質による汚染で問題となっているものに，プラスチック製品の可塑剤のフタル酸エステル，合成原料のビスフェノールAなどが挙げられ，これらは内分泌撹乱物質の可能性が示唆されている．同様に食器用合成樹脂合成原料である塩化ビニルモノマー，ホルムアルデヒド，アクリロニトリルなどの漏出や陶器から溶出した鉛なども食品衛生上問題となる．鉛は他にも鉛製の水道管からの溶出が問題となる．

b. 環境中に排出された重金属・化学物質による汚染　この代表的な実例として，水俣病およびイタイイタイ病が挙げられる．水俣病は，排水中の，触媒として使用した無機水銀から副生されたメチル水銀が，魚介類に蓄積し，ヒトがこれを摂取することにより起きたものとされており，四肢のしびれ，歩行障害などが起きた（熊本県水俣市，1956年）．また有機水銀は，胎盤を介し胎児に移行し，生まれた子どもに脳性麻痺等の症状が出た．その後，新潟県阿賀野川流域においても有機水銀中毒という第二水俣病（新潟水俣病）が発生した（1965年）．現在，魚類に蓄積した有機水銀量については国際的に注意が払われており，日本では自然界で水銀を蓄積しやすいサメ，メカジキ，キンメダイ，クジラ類の一部，マグロ類等の妊婦の摂取について注意が喚起されている．イタイイタイ病は，富山県神通川下流域で，鉱山廃水に含まれて排出されたカドミウムを多量に含む米を摂取したため起きたものとされており，骨が非常に脆くなり骨折しやすい患者が多く出た（1955年）．

近年，船底防汚剤，木材防腐剤などとして使用されたトリブチルスズなどの有機スズ化合物が，水生生物に対して低い濃度で作用し，内分泌撹乱物質である可能性が示唆され，海洋汚染物質として，ヒトへの生体影響が懸念されている．

その他，環境中の化学物質による汚染で問題視されるものとして，ゴミ焼却場などから排出されるダイオキシン類や残留農薬が挙げられる．ダイオキシン類は，ポリ塩化ジベンゾパラジオキシン（polychlorinated dibenzo-o-dioxin, PCDD），ポリ塩化ジベンゾフラン（PCDF）およびコプラナーポリ塩化ビフェニル（coplanar polychiorinated biphenyl, Co-PCB）の総称である（ダイオキシン類対策特別措置法）．塩素置換数と置換位置によってそれぞれ数多くの異性体が存在する．空気中で塩素源と炭素源が300℃程度の不完全燃焼することにより発生する．したがって，この発生はプラスチックの不完全燃焼だけとは限らないこととなる．ダイオキシン類の毒性の強さは異性体によって異なり，もっとも毒性が強いのは2, 3, 7, 8-テトラクロロベンゾ-p-ジオキシン（2, 3, 7, 8-TCDD）である．ダイオキシン類の毒性評価に際しては，2, 3, 7, 8-TCDDに対する毒性等価係数（TEF）がWHOから提唱され，各異性体のTEFと残留濃度の積の合計量（TEQ）を用いて検討されている．モルモットに極めて低用量で急性毒性（2, 3, 7, 8-TCDDによる半数致死量は，600 ng/kg）を示した以外にも，実験動物に対し免疫毒性，発がん性や催奇形性などの毒性を示す．遺伝子改変マウスを用いた実験等から，この作用の多くが芳香族炭化水素受容体（aryl hydrocarbon receptor；Ahレセプター）（ダイオキシン受容体）を介して引き起こされるものと考えられている．

c. かびによる食品汚染[8]　かび等の真菌類による食品汚染の中では，発がん性等ヒトや動物に有害作用を有する二次代謝産物として産生される毒の総称であるマイコトキシン（mycotoxin）による健康被害がとくに問題となる．主なマイコトキシンの種類と原因かび，汚染源，毒性様式について表5.3.2に示した．アスペルギルス属（*Aspergillus*），ペニシリウム属（*Penicillium*），フサリウム属（*Fusarium*）の3属によるものがほとんどである．アフラトキシンB_1には強い肝毒性と強い発がん性がある．ピーナッツ，トウモロコシ，麦などの貯蔵，輸送の管理が不適切だった場合に発生する．マイコトキシンにより引き起される障害は，肝障害，腎障害以外にも，ゼアラレノンのように家畜で不妊を起こすもの，エルゴタミン（麦角アルカロイド）のように血管収縮作用や子宮平滑筋収縮作用を有するものなど多彩である．日本では2009年現在，アフラトキシンB_1，デオキシニバレノール，パツリンの3種について規制している．アフラトキシンB_1は，食品衛生法第6条により，全食品を対象に検出されてはならないと規制されている．パツリンについて

は，りんごの搾汁（ジュース）を対象に含有量が0.050ppmを超えるものであってはならないと規制されている（パツリン告示法）．デオキシニバレノールは，小麦を対象に暫定的な基準値1.1ppmが定められている．

なお，エルゴタミンは片頭痛治療薬として利用され，ゼアラレノンはエストロゲン活性を有し，またフモニシンはスフィンゴ脂質の生合成経路を阻害するが，このようにマイコトキシンのなかには，細胞分子機能解析用の生理活性物質としても研究用試薬として注目されているものがある．

5.3.4　飼料添加物

飼料添加物（feed additive）とは，「飼料の安全性の確保および品質の改善に関する法律」（飼料安全法）において，「飼料の品質の低下の防止その他の農林水産省令で定める用途に供することを目的として飼料に添加，混和，浸潤その他の方法によって用いられる物で，農林水産大臣が農業資材審議会の意見を聴いて指定するものをいう」と定義されている．法の規制対象とする家畜等は経済動物に限定することが妥当と考えられている．2003年に改正された政令で定められた動物（家畜等）は，家畜（牛，豚，めん羊，山羊およびしか）（馬は対象外），家禽（鶏およびうずら），みつばち，養殖魚（ぶり，まだい，まあじ，ひらめ，すずき，すぎ，くろまぐろ，うなぎ，あゆなど23種）の計31種となっている．飼料添加物は，飼料の品質の低下の防止（抗酸化剤，防かび剤，乳化剤等），飼料の栄養成分その他の有効成分の補給（アミノ酸，ビタミン，ミネラルなど），飼料が含有している栄養成分の有効な利用の促進（抗生物質，合成抗菌剤，生菌剤等）を目的として添加される（2004年現在で合計153種）．それぞれ対象飼料や添加量が定められている．また，BSEの新たな発生を防止するため，牛の餌については，骨肉粉などの動物由来タンパク質が混入してはいけないことが定められている．

ヒトへの健康被害との関連では，過剰摂取あるいは抗生物質における薬剤耐性などが問題となるが，抗生物質，合成抗菌剤については，「動物用医薬品の使用の規制に関する省令」によって，投与用量や出荷前の使用禁止期間が定められている．また，動物用医薬品として投与されたホルモン剤や抗生物質等の食肉への残留も問題となるが，これもこの省令により投与用量や出荷前の使用禁止期間が定められている．したがって抗菌性物質はその用途により「飼料添加物」と「動物用医薬品」の両方に含まれ，飼料安全法，薬事法により，それぞれに使用規制がある．ヒトへの健康被害防止に際しては，これらの物質のモニタリングが重要となっている．

食品中の飼料添加物と動物用医薬品の残留は，残留農薬と同様にポジティブリスト制が導入されており，基準値が設定されていないものには，一律基準が適用される．残留基準は，食品安全委員会により定められたADIに基づき，厚生労働省の薬事・食品衛生審議会において定められる．

なお，犬，猫等の愛がん動物用の飼料（ペットフード）は，飼料安全法において規制の対象とされていなかったが，この安全性の確保を図るため，2009年6月1日より「愛がん動物用飼料の安全性の確保に関する法律（ペットフード安全法；農林水産省・環境省共管）が施行された．2009年6月現在，政令により愛がん動物は犬と猫と定められている．

［北嶋　聡］

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An experimental design for judging synergism on consideration to endocrine disruptor animal experiments

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SUMMARY

This paper investigates an appropriate statistical design for an animal experiment to evaluate synergism of two test chemicals. It assumes a certain number of animals are divided into groups, each of which is treated with a combination of dose levels of two chemicals. A design is identified by the set of group size for each combination of doses, including the case where the dose of either one chemical is zero. The power of *t*-test to detect synergism by positive surplus of response on a simultaneous administration group from the additivity plane composed of the responses on single administration groups is adopted as the criterion for the appropriate design. The applicable design is investigated for the application to real cases of endocrine disrupter study conducted at the National Institute of Health Sciences of Japan.

It revealed that the dose level of the simultaneous administration group should be located inside or on the boundary of a triangular region and that the total number of animals should be the same as those for single administration groups. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS: additivity; animal experiment; experimental design; endocrine disruptor; synergism; triangular region

1. INTRODUCTION

In the past, environmental pollutants were regulated according to individual effects. However, recently, there has arisen the problems of combinations of complex pollutants, and regulations that address synergism have become necessary. As a result, experimental researches have been conducted on pollutant synergism. The investigation by Kanno, one of the authors (Kanno *et al.*, 2001) on the synergism of endocrine disruptors, using the rodent uterotrophic assay, is an example of such researches.

In our experiments using multiple test substances, dividing animals such as rats into multiple groups of single administration and simultaneous administration, we estimated the response when there is no synergism based on the response in the single administration group to investigate whether the response in the simultaneous administration group exceeds the estimated response.

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For the data analysis method used in this type of animal experiment to investigate synergism, Kelly and Rice (1990) proposed a method to evaluate the dose–response curve by smoothing method. Gennings and Carter (1995) and Gennings *et al.* (1997), on the other hand, proposed a method to evaluate synergism by using a model in which the response becomes flattened when there is no synergism. Using a similar plan to that of Gennings *et al.* (1997), Matsunaga *et al.* (2003) proposed a method to evaluate the difference in the responses with simultaneous administration of two substances from those estimated by applying an additive model to the data of single administration of each substance. They applied their proposed method to the actual data analysis. Other data analysis methods are also cited in Laska and Meisner (1989) and Machado and Robinson (1994).

For the experimental design evaluating the synergism, Hasegawa *et al.* (1996) proposed the experimental design of animal experiment for five or ten chemical mixture, and Straetemans *et al.* (2005) investigated a fixed-ratio design on *in vitro* study. Abdelbasit and Plackett (1982) and Tan *et al.* (2003) are also related to this issue. However, the situation of these researches differs from our case study. Our interest is limited to simply checking whether the effects of the chemicals are additive or not. And in the animal experiments, some assumptions and limitations are generated for the applicable information and experimental conditions.

In this kind of research, we thought that the problem of the experimental design is to determine appropriate dose levels for simultaneous administration and to select the appropriate number of animals for allocation to the dose levels. However, in the past research, the research directly related to this problem by the animal experiment was not found. Accordingly, based on the analysis methods proposed by Matsunaga *et al.* (2003), we investigated what type of design would be appropriate.

The paper is organized as follows. Section 2 introduces the conditions in the case study that motivated this paper, while Section 3 formulates the issues. Section 4 derived the appropriate design corresponding to the case studies dealt with this paper. Finally, Section 5 provides Conclusions and Discussions for future issues.

2. MOTIVATING CASE

According to the World Health Organization, an endocrine disruptor is defined as "an exogenous substance or mixture that alters function(s) of the endocrine system and causes adverse health effects in an intact organism, or its progeny or (sub)populations."

The effect of endocrine disruptors is not stimulated directly at the site of the adverse effect, but is mediated by the signal and occurs through nuclear receptor. Furthermore, there is more than one signal transduction system in humans and animals. Because nuclear receptors and transcription factors are redundant, there may be an interaction between different pathways, which leads to possible synergistic endocrine disruption action. Here the definition of "synergistic" is that if two chemicals produce the same endpoint, they bring about a larger response as compared to the anticipated response when these chemicals are purely added. Consequently, it is very important to realize the synergism between two endocrine disruptors such as "Genistein" and "bisphenol A (BPA)" through animal experiments for explaining the mechanism of action.

Both Genistein and BPA bind to estrogen receptors and elicit estrogenic responses to an organism including uterotrophic responses. Genistein is a phytoestrogen found relatively abundant in soybeans and its derivative foods. BPA is the basic monomer of polycarbonate plastic and epoxy resins widely used as a lining for food and beverage cans, in hard plastic baby and water bottles, toys, dental sealants, etc. It has been reported that BPA monomer can leached out to food and drinks especially when the

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polymerization process is incomplete and/or the plastic is aged. These two estrogenic compounds can be found very commonly in our food environment. Therefore, it is of great importance from the point of the safety regulations to examine whether the combined effect is additive or synergistic.

For the experimental design of the research based on this background, some assumptions and limitations were generated for the applicable advanced information and experimental conditions. In the interests of simplicity, when explaining synergism research for two substances, the limitations are as follows.

First, before conducting the experiment that investigates synergism, advance knowledge can be obtained to some extent on dose–response curves for the single administration from preliminary experiments using each substance. Therefore, if the dose–response curve is nonlinear, by appropriate transformation of variables for the dose and response, it is possible to assume the dose–response curve to be approximately linear.

Second, the maximum dose of each substance used in the experiment is limited. We, for example, recognize in our experiments that the signal transduction system amplifies the signal at significantly smaller doses as compared to the dose used in normal toxicity studies. Other toxicities appear with higher doses, so that the endocrine disruption effect to be investigated is concealed. Because these maximum doses, $D_{A\max}$ and $D_{B\max}$, generally can be obtained through preliminary experiments, the range of dose levels used in the experiments can be limited.

Third, because various kinds of test substances are to be investigated, the number of animals, n, used in each experiment is relatively small. In our actual experience, the number of animals is approximately 40–50.

Fourth, the fundamental form of the experiment for investigating synergism is roughly decided. In an experiment using two substances A and B, we set single administration groups of G_{00} (at a dose level of 0), G_{A2}, and G_{B2} (at the maximum dose levels of $d_{A2} = D_{A\max}$ and $d_{B2} = D_{B\max}$, respectively), and groups G_{A1} and G_{B1} (at the middle dose levels of d_{A1} and d_{B1}, respectively), and administer the test substances after assigning the same number of animals n_s in every group. Independent of this, we set one or more simultaneous administration groups of G_{AB} with dose levels of the two substances at d_A and d_B. We measure responses by performing the experiment with this type of design, and estimate dose–response curves by forecasting synergism from the single administration group to confirm whether the response obtained in the simultaneous administration group is larger compared to that estimated.

Fifth, the observed response is usually quantitative variable such as the uterine weight of rats, which generally shows normal distribution, because it is difficult to define the additivity/synergism for the response in qualitative values.

Under the above conditions, what should be questioned for the design is what the most appropriate dose for simultaneous administration is, and whether to have more or less animals for the simultaneous administration group as compared to the single administration group. In order to obtain guidelines for these, this paper generalizes and formulates the above mentioned problems to make numerical evaluations under some conditions.

3. FORMULATION OF PROBLEM

3.1. Definition of synergism

In order to simplify the discussion, hereafter, we assume that there are two test substances denoted by A and B.

There have been many discussions in the past for how to define the terms additivity, synergism, and antagonism. Synergism is not defined unconditionally (Hewlett and Plackett, 1959; Berenberm, 1989).

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Table 1. Difference between factorial design and triangular design

	Dose of A		
Dose of B	d_{A0}	d_{A1}	d_{A2}
(a) Factorial design			
d_{B0}	(1)	(2)	(3)
d_{B1}	(4)	(6)	(7)
d_{B2}	(5)	(8)	(9)
(b) Triangular design			
d_{B0}	(1)	(2)	(3)
d_{B1}	(4)	(6)	
d_{B2}	(5)		

Single chemical is administered at (1)–(5), whereas combination of two chemicals is administered at (6)–(9).

In fact, in the general remarks of these studies, many ideas are introduced for discussing synergism such as "independent joint action," "potentiation," "simple similar action," "complex action," and "dissimilar action." We will first explain the definition of synergism that is adopted in this paper.

From the standard statistical viewpoint, the dosages set certain dose level for the respective two substances as shown in Table 1(a). If the response at the simultaneous dose level is the sum of the effects generated by single substances, then the effect is considered to be additive. On the other hand, if it is large, there is a positive interaction and the effect is synergistic.

However, with toxic responses like endocrine disruption action, this point of view is not appropriate. Because, for this toxic response, as pointed out by, for example, Hasegawa *et al.* (1996), it is impossible to establish response linearity at doses that exceed the maximum dose for the respective substances, and thus it is impossible to determine whether a positive interaction is attributable to synergism or nonlinearity. Therefore, the following definition that expresses the tenets of Hewlett and Plackett (1959) by isobologram is adopted in this paper.

Label the expected response at dose d_A and d_B (doses for A and B) as $f(d_A, d_B)$. Also, label the single administration dose of A that results in an arbitrary response E as D_A, so that $f(D_A, 0) = E$. Similarly, label the dose of substance B that has expectation E as D_B. In the cases that motivated this study, A and B generate their responses in a similar stimulation process, the expected effects are proportional to the doses of A and B, and the effects of the two substances are additive. If these conditions hold, then $f(d_A, d_B) = E$ whenever (d_A, d_B) satisfies Equation (1).

$$\frac{d_A}{D_A} + \frac{d_B}{D_B} = 1 \tag{1}$$

The reason is that because a combination of dose levels like this represents simultaneous administration of A and B at an arbitrary ratio, using the amount that brings about a response of the same magnitude. If a response of magnitude E is consequently generated as expected, there is no special combined effect between the two chemicals. In this paper, when this relationship holds, the effect of the two substances is additive, or the two substances satisfy additivity.

On the other hand, if the two substances generate a synergistic response in different stimulation processes, it is considered that $f(d_A, d_B) > E$ is established with respect to an arbitrary (d_A, d_B) that satisfies Equation (1). In this paper, when this relationship is established, the two substances are synergistic, or satisfy synergism.

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3.2. Terminology, notation, and assumption

The two-dimensional plane by plotting d_A (dose of A) on the x-axis and d_B (dose of B) on the y-axis is referred to as the dose plane, and the three-dimensional space by plotting the response on the z-axis above the dose plane is referred to as the response space.

In the experiment, the response is measured for each individual animal. The response that is measured is called the response variable, and is generally expressed by the symbol Z. The response variable Z measured for each individual is set as a random variable that follows a normal distribution independent of other individuals. Since it is assumed that the endpoint is organ weight as a target for application in the case study, such as endocrine disruptor study, it is considered that the assumption of the normal distribution is empirically valid. When the dose of the two substances administered is (d_A, d_B), the expected value is $E\{Z\} = f(d_A, d_B)$.

For single administration, that is, when the dose of one test substance is 0, Equation (2) can be assumed concerning the dose–response curve f.

$$f(d_A, 0) = \beta_0 + d_A\beta_A,\ f(0, d_B) = \beta_0 + d_B\beta_B \tag{2}$$

This assumes the dose–response curve for single administration to be linear. With this assumption, $f(d_A, d_B)$ is expressed by Equation (3). The two substances are additive if the hypothesis H_0 of Equation (4) holds, while synergistic if the hypothesis H_1 holds.

$$f(d_A, d_B) = \beta_0 + d_A\beta_A + d_B\beta_B + \Delta(d_A, d_B) \tag{3}$$

$$H_0 : \Delta(d_A, d_B) = 0,\ H_1 : \Delta(d_A, d_B) > 0 \text{ for all } (d_A, d_B) \tag{4}$$

The value of the dose used in the experiment is called the dose level, the collection of animals allocated for the dose level is called a group, and the number of animals for each group is called the group size, and the dose level of the group on the dose plane is called the group point. With this terminology, it is defined that "design is the set of group point and group size."

For numerical evaluation described in the next section, five groups of group size n_s for single administration and one group of group size n_m for simultaneous administration as described in Table 1(b) are assumed as the design. For the single administration group, the group points are set to be $(0, 0)$, $(d_{A1}, 0)$, $(d_{A2}, 0)$, $(0, d_{B1})$, $(0, d_{B2})$, and the response variables are distributed as normal with variance σ_s^2. For the simultaneous group, the group point is set to be (d_A, d_B), and the response variable is distributed as normal with variance σ_m^2

Let the sample mean of response variable in each group be Z_{00}, Z_{A1}, Z_{A2}, Z_{B1}, Z_{B2}, and Z_{AB}, respectively. It is assumed that $Z_{00}, \ldots, Z_{B2}$ are distributed as normal with the mean of Equation (2) and the variance σ_s^2/n_s, while Z_{AB} is distributed as normal with the mean of Equation (3) and the variance σ_m^2/n_m.

3.3. Criterion for the appropriate design

As criterion for the most appropriate design, it is natural to use the power in the hypothesis test of "H_0 versus H_1." Because the model is a linear model and the hypothesis is a linear hypothesis, a one-sided

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t-test (or Welch test) with Equation (5) is naturally set as the test statistic.

$$T = \frac{\hat{\Delta}}{\sqrt{\hat{V}(\hat{\Delta})}} \tag{5}$$

Here, $\hat{\Delta}$ is the least square estimator of Δ, and the denominator of the statistics is the square root of the variance estimator.

The critical value of this test statistics is $t(\nu, \alpha)$, the upper 100α percentile of the t-distribution with degree of freedom ν, and the test is a one-sided test. In other words, A and B are judged to be synergistic when $T > t(\nu, \alpha)$.

This test, in short, detects the synergism when there is a statistically significant difference between Z_{AB} and the estimate obtained from the single administration groups assuming the dose–response surface under H_0.

4. EXAMPLES OF RECOMMENDED DESIGN

4.1. Real examples motivated the problem

We conducted many experiments and selected endocrine disruptor study as case study. This study was performed using triangular design such as Table 1(b). This design consisted of seven dose groups which included five group points for single administration and two group points for simultaneous administration. The endpoint was uterine weight gain and the main purpose was to evaluate whether the combined effect was synergistic or not. In order to explain the characteristic of the data, we took up two real examples. The details of these data are as follows.

Example 1. *Chemical A: genistein (mg/kg), chemical B: BPA (mg/kg)*

1. Group points for the single administration: $(d_A, d_B) = (0, 0), (12.5, 0), (25, 0), (0, 35), (0, 70)$.
2. Group points for simultaneous administration: $(d_A, d_B) = (6.25, 17.5), (12.5, 35)$.
3. Group size: $n_s = 6, n_m = 6, n = 42$.
4. Mean $\pm$ standard deviation of observed values

$$(d_A, d_B) = (0, 0) : 84.0 \pm 7.1$$
$$(d_A, d_B) = (12.5, 0) : 111.2 \pm 9.3, \quad (d_A, d_B) = (25, 0) : 149.5 \pm 33.7$$
$$(d_A, d_B) = (0, 35) : 138.4 \pm 16.2, \quad (d_A, d_B) = (0, 70) : 181.0 \pm 24.4$$
$$(d_A, d_B) = (6.25, 17.5) : 141.1 \pm 11.4, \quad (d_A, d_B) = (12.5, 35) : 180.2 \pm 26.8$$

5. Estimated value of Δ: 15.6.
6. Result of t-test: Significant in one-sided Welch test with significance level 2.5% $T = 2.19$, $\nu = 19$, $p = 0.02$.

Example 2. *Chemical A: diethylstilbestrol (μg/kg), chemical B: genistein (mg/kg)*

1. Group points for the single administration: $(d_A, d_B) = (0, 0), (0.1, 0), (0.2, 0), (0, 12.5), (0, 25)$.
2. Group points for simultaneous administration: $(d_A, d_B) = (0.05, 6.25), (0.1, 12.5)$.

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3. Group size: $n_s = 6, n_m = 6, n = 42$.
4. Mean $\pm$ standard deviation of observed values

$$(d_A, d_B) = (0, 0) : 91.2 \pm 16.2$$
$$(d_A, d_B) = (0.1, 0) : 92.3 \pm 9.7, \quad (d_A, d_B) = (0.2, 0) : 96.5 \pm 5.4$$
$$(d_A, d_B) = (0, 12.5) : 165.4 \pm 27.0, \quad (d_A, d_B) = (0, 25) : 220.8 \pm 27.5$$
$$(d_A, d_B) = (0.05, 6.25) : 141.8 \pm 12.8, \quad (d_A, d_B) = (0.1, 12.5) : 183.5 \pm 10.3$$

5. Estimated value of Δ: 19.5.
6. Result of t-test: significant in one-sided Welch test with significance level 2.5% $T = 3.98$, $\nu = 31$, $p < 0.01$.

From the above results, we suggested that the combinations for these two agents were synergistic. The synergism was observed in the real situations such as endocrine disruptor study.

4.2. *Recommended group point selection*

The example introduced in the preceding section has two simultaneous administration groups. In this section, we investigate the most appropriate dose level among several dose levels in the case of one simultaneous administration group. The conditions in the investigation are as follows. The conditions are set pursuant to the example in the preceding section except for assuming $\sigma = 1$ without losing any generality.

4.2.1. *Fixed condition.*

1. Group points for single administration: $(d_A, d_B) = (0, 0), (1, 0), (2, 0), (0, 1), (0, 2)$.
2. Group size: $n_s = 6, n_m = 12$.
3. Parameters in the dose–response curve: $\beta_0 = 1.0, \beta_A = 1.0, \beta_B = 1.0$.
4. Variance σ^2: $\sigma_s^2 = \sigma_m^2 = 1.0$.
5. Nominal significance level of t-test: one-sided 2.5%.

4.2.2. *Varied condition.*

6. Group points for simultaneous administration: $d_A = 0.1(0.1)2.0, d_B = 0.1(0.1)2.0$.
7. Strength of synergism Δ:
   Case 1 (constant case): $\Delta = 1.0$
   Case 2 (square root case): $\Delta = 0.8\sqrt{(d_A + d_B)}$
   Case 3 (linear case): $\Delta = 0.6(d_A + d_B)$

Numerical calculations were performed to calculate the power under the above conditions. The left-hand side of Figures 1–3 shows a three-dimensional display on the vertical axis above the dose plane of the power in Cases 1–3, respectively. On the other hand, the right-hand side of Figures 1–3 represents power functions when $d_A = d_B$ at the dose level for simultaneous administration group.

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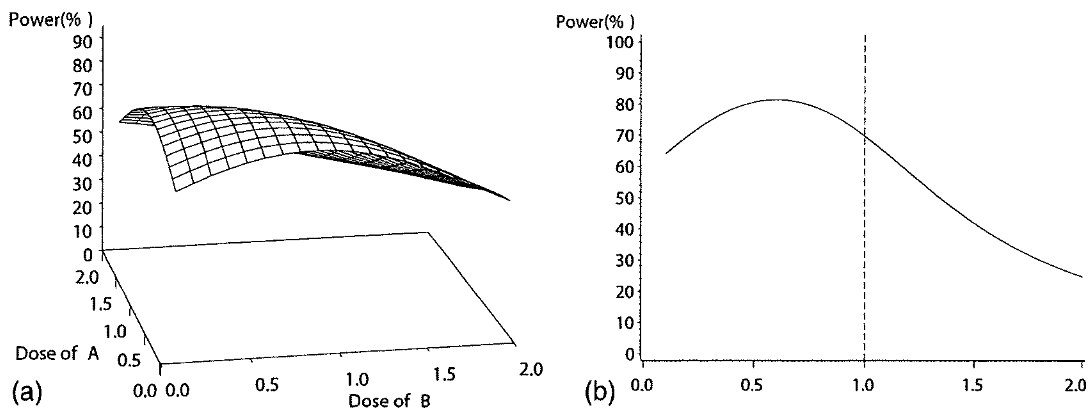

Figure 1. Power surface on the (a) dose plane and power function on the (b) dose for a simultaneous administration group with a constant surplus case (Case 1)

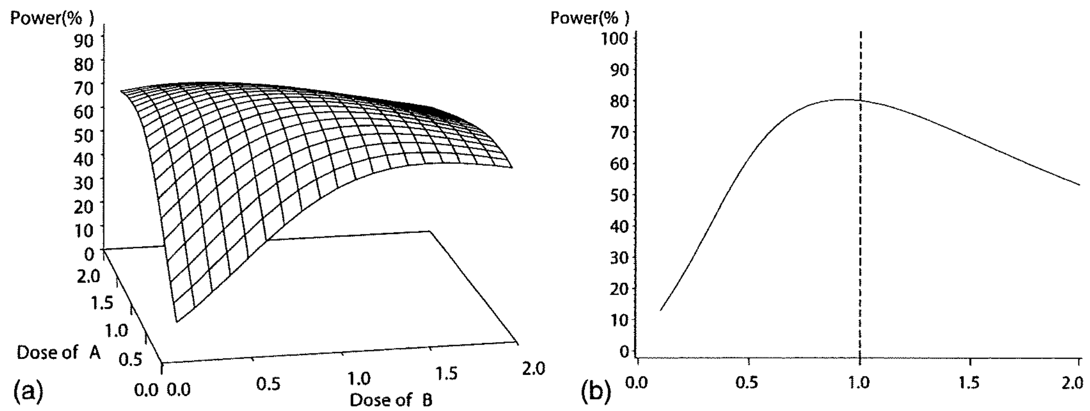

Figure 2. Power surface on the (a) dose plane and power function on the (b) dose for a simultaneous administration group with a square root surplus case (Case 2)

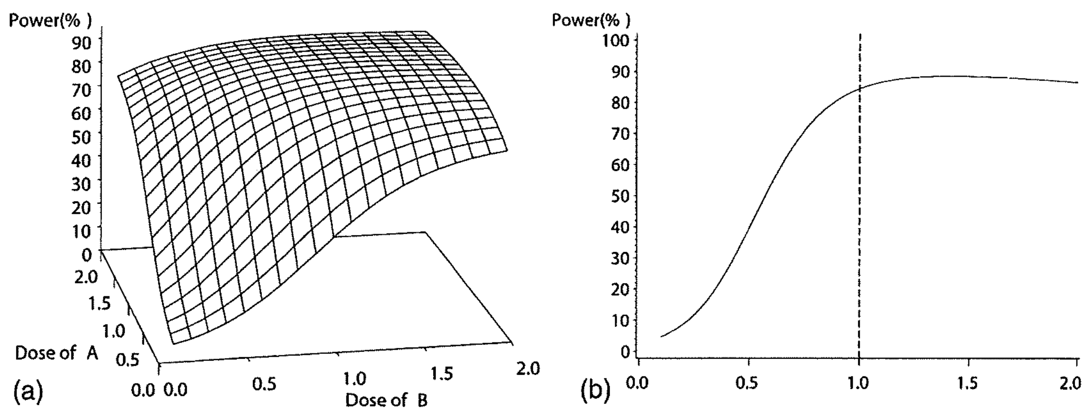

Figure 3. Power surface on the (a) dose plane and power function on the (b) dose for a simultaneous administration group with a linear surplus case (Case 3)

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According to Figure 1, the power is high when the group point of the simultaneous administration group is within the triangular region and the power is low when it is located outside that region. Consequently, the design to evaluate synergism should have the group point within the triangle region. Figure 2 shows the power reaches a peak near the boundary of the triangular region and decreases apart from the triangular region as the dose increases. In consequence, the group point should be located on the boundary of triangle region. Finally, according to Figure 3, the power achieves the steady state on the boundary of the triangular region and reaches a peak slightly outside the triangular region when Δ becomes larger linearly as the dose increases. In numerical example, recommended group point in Cases 1–3 is, respectively, $(d_A, d_B) = (0.6, 0.6), (0.9, 0.9), (1.4, 1.4)$ and the power is 81.4%, 80.4%, 88.4%, respectively.

The sensitivity analysis was conducted to investigate the usefulness of location of the group point in the real study, such as two group points $(d_A, d_B) = (0.5, 0.5)$ and $(1.0, 1.0)$. In this section, only group points for simultaneous administration were changed in the following way and all other conditions were the same.

4.2.3. Group points for simultaneous administration.

(1) $(d_A, d_B) = (0.6, 0.6)$ (recommended in Case 1)
(2) $(d_A, d_B) = (0.9, 0.9)$ (recommended in Case 2)
(3) $(d_A, d_B) = (1.4, 1.4)$ (recommended in Case 3)
(4) $(d_A, d_B) = (1.0, 1.0)$ (on the boundary)
(5) $(d_A, d_B) = (0.5, 0.5), (1.0, 1.0)$ (real study)

Here, the total sample size for simultaneous administration groups is fixed in all group points (1)–(5). Then n_m for two groups' simultaneous design such as (5) is half of that for one group's design.

The numerical calculation was conducted under the above conditions. Table 2 summarizes the reduction of power from a recommended group point. When the group point is located close to the boundary of the triangular region, the reductions of power from a recommended group point are small. Furthermore, in the situation of real studies, the loss of power is negligible compared to that in a recommended group point. It is considered that the configuration of the group points in real study is reasonable from the statistical viewpoint.

4.3. Recommended group size

In this section, under conditions in which the total number of animals is fixed and the effect sizes are varied, we investigate the group size of the simultaneous administration group that maximizes the power.

Table 2. Reduction of power from a recommended group point in three cases

Group point (d_A, d_B)	Case 1 (constant case)	Case 2 (square root case)	Case 3 (linear case)
(0.6, 0.6)	0.0	−10.2	−34.7
(0.9, 0,9)	−7.3	0.0	−9.3
(1.4, 1.4)	−34.6	−9.7	0.0
(1.0, 1.0)	−12.2	−0.4	−5.1
(0.5, 0.5), (1.0, 1.0)	−2.0	−2.6	−19.7

n_m for two group points is half of that for one group.

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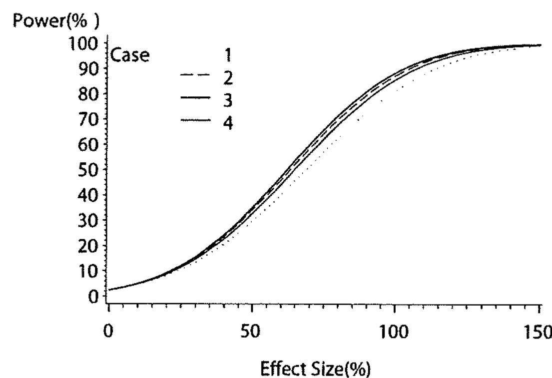

Figure 4. Relation between power and group size in four cases

4.3.1. Fixed conditions.

1. Group points for single administration: $(d_A, d_B) = (0, 0), (1, 0), (2, 0), (0, 1), (0, 2)$.
2. Group point for simultaneous administration: $(d_A, d_B) = (0.6, 0.6)$.
3. Parameters in the dose–response curve: $\beta_0 = 1.0$, $\beta_A = 1.0$, $\beta_B = 1.0$.
4. Variance σ^2: $\sigma_s^2 = \sigma_m^2 = 1.0$.
5. Nominal significance level of t-test: one-sided 2.5%.
6. Total number of animals: $n = 42$.

4.3.2. Varied conditions.

7. Group size:
   Case 1: $n_s = 6, n_m = 12$
   Case 2: $n_s = 5, n_m = 17$
   Case 3: $n_s = 4, n_m = 22$
   Case 4: $n_s = 3, n_m = 27$
8. Effect Size: $\Delta/\sigma_m = 0.1(0.1)1.5$.

Figure 4 shows the results of the numerical calculations, with the power on the vertical axis and the effect size on the horizontal axis. According to Figure 4, regardless of the effect size, Case 3 gives the maximum power. Because the group size is constrained to be an integer, it is not possible to give the most appropriate group size as a continuous value. The power becomes larger when the group size of the simultaneous administration group is set larger than that of the single administration group. When $n = 42$, what is prominent is the fact that the group size of 22 in the simultaneous administration group is nearly equal to the total number of animals in the single administration group, $4 \times 5 = 20$.

5. DISCUSSION

5.1. Conclusion under assumed conditions

In the previous section, we investigated applicable designs assuming that the response variables follow a homoscedastic normal distribution, that the dose–response relationship for single administration is

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linear, that synergism is defined as a larger response obtained with simultaneous administration as compared to the dose plane obtained with single administration by assuming additivity, that there are five groups for single administration and one group for simultaneous administration, etc.

As the results of numerical calculation for the group point, when the departure Δ from additivity is proportional to square root of the dose, it is revealed that the group point for the simultaneous administration group should be on the boundary of the triangle region. Here, the results seemed to be reasonable. Because it is natural to expect that the departure Δ does not continue to increase along with the dose constantly. So, we applied these two situations as the sensitivity analysis. When the departure Δ become larger linearly along with the dose, the group point should be located slightly outside the triangle region. On the other hand, the group point should be set inside the triangle region when the departure Δ is constant. However, the group point should be placed on the boundary of the triangular region, because the reduction of power from a recommended group point is small. This means that the conventional design in the real study is appropriate.

Subsequently, with respect to the group size, we revealed that the total number of animals allocated to the simultaneous administration group should be same size as that in the single administration group.

5.2. *Heteroscedasticity*

When heteroscedasticity in data is expected from the past research, it is required to adjust the degree of freedom by using the Welch test. For the cases discussed in this paper, we used the Welch test, which is robust for heteroscedasticity because there is a tendency in real data for the variance to increase with an increase in responses although the number of animals is small. However, the Welch test does not control a type 1 error below the nominal significance level under heteroscedasticity. It is, therefore, required to confirm, *ex post facto*, the type 1 error when the degree of heteroscedasticity is large.

5.3. *Linearity*

For the cases discussed in this paper, based on advanced information, it was possible to select the dose so that there is linearity with the single administration group. This was easier due to the fact that the number of dose levels in the single administration group was small (three levels). From the experimental results, it was also confirmed that the linearity assumptions were established to some extent. When using the results in this paper, it is important to confirm the dose–response relationship in preliminary experiments or in past experiments using analogous substances. It is necessary to examine, *ex post facto*, the linearity by displaying in figure or by linearity tests.

5.4. *Group size*

The group size must be an integer of at least 1. In addition, the total number of animals must be a comparatively small. Under these conditions, as in this paper, it is necessary to obtain the appropriate design using numerical calculations, separately considering a combination of possible group sizes. However, in order to generalize these results, it is useful to perform power calculations, taking the group size as a continuous value. When calculating the recommended ratio of the total number of animals in the single administration group to the group size of the simultaneous administration group, the results shown in Figure 5 are obtained.

When determining the appropriate group point of the simultaneous administration group, with the departure Δ from the additivity being constant, the highest power is obtained by setting the number of

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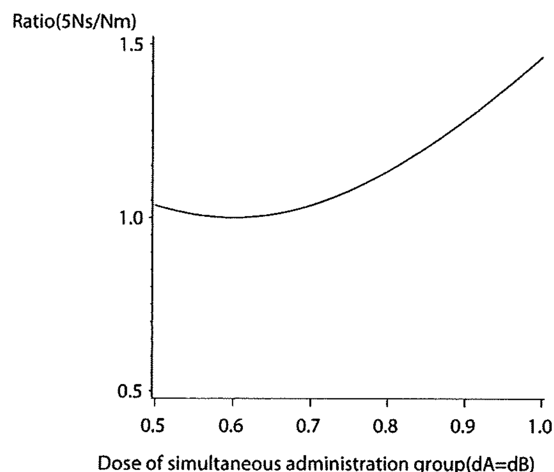

Figure 5. Recommended ratio of the group size between single administration group and simultaneous administration group

animals at a ratio of 1:1 between the single administration group and the simultaneous administration group.

When determining the simultaneous administration group on the boundary of the triangular region, a theoretical calculation shows that the allocation at a ratio of 1.464:1 is appropriate. In other words, it is best to set the total number of animals in the single administration group to be approximately 1.5 times the total number of animals in the simultaneous administration group. The reason is that because it is theoretically favorable for the accuracy of estimates based on additivity to be equal to that based on simultaneous administration.

5.5. Practical consideration on the recommended design

In the numerical examples of the previous section, we showed that the group point for the simultaneous administration on the boundary of the triangular region is not necessarily best and the recommended number of animals for the simultaneous administration group is considerably greater than those for single administration groups.

Although these results speciously imply that the design exemplified in the Subsection 4.1 should be replaced with the recommended design shown in this paper in future, we think it is not always true, because we have to take practical conditions into consideration, which were not incorporated in the assumptions to derive the recommended design.

One of them is the robustness or stability of the result of data analysis in such experiments. The situation is quite similar to that for the recommended design at linear regression analysis, for example, related to a single chemical experiment. Actually, if we can entirely assume the linearity of the dose–response relationship in regression analysis, the recommended design is to allocate a half of animals to the maximum dose and the remaining half to the minimum dose, while such design is really not adopted and animals are evenly allocated to uniformly distributed three or four doses probably to secure the robustness of the result of data analysis. Likewise, when the functional relationship of the Δ is not particularly clear, or when there is concern for the instability in squeezing the simultaneous administration group into one group in our experiments, the design with two or three simultaneous

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administration groups within the triangular region must be practical. Since the mathematical formulation in such condition could not be established up to present due to its difficult nature, we left it for future investigation.

5.6. *Other issues for future investigation*

When the number of test substances is 3 or more, there needs to be strict controls for the number of required animals. Therefore, a design must be determined by examining the design conditions in detail for each case. Under this condition, an investigation following the same approach as in this paper is necessary. This is another problem to be addressed in future research.

In this paper, we introduced a test statistic by using the unweighted least squares method. The weighted least squares method must be considered when the rules for the size of variance are understood from past research, such that variance linearly becomes larger along with the dose. The specific design in this instance is also to be investigated in the future.

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Cellular distributions of molecules with altered expression specific to thyroid proliferative lesions developing in a rat thyroid carcinogenesis model

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To identify differentially regulated molecules related to early and late stages of tumor promotion in a rat two-stage thyroid carcinogenesis model by an antithyroid agent, sulfadimethoxine, microarray-based microdissected lesion-specific gene expression profiling was carried out. Proliferative lesions for profiling were divided into two categories: (i) focal follicular cell hyperplasias (FFCH) and adenomas (Ad) as early lesions; and (ii) carcinomas (Ca) as more advanced. In both cases, gene expression was compared with that in surrounding non-tumor follicular cells. Characteristically, upregulation of cell cycle-related genes in FFCH + Ad, downregulation of genes related to tumor suppression and transcription inhibitors of inhibitor of DNA binding (Id) family proteins in Ca, and upregulation of genes related to cell proliferation and tumor progression in common in FFCH + Ad and Ca, were detected. The immunohistochemical distributions of molecules included in the altered expression profiles were further examined. In parallel with microarray data, increased localization of ceruloplasmin, cyclin B1, and cell division cycle 2 homolog A, and decreased localization of poliovirus receptor-related 3 and Id3 were observed in all types of lesion. Although inconsistent with the microarray data, thyroglobulin immunoreactivity appeared to reduce in Ca. The results thus suggest cell cycling facilitation by induction of M-phase-promoting factor consisting of cyclin B1 and cell division cycle 2 homolog A and generation of oxidative responses as evidenced by ceruloplasmin accumulation from an early stage, as well as suppression of cell adhesion involving poliovirus receptor-related 3 and inhibition of cellular differentiation regulated by Id3. Decrease of thyroglobulin in Ca may reflect dedifferentiation with progression. (*Cancer Sci* 2009; 100: 617–625)

Although clinically recognized thyroid Ca constitute less than 1% of all human malignant tumors, it is the most common endocrine cancer (90% of cases) and is responsible for more deaths than all other endocrine cancers combined.[1] Ca of the thyroid is usually of follicular cell origin, but the medullary carcinoma arises from parafollicular or C cells. In humans, causative factors for thyroid Ca are not well understood except for secondary occurrence after radiotherapy.[2] In rats, on the other hand, thyroid follicular cell tumors can be produced by administration of antithyroid agents, such as by propylthiouracil,[3] methimazole,[4] and 3-amino-1,2,4-triazole,[5] in an initiation-promotion model.

Many chemicals that can induce thyroid tumors in rodents cause disruption of the thyroid–pituitary axis through induction of hypothyroidism.[6] The putative mechanism for this carcinogenesis is believed to be non-genotoxic, decrease in the serum levels of triiodothyronine and thyroxine causing suppression of negative feedback through the pituitary and an increase in serum TSH. TSH then stimulates thyroid functions, including growth and proliferation of follicular cells.[7,8] However, detailed molecular mechanisms remain to be resolved.[6]

SDM is a broad-spectrum antimicrobial sulfonamide that has been shown to effectively induce thyroid follicular cell tumors in a rat two-stage thyroid carcinogenesis model after initiation with DHPN.[9] The anti-thyroidal effects of this drug are mediated through inhibition of iodination reactions catalyzed by thyroid peroxidase, resulting in reduction of thyroid hormone synthesis and increased levels of TSH in the bloodstream.[10]

Histological lesion-specific gene expression profiling provides valuable information on the mechanisms underlying lesion development. We have established molecular analysis methods for DNA, RNA, and proteins in paraffin-embedded small-tissue specimens utilizing an organic solvent-based fixative, methacarn,[11–13] and applied them for analyses of microdissected lesions.[14,15] With regard to mRNA expression analysis, expression fidelity in the methacarn-fixed paraffin-embedded tissues was found to be very close to that in the unfixed frozen tissues in both the real-time RT-PCR and oligonucleotide microarray systems, suggesting a great advantage of methacarn in analyses of microdissected lesions after paraffin embedding.[14,15]

In the present study, to identify differentially regulated molecules related to thyroid carcinogenesis through hypothyroidism, we carried out global gene expression profiling of early and late-stage proliferative lesions obtained after promotion with SDM in a rat two-stage carcinogenesis model. Localization of representative molecules showing altered expression was further analyzed immunohistochemically.

Materials and Methods

Chemicals and animals. DHPN (CAS no. 53609-64-6) and SDM (CAS no. 122-11-2) were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma (St Louis, MO, USA), respectively. Male 5-week-old F344 rats were purchased from Japan SLC (Hamamatsu, Japan) and housed four to five rats per polycarbonate cage with sterilized softwood chips as bedding in a barrier-sustained animal room conditioned at 24 ± 1°C and 55 ± 5% humidity, with a 12:12 h L : D cycle. They received CRF-1 (Oriental Yeast Co., Tokyo, Japan) as a basal diet and water *ad libitum* throughout the experimental period, including the 1 week of acclimation.

Experimental design. At 6 weeks of age, 30 rats were injected subcutaneously with 2800 mg/kg body weight DHPN. Another

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Abbreviations: Ad, adenoma; Ca, carcinoma; Ccnb1, cyclin B1; Cdc2, cell division cycle 2; DHPN, *N*-bis(2-hydroxypropyl)nitrosamine; FFCH, focal follicular cell hyperplasia; Id, inhibitor of DNA binding; IGSF, immunoglobulin superfamily; NTF, non-tumor follicles; PCR, polymerase chain reaction; Pvrl3, poliovirus receptor-related 3; RT, reverse transcription; SDM, sulfadimethoxine; TSH, thyroid-stimulating hormone.

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group of nine animals was injected with the vehicle saline as non-treated controls. One week later, 25 DHPN-initiated animals were administered SDM at 1000 p.p.m. in the drinking water *ad libitum* for up to 15 weeks. The other five DHPN-initiated animals were maintained on tap water for 10 weeks as a DHPN-alone group. At week 10 after SDM treatment, 10 animals were killed for microdissection of FFCH + Ad as well as NTF in each animal. The other SDM-promoted animals were further maintained until week 15, when 12 rats were killed for microdissection of Ca. Five untreated controls and the DHPN-alone group were killed at week 10 of SDM promotion, and the four remaining untreated controls at week 15. All animals were killed by exsanguination from the abdominal aorta under deep anesthesia with ether. The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Preparation of tissue specimens and microdissection. Caudal halves of the bilateral thyroid tissues of SDM-promoted animals were immersed in methacarn solution for 2 h at 4°C.[11] Tissue samples were then dehydrated, immersed in xylene, and embedded in paraffin as described previously.[15] Embedded tissue blocks were stored at 4°C until microdissection.[16]

For microarray analysis, 4 µm-thick sections between 10 16 µm-thick serial sections were prepared. The 16 µm-thick sections were mounted onto PEN-foil film (Leica Microsystems, Welzlar, Germany) overlaid on glass slides, dried in an incubator overnight at 37°C, and then stained using an LCM staining kit (Ambion, Austin, TX, USA). All microdissections were carried out within 2 h of tissue staining. The histopathological identity of each FFCH, Ad, and Ca, as well as NTF, was determined under microscopic observation of the adjacent 4 µm-thick sections stained with hematoxylin–eosin according to published criteria (Fig. 1).[17] In the present carcinogenesis model with SDM promotion, capsular invasive carcinomas are generated in addition to less-frequent parenchymal Ca.[18] In the present study, parenchymal proliferative lesions including the latter were subjected to laser microbeam microdissection (Leica Microsystems). Approximately 20 sections of bilateral thyroids were subjected to microdissection in one animal, and the microdissected samples (NTF, FFCH + Ad, and Ca) were collected and stored in separate 1.5-mL sample tubes at –80°C until extraction of total RNA.

RNA isolation, amplification, and microarray analysis. Total RNA extraction from each histological sample and quantitation of the RNA yield were carried out according to methods described previously.[15]

For microarray analysis, equal amounts of extracted total RNA samples from two animals were mixed (100 ng/sample) and subjected to amplification, consisting of RT and subsequent two-step *in vitro* transcription, using a MessageAmp II aRNA Kit (Ambion).

Second-round-amplified biotin-labeled antisense RNA was subjected to hybidization with a GeneChip Rat Genome 230 2.0 Array (Affymetrix Inc., Santa Clara, CA, USA). RNA samples collected from two animals were subjected to analysis with individual microarrays (n = 5/histological preparation).

Selection of genes and normalization of expression data were carried out using GeneSpring software (ver7.2; Silicon Genetics, Redwood City, CA, USA). To normalize chip-wide variation in intensity, per chip normalization was performed by dividing the signal strength for each gene with the level of the 50th percentile of the measurement in the chip, and dividing the value by the average intensity in the samples of NTF. Genes showing signals judged to be 'absent' in all 10 samples of NTF and each proliferative lesion group (FFCH + Ad or Ca) for comparison were excluded. Then, genes showing expression change with differences at least 2-fold in magnitude in the proliferative lesion groups from the NTF, as well as the 'presence' signal in more than four of five samples in the histological lesion group (NTF or each proliferative lesion group) showing higher expression values in comparison, were selected. Genes showing altered expression in common in both FFCH + Ad and Ca were also selected.

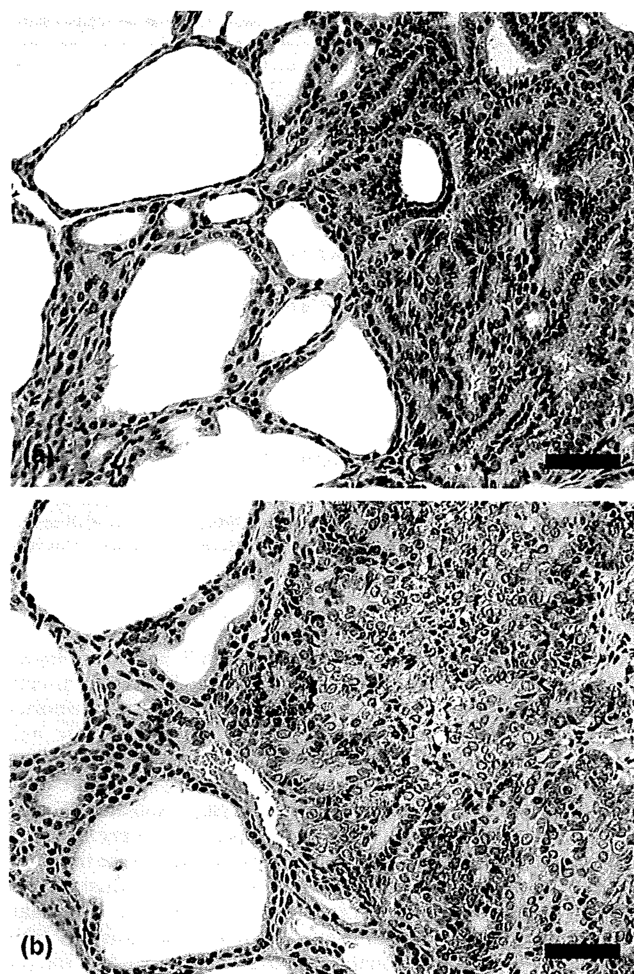

Fig. 1. Representative proliferative lesions developing after promotion with sulfadimethoxine (SDM) for (a) 10 or (b) 15 weeks in a rat two-stage thyroid carcinogenesis model. (a) Adenoma with follicular features, showing expansive growth with minor atypia. (b) Carcinoma showing obvious cellular atypia, consisting of follicular and solid growth elements with structural irregularity. Note focal necrosis and fibrosis. Hematoxylin–eosin staining. Scale bars = 50 µm.

Real-time RT-PCR. Quantitative real-time RT-PCR was carried out for confirmation of expression values obtained with microarrays using an ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA). The following 11 genes (eight upregulated and three downregulated in proliferative lesions) were selected as targets: chitinase 3-like 1, ceruloplasmin, solute carrier family 2 (facilitated glucose transporter) member 3, solute carrier family 16 (monocarboxylic acid transporters) member 6, glucagon, prolactin receptor, phosphatidylinositol 4-kinase type 2α, and actinin α1 as upregulated examples; and *Pvrl3*, retinoic acid induced 3, and glucosaminyl (*N*-acetyl) transferase 1 core 2 as downregulated examples. RT was carried out using first-round antisense RNA prepared for microarray analysis. Real-time PCR analysis of ceruloplasmin, glucagon, and glucosaminyl (*N*-acetyl) transferase 1 core 2 was carried out using ABI Assays-on-Demand TaqMan probe and primer sets from Applied Biosystems (available at https://products.appliedbiosystems.com/ab/en/US/

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