出来ない場合は，現在のところ，人間の身代わりとしてモデル動物を用いることになる。ラット，マウス，ウサギなどが化学物質の毒性試験に，これらに加えてイヌやサルが主に医薬品関係の毒性試験に用いられる。発生毒性，催奇形性，生殖毒性など，様々な試験があるが，一般的なげっ歯類をモデルにした毒性試験は，

1. 対照群（検体を投与するための溶媒を投与する）
2. 低用量処置群
3. 中用量処置群
4. 高用量処置群

を設ける場合が多い。すなわち，薬の場合と異なり，幅広い用量範囲における毒性の用量作用関係を求める実験が行われる。一群の動物匹数は10，20あるいは50近くを用いることもある。また，投与方法（投与経路）には経口，混餌，皮下，静脈内，吸入などがある。投与期間は，単回投与から，28日間反復投与，90日，1年，2年など，目的に応じて選択される。母体に投与し経胎盤的に胎児に暴露する場合もある。観察する項目は体の外表所見（奇型の有無など），体重，臓器重量，血清生化学，血算，病理組織検査など，多岐にわたる。

さて，毒性の判定は通常，対照群の測定値に対して処置群の値が有意に増加（あるいは減少）したか否かによって行われる。毒性の場合はp値が0.05未満をもって有意差有りとすることが多い。すなわち，20回に1回偶然に起こる事象よりも稀な事が起こった場合，投与が何かを引き起こしたと考えるわけである。生物統計の専門家，吉村功・東京理科大学教授のお話では，勝負事（例えば囲碁や将棋）で1回，2回，3回，そして4回連続して負けても，「まだまだ，次は勝つぞ」と思うかもしれない。これが，2の4乗分の1，すなわち，偶然に確率16分の1の事象が起きた段階。しかし，5回連続で負けたら，さすがに自分の方が弱いと観念するのが一般的な感覚であろう。それが，2の5乗分の1，即ち32分の1。p値 = 0.05 = 20分の1という値は，そういう感覚のものであるとのことである。毒性試験では，様々な項目について検討するわけであるが，その中で一番低用量で有意差が認められた用量を最小作用量（LOEL）あるいは最小毒性量（LOAEL）とすると，その下の用量が無作用量（NOEL），あるいは無毒性量（NOAEL）の候補となるわけである。通常，この値を，種差や個体差の不確実性を勘案する「不確実係数あるいは安全係数（uncertainty factor あるいは safety factor）」（通常100）で除して規制に用いる基準値を設定することが多い。

ここで，ちょっと話を変えさせて頂く。ある人が，熱っぽく，だるく，頭が痛いので病院に行ったとする。すると外来で…

医者　「どうなさいました？」

患者　「熱っぽく，だるく，頭が痛いので，風邪かなと思いまして」

医者　「では，正常な人を10人と，そう，それから，あなたと同じ症状の人を9人連れてきて下さい」

患者　「？？」疑問に思いながらも，ちょうどインフルエンザが流行っていて，外来には9人ぐらいそれらしき人が順番待ちしていたので，そこから9人と，健康そうな看護婦さん10人を病院中からかき集めてきて，

患者　「先生，揃いました」

医者　「どれどれ，では，体温は…」，「風邪疑い10名の体温は　38.2 ± 1.1度，健康と思われる10名の体温は36.5 ± 0.4度。t検定で良いですかね，この場合，……，n = 10，p値が…」

医者　「結果が出ました，統計学的に，あなたは健康ではないようですね。」

患者　「？？？？」

実際には，こんな事は行われていない。「正常でない」ことを言い当てる事と「診断」することとは全く異なっていることは明白である。では，医者はn = 1の患者さんに対してどのような手順で診断しているのか。「正常」との対比ではなく，多数の「病気，病状」の知識との対比をしていくわけである。つまり，患者からの「特徴抽出」と，医者の頭の中の「データベース」との比較を行っているわけである。名医と藪医者の差は，特徴抽出能力とデータベースの差にあると考えられる。例えば，新しい病気の発見の論文は，たった数症例で書かれることが多いが，これも（新しい病気のデータベースがない筈であるにもかかわらず）今までの病気のデータベースとの比較によって可能となる訳である。

毒性試験の話に戻る。20匹からなる実験群の血糖値を測定したところに1匹だけ飛び離れた値を示すものがあったとする。用いた動物は近交系（臓器移植がお互いに可能なぐらい遺伝子が均一な実験動物）であるし，飼育条件も何もかも差別なく施してきたにも関わらず，である。こういう場合，統計学的には，「はずれ値（outlier）」として，その測定値をその後の計算から除外することが往々にして行われる。では，その一匹を「診断」したらどうであろうか。全く原因不明で血糖値だけがおかしいのであれば，本当に測定エラーであった可能性が高い。しかし，その動物だけ腫瘍が発生していた，あるいは炎症病変があった，ということが見つかれば，これは「はずれ値」ではない可能性が高い。毒性試験の判定の際には，すなわち，2通りの見方が常に行われる。

1. 同じ処置を受けた群単位の動向として，統計判断に基づく有意差検定を行う，
2. 処置（投与した化合物とその量）によって個々の動物に何が起こっているかを「診断」する，

の2とおりである。1. に係る検査項目は「所定（routine）

の項目」を基本として，2. で問題となった（ad hoc）項目を追加することで強化される。

ある特定の化学物質が悪い影響を及ぼしているかもしれないと，報告されたとしよう。その内容は，「毒性試験をしたところ，統計学的有意差が付いた測定項目がいくつかある」というものであった。追試験を行ったところ，今度は傾向はあるものの有意差が無い。はっきりした科学的裏づけが得られない。それでも，安全を見越して，法的に規制することも考えられる。しかし，p 値 < 0.05 で有意差検定をすることは20項目に1項目は，偶然に有意差有りという判定がなされる可能性を示している。多数の検査項目からなる長期毒性試験においては，偶然に何項目かに有意差が付く事象が起こる確率が決して低くない。もしも，この様な「有意差」だけを根拠に一旦規制を開始してしまった場合，それを解除する試験結果を得る方策は，同じ類の毒性試験からは，事実上無いことになる（偶然はじめたとしたら，偶然やめるしかないわけである）。

これに対して，一群20匹の実験で，処置群の中のたったの1匹にだけ異常な病変があったとする。統計学的には有意差は無いと計算されるであろう。しかし，この1例を「診断」してみると，処置によって引き起こされた変化であることが強く示唆される場合があり得る。この場合，本当に確からしいことを診断学的に示すことが出来れば，統計学的に有意でなくても緊急性に鑑みて規制することは正しいと判断される場合があり得る。この場合，継続的に「下した診断が正しかったか否か」を検討し続けることで，「誤診」であった場合には，規制を中止することが出来る。また，第2例目以降が続々と出現すれば，「診断」は正しかったことになる。ここでの注意点は「診断学は純粋科学では無い」事である。診断基準は日々更新されるものであり，診断については「診断医」あるいは「診断者」がその責任を負うのである。

まとめ：

化学物質の毒性は，生体内に進入した化学物質が引き起こす生命体の複雑な反応を診て，どのような有害作用が起こるかを見届けることにより判断することになる。体内では化学物質が生体による修飾を受けて，その修飾体がさらに生体反応を複雑にすることもしばしばある。生体反応は，人為的に制御不能な変動性を持っているので，例え動物数を無限に増やしてみても，ひとつの値に収束するものでは決してない（ときとして，変動の幅を正確に教えてくれるようにはなるが）。まずは，動物において上述の二通りの見方をした上で，ヒトへの外挿が検討される。実験動物個体として，そして集団として何が起こりうるのかを考察し，それを基にヒトの個体として，亜集団として，そして集団として何が起こりうるのかを考察することが，ヒトに対する毒性を判断するには重要であると考える。

以上，化学の講義ではあまり遭遇されないであろう，生物的な側面からの裏話を一研究者としてご紹介した。より正確な毒性決定手順等は成書に譲る。（人体病理標本は，佐々木研究所附属杏雲堂病院のご厚意による。）

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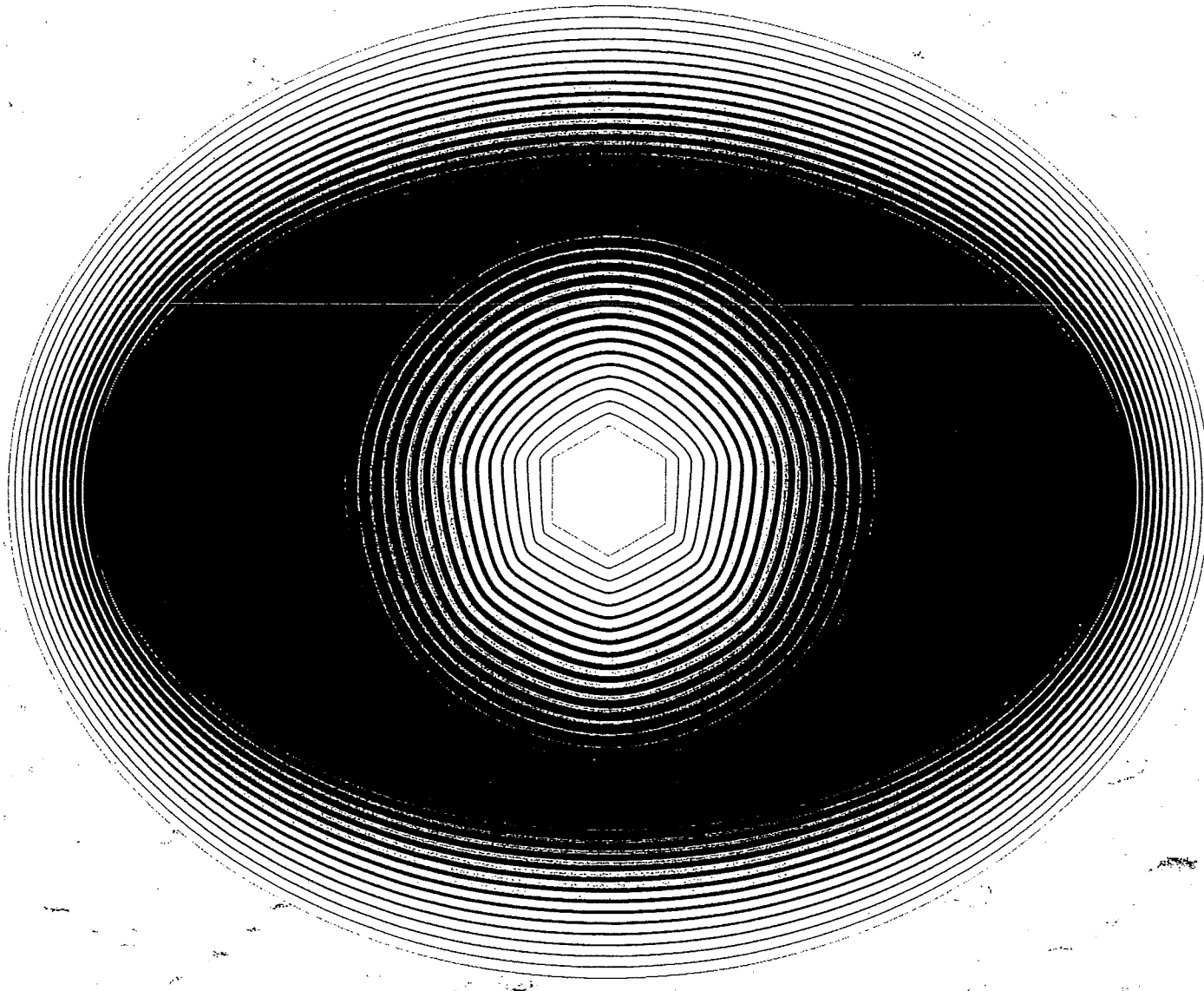

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RESEARCH ARTICLE

Functional changes in dopamine D3 receptors by prenatal and neonatal exposure to an endocrine disruptor bisphenol-A in mice

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Abstract

Bisphenol-A (BPA), one of the most common environmental endocrine disrupters, has been evaluated extensively for toxicity and carcinogenicity. However, little is still known about its action on the central nervous system (CNS). In the previous study, we found that prenatal and neonatal exposure to BPA markedly enhanced the rewarding effect induced by morphine. Here we found that prenatal and neonatal exposure to BPA resulted in the attenuation of dopamine D3 receptor-mediated G-protein activation by 7-OH-DPAT in the mouse limbic forebrain. This treatment also caused a significant decrease in the B_{max} value of [^{3}H]PD128907, a dopamine D3 receptor ligand, in this area. Under these conditions, no change in dopamine D3 receptor mRNA expression in the limbic forebrain and lower midbrain was observed by prenatal and neonatal exposure to BPA. The present data provide further evidence that prenatal and neonatal exposure to BPA leads to the reduction of functional dopamine D3 receptors without affecting the new synthesis of dopamine D3 receptors in the mouse limbic forebrain.

Introduction

Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. It has been reported that administration of bisphenol-A (BPA) to pregnant mice on gestation days at a dose that is within the range typical of the environmental exposure of human produces significant changes in the postnatal growth rate and brings on early puberty in these mice.[1] These results imply that BPA may cause the unpleasant toxicity in the developmental process. In contrast, little is known about the neuronal toxicity induced by BPA in the central nervous system (CNS). More recently, we have reported that prenatal and neonatal exposure to BPA enhances the methamphetamine-induced rewarding effect and central dopamine D1 receptor function in mice.[2]

The dopamine D3 receptor cloned by Sokoloff and colleagues has been characterized extensively.[3] The dopamine D3 receptor shows a distinct distribution in limbic areas of the brain, including the nucleus accumbens and olfactory

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tubercle.[3] Several pharmacological studies with dopamine D3 receptor-preferring agonists such as 7-hydroxy-N,N-di-n-propyl-2-aminotetralin (7-OH-DPAT) suggest that the dopamine D3 receptor regulates the inhibitory effect to produce hyperlocomotion in rodents.[4,5] In addition, we found that the morphine-induced rewarding effect and hyperlocomotion were markedly enhanced in mice lacking dopamine D3 receptor gene.[6] These findings suggest that the dopamine D3 receptor plays a critical role in the psychoeffective functions of dopamine neurotransmission.

The present study was then designed to investigate the changes in the function of dopamine D3 receptors by prenatal and neonatal exposure to BPA in mice.

Materials and method

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. All efforts were made to minimize the number of animals used and their suffering.

Animals

All experiments were performed using male ddY mice (8–12 weeks old) that had been exposed prenatally and neonatally to BPA. Prenatal and neonatal BPA exposure was conducted as described previously.[2] Adult female mice were chronically treated with BPA-admixed powder food containing 0 (B0) and 2 (B2) mg BPA/g of food from mating to weaning. Their pups were prenatally (about 3 weeks) and neonatally (about 2 weeks) exposed to the respective concentration of BPA from their mothers. During the treatment with BPA, animals did not show either weight loss or disrupted maternal behaviours.

Membrane preparations

In the membrane preparation, mice were killed by decapitation and the limbic forebrain was then dissected as described previously.[7] The limbic forebrain was rapidly excised at 4°C, and the tissues were homogenized using a Potter-Elvehjem tissue grinder with a Teflon pestle in 20 volumes (w/v) of ice-cold Tris-Mg^{2+} buffer containing 50 mM Tris-Hcl (pH 7.4), 5 mM $MgCl_2$ and 1 mM EGTA for the [^{35}S]GTPγS binding assay. The homogenate was centrifuged at 4°C for 10 min at 48,000 g. The pellet was resuspended in ice-cold Tris buffer *or* [^{35}S]GTPγS binding assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, and 100 mM NaCl and centrifuged at 4°C for 10 min at 48,000 g. The resultant pellet was resuspended in ice-cold Tris buffer or [^{35}S]GTPγS binding assay buffer and stored at −70°C until used.

[^{35}S]GTPγS binding assay

The membrane homogenate (3–8 μg protein/assay) was incubated at 25°C for 2 h in 1 ml of assay buffer with 10 μM 7-OH-DPAT or a dopamine D_2 receptor agonist N-propylnorapomorphine (NPA), 30 μM guanosine-5′-diphosphate (GDP) and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters presoaked in 50 mM Tris-HCl (pH 7.4) and 5 mM $MgCl_2$ at 4°C for 2 h. Filters were then washed three times with 5 ml of an ice-cold Tris-HCl buffer (pH 7.4), transferred to scintillation counting vials containing 0.5 ml of Soluene-350 and 4 ml of Hionic Fluor, equilibrated for 12 h and the radioactivity in the samples was determined with a liquid scintillation analyser. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. Comparable results were obtained from at least three independent sets of experiments.

Dopamine D_3 receptor binding assay

The dopamine D_3 receptor binding assays were carried out in duplicate with [^{3}H] PD128907 (specific activity, 111 Ci/mmol; Amersham, Arlington Heights, IL) at 0.039 to 5 nM in a final volume of 1.0 ml which contained 50 mM Tris-HCl buffer (pH 7.4), 100 μM guanosine-5′-[β,γ-imido]triphosphate (Gpp(NH)p) and 0.1 ml) of the homogenated membrane fraction. The amount of membrane proteins used in each assay was in the range of 90 to 140 μg, as determined by the method of Bradford.[8] The test tubes were incubated for 2 h at 25°C. The specific binding was defined as the difference in bindings observed in the absence and

presence of 10 μM unlabeled PD128907. The incubation was terminated by collecting the membranes on Whatman GF/B filters using a Brandel cell harvester. The filters were then washed three times with 5 ml Tris-HCl buffer (pH 7.4) at 4°C and transferred to scintillation vials. Then, 0.5 ml of Soluene-350 (Packard Instrument Company, Inc, Meriden, CT) and 4 ml of Hionic Fluor Cocktail (Packard Instrument Company) were added to the vials. After a 12 h equilibration period, the radioactivity in the samples was determined in liquid scintillation analyzer.

RT-PCR assay

Total RNA in the limbic forebrain and lower midbrain were extracted using SV Total RNA Isolation System (Promega, Madison, WI). To prepare first strand cDNA, 1 μg of RNA was incubated in 100 μl of buffer containing 10 mM DTT, 2.5 mM $MgCl_2$, dNTP mix, 200 U of reverse transcriptase II (Gibco-BRL, Grand Island, NY) and 0.1 mM oligo $(dt)_{12-18}$ (Gibco-BRL). The dopamine D_3 receptor was amplified in a 50 μl PCR solution containing 0.8 mM $MgCl_2$, dNTP mix and DNA polymerase with synthesized primers: a sense primer of dopamine D3 receptor, which is at position 391–407 (5′-GCA GTG GTC ATG CCA GTT CAC TAT CAG-3′) of the receptor, and an antisense primer at position 498 to 526 (5′-CCT GTT GTG TTG AAA CCA AAG AGG AGA GG-3′), which were designed according to sequence Accession nos U26915 in GenBankTM.

Samples were heated to 94°C for 2 min, 55°C for 2 min and 72°C for 3 min, and cycled 35 times through 94°C for 1 min, 55°C for 2 min and 72°C for 3 min. The final incubation was 72°C for 7 min. The mixture was run on 1% agarose gel electrophoresis with the indicated markers and primers of the internal standard GADPH. Three independent experiments were performed in this study. Semi-quantitation of the intensity of the bands for dopamine D_3 receptor/GADPH was conducted by using a NIH image.

Results

The attenuation of dopamine D3 receptor-mediated G-protein activation in mice prenatally and neonatally exposed to BPA

The dopamine D3 receptor agonist 7-OH-DPAT (0.001-10 μm) produced a concentration-dependent increase in [^{35}S]GTPγS binding to membranes obtained from the limbic forebrain including the nucleus accumbens of B0 mice.

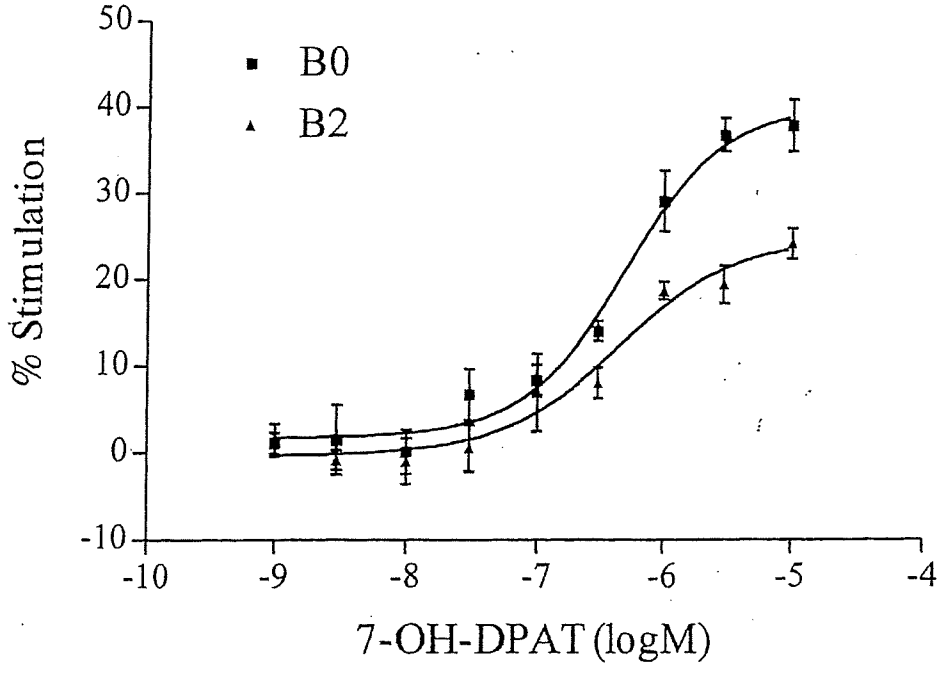

Figure 1. *Comparison of the stimulation of [^{35}S]GTPγS binding by dopamine D_3 receptor agonist 7-OH-DPAT to membranes of the limbic forebrain obtained from control (B0: square) and BPA-treated (B2: triangle) mice. Membranes were incubated with [^{35}]GTPγS (50 pM) and GDP (30 μM) with 7-OH-DPAT (10^{-9}–10^{-5} M). The values are expressed as percentage increase of the value in B0 mice. Data are expressed as the mean ± SEM of three independent samples.*

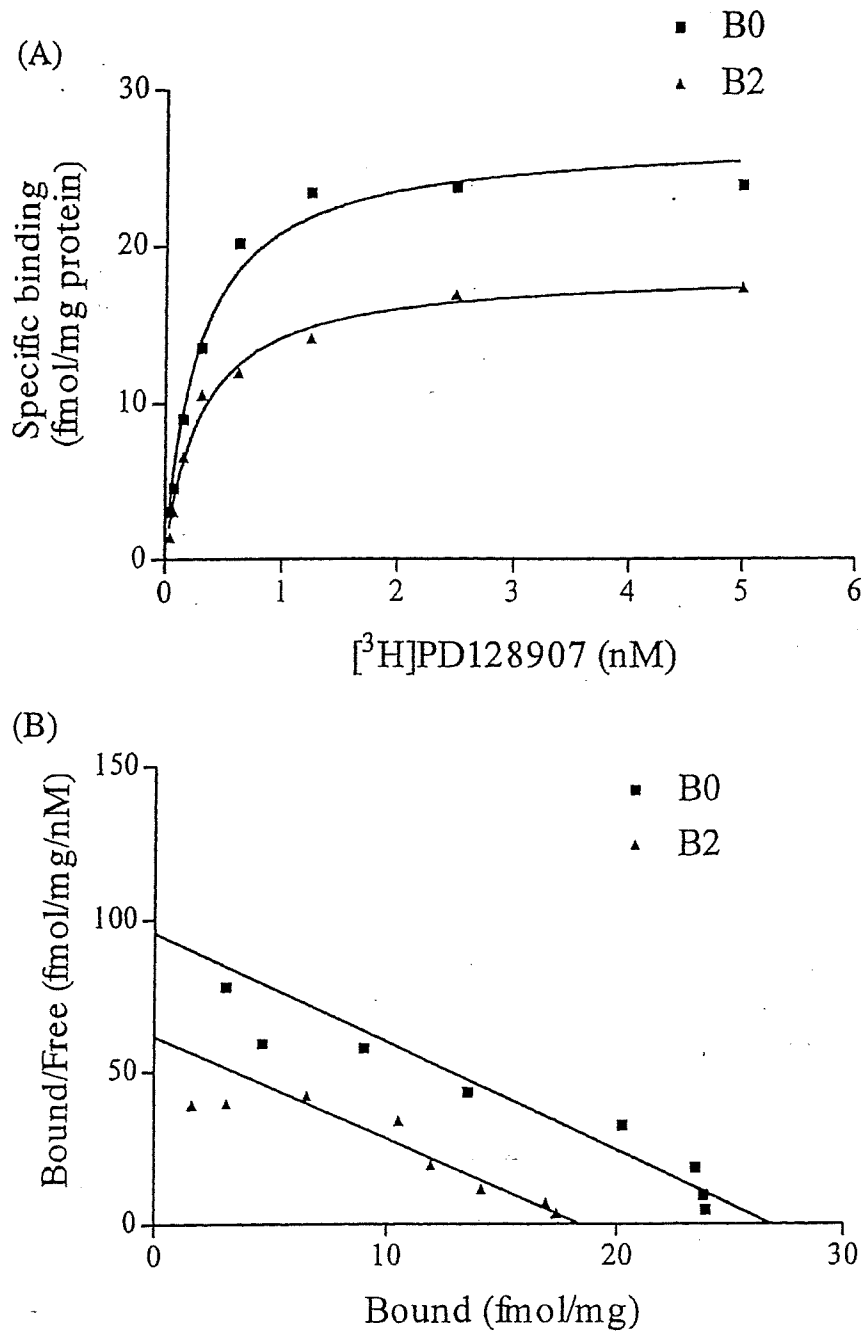

Figure 2. *Decrease of dopamine D3 receptor density in the limbic forebrain obtained from mice prenatally and neonatally exposed to BPA. Saturation binding (A) and Scatchard analysis (B) were performed by receptor binding assay using [^{3}H]PD128907. Membranes were incubated with [^{3}H]PD128907 (0.039–5.0 nM) and Gpp(NH)p (100 μm).*

The key finding of the present study was that the stimulation of [^{35}S]GTPγS binding induced by 7-OH-DPAT was attenuated markedly in B2 mice ($F_{1,\ 174} = 30.45$, $p < 0.001$; Fig. 1). Under these conditions, the G-protein activation induced by the selective dopamine D_2 receptor agonist N-propylnorapomorphine in the limbic forebrain was not affected by prenatal and neonatal exposure to BPA (only 3% increase; figure not shown).

Decrease of dopamine D3 receptor density in the limbic forebrain obtained from mice prenatally and neonatally exposed to BPA

To evaluate the population of dopamine D3 receptors in the mouse brain, we performed the saturation-binding analysis using [^{3}H] PD128907. The saturation-binding curve and Scatchard analysis of [^{3}H]PD128907 are shown in Fig. 2. The B_{max} and apparent K_d value are shown in Table 1. We found that prenatal and

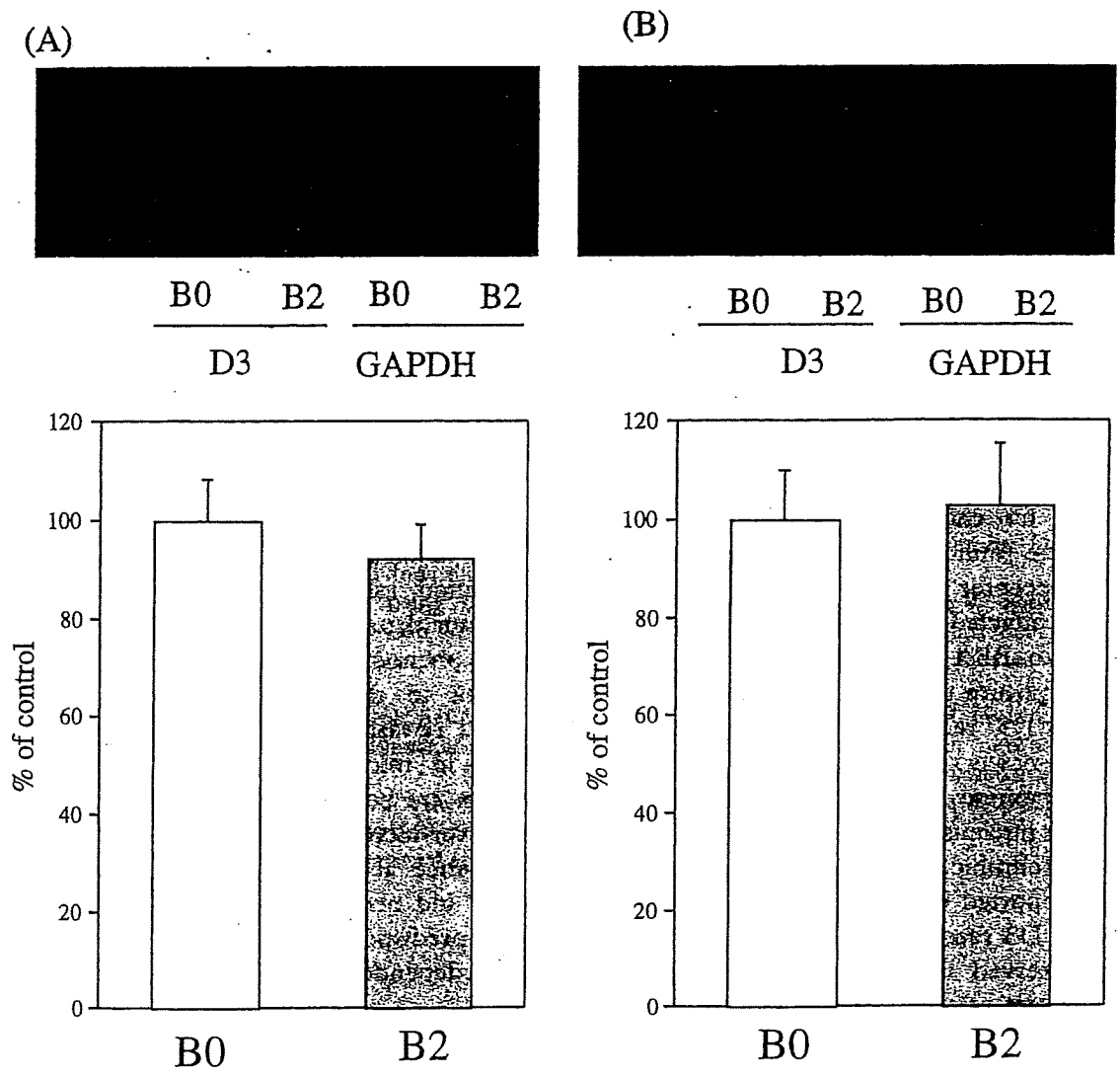

Figure 3. *No changes in the expression of dopamine D3 receptor mRNAs in the (A) limbic forebrain and (B) lower midbrain obtained from B0 and B2 mice. Upper panel: representative reverse transcription-polymerase chain reaction for dopamine D3 receptor mRNAs in the limbic forebrain and lower midbrain obtained from B0 and B2 mice. Lower panel: no changes in the expression of dopamine D3 receptor mRNAs in the limbic forebrain and lower midbrain obtained from B2 mice (hatched bar) compared to that from B0 mice (open bar). The values are expressed as a percentage of the value in B0 mice. Each column represents the mean ± SEM of three independent samples.*

Table 1. *Dopamine D_3 receptor density and affinity performed by [^{3}H]PD128907 binding to the mouse brain membrane preparation from control (B0) and bisphenol A treated (B2) mice*

	Bmax (fmol/mg of protein)	Kd (nM)
B0	28.24 ± 1.57	0.28 ± 0.04
B2	18.60 ± 0.22*	0.29 ± 0.03

Each value represents the mean with SEM of three samples. *$p < 0.05$ vs. B0 group

neonatal exposure to BPA caused the decrease of dopamine D3 receptor density in the mouse limbic forebrain.

No change in the expression of dopamine D3 receptor mRNAs in the limbic forebrain and lower midbrain obtained from mice prenatally and neonatally exposed to BPA

We also found that the expression of dopamine D3 receptor mRNA in the limbic forebrain and lower midbrain was not affected by prenatal and neonatal exposure to BPA (Fig. 3).

Discussion

In the previous study, we demonstrated that prenatal and neonatal exposure to BPA enhanced the methamphetamine-induced rewarding effect associated with the moderate upregulation of

central dopamine D1 receptor function in mice.[2] In the present study, we found that the stimulation of [^{35}S]GTPγS binding induced by 7-OH-DPAT was markedly attenuated in B2 mice. Under these conditions, the G-protein activation induced by the selective dopamine D_2 receptor agonist N-propylnorapomorphine in the limbic forebrain was not affected by prenatal exposure to BPA.

To evaluate the population of dopamine D3 receptors in the membrane surface of the mouse brain, we performed a saturation-binding analysis using [^{3}H]PD128907. In the present study, the density of dopamine D3 receptor was significantly decreased by chronic exposure to BPA. In addition, we found that the expression of dopamine D3 receptor mRNA in the limbic forebrain was not affected by prenatal and neonatal exposure to BPA. These findings suggest that chronic exposure to BPA caused the increase in dopamine D3 receptor turnover without any changes in newly synthesized receptors.

The dopamine D3 receptor is highly distributed in the nucleus accumbens, the terminal sites of the mesolimbic dopaminergic system.[3,9] This can be supported by the present finding that the dopamine D3 receptor expression was predominantly observed in the limbic forebrain. The limbic system-selective expression of the dopamine D3 receptor has led to particular interest in this receptor as a potential mediator of some of the psychoeffective functions of dopamine neurotransmission.[10–13] In the previous study, we demonstrated that the dopamine D3 receptor knockout mice exhibit the enhancement of morphine-induced rewarding effect and hyperlocomotion.[5] Furthermore, prenatal and neonatal exposure to BPA enhances the morphine-induced rewarding effect and hyperlocomotion without direct changes in the μ-opioid receptor function.[14] Thus, these findings provide the possibility that the dramatic suppression of postsynaptic dopamine D_3 receptor function in the nucleus accumbens may be, at least in part, involved in the enhancement of morphine-induced rewarding effect followed by chronic exposure to BPA.

The desensitization of G-protein-coupled receptors is mediated by the phosphorylation of serine and threonine residues within the intracellular domains of receptors.[15] Both second messenger-dependent kinases, such as protein kinase A and protein kinase C, and G-protein-coupled receptor kinases (GRKs) have been shown to contribute to the desensitization and internalization of activated dopamine receptors.[16–18] It has been reported recently that dopamine D3 receptor desensitization can be regulated by GRK3.[19] In the present study, we clearly observed the increase in dopamine D3 receptor turnover without any changes in newly synthesized receptors after chronic treatment with BPA. Taken together, a hypothesis would be advanced that, although further study is required, prenatal and neonatal exposure to BPA may lead to the changes in activities of these kinases associated with the turnover of dopamine D3 receptors.

In conclusion, the present study suggests that chronic exposure to BPA causes the inhibition of dopamine D3 receptor-mediated G-protein activation associated with the decrease in its receptor density in the limbic forebrain of mice. This phenomenon may, at least in part, contribute to the enhancement of morphine-induced rewarding effect by chronic exposure to BPA in mice. Public attention and research efforts are being driven by an understanding of the ever-increasing problems and magnitude of substance abuse. Our findings warn that prenatal and neonatal exposure to BPA in females may predispose their children to the psychological dependence of morphine.

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Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice

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Abstract

Bisphenol-A (BPA), one of the most common environmental endocrine disrupters, has been extensively evaluated for toxicity and carcinogenicity. However, little is still known about its action on the CNS. Here we found that prenatal and neonatal exposure to BPA resulted in the enhancement of the rewarding effect and hyperlocomotion induced by morphine in mice. Under these conditions, no change in the G-protein activation by morphine and μ-opioid receptor expression in the lower midbrain was observed by prenatal and neonatal exposure to BPA. These results suggest that chronic exposure to BPA produces the supersensitivity of the morphine-induced rewarding effect and hyperlocomotion without direct changes in μ-opioid receptor function in the lower midbrain. The present data provide further evidence that prenatal and neonatal exposure to BPA can directly influence the development of the central dopaminergic system.

Keywords: Bisphenol-A; Morphine; Rewarding effect; Hyperlocomotion; μ-Opioid receptor; G-Protein activation

Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. It has been reported that administration of bisphenol-A (BPA) to pregnant mice on gestation days at a dose that is within the range typical of the environmental exposure of humans produces significant changes in the postnatal growth rate and brings on early puberty in these mice [6]. These results imply that BPA may cause unpleasant toxicity in the developmental process. In contrast, little is known about the neuronal toxicity induced by BPA in the CNS. More recently, we have reported that prenatal and neonatal exposure to BPA enhances the methamphetamine-induced rewarding effect and central dopamine D_1 receptor function in mice [19].

Many studies have suggested that the mesolimbic dopaminergic system which projects from the ventral tegmental area (VTA) to the nucleus accumbens is critical for the initiation of opioid reinforcement and hyperlocomotion [7,16,20]. Either DAMGO- or morphine-induced place preference can be blocked by dopamine antagonists receptor [13,14]. As well as the rewarding effect, it has been well recognized that hyperlocomotion induced by morphine can be blocked by treatment with dopamine receptor antagonists in the nucleus accumbens [4,8]. These findings indicate that the dopamine-containing neurons of the midbrain VTA, which has a high density of μ-opioid receptors, play a critical role in the rewarding effects and hyperlocomotion by μ-opioids. In terms of dopamine receptor involvement at the terminal site of the mesolimbic dopamine system, the rewarding effect of abused drug has been shown to be mediated by dopamine D_1 receptors [10,14,17], which could be directly affected by prenatal and neonatal exposure to BPA [19]. The aim of the present study was to investigate whether prenatal and neonatal exposure to BPA in mice could affect the rewarding and locomotor-enhancing effects induced by morphine.

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. All efforts were made to minimize the number of animals used and their suffering. All experiments were performed using male ddY mice that had been prenatally and neonatally exposed to BPA. Prenatal and neonatal BPA exposure was conducted as previously described [19]. Adult female mice were chronically treated with BPA-admixed powder food containing 0 (B0), 0.002

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(B0.002), 0.5 (B0.5) and 2 (B2) mg BPA/g of food from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of BPA from their mothers. During the treatment with BPA, animals did not show weight loss and disrupted maternal behaviors.

Place conditioning was conducted as previously described [18]. The apparatus was a shuttle box (15 × 30 × 15 cm: w × l × h) which was made of acrylic resin board and divided into two equal-sized compartments. One compartment was white with a textured floor, and the other was black with a smooth floor to create equally preferred compartments. For conditioning, mice were confined to one compartment after drug injections and to the other compartment after saline injections. The order of the injection (drug or vehicle) and compartment (white or black) was counterbalanced across subjects. A day after these conditioning sessions, the animal was placed in the test apparatus without any confinements.

The locomotor activity of mice was measured by an ambulometer as described previously [11]. Briefly, a mouse was placed in a tilting-type round activity cage 20 cm in diameter and 19 cm high. Any slight tilt of the activity cage, which was caused by horizontal movement of the mouse, was detected by three microswitches. Total activity counts were automatically recorded for 3 h following the injection of saline (10 ml/kg, s.c.) or morphine (10 mg/kg, s.c.). Total activity was counted for 3 h after the treatment.

In the membrane preparation, mice were killed by decapitation and the lower midbrain was then dissected as described previously [12]. The lower midbrain was rapidly excised at 4 °C, and the tissues were homogenized using a Potter-Elvehjem tissue grinder with a Teflon pestle in 20 volumes (w/v) of ice-cold Tris–Mg^{2+} buffer containing 50 mM Tris–HCl (pH 7.4), $MgCl_2$ and 1 mM EGTA for the [^{35}S]GTPγS binding assay. The homogenate was centrifuged at 4 °C for 10 min at 48,000 × *g*. The pellet was resuspended in ice-cold [^{35}S]GTPγS binding assay buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, and 100 mM NaCl and centrifuged at 4 °C for 10 min at 48,000 × *g*. The resultant pellet was resuspended in ice-cold [^{35}S]GTPγS binding assay buffer and stored at −70 °C until used. The membrane homogenate (3–8 μg protein/assay) was incubated at 25 °C for 2 h in 1 ml of assay buffer with 0.001–10 μM morphine, 30 μM guanosine-5′-diphosphate (GDP) and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters presoaked in 50 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$ at 4 °C for 2 h. Filters were then washed three times with 5 ml of an ice-cold Tris–HCl buffer (pH 7.4), transferred to scintillation counting vials containing 0.5 ml of Soluene-350 and 4 ml of Hionic Fluor, equilibrated for 12 h and the radioactivity in the samples was determined with a liquid scintillation analyzer. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. Comparable results were obtained from at least three independent sets of experiments.

Total RNA in the whole brain was extracted using an SV Total RNA Isolation System (Promega, Madison, WI). To prepare first strand cDNA, 1 μg of RNA was incubated in 100 μl of buffer containing 10 mM DTT, 2.5 mM $MgCl_2$, dNTP mix, 200 units of reverse transcriptase II (Gibco-BRL, Grand Island, NY) and 0.1 mM oligo (dT)12-18 (Gibco-BRL). The μ-opioid receptor was amplified in a 50 μl PCR solution containing 0.8 mM $MgCl_2$, dNTP mix and DNA polymerase with synthesized primers: a sense primer of μ-opioid receptor, which is at position 299–320 (5′-AGACTGCCACCAACATCTACAT-3′) of the receptor, and an antisense primer at position 623–643 (5′-TGGACCCCTGCCTGTATTTTG-3′). Samples were heated to 94 °C for 2 min, 55 °C for 2 min and 72 °C for 3 min, and cycled 35 times through 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min. The final incubation was 72 °C for 7 min. The mixture was run on 1% agarose gel electrophoresis with the indicated markers and primers of the internal standard GAPDH. Three independent experiments were performed in this study. Semi-quantitation of the intensity of the bands for μ-opioid receptor/GAPDH was conducted by using a NIH image.

Data represent the mean counts with SEM. Statistical analyses were performed using two-way ANOVA with Bonferroni/Dunnett's test.

In the previous study, we found that prenatal and neonatal exposure to BPA enhances the methamphetamine-induced pharmacological actions [19]. Here, we investigated the influence of prenatal and neonatal exposure to BPA in the several morphine-induced pharmacological actions. In the present study, morphine produced a dose-dependent place preference in control B0 mice (Fig. 1A), as reported previously [10,17]. Morphine at the low dosage that elicited neither place preference nor place aversion in control B0 mice produced a significant place preference in BPA-treated mice (Fig. 1A). As well as the rewarding effect, a significant increase in the morphine-induced hyperlocomotion was observed by chronic BPA treatment ($F_{1,340} = 6.617$, $P < 0.05$ vs. B0 group, Fig. 1B).

Recently, several investigations have provided evidence that the treatment of adult animals with BPA could not affect the reproductive function and social behaviors [2,3]. We have already confirmed that acute administration of BPA with adult mice could not affect the dopamine-related behaviors (data not shown). These findings indicate that prenatal and neonatal exposure to BPA may cause the neuronal toxicity specifically in the developmental process.

Many studies have pointed to the mesolimbic dopaminergic system, which originates from the VTA projecting mainly to the nucleus accumbens, as a critical site for the initiation of opioid addiction [7,13,14,16,20]. Morphine has been shown to indirectly activate dopamine neurons in the VTA as a consequence of inhibiting non-dopaminergic neurons, presumably γ-aminobutyric acid-containing neur-

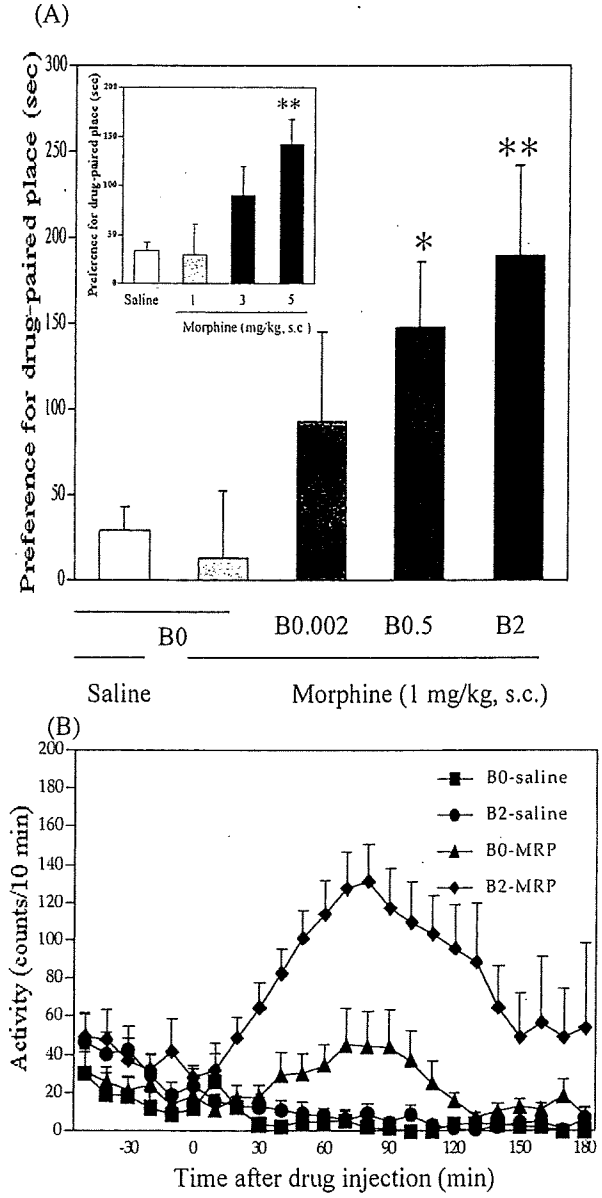

Fig. 1. (A) Effect of prenatal and neonatal exposure to BPA on the rewarding effect of morphine in mice. (Inner) Dose-response for the morphine-induced place preference in control B0 mice (*$P < 0.05$ vs. saline-treated mice). (Outer) Effect of BPA (0.002–2 mg/g of food: B0.002–B2) on the morphine (1 mg/kg, s.c.)-induced place preference. The BPA-non-treated group (hatched bar) did not show any place preference or place aversion with morphine at 1 mg/kg. The BPA-treated group (filled bar) showed a significant place preference induced by morphine at this dosage (*$P < 0.05$, **$P < 0.01$ vs. BPA-non-treated group). Each column represents the mean place preference score with SEM of six to ten mice. (B) Effect of BPA on morphine (10 mg/kg, s.c.)-induced hyperlocomotion. The BPA (2 mg/g of food)-treated group (diamond: B2 mice) revealed a significant potentiation of the morphine-induced locomotor-enhancing effect ($F_{1,340} = 6.617$, $P < 0.05$ vs. B0 group: triangle). Each point represents the mean activity counts for 10 min with SEM of nine to ten mice.

ons, leading to the increased dopamine release in the nucleus accumbens [10]. In order to investigate the mechanism of the enhancement of morphine's effect, we demonstrated whether prenatal and neonatal exposure to BPA could directly affect the opioidergic system associated with the mesolimbic dopaminergic system. In the [^{35}S]GTPγS binding assay, prenatal and neonatal BPA exposure failed to enhance μ-opioid receptor-mediated G-protein activation by morphine in the lower midbrain (Fig. 2A). As well as G-protein activation, the expression of μ-opioid receptor mRNA was not changed by chronic BPA treatment, suggesting that μ-

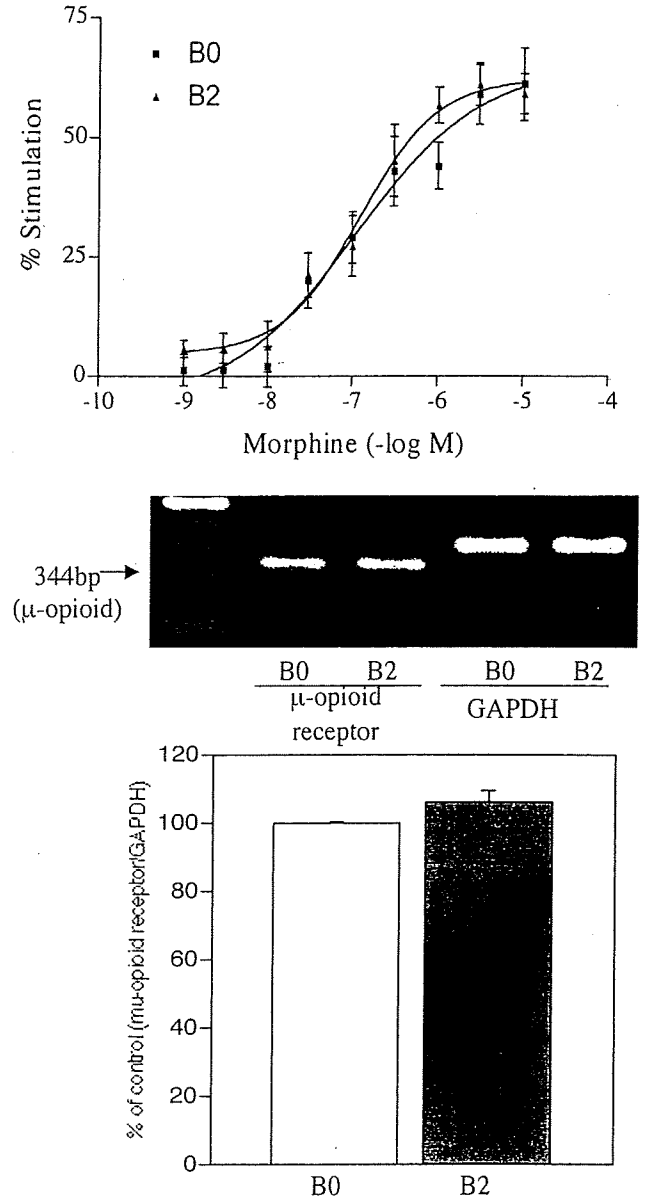

Fig. 2. No changes in the μ-opioid receptor in the mice prenatal and neonatal exposure to BPA. (A) Comparison of the stimulation of [^{35}S]GTPγS binding by morphine to membranes of the lower midbrain obtained from control (B0: square) and BPA-treated (B2: triangle) mice. Membranes were incubated with [^{35}S]GTPγS (50 pM) and GDP (30 μM) with morphine (10^{-9}–10^{-5} M). The values are expressed as % increase of the value in B0 mice. Data are expressed as the mean ± SEM of three independent samples. (B) Effect of prenatal and neonatal exposure to BPA on the expression of μ-opioid receptor mRNA in the mouse brain. (Upper) Representative RT-PCR for the μ-opioid receptor mRNAs in the whole brain minus cerebellum obtained from B0 and B2 mice. (Lower) There were no changes in the expression of μ-opioid receptor mRNAs in the whole brain obtained from B2 mice (filled bar) as compared to that from B0 mice (open bar). The values are expressed as a percentage of the value in B0 mice. Each column represents the mean ± SEM of three independent samples.

opioid receptor function is likely to be directly unaffected in this region. We have already reported that prenatal and neonatal exposure to BPA lead to an up-regulation of dopamine D_1 receptor in the limbic forebrain [19]. Taken together, these results indicate that the enhancement of the morphine-induced rewarding effect and hyperlocomotion may mainly result from the direct up-regulation of the dopaminergic transmission in the limbic forebrain.

BPA is considered to disrupt the endocrine systems via binding to estrogen receptor by mimicking estrogenic action. However, weak estrogenicity of BPA was confirmed with approximately 15,000 times less potency than 17β-estradiol [5]. Furthermore, BPA binds to estrogen receptor with low affinity and transactivates the estrogen responsive element-driven reporter gene in vitro [5]. Recently, it has been reported that BPA acts on thyroid receptor as an antagonist [9]. In addition, Song et al. have reported that BPA induces the expression and steroidogenesis of the orphan nuclear receptor Nur77 gene in mice Leydig cells [15]. Several subfamilies of Nur77, for example NGFI-B or Nurr1, have been shown to be highly expressed in the brain [21]. In particular, it has been accepted that NGFI-B is highly expressed in the basal ganglia, and is involved in the development of dopaminergic and opioidergic systems [1, 22]. These findings suggest that, although we cannot completely exclude the possibility of estrogenic action of BPA, the present action of BPA may mainly result from novel mechanisms associated with dopaminergic transmission.

In conclusion, the present data provide further evidence that prenatal and neonatal exposure to BPA can directly influence the development of the central dopaminergic system in the limbic area. These effects could lead to a change in sensitivity to morphine. Further investigation is necessary to fully understand the molecular mechanism of BPA in the CNS.

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Industrial Health 2004, 42, 219–225

Original Article

Comparative Investigation of Several Sperm Analysis Methods for Evaluation of Spermatotoxicity of Industrial Chemical: 2-Bromopropane as an Example

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Abstract: **Reproductive toxicity of 2-bromopropane (2BP), a substitute for ozone layer-depleting chloro-fluorocarbon, was found among the workers in an electronics factory in Korea in 1995. Furthermore the importance of testicular toxicity has been realized since the problem of endocrine disruptors arose all over the world, but manual methods must rely on subjective assessment. Recently, computer-assisted sperm analysis (CASA) was proposed but this system requires vast investment. We then investigated the applicability of the MTT method with a microplate and sperm quality analyzer (SQA) as simple, rapid, and economic instrumental methods for the examination of sperm quality in rats, comparing it with the manual microscopic method and CASA. Epididymal fluid derived from male F344/N Slc (Fischer) rats intraperitoneally injected with 2BP in the dose range of 125–1,000 mg/kg/d twice a week (total 8 times) were examined by these methods as a model experiment. Sperm count measured by the manual method and CASA in the epididymal fluid, absorbance by the MTT method and sperm motility index value by the SQA method were significantly lower in the 2BP 1,000 mg/kg administered group than in the control group. This result suggests that the MTT method can detect oligospermia. With the microplate and microplate reader, the efficiency of detection becomes much better. Sperm analyses by the MTT method with the microplate reader and the SQA method are available for reproductive toxicity study in rats.**

Key words: 2-Bromopropane, Tetrazolium salt, MTT (3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyl tetrazolium bromide), SQA (sperm quality analyzer), CASA (computer-assisted sperm analysis), Manual microscopic method, Reproductive toxicity, Rat

Introduction

Certain substances found in the environment can upset normal endocrine balance and become a health hazard. An example of growing concern is their effect on sperm[1]. Some workers in semiconductor factories in Korea were found to have affected in their reproductive functions after exposure to 2-bromopropane (2BP)[2,3]. Subsequently, the reproductive effects of 2BP were confirmed to the animal experimental studies[4–7]. These reports prompted close reappraisal of the efficacy and feasibility of mass screening for toxicity to male reproductive functions in industrial populations.

Surveying the method of investigating sperm activity, each method now in use has its own serious shortcomings. For instance, the conventional, manual method of sperm count and assessment of motility under the optical microscope is fraught with inevitable subjective variations which would make inter-institutional comparison of data practically

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impossible. The introduction of computer-assisted sperm analysis (CASA) eliminated variations due to subjective evaluation but this apparatus is expensive[8–13] and has not enjoyed wide acceptance. Meanwhile, the sperm quality analyzer (SQA), which measures sperm count and motility by the optical method, was introduced as a simple and inexpensive alternative[14, 15]. The third approach is the biochemical method (MTT method) which measures color changes in the tetrazolium reaction to mitochondrial reductase by absorption spectrometry reflecting the overall numerical and functional power of sperm activity[16, 17]. Although these methods of sperm testing have proliferated, their performance and efficacy have been evaluated individually and never collectively using the same test samples. In particular, comparative investigation of the MTT method with CASA has never been performed. Their performance in terms of mass handling of large numbers of samples has not been evaluated properly.

We attempted to develop a method for measuring toxicity to the sperm by combining an absorption spectrophotometer with a microplate reader so that a large number of specimen can be processed rapidly. The method is objective, simple, inexpensive and efficient and can be applied to mass screening of workers in suspicious environments. In the days when more and more clinical tests for male reproductive disability need to be performed on an everyday basis, the ability to processing a large number of samples will be an important prerequisite in the selection of test methods. Furthermore, the MTT method, with its speed and simplicity to deal with a large number of facilities, is a technique suitable for the animal studies of male reproductive disturbance induced by various chemicals. In this study, we induced reproductive toxicity with 2BP as a representative of bromopropanes, which are used widely in the industrial workplace.

We report the results of a study carried out on rats given repeated doses of 2BP using the MTT method with a microplate reader in comparison with other methods of sperm testing including CASA. The advantages and merits of various methods were compared and problems in performing the tests will be discussed.

Materials and Methods

Chemicals and supplements

2BP and MTT were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and Dojin (Kumamoto, Japan), respectively. Olive oil, HCl and isopropyl alcohol were from Wako Pure Chemical (Osaka, Japan). Bovine serum albumin Fraction V (BSA), Medium 199 and phosphate buffered saline (PBS) were from Seikagaku Kogyo (Tokyo, Japan), GIBCO (Grand Island, USA) and Nissui Pharmaceutical (Tokyo, Japan), respectively.

Instruments

The semen analyzer (HTM-IVOS Ver. 10.9i) was from Hamilton Thorne Research (Beverly, MA, USA). SQA was the product of Medical Electronic Systems (Migdal Haemek, Israel). The microplate reader (Immunoreader NJ2000) was purchased from Nalge Nunc (Tokyo, Japan). The optical microscope (Eclipse E600) was from Nikon (Tokyo, Japan).

Experimental protocol

F344/N Slc (Fischer) male rats (11 wk of age) from Japan SLC (Shizuoka, Japan) were kept in cages under standard conditions and received pellets (Oriental Yeast, Tokyo, Japan) and water *ad libitum*. The body weight was monitored just before each administration and sacrifice. Each of 4–5 rats (12 wk of age)/group received intraperitoneal instillations of 2BP dissolved in olive oil twice a week for 24 d in doses of 125, 250, 500 and 1,000 mg/kg. Control rats received an equal volume of olive oil. So each rat received a total of 8 injections. After a one week rest period following the last dose, the animals were sacrificed under ether anesthesia and the testes, epididymis and epididymal cauda were separated and weighed immediately. And then relative organ weights were calculated.

Preparation of epididymal cauda sample

Epididymal cauda was minced with scissors to release sperm in 2 ml of Medium 199 containing 0.5% BSA at 37°C. This sperm suspension sample served for the MTT and SQA methods. The aliquot of this sample was stored at –80°C. Before sperm count analysis, this aliquot was diluted 1:4 with PBS, further homogenized at room temperature, and served as a sample for the manual method and CASA.

Manual method

After staining with trypan blue the specimen was spread on a hemocytometer and the sperm heads were counted manually under the optical microscope. The data were expressed as the total number of sperm per one cauda epididymal tissue.

CASA

Each sample was stained with the attached staining kit (Supra Vital IDENT Stain Kit, Hamilton-Thorne Research, Bevely, MA, USA), dropped into a disposable counting chamber CELL-VU (Millennium Sciences Corp. NY, USA) and

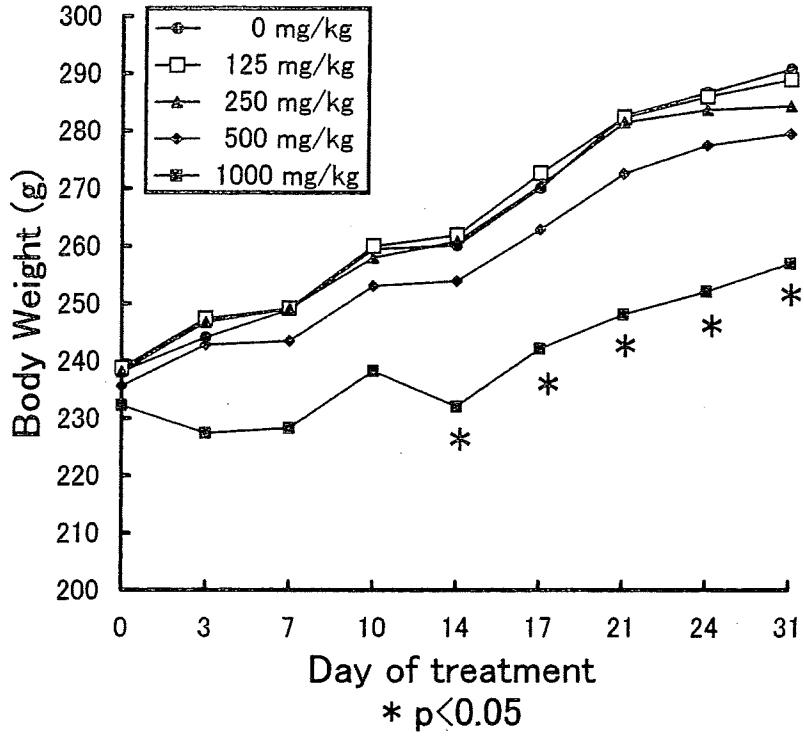

Fig. 1. Mean body weight of F344 rats exposed to 2BP ip.

mounted on the Semen Analyzer. Sperm heads fluorescence under an ultra-violet beam were counted in RAT-IDENT mode automatically. The data were expressed as the total number of sperm per cauda epididymal tissue sample.

SQA method

The disposable SQA capillary (Medical Electronic Systems, Migdal Haemek, Israel) containing the specimen was inserted into the slot in the SQA and the sperm motility index (SMI) was determined.

MTT method

Fifty microliters of the sperm suspension sample in the sterile 96-microplate well was incubated with MTT reagent (5 mg/ml in PBS, 25 micro liter) at 37°C for two hours. Then the reaction was stopped by rapid cooling. After the addition of 0.04 M HCl-isopropylalcohol and pipeting exhaustively to dissolve the formazan thus formed in the process, the absorbance in each well was estimated at 574 nm by the microplate reader.

Correlation among data from various methods

In an attempt at evaluation of various methods, we carried out the following experiments. Epididymis cauda obtained from an untreated rat (17 wk of age) was dissected with a pair of scissors in 2 ml of medium to suspend sperm. The sample consisted of this undiluted suspension and its dilutions with Medium 199 containing 0.5% BSA to 4 strength. Data on sperm count, SMI and absorbance obtained by pair of investigating methods were compared and correlation was sought.

Statistical analysis

The data were analyzed by one-way ANOVA. The statistical significance of difference between the control and 2BP-treated groups was determined with Fisher's PLSD test. In all cases, $P < 0.05$ was considered statistically significant.

Results

Body and organ weights

Body weight decreased in the groups with a dose of 1,000 mg/kg (Fig. 1) on and after day 14 as compared to the control group. The relative weights of testis (TE, right (R) and left (L)), epididymis (EP, right (R) and left (L)), and epididymis cauda (EPC, right (R) and left (L)) are shown in Table 1. The weight of both the right and left testis decreased in the 500 and 1,000 mg/kg dose groups. In the 250 mg/kg dose group, only the left testis weight decreased significantly, but no significant difference was found in the right testis.

Table 1. Relative weight (%) of reproductive organ in 2BP-treated (mg/kg) rats

2BP (mg/kg)	TER	TEL	EPR	EPL	EPCR	EPCL
0	0.503 ± 0.010	0.521 ± 0.018	0.172 ± 0.007	0.176 ± 0.011	0.080 ± 0.004	0.076 ± 0.005
125	0.505 ± 0.020	0.512 ± 0.020	0.186 ± 0.012	0.176 ± 0.017	0.089 ± 0.003*	0.082 ± 0.006
250	0.493 ± 0.023	0.480 ± 0.025*	0.183 ± 0.011	0.184 ± 0.014	0.087 ± 0.006	0.089 ± 0.011*
500	0.407 ± 0.021*	0.409 ± 0.038*	0.168 ± 0.012	0.179 ± 0.008	0.086 ± 0.006	0.076 ± 0.003
1000	0.193 ± 0.010*	0.199 ± 0.019*	0.131 ± 0.009*	0.130 ± 0.007*	0.056 ± 0.006*	0.055 ± 0.004*

TER: Right Testis, TEL: Left Testis, EPR: Right Epidydymis, EPL: Left Epididymis. EPCR: Right Epididymal Cauda, EPCL: Left Epididymal Cauda. Each value represents the mean ± SD. *: Significantly different at $p<0.05$.

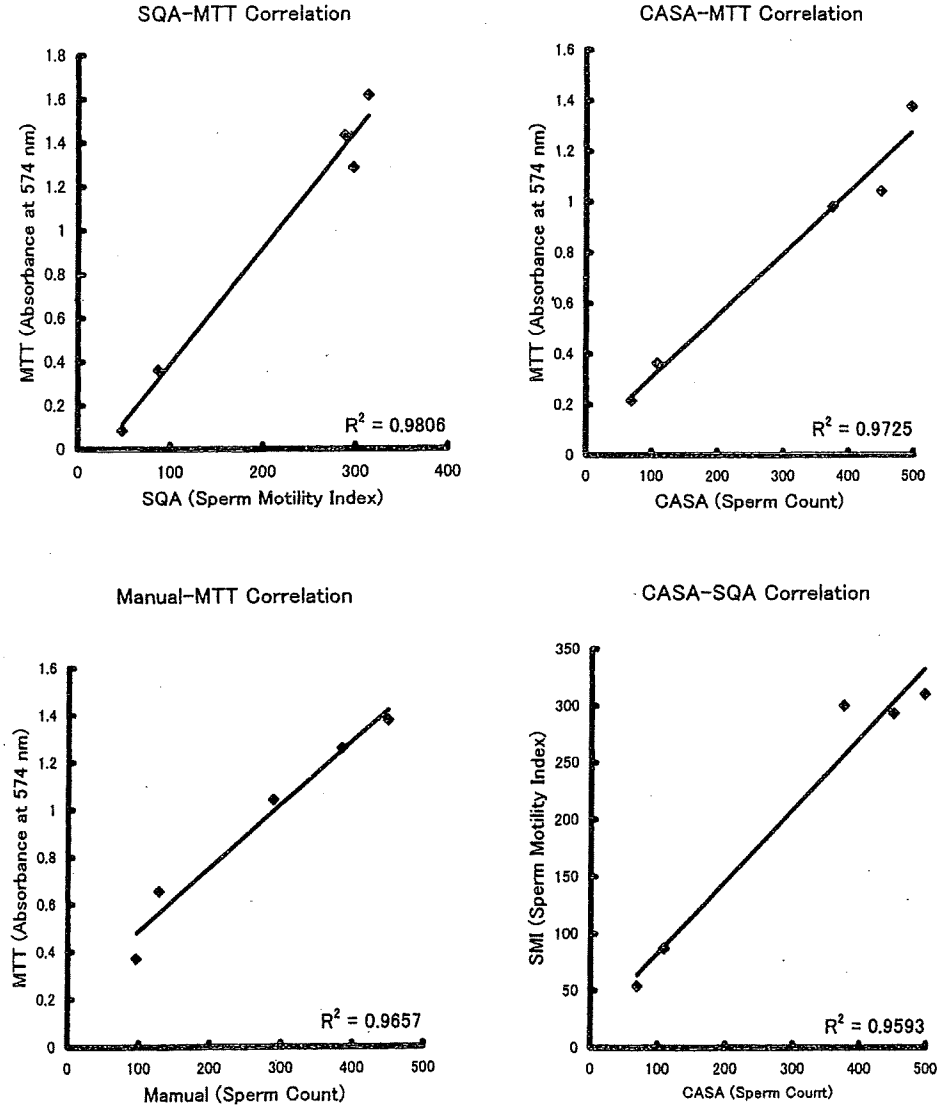

Fig. 2. Relationship between parameters of several methods.

And the weights of the epididymis and epididymal cauda decreased in the 1,000 mg/kg group, but some reverse results were found in lower dose groups (EPCR: 125 mg/kg, EPCL: 250 mg/kg), but they were not in both sides of the tissues.

Correlation between the sperm analysis methods

Figure 2 shows the correlation between the two methods. A high correlation was found between the SMI value found by the SQA method and absorption by the MTT method

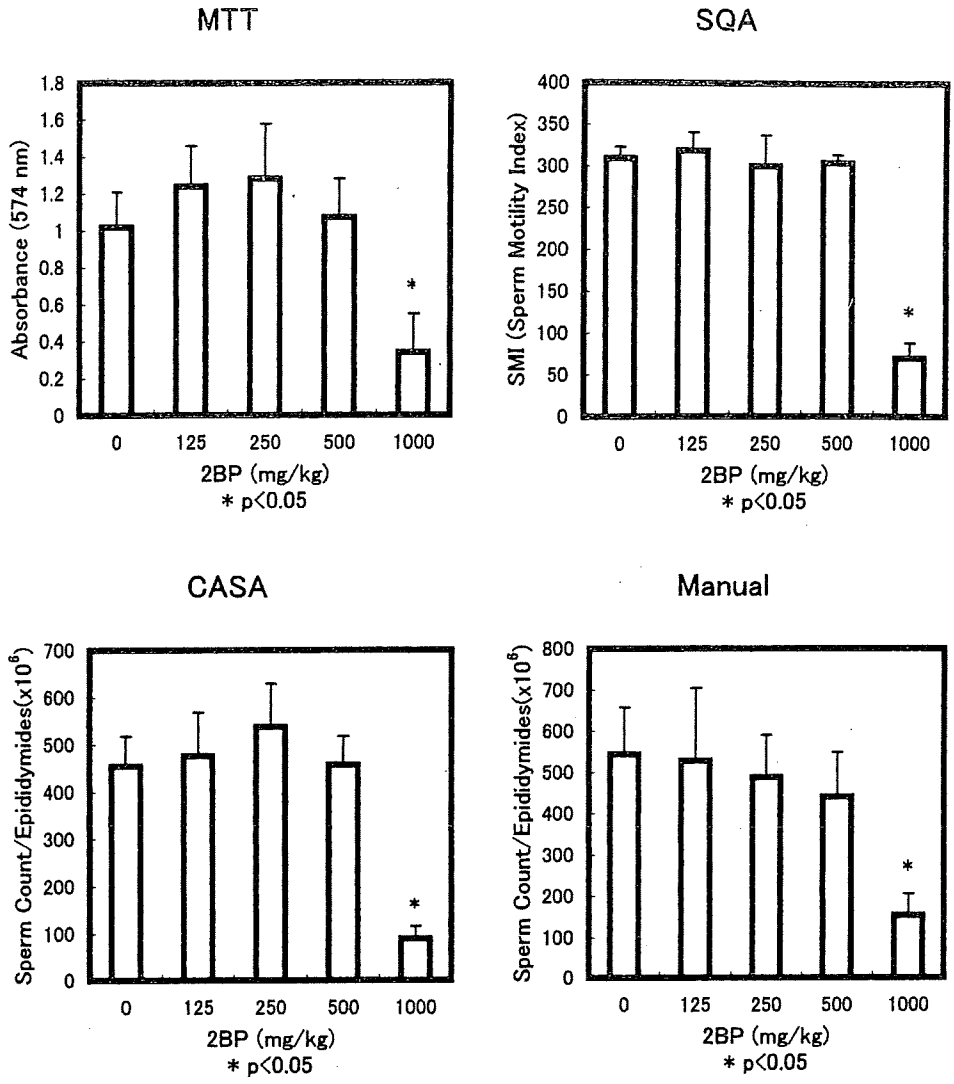

Fig. 3. Effect on rat sperm of 2BP (Comparison of results obtained by the MTT, SQA, CASA and Manual methods).

(R^2=0.9806). Similarly, MTT absorbance correlates well with the Sperm count by CASA (R^2=0.9725) and the manual method (R^2=0.9657). All the methods had common linearity mutually.

Detection of sperm toxicity of 2BP by several methods

As shown in Fig. 3, sperm count by the manual method, sperm count by CASA, SMI by the SQA method and absorbance by the MTT method were similarly low only in the 1,000 mg/kg group as compared with the control group. There was no significant reduction in the parameters in the groups given 2BP at the dose of less than 500 mg/kg.

Discussion

Only the testes weight could indicate sperm toxicity in the 500 mg/kg dose group, whereas while other methods failed to detect the change (Table 1 and Fig. 3). Although the weight of testes was the most sensitive parameter indicating the sperm toxicity, this requires autopsy and therefore has no clinical usefulness. A non-invasive method such as sperm count under an optical microscope is more practical and is widely accepted, but inherent shortcomings of this method are obvious: counts are susceptible to subjective variations and their inter-institutional or inter-observer comparisons and analyses are unreliable if ever possible, and it is not suitable for processing a large number of specimens in mass screening.

SQA is a simple and practical method, but it is no match in efficiency for the MTT method. In comparison, the MTT method excels SQA on account of rapidity and simplicity and therefore its ability to handle a large number of samples simultaneously. CASA gives not only the sperm count, but other information such as the motility rate and even morphological indexes[8–13]. Unfortunately this requires rather expensive equipment and has not achieved wide acceptance. MTT, one of the tetrazolium salts, is known to form formazan and is turned blue in the somatic cells by mitochondrial reductase[16]. The same reaction is observed in a suspension of sperm and the extent of coloring reflects the number and viability of the sperm[17]. The MTT method is utilized in many toxicity studies on somatic cells, but its use in sperm cell studies has been reported in only one paper. We introduced the use of the microplate and established a distinctly more efficient measurement system. If only qualitative analysis is required, direct observation may suffice, dispensing with absorption spectrometry. Possibly other tetrazolium salts may be found equally or more useful as the substrate and may replace MTT. We proposed the use of the MTT method for sperm analysis and established the protocol with microplates to facilitate processing a large number of samples rapidly as is required in mass screening. In the sperm count we obtained high correlation between the results by the MTT method and those by SQA, CASA, and the manual method as shown in Fig 2. We believe that the MTT method can replace these other methods where only the sperm count is required, but the MTT reaction is dependent on the activity of mitochondrial reductase in the sperm. This method cannot be expected to distinguish those sperm with abnormal morphology or diminished motility from normal, healthy sperm, as long as they have metabolic activity. On the other hand, the SMI value which is obtained by SQA has a positive correlation with both sperm count and sperm motility and is recognized as a strong predictor of fertility of the semen. The manual method can distinguish sperm deformity as well as give the sperm count. The advantage of the MTT method, on the other hand, is the efficiency in processing a large number of specimens and therefore may be a powerful tool for preliminary screening. The results of our animal experiments also established that the MTT method could detect sperm toxicity caused by introduced chemical agents to an extent comparable to other methods such as the manual method or CASA.

In conclusion we assert that the MTT method using the microplate reader provide a new tool in detecting sperm toxicity with sensitivity comparable to conventional or more expensive methods and is especially suitable for workplace mass screening. But manual dissolution of formazan is an extra step required in the MTT method. It is not readily amenable to automation. And this is a cause of errors in measurement. Recently tetrazolium salts which produce water-soluble formazan have been developed and their usefulness in toxicity tests on somatic cells has been reported. When these newer salts are used the process of dissolution of formazan is not necessary. Enhanced simplicity and improved accuracy of the method are expected. We plan to continue further studies on sperm toxicity using various tetrazolium salts to replace MTT.

In our experiments, distinct sperm toxicity was observed only in the group of rats given a large dose (1,000 mg/kg) of 2BP whereas a significant reduction in the weight of testes had already been found in the 500 mg/kg group (Table 1). This suggests that 2BP exerts its effect in the spermatogenesis stage. And reverse effects were observed in several cases in organ weight (EPCR: 125 mg/kg, EPCL: 250 mg/kg). This phenomenon is thought to result in a transitory effect.

Furthermore, we have started the study of reproductive toxicity induced by bromopropanes other than 2BP and have found that the MTT method is equally applicable to in these studies. We plan to present additional data on 2BP and other chemicals and further discussion on the mechanism of reproductive toxicity in our ensuing reports.

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